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CHANGES IN AXON BIREFRINGENCE DURING THE ACTION POTENTIAL

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SUMMARY

1. Observations have been made on the changes in optical retardation accompanying the passage of impulses along crab leg nerves and squid giant axons.

2. The nerves were mounted on the stage of a polarizing microscope, at 45° to the planes of polarization and analysis, brightly illuminated with white light. During the nerve impulse the intensity of the light passing the analyser decreased temporarily by 1 part in 10^3–10^6. Signal-averaging techniques were used to obtain an acceptable ratio of signal to noise.

3. The changes in light intensity recorded under these conditions were shown to arise almost entirely from alterations in retardation, with little or no interference from scattering, absorption, linear dichroism or optical rotation effects; the occurrence of stimulus and coupling artifacts was also ruled out.

4. In the squid giant axon, the retardation change was shown to be located in a thin cylinder immediately surrounding the axoplasm, and to have a radially oriented optic axis.

5. The time course of the decrease in optical retardation was very similar to that of the action potential recorded with an intracellular electrode, suggesting that the retardation closely followed the electrical potential across the membrane.

INTRODUCTION

Little is known about any changes in physical structure that may occur during electrical activity in nerves. Cole & Curtis (1938) reported that in

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Nitella the membrane capacity decreased by as much as 15% during the action potential, but in the squid giant axon the capacitance decrease was apparently under 2% (Cole & Curtis, 1939). A simple method of detecting structural changes is to apply optical techniques, but early attempts (Schmitt & Schmitt, 1940) to record birefringence changes in squid axons were unsuccessful through lack of instrumental sensitivity. Some years later, Hill & Keynes (1949) discovered that there were cumulative slow changes in the amount of light scattered by crab nerves during and after stimulation, and Hill (1950*a*) suggested that these were related to a swelling of the fibres which he was able (1950*b*) to measure directly in *Sepia* axons. In the course of a recent re-investigation of light scattering in crab nerve, we also made some observations with polarized light, and having a signal-averaging computer available in the laboratory, we quickly found that with its help we could record with relative ease a rapid change in retardation that appeared to be synchronous with the spike. In this paper we describe experiments on crab leg nerves and squid axons in which the magnitude of the retardation change during the conducted action potential was determined, and in which its localization and the orientation of its optic axis were established. In a later paper (Cohen, Hille, Keynes, Landowne & Rojas, 1971) we discuss the application of voltage-clamp techniques to analyse in greater detail the responses of membrane retardation to abrupt changes in potential.

An object is said to be birefringent or optically anisotropic if its refractive index as observed in polarized light is not the same in every plane. In a uni-axial object, the birefringence is defined as the difference between the refractive index n_e for light whose electric vector is parallel to the optic axis and the refractive index n_o for light polarized perpendicular to the optic axis. The magnitude of $(n_e - n_o)$ is related to the degree of structural order in the object: myelinated axons are more birefringent than non-myelinated axons which are more birefringent than egg cell cytoplasm. The retardation R of a birefringent object of thickness l is related to the phase difference θ (in radians) between coherent beams of light of wavelength λ polarized parallel and perpendicular to the optic axis, and is given by

$$R = \frac{\theta}{2\pi}\lambda = l(n_e - n_o). \tag{1}$$

If such an object is placed in a beam of linearly polarized light, with an angle ψ between its optic axis and the plane of polarization, the intensity of the light emerging from an analyser oriented at right angles to the plane of polarization is given by Fresnel's equation

$$I = I_0 k \sin^2 2\psi \sin^2 \tfrac{1}{2}\theta, \tag{2}$$

where I_0 is the incident intensity and k is a constant. Normally ψ is arranged to be 45°, so that

$$I = I_0 k \sin^2 \tfrac{1}{2}\theta = I_0 k \sin^2 \frac{\pi R}{\lambda}. \tag{3}$$

If now we are concerned with the measurement of a small change in retardation ΔR for an object whose 'resting' retardation is R, we determine the corresponding change in intensity ΔI, and the resting intensity I_r, and differentiate eqn. (3) to obtain

$$\frac{\Delta I}{I_r} = \cot \tfrac{1}{2}\theta \, \Delta\theta. \tag{4}$$

Using the approximation that, for sufficiently small values of θ, $\cot \frac{1}{2}\theta$ can be replaced by $2/\theta$, ΔR can be calculated from

$$\Delta R = \tfrac{1}{2} R \left(\frac{\Delta I}{I_r} \right). \tag{5}$$

Preliminary reports on this work have been published by Cohen, Keynes & Hille (1968), Cohen & Keynes (1969*b*), Hille (1970), Keynes (1970) and Cohen & Landowne (1970); and the methods have been demonstrated to the Physiological Society (Cohen & Keynes, 1969*a*). Several of our findings have been confirmed elsewhere (Tasaki, Watanabe, Sandlin & Carnay, 1968; Berestovsky, Lunevsky, Razhin, Musienko & Liberman, 1969; Berestovsky, Liberman, Lunevsky & Frank, 1970). An account of similar observations on birefringence changes in the electric organ of *Electrophorus* has been given by Cohen, Hille & Keynes (1969).

METHODS

Nerves were dissected from the walking legs of *Maia squinado* by the 'pulling out' method of Furusawa (1929). Giant axons 500–1040 μm in diameter were obtained from the hindmost or second hindmost stellar nerve of *Loligo forbesi* or, occasionally, *L. vulgaris*. The small fibres were removed as completely as possible from the part of the axon which was to be examined optically; the remainder of the axon was less thoroughly cleaned. Most of the squid mantles had been stored for some hours in cold sea water, as described by Caldwell, Hodgkin, Keynes & Shaw (1960).

