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# Changes in ANS and TNS Fluorescence in Giant Axons from *Loligo*

H. V. Davila*, L. B. Cohen, B. M. Salzberg and B. B. Shrivastav**

Department of Physiology, Yale University School of Medicine, New Haven, Connecticut and Marine Biological Laboratory, Woods Hole, Massachusetts

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*Summary.* Changes in the fluorescence of two N-arylaminonapthalenesulfonate dyes, ANS and TNS, were measured during the action potential and during voltage clamp steps in giant axons from the squid. We hoped to obtain information about alterations in membrane structure that occur during activity.

The fluorescence changes of both dyes appeared to be closely related to membrane potential. ANS fluorescence changes were measured with the dye added to both sides of the membrane. The changes were opposite in sign and of comparable magnitude. Neither was related to membrane conductance. Using axons microinjected with TNS and illuminated with polarized quasi-monochromatic light, we were able to separate two polarization-dependent components of a fluorescence change. However, both of these were also potential-related and, therefore, gave no information about the structural basis of the conductance mechanisms. Previous suggestions that the fluorescence of these dyes is correlated with membrane conductance were therefore not confirmed. Possible explanations for this disparity are considered.

Changes in extrinsic fluorescence that occur during the action potential (Tasaki, Carnay & Watanabe, 1969) might provide new information about the changes in axon structure that occur during activity. In analyzing the fluorescence changes, we have found it helpful to divide the electrically measurable events of the action potential into three classes, the changes in membrane potential, the ionic currents, and the transient increases in membrane conductance, and have attempted to determine which of the electrical events is most directly responsible for a given optical change. Since the previously described changes in axon birefringence and light scattering depended either on membrane potential or ionic current (Cohen, Hille, Keynes, Landowne & Rojas, 1971; Cohen, Keynes & Landowne, 1972*b*),

* Fellow of the Venezuelan Institute for Scientific Research (I.V.I.C). *Present address:* Dept. de Fisiologia, Facultad de Medicina, Universidad Los Andes, Merida, Venezuela.
** *Present address:* Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina.

we were particularly interested in the reports that several fluorescence changes were not potential dependent (Tasaki *et al.*, 1969; Watanabe, Tasaki & Hallett, 1970; Conti, Tasaki & Wanke, 1971; Tasaki, Watanabe & Hallett, 1972). In particular, it was claimed that the changes in fluorescence of ANS (1-anilino-8-naphthalene sulfonate) and TNS (2-*p*-toluidinyl-6-naphthalene sulfonate) were related to changes in membrane conductance. In this paper we present the results of experiments with ANS and TNS. Some of our results were qualitatively different from those obtained by Tasaki and collaborators. The reader is also referred to several excellent reviews of the application of fluorescent probes to the study of biological membranes (e.g., Radda, 1971; Weber, 1972).

Preliminary reports of these experiments have been published (Cohen, Landowne, Shrivastav & Ritchie, 1970; Davila, Cohen & Waggoner, 1972; Davila, Salzberg, Cohen & Waggoner, 1972; Cohen, 1973).

## Materials and Methods

Giant axons with diameters of 370 μm to 620 μm were dissected from the hindmost stellar nerves of *Loligo pealii*. The small fibers were completely removed from a 20-mm length of axon. In most experiments the axons were dissected from freshly killed squid, but occasionally the mantles had been stored for several hours in cold seawater (Caldwell, Hodgkin, Keynes & Shaw, 1960). Axons from these mantles gave results similar to the results from freshly killed squid.

A schematic diagram of the experimental apparatus used for axon fluorescence measurements is shown in Fig. 1. The light source was a Hanovia, 901 B11, mercury-

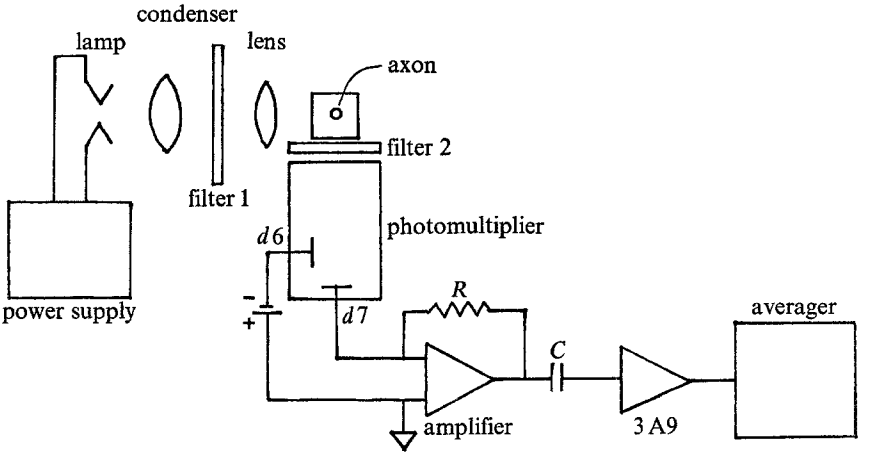


Fig. 1. Schematic drawing of apparatus used to measure fluorescence changes in axons. By using a high quality constant current power supply, acceptable signal-to-noise ratios were achieved without beam splitting and differential amplification. The 9658A photomultiplier has an internally corrugated end window to improve the quantum efficiency of the cathode. The amplifier used for current-to-voltage conversion was battery powered

