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SPECTRAL ANALYSES OF EXTRINSIC FLUORESCENCE OF THE NERVE MEMBRANE LABELED WITH AMINONAPHTHALENE DERIVATIVES

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SUMMARY

A fluorescence spectrophotometer was constructed to determine the emission spectrum of a nerve labeled with various fluorochromes. Using this spectrophotometer, the spectra of 2-*p*-toluidinylnaphthalene 6-sulfonate (2,6-TNS) and other aminonaphthalene derivatives in squid giant axons were determined at the peak of nerve excitation, as well as in the resting state of the axons. During nerve excitation the fluorescent light deriving from the 2,6-TNS-stained nerve undergoes a transient change in intensity. The spectrum of the light contributing to this change in intensity was found to be much narrower and sharper than the fluorescent spectrum of the light arising from labeled axons at rest. This narrow and sharp spectrum is interpreted as being derived from a transient variation in the polarity of the 2,6-TNS binding sites in the axon. In the Appendix, the results of a physicochemical investigation into the factors affecting the fluorescence of 2,6-TNS *in vitro* are described.

INTRODUCTION

In recent years the method of fluorescence labeling has been used to study conformational changes in various macromolecules[1–3]. This method has been applied to the nerve membrane in an effort to elucidate the process of action potential production[4–6]. It is now established that when a nerve labeled with various fluorescent probes is electrically stimulated, there is a transient change in the intensity of the fluorescent light deriving from the probes. However, the molecular basis of this fluorescence signal associated with an action potential is not yet clearly understood.

To clarify the molecular basis of the production of fluorescence signals, we constructed a spectrofluorometer specifically suited for studies of the nerve membrane and carried out detailed spectral analyses of the fluorescent light. Such a special spectrofluorometer is needed because the time-resolution of the commercially available machines is too slow to be used for detection of the rapid changes in fluorescence occurring during nervous activity. We found, by using aminonaphthalene derivatives[7,8]

Abbreviations: 2,6-TNS, 2-*p*-toluidinylnaphthalene 6-sulfonate; 2,6-ANS, 2-aminonaphthalene 6-sulfonate; 1,8-ANS, 1-aminonaphthalene 8-sulfonate.

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in conjunction with this new spectrofluorometer, that the spectra of the fluorescent light contributing to the transient changes during action potentials are generally very different from the spectra in the resting state. In this paper, the results of these spectral analyses are presented and their possible significance are discussed on the bases of the physicochemical studies of the factors which determine the wavelength, intensity and lifetime of fluorescence of 2-*p*-toluidinylnaphthalene 6-sulfonate (2,6-TNS) *in vitro*.

METHODS

An original model of our high time-resolution spectrofluorometer was constructed in 1970 (ref. 5). In the following improved model many of the shortcomings that existed in the original model were eliminated. The spectrofluorometer consists of a device to optically excite the fluoroscent probe in the nerve (see Diagram A, bottom in Fig, 1) and an arrangement to collect the emitted fluorescent light following nerve stimulation (Diagram A, top). The light source used for optical excitation was a 200-W xenon–mercury lamp (Engelhard Hannovia, Inc.). The power supply for the light source, the photomultiplier for detection of fluorescent light and the setup for recording transient changes in fluorescence are similar to those described previously[6].

The emission spectra of fluorescent probes in nerves were determined by inserting a series of interference filters in the pathway of the emitted light. The voltage outputs of the photomultiplier for different wavelengths of emitted light were compared by making the following corrections: (i) subtraction of the photomultiplier output produced by extraneous light, (ii) multiplication of correction factors to account for the variation of the sensitivity of the photo-cathode with the

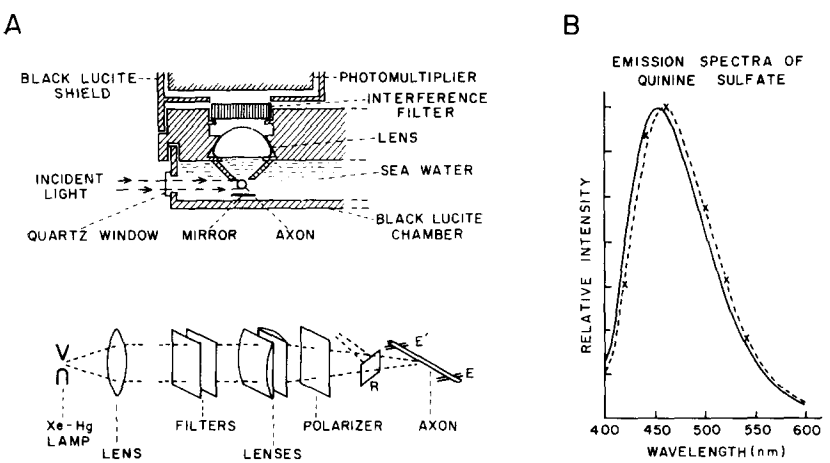

Fig. 1. (A) Schematic diagram of a fluorescence spectrophotometer for determination of emission spectra of an axon labeled with a fluorescent probe. R represents a quartz cover slip used to reflect a small portion of the incident light to the reference photomultiplier tube. E and E′ represent electrodes for stimulating the axon and for recording the action potentials extracellularly. (B) Comparison of the emission spectrum of quinine sulfate determined by the spectrophotometer shown by Diagram A (broken line) and that taken from ref. 9.

