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STUDIES OF LIGHT EMISSION, ABSORPTION AND ENERGY TRANSFER IN NERVE MEMBRANES LABELLED WITH FLUORESCENT PROBES

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Physicochemical properties of fluorescent membrane probes, 2-p-Cl-anilinonaphthalene-6-sulfonate (p-Cl-ANS), 2-p-Br-anilinonaphthalene-6-sulfonate (p-Br-ANS), merocyanine-540, methyl violet, etc., were examined because of the possibility of demonstrating resonance energy-transfer between probes. The emission psectra of p-Cl-ANS and p-Br-ANS and the absorption (or fluorescence excitation) spectra of Eastman Kodak merocyanine-540 (M-540) and other probes were found to be very sensitive to changes in the solvent polarity. The spectra of these probes incorporated in the nerve membrane were determined and compared with the corresponding spectra in various organic solvents and macromolecules. This comparison suggests that the polarity of the binding sites for p-Cl-ANS (and p-Br-ANS) in the membrane is high and that of M-540 is very low. The spectrum of the portion of p-Cl-ANS fluorescence contributing to production of optical responses (i.e., transient changes during action potentials) was determined. Both absorption responses and fluorescence responses were detected from M-540 in the nerve membrane. It was possible to demonstrate resonance transfer of electronic energy from p-Cl-ANS to M-540 incorporated in lysolecithin micelles and in crab nerves. During action potentials, the intensity of M-540 fluorescence excited by energy transfer was found to undergo transient changes. Based on these and other experimental findings, properties of the binding sites for these probes in the nerve membranes are discussed.

1. Introduction

Recent studies of extrinsic fluorescence of the nerve membrane during nerve excitation have yielded very interesting information as to the physicochemical properties of the membrane macromolecules [1,2]. At present a large number of fluorescent probes are known to produce fluorescence responses, i.e., transient changes in fluorescence intensity associated with action potentials, when incorporated in the nerve membrane [3,4]. To analyze the properties of the nerve membrane by this method, a comprehensive knowledge of the physicochemical properties of the probe employed is required. Only by comparing the optical properties of the probes in the nerve membrane with those in various organic solvents and macromolecules, can the nature of the binding sites of the probe molecules in the membrane be inferred.

Among a large number of membrane probes known at present, it is possible to select a pair of probes that can be used to examine the process of resonance transfer of electronic energy [5–7] within or across the nerve membrane. One of the conditions which have to be satisfied by such a pair is that the emission spectrum of one probe significantly overlaps with the absorption spectrum of the other. An example of such a pair is p-Cl-anilinonaphthalene-sulfonate and merocyanine-540 (abbreviated as p-Cl-ANS and M-540). The physicochemical properties of the former probe were examined recently by Kosower and Dodiuk [8]. The usefulness of the latter probe for membrane studies was demonstrated by Davila et al. [9]. Examination of energy transfer between such a pair of probes is expected to yield significant information about the separation between the two kinds of probe molecules in the nerve membrane.

The present study was undertaken with a view toward elucidating physicochemical properties of the fluorescent membrane probes, p-Cl-ANS, p-Br-ANS, M-540, methyl violet, etc., in the membrane of crab nerves and squid giant axons. We first examined physicochemical properties of these dye molecules dissolved

in various organic solvents. Then we analyzed the behavior of these dyes incorporated into the nerve membrane and examined the mechanism of production of fluorescence responses with these probes. Next, we examined the possibility of energy transfer between these dye molecules in the nerve membrane. Finally, we examined the process of production of fluorescence responses mediated by energy transfer between these dyes.

A portion of the experimental results described in this paper was presented at the Annual Meeting of the Marine Biological Laboratory in Woods Hole, Mass. [10] (see also ref. [11]). When the manuscript of the present paper was almost ready for publication, an article by Ross et al. [12] appeared, describing the properties of M-540. The results obtained by these investigators seem to be consistent with those described in section 3 under the results of the present paper.

2. Methods

Two of the fluorescent dyes used, p-Cl-ANS and p-Br-ANS were a gift of Dr. Edward M. Kosower, University of Tel Aviv. The other dyes (M-540, methyl violet, etc.), were purchased from Eastman Kodak Co. When examined by acrylamide gel-electrophoresis (glycine-tris buffer at pH 8), these dyes yielded a single, well-defined band. Most of the organic solvents used in the present study are those employed previously [1], and did not contain any light-absorbing or fluorescent contaminants. Dehydrated ethanol (purchased from Ferry Point) was used after re-distillation. Chloroform (Baker Chem. Co.) was used after passing through a column of aluminum oxide (Woelm basic, M. Woelm) to remove the stabilizing additive, ethanol. Measurements of electric conductivity of aqueous solutions were carried out with a General Radio Impedance bridge (type 1650A).

The extinction coefficients of dye solutions were measured in most case with a Beckman spectrometer (model DU); when a 10 cm long lightpath was required a Cary spectrophotometer (model 14) was employed. Measurements of light absorption by a stained crab nerve (see the broken line in fig. 3b) was carried out with a Beckman spectrometer by mounting the nerve on a special nerve-holder made of black lucite (constructed in this laboratory) which permitted only the portion of light passing through the nerve to reach a photomultiplier tube. A plate of frosted glass was placed near the nerve to produce scattering of light before reaching the photomultiplier. The advantage of inserting a frosted (or opalescent) glass in the lightpath was pointed out by Shibata [13]. In this spectral measurement, the effect of light scattering by the nerve was taken care of by inserting an unstained nerve in the pathway of the reference light beam. Transient changes in light absorption by a stained nerve during excitation (i.e., absorption responses) were measured with a Leitz microscope (Ortholux Pol) with an attachment for Köhler's illumination. With the sharp image of the diaphragm focussed in the middle of the nerve, it was possible to completely block the light reaching the photomultiplier without passing through the nerve. This method is essentially the same as that used in a previous study [14], except that the polarizer and analyzer were removed from the lightpath.

