128. Changes in Light Absorption, Emission and Energy Transfer Produced by Electric Stimulation of Nerves Labeled with Fluorescent Probes

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In recent years a great variety of fluorescent membrane probes have been used to study changes in membrane properties during the process of nerve excitation. The present communication is designed to demonstrate three distinct modes of production of optical responses in crab nerves labeled with fluorescent membrane probes, namely (i) by absorbance changes, (ii) by emission intensity changes and (iii) by changes in the efficiency of transfer of energy from a different probe. It is expected that comprehensive studies of these optical responses lead to a better understanding of the macro-molecular mechanism of the process of action potential production.

Methods. Most of the dyes used in the present studies were purchased from Eastman Kodak Co. The dye used in one series of experiments, p-Br-anilinonapthalene-6-sulfonate, was a generous gift of Dr. Edward M. Kosower of Tel Aviv University, Israel. Initially, purchased dyes were dissolved both in water and in ethanol and the absorption spectra of the solutions were determined. The dyes were then dissolved in artificial sea water at a level of 20 to 50 μM and the stainability of a crab nerve (see below) with each of the dyes was examined. Next, the purities of dye preparations were examined by thin-layer chromatography (Brinkman cellulose and silica). After establishing the purity of a preparation, an exhaustive examination of dye-stained nerve fibers was carried out.

Claw nerves of the crab, Libinia emarginata or Callinectes sapidus, were used in most of the present studies. After dissection, the connective tissue sheath around the nerve was removed under a dissecting microscope. Then individual fibers in the nerve were divided with dissecting needles into small bundles, so that the dye could readily penetrate into the interior of the nerve bundle.

Transient changes in the intensity of the fluorescent light (i.e., fluorescence responses) of the nerve were measured by the method described in detail previously. Transient changes in the intensity...
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of light transmitted through a stained nerve during action potentials were measured by placing the nerve directly between the light source (a 100 W quartz-iodine lamp) and the detector (see Fig. 1, B). The portion of the light which did not penetrate the nerve was completely blocked by thin plates of black Lucite (D) placed on two sides of the nerve. The output of the photodetector (either a photomultiplier or a photodiode) was led to a CAT computer after amplification with an A.C.-coupled amplifier.

Results. Absorption responses. Transient changes in the intensity of light transmitted through a stained nerve during action potentials are called “absorption responses” in this communication. Fig. 2 shows two examples of absorption responses, one obtained from a crab nerve stained with rhodamine B (top) and the other with merocyanine-540 (bottom). An upward deflection of these com-
puter records represents an increase in the light intensity (a decrease in light absorption in this case). It is seen in the figure that the sign of the responses reversed at about 570 nm in the upper records and at 555 nm in the lower records.

The contributions of fluorescence changes to the observed responses were estimated by inserting proper cut-off filters (e.g., Wratten filters) between the stained nerve and the photodetector. In the case of merocyanine-540 the contribution of fluorescence to the observed responses was estimated to be about 10% or slightly less. In the case of rhodamine B, this contribution was estimated to reach about 40%. With these dyes, the sign of the fluorescence responses are known not to vary with wavelength; therefore, it is evident that the observed signal-reversal is the property of the absorption responses.

In addition to merocyanine-540 and rhodamine B, a variety of dyes could be used to demonstrate absorption responses in crab nerves. So far, we have observed absorption signals with 21 different dyes. Among these dyes, those which gave rise to absorption responses with a signal-to-background light-intensity ratio greater than $10^{-4}$ are as follows: crystal violet, 2-(p-dimethylamino-styryl)-1-ethylprimidium iodide, ethyl violet, merocyanine-540, methyl violet, Nile blue, pyronine Y and rhodamine B. On many occasions the responses were seen to reverse their sign within the range of the absorption spectrum of the dye. With some dyes (e.g., Nile blue), the sign remained unaltered over the entire range of the absorption spectrum.

Optical responses mediated by energy transfer. Transient changes in the intensity of fluorescence from nerve fibers labeled with various dyes during action potentials were extensively investigated previously.\(^1\).\(^2\).\(^4\) The probes examined previously were chosen as candidates for donors in the present attempt to demonstrate energy transfer between two dyes in the nerve membrane. Resonance transfer of electronic energy\(^5\) can take place only when the emission spectrum of the donor overlaps the absorption spectrum of the acceptor.