wavelength, and (iii) multiplication by correction factors to account for the difference in the transmission characteristics of the interference filters used. The correction terms of the first type were obtained by measuring the photomultiplier outputs as a function of the wavelength with an unlabeled nerve in the chamber. The photomultiplier tube employed had an S-20 response; the data needed for correction of the second kind were obtained from RCA. The interference filters were purchased from Infrared Industries (Thin Film Products Division); the correction factors of the third kind were determined by measuring their transmission characteristics with a Beckman Spectrometer (Model 1093). The spectrofluorometer was calibrated with a well documented fluorochrome[9], a 30-μM quinine sulfate solution (dissolved in 0.05 M H_2SO_4) enclosed in a glass tubing of 1.4 mm inside diameter (Diagram B in Fig. 1).

The sources of the fluorescent probes, the technique of injecting probe molecules into giant axons of *Loligo pealii*, and the procedure of staining nerve bundles of *Labinia marginata* have been described previously[6]. In the experiments described in the Appendix, the excited state lifetimes of probe molecules were measured with an Ortec Nanasecond Fluorescence Spectrometer (Model 9200).

RESULTS

The diagram on the left in Fig. 2 shows the results of measurements on squid giant axons labeled internally with 2,6-TNS. The probe molecules in axons were excited with polarized quasi-monochromatic light, having a wavelength of 365 nm and its electric vector directed parallel to the longitudinal axis of the axons. The observed emission spectrum in the resting state of the axon was determined in the manner described under Methods and was normalized to unity at the wavelength of the emission maximum. The variation in the results obtained from different axons was very small in these resting state measurements.

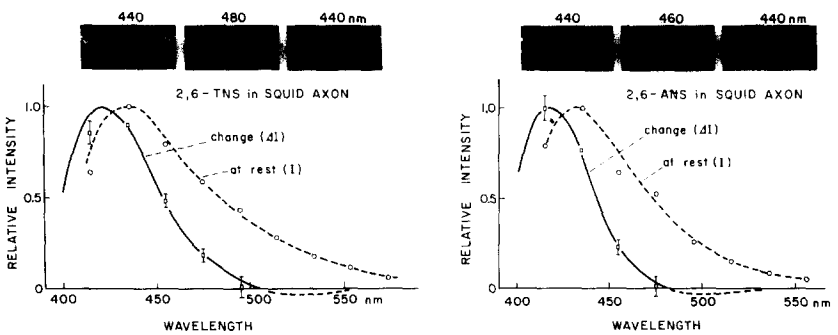

Fig. 2. Left: Emission spectrum of 2,6-TNS in squid giant axons at rest, (I,○), and that of the portion of the fluorescent light that changes during nerve excitation (ΔI,□). Computer records (taken from one axon) showing fluorescence changes associated with action potentials are presented at the top of the figure. The center-band wavelength (for normal incidence) of the secondary filter used is indicated above each record. The number of trials averaged was the same for the three records. The vertical lines indicate 4-ms intervals. The broken and continuous lines are constructed by the procedure described in the text. Right: Similar spectra for 2,6-ANS in squid giant axons. Temperature, 6 °C.

The spectrum of the component of the fluorescent light that changes at the peak of the action potential (ΔI) was measured by the following procedure: First, we determined the wavelength at which the fluorescence signal is largest. Then, we recorded signals at this and another wavelength alternately under constant illuminating and recording conditions and compared the magnitudes of signals at these two wavelengths. An example of such records taken from an axon labeled internally with 2,6-TNS is shown at the top of the figure. The standard errors of these measurements (using 10 different axons) are shown by the short bars in the figure. The continuous line in the figure was constructed from the emission spectra of 2,6-TNS dissolved in model solvents (ethanol and dioxane) by the procedure described in Discussion.

Similar measurements were carried out on six axons internally labeled with 2-aminonaphthalene 6-sulfonate (2,6-ANS) (see the diagram on the right in Fig. 2). The behavior of this probe in axons was found to be very similar to that of 2,6-TNS. The spectrum for the fluorescence signals, ΔI, was found to be far narrower and sharper than that of the resting state fluorescence (I). In the range of wavelengths longer than about 490 nm, no signals were observed with these 2,6-derivatives.

The spectra of 1-aminonaphthalene 8-sulfonate (1,8-ANS) incorporated into squid axons are shown on the left in Fig. 3. Since insertion of a polarizer between the light source and the axon has no effect on the emission characteristics of this probe (except for decreasing the intensity), the exciting light was not polarized in these measurements (using 15 different axons). The emission spectrum of the light intensity in the resting state (I) of the axons was not very different from that obtained with 2,6-ANS. However, the spectrum of ΔI was found to be much broader than the ΔI spectrum of 2,6-ANS.

