11. Fast and Slow Rotation of Dye Molecules in Squid Axon Membrane during Excitation\textsuperscript{a,\textdagger}

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In a recent communication,\textsuperscript{1) experimental evidence was presented indicating that the orientation of dye molecules incorporated non-covalently into a carb nerve can be changed with electric stimuli applied to the nerve. In the present communication, the conclusions reached from the previous studies were substantiated by using squid giant axons stained with a variety of dyes. Furthermore, it was found that a prolonged change in the membrane potential can produce a slow type of rotation which is quite distinct from the process of re-orientation of dye molecules during normal action potentials.

Demonstration of rotation of dye molecules in the nerve membrane is based on the following principle. Absorption of light by a dye molecule reaches a maximum when the orientation of the absorption oscillator (or the transition moment) of the molecules coincides with that of the electric vector of the incident lightwave. When a number of dye molecules with nearly random orientation rotate and eventually assume orientations roughly perpendicular to the membrane surface, the absorbance measured with light polarized in the direction parallel to the membrane must fall, and simultaneously the absorbance determined with light polarized in the perpendicular direction must rise. In squid axon membranes stained with various dyes, such reciprocal changes in light absorption can actually be observed during the process of nerve excitation. Long pulses of electric currents through the membrane can also be used to induce similar dichroic changes in light absorption.

Giant axons taken from squid, \textit{Loligo pealei}, available in Woods Hole, Mass., U. S. A., were used in most of the present experiments. Following extensive cleaning of a short portion, the axon was stained with various dyes either by immersing dye-containing artificial sea water or by injecting an isotonic potassium fluoride solution (at pH 7.3) containing a dye. Dyes were obtained from both Eastman

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Kodak Company and Nippon Kankoh-Shikiso Kenkyusho. The experimental setup used was similar to that employed previously.\textsuperscript{1)} In a series of experiments (see Fig. 1), a small piece of cover-glass partially covered with black tape was placed on the portion of the axon under study. A narrow (0.1–0.2 mm wide and 3–4 mm long) slit in the black tape allowed only a narrow beam of light to pass through to be detected by the photo-diode. Two examples of the results obtained from axons stained externally with crystal violet (1 mg in 250 ml of sea water) are shown in Fig. 1. After a staining period of approximately 30 min, the cover-glass with a narrow slit was placed on the axon in such a manner that the slit was located either along the middle portion, or along the edge of the axon (Fig. 1, insets). Fig. 1 represents the relative size of the change in light intensity ($\Delta I/I$) associated with action potentials, plotted against the wavelength of the light beam employed. The two sets of the observed values in each diagram show the results obtained with light polarized either in the parallel or perpendicular direction relative to the long axis of the axon. At the edge of the axon, the effect of polarization of the light beam on the spectrum of the optical responses was very strong, but in the middle of the axon, the effect was very small or absent. A similar observation with other dyes was made by Ross.\textsuperscript{2)} The dichroism was distinctly seen in a limited range of the wavelength (Fig. 1, left). The optical response recorded under parallel polarization, $\Delta I_{\|}$, was always positive in the dichroic range of the wavelength, representing a transient increase in the light intensity, or a transient decrease in light absorption, during action potentials. In agreement with the expected reciprocity between $\Delta I_{\|}$ and $\Delta I_{\perp}$ at the edge of stained axons, the optical responses recorded

![Fig. 1. Wavelength dependence of the optical responses recorded from squid giant axons stained with crystal violet. The ordinate represents the relative intensity of the light beam transmitted through the edge (left) and the middle (right) of the axons, 1.0 corresponding roughly to $1.7 \times 10^{-8}$ times the background light intensity. Examples of the optical responses obtained at 540 nm are shown; the time markers represent 5 msec intervals.](image-url)
under perpendicular polarization, \( \Delta I_\perp \), were negative at the wavelength of maximum dichroism. The following dyes yielded distinctly dichroic spectra: crystal violet, ethyl violet, 2-(p-dimethylamino-styryl)-1-ethylpridinium iodide and its analogous compounds (NK-90, NK-630 and NK-857 synthesized at Nippon Kankoh-Shikiso Kenkyusho). Bis-[1, 3-diethyl-2-thiobarbituric acid-(5)]-pentamethinoxonol and its trimethin analogue also showed remarkably dichroic spectra in crab nerves. With all these dyes, \( \Delta I_{II} \) was positive and \( \Delta I_\perp \) negative at the wavelength of maximum separation between \( \Delta I_{II} \) and \( \Delta I_\perp \).

The duration of the action potential in a squid giant axon can be prolonged by intracellular injection of tetraethylammonium (TEA) ion. By using giant axons into which a mixture of TEA and a dye was injected, slowly developing optical responses were discovered. In general, the optical responses observed under these conditions consisted of two components, fast and slow. The slow component predominated when the wavelength of the light was close to the "reversal point" of the spectrum obtained without TEA (e.g., 600 nm in axons stained with crystal violet, see Fig. 1). Examples of slow responses recorded from axons with TEA and dyes internally are shown in Fig. 2A and B. The optical responses developed far more slowly than the intracellularly recorded electric responses. With most of the dyes examined, the sign of the optical responses produced by hyperpolarizing voltage pulses was opposite to that of the responses associated with action potentials. The optical responses associated with hyperpolarizing voltage pulse (see Fig. 2C and D) had a time constant between 10 and 15 msec (17°C). The optical responses to hyperpolarizing voltage pulses recorded in a Ca-free medium (containing 100 mM MgCl₂, 400 mM NaCl and 5 mM EGTA, pH 8.0) were compared with those obtained from the same axon immersed in a Ca-rich medium (containing 100 mM CaCl₂ and 405 mM NaCl, pH 8.0). The optical response to voltage pulses of -100 mV recorded in the Ca-rich medium was 1.6–3.0 times as large as that observed in the Ca-free medium (see Fig. 2D). This reversible effect of Ca-Mg exchange was demonstrated in giant axons stained with crystal violet and with 1, 1', 3, 3, 3', 3'-hexamethylindo-dicarboxyamine. A fall of the ambient temperature from 17° to 6°C reduced the response amplitude to a level of 0.4–0.5, and increased the time constant slightly (less than 20%).

Changes in the absorbance of dye molecules incorporated in squid giant axons can be described by slightly modified expressions of the mathematical formulae described previously, and will be pre-
sented elsewhere. The kinetic aspect of the process of the slow rotation of dye molecules in the nerve membrane is not well understood at present. The small effect of lowering the temperature suggests that the process is diffusion controlled. The observed effect of Ca-ions indicates that this process resembles that of production of slow birefringence signals.\textsuperscript{3} It seems plausible that the ordered fibrous protein layer which is known to exist underneath the axolemma\textsuperscript{4} is involved in production of these slow processes.

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