A schematic diagram of the experimental arrangement is shown in Fig. 1. In most of the experiments the polarizer and analyser formed part of a Reichert Zetopan polarizing microscope as described previously (Cohen *et al.* 1969). The light source was a 24 V 150 W tungsten–halogen lamp mounted in the lamp housing of the microscope and connected to lead accumulators. In the absence of the polarizer, the axon was illuminated with about 10 mW of light; this intensity did not raise its temperature by more than 2° C. Since the attainment of an acceptable ratio of signal to noise necessitated the use of as high an intensity as possible, as explained by Cohen *et al.* (1969), white light was normally used, and retardations were calculated assuming a wave-length of 550 nm. For measurement of the wave-length dependence of the retardation change, Balzers Type K wide-band interference filters were in-

serted in the light path. The photodetectors used in the earlier experiments were Mullard 53 CV and 53 CG vacuum phototubes, or a Mullard XP 1114 4-stage photomultiplier tube. Later, type SGD 100 or SGD 444 silicon photodiodes (E.G. & G. Inc., Boston) were substituted. These were preferred because of their appreciably greater quantum efficiency as light detectors, and because they could be used without danger of saturation over a wider range of light intensities. Both the XP 1114 tube and the photodiodes were used with an anode load of 100 kΩ, capacity-coupled to a

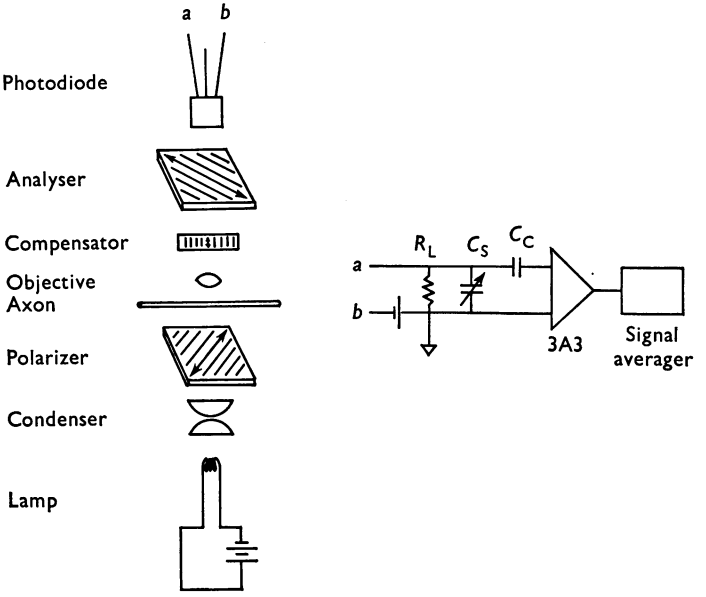

Fig. 1. Schematic diagram of the experimental arrangement for measuring retardation changes in nerve. Parallel light from a tungsten–halogen bulb was passed through a Glan–Thompson prism polarizer and focused by a long-working-distance condenser on to the nerve. An image of the nerve was formed by a 10× strain-free objective, above which was a slot for introduction of a Brace–Köhler compensator or a quarter-wave plate, and then a Polaroid analyser. The output from the photodetector was measured across the load resistor R_L. The d.c. and low-frequency components of the signal were eliminated by the coupling capacitor C_C. The high-frequency response was regulated by the smoothing capacitor C_S.

Tektronix 3A3 amplifier in a 565 oscilloscope. The coupling time constant was always at least 10 times the sweep duration of the signal-averaging computer. A smoothing capacitor, C_S, was used to increase the response time to a step change in light to the values given in the figure legends. The output from the 3A3 amplifier was fed to one of two signal averagers, a TMC Computer of Average Transients (CAT 400 C) or a Princeton Waveform Eductor (type TDH-9). The averaged signals were plotted on an X–Y potentiometric recorder.

Since for observation under the microscope the squid axons had to be mounted horizontally rather than vertically, the method of inserting internal electrodes devised by Hodgkin & Huxley (1945) had to be modified, although the basic principle

for steering the tip of the electrode down the centre of the axon was retained. The cannula to which the axon was tied projected horizontally through the centre of one wall of a rectangular Perspex chamber whose internal dimensions were 3 cm deep, 8 cm wide and 9 cm long. The electrode was attached to a Prior micromanipulator fixed to the same wall so that it could be advanced horizontally into the axon through the cannula. The electrode tip could be observed in two planes with the help of a small 45° mirror also attached to the micromanipulator movement, and its progress was kept central by moving the free end of the axon up, down or sideways. When the electrode had reached the desired position, the end of the axon was tied to a support on the opposite wall of the chamber, and the bottom of the chamber was removed. The chamber was then reassembled for the optical measurements with a different bottom and lid in which there were windows of strain-free glass, a slotted partition across which shocks could be applied to stimulate the axon, and channels through which cooling water flowed to control the temperature. The bottom also incorporated a V-slide which enabled the chamber to be fastened securely to the rotating stage of the microscope and positioned with the electrode tip in the centre of the field.

The electrodes were KCl-filled pipettes 100 μm in diameter, similar to those described by Hodgkin & Katz (1949*a*, Fig. 2*c*) except that a bright platinum wire was used in place of silver. The cathode follower was similar to one described by Hodgkin & Huxley (1945), or was a Bioelectric Instruments PF-1. For stimulation a Grass Instruments SD5 stimulator was used, or a Devices Digitimer with a Mark IV stimulus isolator. A Panax decade counter was used to record the number of sweeps that were averaged in each group. The microscope was fitted with a 10× (N.A. 0·25) strain-free objective. Two adjustable shutters controlled by micrometer screws were mounted at the top of the microscope in the plane of the image, so that the optical field examined was a well-defined slit parallel to the axon. A centre stop could also be added, in order to block out the image of the electrode and the middle part of the axon.

The chamber was filled with a bathing medium which was normally either filtered sea water or an artificial sea water, 10 K (Na)ASW (Baker, 1965).

RESULTS

The resting retardation

Several of the structural components of a nerve fibre are birefringent. Some parts have an optic axis parallel to the long axis of the fibre, but others have a radial or circumferential optic axis. We shall in general refer to the sign of the retardation that is observed as if the axon were uniaxially birefringent with a longitudinal optic axis. This is a useful simplification, but the composite nature of the system that we are studying must not be forgotten.

