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CHANGES IN LIGHT SCATTERING THAT ACCOMPANY THE ACTION POTENTIAL IN SQUID GIANT AXONS: POTENTIAL-DEPENDENT COMPONENTS

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SUMMARY

1. To obtain information about structural events that occur in axons, changes in light scattering from squid giant axons were measured during action potentials and voltage-clamp steps.

2. The scattering changes were measured at several scattering angles. Because the changes in scattering divided by the resting scattering were between 10^{-6} and 10^{-5}, signal-averaging techniques were used to increase the signal-to-noise ratio.

3. The scattering changes during the action potential were different at different angles. Two types were found, one at 10–30° (forward angles) and the other at 60–120° (right angles).

4. At forward angles, there was a transient scattering *decrease* during the action potential. The time course of the change was similar to that of the action potential; this change was thought to be potential-dependent.

5. At right angles, there was a transient scattering *increase* during the action potential followed later by a second, longer-lasting increase. Indirect evidence indicated that neither component could be totally potential-dependent.

6. To further analyse these effects, scattering was measured during voltage-clamp steps. The changes seen during hyperpolarizing steps were presumed to be potential-dependent; again two different changes were found, one at forward angles and one at right angles.

7. The potential-dependent change at right angles occurred with a time course that could be approximated by a single exponential with a time constant $\tau = 24$ μsec. The change at forward angles required two exponentials, $\tau_1 = 23$ μsec, $\tau_2 = 900$ μsec, to represent its time course.

8. The size of both potential-dependent changes was proportional to the square of potential. The change at right angles, but not that at forward angles, was increased in size by the addition of butanol or octanol to the bathing solution.

INTRODUCTION

While the ionic basis of action-potential propagation has been accepted for some time (Hodgkin & Huxley, 1952), investigation of the concomitant structural events has proceeded slowly. In the hope of obtaining structural information, we have measured changes in light scattering from squid giant axons during action-potential propagation and during voltage-clamp steps.

The electrical events occurring during the action potential were divided into three types: first, the changes in membrane potential; secondly, the ionic currents; and thirdly, the transient increases in membrane conductance. An attempt was made to determine which of the three types of events was giving rise to each scattering change. That is, we tried to determine the type of electrical event on which each kind of optical change depended most closely. When the axon birefringence changes (Cohen, Hille, Keynes, Landowne & Rojas, 1971) were characterized as potential-dependent, we meant that some optical properties of the axon changed in response to the change in potential.

Light-scattering changes were first seen in crab leg nerves by Hill & Keynes (see Hill, 1950). With the techniques then available, trains of 100–500 stimuli were required to measure a change in scattering, and no information was obtained about the time course of the change during a single action potential. More recently, Cohen & Keynes (1971) and Cohen, Hille & Keynes (1969) were able to measure scattering changes following single shocks in crab nerves and in slices of eel electric organ. Analysis of the scattering changes in these preparations was difficult, but there was no doubt that some of them were different in origin from the potential-dependent changes in birefringence. These experiments also suggested an investigation of scattering as a function of scattering angle and external refractive index, two variables that we tried to control in the measurements presented here. The present paper describes the light-scattering changes that occur during the action potential in the squid giant axon, and analyses that portion of the changes which is potential-dependent. A second paper (Cohen, Keynes & Landowne, 1972) is concerned with the contribution of changes which are not potential-dependent.

By light scattering we mean those processes which cause light to leave the transmitted beam and thus be measurable by a photodetector placed outside the transmitted beam (see Van de Hulst, 1957). Clearly, there may

have been contributions from reflected and refracted light in our measurements.

Preliminary reports of this work have been published by Cohen, Keynes & Hille (1968); Cohen & Keynes (1970); and Cohen & Landowne (1970).

METHODS

Giant axons were dissected from the hindmost stellar nerves of *Loligo pealii* and *L. forbesi*. The axons from *L. pealii* were 410–660 μm in diameter; those from *L. forbesi* were 590–975 μm. A few experiments (five) were made on axons from *L. vulgaris*. The small fibres were removed as completely as possible from a 20 mm length of axon; only the central 4 mm of this length was illuminated. For some experiments, the squid mantles had been stored for several hours in cold sea water as described by Caldwell, Hodgkin, Keynes & Shaw (1960). Axons from these mantles gave the same results as those from freshly killed squid.

