REVIEW

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Rapid Structural Changes in Nerve Fibers and Cells Associated with Their Excitation Processes

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Summary: In a variety of nerve fibers, cells and other excitable tissues, the electric responses to electric stimuli were found to be accompanied by a transient swelling of the tissues. The rising phase of this swelling coincides with that of the electric response. By use of a heat-sensor made of polyvinylidene fluoride film, it was also shown that the electric responses of many types of excitable tissues are accompanied by simultaneous heat production. To elucidate the origin of this swelling and heat production, the process of Ca^{2+}–Na^{+} exchange in synthetic anionic gel beads and rods was investigated. It is asserted that the observed signs of nerve excitation are manifestations of a rapid structural change of the cortical gel layer of the protoplasm, plasmalemma-ectoplasm complex. The importance of the rapid movement and rearrangement of water molecules in the cortical gel layer in association with excitation processes is emphasized. [Japanese Journal of Physiology, 49, 125–138, 1999]

Key words: cooperative structural changes, nervous tissues, calcium–sodium ion-exchange.

In recent years, we have conducted extensive studies of mechanical and thermal changes associated with the excitation process in nerve fibers, cells and other nervous tissues. In light of these studies, it is now firmly established that the excitation processes in nervous tissues are invariably accompanied by an increase in the volume (or pressure) and temperature of the superficial gel layer of the tissues. We have seen that these mechanical and thermal changes start simultaneously with the electric responses of the tissues. These findings give us important clues for elucidating the mechanism of the excitation process on a sound physical basis.

In classical neurophysiology, great importance was placed on transient changes in the electric parameters (potential, current, resistance, etc.) of excitable tissues associated with the excitation processes. Indeed, with modern electronic devices that are often commercially available, it is relatively easy to record small, transient changes in electric potentials or currents with a sufficiently high time-resolution. Under these circumstances, investigations into the excitation process have relied almost exclusively on the results of measurements of electric parameters of the tissues.

Nevertheless, in order to explain the excitation processes on a sound physical basis, it is desirable, or rather absolutely necessary, to acquire information about non-electric manifestations of the processes. It is to be noted that the cortical layer of the protoplasm of the nerve fiber, which Metuzals *et al.* [1] termed "axolemma-ectoplasm complex" (discussed later), is a soft gel layer composed of highly structured macromolecular material containing a considerable amount of water and inorganic cations, principally Ca^{2+} and Na^{+}, under normal experimental conditions. According to Williams [2], oxygen-anions of biomacromolecules are the sites of the binding of these cations. Exchange between Ca^{2+} and Na^{+} at the binding sites is moderately fast (see also Levine and Williams [3]). In elucidating the mechanism of nerve excitation, it is necessary to deal directly with the interaction between these cations and the macromolecules located in and near the axolemma.

On the basis of the fact that protein molecules are capable of binding Ca^{2+} selectively and reversibly, and also that Ca^{2+} readily acts as a bridge between

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the anionic sites of neighboring macromolecules, Williams made a bold prediction in 1970. In his Tilden lecture entitled "The Biochemistry of Sodium, Potassium, Magnesium and Calcium," he said that a propagated nerve impulse may actually be a wave of running structural change—displacement of the membrane surface caused by Ca^{2+} binding—rather than electrostatic changes altering the membrane from a potassium to a sodium permeable condition (see p. 362 in William [2]).

Ten years after Williams' prediction, an unmistakable sign of reversible structural changes—swelling followed by shrinkage of the nerve fiber (Fig. 1)—was actually observed in association with the production of a propagated action potential [4]. However, it should be noted here that this was not truly the first observation indicating the existence of a structural change in the nerve fiber. For example, Cohen *et al.* [5] had found already, in 1968, that there is a rapid, transient change in nerve birefringence coincidentally with the production of action potential. We now know [6, 7] that this optical change is, as a matter of fact, a manifestation of a decrease in the form-birefringence, indicating a profound structural change of the longitudinally oriented fibrous elements located in the ectoplasm (discussed later). At present, many earlier findings must also be regarded as indicative of the existence of structural changes in the nerve fiber.

In 1968, we began our studies of optical (fluorescence, light absorption, etc.) signals from nerve fibers stained vitally with various dyes [8, 9]. Then, we started examining volume, pressure and temperature changes associated with the process of excitation in a variety of nerve fibers and cells [4, 7]. In the years that followed, we examined excitatory processes in the synaptic zone of the sympathetic ganglia and in the fish electric organs by using the same optical, mechanical and thermal devices [10, 11]. Furthermore, we have investigated a model system that reproduces some aspects of the behavior of the excitable tissues [12, 13]. In this article, we briefly review the results of these investigations which led us to the following conclusion: All the observed signs of excitation processes are manifestations of rapid structural transformation of the cortical gel layer of the protoplasm, "plasmalemma-ectoplasm complex," involving exchange among weakly bound Ca^{2+}, Na^{+} and K^{+}. Our investigations also emphasize the great importance of the role played by water molecules in the process of nerve excitation.

Swelling of Nerve Fibers and Cells Evoked by Electric Stimulation

A simple, but quite serviceable device for detecting a transient change in the pressure at the surface of excitable tissues can be constructed with a piezoelectric bender transducer and an operational amplifier [14]. Piezoceramic bars (G-1195) purchased from Gulton Industries, Inc. (NJ) were of the lead-zirconate-titanate type and roughly 0.45 mm thick, 1.5 mm wide and 15 mm long. They had a relatively large capacitance ($\approx$1.5 nF) and very high resistance. One end of a bender was connected to the input of an operational amplifier, OPA128 (Burr-Brown, Tucson, AZ), with a high-feedback resistor ($\approx 10^{10}\,\Omega$) and a capacitor, the product of the capacitance and resistance being chosen to be several times the duration of the electric response of the tissue under study. To the other end of the bender, a light wooden stylus was attached at a right angle to the bender. With the aid of a rack-and-pinion device, the bender set was lowered from above and the flattened end of the stylus was brought into firm contact with the surface of the tissue under study (Fig. 1, left).

A small transient displacement of the surface of an excitable tissue was measured optically with a Fotonic sensor (KDP 032R, Mechanical Technology, Inc., Latham, NY). This device consisted of two bundles of optical fibers, mixed evenly at the sensing end (roughly 0.5 mm in diameter) of the device (Fig. 1, right). One of the bundles was used for carrying light from the source, a 100 W Osram quartz iodine lamp, to the object under study. The other bundle was utilized to transmit light from the object to a photodetector (Pin-10, United Detector Technology, Inc., Culver City, CA). In order to enhance the reflection of light at the surface of the tissue, a small piece of platinum or aluminum foil was placed on the surface of the tissue. With this device, the intensity of the photocurrent detected strongly depends on the distance between the metal foil and the sensing end of the Fotonic sensor. The procedure of calibrating the piezoceramic bender and the Fotonic sensor is described elsewhere [14].

It is seen in the left-hand record in Fig. 1 that, during the rising phase of the action potential of a squid giant axon, there was a rapid increase of the force tending to push the bender upward. The peak of this mechanical response coincided fairly accurately with the peak of the action potential (note that this mechanical response was recorded directly from the site where the propagated action potential was recorded). The falling phase of the mechanical response was also found to coincide with that of the action potential.

