gents like 2-phenoxyethanol make cooling facilities unnecessary in the field or during transportation to the laboratory (*20*).

MIKIYE NAKANISHI
ALLAN C. WILSON, RICHARD A. NOLAN
GEORGE C. GORMAN
GEORGE S. BAILEY
Department of Biochemistry and
Museum of Vertebrate Zoology,
University of California, Berkeley

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7. Phenoxyethanol, even at a final concentration of 2 percent in the reaction mixture, does not interfere with the enzyme assay.
8. An enzyme unit is defined as that amount of lactate dehydrogenase which produces an absorbance change of 1.0 per minute at 340 nm in a 3-ml reaction volume at 23°C and is approximately equal to 1 μg of lactate dehydrogenase or 1 international unit (at 30°C).
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11. Serum albumin was purified from chicken serum by ion exchange chromatography and gel filtration on Sephadex G-100. A rabbit was immunized by a primary injection with 0.5 mg of purified albumin emulsified in Freund's complete adjuvant. Twenty-three days later the rabbit was bled. The resulting antiserum appeared to be directed against only albumin, as judged by the results of immunodiffusion, immunoelectrophoresis, and quantitative microcomplement fixation tests with both pure albumin and crude serum (R. A. Nolan and A. C. Wilson, unpublished data).
12. After storage of chicken tissues for 1 year in 2 percent phenoxyethanol at room temperature, H_4-lactate dehydrogenase and serum albumin were still readily detectable but less than 1 percent of the original malate dehydrogenase and M_4-lactate dehydrogenase remained. Moreover, the surviving lactate dehydrogenase and serum albumin were now slightly modified in their electrophoretic and immunological properties. There was no gross evidence of microbial contamination, and, except for slight discoloration, the tissue appeared normal. After a year, the tissue weighed 95 percent of the starting value, indicating that little autolysis had occurred.
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17. Thermostability studies were carried out with chicken H_4-lactate dehydrogenase, as in (*6*), except that phenoxyethanol was included at a final concentration of 2 percent in the heating mixture. Phenoxyethanol made the enzyme more sensitive to thermal denaturation. Thus, the temperature required for 50 percent inactivation in 20 minutes was lowered by 15°C in the presence of 2 percent phenoxyethanol. If sucrose (0.25*M*) was also present in the heating mixture, the enzyme was partially protected from the denaturing effect of phenoxyethanol.
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20. Once in the laboratory, samples should be refrigerated or frozen. When frozen, each of the proteins discussed, including the least stable one, mitochondrial malate dehydrogenase, survives for many years.
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23. Supported by research grants awarded by NSF (GB-6420 and GB-6944), a training grant awarded by NIH (T1-GM-31-10) and the Work Study program. Two of us (R.A.N. and G.C.G.) were supported by postdoctoral fellowships from NIH. We thank T. R. Howell and G. L. McLean for their help in obtaining seed snipe tissues and A. G. C. Grandison and R. W. Sims for informing us about the observations that zoologists at the British Museum have made with phenoxyethanol as a preservative. We also thank G. F. Sensabaugh for helpful discussions.

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Fluorescence Changes during Conduction in Nerves Stained with Acridine Orange

Abstract. *Nerves from spider crabs and squid fluoresce when stained with Acridine Orange. The intensity of fluorescence increases during nerve conduction. Prolongation of the electric response in the squid axon is associated with a fluorescence change of similar duration. These findings suggest that the physicochemical properties of the macromolecules around the dye molecules in the nerve membrane drastically change during the process of nerve conduction.*

The process of conduction of a nerve impulse is considered by several investigators (*1*) to involve a reversible cooperative change in conformation of the macromolecules in the nerve membrane. Recent studies of various optical properties of nerves (*2*) support the view that some kind of conformational change does take place in the membrane during conduction. This report presents our findings that the fluorescence yield of a nerve stained with Acridine Orange (AO) changes during nerve conduction, and suggests that this change is a sign of a conformational change of the membrane macromolecules.