The diagram on the right in Fig. 3 shows the results obtained from eight crab nerves stained externally with 1,8-ANS. It was found that the spectrum of ΔI (transient increase in fluorescence) was not very different from that of I observed in the resting

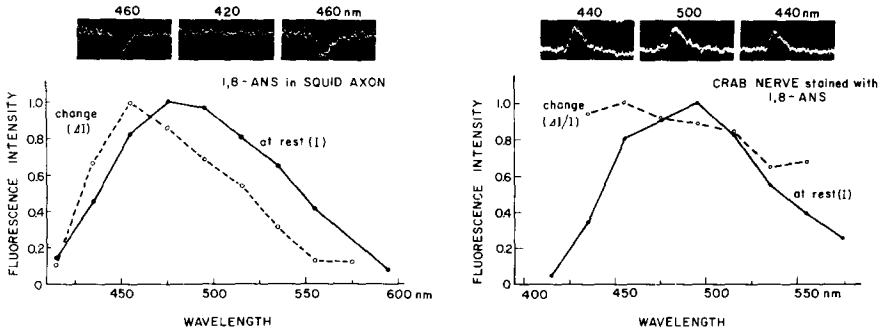

Fig. 3. Left: Emission spectra of squid giant axons internally labeled with 1,8-ANS. Computer records (taken from one axon) are shown on the top. The vertical lines in the records indicate 4-ms intervals. Right: Similar spectra of crab nerve externally labeled with 1,8-ANS. The ratio of the change in intensity during nerve excitation to the intensity at rest ($\Delta I/I$) is plotted against the wavelength. (Note that the computer records at the top of the figure show a transient increase in fluorescence.) Photographic records (taken from an axon) of fluorescence changes associated with action potentials are shown above. The vertical lines indicate 32.5-ms intervals. Temperature 6 °C.

state of the nerve. In order to indicate that there is a small, but definite difference between the spectrum of I and that of ΔI, the values of the ratio of $\Delta I/I$ were plotted against the wavelengths in the figure.

DISCUSSION

In the following discussion, we deal mainly with the behavior of 2,6-TNS in squid giant axons. We have examined the properties of 2,6-TNS dissolved in various solvents or bound to various macromolecules (see Appendix). Based on the knowledge concerning the *in vitro* behavior of the probe molecules, we infer the physicochemical properties of the binding sites for the probe molecules in the axon.

In general, it is possible to reproduce the emission spectra of the probe molecules bound to various macromolecules by using a single mixture of organic solvent and water. For example, the fluorescence of 1 μM 2,6-TNS bound to 5 mg/ml bovine serum albumin is very similar to that emitted by 2,6-TNS of the same concentration dissolved in a mixture of 95% ethanol, 4% water and 1% pyridine. The similarity in the emission involves such quantities as (i) the wavelength of maximum emission, (ii) the band-width, and (iii) the quantum yield. In this case a variation in the water content was used to match the peak wavelength of 2,6-TNS in the mixture with that in bovine serum albumin. Pyridine, a quencher, was used to adjust the quantum yield of the probe molecules in the mixture. The spectra can be matched in this manner because in a wide range of solvent polarities, both the wavelength of maximum emission and the band-width of the spectrum are determined almost uniquely by the solvent polarity (see McClure and Edelman[8]; Turner and Brand[10]; and the Appendix of this paper). Chemical quenchers reduce the fluorescence intensity without affecting the peak wavelength or the band-width.

It was not possible to reproduce the spectrum in the resting state with a single mixture of ethanol, water and pyridine. This fact may be regarded as indicating that there is more than one kind of binding site for the probe molecules in the axon interior. Under the assumption that there are only two kinds of binding sites, the best fit was obtained by taking a linear combination of two spectra in the following form:

$$I(\lambda) = I_{100}(\lambda) + 5.8 \cdot I_{40}(\lambda) \tag{1}$$

where $I(\lambda)$ is the spectrum of 2,6-TNS in the axon at rest (expressed in an arbitrary intensity scale), $I_{100}(\lambda)$ and $I_{40}(\lambda)$ are, respectively, the spectra of the probe molecules dissolved in 100 and 40% ethanol shown in Fig. 4, left. As can be seen in Fig. 2, left, the agreement between the observed spectrum and the one obtained by reconstruction is very good. Since the spectrum of 2,6-TNS in a dioxane–water mixture is not different from that in an ethanol–water mixture with the same solvent polarity, equally satisfactory agreement could be obtained by using two spectra in dioxane–water mixtures with the corresponding solvent polarities. By taking the relation expressed by Eqn 1 literally, it may be suggested that the major portion of binding sites for 2,6-TNS molecules in the axon are divided into two classes. One class of sites has a relatively low polarity with about 75 in Kosower's Z value[11] (corresponding to 100% ethanol) and the other class has a high polarity with about 90 in Z value (corresponding to 40% ethanol). The coefficients of the equation suggest that there