With this convention, the net retardation of a squid axon or a crab nerve trunk is positive. The average retardation measured at the centre of six squid axons whose mean diameter was 850 μm was 56 nm, which represents a birefringence of $6{\cdot}6 \times 10^{-5}$ (cf. Forman, 1966). The average retardation of crab leg nerves whose mean diameter was 600 μm was 32 nm. In the squid axon the retardation did not vary greatly across the axon, as may be seen in Fig. 2*b*. Most of the resting retardation at the

centre of the axon was apparently contributed by longitudinally oriented fibrils and tubules in the axoplasm, because removal of the axoplasm and perfusion with potassium fluoride solution reduced the retardation through the centre by about 90 %. At the edges of the axon the retardation was unaffected by perfusion, because of the appreciable contribution made by the form and intrinsic birefringence of the Schwann cell and connective tissue (Schmitt & Bear, 1939).

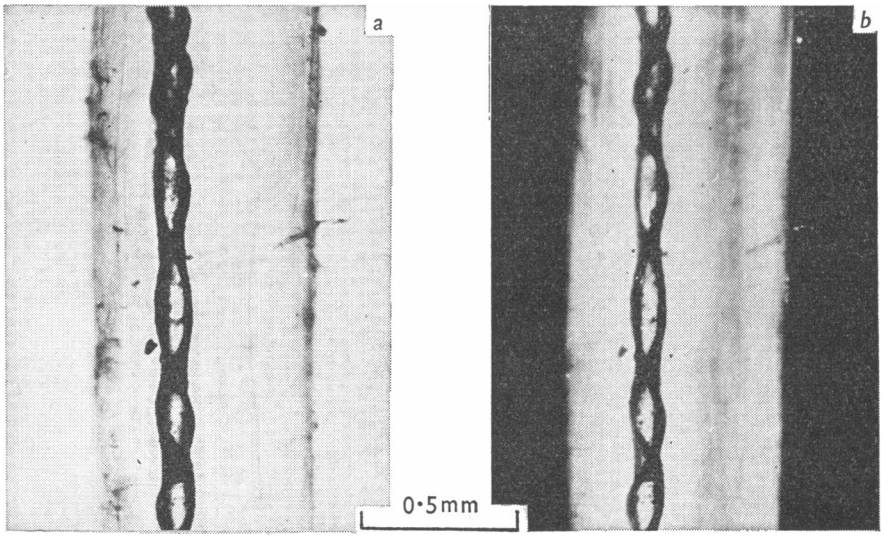

Fig. 2. Photomicrographs of a squid giant axon with a double-spiral internal electrode (see Cohen *et al.* 1971). The axon was mounted horizontally on the stage of the Reichert Zetopan polarizing microscope, at 45° to the plane of polarization of the incident light. Left-hand picture: planes of polarization and analysis parallel. Right-hand picture: perpendicular. Note that the intensity of light, and hence the resting retardation, was nearly uniform at all positions across the giant axon.

The axon membrane itself probably contributes relatively little to the net resting birefringence. If it had a structure similar to that of an erythrocyte ghost, it would have a positive intrinsic birefringence and a negative form birefringence *both with a radial optic axis*, so that referred to the longitudinal axis its intrinsic birefringence would make a negative contribution and its form birefringence a positive one. However, the retardation of the edge of an erythrocyte ghost, placed in glycerol to minimize the contribution of form birefringence, is only about 0·4 nm (Mitchison, 1953), or less than 1 % of the average retardation of a squid axon. Since in nerve fibres the membrane is thus a rather minor com-

ponent as far as the overall birefringence is concerned, there is no direct way in which the absolute magnitude of its retardation can be determined.

Evidence that the observed intensity changes represent changes in retardation

In the experiment illustrated in Fig. 3, the axon was stimulated and the resulting change in intensity was recorded when the angle between the axon and the plane of polarization of the incident light was either 0 or 45°. At 0° the intensity did not change, but at 45° it first decreased and

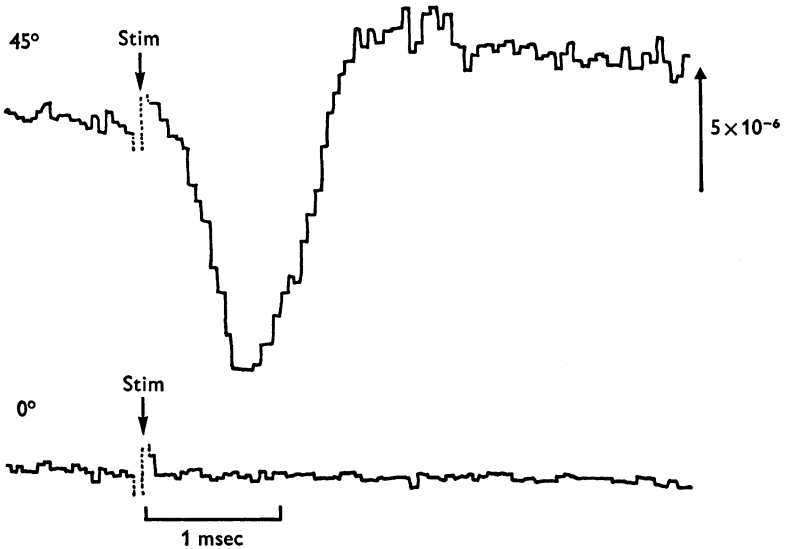

Fig. 3. The intensity changes recorded during propagation of an impulse along a giant axon from *Loligo forbesi* when the angle ψ between the axon and the plane of polarization of the incident light was 45° (above) and 0° (below). Similar results were obtained in experiments on crab leg nerves (Cohen *et al.* 1968, fig. 3). In this and other figures the arrow labelled 'Stim' indicates the time when the axon was stimulated. The direction of the arrow to the right of the tracings indicates an increase in light intensity, and the length of the arrow corresponds to the stated value of $\Delta I/I_r$ for a single sweep. All such records were traced from the original $X-Y$ recorder plots. Temperature 13° C; number of sweeps averaged was 3000 in each case. The response time constant to a step change in the light was 30 μsec.

then increased. The length of the arrow to the right of the optical records corresponds to the stated value of change in intensity (ΔI) for a single sweep divided by the resting intensity (I_r). At the beginning of twenty-seven experiments on squid giant axons, the peak value of $\Delta I/I_r$ per sweep at 45° averaged $8{\cdot}1 \pm 0{\cdot}9 \times 10^{-6}$ (S.E. of mean). Six consecutive measurements

of $\Delta I/I_r$ per sweep made on the same axon averaged $11{\cdot}3 \pm 0{\cdot}7 \times 10^{-6}$ (S.D. of a single observation). The average value in measurements on eleven crab nerves was $4{\cdot}5 \pm 0{\cdot}8 \times 10^{-4}$ (S.E. of mean). During these experiments the chamber temperature was controlled between 5 and 22° C.