Fluorescence emission spectra of the dyes in vitro were determined with a Hitachi Perkin–Elmer spectrofluorometer (MPF 2) in this laboratory or with an Aminco–Bowman spectrofluorometer which belongs to Drs. Egyud and Szent-Gyorgyi in Woods Hole. Corrected excitation spectra were kindly recorded for us by Drs. Chen and Cathou at Tufts University in Boston and by Dr. R. Chen at NIH, Bethesda. The emission spectra of the fluorescent probes incorporated into the nerve membrane was determined both with these commercially available spectrofluorometers and by the use of a special instrument constructed in this laboratory [1]. The method of recoding fluorescence responses (i.e., transient changes in fluorescence intensity associated with action potentials) was described in detail in previous papers [15]. A CAT computer (Mnematron Corp.) was used to improve the signal-to-noise ratio. The spectrum of the portion of light contributing to production of fluorescence responses (see fig. 1b) were determined by introducing a series of interference filters between the nerve and the photomultiplier. The excitation spectrum of a fluorescence response (fig. 3a) was determined by introducing interference filters between the light source and the nerve. These spectra were corrected to account for the difference in the transmission properties of the interference filters used and, in determination of emission spectra, for the spectral sensitivity of the photomultiplier used (RCA 6643). In determining excitation spectra, correc-

tions were made to take care of the spectral distribution of the light energy from the source (200 W xenon lamp, Hannovia).

In the present studies, either squid giant axons (taken from *Loligo pealei*) or crab nerves (taken from *Libinia emarginata* or *Callinectes sapidus*) available in Woods Hole, Mass., were used. When external staining of the nerve membrane with M-540 was required, a cleaned giant axon or a de-sheathed crab nerve was immersed in sea water saturated with the dye for a period of about 1 hr. The method of application of aminonaphthalene derivatives to squid giant axons and crab nerves has been described previously [15]. When repetitive stimulation of a stained nerve was required, sea water saturated with nitrogen gas was used externally.

Most physicochemical studies of the dyes in vitro and in the nerve membrane were carried out at room temperature (20–23°C). Fluorescent responses were usually recorded at about 6° C.

3. Results

3.1. Physicochemical properties of p-Cl-ANS and p-Br-ANS in vitro and in nerve membrane

When dissolved in water, both p-Cl-ANS and p-Br-ANS fluoresce very weakly. They fluoresce intensely when they are dissolved in organic solvents or bound to various macromolecules. Like other anilinonaphthalene derivatives (see refs. [16,17], their emission spectra are affected by the polarity of the solvent used (see ref. [8]). In solvents with high polarity, the wavelengths of maximum emission are red-shifted, the quantum yields are reduced and the bandwidths of the emission spectra are broadened. The absorption spectra are quite insensitive to changes in the solvent polarity. Qualitatively, the behavior of these probes in vitro is very similar to that of other 2,6-ANS derivatives studied extensively by McClure and Edelman [18] and by Tasaki et al. [1].

When applied extracellularly to crab nerves or to squid giant axons, p-Cl-ANS was found to produce a negative *fluorescence response* (i.e., a transient decrease in intensity) during nerve excitation. The amplitude of the observed response (ΔI) in crab nerve was $(3-7) \times 10^{-4}$ times the intensity at rest (I). Polarization properties of the fluorescence responses were examined by inserting dichroic filters in the lightpath, a polarizer between the light source and the nerve and an analyzer between the nerve and the detecting photomultiplier [2]. With the electric vector of the exciting light beam oriented perpendicularly to the long axis of a crab nerve, electric stimulation of the nerve produced a response representing a large transient reduction in intensity of the fluorescent light polarized perpendicularly with respect to the nerve axis. On the basis of the experimental results obtained under four different combinations of the polarizing axes of the polarizer and analyzer [2], it was concluded that the major portion of the p-Cl-ANS and p-Br-ANS molecules at the external surface of the crab nerve is aligned with their transition moments oriented perpendicularly to the membrane surface.

Using squid giant axons, fluorescence responses produced by intracellularly applied p-Cl-ANS and p-Br-ANS were examined. These responses were found to be positive regardless of the directions of the polarizer and analyzer in the optical path. In other words, the sign of the response was found to be reversed when this dye was applied intracellularly instead of being applied extracellularly. This reversal of the sign of the responses indicates that the axonal membrane constitutes a major diffusion barrier for these negatively charged dye molecules and, consequently, that the internally applied dye molecules do not occupy the same binding sites as those administered extracellularly. The amplitude of the fluorescence responses observed in axons with p-Cl-ANS internally (excited at 365 nm) was roughly 5×10^{-5} times the background intensity.

Further information about the behavior of this dye in the nerve membrane was obtained by comparing the *emission spectra* of crab nerves stained externally with p-Cl-ANS with those of this probe in various organic solvents (see fig. 1). Reflecting the insensitivity of the absorption spectrum of p-Cl-ANS to changes in solvent polarity, the excitation spectrum of this probe was found to be quite insensitive to changes of the solvent. In contrast, the wavelength of maximum fluorescence emission was strongly influenced by the polarity of the solvent employed. The following list illustrated this solvent effect on the wavelength of maximum emission: 396 nm in octanol, 405 nm in butanol, 405 nm in propanol, 407 nm in ethanol, 417 nm in methanol, 425 nm in glycerol, 430 nm in ethylene glycol, 445 nm in formamide. Incorporated into lysolecithin

