

# CHANGES IN FLUORESCENCE, TURBIDITY, AND BIREFRINGENCE ASSOCIATED WITH NERVE EXCITATION

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*Communicated by Hallowell Davis, September 3, 1968*

The objective of this paper is to offer experimental evidence which shows that the process of excitation in the nerve is accompanied by a transient change in optical properties of the nervous tissue. The optical properties examined include fluorescence, turbidity, and birefringence.

Changes in fluorescence were examined after nervous tissues were stained with the dye 8-anilino-naphthalene-1-sulfonic acid (ANS).<sup>1</sup> Our search for fluorescence under these conditions was prompted by the work of Aronson, Detert, and Morales,<sup>2</sup> who demonstrated that the fluorescence of ANS is extremely sensitive to conformational changes of various macromolecules.

Our attempt to measure turbidity changes of the nerve during excitation was made with a view to extending the work reported by Cohen and Keynes<sup>3</sup> by the use of monochromatic light.

The observation by Inoue and Sato<sup>4</sup> of rapid changes in the mitotic figure of the *Pectinaria* oöcyte under a polarizing microscope aroused our interest in the birefringent properties of the nerve during excitation. Our experiments along this line were greatly accelerated when we encountered a very significant paper by Cohen *et al.*<sup>5</sup> on this subject in the early stages of our investigation.

Changes in the optical properties of the nerve during excitation are very small. In fluorescence and turbidity studies, optical signs of nerve excitation could not be measured without the use of a computer to average multiple responses. However, in birefringence studies, it was possible to record optical signs of nerve excitation directly on an oscillograph screen.

*Method.*—Most of the present fluorescence studies were carried out with nerve trunks from the legs of lobsters (*Homarus americanus*) and spider crabs (*Libinia emarginata*) and with the fin nerves of squid (*Loligo pealii*). Nerves were immersed in sea water containing 0.05 mg/ml ANS (Baker Chemical Co. or Eastman Organic Chemicals) for a period of 15–20 min. The nerves were then transferred to a chamber made of black acrylic plastic filled with sea water without dye as shown diagrammatically at the top of Figure 1. The nerve chamber was provided with two pairs of platinum electrodes (not shown in the figure): one pair for delivering electric stimuli from a Grass stimulator to the overlying nerve, and the other pair for recording action potentials near the end of the nerve.

Various types of light sources were used during the course of the present experiments; an Osram quartz-iodine lamp operated at d-c 15 volts and approximately 150 watts was used in the later stage of the present study as a source of near-visible ultraviolet light. Quartz lenses  $L_1$  and  $L_2$  (Fig. 1) were used to condense the light on a 1–3-mm portion of the nerve. Optical filter  $F_1$ , used to absorb visible light, was either a Corning glass filter (CS 7-83) or a Bausch and Lomb interference filter for 365 m $\mu$ . Visible light emitted by the nerve was detected with a photomultiplier tube (RCA 4463) at a right angle to the direction of the incident light. Filter  $F_2$ , used to absorb the incident light scattered by the nerve, was either a Corning filter CS 3-72 (transmitting visible light longer than 430 m $\mu$  in wavelength) or a Corning filter CS 5-75 (transmitting a narrow spectrum around 460 m $\mu$ ).

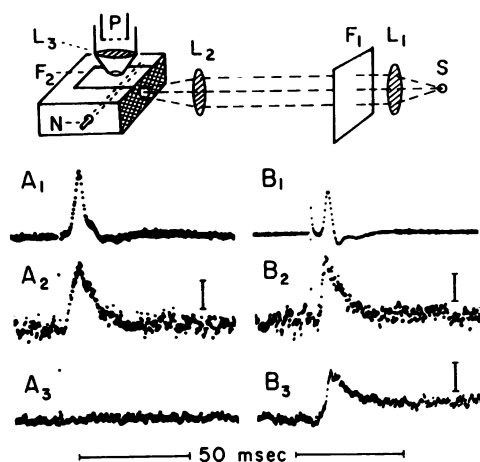


FIG. 1.—Demonstration of fluorescence changes associated with nerve excitation.