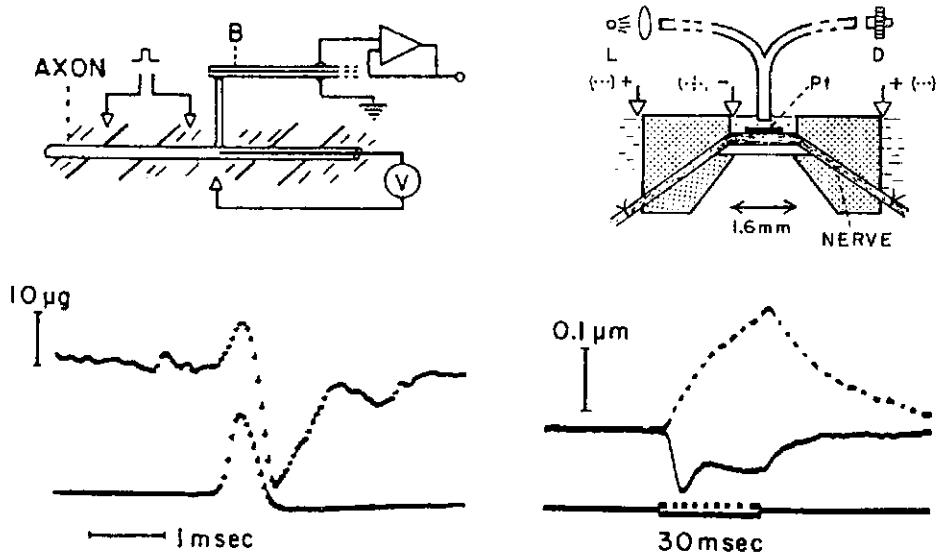

Fig. 1. Mechanical responses of nerve fibers. Left top: Schematic diagram of the setup for detecting mechanical responses of the squid axon. B, Gulton bender. **Left bottom:** Mechanical response (upper trace) and intracellularly recorded action potential (bottom trace). **Right top:** Diagram of the setup for recording surface displacements of the garfish olfactory nerve with a Fotonic sensor. L, light source; D, photo-detector; Pt, platinum foil placed on the surface of an olfactory nerve bundle. **Right bottom:** Displacement of the nerve surface evoked by an outwardly directed current pulse (continuous lines) and by an inwardly directed pulse (broken lines) (from Tasaki [18] and Tasaki and Byrne [39]).

However, the amplitude of the negative deflection of the mechanical trace was far greater than that of the corresponding negative deflection of the potential trace. There is little doubt that the negative defection of the mechanical trace in the figure is in accordance with the prediction made by Williams [2]; it is an indication of the shrinkage of the axon caused by the increased influx and subsequent binding of Ca^{2+}.

(It is known that, during repetitive stimulation at 50 Hz of a squid giant axon under internal perfusion, the influx of Ca^{2+} measured by the amount of radioactive tracer appearing in the internal perfusion solution rises to about 5 times its resting level [15]. Since this rise in influx occurs only during the excited state of the axon, which lasts about 1 ms, and since the axon was excited only once in 20 ms under these conditions, there was a 100 to 200-fold increase in the Ca^{2+} influx at the peak of excitation of the axon.)

These findings, obtained with a piezoceramic bender, were confirmed and extended by the use of a Fotonic sensor [16]. During the rising phase of the action potential, the axon surface was found to move outwards. The displacement of the surface was generally in the range between 0.5 and 2 nm at the peak of excitation.

(The following calculation indicates that the exchange of extracellular Na^+ for intracellular K^+ does not account for the observed displacement of the axon surface. The difference between the molar volume of Na^+ and that of K^+ is known to be about 23 cm^3 [17]. An exchange of 12 $pmol/cm^2$ per impulse (see p. 221 in Tasaki [18]) can bring about a surface displacement of only 0.0028 nm. Furthermore, such an exchange process does not explain the appearance of a large shrinkage of the axon at the end of the action potential.)

The right-hand record in Fig. 1 shows the movement of the surface of a nerve at the site where a long pulse of stimulating current was delivered. In this experiment, a garfish olfactory nerve was used instead of a squid giant axon. This nerve is known to be composed of a large number of very small non-myelinated fibers. A short middle portion of the nerve was immersed in a small pool of saline solution at the center of a plastic chamber. To deliver a long pulse of electric current to the short middle portion of the nerve, three large Ag–AgCl electrodes were used, one electrode placed in the middle pool and two in the lateral pools separated from the middle pool by 6 mm–wide partitions.

The downward deflection of the solid line in this figure shows that there was a transient decrease in the distance between the sensing end of the sensor and the target (marked Pt). Unquestionably, this is an indication of swelling of the nerve brought about by the stimulating current. It is of great interest that the time-course of this swelling is not a smooth, monotonic one. Note also that, when the applied current is reversed (see the broken lines in the figure), the displacement of the nerve surface is also reversed and that the time-course of this displacement (representing shrinkage of the nerve) is smooth and monotonic. As is well-known, an outwardly directed current through the surface of the nerve fiber is capable of evoking an action potential, but an inwardly-directed current is not. It is evident, therefore, that the initial, sharp, downward deflection in the figure represents the swelling of the nerve associated with the production of an action potential. A sustained outwardly directed

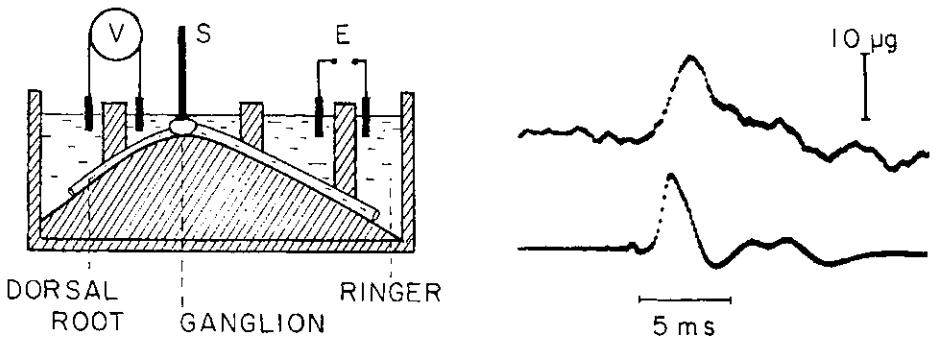

Fig. 2. Mechanical response of ganglion cells. Left: Diagram of the setup for recording mechanical responses of a dorsal root ganglion. S, stylus of a Gulton bender; E, stimulating electrodes; V, input of an operational amplifier for recording the action potential. **Right:** Mechanical response of a ganglion (top trace) and action potential (bottom trace) (from Tasaki and Byrne [20]).

current causes relatively small swelling of the nerve. Similar observations have been made on squid giant axons [19].

In recent years, we have examined excitation processes of a large number of nervous tissues using the piezoelectric and optical devices mentioned above. The materials examined include, besides the invertebrate and vertebrate nerve fibers mentioned above, amphibian and mammalian ganglion cells [20–22], invertebrate photoreceptor cells [23, 24] and amphibian skin glands [25]. All the materials examined, without exception, were found to respond to electric, photic or chemical stimuli with rapid swelling.