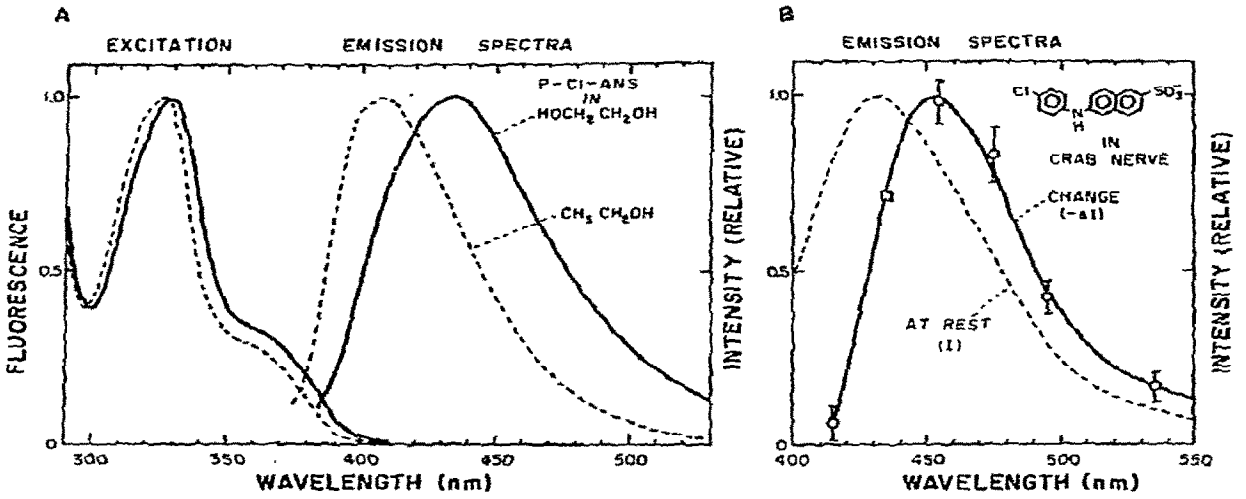

Fig. 1. (a): fluorescence excitation and emission spectra of 2-p-chloroanilinonaphthalene-6-sulfonate (2.8 μM) dissolved in ethanol (broken line) and in ethylene glycol (continuous line). (b): emission spectrum of the same dye in crab nerve at rest (broken line) and spectrum of the light contributing to production of fluorescence response of the probe (continuous line). Note that the spectrum in a crab nerve at rest is very similar to that observed in ethylene glycol.

micelles, the wavelength of maximum emission of this probe was 428 nm. In erythrocyte ghost membranes, it was 414 nm. Under these experimental conditions, the bandwidth of the spectrum increased monotonically with the wavelength of maximum emission. The spectrum of p-Cl-ANS incorporated into a crab nerve at rest (I in fig. 1b) was found to be very similar to that in ethylene glycol, the wavelength of maximum emission being about 430 nm and the half-bandwidth being about 77 nm.

The spectrum of the portion of the emitted fluorescent light involved in production of fluorescence responses in shown by the solid line (ΔI) in the figure. This spectrum was constructed from the data obtained from 20 different nerves and indicates essentially the relative amplitudes of the fluorescence responses observed at various wavelengths. [Note that the response amplitude at a given wavelength is proportional to the light intensity.] It is seen in the figure that this spectrum (ΔI) is slightly red-shifted relative to the spectrum at rest (I).

3.2. Behavior of M-540 in solvents and in crab nerve

M-540 is poorly soluble in sea water. In distilled water at 20°C, this dye may be dissolved at a level of about 1 mM (570 mg/ℓ) or slightly more. Addition of inorganic salts profoundly reduces the solubility of M-540 in water. Organic solvents, such as methanol, ethanol, ethylene glycol, etc., are far better solvents. This dye is practically insoluble in apolar solvents, such as benzene, xylene, octane, etc. It is sparingly soluble in chloroform and dioxane.

In water, the absorbance of this dye was found not to obey Beer's law except at a particular wavelength, i.e., isosbestic point (aproximately 500 nm). With decreasing concentration, the band intensity (i.e., molar extinction) on the longer-wave side of the isosbestic point gradually increased and the band on the other side decreased. At about 0.25 mM, the wavelength of maximum absorption was found to be approximately 490 nm. When this aqueous solution of M-540 was diluted by a factor of 1000, a new absorption maximum appeared at about 540 nm. Measurements of electric conductivity of this probe in distilled water showed that, in the range of concentration between 1.2 and 0.3 mM, the equivalent conductivity of M-540 anions was concentration-independent, being approximately 22 $ohm^{-1}\cdot cm^2\cdot equiv^{-1}$ at 20°C. This equivalent conductivity is close to that of tetrapropylammo-

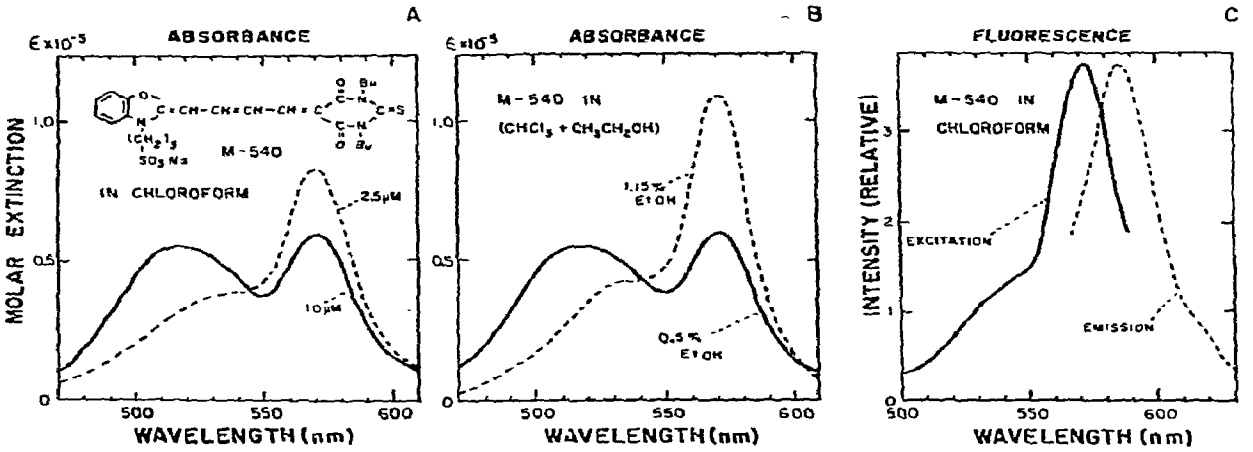

Fig. 2. Absorption, fluorescence excitation and emission spectra of Eastman Kodak merocyanine-540 dissolved in chloroform. In A, the effect of varying the concentration of probe in chloroform (containing 0.5% ethanol) on the molar extinction coefficient is shown. In B, the effect of varying the ethanol content on the absorbance of M-540 (10 μM) is illustrated. In C, the extinction and emission spectra of the probe in chloroform are shown. Note that the "dimer" band at about 520 nm is absent in the excitation spectrum.

nium-ion.