*Top:* Schematic diagram of the optical arrangement used. *S* represents the light source; *L*<sub>1</sub>, *L*<sub>2</sub>, *L*<sub>3</sub> the lenses; *N* the nerve; *P* the photomultiplier; *F*<sub>1</sub> the ultraviolet-transmitting filter; and *F*<sub>2</sub> the ultraviolet-absorbing filter. (Electrodes for stimulating the nerve and for recording action potentials are not shown.)

*Bottom:* (*A*<sub>1</sub> and *B*<sub>1</sub>) Action potentials and (*A*<sub>2</sub> and *B*<sub>2</sub>) optical signals observed with nerves from spider crabs. (Right- and left-hand columns were from two different nerves.) A CAT computer was used to record both action potentials and optical signals. The amplitude of these extracellularly recorded action potentials was approximately 2 mv. The vertical bars in the second row represent (*left*)  $10^{-4}$

and (*right*)  $2 \times 10^{-5}$  times increase in light intensity. Record *A*<sub>3</sub> was taken with filter *F*<sub>2</sub> moved to a position between *L*<sub>2</sub> and *N*. Record *B*<sub>3</sub> represents the time course of change in light scattering at 90° observed in the stained nerve; the vertical bar represents a  $4 \times 10^{-6}$  times increase in light intensity. The temperature was 19°C.

The optical arrangement used for studying turbidity changes was similar to that for the fluorescence studies. A Bausch and Lomb interference filter was used (at position *F*<sub>1</sub> in Fig. 1) to obtain monochromatic light of the desired wavelength. No optical filter was placed between the nerve and the photomultiplier tube. A 3–4-mm portion of unstained nerve was exposed to the monochromatic light, and the scattered light was detected at 90°. Maximal stimuli to the nerve were repeated at a rate of 1–10 per second.

In the birefringence studies, the nerve was illuminated from below through a hole in the nerve chamber. A small image (approximately 0.3 mm in diameter) was focused on the nerve, and the transmitted light was detected at 0°. A polarizer (Polaroid film) was positioned between the focusing lens and the nerve, and an analyzer between the nerve and the photomultiplier tube. In agreement with the results reported by Cohen *et al.*,<sup>5</sup> the best optical signs were obtained with the polarizer and the analyzer in crossed positions and the nerve at a 45° angle to both. (For the theoretical background of this crossed polar arrangement for detection of weak birefringence, see p. 346 in Drude.<sup>6</sup>) In some instances a quarter-wave plate (Spindler and Hoyer, Göttingen) was introduced between the nerve and the analyzer.

In most of our birefringent experiments, the output of the photomultiplier was fed to a Bak unity gain electrometer and then to a Tektronix oscilloscope. In the fluorescence and turbidity experiments, the optical signal detected by the photomultiplier tube was amplified with a capacity-coupled amplifier (Tektronix type 122) and was fed to a Nema-tron CAT computer. A Grass camera was used to photograph the images on the oscillograph or computer screen.

*Results.—Fluorescence:* Nerves from lobster, crab, and squid emit visible light when they are stained with ANS and then irradiated with ultraviolet light; a faint greenish color can be seen in the region irradiated with 365 m $\mu$  light. The intensity of the fluorescence was found to increase when the nerve was stimulated electrically (see records *A*<sub>2</sub> and *B*<sub>2</sub> in Fig. 1). The increase in fluorescence started at about the time of arrival of the nerve impulse at the site of ultraviolet irradiation. The light intensity rapidly rose to its maximum and then gradually declined toward the resting level of fluorescence. (Since we have used nerve trunks

in these studies, the precise temporal relationship between the action potential and the transient fluorescent change could not be determined.)