xenon arc lamp in a Schoeffel LH 150 lamp housing. The lamp was powered by a KEPCO JQE 75–15 (M)HS power supply (KEPCO, Inc., Flushing, N.Y.) operated in the constant current mode according to the manufacturer's instructions. In our experience, fluctuations in the output of the lamp were inversely related to the quality of the constant current supply. With the KEPCO supply the peak-to-peak noise (0.5–1,000 Hz) during a 20-msec sweep was spectrally uniform and less than $4 \times 10^{-4}$ of the total light, so that acceptable signal-to-noise ratios were achieved without recourse to the split beam technique used by Tasaki *et al.* (1972). The output of the lamp was focused onto a 4-mm length of axon by the condenser and a second quartz lens. Between the two lenses were two heat filters (G-776–7100, Oriel Optics Corp., Stamford, Conn.) and an interference filter (filter 1, peak transmission at 375 nm, bandwidth of 25 nm at half height, Oriel Optics Corp.) that transmitted only light of the wavelengths absorbed by ANS and TNS. For the TNS experiments a polarizer (HNP'B, Polaroid Corp., Cambridge, Mass.) was inserted between the lens and the chamber. The axon was held vertically in a temperature controlled chamber made from a 1-cm path length quartz cuvette. The solution surrounding the axon was filtered seawater or seawater plus dye, and was bubbled with nitrogen before it was introduced into the chamber. The secondary filter (filter 2, GG420, Schott Optical Glass, Inc., Duryea, Pa.) allowed only the longer wavelength-emitted light to pass to the photodetector, an EMI 9658A photomultiplier tube used as a six-stage multiplier. The current in the last stage (dynodes 6 and 7) was converted to a voltage signal using a Burr Brown, 3420L low-noise amplifier. The ratio of voltage to current was adjusted by changing the resistance $R$ (usually set at 100 KΩ). The coupling capacitor $C$ blocked the D-C and low-frequency components of the optical signal; to measure the resting intensity $I_r$ the capacitor was removed from the circuit. The coupling time constant was about ten times the sweep duration. The high frequency response time constant was limited to the values given in the figure legends by using the high frequency cut off on a Tektronix 3A9 amplifier. The response time constant was measured using a Monsanto MV-50 light-emitting diode to provide a step change in light intensity. The output of the 3A9 amplifier was fed into one of two signal averagers, a Data Laboratories Ltd., Biomac 1000, or, when the Biomac was not functioning, a Princeton Waveform Eductor (type TDH-9). Waveform eductors were very kindly lent to us by Drs. I. Tasaki and R. Byck. The averaged signals were recorded on an X-Y potentiometric recorder. The apparatus, from lamp housing to photomultiplier, was bolted to the Benelex top of a vibration isolation system having a 1.1 Hz resonant frequency (Lansing Research Corp., Ithaca, N.Y.).

For the experiments with Capri blue the apparatus shown in Fig. 1 was modified somewhat. A 12 V, 100 W tungsten-halogen lamp was used in place of the mercury-xenon lamp; a SGD 444 (E.G. & G. Inc., Salem, Mass.) silicon photodiode was used in place of the photomultiplier tube; filter 1 had a peak transmission at 600 nm; and filter 2 (Schott, RG 645) passed light of wavelengths longer than 645 nm. The improved signal-to-noise ratios in the Capri blue experiments mainly resulted from the greater stability provided by the tungsten filament lamp and the higher quantum efficiency of the silicon photodiode. Capri blue was used for the experiments in Figs. 8 and 9 because of its larger signal-to-noise ratio and the absence of photodynamic damage made it easier to do bracketed measurements.

Conti and Tasaki (1970) have presented substantial evidence that axon fluorescence measurements are not seriously contaminated by artifacts from light-scattering changes. It is clear that the fluorescence changes presented below have a time course that is qualitatively different from the light-scattering changes (Cohen *et al.*, 1972*a*, *b*) measured under similar conditions. No intensity changes could be detected before the dye was added; when the barrier filter (filter 2, Fig. 1) was omitted, large light-scattering signals

were obtained. It thus seems extremely unlikely that light-scattering phenomena were a source of difficulty. In the experiments reported here, no precautions were taken to prevent light from dye bound to the electrode from reaching the photodetector. However, in one experiment with another dye, rhodamine B, fluorescence changes were measured before and after blocking the light from the electrode (*see* Cohen *et al.*, 1972*a*, for method). The changes were not significantly altered by blocking the light from the electrode.

The electrodes and amplifiers used for voltage clamp experiments were the same as previously described (Cohen *et al.*, 1972*a*). Compensation for the resistance in series with the axon membrane $r_s$ (Hodgkin, Huxley & Katz, 1952), was used except where noted. Complete compensation was considered to be approached at the setting of the potentiometer (*p*, in Fig. 6, Hodgkin *et al.*, 1952) which caused the current to oscillate during the entire sweep. The potentiometer was immediately turned back 10%. When $r_s$ was estimated from the potentiometer setting for 100% compensation, its values were within the range of those reported by Hodgkin *et al.* (1952). Microinjection of solutions into axons was carried out using the technique of Hodgkin and Keynes (Keynes, 1963). About 0.35 μliter of solution was injected along a 2-cm length of axon. The internal dye concentration was estimated from the volume injected and the volume of the 2-cm length of axon calculated from a measurement of the diameter. The TNS microinjection solutions were made up as follows. Forty mg of TNS were slurried with 0.4 ml of acetone and heated to 60 °C with 380 mg Pluronic F-127, a nonionic surfactant polyol which, at this concentration, is not biologically active. Then a solution of 4% glycerol (by volume) in 0.4 M KCl was added to give a final concentration of 1.0 mg/ml TNS, 1% Pluronic F-127 (by weight), and 4% glycerol (by volume). The TNS, KCl, and glycerol concentrations were the same as those used by Tasaki *et al.* (1972). The ANS (K & K Laboratories, Inc.) was purified by recrystallization as the magnesium salt. The TNS (Sigma Chemical Co., St. Louis), and Capri blue (Chroma-Gesellschaft Schmid & Co., Stuttgart) were used without further purification. The tetrodotoxin was obtained from the Sankyo Co., Ltd., Tokyo. The chamber temperature was held constant at temperatures between 9 and 13 °C.

## Results

### *Changes in ANS Fluorescence*

*ANS Added Outside.* A simple experiment which provides an important clue about the physiological origin of any optical change is to measure the change during the action potential and carefully compare its time course with that of the membrane potential. Such an experiment is illustrated in Fig. 2 where the fluorescence intensity is the heavy, noisy trace and the membrane action potential is shown as the dotted trace. There clearly is an increase in ANS fluorescence at the time of the action potential. However, to reduce noise, the high frequency response of the light-measuring system was limited to a time constant of 170 μsec; therefore, the intensity change and potential measurements are not directly comparable. When the potential measurement was slowed equivalently, or "lagged", with the time constant of 170 μsec, the result shown by the solid thin line was obtained. The thin line and the intensity trace had essentially identical time courses, and thus,