In ethanol, this dye was found to obey Beer's law in a wide range of concentration. Dissolved in chloroform, Beer's law was not obeyed by M-540 (fig. 2a). With a decrease in the concentration, the molar extinction at the absorption band at about 570 nm was intensified at the expense of the band at 510 nm. Since many merocyanines are known to show a strong tendency to form dimers (see ref. [19]), the shorter waveband of the spectrum shown in fig. 2a is considered to represent absorption by dimers.

The solvent polarity was found to affect the wavelength of maximum absorption of M-540 to a considerable degree, a rise in the polarity bringing about a blue-shift. The solvent polarity was varied systematically by using a homologous series of organic solvents. The absorption maxima were found at 555 nm in methanol, 561 nm in ethanol, 562 nm in propanol and 566 nm in octanol. This fact can readily be explained on the assumption that the dipolar form of the two resonance structures of the dye molecule dominates even in organic solvents. It is known that this is the case with many other merocyanine dyes (see Brooker et al. [20]).

It is interesting to compare the wavelength of maximum absorption of M-540 incorporated into a crab nerve with the maxima observed in various organic solvents and bio-macromolecules. The *absorption spectrum* of this probe in a crab nerve (at rest) is shown by the broken line in fig. 3b. Based on the similarity between this spectrum and that shown in fig. 2a, the band with a maximum at about 570 nm is regarded as deriving from the monomeric form of the probe molecules. In general, the wavelengths of maximum absorption observed in organic solvents and bio-macromolecules are shorter than 570 nm seen in crab nerves. [Note that they are 559 nm in glycerol, 559 nm in ethylene glycol, 560 nm in bovine serum albumin, 563 nm in lysolecithin micelles, 563 nm in gelatin-gel (10%).] The binding sites for M-540 in crab nerves must then have properties of low dielectric media in which the probe is only sparingly soluble. [Note in fig. 2 that the monomer band of M-540 in chloroform has a maximum at about 570 nm.]

The *fluorescence excitation spectra* of M-540 in organic solvents and in crab nerves were determined by reading the emission intensity in the range of wavelengths between 600 and 640 nm. In ethanol, the spectrum was found to be an almost exact replica of the absorption spectrum (when the vertical scale was properly adjusted); this is expected since the absorbance measurement has indicated that only one (monomeric)

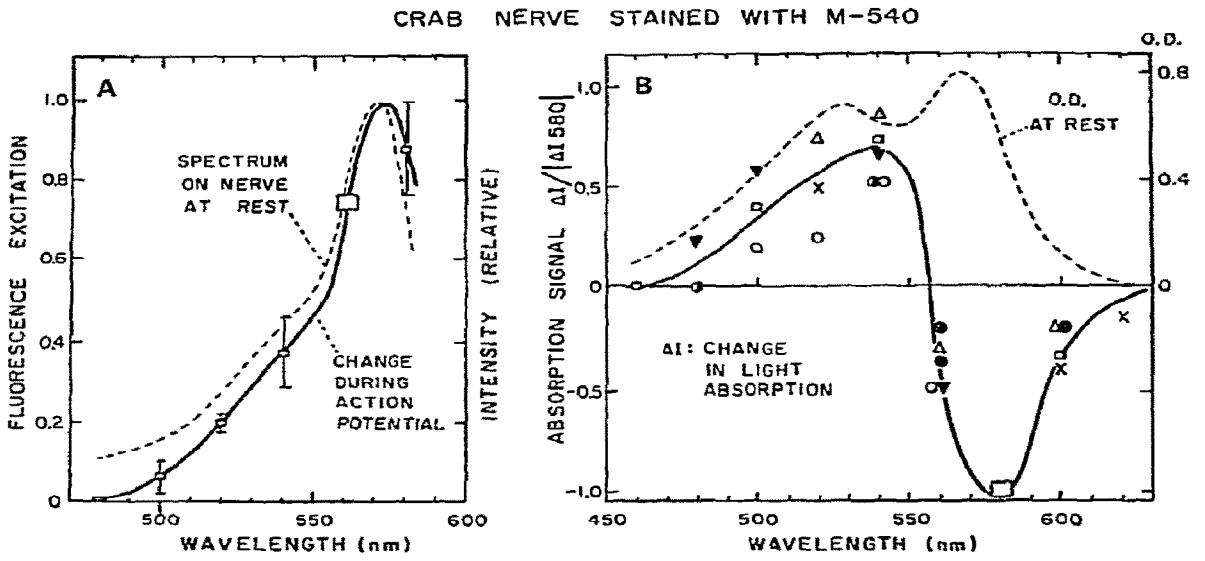

Fig. 3. Left: The broken line shows an example of fluorescence excitation spectrum of M-540 in a crab nerve at rest; the continuous line shows the excitation spectrum of M-540 contributing to production of fluorescence responses. Right: Absorption spectrum of M-540 in a crab nerve at rest (broken line) and spectrum of M-540 determined by taking absorption changes (or signals) associated with action potentials as an index (continuous line). Different symbols were used to show the results obtained from different nerves.

form of M-540 molecules exists in ethanol. In chloroform, the excitation spectrum was far narrower than the absorption spectrum; this difference between absorption and excitation spectra can reasonably be attributed to the existence of dimers (or of aggregates) in this medium. By comparing fig. 2a with 2c, it is concluded that the fluorescence yield of dimers is far lower than that of monomers. An example of the excitation spectrum of M-540 in crab nerve is shown in fig. 3. This spectrum is very similar to that observed in chloroform (fig. 2c), the maximum being located at about 570 nm.

The *emission spectra* of M-540 in chloroform is shown in fig. 2c. The emission spectra were found to be slightly affected by changes in solvent polarity. The wavelength of maximum emission was approximately 578 nm in ethanol and 584 nm in chloroform. These wavelengths are only slightly longer than the wavelength of maximum absorption, the Stokes shift being approximately 18 nm in ethanol and about 14 nm in chloroform. The effect of addition of a small amount of ethanol to chloroform solution of M-540 is also shown in fig. 2b. The large increase in the fluorescence intensity was brought about by ethanol, indicating that a very small enhancement of the solubility of M-540 is effective in converting a significant fraction of the dimeric form of M-540 into its monomeric form.