An example of the records taken from a frog dorsal root ganglion [20] is presented in Fig. 2. A ganglion was dissected out together with the sciatic nerve and the dorsal root connected. The preparation was placed in a shallow pool of saline solution in a plastic chamber provided with two partitions at a distance of about 2 mm from the ganglion. When a stimulating shock was delivered to the sciatic nerve by way of the electrodes, E, in the figure, an action potential could be recorded from the dorsal root (by using the electrodes placed across the other partition). At about the same time, rapid transient swelling of the ganglion could be detected with the piezoceramic bender pushing the surface of the ganglion. It is to be noted that here we are dealing with an assembly of nerve fibers and cells with non-uniform size distribution. Since the responses of large fibers and cells dominate in electric recording, and since the conduction velocity is greater in the larger fibers, the peak of the electric response (lower trace) is reached before the peak of the mechanical response (upper trace).

Heat Production Associated with Excitation Processes

In 1958, using a thermopile in conjunction with a galvanometer, Abbott *et al.* [26] conducted a careful study of the time-course of temperature changes associated with a nerve impulse and discovered that the production of heat is followed immediately by the reabsorption of heat by the nerve. This discovery was confirmed and extended by a number of subsequent investigations [27, 28]. During the course of these investigations, the time-resolution of the thermopile recording system was greatly improved. Nevertheless, since the response of the recording system employed was still far slower than the thermal responses of the nerve, it was necessary to rely on the "heat block" method combined with cooling of the nerve to compare the time-course of the heat production with that of the action potential.

Not long ago, we noticed that the time-resolution of heat detection could be greatly improved by use of synthetic pyroelectric polymer as a heat-sensor. We constructed a new type of heat detector utilizing 6 and 9 μm–thick films of polyvinylidene fluoride (PVDF), which were kindly donated to us by Kureha Chemical Company in Tokyo [29–31]. According to the data supplied by the company, this polymer material has the following physical properties: density 1.78 g/cm^3, dielectric constant 13, volume resistivity roughly $9\times10^{14}\,\Omega\cdot$cm, heat capacity 0.6 cal$\cdot$cm$^{-3}\cdot$deg^{-1}, and thermal diffusivity 0.53×10^{-3} cm^2/s. The pyroelectric coefficient (i.e., the change in the electric charge on the surface of the film induced by 1 deg change in the temperature) is roughly 4×10^{-9} coulombs per deg$\cdot$cm^2. The film has an approximately 10 nm–thick aluminum layer deposited on each surface.

The advantage of using a PVDF film for measuring heat produced by nervous tissue lies in the fact that its heat capacity is small as compared with that of the nervous tissues and also that the temperature-sensitive area of the detector can be varied readily depending on the size of the tissue under study. One troublesome feature of this pyroelectric film is that it is also piezoelectric. The sensitivity of a PVDF heat detector to mechanical disturbances can be reduced to a great extent by inserting a thin metal layer or a thin stretched Mylar film between a doubly-folded PVDF film and the nerve (see Fig. 3, top).

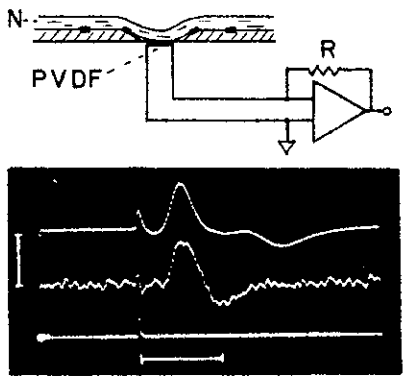

Fig. 3. Detection of thermal response of nerve. Top: Diagram of the setup for recording thermal responses of a nerve trunk (N). PVDF, thin pyroelectric film of polyvinylidene fluoride; R, feedback resistor of an operational amplifier. **Bottom:** action potential (top trace) and thermal response (bottom trace) of a garfish olfactory nerve. The calibrating vertical bar represents the rate of temperature rise of 4 mdeg/s; the horizontal bar, 30 ms (from Tasaki *et al.* [31]).

With this thermal detector, the output voltage of the operational amplifier connected to the film is proportional to the rate of temperature rise at the surface of the tissue. A change in the temperature can easily be obtained by integrating the output voltage with respect to time. When the sensor is warmed by the sudden onset of a constant Joule's heat, the time, t, required for the voltage output of the amplifier to rise to 70% of the final level may be roughly estimated by the formula $t=0.5\,l^2/D$; where l is the effective thickness of the film and D is its thermal diffusivity (see p. 102 in Carslaw and Jaeger [32]). This formula gives $t=1.4$ ms for a 12 μm–thick layer of film.

The middle trace in the photograph reproduced in Fig. 3 is an example of thermal responses taken from a garfish olfactory nerve at room temperature (21°C). The top trace shows the action potential recorded from the middle of the 2 mm–long portion of the nerve from which the thermal response was recorded. The bottom trace indicates the time at which a 0.5 ms–long stimulating pulse was delivered to the nerve at a point 1.2 mm away from the edge of the PVDF film. It is seen in the figure that the thermal response started and reached a peak nearly simultaneously with the electric response. As expected from the previous observations made by Howarth *et al.* [28], the phase of heat production was followed immediately by the phase of heat absorption. The temperature of the nerve was found to rise to 23 (±4)μ°C above its resting level. Under these experimental conditions, between 50 and 85% of the heat generated during the positive phase was reabsorbed during the negative phase.

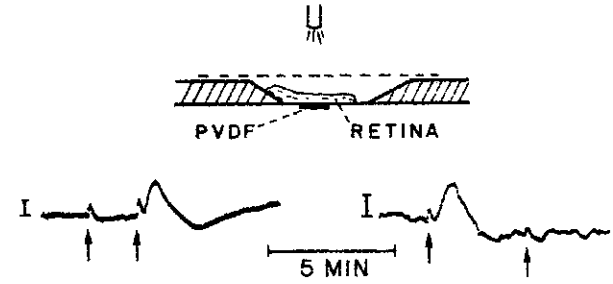

Fig. 4. Heat production associated with spreading depression in bullfrog retina. Top: Diagram showing the setup for demonstrating heat production by the retina with a PVDF sensor (2×2 mm in area) triggered by light pulses. **Bottom:** Records of all-or-none thermal responses of a retina to brief trains of strong light pulses. The first arrow indicates delivery of 5 pulses repeated at 0.5 s intervals, the second, third and fourth arrows, 10 pulses. Note that the retina was refractory to the fourth train of light pulses. The vertical bars represent the rate of temperature rise of about 10 mdeg/min (from Tasaki and Byrne [35]).

Using our PVDF thermal detectors, we have recorded thermal responses from the following materials: bundles of non-myelinated and myelinated nerve fibers [31, 33], electric organs of electric fish [11, 34], amphibian retina [35], and others. In many of the records obtained, the appearance of the phase of heat reabsorption following the phase of heat production was clearly recognized.

The records in Fig. 4 show the production of heat associated with the phenomenon of spreading depression in an isolated vertebrate retina [35]. We see in the figure that the heat generation triggered by a train of light pulses was followed immediately by heat reabsorption by the retina. Previously, Martins-Ferreira and Castro [36] demonstrated changes in the thickness of the retina associated with spreading depression. Bures [37] has already shown the existence of competition between divalent and monovalent cations in this process. Therefore, except for the scale of time and space involved, there is a remarkable similarity between the processes of nerve excitation and the spreading depression.