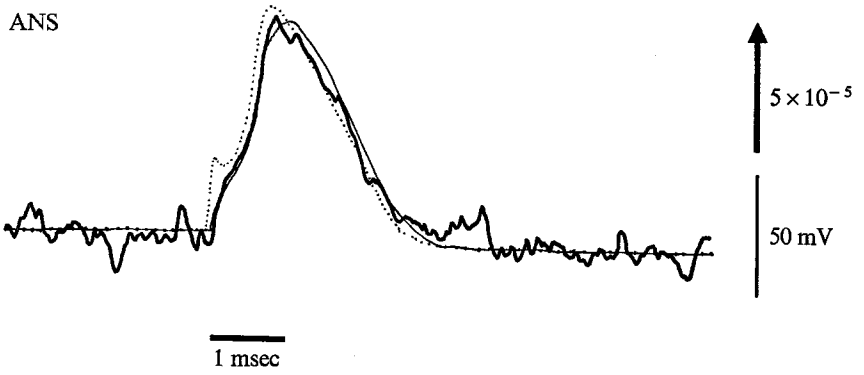


Fig. 2. Change in ANS fluorescence (heavy, noisy trace) during a membrane action potential (dotted line). When the action potential was lagged with a time constant of 170 μsec (thin line) the lagged action potential and the fluorescence increase had identical time courses. In this and subsequent figures, the direction of the vertical arrow to the right of the tracings indicates the direction of an increase in intensity, and the size of the arrow represents the stated value of the change in intensity $\Delta I$ divided by the resting intensity $I_r$ for a single sweep. All records are traced from the originals. The response time constant of the light-measuring apparatus to a step change in light intensity was 170 μsec. The ANS concentration in the external seawater was 64 μM; 4,000 sweeps were averaged

when both light intensity and potential changes were measured with the same bandwidth, they occurred at the same time.

The similarity in time course suggested that the ANS fluorescence change was probably not related to the ionic currents or membrane conductance increases because these (Cole & Curtis, 1939; Hodgkin & Huxley, 1952) have time courses different from that of the potential. Thus, this simple experiment indicated that the ANS fluorescence change was dependent on membrane potential. But this argument can not be very strong because the time course of the conductance increase is not very different from that of the potential, and noise in the optical records might obscure a relationship between the intensity change and membrane conductance. This difficulty is surmounted by measuring fluorescence during voltage clamp steps, and the results of such an experiment are illustrated in Fig. 3. During the hyperpolarizing step, on the left, the ionic currents were very small and there were no obvious conductance changes; only the membrane potential changed. There was a fluorescence decrease during the hyperpolarizing step and it had a time course similar to the potential, so this fluorescence change depended on membrane potential, and not on ionic current or membrane conductance. During the depolarizing step there were large currents and conductance increases, but the fluorescence change during the depolarizing step had the size and time course that would have been

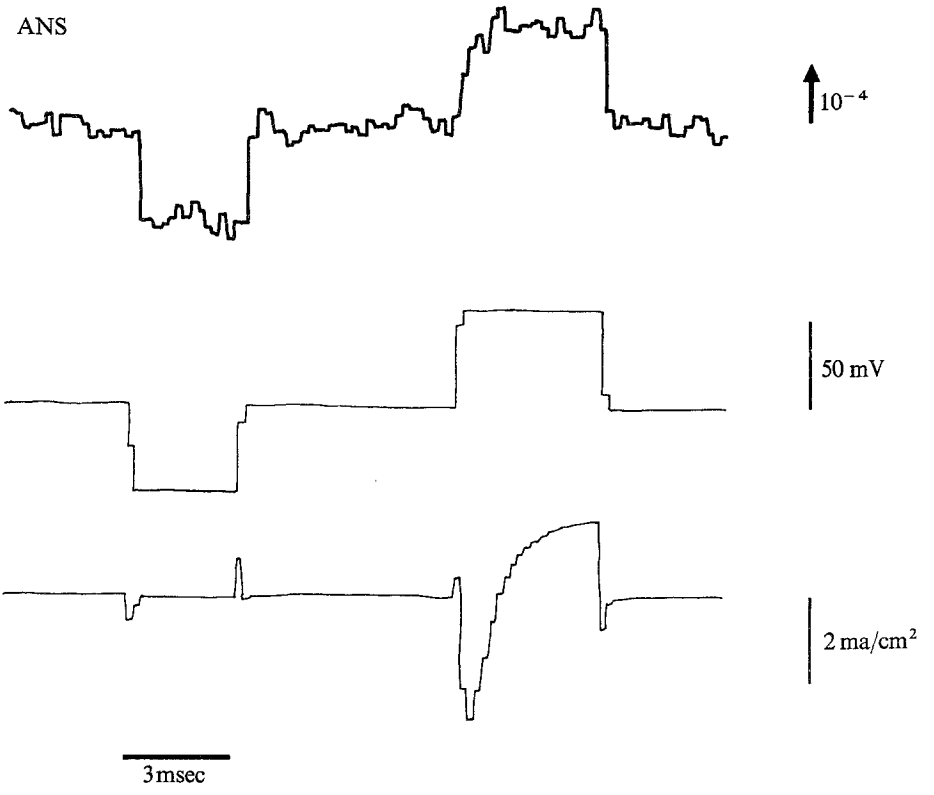


Fig. 3. Changes in ANS fluorescence (top trace) during hyperpolarizing (on the left) and depolarizing (on the right) potential steps (middle trace). The fluorescence changes had a time course similar to the potential during both steps. The bottom trace is current density. In this and subsequent figures the holding potential was the resting potential, hyperpolarizing is downward, inward current is downward. The ANS concentration in the external seawater was 32 μM. The time constant of the light-measuring apparatus was 60 μsec; 2,000 sweeps were averaged

predicted from the decrease during the hyperpolarizing step, if one assumed that the ANS fluorescence was linearly dependent on membrane potential.