Davila et al. [9] found that M-540 is a very effective probe for detecting *transient fluorescence changes* associated with action potentials. Taking advantage of this property of M-540 in the nerve membrane, the excitation spectrum contributing to production of such fluorescence responses was determined. The result of such measurements on crab nerves is shown by the solid line in fig. 3a. The method of determination was essentially the same as that employed previously [1]. The excitation wavelength of 560 nm was selected as the reference point. The response amplitude (ΔI) determined at this wavelength (on 9 nerves) was $(7.0 \pm 1.8) \times 10^{-4}$ times the background light intensity (I). It is seen that this excitation spectrum (ΔI) is very similar to that taken in the resting state of the nerve (I). Since this response represents an increase in the intensity of fluorescence, the result obtained has to be taken as representing a transient increase in emission by monomers of M-540 in the nerve. Such an increase in monomer emission can take place if there is a transient conversion of dimers into monomers.

Direct evidence for dimer-monomer conversion was obtained by measuring *transient changes in absorption* associated with production of action potentials (fig. 3). The absorption spectrum of M-540 in a crab nerve (see the broken line in the figure) was found to show two bands, one corresponding to the monomer band and the other to the dimer band. Furthermore, it was found that production of an action potential was accompanied by an increase in absorption in the region of wavelength corresponding to the monomer band (see the solid line in the figure). Simultaneously, there was a decrease in the region of the dimer absorption band. The neutral point at which no change in absorption was observed was close to 560 nm. In determining this change in the absorption spectrum (see section 2), 580 nm was selected as the reference wavelength. At this wavelength, the observed reduction in the light intensity during action potentials was of the order of 10^{-4} times the background light intensity. This ratio of the change to the background intensity is expected to vary with the amount of the dye in the nerve. Furthermore, it is possible that this spectrum is distorted by a change in light scattering during nerve excitation [21,22]. Nevertheless because the sign of the responses produced by a light scattering change is not influenced by a change in the wavelength, it was concluded that fluorescence responses in nerve stained with M-540 are produced by conversion of dimers of the probe into monomers. A similar conclusion was reached by Ross et al. [23] using a different merocyanine as a probe.

3.3 Estimation of the reference distance for energy transfer from p-Cl-ANS to M-540

According to Förster's theory of resonance energy transfer [5–7,24], the efficiency of transfer is given by

$$T = \frac{(R_0/R)^6}{1 + (R_0/R)^6},$$

where R is the distance between the donor and acceptor molecules and R_0 is the reference distance. R_0 is determined by the overlap integral, the geometric factor, the quantum yield of the donor molecules and the refractive index.

The corrected emission spectrum $f(\lambda)$ of p-Cl-ANS on the external surface of a crab nerve shown in fig. 1b can be used to calculate the overlap integral. The wavelength dependence of the overlap, $f(\lambda)\epsilon(\lambda)\lambda^4$, was calculated from the absorbance of M-540 in ethanol, $\epsilon(\lambda)$. The integral of this overlap yielded

$$J = 6.5 \times 10^{-14} \ (\mathrm{M^{-1}cm^3}).$$

By using this value of J, R_0 was found to be 53 Å when the geometric factor is assumed to be 2, the refractive index to be 1.5 and the quantum yield to be 0.5. [Note that the transition moments of the major portion of the donor and acceptor molecules are normal to the membrane surface.] There is some uncertainty in the estimation of R_0, because the values of the geometric factor and the refractive index cannot be directly measured.

3.4. Energy transfer from p-Cl-ANS to M-540

After this rough estimation of R_0, it seemed highly desirable to show that energy transfer of the non-trivial type (see ref. [24]) can actually take place between p-Cl-ANS and M-540 in vitro. It is known that lysolecithin dissolved in water form micelles consisting of about 300 single molecules (see ref. [25]). The diameter of such micelles is considered to be about 70 Å which is not much larger than the value of R_0 estimated above. If both p-Cl-ANS and M-540 molecules could be incorporated into one and the same micelle, the separation between the two molecules would be comparable to R_0. Addition of lysolecithin to an aqueous solution of M-540 (without mixing with p-Cl-ANS) increases the fluorescence of M-540 molecules excited at the peak of their monomer absorption band. The excitation spectrum of M-540 incorporated in lysolecithin has a maximum of 563 nm. Incorporated into lysolecithin micelles, p-Cl-ANS (without M-540) emits strong fluorescent light with a wavelength of maximum emission at about 428 nm.

To demonstrate energy transfer between these dyes incorporated in lysolecithin micelles, a series of test tubes were prepared containing constant amounts of p-Cl-ANS (8 μM) and of M-540 (2 μM). The concentration of lysolecithin added was varied logarithmically from 3 μM up to 6.2 mM. The excitation spectrum of the mixture was determined with a Perkin–Elmer spectrofluorometer by reading the emission of M-540 in the range of wavelength longer than 600 nm. In this