Various combinations of candidates for donors and acceptors were tried on crab nerves. Obviously, the conditions that have to be satisfied by these two dyes are quite demanding. Even when a pair of membrane probes were found to stain the nerve membrane rather well, frequently no evidence was obtained indicating transfer of energy between the two probes. When a fluorescence response was observed suggesting significant contribution of transfer of energy from one dye to the other, the following two questions had to be
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answered: (i) whether or not the secondary filter (F2 in Fig. 1, A) was effective in blocking the fluorescent light emitted by the donor molecules, and (ii) whether or not the observed response was produced by direct excitation of the acceptor molecules by the incident light. Unless both of these questions are answered, it is not possible to prove or disprove that the response in question is mediated by energy transfer. We believe that the following observations in which 2-p-Br-anilinonaphthalene-6-sulfonate (abbreviated as p-Br-ANS) and merocyanine-540 (M-540) were chosen as the donor and the acceptor, respectively, have satisfactorily demonstrated the existence of responses mediated by energy transfer.

The absorption and emission spectra of p-Br-ANS are very similar to those of 2-p-toluidinynaphthalene-6-sulfonate studied by McClure and Edelman.6) Adsorbed to a crab nerve, p-Br-ANS had an emission maximum at around 420 nm. M-540 is known to produce large fluorescence responses when electrically stimulated under illumination by lightwave at 550 nm.7) One of the reasons for choosing these dyes is that externally applied p-Br-ANS produces negative responses, whereas the responses produced by extracellularly administered M-540 are positive. Based on this difference in sign between the two dyes, it was possible to determine which one of the dyes in the nerve membrane was contributing to optical responses.

A typical example of the records obtained from 9 different nerves is shown in Fig. 3. A desheathed crab nerve was immersed in artificial sea water containing M-540 (1 mg/50 ml) for a period of about 60 min. Next, the stained nerve was transferred into a nerve chamber (see ref. 4), and by inserting a Wratten 29 filter (which cuts off lightwaves shorter than 600 nm in wavelength) between the photomultiplier and the M-540 stained nerve, a record
of fluorescence responses was obtained (see the 1st record in the figure). At this stage (see the 2nd record), practically no fluorescence response was observed when 365 nm excitation wavelength was chosen. Then, the dye-free artificial sea water in the chamber was replaced with one containing p-Br-ANS (1 mg/100 ml). During the course of progressively deeper staining with p-Br-ANS, the procedure was repeated from time to time to record fluorescence responses at the same excitation wavelength (365 nm) and with the same secondary filter (Wratten 29). As can be seen in the last two records, definite responses were observed from a nerve stained both with M-540 and p-Br-ANS. Furthermore there was a gradual increase, with time, in the amplitude of the recorded responses.

There was a small, gradual loss of M-540 bound to the nerve during this period of progressive staining with p-Br-ANS; hence, the increase in the amplitude of the observed response cannot be attributed to direct excitation of M-540 by the incident light. The sign of the fluorescence response produced by a crab nerve stained with p-Br-ANS alone is negative. Since the sign of the observed responses is positive (representing an increase in the fluorescence intensity during action potentials), we believe that the response shown by the 3rd and 4th records in the figure can safely be attributed to changes in M-540 fluorescence excited by resonance transfer of electronic energy from p-Br-ANS.

**Discussion.** Since fluorescence probes have been introduced to study properties of the axonal membrane during action potential, we have concentrated mainly on physicochemical analyses of optical responses produced by changes either in the fluorescence quantum yield or in the emission spectrum of the probes in the nerve membrane. Investigations into the mechanism of production of absorption responses are relatively new. The absorption spectrum of a crab nerve stained with M-540 was found to show two distinct maxima, one at 528 nm and the other at 565 nm. An extensive study of this dye *in vitro* indicated that the absorption band with a maximum at 528 nm represents dimers and that at 565 nm represents monomers of the dye molecules. With this dye, the absorption responses were produced by conversion of a portion of dimers into monomers. Similar results were reported recently by Ross *et al.*

In general, the dyes of which the absorption spectra are sensitive to the polarity of the solvents are considered to possess a large dipole moment in the ground state of the molecules. This class of dyes could be used to detect absorption changes during action potentials. A large change in the absorption spectrum during action potentials, sometimes associated with conversion of dimers into monomers, is
considered to be the cause of production of absorption responses with these dyes. In some dyes (e.g., methyl violet), the dependence of the absorption responses on the wavelength of the measuring light was found to be very complex.

The fluorescence responses shown in Fig. 3, right, are interpreted as being mediated by transfer of energy from one dye species to another in the nerve membrane. To prove that Förster's criteria for resonance transfer of electronic energy \(^{12}\) are satisfied in these experiments, we have examined these dyes in vitro extensively and determined the overlap of the donor emission spectrum and the acceptor absorption spectrum. The results of those studies, which strongly support the interpretation stated above, will be published elsewhere.\(^9\) Demonstration of energy transfer within and across the nerve membrane is expected to lead to a better understanding of the macromolecular organization of the excitable membrane.

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