Optical properties of complexes of AO with polynucleic acids, polypeptides, and other macromolecules have been studied as a means of elucidating the secondary and tertiary structure of the macromolecules (*3–5*). Variations in the absorption and emission spectra of AO-macromolecule complexes have been analyzed on a statistical and quantum-mechanical basis (*5*). Acridine Orange has also been used as a vital stain for nerve cells and fibers (*6*). Many biologists believe that the fluorescence of tissues stained with AO is a sensitive indicator of the state (or "vitality") of the cells (*7*). For these reasons, a study of the changes in fluorescence of the AO-membrane complex is expected to yield significant information concerning the state of the membrane macromolecules during nerve conduction.

Nerves taken from spider crabs (*Libinia emarginata*) and from North Atlantic squid (*Loligo pealei*) were used in the present studies. The dye, AO, was dissolved in either artificial seawater (for external application) or a potassium phosphate–glycerol solution containing 300 meq of K^+ per liter (for internal application). The concentration of AO was between 0.05 and 0.1 mg/ml. Crab nerve fibers were stained by immersion of a 5- to 8-mm portion of the nerve in the AO solution for approximately 10 minutes and subsequently washed with seawater free of dye. Intracellular application of AO was done by perfusing the interior of a 4-mm-long portion of a squid giant axon with the AO-containing solution for a period of 5 to 10 minutes; the AO solution was kept in the axon interior during the fluorescence measurements. The technique of intracellular perfusion has been described previously (*8*).

A stained nerve was mounted in a chamber (made of black lucite) filled with artificial seawater (see Fig. 1, top). The chamber was provided with two pairs of platinum electrodes. One pair of the electrodes was used to

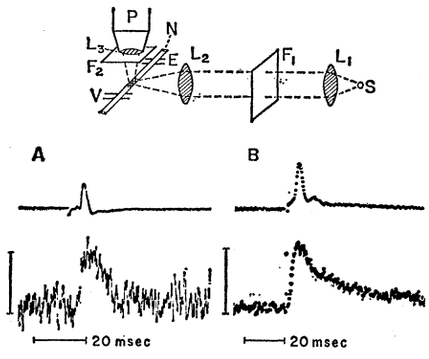

Fig. 1. (Top) Schematic diagram of the experimental arrangement used for the detection of fluorescence changes associated with nerve conduction. The letter S represents the light source; L_1, L_2, and L_3, lenses; F_1 and F_2, optical filters; N, nerve; E and V, stimulating and recording electrodes, respectively; and P, photomultiplier tube. (Bottom) Electric (top) and optical (bottom) responses obtained from crab nerves stained with Acridine Orange; the vertical bars indicate an increase of 5×10^{-4} (A) and 2.5×10^{-4} (B) times the background intensity. In record B, the noise level of the optical signal was reduced by the use of a CAT computer. Temperature was 19°C.

stimulate the nerve with brief electric pulses and the other pair to record the electric response of the nerve. The portion of the nerve stained with AO was illuminated with quasi-monochromatic light of 465 mμ wavelength obtained with a d-c–operated 120-watt quartz-iodine lamp (S) and an interference filter (F_1). The fluorescent light emitted by the nerve was detected with a photomultiplier (RCA 4463) at approximately 90°C. A secondary filter (F_2) was placed between the nerve and the photomultiplier to cut off light waves shorter than 507 mμ in wavelength. The output of the photomultiplier was led to a Bak electrometer (Life Science) and then to a cathode-ray oscillograph (see record A in Fig. 1). Because of the high noise level superposed on the optical signal, a CAT computer (Mnemotron) was frequently used in conjunction with a Tektronix amplifier to increase the signal-to-noise ratio.

Two examples of the records obtained from spider crab nerves by this technique are presented in Fig. 1. The upper oscillograph trace in record A shows the electric response of the nerve. The optical signal superposed on a random noise is shown by the lower trace. In record B, the signal-to-noise ratio of the optical signal was increased by the use of a CAT computer. The optical signal observed in this manner represented an increase in the intensity of fluorescence. The peak value of the increase, expressed as a ratio of the change in light intensity to background intensity, was on the order of 2×10^{-4}.

The optical signal observed under these conditions showed a sharp rising phase which started at about the time of arrival of the nerve impulse at the site of optical recording. After reaching a relatively sharp peak, the light intensity returned gradually to the resting level. (The slow rate of this return can be attributed, at least in part, to the presence of many fibers in the nerve conducting at different velocities.)