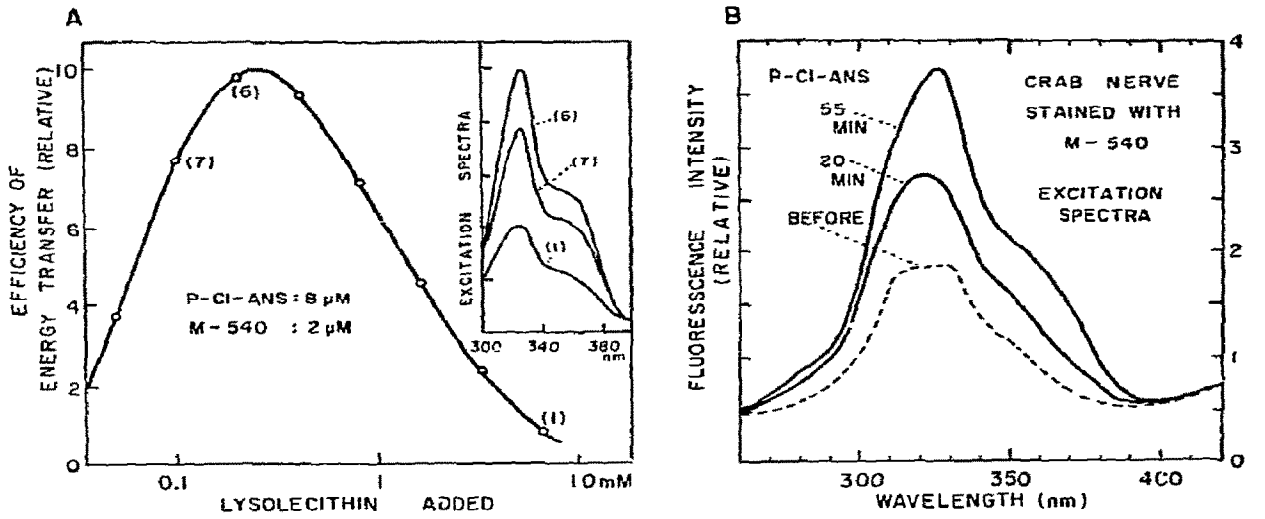

Fig. 4. A: demonstration of resonance transfer of electronic energy from p-Cl-ANS to M-540 incorporated in lysolecithin micelles (see text). Three examples of the fluorescence excitation spectra from which the efficiency curve was constructed are shown in the inset. B: demonstration of energy transfer between p-Cl-ANS and M-540 incorporated in a crab nerve. The broken line shows the excitation spectrum observed before introduction of the donor (p-Cl-ANS) into the nerve. Note that there was a gradual increase in the intensity of the excitation band at 330–360 nm as the nerve (pre-treated with M-540) was stained with p-Cl-ANS. The spectra in this figure are uncorrected.

range of wavelength, p-Cl-ANS makes very little direct contribution to the excitation spectrum. Therefore, an increase in the intensity of the excitation band in the 330–360 nm region (which corresponds to an absorption of maximum of p-Cl-ANS) can be taken as a measure of energy transfer from p-Cl-ANS to M-540.

The direct contribution of M-540 to the excitation band in the 330–360 nm region was estimated by carrying out similar measurements on a series of test tubes containing M-540 (2 μM) and lysolecithin (variable concentration) but without adding p-Cl-ANS. Similarly, the direct contribution of p-Cl-ANS was determined by measuring the band intensity in the same region using a series of test tubes containing p-Cl-ANS (8 μM) and lysolecithin (variable concentration) but no M-540. These contributions, which do not involve energy transfer, were subtracted from the observed amplitude of the 330–360 nm excitation band. Possible effects of competition between two kinds of dye molecules at the dye-binding sites were ignored.

It is seen in fig. 4a that energy transfer does take place when lysolecithin is added to the mixed solution of p-Cl-ANS and M-540. The efficiency of energy transfer was found to increase with increasing lysolecithin concentration. The efficiency reached a maximum at a definite concentration level of lysolecithin. The fall in the efficiency at high concentration in the figure is expected from the following consideration. Energy transfer takes place only when both donor and acceptor molecules occupy the same micelles. At very high concentrations of lysolecithin, the number of micelles is so great that the probability of finding micelles containing both p-Cl-ANS and M-540 decreases; this explains the existence of a maximum in the efficiency. When the micelle concentration is very low, dye molecules tend to remain free in the aqueous phase; hence, the efficiency of transfer is low. No sign of energy transfer was observed in an aqueous or ethanol solution containing both p-Cl-ANS and M-540 at a level of approximately 10 μM.

We made an unsuccessful attempt to describe the curve shown in fig. 4a quantitatively by using the mass-action law applied to the process of binding of two kinds of dye molecules to lysolecithin micelles. The difficulty of such an attempt is expected since it is known that partition of cyanine dyes between water

and oil cannot be described on the basis of the mass-action law (see ref. [19]). A complex interaction between lysolecithin micelles and M-540 molecules appears to lead to a sharp fall in the efficiency of energy transfer at the lower side of the lysolecithin concentration.

In a suspension of lysolecithin micelles to which both p-Cl-ANS and M-540 are added, the dye molecules exist in the aqueous phase as well as in micelles. Under these conditions, evaluation of the efficiency of energy transfer is not very simple. [This is the reason why only a relative scale is used in fig. 4a.] Measurements of the fluorescence of the mixture suggest that, at 0.8 mM in lysolecithin concentration, the major portion of the dye molecules is incorporated into micelles. Then, the following formula (see eq. (61b) in ref. [7]) can be used to estimate the efficiency.

$$T = \frac{\text{OD of M-540 at 563nm}}{\text{OD of p-Cl-ANS at 530 nm}} \times \frac{\text{emission of M-540 excited at 330 nm}}{\text{emission of M-540 excited at 563 nm}}.$$

The OD (optical density) ratio in this equation was found to be approximately 2.1. The ratio of the M-540 emission increment (excited at 330 nm in the presence of p-Cl-ANS) to the M-540 emission intensity (excited at 563 nm in the presence of p-Cl-ANS) was estimated to be roughly 0.17. Thus, the efficiency at this lysolecithin concentration is estimated to be roughly 0.36. Qualitatively, an efficiency of this order of magnitude is expected from the diameter of the micelles.

The process of energy transfer between p-Cl-ANS and M-540 in the nerve membrane was examined using a commercially available spectrofluorometer with a nerve introduced into a standard cuvette. A nerve was fastened, after staining with a dye, to a small frame made of black Lucite in such a way that replenishment of the sea water in the cuvette did not produce any significant change in the intensity of the fluorescent light from the nerve. Initially, the nerve was stained with M-540 by immersion in artificial sea water saturated with the fluorochrome for a period of 60 to 90 min. Subsequently, the nerve was rinsed thoroughly with dye-free sea water and the fluorescence excitation spectrum of the nerve was examined. [Under these conditions, the intensity of the fluorescence of the nerve remained roughly constant even when the saline solution in the cuvette was repeatedly replenished.] The excitation spectrum of the nerve was examined by reading the emission in the range of wavelength between 600 and 640 nm. Then, the nerve was transferred into sea water containing p-Cl-ANS (10 μM). After a period of about 6 min, the excitation spectrum was recorded from the nerve in dye-free sea water. By repeating this procedure, the effect of a gradual increase in the amount of p-Cl-ANS incorporated into the nerve on the excitation spectrum was investigated. In most axons examined, incorporation of p-Cl-ANS brought about a slight reduction in the fluorescence of M-540 excited at 570 nm. [This reduction is probably attributable to competition of these two dye molecules in the nerve membrane.]