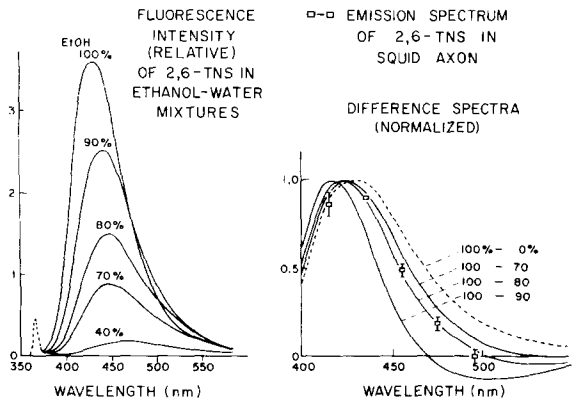

Fig. 4. Left: Corrected emission spectra of 2,6-TNS in ethanol–water mixtures. Right: Curves obtained by taking differences between the emission spectra shown on the left (normalized to unity at peak). The squares show the data taken from Fig. 2, left.

are nearly six times as many high-polarity sites as low-polarity sites in the axon.

We now turn to the spectrum of ΔI of 2,6-TNS in an axon at the peak of nervous activity (see Fig. 2, left). A close inspection of this spectrum indicates that its wavelength of emission maximum is around 420 nm and its long-wavelength portion terminates abruptly at about 485 nm. We note that almost no *in vitro* system produces such a sharp and narrow spectrum (see Appendix). Known examples of such a narrow spectrum are that of anhydrous crystals of 2,6-TNS (ref. 12) and that of those probe molecules incorporated in dried sheets of polyvinylalcohol; obviously such anhydrous forms of 2,6-TNS do not exist in the axon. The spectrum of a hydrated crystal is very broad and red-shifted.

It is possible to reconstruct this sharp and narrow spectrum by taking the difference between two spectra. It is seen on the right of Fig. 4 that the spectrum of $\Delta I(\lambda)$ at the peak of nervous activity agrees very well with the difference between the spectrum in pure ethanol and that in 80% ethanol:

$$\Delta I(\lambda) = I_{100}(\lambda) - I_{80}(\lambda) \tag{2}$$

It is not possible to obtain satisfactory agreement by substraction of two spectra which are very different from those chosen above. By comparing Eqn 2 with 1, we interpret these results as indicating that only the low-polarity binding sites for 2,6-TNS in the axon are involved in production of fluorescence signals (ΔI) during nerve excitation. The polarity of these sites are considered to change from about 80 in Z value to about 85 at the peak of nerve excitation. (Note that the Z value of pure ethanol is 79.6 and that of 80% ethanol is 84.8.) It is evident that such a process can give rise to a decrease in the fluorescent light with a spectrum similar to the observed one.

It is to be noted that the observed spectrum cannot be produced by a transient increase in the effect of some unknown quencher in the membrane during nervous activity. Typical quenchers decrease the quantum yield without shifting the wavelength of the emission maximum (see Appendix). Therefore, the transient fluorescence change caused by a quencher should have the same spectrum as that in the resting

state of the binding sites. Evidently, the observed spectrum is too sharp to be explained on the basis of such mechanisms. Similarly, a reduction in the number of binding sites would yield a broader spectrum than we observed. Note also that the observed sharp spectrum cannot be explained in terms of a fall in local viscosity. A large change in viscosity also alters the quantum yield of fluorescence without affecting the wavelength of maximum emission appreciably (see Appendix). Since the details of the molecular architecture of the axon membrane are presently unknown, we cannot exclude the possibility that some factors other than the proposed polarity change are involved in production of 2,6-TNS signals in the axon membrane. Nevertheless, we believe that a change in the polarity around the binding sites is at the moment the most plausible explanation of the experimental results shown in Fig. 2. The mechanism whereby such a change in polarity is brought about by a transition variation in the membrane potential is not clear at present.

The spectra of 2,6-ANS in the axon interior are shown on the right in Fig. 2. The spectrum in the resting state of the axon is broad and the spectrum of *AI* is narrow as in the case of 2,6-TNS. It is therefore possible to explain this spectrum in a manner similar to that for 2,6-TNS. In this case, however, the best fit is obtained by choosing the Z value of the low-polarity binding sites to be around 83. It seems possible that this slight difference in behavior of 2,6-ANS is due to a very small difference in the location of the binding sites created by the absence of the hydrophobic side-group, CH_3.

The spectral data obtained with 1,8-ANS inside the axon is shown in Fig. 3, left. The spectrum of this probe at the peak of nerve excitation is much broader than that of 2,6-derivatives. Since the spectra of 1,8-ANS in ethanol–water mixtures are much broader than those of 2,6-derivatives, the procedure used to analyze the data of 2,6-TNS (Fig. 4) did not yield a clear result. The difference in the properties between 1,8- and 2,6-derivatives of naphthalene has been studied previously by Baba and Suzuki[13] and also by Mataga[14].