The wavelength of the light emitted during nerve excitation was estimated by the use of a series of Corning sharp-cut yellow and red filters, Corning blue filters, and Bausch and Lomb interference filters. The optical signal associated with nerve excitation was strongly reduced or completely suppressed when emission in the range between 400 and 500  $m\mu$  was prevented by the secondary filter  $F_2$  from reaching the photomultiplier tube. Large optical signals were obtained when the secondary filter transmitted blue light of approximately 450  $m\mu$ . No optical signal was observed when filter  $F_2$  (ultraviolet light-absorbing) was inserted between lens  $L_2$  and the nerve (see record  $A_3$  in Fig. 1); this observation was made to demonstrate that the phenomenon under study is not due to the scattering of light leaking through the filters. No transient optical signal was observed when ANS pretreatment of the nerve was omitted in the experimental procedure adopted in the present fluorescence studies.

The increase in fluorescence during excitation of a crab nerve was, in most cases, in the range  $2-3.5 \times 10^{-4}$  times the light intensity observed before stimulation. The corresponding value for the lobster leg nerve was approximately  $5 \times 10^{-5}$ . Our attempt to determine the degree of depolarization of the fluorescence yielded no significant results because of insufficient light intensity.

With crab nerves pretreated with ANS, it was possible to demonstrate a transient change in the intensity of light scattered by the nerve during excitation (see record  $B_3$  in Fig. 1). The differences in the time course and magnitude between light scattering and fluorescence will be discussed below.

*Turbidity:* Changes in light scattering during excitation were observed in nerve trunks from lobster, crab, and squid. With 550  $m\mu$  monochromatic light, the change in the light intensity scattered by crab nerves was found to be within a range of  $0.8-5.7 \times 10^{-5}$  times the intensity at rest. This value is in good agreement with that obtained by Cohen *et al.*<sup>5</sup> using white light. The corresponding values for lobster and squid fin nerves were  $1.0-7.6 \times 10^{-5}$  and  $0.9-5.8 \times 10^{-5}$ , respectively (see Fig. 2).

The dependence of the change in light scattering during excitation of crab nerves upon the wavelength of the light employed was examined with a series of interference filters (Bausch and Lomb). Distinct optical signals were observed with monochromatic light of all the wavelengths examined between 365 and 650  $m\mu$ . Neither the magnitude nor the time course of the optical signal was significantly affected by the difference in wavelength.

On several occasions polarized light was used to measure scattering. The intensity of light scattered at  $90^\circ$  was found to be much stronger when the electric vector of the incident light was parallel to the long axis of the nerve than when it was at a right angle. However, neither the time course nor the magnitude (expressed as a ratio of change in light intensity to background intensity) appear to depend on the plane of polarization relative to the axis of the individual nerve fibers.

The duration of the light-scattering change (at  $90^\circ$ ) during excitation was longer than the fluorescence or the birefringence changes associated with nerve stim-

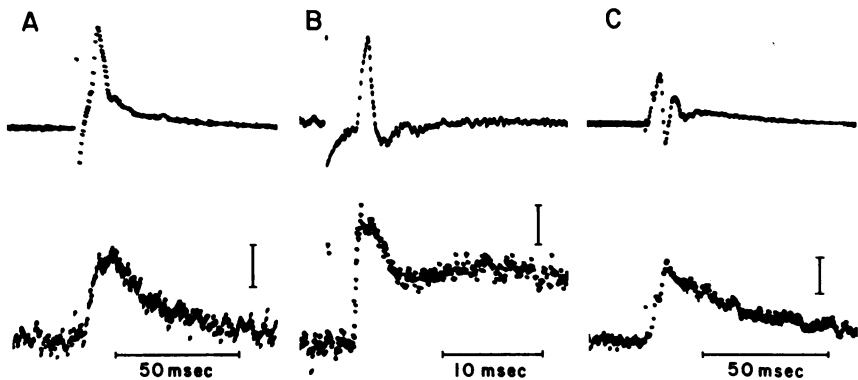


FIG. 2.—(Top) Action potentials and (bottom) changes in light scattering at  $90^\circ$  associated with stimulation of (A) a spider crab nerve, (B) a squid fin nerve, and (C) a lobster leg nerve immersed in natural sea water. Action potentials were recorded extracellularly and were 1–2 mv in amplitude. The vertical bars represent  $10^{-5}$ ,  $2 \times 10^{-5}$ , and  $3 \times 10^{-5}$  times increase in light intensity for A, B, and C, respectively. A CAT computer was used for recording. The temperature was  $19^\circ\text{C}$ .