In Fig. 4, the relative peak size of $\Delta I/I_r$ measured in two experiments of this kind is plotted against ψ. If the change in light intensity resulted purely from a change in either retardation or linear dichroism, then the

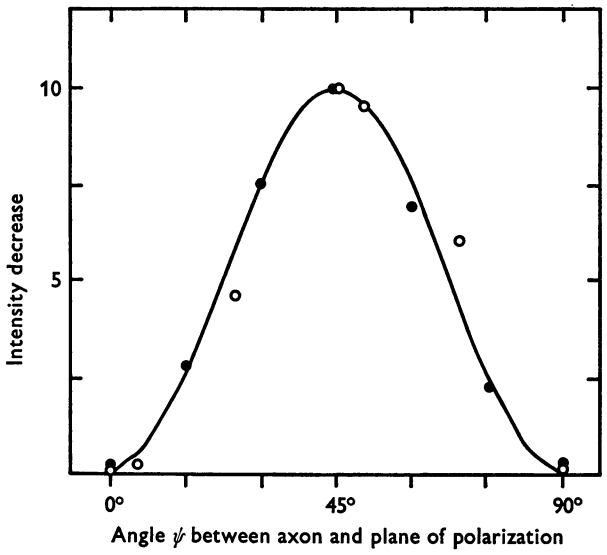

Fig. 4. The normalized intensity change [ΔI] in squid giant axons, ○, and crab leg nerves, ●, as a function of the angle ψ. The continuous line is the curve for $\sin^2 2\psi$ on which the points would fall for a change in either retardation or linear dichroism. At 0° and 90° ΔI was less than 0·5. Temperature 13·5° C. Number of sweeps averaged was 10,000 for squid, 10 for crab.

experimental points would fall on the continuous line calculated from eqn. (2). The coincidence is quite good, which it would not be if there were appreciable contributions from changes in light scattering, optical rotation or absorption. Under the experimental conditions of Figs. 3 and 4 the whole of the observed change in intensity must therefore represent either a retardation or a dichroism change.

A simple way of distinguishing between these two possibilities is to add a variable optical retardation between the axon and the analyser. A Brace–Köhler rotating mica plate compensator with a maximum retardation of $\frac{1}{10}\lambda$ was used in the case of crab nerve, but for squid axons a greater retardation was usually needed, so that the quarter-wave plate method of

de Sénarmont-Friedel (Bennett, 1950) had to be used. Fig. 5 shows three tracings of the changes in light intensity during the spike in an optical compensation experiment on a squid axon. In the top trace, no compensation was introduced; in the middle trace there was just enough series

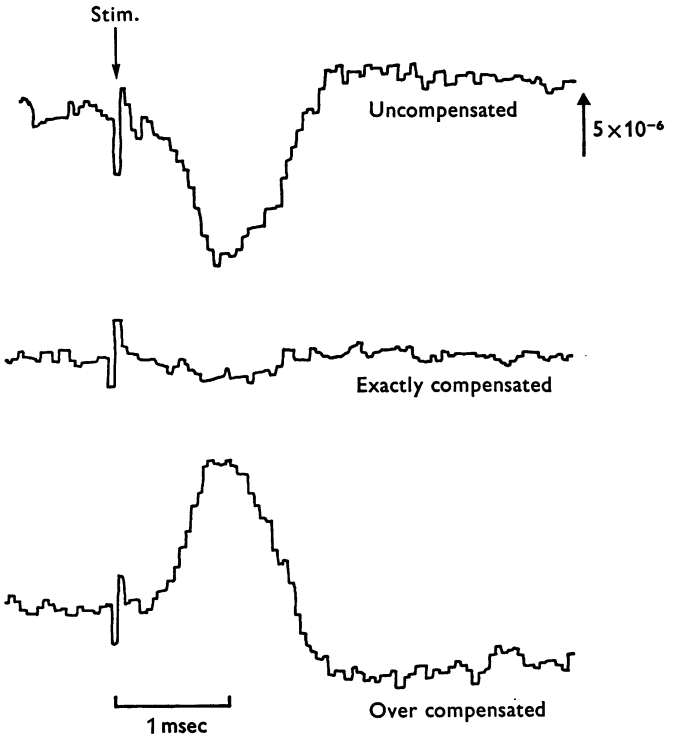

Fig. 5. Intensity changes during the spike in a giant axon with and without additional fixed retardation introduced between the axon and the analyser by the quarter-wave plate method of de Sénarmont-Friedel (Bennett, 1950). Top tracing: no compensation. Middle tracing: resting retardation exactly compensated. Bottom tracing: total resting retardation roughly the same as in top tracing, but reversed by overcompensation. Adjustable shutters in the image plane of the objective were used to block the light that did not pass through the axon. In this and subsequent figures ψ was 45°. Temperature 13° C; time constant 45 μsec; number of sweeps averaged was 3000. Both in this experiment and in similar tests on crab nerves, the time course of the intensity change when the resting retardation was reversed by overcompensation was almost exactly the inverse of that with no compensation.

compensation to reduce the resting retardation to zero; and in the bottom trace the compensation was further increased until the resting intensity was about equal to that in the absence of compensation—i.e. the compensation just reversed the resting retardation. As may be seen, the record for

the overcompensated axon was almost exactly a mirror image of that for the uncompensated axon. A small difference found on two occasions was that the extent of the return of the intensity past the base line at the time corresponding to the positive phase of the action potential (Frankenhaeuser & Hodgkin, 1956) was relatively greater during overcompensation than it was without compensation. This suggests that there was a small amount of interference from other types of optical effect.