Finally, the spectra of the fluorescent probes applied to the external surface of axons (Fig. 3, right) are discussed briefly. Under these circumstances, the spectra at the peak of nervous activity are broad and very similar to those observed in the resting state of the axons. Therefore, the procedure used to analyze the spectra of 2,6-derivatives is not applicable to this case. Since the intensity of the fluorescent light of these probes bound to macromolecules are affected by a large number of factors, such as changes in the binding constant or in the rigidity, *etc.*, it is at present very difficult to offer an unambiguous explanation of these spectra (Fig. 3).

APPENDIX: PHYSICOCHEMICAL PROPERTIES OF 2,6-TNS FLUORESCENCE

In this Appendix we describe properties of 2,6-TNS fluorescence in various solvents and macromolecules. It is well known that a change in the solvent polarity affects both the wavelength of maximum emission and the half band-widths of 2,6-TNS fluorescence in a characteristic manner. To express the solvent polarity quantitatively, Turner and Brand[10] used an empirical scale called the Z value proposed by Kowoser[11]. The emission spectrum of 2,6-TNS dissolved in a dioxane–water mixture is almost identical to that in an ethanol–water mixture with the same Z value. The red shift of the spectrum with increasing solvent polarity has been

attributed to an increase in the dipole moment of the probe molecules in the lowest excited singlet state (McClure and Edelman[8]; Seliskar and Brand[15]; Kosower and Tanizawa[16]). Polar solvent molecules interact more strongly with the probe molecules in the excited state than in the ground state, thus lowering the energy difference between the two electronic states of the probe molecules. Quite recently, Penzer[17] questioned the validity of this interpretation applied to 1,8-ANS. The investigations described below were conducted in an effort to gain a better understanding of the effect of the solvent polarity on 2,6-TNS fluorescence emission.

According to the theory advanced by Lippert[18] and by Mataga *et al.*[19], the effect of solvent polarity on the wavenumber of emission and absorption is described by

$$hc(\nu_a - \nu_e) = 2f(\varepsilon,n)\cdot(\Delta\mu)^2/a^3 + \text{constant} \tag{3}$$

In this equation, h represents Planck's constant, c the light velocity, ν_a and ν_e the wavenumbers (in cm^{-1}) of the peaks of the absorption and emission band, respectively, $\Delta\mu$ the difference between the dipole moment in the excited state and that in the ground state, a Onsager's cavity radius, and $f(\varepsilon,n)$ a function of the dielectric constant, ε, and the refractive index, n, of the solvent defined by

$$f(\varepsilon,n) = \frac{\varepsilon-1}{2\varepsilon+1} - \frac{n^2-1}{2n^2+1} \tag{4}$$

Fig. 5, left, shows the result of our measurements of the Stokes shift, $(\nu_a - \nu_e)$, as a function of the value of $f(\varepsilon,n)$. To vary the value of $f(\varepsilon,n)$ of the solvent, we used mainly the series of solvents used by Mataga in his study of α- and β-naphthylamines[14]. It is seen in the figure that there is actually an approximately linear relationship between the Stokes shift $(\nu_a - \nu_e)$ and the $f(\varepsilon,n)$ value of the solvent. If one assumes,

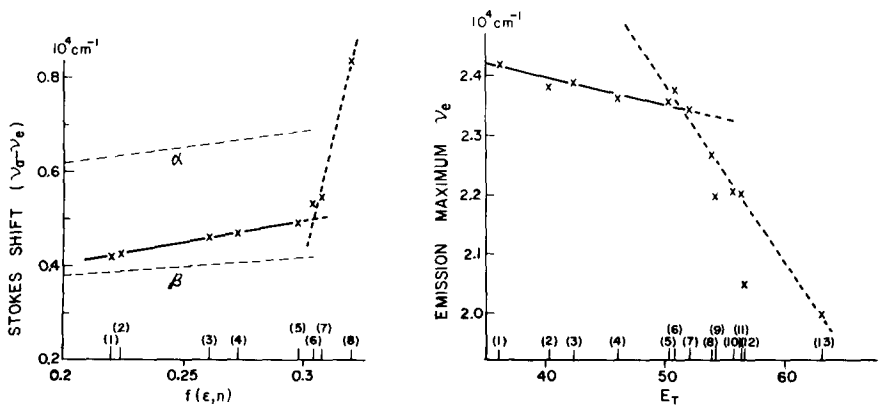

Fig. 5. Left: Stokes shifts of 2,6-TNS dissolved in various solvents plotted against the values of $f(\varepsilon,n)$ of the solvents defined by Eqn 3 in text. The solvents used are: (1) methyl acetate, (2) n-octanol, (3) n-butanol, (4) n-propanol, (5) ethanol, (6) acetonitrile, (7) methanol, and (8) water. The broken lines marked α and β indicate the slopes observed with α-and β-naphthylamine, respectively, confirming Mataga's results[14]. Right: Relationship between the wavenumber of emission maximum and the solvent polarity, E_T(30). The solvents used are (1) dioxane, (2) pyridine, (3) acetone, (4) acetonitrile, (5) n-butanol, (6) n-propanol, (7) ethanol, (8) diethylene glycol, (9) N-methyl formamide, (10) methanol, (11) ethylene glycol, (12) formamide, and (13) water.

after Seliskar and Brand[15], a to be 5Å, $\Delta\mu$ is found to be roughly 11.2 D; this is in good agreement with the value suggested by McClure and Edelman[8]. The value for 1,8-ANS was found to be about 11.4 D. However, in the range of $f(\varepsilon,n)$ greater than 0.3, a large deviation from linearity is noted.