Incorporation of p-Cl-ANS in a crab nerve pre-stained with M-540 brought about a definite increase of the excitation band in the 330–360 nm range (fig. 4b). In principle, this excitation band consists of (*i*) the contribution of M-540 molecules absorbing light in the range of 330–360 nm, (*ii*) the contribution of p-Cl-ANS fluorescence extending toward the M-540 emission band, and (*iii*) the contribution from the energy absorbed by p-Cl-ANS and transferred to M-540 molecules. Obviously, staining with p-Cl-ANS does not increase the magnitude of contribution (*i*). The fluorescence of p-Cl-ANS bound to the nerve makes little or no contribution to the range of wavelength over 620 nm; hence, contribution (*ii*) is negligible. Thus, the observed increase in the amplitude of the excitation band at 330 nm must derive from (*iii*), namely, from the process of non-radiative transfer of electronic energy between p-Cl-ANS and M-540. The increase in the amplitude of the excitation band at 330 nm was, in most of the crab nerves examined, in the range between 2 and 3-fold (see fig. 4b).

It is important to note that the excitability of the nerve under study was maintained during the entire course of these experiments. Since dye-stained nerves are known to lose their excitatbility when exposed to intense light in the presence of oxygen (see e.g., refs. [26,27]), a precaution was taken to stain the nerve and to test its excitability in complete or partial darkness. When excitability of the nerve was suppressed by exposure to strong light in the presence of O_2, there was a rapid increase in the excitation band at 330 nm.

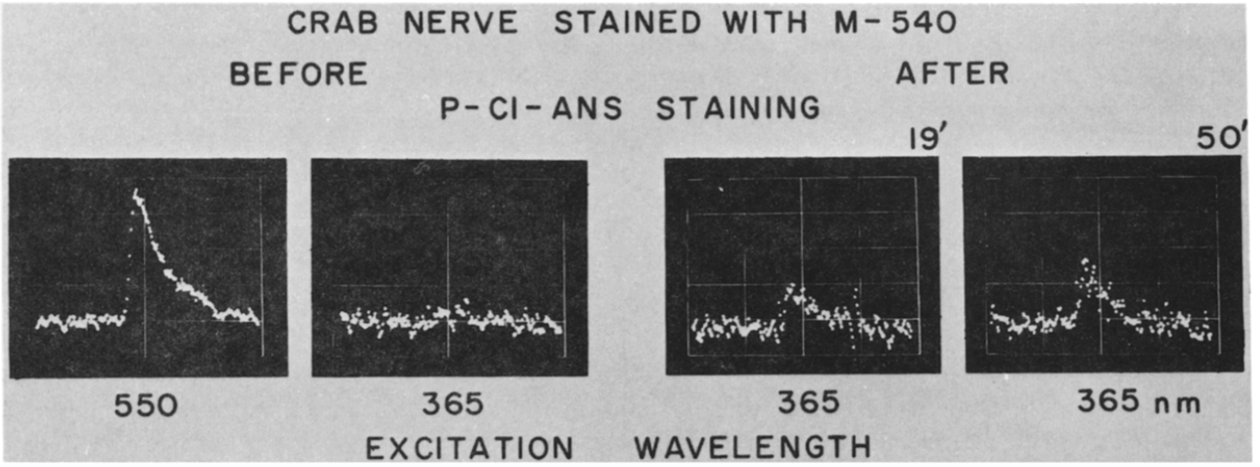

Fig. 5. Fluorescence responses recorded from a crab nerve (pre-treated with M-540) during the course of staining with p-Cl-ANS. The central wavelengths of the interference filters inserted between the light source and the nerve are indicated. The amplitude of the large signal in the first record was 9.4×10^{-4} times the background light intensity. The corresponding value for the last record was 6.1×10^{-4}. The three records obtained at 365 nm were taken at the same detector sensitivity.

3.5. Fluorescence responses mediated by energy transfer

In the presence of both p-Cl-ANS and M-540 in the nerve membrane, exciting light in the range of wavelength between 330 and 360 nm is absorbed mainly by p-Cl-ANS (see fig. 4b). If the light energy absorbed by p-Cl-ANS is transferred to M-540 under these conditions, it should be possible to record changes in the intensity of the fluorescent light emitted by these M-540 during an action potential. In fact, when a doubly stained crab nerve was electrically stimulated, a response representing a transient rise in M-540 emission was observed. Since, however, M-540 has a weak, but finite absorbance at about 330 nm, it was necessary to show that the observed signal did not derive directly from the light energy absorbed by M-540.

When a crab nerve pre-stained with M-540 is immersed in sea water containing p-Cl-ANS, there is a gradual increase in the 330–360 nm excitation band observed by reading emission in the range of wavelength between 600 and 640 nm (see fig. 4b). During the course of this gradual increase, the amplitude of the response mediated by energy transfer is expected to increase; but no increase is expected in the amplitude of the response resulting from direct light absorption by M-540. [Note that the number of p-Cl-ANS molecules does increase and the number of M-540 molecules does not increase in the nerve membrane during this period.] Thus, two possible modes of signal production, one direct and the other mediated by energy transfer, can be distinguished from each other by measuring the response amplitudes during this period of increasing incorporation of p-Cl-ANS.

The results of actual measurement indicated that there was a gradual increase in the response amplitude (fig. 5). The sensitivity of the recording system was such that practically no response was recorded from the nerve (pre-stained with M-540) before addition of p-Cl-ANS (see the second record in the figure). [Note that the wavelength of exciting light was 365 nm and emission of fluorescence was detected at the extreme red-end of the M-540 emission spectrum.] Then, the nerve was immersed in artificial sea water containing p-Cl-ANS. During the following period, there was a gradual increase in the intensity of the fluorescence of the nerve at rest. Electric stimulation of the nerve during this period was found to produce distinct fluorescence responses detected at about 620 nm. Furthermore, the response amplitude was found to increase roughly in proportion to the intensity of the background fluorescent light (see the third and fourth records), the ratio of the response amplitude to the background light intensity remaining at an approximately

constant level. Based on the results of these experiments, it was concluded that the electronic energy transferred from p-Cl-ANS to M-540 does contribute to production of fluorescence responses.

It is also important to note that the fluorescence response produced by a nerve stained externally with p-Cl-ANS alone are negative (representing a transient decrease in light intensity). The finding that p-Cl-ANS applied externally after pre-staining with M-540 brings about positive responses (fig. 5) can not be explained on the assumption that these responses are produced by p-Cl-ANS alone.