Observations made with squid fin nerves yielded results similar to those obtained from spider crab nerves. The peak value of the increase in fluorescence was found to be roughly 2.5×10^{-5} times the intensity at rest.

An example of the optical signals obtained from a squid giant axon after internal application of AO is presented in Fig. 2 (see the lower traces). The electric response (upper traces) was recorded from the external surface of the axon at a point about 2 mm away from the site of optical recording. When the intensity of the stimulating pulse (10 μsec in duration) was varied, both the electric response and the optical signal changed in an all-or-none manner. The optical signal of a squid giant axon had a fast rising phase and a slow falling phase. The peak value of the signal was on the order of 2×10^{-5} times the level of the light intensity before stimulation.

It is well known that addition of a small amount of tetraethylammonium salt to the internal perfusion fluid prolongs the electric response of a squid giant axon (*8*). When a short (approximately 4 mm) portion of an axon was perfused with the internal fluid containing both 10 mM tetraethylammonium and AO, the electric response recorded from the perfusion zone with an internal electrode showed a complex time course. This time course is regarded as a long, rectangular response with superposed repetitive, brief responses derived from the lateral unperfused zones. Record B in Fig. 2 was obtained from such an axon. (During the recording of the optical signal, both the perfusion cannulas and the internal electrodes were removed.) The upper trace (B_1) shows that the time course of the

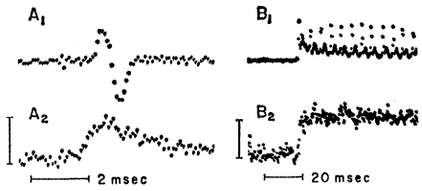

Fig. 2. Electric responses (top) and optical signals (bottom) in squid giant axons stained with Acridine Orange by internal perfusion. Record A was obtained from an axon internally perfused with a potassium phosphate solution containing Acridine Orange. Record B shows the results obtained when 10 mM tetraethylammonium was added to the perfusion fluid. The electric response was recorded extracellularly; there was some distortion in these electric responses due to the variation in the volume conductor (that is, in the amount of shunting seawater) near the recording electrodes. A CAT computer was used to reduce the noise level in the optical signal. The vertical bars represent an increase 4×10^{-5} (A_2) and 5×10^{-5} (B_2) times the background light intensity. Temperature, 19°C.

electric response (recorded externally near the site of optical recording) was very complex. The optical signal observed under these conditions (B_2) had a simple, approximately rectangular time course; the duration of the signal was close to the duration of the electric response at the site of optical recording.

On several occasions, prolongation of the electric response of the squid giant axon was brought about by addition of cesium fluoride to the usual perfusion solution or by using sodium phosphate as the sole intracellular salt (*8*, *9*). The optical responses were always prolonged and roughly rectangular under these circumstances.

It is interesting to note that the prolonged electric responses observed under these conditions have a distinct initial peak (*8*, *9*). There was no detectable initial peak in the prolonged optical responses. Since the averaging technique was used for recording, there is some uncertainty as to the time course of the unaveraged optical response.

Finally, a few control experiments will be described showing that the optical signal described in this report actually represents changes in fluorescence. (i) When the step of AO pretreatment was omitted, no optical signal could be obtained with the experimental setup of Fig. 1. (ii) The optical signal from an AO-stained crab nerve disappeared when filter F_2 (see Fig. 1) was moved to a position between the light source

and the nerve; this finding indicates that the properties of both F_1 and F_2 are adequate to eliminate optical signals due to changes in light scattering. (iii) With an AO-stained crab nerve, optical signals similar to those in Fig. 1 could be observed at 0°, namely, when S, F_1, N, F_2, and P were arranged in one straight line; this indicates that the fluorescent light emitted at both 0° and and 90° increases during nerve conduction. (Note that an increase in light scattering during nerve conduction tends to reduce the light intensity observed at 0°.) From these observations, we conclude that possible sources of artifact in our detection of small fluorescence changes are eliminated.

The reason for the small size of our optical signal is easy to understand. The plasma membrane, where the primary event in the process of nerve conduction takes place, is extremely thin (roughly 5 to 10 mμ in thickness). The dye molecules contributing to the optical signal during conduction must be limited to those bound to the plasma membrane and the structures in its immediate vicinity. The major portion of the background fluorescent light derives from the dye molecules bound to the connective tissue in the nerve. Therefore, the level of fluorescence change in the critical layers of the nerve is considered to be several orders of magnitude greater than those described in this report.