Nerve Birefringence and Axolemma-Ectoplasm Complex

When we examine a garfish olfactory nerve under illumination with polarized light, we find that its refractive index measured with the light polarized in the longitudinal direction is larger than the index for light polarized in the transverse direction. That is to say, the nerve is positively birefringent. Since the olfactory nerve is composed of a large number of very small non-myelinated fibers (0.2–0.3 μm in diameter), all running in the direction parallel to the long axis of the

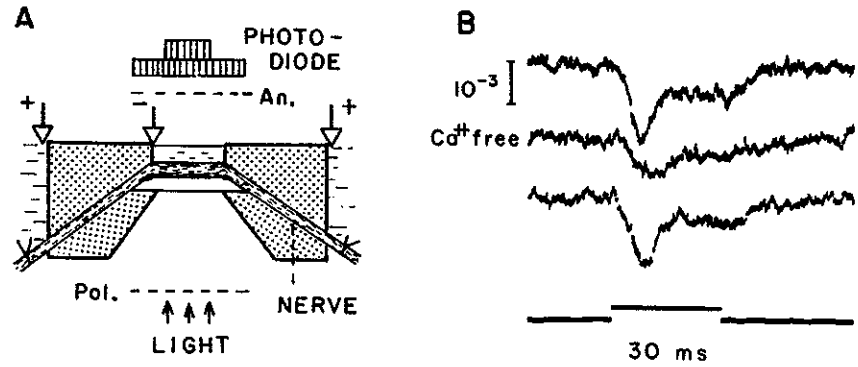

Fig. 5. Demonstration of changes in nerve birefringence evoked by electric current pulses. A: Diagram of the setup employed. Pol represents a polarizer; An, an analyzer. The symbols + and − in the figure represent the source and sink of the applied current pulses, respectively. **B:** Changes in birefringence of an olfactory nerve evoked by 30 ms-long current pulses (top and bottom traces). The middle trace shows suppression of the fast component of the birefringence signal by immersion of the nerve in a Ca^{2+} free medium (from Tasaki and Byrne [39]).

nerve, it is expected, from Wiener's theory of form-birefringence (see p. 707 in Born and Wolf [38]), that the nerve has positive birefringence.

When we deliver a stimulating shock to the nerve, there is a transient change in the nerve birefringence [5, 8]. This transient change can be easily detected by placing the nerve between two sheets of Polaroid film arranged in a crossed position and measuring the intensity of monochromatic light transmitted through the nerve. When the direction of the E-vector of the incident light is at 45° with the long axis of the nerve, we see that the light intensity falls simultaneously with the production of an action potential. This fall is also expected from the fact that the nerve fibers swell during excitation and approach, to a small extent, an optically homogeneous state.

Again, we are interested in knowing what happens to the birefringence of the nerve at the site of application of an electric current [39]. In the experimental setup schematically illustrated in Fig. 5A, a short, middle portion of a garfish olfactory nerve was immersed in a small pool of saline solution in which a large Ag–AgCl electrode was placed. The two ends of the nerve were immersed separately in the lateral pools of saline. A long pulse of electric current was applied to the nerve by way of the middle and lateral electrodes, and changes in the nerve birefringence were detected by measuring the intensity of light transmitted through the middle portion of the nerve.

When the middle pool of saline is connected to the sink and the two lateral pools to the source of the current, the portion of the nerve under study is traversed by an outwardly directed current. Under these circumstances, both Na^+ and Ca^{2+} in the external saline are transported by the current away from the surface of the nerve fibers. Within the ectoplasm of the fibers, however, the current is carried predominantly by outwardly moving monovalent cations, K^+ and Na^+, because the free Ca^{2+} concentration in the endoplasm is very low [2]. Consequently, the applied current causes a gradual fall in the Ca^{2+} concentration in the superficial gel layer of the fiber. The rapid fall in the nerve birefringence seen in Fig. 5B is an indication of sudden swelling (i.e., a rise in the water content) of the superficial gel layer associated with a gradual loss of Ca^{2+} in the layer. The effect of Ca^{2+} on the water content of the gel layer will be discussed in greater detail later.

The middle trace in Fig. 5 shows that the amplitude of the birefringence signal is reduced markedly when Ca^{2+} in the surrounding saline solution is replaced with Na^+. This effect is reversible. The bottom trace, taken after bathing the nerve in normal saline solution again, shows the recovery of the birefringence signal.

The importance of form-birefringence in fresh, excitable nerve fibers cannot be emphasized enough. By the method of differential interference microscopy, Metuzals and Izzard [40] have demonstrated the existence of closely packed, longitudinally oriented, thread-like elements in the ectoplasm of fresh squid giant axons. Hence, the birefringence of the squid giant axon at rest [41, 6] has to be regarded as a manifestation of form-birefringence of the ectoplasm of the axon (see also Fig. 7B). The birefringence signals recorded by Sato *et al.* [6] from "surgically flattened" giant axons is, without question, proof that thread-like elements in the cortical gel layer of the axon undergo a transient structural change when an action potential is generated in the axon (see also Tasaki and Byrne [7]).

Figure 6 is a scanning microscope photograph showing the external surface of the axolemma of a squid giant axon from which the connective tissue and Schwann sheath have been surgically removed [1]. Since this axon had been mildly fixed with glutaraldehyde, it does not represent the normal, excitable state of the axon. Nevertheless, it clearly demonstrates how closely the axolemma is adhered to the underlying ectoplasm, and also the prominence of the longitudinally oriented bundles of thread-like elements in the ectoplasm.