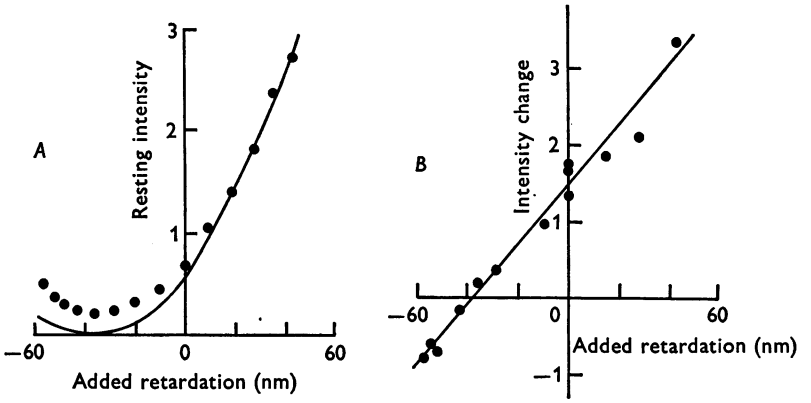

Fig. 6. *A*. The resting intensity (I_r) in a crab nerve as a function of added retardation. The line is the value calculated for a homogeneous birefringent object with a resting retardation of 35 nm, drawn to fit the point at which the added retardation was +20 nm.

B. The change in intensity (ΔI) on stimulation of a crab nerve, plotted as a function of retardation added by means of a Brace–Köhler compensator. The points lie close to the line calculated for a pure retardation change. Temperature 16° C; number of sweeps averaged for each point was twenty. Similar results were obtained for squid giant axons (see also Cohen *et al.* 1971).

The results of experiments with optical compensation can be treated quantitatively in the manner illustrated in Fig. 6. The points plotted on the left (Fig. 6*A*) show the variation in resting intensity for different amounts of added retardation. The solid line is the intensity calculated from eqn. (3) for a nerve behaving as a homogeneous pure retardation of 35 nm. The experimental points lie close to the theoretical curve for extra retardations whose sign is the same as that of the nerve, but in the other direction the fit is less good, and exact compensation was not achieved at any setting of the compensator. This is partly attributable to the optical inhomogeneity of the nerve, which made it impossible to match the resting retardation simultaneously over the whole field, so that the intensity could never be reduced to zero. This effect was estimated to account for about half the discrepancy between theory and practice. The remainder

may have resulted from partial depolarization of the incident light by scattering effects.

In Fig. 6*B* the change in intensity during the spike is plotted against the added retardation. Since from eqn. (3) it follows that

$$\Delta I = \tfrac{1}{2} I_0 k \sin\theta \, \Delta\theta, \tag{6}$$

then, if the retardation change in the axon ($= (\lambda/2\pi).\Delta\theta$) is constant and small, ΔI will be directly proportional to $\sin\theta$ and will thus vary almost linearly with added retardation in the range examined. The experimental points in Fig. 6*B* do lie on a straight line, whereas if the intensity change had been caused by a change in linear dichroism, the plot would have been parabolic. We therefore conclude that the changes in light intensity observed during conducted action potentials represent changes in optical retardation rather than any other type of optical effect. Retardation is a function both of path length and of birefringence. We have no evidence at present to show whether the retardation change arises from an alteration in membrane thickness or in its birefringence.

Controls

The compensation experiments just described provided a control against the possibility that the observed signals resulted from an electrical coupling between the action potential and the light-detection system. Electrical coupling would not be reversed in sign by rotating an optical compensator, and therefore cannot account for the results. Further controls were carried out to prove that the apparent retardation change was not a stimulus artifact, nor a spurious result of electrical coupling between the electrical and optical recording systems, nor an artifact caused by movement of the nerve.

Two kinds of test showed that the intensity change was not a stimulus artifact. First, the propagation of the action potential was blocked by raising the external [K] to 100 mM, or by replacing all the sodium in the artificial sea water with choline, or by treatment with tetrodotoxin 10^{-6} g/ml. In each case the change in light intensity was abolished, although the stimulus voltage and current were unaltered. These experiments were done both on crab nerves and on squid axons; Fig. 7*A* shows an example for a crab nerve tested with tetrodotoxin. Secondly, the intensity changes were measured as a function of stimulus strength. In crab nerves (Fig. 8*A*) the optical signal increased gradually with increasing shock strength at low voltages, but then reached a plateau. In squid axons (Fig. 8*B*) the optical signal appeared as soon as the threshold for electrical excitation was exceeded, and was constant in size for larger shocks. These observations are, of course, what would be expected for a multi-fibre preparation on the one hand, and for a single axon on the other, and they provide further proof that the change in light intensity was not a stimulus artifact.

Two types of control showed that the retardation changes did not result from electrical pick-up of the conducted action potential by the amplifier connected to the photodetector. One such control was the optical compensation experiment

described above. In the other, a stop was placed at the image plane of the objective to block the image of the nerve, and the analyser was slightly rotated so that the resting intensity recorded by the photodetector was the same as before. As may be seen in Fig. 7*B* blockage of the image in this fashion caused a complete disap-

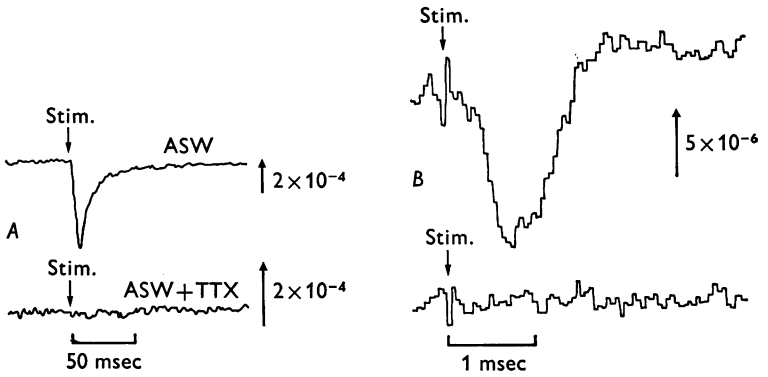

Fig. 7. *A*. Intensity changes in a crab nerve stimulated in artificial sea water in the presence (below) and absence (above) of tetrodotoxin 10^{-6} g/ml. Temperature 13° C; time constant 2 msec; number of sweeps averaged was 30 in ASW, 60 in ASW + TTX.