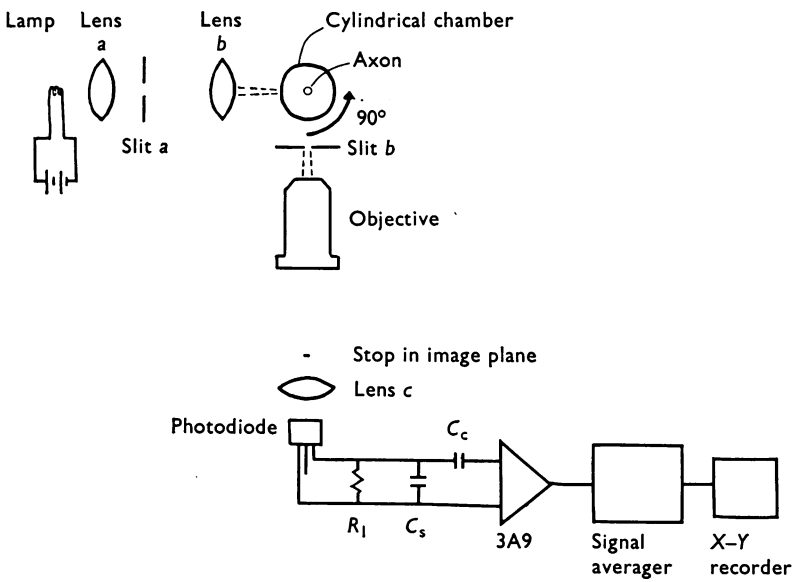

Fig. 1. A schematic diagram of the experimental apparatus for measuring light scattering, shown in the position for measuring 90° scattering. Two Balzers B_1/K_1 heat filters were placed between lens *a* and slit *a*. The cylindrical chamber was equipped with a positionable gate about 20 mm below the illuminated portion of the axon. For action potential experiments, the stimulus currents were passed between electrodes on either side of this gate.

A schematic diagram of the experimental apparatus is shown in Fig. 1. The 12 V, 100 W tungsten halogen lamp was powered by a lead accumulator, the voltage of which was kept constant with a Hewlett–Packard 6267 B direct-current power supply. The light was collected by a condenser lens (lens *a*), passed through slit *a*, and focused by lens *b* into a 4 mm-diameter spot which illuminated the axon. The axon was per-

pendicular to the plane of observation (plane of the paper in Fig. 1) in the centre of the cylindrical chamber made from glass tubing (10 mm i.d., 12 mm o.d.). The solution surrounding the axon was usually filtered sea water; exceptions are noted in the text. A 10 × microscope objective formed an enlarged image of the axon with the scattered light passing through slit *b*. A stop placed in the objective image plane blocked the light coming from the electrode. Lens *c* collected the light from the axon on to the SGD 100 A (E.G. & G. Inc., Boston) photodiode used in the photoconductive mode. To change the angle being measured, the photodetection system (slit *b* through photodiode) was rotated about the axon axis in the plane of observation. The photocurrent from the SGD 100 A was measured as the voltage drop across the resistor, R_l, usually 100 kΩ. A variable capacitor C_s limited the high-frequency response time constant to the values indicated in the Figure legends. The coupling capacitor, C_c, blocked the DC and low-frequency components of the signal. The coupling time constant was at least ten times the sweep duration. The filtered signal was amplified by a Tektronix 3A9 amplifier, the output of which was fed to one of three signal averagers, a Data Laboratories Ltd. Biomac 1000, a TMC Computer of Average Transients (CAT 400C) or a Princeton Waveform Eductor (type TDH-9). The averaged signals were recorded on an $X-Y$ potentiometric recorder. White light was used in all of the experiments.

Slit *a* was used to make the illuminating light more parallel, and slit *b* was used to limit the scattering angles over which the measurements were made. These slits were relatively wider for the measurements at Plymouth on *L. forbesi* and *L. vulgaris* axons than for the measurements at Woods Hole on *L. pealii* axons. The scattering angle was thus much better defined in the *L. pealii* experiments. The dashed lines in Fig. 1 indicate the actual convergence of the incident light and the actual divergence of the scattered light admitted to the detection system in most *L. pealii* experiments. The angle between the most divergent rays of incident light (using the axon centre as the origin) and the angle between the most divergent rays of scattered light admitted to the detection system were added together and called the sum of the divergences. Except where mentioned, for *L. pealii* experiments this sum was 9–14°; and for the *L. forbesi* experiments the sum was 60°. Because of the wide slits in the *L. forbesi* experiments, at a stated scattering angle of 35°, the measurements included light scattered from 5 to 65°. Thus the results of experiments measuring scattering changes as a function of scattering angle in *L. forbesi* cannot be directly compared with the results in *L. pealii*. Using the results from *L. pealii*, where the angular definition was better, the scattering changes were characterized by their various time courses, and using this system, we then labelled the changes found in *L. forbesi* according to their time course. There was probably more interference between types of scattering and more transmitted light measured as scattered light in the *L. forbesi* experiments.

The preceding discussion concerned the divergence of light in the plane of observation. No steps were taken to limit the divergence perpendicular to that plane, whose sum was about 60° in both *L. forbesi* and *L. pealii* experiments.

A few birefringence measurements were made in the light-scattering chamber with the photodetection system rotated to 0°. Perpendicular polarizers were introduced into the chamber itself, one in front of, and one behind the axon. No signal was found when the angle between polarization axis and axon axis was 0°; the signals shown in Fig. 11 were obtained when this angle was 45°.