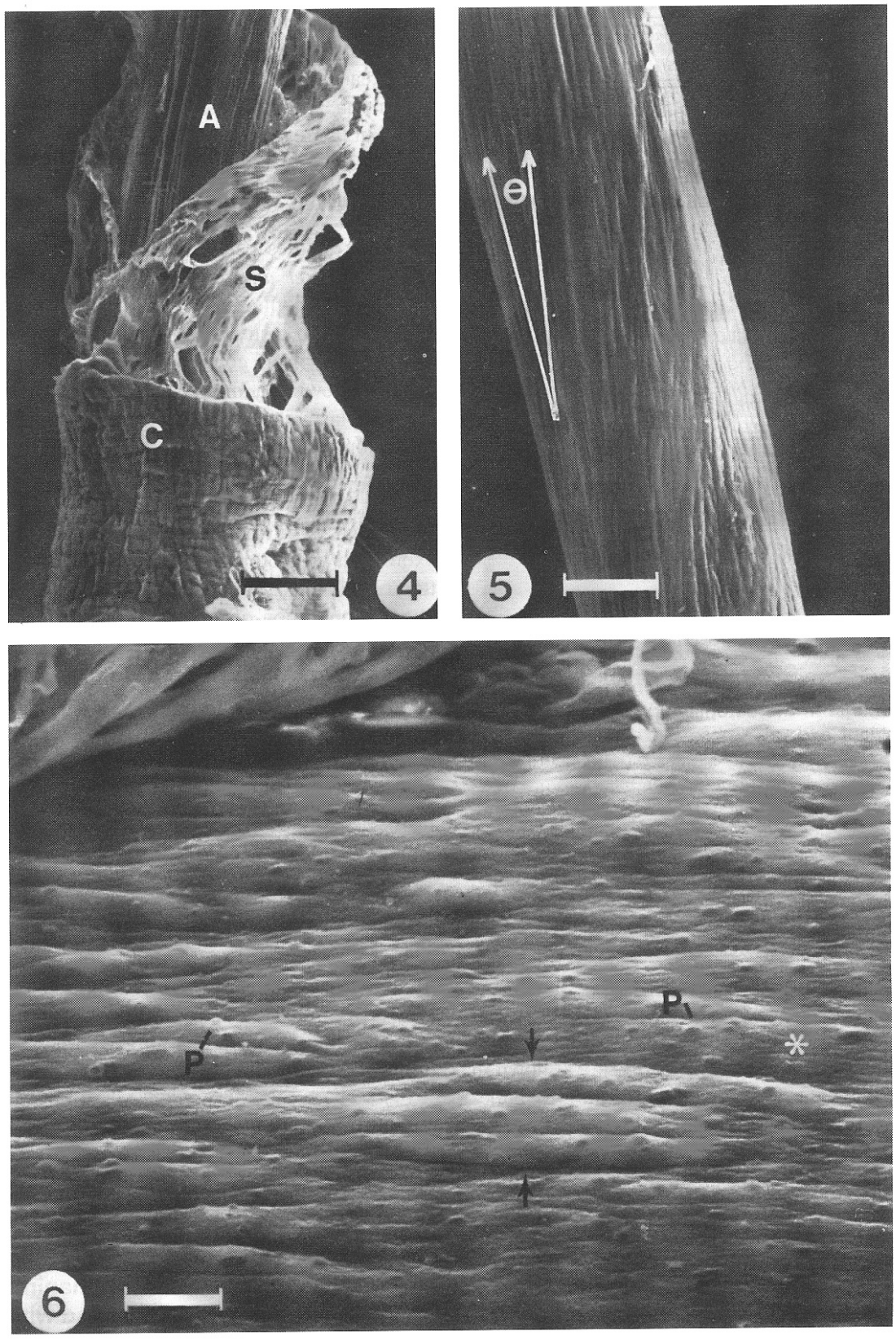

Fig. 6. Scanning electron micrograph of desheathed squid giant axon showing a ridge-and-groove pattern on the surface. A represents exposed surface of the axolemma; S, Schwann sheath; C, everted layer of Schwann sheath; P, protuberances. Note that the ridges are oriented in a right-handed helix with a tilt angle of about 10°. Bars: 100 μm in micrograph 4, 50 μm in micrograph 5, and 10 μm in micrograph 6 (from Metuzals *et al.* [1]).

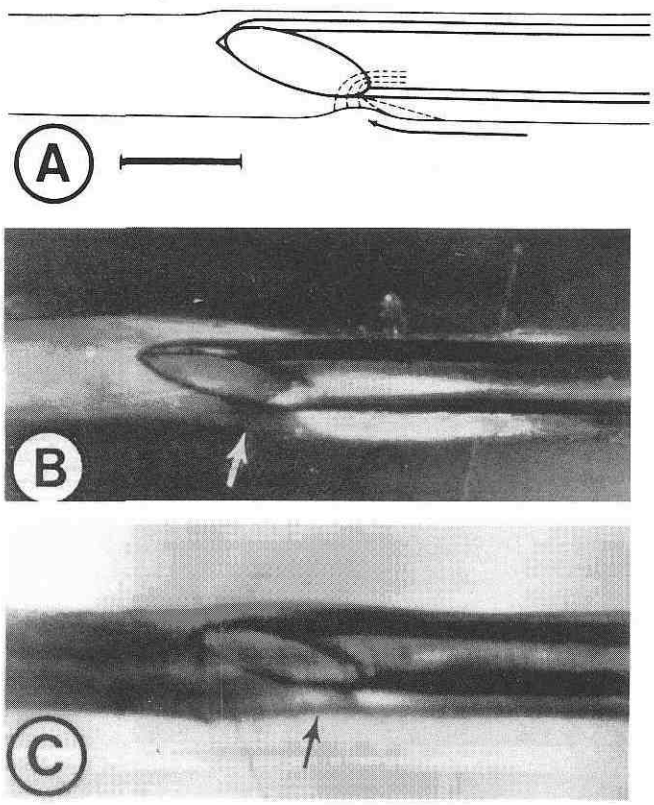

Fig. 7. Demonstration of direct connection of the filamentous material in the ectoplasm with the axolemma of a fresh giant axon A: Schematic diagram illustrating suction of the protoplasm into a glass pipette inserted into a squid giant axon. **B, C:** Photographs taken from a fresh excitable axon, placed between a polarizer and an analyzer in a crossed position, showing strong birefringence of the protoplasmic gel in the vicinity of the rear end of the orifice of the glass pipette. The broken lines in diagram A show the orientation of the filaments revealed by the birefringence of the protoplasm (from Metuzals and Tasaki [42]).

The close connection between the axolemma and ectoplasm in a fresh, excitable giant axon is demonstrated by the following observation [42]. As shown in Fig. 7, a freshly excised axon, about 0.5 mm in diameter, was placed horizontally in a shallow layer of seawater on a glass plate. The two ends of the axon were tied with a thin thread and a small tension was applied to the axon. Through an opening in the axon surface made near one end of the axon, a glass pipette, about 0.3 mm in outside diameter and beveled as illustrated by the diagram in the figure, was slowly pushed into the axon interior. To prevent a rise of pressure inside the axon, it was necessary to suck the endoplasm into the pipette during this insertion. When the orifice of the pipette approached the axolemma during this procedure, we recognized the formation of a small depression on the axon surface in the vicinity of the orifice. By placing an axon in this condition between a polarizer and analyzer, we were able to demonstrate that the depression on the axon surface is produced by the thread-like elements in the ectoplasm pulling the axolemma. Note that the brightness (in B) or darkness (in C) in the region close to the orifice of the pipette is an indication of the birefringence of the gel containing closedly packed, orderly aligned, filamentous elements. This observation, corroborated by electron-microscopic studies performed by Metuzals *et al.* [43] and Tsukita *et al.* [44], indicates that the approximately 0.5 μm–thick superficial gel layer of the axon is formed into a structurally integrated entity, the axolemma-ectoplasm complex.

The following additional observations indicate that the ectoplasm should be treated as an integral part of the "axon membrane."

(1) During the course of development of the method of internal perfusion (discussed later), it was found that perfusion of the interior of a squid giant axon with an isotonic chloride or bromide salt solution of K^+ slowly dissolves the ectoplasm and brings about suppression of the excitability of the axon [18, 42]. This indicates that the intactness of the ectoplasm is a necessary condition for the production of an action potential.

(2) When the proteins in the ectoplasm are digested by the internal perfusion of a giant axon with a dilute pronase solution, there is a gradual decrease in the maximum intensity of the inward current measured under voltage clamp [6] and soon the axon becomes inexcitable [6, 42].