ulation. Record  $B_3$  in Figure 1 was taken from the same axon that had been used previously to show a transient change in fluorescence (record  $B_2$  in Fig. 1). For  $B_3$  the light source was visible monochromatic light of  $550 \mu$  in wavelength. As seen in Figure 1, the time course of the light-scattering change was longer than that of the fluorescence change. Furthermore, the magnitude of the light-scattering change (expressed as a ratio of change in light intensity to background intensity) was much smaller than that of the fluorescence change.

In squid fin nerves, the falling phase of the turbidity signal (see record B in Fig. 2) was long and complex. A small secondary component that rises after the initial response is noted. (See *Discussion*.)

*Birefringence:* When a nerve from a lobster or a crab was placed at an angle of  $45^\circ$  to the plane of polarization of the monochromatic incident light, there was a transient decrease during excitation in the intensity of the transmitted light seen through an analyzer at an angle of  $90^\circ$  to the polarizer. The optical signal associated with nerve excitation under these conditions was large enough to be recorded with a cathode-ray oscillograph without the use of a computer (see records A and B in Fig. 3). No clear optical signal was obtained when the nerve was parallel to either the principal axis of the polarizer or to that of the analyzer. These findings are quite consistent with those recently reported by Cohen, Keynes, and Hille.<sup>5</sup>

It was possible to reverse the sign of the optical signal by introducing a quarter-wave plate between the nerve and the analyzer (see record C in Fig. 3). This reversal was achieved by placing the zero axis of the quarter-wave plate parallel to the plane of polarization of the incident light and then rotating the plate beyond the point of complete cancellation of the birefringence of the resting nerve. A similar reversal of the optical signal was reported recently by Cohen *et al.*<sup>5</sup>

The temporal relationship between the action potential and the optical signal under birefringence conditions was examined in squid giant axons. A potential-

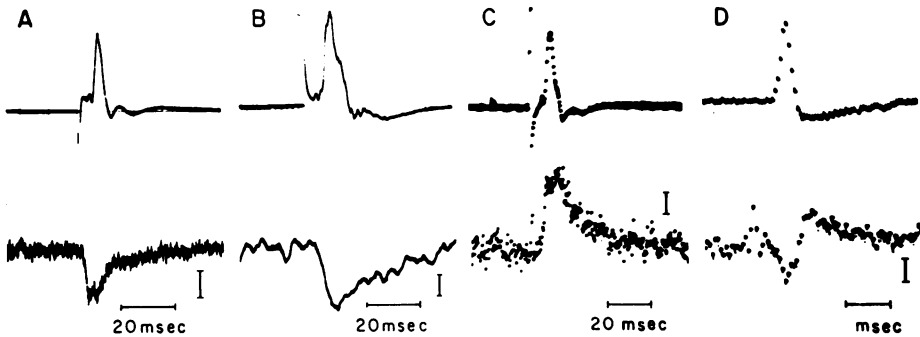


FIG. 3.—(Top) Action potentials and (bottom) birefringence changes associated with stimulation of (A, B, and C) nerves from spider crabs and (D) nerve of a squid giant axon.