The fluorescence of AO is strongly influenced by changes in the microenvironment of the dye molecules (*4*, *10*). The change in the intensity of fluorescence during nerve conduction is therefore indicative of changes in the physicochemical properties of the microenvironment of the dye molecules bound to the nerve membrane. The observed change in fluorescence suggests that the macromolecules in the nerve membrane undergo drastic conformational changes during nerve conduction. This report indicates that the use of fluorescence is a powerful means of studying the process of nerve excitation and conduction.

I. TASAKI
L. CARNAY
R. SANDLIN

Laboratory of Neurobiology, National Institute of Mental Health, Bethesda, Maryland 20014

A. WATANABE

Tokyo Medical and Dental University, Tokyo, Japan

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Macromolecular Subunits in the Walls of Marine Nitrifying Bacteria

Abstract. *The outermost layer of the cell wall of all marine ammonia-oxidizing bacteria so far isolated is made up of protein subunits arranged in a regular manner and linked together through metal-oxygen bonds. This sculptured, outer wall layer appears to be unique to the marine forms and is not found in the terrestrial ammonia-oxidizing bacteria.*

The presence of macromolecular subunits in the outer cell wall of bacteria has been known for some time. These structures were first described in *Spirillum* sp. (*1*) and later in a photoorganotrophic bacterium (*2*), *Bacillus cereus* (*3*), *Halobacterium cutirubrum* (*4*), and most recently in a marine photosynthetic bacterium, *Ectothiorhodospira mobilis* (*5*). It was not surprising, therefore, to discover that marine ammonia-oxidizing bacteria also have an outer layer of regularly arranged subunits. This layer was not found in their terrestrial counterparts or in marine or terrestrial nitrite-oxidizing bacteria.

Using the freeze-etching technique

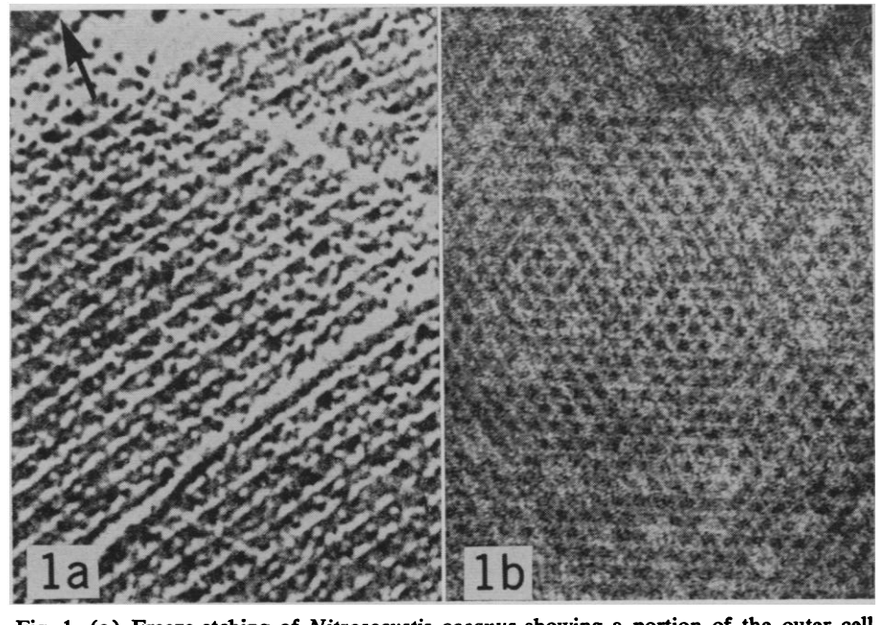

Fig. 1. (a) Freeze-etching of *Nitrosocystis oceanus* showing a portion of the outer cell wall layer consisting of small particles (about 20 Å) forming a diamond-shaped unit with side dimensions of about 110 Å. The arrow in the upper left corner indicates the direction of platinum-carbon shadow (× 750,000). (b) Negatively stained outer cell wall fragment of *N. oceanus* showing the regular pattern of subunits (× 260,000).