The voltage-clamp amplifier was similar in design, but not in components, to that developed by Hodgkin, Huxley & Katz (1952). The internal electrode was similar to that used by Hodgkin *et al.* (1952), except that 20 μm platinum wire was used in place of silver. The wire was platinized according to the recipe given by Moore &

Cole (1963). The voltage wire was platinized for 3 mm and the current wire for 10 mm, the platinized portion of the voltage wire being in the centre of the illuminated zone. Although the initial electrodes had diameters up to 225 μm, it was possible with practice to make them with 120–130 μm diameters. The total current was measured across a 10 Ω resistance placed between a bath electrode and ground. The bath electrode was also platinized platinum, and its resistance, at the end of a 0·5 msec current pulse, was less than 2 Ω. Current densities were calculated on the assumption that the currents were evenly distributed over a 10 mm length of axon. The potential between internal and external electrodes followed the control voltage with a lag of less than 10 μsec. Compensation for the series resistance R_s (Hodgkin *et al.* 1952) was not used, except in experiments measuring potential dependence (Fig. 13). Complete compensation was considered to be approached at the setting of the potentiometer (p in Fig. 6; Hodgkin *et al.* 1952) which caused the current to oscillate during the entire sweep. The potentiometer was immediately turned back 10 %. When r_s was estimated from the potentiometer setting for 100 % compensation, its values were within the range of those reported in Table 1 of Hodgkin *et al.* (1952). Action-potential measurements were made either with the voltage wire of the voltage–clamp electrode or with an electrode similar to that described by Hodgkin & Katz (1949, Fig. 2*C*), except that a bright platinum wire was used in place of silver, and 0·57 M potassium chloride solution was used in place of sea water. Voltage command steps and their timing were obtained from a Digipulser digital stimulator (W. P. Instruments, Inc., Hamden, Conn.)

Axons were internally perfused, using the method of Baker, Hodgkin & Shaw (1962). The perfusion solution was 570 mM potassium fluoride, 35 mM potassium phosphate buffer, pH 7·4 (Tasaki, Singer & Takenaka, 1965). Sometimes partial removal of the small fibres was attempted after perfusion.

Micro-injection of solutions into axons was carried out, using the technique of Hodgkin & Keynes (Keynes, 1963). The micro-injections and electrode insertions were performed in a vertical chamber, using a mirror as described by Hodgkin & Huxley (1945) and Hodgkin & Katz (Fig. 1) (1949).

The artificial sea water occasionally used as the bathing solution was 10 K (Na) ASW (Baker, 1965). Tetrodotoxin was obtained from Sankyo Co., Ltd., Tokyo. Tetraethylammonium bromide was obtained from Hopkins & Williams, Ltd, or J. T. Baker Chemical Co; tetraethylammonium chloride was Etamon chloride, from Parke Davis & Co. Ouabain (Strophanthin G) was obtained from Sigma Chemical Co., and 1-octanol and 1-butanol from Fisher Scientific Co. The inorganic chemicals were analytical grade reagents.

The chamber temperature was held constant at 12 $\pm$ 1° C, except where noted.

RESULTS

Resting scattering

The giant axon scattered much more light in the forward direction than at right angles (Fig. 2), with a resting intensity at 10° about 1000 times greater than that at 90°. This kind of angular dependence is the expected result for scatterers much larger than the wave-length of the light; thus, B. B. Shrivastav found a similar dependence in the scattering from intact erythrocytes (Cohen & Landowne, 1971).

When the axon was illuminated with unpolarized light, the scattered light at 90° was mainly polarized in the direction perpendicular to the

plane of observation (parallel to the axon's long axis). In measurements on three *L. pealii* axons, the resting intensity of the scattered light with an electric vector perpendicular to the plane of observation averaged 4·3 times the resting intensity of the light with an electric vector parallel to the plane. With a scattering angle of 10°, the resting intensities of the scattered light polarized parallel and perpendicular to the plane of observation were about equal.

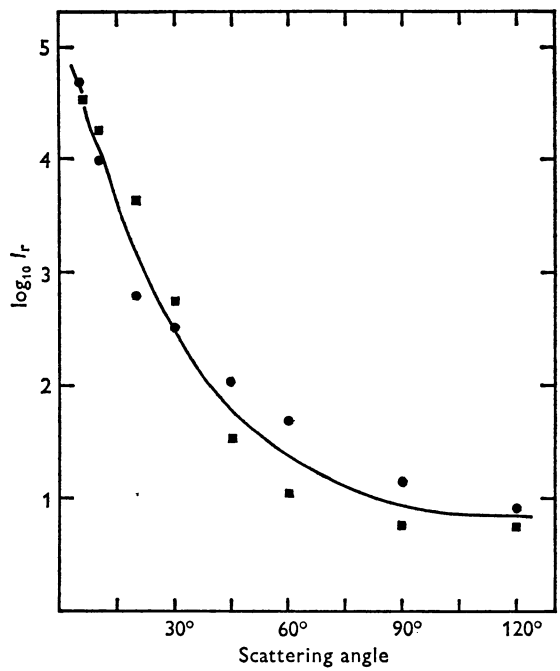

Fig. 2. Resting scattering, I_r, from the axon as a function of scattering angle using unpolarized light. Measurements from two axons are plotted as $\log_{10} I_r$ versus scattering angle. Axon diameters were 420 μm (●) and 610 μm (■). *L. pealii*. The actual intensity of light reaching the photodetector at 20° was about 10 μW.