The exact dependence of the ANS fluorescence change on potential was determined in experiments using a sweep with four different potential steps. The results of such an experiment are shown in the solid squares in Fig. 4. The experimental points did fall near a straight line indicating that the ANS fluorescence changes depended linearly on potential. The circles in Fig. 4 represent the results from another axon. The open circle is the result obtained during the 100-mV action potential shown in Fig. 2 while the filled circles are from subsequent voltage clamp steps. Because the axons deteriorated during these measurements, the physiological condition of the axon during the voltage clamp measurements was not the same as that during

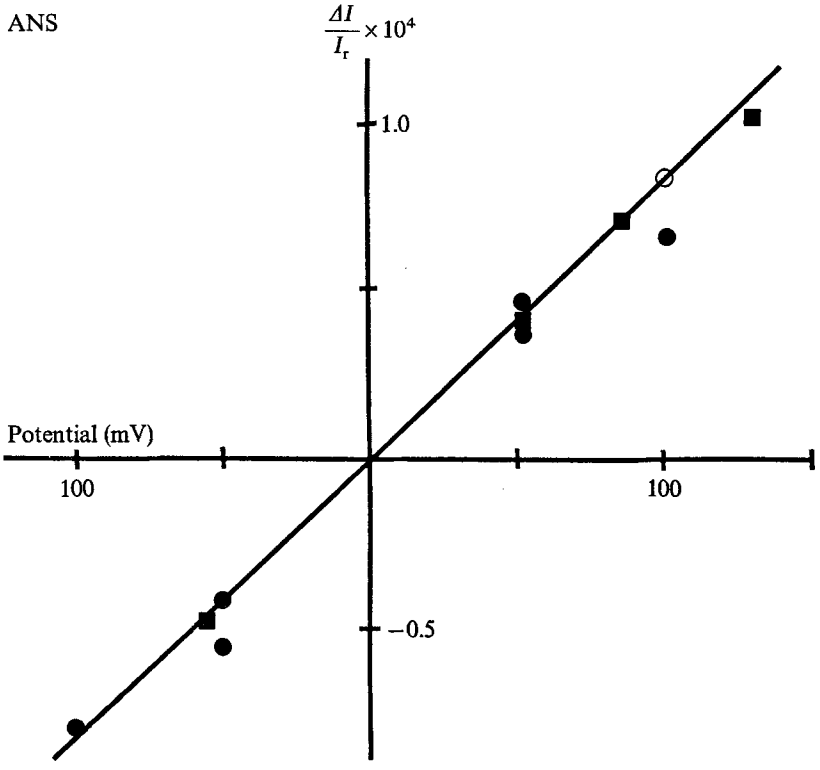


Fig. 4. The size of ANS fluorescence changes plotted *vs.* potential for ANS added to the outside of the axon membrane. The experimental points fell near a straight line. The origin on the abscissa is the resting potential (about −60 mV, inside negative). The results from two axons are shown. The measurements indicated by the filled squares were obtained from a sweep with four steps; the peak inward current for the 50-mV depolarizing step was 1.3 mamp/cm$^2$. The measurements indicated by circles are from the same axon as Fig. 2 with the filled circles the results of subsequent voltage clamp measurements. The axon had deteriorated considerably by the time the voltage clamp measurements were made. The ANS concentration was 320 μM for ▪ and 64 μM for ○ and •

the action potential measurement, but the results show that a 100-mV action potential and a 100-mV depolarizing step gave rise to fluorescence changes of the same size.

Altogether the potential dependence of the ANS fluorescence change was measured in six axons. In five, results similar to those in Fig. 4 were obtained, but in one, the experimental points were somewhat better fit by two straight lines with differing slopes meeting at the origin. However, even in this case the fluorescence change during the depolarizing step had a time course similar to the potential, so there seems little doubt that the fluorescence changes with externally added ANS were potential dependent.

To compare the results from different axons, we calculated the relative intensity changes, the change in intensity divided by the resting intensity

in a single sweep. The relative intensity change for 50-mV hyperpolarizing potential steps averaged $-9.1 \pm 1.8 \times 10^{-5}$ (standard error of the mean) when the measurement was made with the axon incubated in 320 μM ANS. When the ANS was washed out the relative intensity change was about five times smaller.

*ANS Added Inside.* When, as above, the ANS was added to the outside of the axon membrane, depolarizations always (32 axons) led to an increase in fluorescence. However, when ANS was microinjected into the axoplasm, depolarizations always (11 axons) led to a decrease in fluorescence. Although the sign of the fluorescence change was reversed when the ANS was added to the inside of the axon, further experiments led to results similar to those in Fig. 3 and similar to those in Fig. 3 of Conti and Tasaki (1970) indicating that these changes were also potential dependent. The size of the relative intensity changes for ANS added to the inside of the axon was similar to the changes with ANS added to the outside. In one experiment with ANS, 100 nM tetrodotoxin was added to the external seawater. The addition of tetrodotoxin had no effect on the fluorescence change. Thus we conclude that most, if not all, of the ANS fluorescence changes we measured were potential dependent, and were not related to either membrane current or conductance.

### *Changes in TNS Fluorescence*

The experiments with ANS were difficult because of rapid axon deterioration and because of fluctuations in the output of the arc lamp needed to provide light of the appropriate wavelength to excite the dye molecules. With TNS these difficulties were compounded by the smaller size of the fluorescence change and by its lability. Often, with the incident light polarized parallel to the axon's axis, only the first trial resulted in a measurable signal; as the axon deteriorated this signal disappeared. Because of the smaller size of the TNS signals, the high frequency response of the light measuring system was further limited to achieve acceptable signal-to-noise levels.

Since the solubility of TNS in normal microinjection solutions was quite low, in our experiments the TNS microinjection solution contained Pluronic F-127, a nonionic detergent (*see* Materials and Methods). To show that TNS introduced in this way was binding to the axon membrane similarly to TNS introduced as microcrystals (Tasaki *et al.*, 1972), a polarizer was introduced between the light source and the axon so that the incident light could be polarized either parallel or perpendicular to the axon's axis. Tasaki *et al.* (1972) had shown that the fluorescence changes would be opposite in sign for the two orientations of the polarizer, and Fig. 5 shows