(3) Application of tetrodotoxin or saxitoxin to the external surface of an internally perfused giant axon causes the release of protein molecules from the ectoplasm into the stream of internal perfusion solution [45]. This indicates that there is a strong interaction between the intrinsic membrane proteins (channel proteins) and the protein strands in the ectoplasm.

(4) The results of many observations made on the optical (fluorescence and light absorption) signals recorded from nerve fibers vitally stained with various dyes can be understood only on the assumption that dye molecules located in the ectoplasm participate in the production of optical signals [7, 9].

Swelling Phenomena in Inanimate Anionic Gels Associated with Ca^{2+}–Na^+ Exchange —A Model System

A significant consolidation of the concept of cooperative cation exchange in the cortical gel layer of excitable tissues has come from studies of swelling phenomena in a synthetic negatively-charged polymer gel

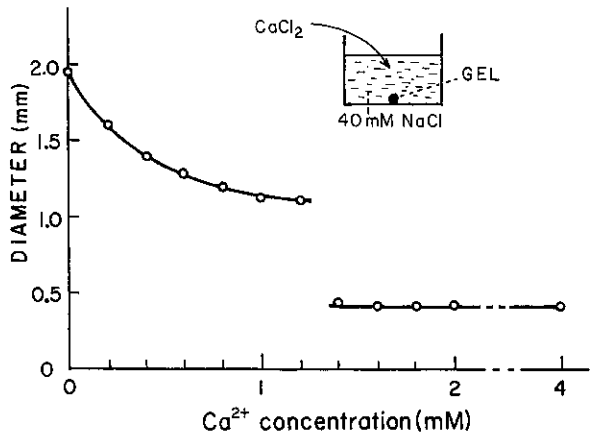

Fig. 8. Demonstration of discontinuous volume transition in cross-linked Na-polyacrylate gel beads. Swollen gel beads with practically the same diameter were introduced into glass vessels containing 40 mM NaCl solution. After addition of $CaCl_2$ to the solution in small increments, the diameters of the beads in equilibrium with the solution were determined. Note the discontinuity in the gel diameter–Ca^{2+} concentration relation.

[12, 13]. Our investigation along this line, carried out in collaboration with P. Basser of the Laboratory of Integrative and Medical Biophysics, NICHD, is still in progress. Here, we describe some of the relevant features of our findings.

Following the procedure described by Tanaka and his collaborators [46, 47], we synthesized various polyelectrolyte gels made of cross-linked polyacrylic acid, polymethacrylic acid, acrylamide-acrylic acid copolymers, etc. Small gel beads or rods of these anionic polymers were immersed in aqueous solutions (at pH 7.4) containing the salts of Ca^{2+} and Na^+ (or K^+) at varying mol ratios (Fig. 8).

When immersed in a 40 mM NaCl solution without the addition of $CaCl_2$, the gel stays in a highly swollen state. Addition of $CaCl_2$ to the solution in small increments brings about a decrease in the volume of the gel, indicating the occurrence of an exchange of a portion of Na^+ in the gel with Ca^{2+}. The solid lines in Fig. 8 show the diameter of the gel bead plotted against the Ca^{2+} concentration in the solution in which the gel is kept until equilibrium is reached. Interestingly, we find that the gel volume does not change continuously with the amount of Ca^{2+} added. Instead, when the mol ratio, $[Ca^{2+}]/[Na^+]$, in the solution rises to a level of about 1/30, there is a discontinuous decrease in the gel volume. The volume of the gel decreases by a factor of nearly 1/10 at this critical point. In the range of $[Ca^{2+}]$ above the critical point, the gel volume remains nearly constant.

A further study of the phenomena of discontinuous volume transition has revealed that the amount of Ca^{2+} inside the gel bead does not show noticeable discontinuity over the entire range of mol ratio, $[Ca^{2+}]/[Na^+]$, in the external solution. We estimate the negative fixed-charge density inside the swollen gel to be roughly 200 meq/*l*. Hence, we expect that Cl^- is effectively excluded from the gel bead (see "co-ion exclusion" in Helfferich [48]). In fact, the sum of the quantities (expressed in equivalent units) of the two cations, Na^+ and Ca^{2+}, in the gel bead was found to remain practically unaltered over the entire range of the Ca^{2+} concentration outside. The discontinuous volume change takes place when Ca^{2+} inside the gel bead rises to roughly 80% of the final level.

From these findings, the following picture of the phenomenon of discontinuous volume transition emerges. An anionic gel bead immersed in a pure NaCl solution is expanded by virtue of the osmotic pressure exerted by the counter-ions, Na^+, within the gel (note that the osmotic coefficient of Na^+ inside the gel is expected to be roughly 0.2 [49]). The polymer strands located near the surface of the gel are pushed by this pressure toward the surface of the gel. The pressure is counteracted by the mechanical tension exerted by the polymer strands running in the direction perpendicular to the gel surface. When the Ca^{2+} concentration outside is very low, the polymer strands are widely separated from one another (on average). This situation is unfavorable for the formation of Ca^{2+} cross-bridges in the gel; a bridge formed under such circumstances is readily broken by the incessant thermal motion of the polymer strands. The situation is quite different when the cations surrounding the polymer strands are predominantly Ca^{2+}. Under such circumstances, the formation of one Ca^{2+} bridge enhances the probability of the neighboring anionic sites of the strands to form a second cross-bridge. In other words, the free energy required to form one cross-bridge is smaller when the neighboring sites are already cross-linked than when they are not [18]. The process of Ca^{2+}-bridge formation can now proceed in a cooperative fashion until the entire gel bead is involved in the process. In the equilibrium, a large number of water molecules around the anionic sites and Ca^{2+} within the gel are displaced and the gel becomes very compact.

The process of Ca^{2+}–Na^+ exchange in the anionic gel is reversible. When the mol ratio, $[Ca^{2+}]/[Na^+]$, in the external solution is reduced, the volume of a shrunken gel rises to its new equilibrium value (which depends only on the new concentration ratio). Similar results were obtained using Ba^{2+}, Sr^{2+}, K^+, etc. in these experiments [12]. We have also seen that exchange of Na^+ for Ca^{2+} in the gel is exothermic, ac-

companied by an enthalpy change of roughly 2 kcal per mol of Ca^{2+} [12].

At this point in our study, it appeared desirable to investigate the electrical properties of a thin gel layer. It is well-known that the electric conductance of a cation-exchanger membrane can be markedly enhanced by a rise in the water content of the membrane (see p. 280 in Helfferich [48]). We have seen that the conductance of an oxidized collodion membrane can be varied by a factor of almost 1,000 by changing its water content (see Appendix in Tasaki [50]). Both the membrane conductance and potential are governed by the mobilities of the counter-ions in the membrane. It is therefore expected that the potential difference across a gel layer is strongly affected by Na^+–Ca^{2+} exchange in the gel.

Our investigations along this line have been met with only partial success. With a thin layer of swollen gel, it was difficult to construct a leak-proof partition between two solution compartments of a plastic chamber in which measurements of the potential difference across the layer could be made. Judging from the diffusion coefficient of Ca^{2+} (1.34×10^{-5} cm^2/s in water), it is evident that a stationary cation distribution in the gel layer cannot be achieved within several hours when the thickness of the layer exceeds a few millimeters. Under these circumstances, the strategy we adopted was to carry out potential and conductance measurements in a non-stationary state using thick layers of these anionic gels. Since it is impossible to make quantitative analyses of the potentials observed under non-stationary conditions (see p. 343 in Helfferich [48]), our study is limited to an empirical, qualitative description of the results.