B. Intensity changes in a stimulated giant axon. For the lower tracing the image of the axon was blocked, and the analyser was rotated slightly so that the amount of light reaching the photodiode was the same as for the upper tracing, but none of it passed through the nerve. Temperature 13° C; time constant 30 μsec; number of sweeps averaged was 3000 in each case.

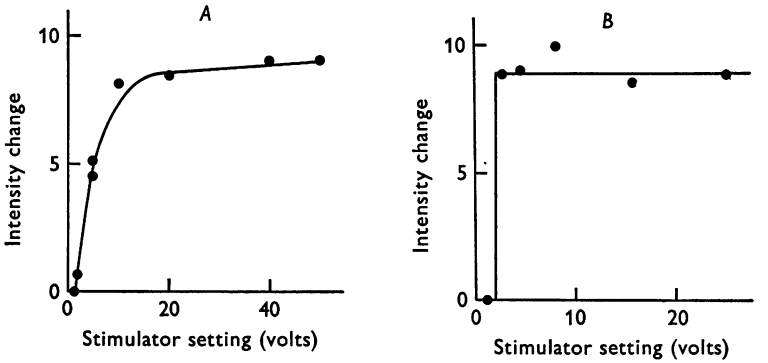

Fig. 8. Variation in intensity change (arbitrary units) with stimulus voltage. *A*. Crab leg nerve, ten sweeps averaged. *B*. Squid giant axon, 10,000 sweeps averaged. Temperature 13° C.

pearance of the change in light intensity, although the action potential was being conducted as before.

Bryant & Tobias (1955) reported that *Carcinus* leg nerves shortened by 1 part in 3×10^7 when given a train of stimuli at 30/sec. Since a nerve has somewhat different

retardations along its length, a shortening during the action potential might conceivably cause a change in light intensity. However, in several tests the intensity changes were the same whichever end of the nerve was stimulated, which provides some evidence against the occurrence of measurable movement artifacts.

Wave-length dependence

In one experiment, $\Delta I/I_r$ per sweep was measured in a crab nerve at several wave-lengths between 400 and 700 nm; its value was roughly constant over this range. Two experiments on squid axons subjected to hyperpolarizing voltage pulses (see Cohen *et al.* 1971) gave the same result.

Localization of the retardation change

The axoplasm of a giant axon can be removed and replaced with a salt solution without destroying its ability to conduct action potentials (Baker, Hodgkin & Shaw, 1961, 1962; Oikawa, Spyropoulos, Tasaki & Teorell, 1961). This permits a simple test to be made to see whether the retardation change is located in the bulk of the axoplasm, or only in the vicinity of

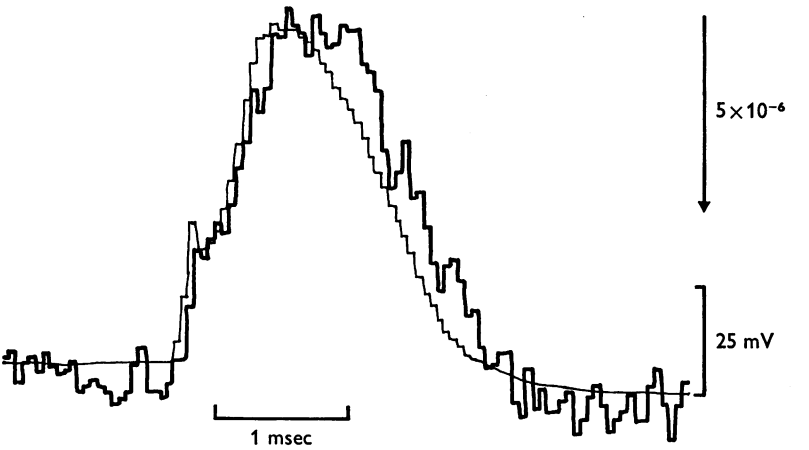

Fig. 9. Simultaneous recordings of the retardation change (thick line) and action potential (thin line) in a squid giant axon perfused with a potassium fluoride solution. Temperature 14° C; time constant for optical record was 24 μsec; number of sweeps averaged was 2030.

the membrane. Fig. 9 shows the results of an experiment in which the retardation change and the action potential were measured simultaneously in a perfused axon; the internally recorded spike is shown conventionally as an upward deflexion, and the optical record has therefore been inverted to facilitate comparison. The value of $\Delta I/I_r$ per sweep averaged $13{\cdot}8 \pm 3{\cdot}1 \times 10^{-6}$ (S.E. of mean) in experiments on three perfused axons and was

thus slightly larger than the corresponding figure for intact axons ($8{\cdot}1 \times 10^{-6}$), possibly because removal of the axoplasm reduced I_r without affecting ΔI. The 'garden roller' method of perfusion which we used removes up to 97% of the axoplasm, but leaves a layer 1–12 μm thick just inside the axon membrane (Baker *et al.* 1962). Although these experiments demonstrate that the central core of the axoplasm does not make a major contribution to the retardation change, they do not exclude the