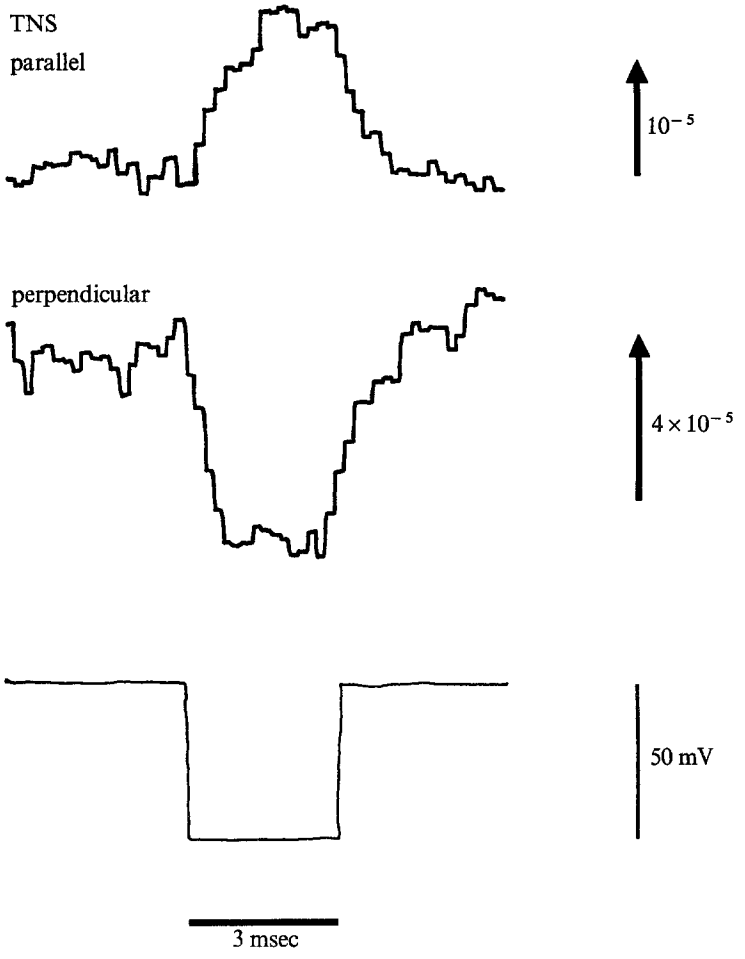


Fig. 5. TNS fluorescence changes during a hyperpolarizing potential step (bottom trace) with the incident light polarized parallel to the axon's axis (top trace) or perpendicular to the axon's axis (middle trace). The fluorescence changes were in opposite directions for the two positions of the polarizer. The current at the end of the 50-mV hyperpolarizing step was less than 0.1 mamp/cm$^2$. The TNS concentration in the axoplasm was 220 μM. The time constant of the light-measuring apparatus was 560 μsec; 2,000 sweeps were averaged

that we obtained the same result in our experiments. During a hyperpolarizing step there was a fluorescence increase when the incident light was polarized parallel to the axon's axis and a decrease when the incident light was polarized perpendicular to the axon's axis. These results were identical to those obtained by Tasaki *et al.* (1972) and provided strong evidence that we were studying the same fluorescence change.

*Incident Light Parallel.* When the TNS fluorescence changes were measured during both hyperpolarizing and depolarizing steps, the results

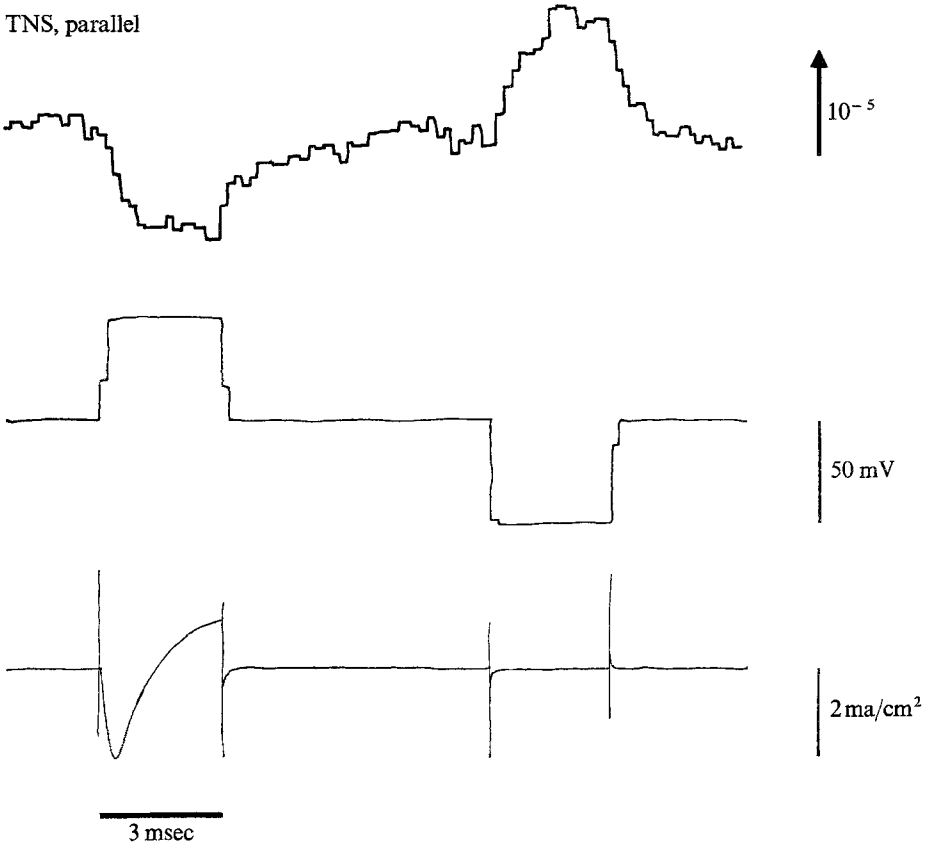


Fig. 6. TNS fluorescence changes (top trace) during hyperpolarizing and depolarizing potential steps (middle trace). The fluorescence changes during the two steps had the same shape, indicating that the TNS fluorescence was also potential dependent. The current densities are shown in the bottom trace, same axon as Fig. 5. The time constant of the light measuring apparatus was 560 μsec; 2,000 sweeps were averaged

shown in Fig. 6 were obtained. Again the fluorescence changes during both steps were about the same size and had similar time courses; suggesting that the TNS fluorescence changes were potential dependent. The changes that we measured during the depolarizing step were qualitatively different from those obtained by Tasaki *et al.* (1972). In our experiments the fluorescence decrease was maintained until the end of the depolarizing step, it did not return to the base line during the step as shown in Figs. 3 and 5 of Tasaki *et al.* (1972).