It was clear from looking at a cleaned *Loligo* axon that it scattered a much smaller percentage of the incident light than did eel electric organ or crab nerve. This suggested that the axon would transmit more than 75% of the incident light and thus that the light reaching the photodetector would be scattered only once (Van de Hulst, 1957). The percentage transmission by one axon was measured by placing slits in the illuminating system so that all of the light passed through the axon, and by moving the photodetector to 0°. The presence of the axon reduced the amount of light reaching the photodetector by 17%, and therefore, by this criterion, the axon was a single scatterer. However, this method of measuring percentage

transmission is far from ideal. If parallel light had been used, and if the photodetector had been placed far from the axon, the percentage transmission would have to be zero for a cylinder (axon) with a refractive index greater than that of the solution. Thus the interpretation of our measurement was equivocal, and we cannot say positively that we were measuring only single scattering. The experimental results presented below do, however, argue against extensive interference from multiple scattering.

Changes in scattering during the action potential

Because the intensity of the resting scattering of the axon was strongly dependent on scattering angle, scattering changes were measured as a function of angle. The results illustrated in Fig. 3 showed that changes in light scattering during the action potential could be measured and that they did depend on angle. At 10 and 27°, there was a decrease in scattering during the spike; while at 70 (not illustrated), 90, and 120°, there was an increase. At 45°, no scattering change was measured; the cross-over angle was between 40 and 80° in different axons. Experiments on many other axons gave similar results; the scattering changes at 10–40° (called forward angles) were consistently different in direction and shape from the changes at 80–120° (called right angle because they were usually measured at 90°). The differences suggest that the two types of scattering change have different structural origins, and this suggestion has been confirmed in subsequent voltage-clamp experiments (see below, and Cohen *et al.* 1972). In the following sections, the scattering changes during the action potential at forward angles and at right angles will be discussed separately.

Ludkovskaya, Emeljanov & Lemazhikhin (1965) carried out light-scattering experiments on giant axons, using a geometry similar to ours. With a photographic method of light measurement, they reported intensity changes of $2–3 \times 10^{-1}$ during the action potential. The changes we measured (Fig. 3) were approximately 10^5 smaller, and we therefore presume that their measurements must have resulted from an artifact.

Forward angles. The light-scattering decrease that occurred during the spike was measured in *L. pealii* axons, using the narrow slits, and in *L. forbesi* axons, using the wide slits (see Methods). The peak change in scattering intensity per sweep, ΔI, divided by the resting scattering I_r averaged $-7{\cdot}5 \times 10^{-6}$ for the *L. pealii* axons and $-0{\cdot}56 \times 10^{-6}$ for the *L. forbesi* axons (Table 1*a*). While it is possible that the difference between Woods Hole and Plymouth squid was a real species difference or was related to the smaller diameter of *L. pealii* axons, it seems more likely that the difference resulted from the narrower slits used in *L. pealii* experiments, which would help to prevent interference from the light-scattering increase occurring at large angles. In fact, the relative changes for all of the