The drawing in Fig. 9, top, schematically illustrates our experimental arrangement. Cross-linked sodium polyacrylate gel was synthesized in glass capillaries of about 1 mm (i.d.). Then, the capillaries were cut into pieces roughly 30 mm in length and were kept in a large volume of distilled water for about 5 d (to remove the major portion of unreacted compounds from the gel). Finally, the capillary was broken in the middle (without breaking the gel strand inside) and placed in a plastic chamber provided with two partitions separated by about 10 mm. The partitions were sealed by use of silicon rubber and vaseline. Keeping the two ends of the capillary in a 40 mM NaCl solution, a $CaCl_2$ solution was introduced into the middle compartment, whereby exposing the 0.3–0.4 mm long cylindrical gel surface in the middle to the Ca^{2+}-rich solution. A pair of large Ag-wire electrodes, one in the middle and the other in one of the lateral compartments, were employed to deliver electric current

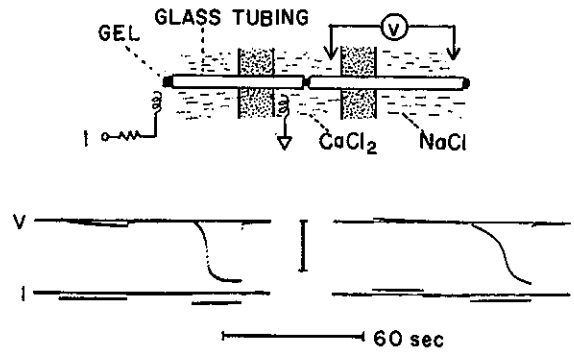

Fig. 9. Demonstration of cooperativity of the Na^+–Ca^{2+} exchange in cross-linked Na-polyacrylate gel. First, the gel, in glass tubing (1 mm in inside diameter and 30 mm in length), was equilibrated with 40 mM NaCl solution. Next, by gently breaking the glass tubing, a very short segment of the gel surface in the middle was exposed to 100 mM $CaCl_2$ solution. Subsequently, a constant current pulse was delivered between the middle and left-hand pools of solutions, and the continuous potential change developed across the exposed gel surface in the middle was recorded (see the trace marked *V*). The current intensities (indicated by the trace marked *I*) were, from the left, −0.3, −0.6, +0.6, and −0.6 mA. Note that the rapid change in the potential is preceded by a period of gradual change. Bar: 1 V.

pulses to the gel. The potential variation induced between the middle and the other lateral compartment was recorded with a pair of Ag–agar electrodes connected to the input of a differential amplifier.

With this arrangement, an inwardly directed current through the exposed gel surface in the middle compartment tends to drive Ca^{2+} into the gel. The effect of the resulting Ca^{2+}–Na^+ exchange in the superficial gel layer is uncovered by recording the potential variation developed across the superficial gel layer in the middle compartment.

The examples of the records shown in Fig. 9 indicate that the process of exchange of Ca^{2+} for Na^+ in the gel is indeed cooperative. Note that the rapid change in the observed potential is preceded by a period of much slower potential change. This delay in the onset of the rapid potential change indicates that, when the Ca^{2+} concentration reaches a certain level, a rapid transformation of the superficial gel layer from swollen to compact state is initiated. To induce this rapid potential change, a certain (threshold) intensity of current is required. As expected, an outwardly directed current does not produce a rapid potential change (see the first half of the right-hand record in Fig. 9).

Previously, we conducted an extensive study of an entirely analogous phenomenon in nerve fibers, known as "generation of a hyperpolarizing response by inward current" (see p. 183 in Tasaki [18]).

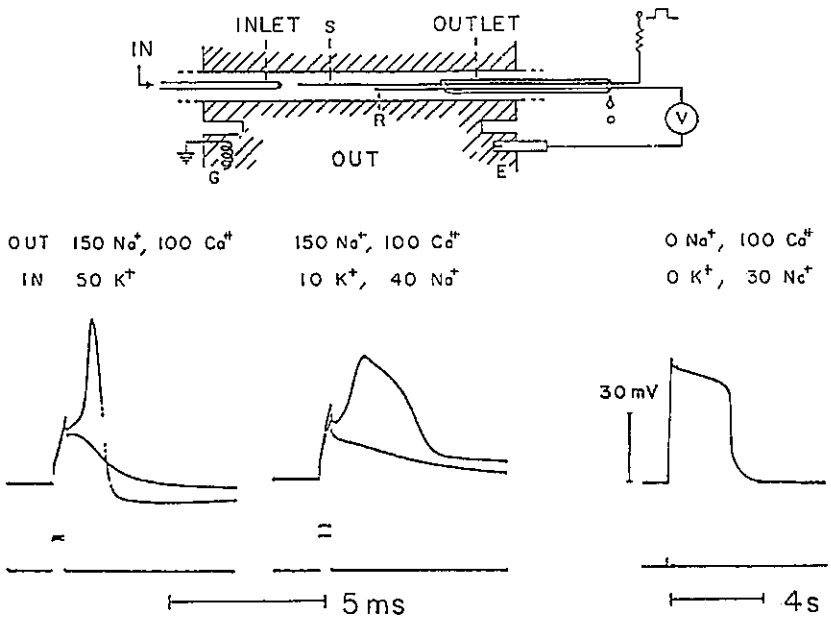

Fig. 10. Action potentials recorded from squid giant axon under internal perfusion. Top: Schematic diagram of a squid axon into which an inlet and an outlet perfusion cannulae are inserted. With the flow of internal salt solution maintained, brief stimulating current pulses were delivered to the perfused portion of the axon by way of a long Ag-wire electrode (S), and the electric responses of the axon were recorded with an internal glass-pipette electrode (R). E represents the ground electrode. **Middle and bottom:** The compositions (mM) of the salt solutions in the outside medium (OUT) and the internal perfusion solution (IN) are given above each record (from Tasaki [18]).

Ca^{2+}–Na^+ Exchange in Internally Perfused Axon

It is well-known that the protoplasm of the squid giant axon contains a variety of cations (potassium, sodium, magnesium, calcium and organic bases) and a large number of organic anions [51]. Undoubtedly, the concentrations of these ions are strongly influenced by the metabolic activity even after excision of the axon. Hence, it is desirable to be able to investigate the excitation process of the axon under conditions which permit direct experimental control of the ion concentrations in the axon interior. In fact, it was found possible to maintain the ability of the axon to produce all-or-none action potentials under continuous perfusion of the axon interior with an artificially prepared salt solution [52, 53].

The records presented in Fig. 10 were taken from giant axons under continuous internal perfusion with salt solutions of which the compositions are indicated above each record. The record at the left was obtained from an axon with a solution of K^+-salt internally and a mixture of Na^+- and Ca^{2+}-salt solutions externally. The time-course of this brief action potential is almost indistinguishable from those taken from an intact giant axon (without internal perfusion). When K^+-salts of fluoride, phosphate or sulfate ions (anions with a low lyotropic number [18, 50]) are used internally, the ability of such axons to produce full-size action potentials can be maintained for more than 24 h. Note that no biochemical energy source is added to the solutions. In these internally perfused axons, the free energy of cation exchange and mixing, required for the production of action potentials, is supplied artificially by maintaining large salt concentration differences between the internal and external solutions.