The TNS changes illustrated in Fig. 6 seemed to be slow compared to the potential changes. However, a large fraction of this difference could have been due to the fact that the light measurement was limited in bandwidth, with a response time constant of 570 μsec. The top trace in Fig. 7 shows

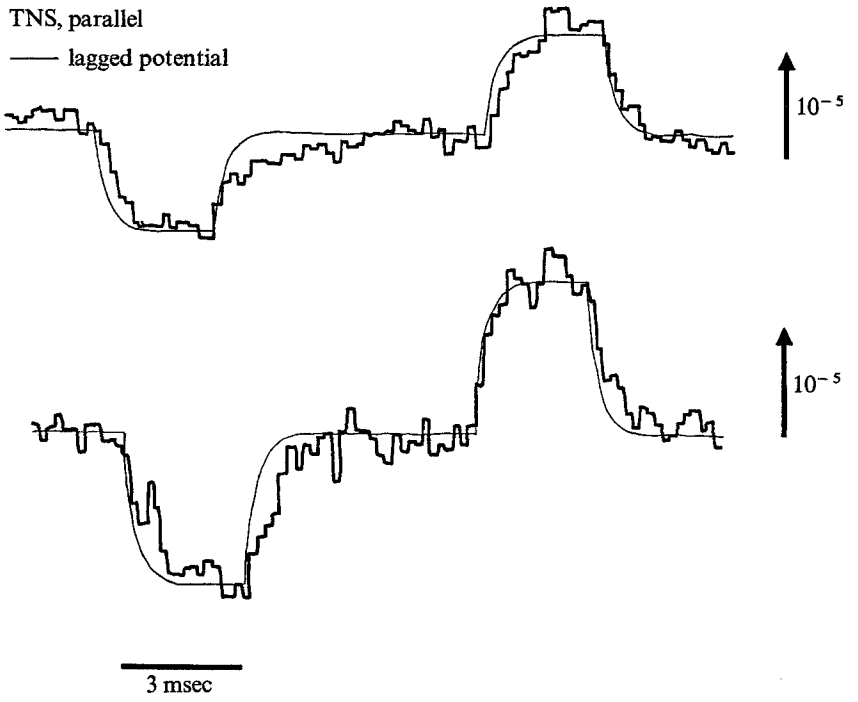


Fig. 7. Comparison of TNS fluorescence changes (heavy lines) and potential when both were recorded with the same high frequency response. When the 570-μsec time constant used in the light measurement was imposed on the potential measurement, then TNS fluorescence and potential had similar time courses. The lagged potential traces (thin lines) were inverted to facilitate comparison with the intensity. 50-mV depolarizing (on the left) and hyperpolarizing (on the right) potential steps were used in both experiments. The results in the top trace are the same as in Fig. 6. In the experiment illustrated in the bottom trace the peak inward current during the depolarizing step was 1.8 mamp/cm$^2$. The TNS concentrations in the axoplasm were 220 μM, top, and 260 μM, bottom. The time constant of the light-measuring apparatus was 570 μsec; 2,000 sweeps were averaged

the same intensity change as in Fig. 6 but in this Figure the potential changes (thin line, inverted to facilitate comparison with intensity) have been lagged by 570 μsec, so that potential and intensity are now measured with the same bandwidth. The bottom trace is a similar measurement on another axon. These were our two best experiments in terms of signal-to-noise ratio of the fluorescence change and in terms of reasonably large currents during the depolarizing step. In both experiments the lagged potential and the intensity trace had very similar time courses. While it is possible that the differences in time course all resulted from noise in the optical records, in most cases the fluorescence changes were slightly slower than the potential change, and this may indicate that the TNS fluorescence lagged behind the potential with a time constant of a few hundred μsec. In any case, it is clear from Fig. 7 that the TNS fluorescence and the potential

had similar time courses and thus we conclude that the TNS fluorescence changes were potential dependent.

The relative TNS intensity changes for a 50-mV hyperpolarizing step with parallel incident light were two to five times smaller than the changes in ANS fluorescence.

*Incident Light Perpendicular.* Only a few experiments were done with light polarized in this plane. In one experiment similar to that in Fig. 6, a similar result was obtained, the signals during hyperpolarizing and depolarizing steps were symmetrical and so this fluorescence change also seemed to be potential dependent. The size of the fluorescence changes with light polarized perpendicular to the axon axis was approximately the same as that found with parallel light.

## Discussion

### *ANS*

The fluorescence changes we measured with ANS added to the outside of squid axons were quite similar to those obtained by Patrick, Valeur, Monnerie, and Changeux (1971) with ANS added to the outside of eel electric organ. Our observations support their conclusion that the ANS fluorescence changes were linearly related to membrane potential. The present evidence however, is somewhat stronger since the experiment of Patrick *et al.* (1971) was done in the presence of tetrodotoxin which abolishes the sodium conductance increase, while our result, from voltage clamp measurements, was obtained in unpoisoned axons.

The fluorescence changes we measured with ANS added internally were quite similar to those obtained previously by Tasaki *et al.* (1972), Conti *et al.* (1971), Conti and Wanke (1971) and Conti and Tasaki (1970), although we concluded that the ANS fluorescence changes were potential dependent. This conclusion is supported by the finding of Conti and Malerba (1972) that similar ANS fluorescence changes could be measured in black lipid membranes where there were no conductance changes.

The ANS fluorescence changes were opposite in sign when the dye was added to opposite sides of the membrane. This finding, which has also been confirmed in black lipid membranes (Conti & Malerba, 1972), suggests that the ANS permeability of the axon membrane is low.

### *TNS*

The fluorescence changes we have measured with the incident light polarized parallel to the axon's axis were qualitatively different from those reported by Tasaki *et al.* (1972). During brief 15- and 43-mV depolarizing

steps they found an intensity decrease that had a time course similar to the ionic current or an action potential, while the intensity decrease we found (Figs. 6 and 7) was maintained until the end of the potential step. We have considered three possible explanations for this discrepancy in results.