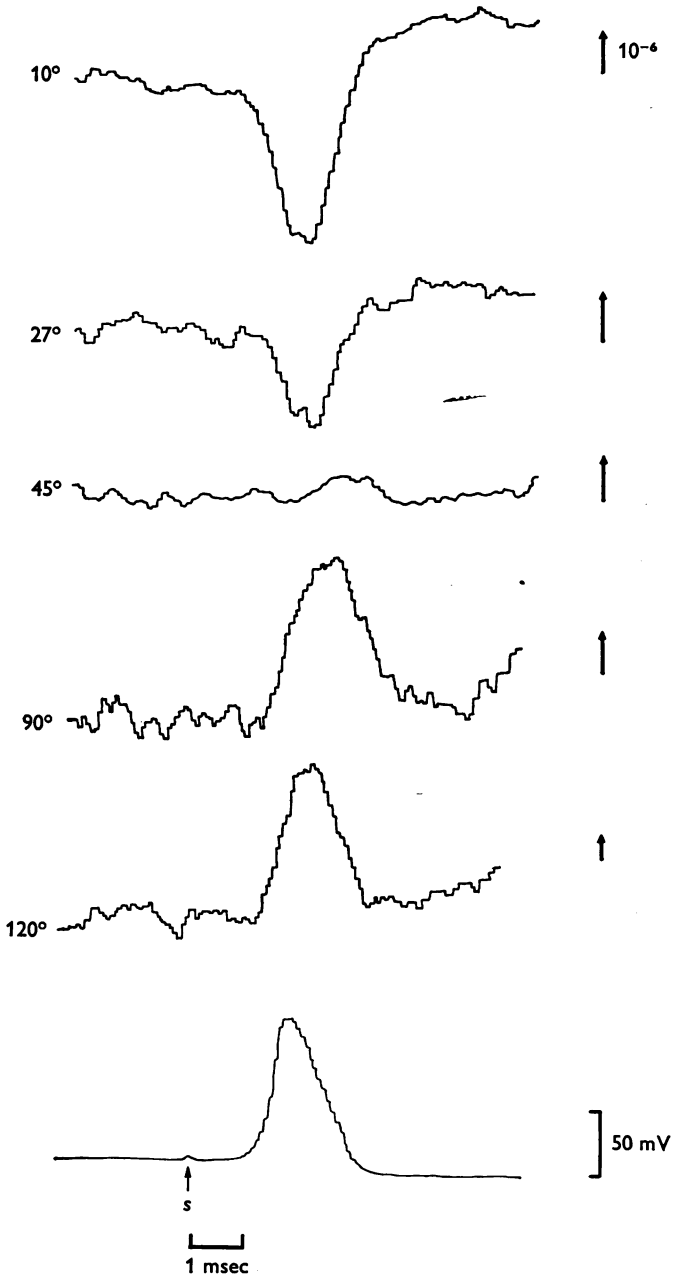

Fig. 3. For legend see foot of facing page.

measurements reported in these two papers were larger in *L. pealii* than in *L. forbesi* axons, presumably for the same reasons.

To help to determine the origin of the scattering change at forward angles, we compared the time course of the scattering change with the time course of the action potential measured simultaneously with an internal electrode whose tip was in the centre of the 4 mm illuminated region. In Fig. 4, where the light-scattering traces have been inverted to facilitate comparison with the potential traces, the scattering change has a

TABLE 1. Mean ΔI/sweep-I_r. The number of axons is in brackets

	Species	Mean ± S.E. of mean ($\times 10^6$)
a. Peak decrease during spike, forward angles	*L. pealii*	−7·5 ± 3·2 (3)
	L. forbesi	−0·56 ± 0·17 (5)
b. Peak increase during spike, right angles	*L. pealii*	+3·3 ± 0·5 (8)
	L. forbesi	+1·3 ± 0·3 (7)
c. Peak slow increase after spike, right angles	*L. pealii*	+5·2 ± 0·9 (5)
	L. forbesi	+1·7 ± 0·7 (7)
d. 100 mV hyperpolarizing step, right angles	*L. pealii*	−19·3 ± 1·6 (20)
	L. forbesi	−8·7 ± 1·9 (5)
e. 100 mV hyperpolarizing step, forward angles	*L. pealii*	+19·4 ± 4·0 (14)
	L. forbesi	+5·5 ± 1·5 (5)

time course that was for the most part similar to that of the action potential, both during the spike (Fig. 4*A*) and during the longer-lasting positive phase (Frankenhaeuser & Hodgkin, 1956) of the action potential (Fig. 4*B*). This result suggested that the scattering changes that occurred during the action potential depended mainly on the potential across the axon membrane, and not on the ionic currents or membrane conductance changes. This suggestion was supported by the results of an experiment where the scattering change was measured seventeen times as the axon was stimu-

Fig. 3. Changes in light scattering during the action potential measured at five scattering angles (heavy lines). The bottom trace is the action potential. The scattering changes at 10 and 27° were in a different direction from those at 90 and 120°. In this and subsequent Figures, the direction of the vertical arrow to the right of the tracings indicates the direction of an increase in scattering, and the size of each arrow represents the value of ΔI/sweep-I_r shown against one of them. The arrow labelled *s* indicates instant of stimulus. All records are traced from the originals. Unless stated otherwise, all traces in each Figure are from one axon. The action potential height was 108 mV for the 10° experiment, 105 mV for 27°, 100 mV for 45°, 80 mV for 90°, and 72 mV for 120°. The response time constant to a step change in light intensity was 250 μsec for 10, 27 and 45°, and 630 μsec for 90 and 120°. *L. pealii*; 2048 to 8196 sweeps averaged.

lated to exhaustion. In Fig. 5, the size of the peak scattering decrease was plotted against the mean size of the action potential during the scattering measurement. The experimental points fell reasonably close to the line, which extrapolated to zero-scattering change for zero-action potential. Thus, over the range 70–110 mV the size of the scattering change was proportional to the size of the potential change.