The value of $\Delta\mu$ obtained by Seliskar and Brand[15] for 2,6-TNS is 44 D. Since they examined the Stokes shift only in the range larger than 0.3 in $f(\varepsilon,n)$, this difference may reflect the existence of two kinds of excited states of the 2,6-TNS molecule. Kosower and Tanizawa[16] have shown the existence of the two states by examining the relationship between ν_e and the solvent polarity expressed in the scale known as $E_T(30)$ (see Reichardt and Dimroth[20]) using a series of mixtures of dioxane and water to vary the solvent polarity. Fig. 4B shows the result of our measurement of the ν_e–$E_T(30)$ relationship using a series of organic solvents. (To avoid the solvent sorting effect in mixed solvents[21], only pure solvents were used in this experiment.) It is clear that most of the observed points do lie in two distinct straight lines. This finding is consistent with the view that a different, or a second state of the excited molecules appears in solvents with high polarity. A sharp break in the Stokes shift–$E_T(30)$ relationship was encountered not only with 2,6-TNS but also with naphthylamines, 1,8- and 2,6-aminonaphthalene sulfonate and *N*-methyl-2,6-ANS.

The second state of the excited 2,6-TNS molecules is extremely sensitive to the solvent polarity. Addition of various molecules with large dipole moments to an ethanol (or dioxane) solution of 2,6-TNS produces a distinct red shift in the emission spectrum. Formamide, acetamide, phenol, ^2H_2O are examples of chemicals which exhibit a polarity effect on the 2,6-TNS fluorescence.

The solvent polarity is known to affect not only the wavelength of maximum emission but also the half band-width of the emission spectrum[8,10]. In the range of wavelengths of maximum emission between 420 and 480 nm, the band-width increases monotonically with the wavelength. The intensity of fluorescence is also affected by the solvent polarity. A high solvent polarity tends to reduce the quantum yield of fluorescence and the lifetime of the excited molecules by facilitating transition from the excited singlet state to the triplet state. However, there is in general no simple relationship between the quantum yield and the Z value of $f(\varepsilon,n)$, because the quantum yield is affected by factors other than the solvent polarity (see below).

A group of compounds known as dynamic quenchers (see *e.g.* Radda and Vanderkooi[3]) can affect the quantum yield of fluorescence without changing the wavelength of maximum emission. Within a certain range of concentration, the following compounds were found to lower the quantum yield and shorten the lifetime of the excited state of 2,6-TNS without producing any detectable change in peak wavelength and band-width: pyrimidine, pyridine, dipropylamine, I^-, *etc.* Fig. 6 shows an example of the results obtained with pyrimidine affecting the fluorescence of this probe dissolved in ethanol.

Dynamic quencher molecules are considered to inactivate fluorescent molecules by collision during their excited state. The rate of loss of probe molecules in the excited state is determined by the following three processes: (i) emission of fluorescent light, (ii) non-radiative inactivation without involving the quencher, and (iii) collision with quencher molecules. We denote the rate constants of these three processes respectively by k_1, k_2, and $k_3 \cdot [Q]$, where [Q] represents the concentration of the quencher. In this case both the ratio of the fluorescence intensities in the

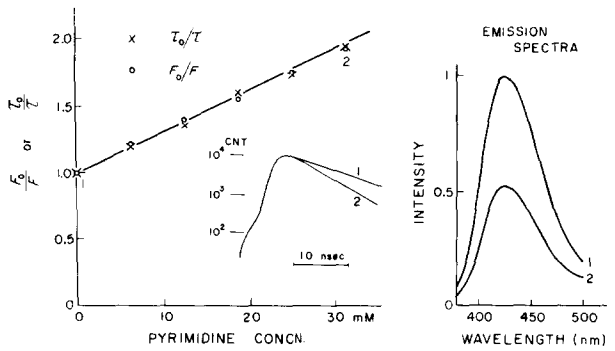

Fig. 6. Quenching of fluorescence of 2,6-TNS dissolved in ethanol by addition of pyrimidine. F_0 and τ_0 represent the fluorescence intensity and the excited state lifetime, respectively, observed in the absence of the quencher; the emission spectrum and the record of single photon counting for this case are marked "1". F and τ are the corresponding values observed in the presence of the quencher. The spectrum and the record of single photon counting marked "2" were taken at a pyrimidine concentration of 32 mM.

absence and presence of the quencher, F_0/F, and the corresponding ratio of the lifetimes, τ_0/τ, are expected to satisfy the relationship known as the Stern–Volmer equation[22]:

$$\frac{F_0}{F} = \frac{\tau_0}{\tau} = 1 + \frac{k_3}{k_1 + k_2} \cdot [Q] \tag{5}$$

The experimental results obtained indicate that, with a given quencher dissolved in a given solvent, both F_0/F and τ_0/τ in fact vary linearly with [Q] in the manner described by Eqn 5. Similar results were obtained by using pyridine or dipropylamine. However, with these quenchers, the concentration required to reduce the fluorescence intensity by 50% was nearly ten times as high as that for pyrimidine, indicating that the rate-constant k_3 depends on the nature of the quencher. It has also been shown that this constant decreases, as expected, with an increase in the solvent viscosity (see also Lakowicz and Weber[23]).