The middle record in the figure was taken after a large portion of K^+ in the internal perfusion solution was replaced with Na^+. It is seen that this replacement reduced the action potential amplitude and increased its duration. Interestingly, the ability of the axon to produce action potentials could be maintained even after the complete replacement of intracellular K^+ with Na^+, as long as the external Ca^{2+} concentration was kept at a high level.

The action potential record on the right was taken from a giant axon immersed in a 100 mM $CaCl_2$ solution and internally perfused with a 30 mM NaF solution (the pH of the internal solution was kept at 7.4 with Na-phosphate buffer, and the osmolarity was maintained by the addition of glycerol). Note that the Na^+-salt in the external solution had been completely eliminated in this experiment. The production of action potentials under these totally simplified ionic conditions has been observed using giant axons taken from *Loligo pealii* available in Woods Hole, MA [18, 54], axons of *Loligo forbesi* available in Plymouth, England [55], as well as axons excised from *Doryteuthis bleekeri* caught in the northern part of the Sea of Japan [56].

It is of deep significance that the ability of the axon to produce action potential is immediately suppressed when the Ca^{2+}-salt is eliminated leaving only the Na^+-salt in the external solution. Addition of 1 mM Ca^{2+} to the internal solution suppresses the axon excitability within a brief period of time.

It is worth mentioning in this connection that solutions of $CaBr_2$, $SrCl_2$ or $BaCl_2$ can be used in place of

the extracellular $CaCl_2$ without suppressing the axon excitability [18]. The internal perfusion solution favorable for the production of large action potentials includes dilute phosphate or fluoride salt solutions of Cs^+, tetraethylammonium, choline, etc. Although the presence of a monovalent cation salt in the external solution is not essential for the maintenance of axon excitability, addition of the salt of hydrated or hydrophilic cations (Na^+, hydrazine, guanidine, etc.) to the external Ca^{2+}-salt solution enhances the action potential amplitude significantly [18].

It is not difficult to understand the mechanism of action potential production in the axon under the totally simplified experimental condition illustrated in Fig. 10, right. The axolemma-ectoplasm complex is sandwiched, under such circumstances, between the Na^+-containing internal solution and the Ca^{2+}-rich external solution. The high external Ca^{2+} concentration warrants the presence of a compact Ca-rich layer near the external surface of the complex. To elicit an action potential from the axon, a pulse of outwardly directed electric current has to be delivered to the axon. Such a stimulating current pulse tends to lower the ratio of Ca^{2+} to Na^+ ions in the layer. When the pulse is strong enough, the Ca cross-bridges in the layer are expected to be broken in a cooperative manner.

When a stimulating current pulse of the threshold strength is employed to excite an axon, a rapid rise of the potential in the axon takes place in the absence of an electric current through the complex. What happens during this rising phase of action potential is a massive movement and rearrangement of water molecules associated with conversion of the osmotically inactive (cross-linking) form of calcium ions into their osmotically active (free and weakly bound) form. This structural transformation of the complex causes a rise in the mobilities of the counter-ions in the complex. The potential difference between the internal and external salt solutions is determined predominantly by the distribution of counter-ions with different mobilities between the two solutions. We emphasize here that the presence of a potential difference between two electrolyte solutions is a consequence of the electroneutrality requirement (see p. 368 in Helfferich's monograph "Ion Exchange" [48]).

The relaxation process that occurs in the excited state of the axon can also be easily understood. At the moment when the external layer of the complex is transformed from a compact to swollen state, there is an abrupt rise in the cation-interdiffusion in the complex, which brings about a gradual change in the cation distribution within the complex. The interdiffusion fluxes are by far weaker in the case involving migration of only two cations, Ca^{2+} and Na^+, than in the case involving all three cations, Ca^{2+}, Na^+ and K^+. Without going into detail, it may be concluded that the difference in duration among the three action potentials presented in Fig. 10 is a reflection of this difference in the intensities of the interdiffusion fluxes (see Tasaki [18] for further discussion on this problem).

From what has been stated above, it should be possible to induce an action potential simply by lowering the external Ca^{2+} (without using electric current). In fact, it is well-known in classical neurophysiology that lowering of the Ca^{2+} concentration in the external medium can induce "repetitive firing of action potentials" without electric current (see p. 186 in Tasaki [18]). This is also the case in internally perfused squid giant axons.

Overview

When a nerve fiber is repetitively firing action potentials, with or without electric stimulation, the electric potential inside the fiber rises, then falls, and in the end, returns to its initial level. We have seen that swelling of the nerve fiber is followed by shrinkage, and at the end of each excitation cycle, the diameter of the fiber returns nearly to its original value. Similarly, the production of heat is followed by absorption of heat and eventually the temperature of the fiber goes back roughly to the initial level. These facts suggest that we may regard the underlying rapid structural change in the fiber as, in essence, a reversible process. Naturally, this reversible process is superposed on the irreversible aspects of the excitation process, namely, on the dissipation of free energy associated with the generation of electric current and diffusion of cations. In the past, great efforts were made to examine these irreversible aspects of the process.

We have seen that the excitation process in a nerve fiber involves the rapid spreading of a structural change occurring at one location of the fiber to a neighboring location. An exchange of cations at one binding site or domain has a strong influence over the behavior of a neighboring site or domain. To use biochemists' terminology, a nerve fiber is an "allosteric system" [57]. In physiology, the existence of a long-range electric interaction between neighboring sites of the nerve fiber has been known for more than a century, namely, since the time of Hermann [58].

Our observations of the mechanical and thermal changes in synapses [10, 25, 59, 60] and sensory and glandular tissues [23, 25, 30, 61] suggest the possibility of extending the notion of cooperative structural

change to the excitation processes in these tissues. If the cortical gel layers of these tissues are maintained, by virtue of the cellular metabolism, in a state very close to the threshold for abrupt transition, it should be possible to induce a large structural change in these tissues by a minute perturbation of the system, such as the binding of a few transmitter molecules [62] or the absorption of a small number of light quanta. The exceedingly high sensitivity, efficiency and rate of development of a response encountered in the stimulation of these tissues have been a perplexing problem in neurophysiology for ages. The notion of cooperative ion-exchange at the binding sites appears to offer a new approach to the problem.

In closing, we emphasize the need for more information about the non-electrical manifestations of excitation processes.

Shortly after the manuscript of this article was completed, I learned that Sir Alan Hodgkin died after a long illness. I take this opportunity to acknowledge my deep indebtedness to Sir Alan. When I was in postwar Tokyo, he graciously arranged for me to study in England. I spent a marvelous and memorable time in his laboratory in 1951. Numerous papers he published have provided starting-points for my research on myelinated and nonmyelinated nerve fibers. Additionally, I express my acknowledgment to Dr. Peter Basser and to Dr. Ralph Nossal of the Laboratory of Integrative and Medical Biophysics, NICHD, for their continuous support.

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