However, one feature of Fig. 4*A* seemed contradictory to the conclusion that the scattering change depended solely on membrane potential. If the

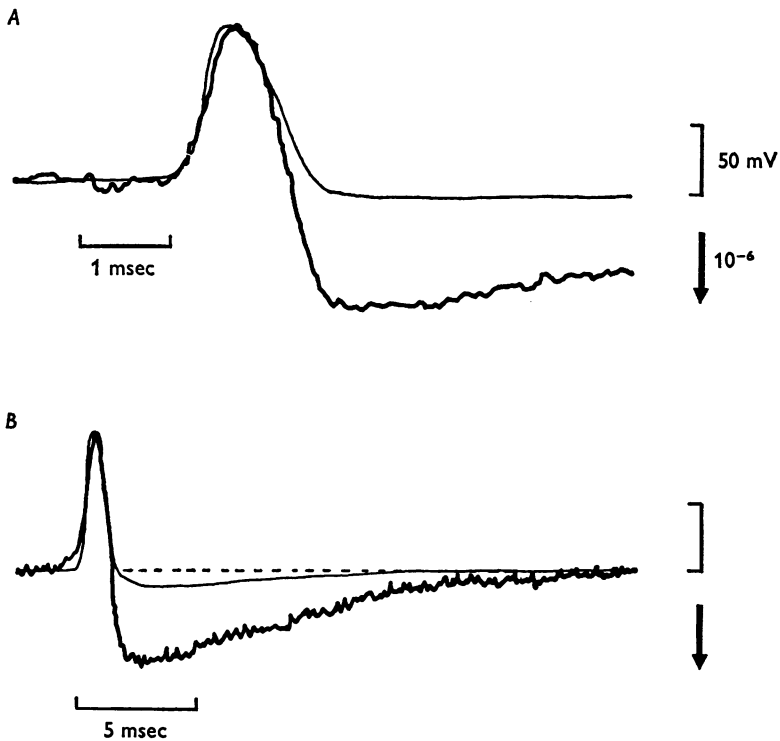

Fig. 4. Forward-angle scattering changes (heavy lines) compared with action potentials (thin lines). The changes had time courses similar to those of the action potentials at the faster (*A*) and slower (*B*) sweeps. The scattering traces were inverted to facilitate comparison with the action potential. Potential-measuring electrode. *L. forbesi*: time constant, 50 μsec; 23,000 sweeps averaged.

scattering change were dependent on potential in a simple manner, then the scattering should have returned past its resting level at the same time as, or later than, the potential returned past the resting level on its way into the positive after-potential. But in Fig. 4*A* the light intensity reached its resting level about 0·25 msec before the potential did. Similar results

were obtained from *L. pealii* axons. This discrepancy was exaggerated when the action potential was prolonged by internal micro-injection of tetraethylammonium ions (Fig. 6). Here, the scattering returned to the resting level 1 msec earlier than the potential. This behaviour could be explained if a step depolarization led not to a step decrease in light scattering, but to a change in scattering which reached a maximum and

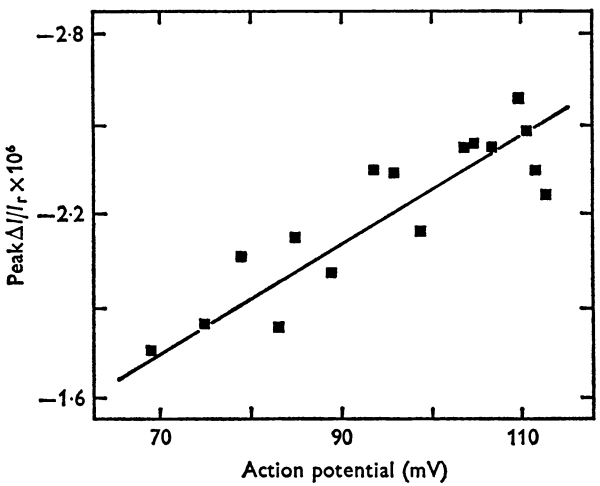

Fig. 5. The size of the forward-angle scattering change *vs.* action potential size. The experimental points fall near the straight line, which extrapolates to zero scattering change for a zero mV action potential. Same axon as in Fig. 4.

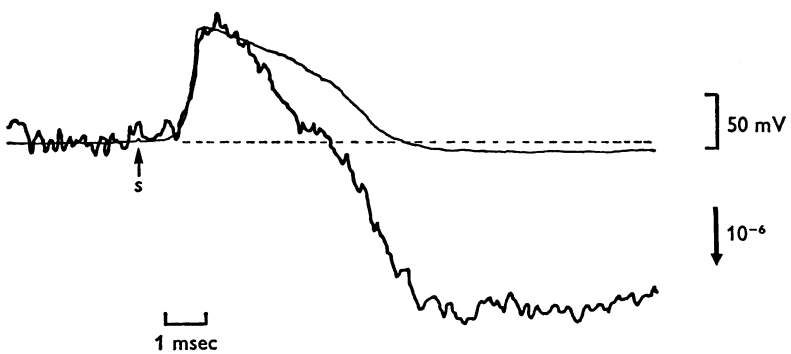

Fig. 6. Forward-angle scattering change (heavy line) during the action potential (thin line) in an axon internally injected with tetraethylammonium chloride. The scattering change returned to its base line almost one msec before the positive after-potential began. The light scattering trace was again inverted. Final tetraethylammonium chloride concentration was 15 mM. Voltage-clamp electrode. *L. forbesi*; time constant, 75 μsec; 1100 sweeps averaged.