There is another distinct type of process that can affect the intensity of fluorescence. In a mixture of 2,6-TNS and some macromolecules, a factor that alters the degree of 2,6-TNS binding to the macromolecule can affect the fluorescence intensity without influencing either the wavelength of maximum emission or the lifetime of the excited state. An example of this type of process is shown in Fig. 7, in which the effect of $CaCl_2$ on the 2,6-TNS fluorescence in an aqueous solution of lysolecithin was examined; this system was studied previously by Vanderkooi and Martonosi[24] in a different manner.

In such a system, equilibrium between the dye molecule D and the macromolecule (or lysolecithin in this case) M may be described by

$$[D][M]/[DM] = K \tag{6}$$

where [D] and [M] are the concentrations of the free dye molecules and that of free macromolecules, respectively, [DM] is that of 1:1 complex of D and M, and K is an equilibrium constant. The total (or initial) concentration of the dye and

macromolecule are denoted by d and m, respectively. Under the condition of the experiment shown in Fig. 7, the concentration of lysolecithin is far greater than that of 2,6-TNS; namely, $m \gg d, >$ [DM]. Since the observed intensity of fluorescence, F, is proportional to the concentration [DM], we have $F = k$ [DM] where k is a constant which may depend on the property of the binding sites. Under these conditions, Eqn 6 can be rewritten in the following form:

$$\frac{1}{F} = \frac{1}{kd} + \frac{K}{kd}\frac{1}{m} \tag{7}$$

Thus, a plot of $1/F$ against $1/m$ is expected to yield a straight line.

The data furnished in Fig. 7 shows this is actually the case. When the value of m was fixed, F was proportional to d over a wide range of d, as expected from Eqn 7. The value of K can be determined from the intercept of the observed straight line with the abscissa. It is seen in the figure that addition of $CaCl_2$ had affected the equilibrium constant K (by a factor of about 3) without significantly influencing the proportionality constant K. It is important to note that over the entire range of concentrations of $CaCl_2$ and m examined, both the lifetime of the excited state (5.3 ns at 20 °C) and the wavelength of maximum emission (444 nm) remained constant in spite of the large change in the fluorescence intensity.

Addition of $CaCl_2$ to erythrocyte ghost membrane labeled with aminonaphthalene derivatives is known to increase the fluorescence intensity[25,26]. Again, we found that both the fluorescence lifetime (9.9 ns at 20 °C) and the peak wavelength (426 nm) remained unaltered when the intensity fluorescence of 2,6-TNS was increased by addition of $CaCl_2$.

Based on the experimental facts described above, the effects produced by

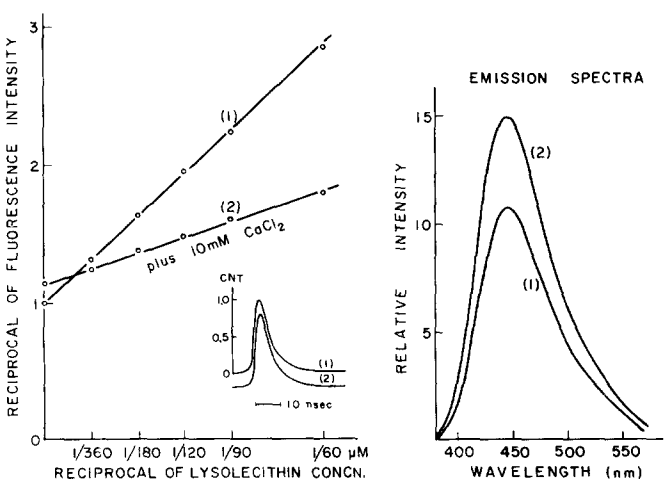

Fig. 7. Addition of lysolecithin to a 5-μM aqueous solution of 2,6-TNS in the presence and absence of 10 mM $CaCl_2$. The emission spectra and the records of single photon counting (marked "1" and "2") were taken at a lysolecithin concentration of 90 μM. The two records of single photon counting are normalized and displaced from each other. Note that the wavelength of maximum emission, the band-width of spectrum and the excited state lifetime are practically independent of the lysolecithin and $CaCl_2$ concentrations.

various chemicals affecting the fluorescence intensity of 2,6-TNS may be divided into three of the following categories. (A) The solvent polarity effect which affects all three of the parameters of fluorescence, the peak wavelength, the lifetime and the intensity; (B) the dynamic quenching effect which decreases the intensity and the lifetime but does not affect the peak wavelength; and (C) the static effect which changes the fluorescence intensity without affecting the lifetime or the peak wavelength.