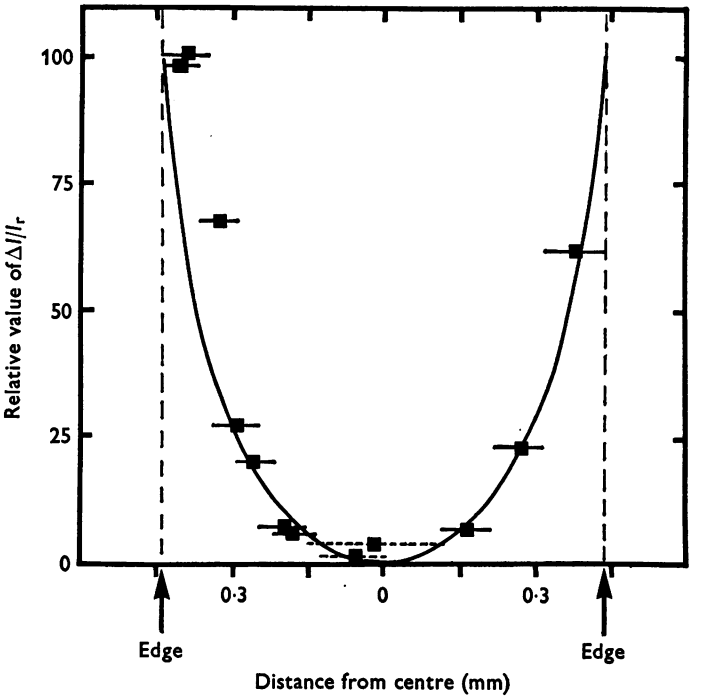

Fig. 10. The relative values for $\Delta I/I_r$ recorded for narrow strips of the image parallel with the edge of a squid giant axon at various positions across the axon. The lengths of the horizontal bars indicate the width of the strip for each point. At the centre of the axon no change in intensity could be seen, although any signal greater than one twentieth of that at the edge would have been detectable. The line is the predicted retardation for a thin birefringent cylinder with a radial optic axis. Temperature 14° C; axon diameter 890 μm; number of sweeps averaged was 2000 for each point.

possibility that it originates at least partly in axoplasmic components in the immediate neighbourhood of the inner surface of the membrane. Observations have not yet been made with perfusion fluids containing proteases to remove the last traces of axoplasm (Takenaka & Yamagishi, 1969).

In order to obtain further evidence on the localization of the retardation change and on the orientation of its optic axis, a narrow slit was placed in the image plane of the objective parallel to the long axis of the axon so that the light reaching the photodetector came only from a section of the axon that was 75–95 μm wide and 2 mm long. Measurements of the retardation change during the spike were then made at a succession of positions across the axon. Fig. 10 shows the results of one such experiment, from which it is seen that the value of $\Delta I/I_r$ was greatest at the extreme edge of the axon, and fell to zero at the centre. The continuous line is the prediction of a theory outlined in the next paragraph which assumes that the retardation change arises in a cylindrical shell of birefringent material with a radial optic axis, which lies roughly in the position of the axon membrane. The experimental points fall close to this theoretical curve. This establishes unequivocally not only that the retardation change is located close to or in the membrane but also that it has an optic axis which is mainly radial. For if the change had a longitudinal axis, $\Delta I/I_r$ would be greater at the edge than in the centre, but would not be zero in the centre; and if the optic axis were circumferential, $\Delta I/I_r$ would be greatest at the centre and zero at the edges. The precision of the measurements was, of course, insufficient to determine the *exact* localization of the change, which might have occurred either in the membrane itself, or in the regions close to its surface on either side, or even in the Schwann cell; and the participation of a minor component with an optic axis other than radial cannot be entirely ruled out. But the approximate localization of the retardation change and its predominant optic axis are not in doubt.

The curve for $\Delta I/I_r$ in Fig. 10 was calculated from the following considerations. Consider a birefringent membrane of thickness t whose optic axis is normal to its surface, immersed in a solution of refractive index n_s. Light striking the membrane at an angle ϕ_1 from the normal enters the membrane at an angle ϕ_2, where according to the law of refraction

$$n_s \sin\phi_1 = n_o \sin\phi_2, \tag{7}$$

n_o being the refractive index for the ordinary ray. The optical path length in the membrane is $t/\cos\phi_2$, and its retardation R increases with the angle ϕ_2 according to the relation

$$R = \frac{t}{\cos\phi_2}(n_e - n_o)\sin^2\phi_2, \tag{8}$$

where n_e is the refractive index for the extraordinary ray. Eqns. (7) and (8) can readily be used to find the retardation for beams of light striking a cylinder of birefringent membrane at different distances from the centre and passing twice through the membrane. Using values of 1·34 for n_s and 1·48 for n_o, the maximum retardation at the edge of the cylinder works out to be $3{\cdot}86t(n_e - n_o)$ (see p. 513). This derivation neglects any reflexions, and assumes that t is much less than the radius of the axon and that $(n_e - n_o)$ is very small in comparison with n_o.

Comparison of the retardation change with the action potential

An observation that provided an immediate clue to the origin of the decrease in retardation consisted in comparing its time course as accurately as possible with that of the intracellularly recorded action potential. The tip of a 100 μm axial electrode was placed at the centre of the illuminated

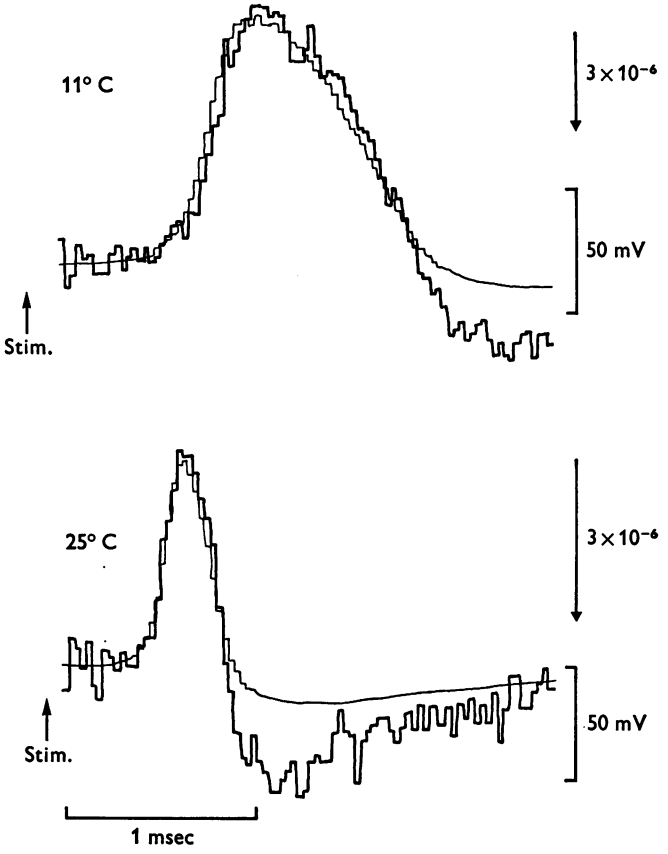

Fig. 11. Comparison of the intensity changes (thick lines) and the action potentials (thin lines) recorded at the same point in a squid giant axon at two temperatures, and scaled to coincide at the peak of the spike. The intensity traces have been inverted, so that an increase is downwards. Light response time constant 16 μsec; number of sweeps averaged was 10,000 at 11° C, 11,000 at 25° C.