then relaxed to the base line with a time constant of a few msec. The return of scattering to its resting level would then precede the return of the potential. A relaxation of the birefringence change was sometimes found (Cohen *et al.* 1971). However, it was not possible to measure potential-dependent scattering changes for depolarizing steps longer than 0·3 msec, and the scattering changes during long hyperpolarizing steps did not show such relaxation (see below). Thus, while much of the data indicated that the change in forward scattering during the action potential depended mainly

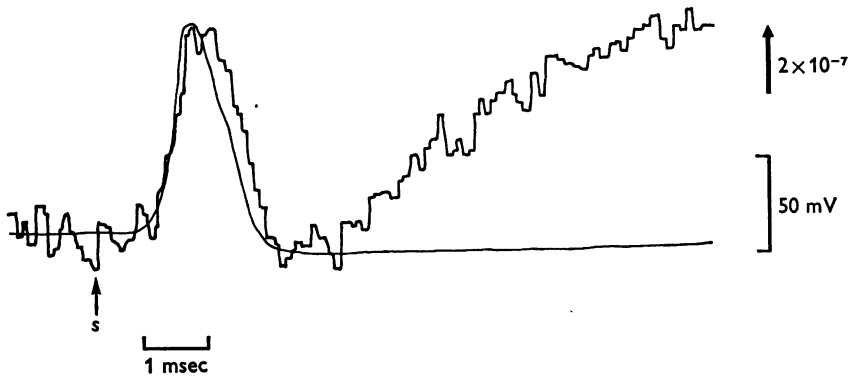

Fig. 7. The right-angle light-scattering change (heavy line) compared with the simultaneously recorded action potential (thin line). The light-scattering change had two phases, an early increase which accompanied the spike and a slow, long-lasting increase which continued long after the action potential. Voltage-clamp electrode. *L. forbesi*; time constant, 120 μsec; 8000 sweeps averaged.

upon membrane potential, this conclusion must be tentative. The differences in time course seen in Fig. 6 cannot easily be explained in terms of scattering changes dependent on current because voltage-clamp experiments (Cohen *et al.* 1972) indicated that such changes would have been much too small. Further experiments showed that this scattering change was not affected by internal perfusion with fluoride solutions or by addition of 3×10^{-5} M ouabain to the sea water surrounding the axon.

Right angles. When the scattering change at 80–120° was compared with the action potential (Fig. 7), two components were found: an early transient increase which occurred during the spike and a slower, longer-lasting increase which began at the time of the spike and continued long afterwards. The sizes of both changes (Table 1, *b* and *c*), as measured on the first trial on each axon, were similar to the sizes of the forward-angle changes, but, because the resting intensity at right angles was much lower, the measurements were more difficult. Similar changes were found in axons internally

perfused with fluoride solution. Neither the early nor the late change appeared to be affected when 10^{-4} M ouabain was added to the sea water surrounding the axon.

Since the time course of the early scattering increase was similar to that of the action potential, it was first thought that this change depended on membrane potential. While this may, in part, be true, one experiment suggested that other factors were contributing. When the size of the early change was measured as the axon was stimulated to exhaustion (Fig. 8,

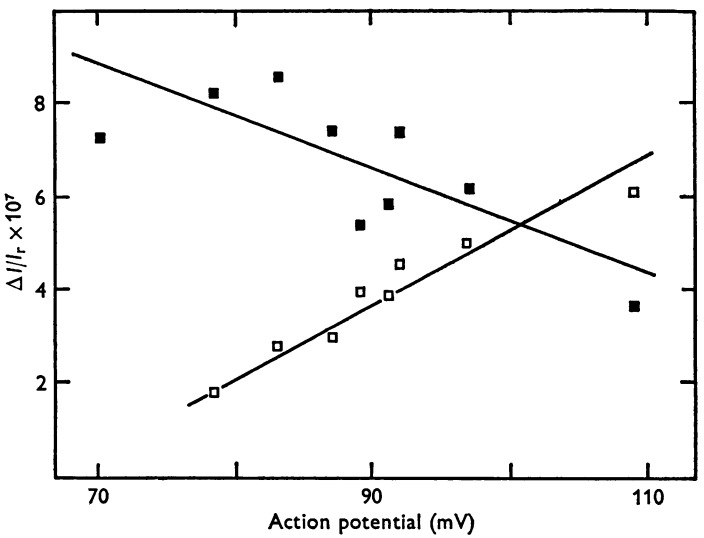

Fig. 8. Peak intensity change of the early (■) and late (□) scattering changes at right angles as a function of action potential size. The early change grew larger as the action potential became smaller. The line for the early change was computed with the least squares method. It had a negative slope of $0{\cdot}11 \pm 0{\cdot}035$ (S.E. of mean). All other lines were drawn by eye.

filled squares), the scattering change was largest for the smallest action potential. One possible explanation is the following. If the scattering change during the spike had a potential-dependent component (an increase) and a second component dependent on current or conductance (a decrease), then, in a healthy axon with a 110 mV action potential, the second component would be relatively large and subtract considerably from the potential-dependent component; in a fatigued axon, the currents and conductances would be barely large enough to move the membrane potential, and therefore the second component would subtract relatively little from the potential-dependent component. While this explanation is speculative,

the experiment in Fig. 8 does indicate that the early scattering increase was not solely potential-dependent.