However, the effects of some factors on the fluorescence intensity do not automatically come under one of these three categories. An example of such a factor is temperature. We investigated the cooling of 2,6-TNS dissolved in glycerol, ethylene glycol, propylene glycol, or in aqueous solution of sucrose (8%). Cooling is known to enhance the intensity of fluorescence of these solutions significantly[8,15]. Lowering of the temperature of 2,6-TNS–glycerol solution from 20 to 8 °C was found to increase the fluorescence intensity by a factor of about 3. Associated with this fluorescence enhancement, there was a small blue shift of the wavelength of maximum emission of about 4 nm. The results of time-resolved spectral analyses by Brand and Gohlke[27], confirmed in this laboratory, indicates that this blue shift is related to the time-dependent aspect of the value of $f(\varepsilon,n)$ in Eqn 3. (Note that the dielectric constant of a medium, ε, varies with the frequency of the high-frequency a.c. used for measurement.) During the lifetime of the excited state, the difference between ε and n^2 increases gradually, because the reorientation of polar solvent molecules in the electric field takes place slowly. For this reason, the influence of viscosity[9] is not treated here as an independent factor.

The fluorescence of an ethanol (or dioxane) solution of 2,6-TNS is reduced in intensity and red-shifted in spectrum by addition of heavy water. Replacement of H_2O for this ^2H_2O brings about an additional reduction in the fluorescence intensity. This replacement, however, does not produce any change in the wavelength of maximum emission (see Stryer[1]). In the present terminology, therefore, the solvent polarity effect of ^2H_2O is not different from that of H_2O. Note that there is almost no difference in the dielectric constant or in the refractive index between ^2H_2O and H_2O. The physicochemical nature of the large difference in dynamic quenching effect between these two isotopes is not well understood at present. Nevertheless, the increase in fluorescence intensity produced by replacement of ^2H_2O for H_2O has been used to determine the accessibility of water molecules to the binding sites of various macromolecules[3]. The 2,6-TNS binding sites in most macromolecules examined are accessible to water molecules.

The emission spectra and the quantum yield of 2,6-TNS bound to proteins and phospholipids have been investigated by previous investigators[8,10]. In order to facilitate a comparison of the emission characteristics of 2,6-TNS in a number of commercially available macromolecules with the squid data described under Results, the peak wavelength (in nm), the band-widths (expressed in ratio of the intensity at 480 nm to the maximum intensity) and the lifetime (in ns) are listed below. The following proteins were dissolved in potassium phosphate buffer (at pH 7.3) at concentrations between 0.2 and 20 mg/ml and mixed with 2,6-TNS of 2–10 μM: (1) albumin (bovine serum), 435 nm, 0.24, 10.9 ns; (2) ovalbumin, 420, 0.39, 10.1; (3) albumin (sheep serum), 430, 0.36, 8.9; (4) α-casein, 428, 0.35, 10.9; (5) chymotrypsin, 450, 0.36, 10.1; (6) globulin (cucurbit seed), 430, 0.23, 10.9; (7) β-lactoglobulin, 428, 0.25, 10.7; (8) glycoprotein (bovine), 418, 0.25, 11.6; (9) trypsin, 442,

0.50, 9.7. In the following cases histidine buffer (pH 7.2) was used: (10) alcohol dehydrogenase, 440, 0.56, 10.1; (11) hexokinase, 438, 0.48, 11.9.

Similar measurements were made on the following lipids and lipoproteins labeled with 2,6-TNS: (1) β-lipoprotein, 5 μM in potassium phosphate buffer (pH 7.2), 433 nm, 0.39, 10.8 ns; (2) lysolecithin, 0.05 mg/ml in Tris buffer (pH 7), 444, 0.54, 5.3; (3) bovine serum albumin *plus* cholesterol (each 30 μM) in potassium phosphate buffer (pH 7.3), 432, 0.43, 11.1; (4) ovalbumin *plus* cholesterol (each 30 μM) in potassium phosphate buffer (pH 7.3), 425, 0.43, 7.6; (5) erythrocyte ghost membrane in Tris buffer (pH 7.4), 426, 0.34, 9.9; (6) lobster nerve in artificial sea water (pH 8.1), 425, 0.37, 8.8.

These measurements indicate that the emission spectrum of 2,6-TNS in the squid axon at rest is not very different from the spectra of the probe molecules bound to various macromolecules. The spectra listed above may be compared also with the spectrum (ΔI) at the peak of nerve excitation. It is noted that the ratio of the light intensity at 480 nm to that at emission maximum varies in these macromolecules in the range between 0.23 and 0.54. The corresponding ratio for spectrum ΔI is estimated to be about 0.15. The interpretation of ΔI described under Discussion is based on this comparison.

The lifetime of the excited state of 2,6-TNS bound to the axon membrane is excepted to change during the process of nerve excitation. However, it is not possible at present to demonstrate such a change occurring during a period of time of the order of 10^{-3} s.

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