Records A and B were taken by means of single sweeps of a dual-beam oscillograph (without a computer); records C and D were taken with a CAT computer. In A, C, and D, the external medium used was natural sea water. In B, the medium contained 300 mM hydrazinium chloride and 100 mM calcium chloride; a 1-kc low-pass electric filter was used to reduce noise. Record C was obtained by inserting a quarter-wave plate between a nerve and the analyzer. (Note that the optical signal is reversed.) The vertical bars represent (A and B)  $5 \times 10^{-4}$ , (C)  $5 \times 10^{-5}$ , and (D)  $10^{-6}$  times the background light intensity. A downward deflection of the lower trace represents a decrease in light intensity. The temperature was 19°C.

recording electrode was placed within 2 mm of the site of optical recording (record D in Fig. 3). The optical signal observed was found to start approximately at the time of arrival of the nerve impulse at the site of optical recording, and the peak of the optical signal coincided with the peak of the action potential. The optical signal was found to show much more distinct biphasicity than the intracellularly recorded action potential.

The dependence of birefringence changes upon the wavelength was examined in crab nerves with a series of interference filters. As in the turbidity experiments, neither the magnitude nor the time course of the optical signal was significantly affected by varying the wavelength between 450 and 650  $m\mu$ .

Birefringence changes were also examined in crab nerves immersed in a medium containing 300 mM NaCl and 100 mM  $CaCl_2$  instead of natural sea water; no significant difference in the magnitude and time course of the signals was observed in the two different external media. Substitution of  $SrCl_2$  for  $CaCl_2$  in the external medium brought about little change in either the optical or the electrical signals associated with nerve excitation. However, substitution of hydrazinium chloride for NaCl produced a distinct prolongation in both electrical and optical signals (see record B in Fig. 3). Replacement of approximately 50 per cent of the water with  $D_2O$  did not produce any drastic change in the optical signal.

*Discussion.*—The experimental findings described in this paper showing the presence of optical changes during nerve excitation are consistent with the view that the process of excitation is accompanied by conformational changes in macromolecules of the nerve.<sup>7-9</sup> However, it is very difficult, at least at the present stage of our investigation, to determine the anatomical structure in which the suspected conformational changes take place. There is an enormous

increase in the rate of interdiffusion of cations across the nerve membrane during excitation.<sup>10</sup> Consequently, it is expected that the ionic composition in the immediate neighborhood (within 1  $\mu$ ) of the nerve is rapidly and drastically altered during excitation. Such an alteration could bring about the observed changes in optical properties of the nerve.

Record *B* in Figure 1 shows that the change in turbidity following nerve stimulation outlasts the fluorescence change under the same experimental conditions. This fact can be interpreted as an indication that the zone involved in the turbidity change is larger than that involved in the fluorescence change. A somewhat similar view has been stated recently by Cohen *et al.*<sup>5</sup>

The intensity of light observed with the nerve at rest under the conditions of our turbidity and birefringence studies is strongly affected by small changes in the Na-Ca concentration ratio in the external medium. Since such small changes in the Na-Ca concentration ratio are not expected to alter the membrane potential, the optical changes observed under these conditions appear to be unrelated to the membrane potential.

It is known that the dye in our fluorescence studies, 8-anilino-naphthalene-1-sulfonate (ANS), does not fluoresce unless the dye molecules are bound to the hydrophobic sites of the macromolecules.<sup>1</sup> When ANS is introduced into the interior of a squid giant axon by the internal perfusion technique, it is localized within the axon interior, which indicates that the dye molecules do not penetrate the axon membrane. From this fact, it may be inferred that the extracellularly applied ANS remains outside the axoplasm and consequently that the observed fluorescence change during excitation does not involve the axoplasm.

Further studies are required to determine the physical-chemical nature of the optical changes associated with nerve excitation.

*Summary.*—Nerves from crabs and lobsters stained with 8-anilino-naphthalene-1-sulfonate fluoresce under ultraviolet illumination; this fluorescence increased during excitation of the nerve. Changes in light scattering and in birefringence were also observed during excitation of various invertebrate nerves.

It is a pleasure to acknowledge our debts to Dr. M. Morales who suggested using ANS for detecting fluorescence changes, to Dr. S. Inoue who advised us in our birefringent studies, and to Dr. William Barry who helped to perfect our experimental methods.

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