*Compensation for Series Resistance.* In the ANS and TNS experiments reported above we attempted to provide electrical compensation for the resistance in series with the axon membrane (*see* Materials and Methods). Tasaki *et al.* (1972) did not use compensation. Fig. 8, an experiment using the dye Capri blue because of its larger signal-to-noise ratio and absence of photodynamic effects, shows fluorescence changes with (third trace) and without (second trace) compensation for the series resistance. Without compensation the fluorescence change during the depolarizing step had components with the time course of the current. This is the expected result, since in the absence of compensation, the membrane potential will have components with a time course identical to that of the current (Fig. 17, Hodgkin *et al.*, 1952). When 90% compensation was used the fluorescence change had a shape much more like that of the potential measured between internal and external electrodes. Thus, this difference in experimental procedure may account for some of the difference in results, but the uncompensated record does not have a shape sufficiently different from that of the potential to completely account for the disparity [unless the series resistance in the experiments of Tasaki *et al.* (1972) was very large]: therefore other factors must be involved.

*Spatial Nonuniformity in Potential Control.* In our experiments a 4-mm length of axon was illuminated, compared to a 12-mm length used by Tasaki *et al.* (1972). It is known that satisfactory spatial control is very difficult to achieve (Cole & Moore, 1960), and the possibility of spatial nonuniformity in membrane potential is considerably greater in a longer length of axon. If spatial control of potential were poor, then in some regions the membrane potential would react to small suprathreshold depolarizations with an action potential-like response. One aspect of the results of Tasaki *et al.* (1972) suggests that spatial nonuniformity may have been a problem. In their Fig. 5 the peak inward current for the 15-mV step was slightly more than half as large as the peak current resulting from the 43-mV step, while other investigators (e.g. Hodgkin, *et al.*, 1952; Cole & Moore, 1960; Armstrong, 1969), reported that the peak current for a 15-mV step was less than one-tenth of that for a 43-mV step. Since one explanation for the relatively large current found by Tasaki *et al.* during the 15-mV step is an inadequate space clamp, in another experiment with Capri blue, shown in Fig. 9 we

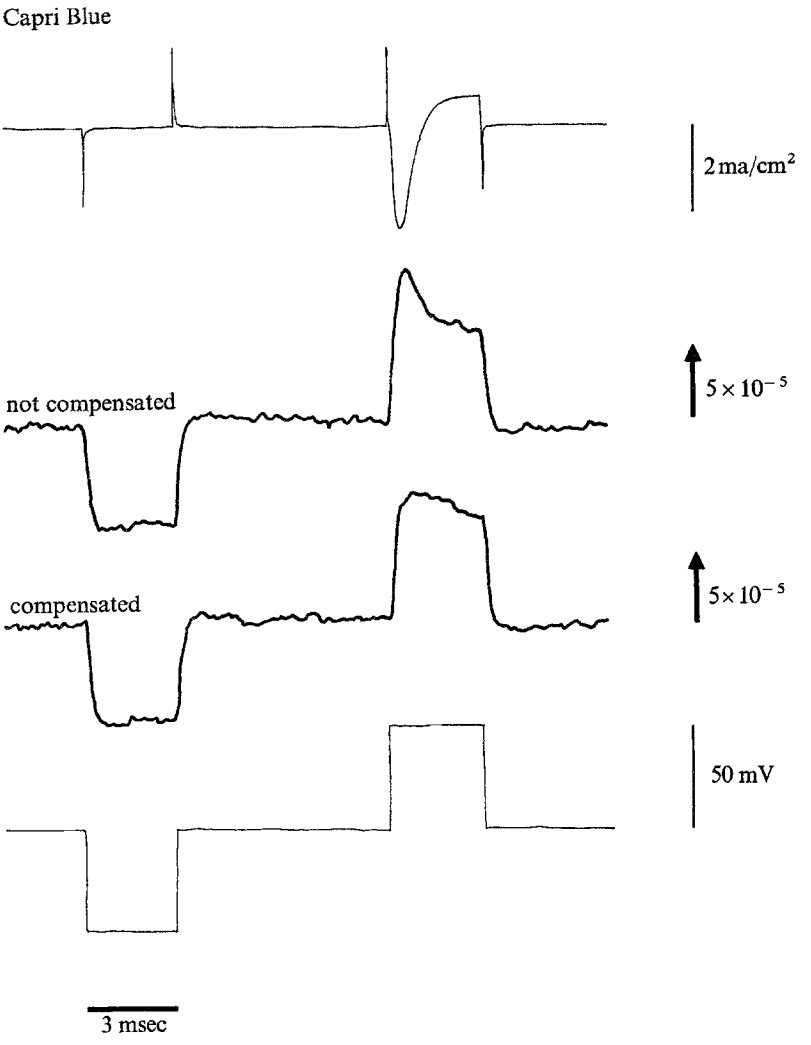


Fig. 8. Changes in fluorescence of the dye Capri blue with (third trace) and without (second trace) electrical compensation for the resistance in series with the membrane. Without compensation there was a component of the fluorescence change which had the same time course as the current (top trace). Compensation removed this component and the fluorescence change had a time course very similar to that of the potential measured between internal and external electrodes (bottom trace). Similar results with and without compensation were obtained in previous birefringence measurements (Cohen *et al.*, 1971). The current and potential traces were recorded when compensation was not used. The axon was soaked for 10 min in seawater with 120 μM Capri blue; the dye solution was replaced with seawater and the measurements then made. The time constant of the light-measuring apparatus was 180 μsec; 250 sweeps were averaged

compared fluorescence changes measured when the incident beam illuminated the middle portion of the internal electrode where the potential control should be adequate (position *A*), and when the incident beam was moved