region of the axon; its presence did not affect the retardation change. Fig. 11 shows superimposed optical and electrical records for the same squid axon at two different temperatures; as in Fig. 9 the optical records have been traced upside down to facilitate comparison. The action

potentials were recorded both before and after measuring the retardation change, and there was no discernible alteration in their time course over this short interval. It is clear that at both temperatures the time course of the retardation change closely paralleled that of the action potential, though at the lower temperature the optical record was slightly but consistently delayed (cf. also Fig. 9). This conclusion still held good when the illuminated region was reduced in length from 1·5 to 0·75 mm in order to minimize errors from time-lags in conduction of the spike, and when conduction effects were eliminated altogether by making the observations on a 2·5 mm length of axon which was space-clamped by a platinized platinum internal electrode.

When the optical and electrical records were adjusted to correspond in size during the main part of the spike, the retardation change appeared to be relatively accentuated during the positive phase at the tail of the spike which is characteristic of dissected squid axons, especially at the higher temperatures (Hodgkin & Katz, 1949*b*). This accentuation can be attributed partly to the non-linearity of the relationship between retardation and membrane potential (Cohen *et al.* 1971), but as mentioned earlier there may also be some interference from optical effects other than a retardation change. However, the general conclusion is warranted that the retardation change must be more directly dependent on membrane potential than on any other factor.

DISCUSSION

It is clear from the results presented here that during the action potential there is an appreciable change in the retardation of a structure which would appear, from its location, to be the nerve membrane or molecules closely associated with it. The experimental evidence does not in fact exclude the Schwann cell as the site of the retardation change, but this possibility seems rather unlikely for two reasons. First, much the greater part of the potential gradient that seems to be the principal or only controlling factor for retardation is located across the axon membrane. Secondly, very similar retardation changes have been observed in the electric organ, where there are no Schwann cells (Cohen *et al.* 1969). It would doubtless be unwise to dismiss the Schwann cell altogether from consideration, because the potential drop across it arising from the flow of current during the propagated action potential or the non-propagated depolarizing voltage-clamp pulse is not negligible. But any current-dependent component contributed by the Schwann cell to the retardation change must be a relatively minor one, since both propagated and membrane action potentials (recorded with an axial wire) give rise to similar retardation changes,

despite the fact that after the initial stimulus no current flows across the Schwann cell during a membrane action potential.

The experimental evidence gives no indication as to what kinds of molecule produce the retardation change. We only know that it is rather small, has a radial optic axis, and does not involve groups with strong absorption bands between about 400 and 700 nm. This conclusion does not enable us to distinguish between proteins, phospholipids and other molecules, or between changes in form or intrinsic birefringence. Since retardation is the product of optical path length and birefringence, it depends both on the thickness of the membrane and on its refractive indices, any of which might be altering during the spike. If it is correct to allocate to the nerve membrane optical properties similar to those of the erythrocyte membrane (Mitchison, 1953), then the decrease in retardation referred to the longitudinal axis which is seen during the spike, corresponds either to an increase in the intrinsic positive birefringence of the membrane or to a decrease in its negative form birefringence, because both have a radial optic axis. Since a reduction of the electric field across the membrane might have been expected to result in a worse alignment of its component molecules rather than a better one, an increase in intrinsic birefringence with a decrease in membrane potential could be regarded as improbable. But the entropy change that appears to accompany depolarization (Howarth, Keynes & Ritchie, 1968) corresponds similarly to a greater orderliness, and entropy and birefringence may somehow be related. Alternatively, if the retardation change involves a polarization or reorientation of groups in the membrane analogous with the Kerr effect, the Kerr coefficient might be negative rather than positive. It should perhaps be recalled in this connexion that the sign of the retardation change observed in the electric organ (Cohen *et al.* 1969) seems at first sight to be the opposite of that found in nerve; but because of the multiple invagination of the faces of the electroplates, there is some doubt as to what is the predominant plane of orientation of the membrane.

If the whole of the observed retardation change is assigned to the axon membrane, we can try to estimate how much the structure of the membrane changes. The percentage change in membrane retardation is certainly much greater than the percentage change in total retardation, since most of the resting retardation arises from sources other than the membrane itself. The product $t(n_e - n_o)$ of the thickness and birefringence of a squid axon membrane is not known, but for a human erythrocyte ghost membrane it is 0·08 nm (Mitchison, 1953). For the myelin of frog nerves, the birefringence is about 0·011 (Schmitt & Bear, 1937), so that if the thickness of a single membrane is 8·5 nm (Finean & Burge, 1963), the product is 0·09 nm. The average value of $\Delta I/I_r$ for the action potential in a squid

axon was 8×10^{-6}, but in many of these experiments the recording was not restricted to the extreme edge of the axon, where, as Fig. 10 shows, the change was appreciably greater. A value of 2×10^{-5} at the edge would therefore be more realistic. Taking the resting retardation as 56 nm (see p. 499), ΔR is, from eqn. (5), $5{\cdot}6 \times 10^{-4}$ nm. The theory discussed on p. 509 predicts that at the edge of the axon $\Delta R = 3{\cdot}86\Delta\{t(n_e - n_o)\}$, whence the calculated change in the product of birefringence and membrane thickness was $1{\cdot}45 \times 10^{-4}$ nm. On this basis the optical properties of the membrane change by about 0·15 % during the action potential. This estimate is unavoidably subject to considerable uncertainty, but it serves to indicate the order of magnitude that might be anticipated for concomitant changes in other parameters of the membrane. Clearly, a capacitance change of this size would have been well below the limits of resolution in the experiments of Cole & Curtis (1939).

Further discussion of the mechanism of the retardation change and the nature of the underlying molecular rearrangements is best postponed until the results of voltage-clamp studies have been presented (Cohen *et al.* 1971).

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