In a series of experiments using squid giant axons, we made an attempt to demonstrate fluorescence responses produced by energy transfer between p-Cl-ANS and M-540. We also examined energy transfer between p-Br-ANS and M-540 and other pairs of membrane probes. The preliminary account of these experiments may be found elsewhere [11].

4. Discussion

The ultimate goal of the present investigation is to elucidate, by the use of fluorescent probe molecules, the physicochemical properties of the membrane macromolecules in the resting as well as in the active state of the nerve. The probe molecules are thought to inform us to the physicochemical state of the macromolecules to which these probes are bound. However, the information acquired by the use of fluorescent probes is of a somewhat indirect nature (see e.g., ref. [28]).

It is obvious that a detailed knowledge as to the behavior of probe molecules in well-defined media is essential to decipher the information given to us by the membrane probes. This is the reason why a great portion of our effort was put forth to analyze physicochemical properties of the probe molecules in water and various organic solvents. At the dye-binding sites in the nerve, the factors like refractive index, dielectric constant, rigidity (or fluidity), accessibility of water, presence or absence of quenchers in the vicinity, etc. are expected to determine the properties of the fluorescent light emitted. The effects of these factors may be analyzed far more easily and directly in vitro than in isolated nerve fibers. The fluorescence properties of the dye molecules bound to various macromolecules (lysolecithin micelles and proteins) in water were also examined and compared with their behavior in organic solvents. In these experiments, it is possible to express the microenvironment around the dye-binding sites in macromolecules in terms of the polarity of the solvent which gives rise to very similar excitation and emission spectra. Various scales of solvent polarity are proposed by Mataga et al. [29], Lippert [30], Kosower [31], Reichart and Dimroth [32]. Although the question may be raised as to the legitimacy of including such factors as the depth of penetration of a dye molecule into the membrane, the effects of viscosity or temperature changes, etc. in a one-dimensional solvent --polarity scale, it seems to us this approach is the only practical means of characterizing the properties of the dye-binding sites in the nerve membrane. As has been pointed out in section 3, the fluorescent properties of the probes used in the present study vary monotonically with the polarity scale in organic solvents.

The spectrum of the fluorescent light (I) from p-Cl-ANS in a crab nerve is very similar to that of the dye dissolved in ethylene glycol (dielectric constant $D = 37$ and refractive index $n = 1.32$). Both the wavelength of maximum emission and half-bandwidth of this spectrum is intermediate between those observed in methanol ($D = 33, n = 1.330$) and in formamide ($D = 108$, $n = 1.447$). [According to Mataga and Lippert, the solvent polarity increases monotonically with the difference $(D-n^2)$.] This high value of the (effective) solvent polarity of the p-Cl-ANS binding sites may be taken as indicating that the probe molecules are only partially buried in the membrane.

The fluorescence response produced by externally applied p-Cl-ANS represents a transient reduction in the intensity of fluorescence from dye molecules with their transition moment roughly normal to the membrane surface. The electrolyte-dilution experiment reported previously [33] has indicated that at the external surface of the nerve membrane there is a layer with negative fixed charges which prevents permeation of negatively charged probes. These dye molecules may therefore be visualized as being bound to the Stern layer [34] of the membrane with the sulfonate-group directed toward the queous phase. At the peak of an action potential, the properties of the external surface layer of the membrane is expected to change. The observed spectrum, ΔI, could be explained by assuming a change in the polarity of the environment of

the dye molecules from the value for ethylene glycol to that for ethanol.

In the case of M-540, there is good reason to postulate that both monomers and dimers of the dye molecules exist in the membrane at rest. When an action potential is produced, the environment of the dye molecule changes in such a manner that a portion of dimers are converted into monomers (see fig. 3). It was possible to demonstrate such dimer-to-monomer conversion in vitro (see fig. 2c). This process of dimer-to-monomer conversion does not necessarily indicate that there is a gross displacement of the dye molecules in the nerve membrane during action potentials. The dimer band in the absorption spectra shown in figs. 2 and 3 may be regarded as arising from a parallel arrangement of the π-electron systems of the two dye molecules (see p. 250 in ref. [35]). If the separation between the two dye molecules of a dimer is of the order of 4 Å (see p. 316 in ref. [19]), a displacement of this separation appears to be sufficient to bring about the observed change in the absorption spectrum.

The excitation spectrum of M-540 in the external layer of a crab nerve (ΔI and I in fig. 3b) indicates that the wavelength of maximum absorption is at about 570 nm. This finding seems to suggest that the binding sites of M-540 are of an extremely low polarity. It is to be noted that such a medium with a low solvent polarity is favorable for formation of M-540 dimers.

The relationship between a change in the membrane potential and the fluorescence responses is not well understood at present. It is important to note that a change in the membrane potential is a macroscopic (i.e., averaged) quantity; in contrast, fluorescent membrane probes yield information about their environment of a molecular dimension. In the molecular dimension, the nerve membrane is heterogeneous. Therefore we do not anticipate finding a simple and precise relationship between the membrane potential and the optical responses. We have seen that the fluorescence responses produced by high-frequency stimulation of a crab nerve stained with M-540 "summate". This situation is analogous to that encountered by Watanabe and Terakawa [37,38]. These investigators found summation of birefringence signals in response to repetitive stimulation and concluded that there is no simple relationship between the membrane potential and the optical responses.

Demonstration of energy transfer between two kinds of fluorescent membrane probes in the external layer of a crab nerve is probably not very surprising. It indicates that the low-polarity binding sites for M-540 are surrounded by binding sites of p-Cl-ANS. Most of these p-Cl-ANS molecules are probably bound to sites which are not involved in production of fluorescence responses, because the sign of the responses mediated by energy transfer is dominated by M-540. There are more non-responsive sites in the membrane than responsive sites [15].

It seems to us that the demonstration of energy transfer in the nerve membrane has opened up a new approach toward analyzing physicochemical properties of excitable membranes. Furthermore, the present investigation has shown that measurements of light absorption during action potentials can yield significant information as to the active state of the nerve membrane. Further studies are required to find how much information can be acquired by these new approaches.

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