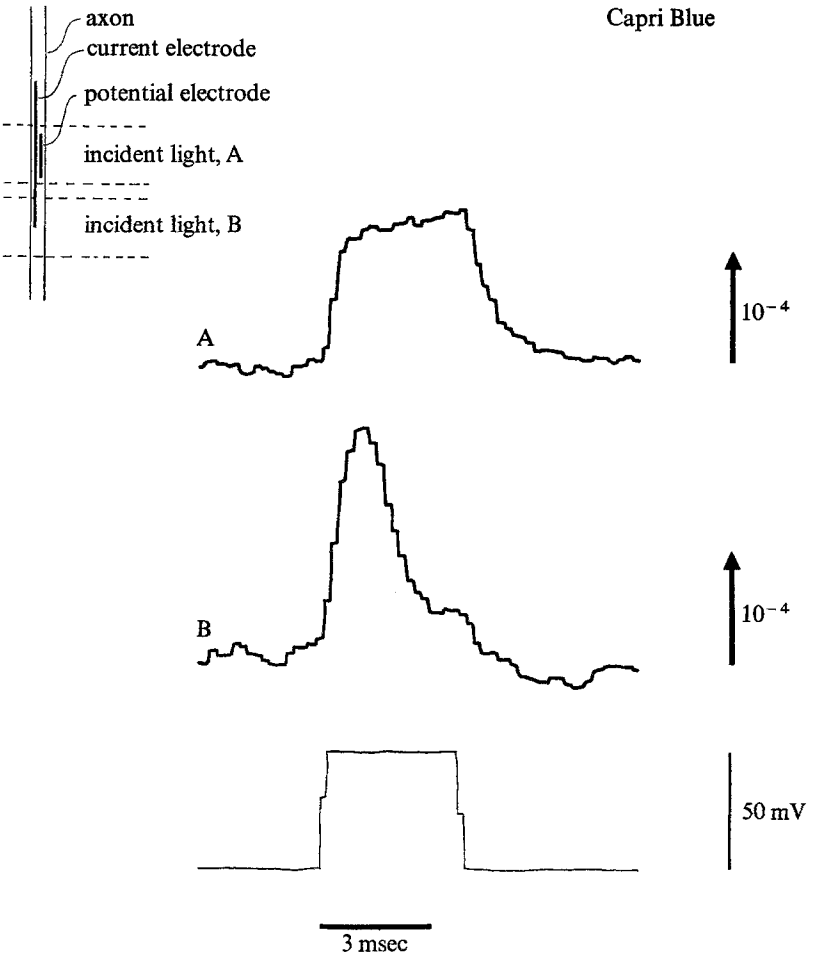


Fig. 9. Changes in Capri blue fluorescence when the portion of the axon that was illuminated included the potential sensing electrode *A* and when the portion illuminated was below the potential sensing electrode *B*. The fluorescence changes in the region below the electrode, where the potential control is expected to be poor, had a time course very different from that of the measured potential (bottom trace). The peak inward current density during the depolarizing step was 1.8 mamp/cm$^2$. The axon was soaked for 10 min in seawater with 120 μM dye; the dye solution was then replaced with fresh seawater and the measurements made. The time constant of the light-measuring apparatus was 240 μsec; 620 sweeps were averaged

(position *B*) to the edge of the electrode where the potential control is known to be poor. The time course of the change during the depolarizing step in trace *B* was similar to the results obtained in Figs. 3 and 5 of Tasaki *et al.* (1972). Poor spatial control can therefore lead to results similar to those obtained by Tasaki *et al.* and, thus, spatial nonuniformity may partly explain the difference in results.

*Current and Light Not Measured from Same Length of Axon.* In our experiments only the central 4 mm of a 10-mm clamped region was illuminated. Since illumination was harmful to the axon in these experiments, it is possible that our current records were misleading. The illuminated 4 mm of axon might have had reduced currents even though the currents measured over the total 10 mm seemed relatively good. If this were the case, our optical records might not show any changes related to conductance because the conductance increases were no longer present in the illuminated region. There are two reasons for thinking that this explanation will not account for the difference between our results and those of Tasaki *et al.* (1972). First, Tasaki *et al.* measured light from a 12-mm length of axon while the currents were measured from a 22-mm region, so their experiment would seem to be subject to the same criticism as ours. A second argument comes from experiments with the dye pyronin B, where the illuminating light did not have deleterious effects. In our experiments, the fluorescence changes with this dye were potential dependent (H. V. Davila, B. M. Salzberg and L. B. Cohen, *unpublished*) while Fig. 2 of Tasaki *et al.* (1972) shows pyronin B fluorescence changes with time courses identical to those that they found with TNS. Since our results were qualitatively different from those of Tasaki *et al.* (1972) with both harmful and nonharmful dyes, the disparity is probably not related to errors resulting from inappropriate current measurement.

It is our conclusion that, together, the lack of compensation for the series resistance and spatial inhomogeneities in potential in the experiments of Tasaki *et al.* (1972) could account for the differences in our results. We feel that it is most likely that the TNS fluorescence changes are potential dependent.

Thus the ANS and TNS fluorescence changes could provide information about changes in membrane structure that result from changes in membrane potential, but give no information about the structural bases of the conductance increases. Since the fluorescence of both dyes is very sensitive to solvent polarity, it might be supposed that the dyes are responding to changes in polarity of the environment within the membrane. However, in its simplest form, this hypothesis isn't particularly attractive since it would suggest that the internal surface of the axon membrane becomes both more (TNS perpendicular) and less (TNS parallel, ANS) hydrophobic during depolarizations. But, in addition, there is no evidence to show that polarity is the environmental variable sensed by the ANS and TNS in these experiments. The fluorescence of both dyes is also sensitive to viscosity and the presence of unpaired electrons (Brand, Seliskar & Turner, 1971; Chakrabarti & Ware, 1971). Furthermore, no evidence has been presented that

would rule out possible trivial (i.e. unrelated to membrane modification) mechanisms (Cohen, 1973), such as movement of the dye within the membrane, that might account for the fluorescence changes. The intensity changes with ANS on both sides of the membrane, and of TNS with the incident light polarized parallel might all be accounted for by electric field induced movement of the negatively charged dye molecules within a membrane whose center was more hydrophobic, or viscous, or had a lower concentration of unpaired electrons. (It is worth noting that this mechanism is not limited to charged dyes; if the field is inhomogeneous, molecules having a net dipole moment will, of course, migrate.) To accommodate the TNS changes with perpendicularly polarized incident light into this scheme, we would have to assume that these dye molecules were bound to a molecule with a net positive charge which was moving in an electric field. Other trivial mechanisms include the direct effect of the electric field on spectral properties of the dye (Platt, 1962; Bücher, Wiegand, Snavely, Beck & Kuhn, 1969), and changes in the number of molecules bound at membrane surfaces. Indeed, there is presently evidence that potential dependent changes in the fluorescence of certain cyanine dyes bound to mammalian erythrocytes (P. Sims, A. S. Waggoner, C.-H. Wang, and J. F. Hoffman, *personal communication*) result from alterations in the binding. Since it is possible to account for the fluorescence changes by a variety of mechanisms, in the absence of experimental evidence to suggest which is correct, the structural bases of the ANS and TNS fluorescence changes cannot presently be determined.

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