In two experiments, longer sweeps were used in an attempt to measure the time course of the long-lasting increase. It reached a peak 10–20 msec after the stimulus and returned halfway to the resting level 50–100 msec later. Since the long-lasting increase occurred after and continued much longer than the action potential, it cannot be potential-dependent. When the axon was stimulated to exhaustion, the size of the long-lasting change

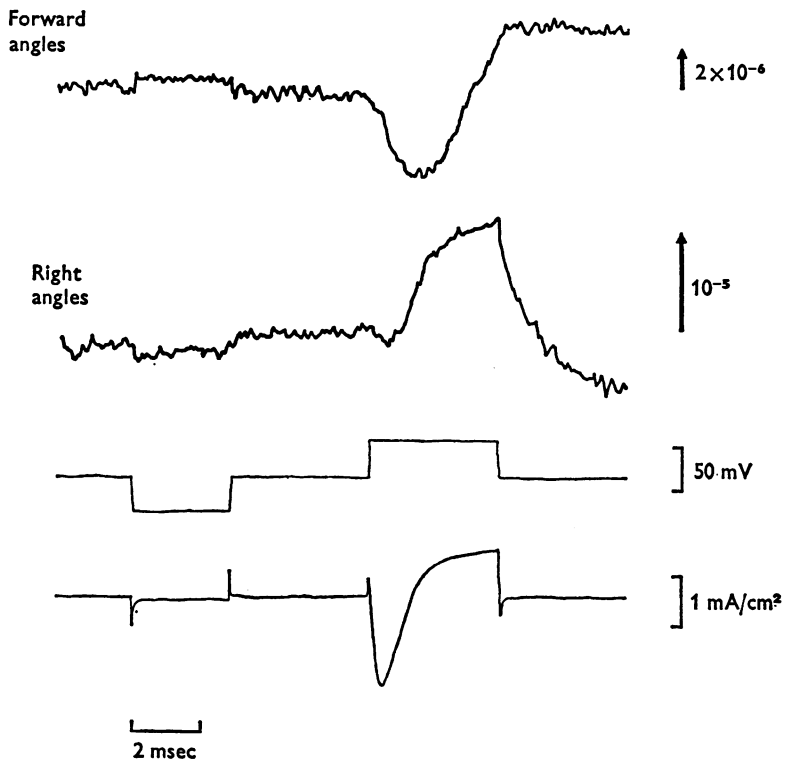

Fig. 9. Light-scattering changes (heavy lines) at forward angles and at right angles during voltage-clamp steps. At both angles, there were large changes during the depolarizing step and only small changes during the hyperpolarizing step. Top two traces, scattering; middle trace, potential; bottom trace, current density. In this and subsequent Figures, the holding potential was the resting potential, hyperpolarizing was downward, inward current was downward. The light-scattering traces are from different axons. The current trace is the actual trace for the experiment at forward angles. The currents for the experiment at right angles were similar in magnitude, but had a somewhat more rapid time course because the temperature was higher. *L. forbesi*; time constant, 70 μsec; temperature, forward angles, 13° C, right angles, 19° C; 900 sweeps averaged.

decreased as the axon fatigued (Fig. 8, open squares). Extrapolating the curve indicates that there would be no long-lasting increase when the action potential size had deteriorated to 68 mV.

Initial measurements during voltage-clamp steps

While experiments during the action potential provide useful clues concerning the origins of the scattering changes, the only way in which we could hope conclusively to sort out changes dependent on potential, current, or conductance was to measure light-scattering changes during voltage-clamp steps. To avoid possible artifacts arising from changes in scattering by the electrode, we always used a microscope objective to form an image of the axon and electrode, and then blocked out the light coming from the electrode. Fig. 9 illustrates scattering changes at forward and at right angles during both hyperpolarizing and depolarizing voltage–clamp steps. During the depolarizing step, when there were large currents and conductance changes, there were large light-scattering changes. Discussion of these large changes, which presumably depend on either current or conductance, will be deferred to a subsequent paper (Cohen *et al.* 1972). But, in addition, an imaginative scrutiny of the light-scattering traces during the hyperpolarizing step in Fig. 9 suggests that there were smaller changes occurring at that time, an increase in scattering at forward angles and a decrease at right angles. By averaging more sweeps, these changes could be measured in greater detail.

Potential-dependent scattering changes

Identification. Fig. 10 illustrates the changes in scattering that occurred during hyperpolarizing potential steps: these were an increase in forward scattering and a decrease in right-angle scattering. Since these changes occurred when the currents and conductance changes were small, and since their time courses were similar to the time course of the potential, we conclude that they depended primarily upon the changes in membrane potential.

One might argue that they could have depended upon current, since the ionic 'leak' current also had the same time course as the potential. However, the other scattering changes that we have definitely identified as current-dependent (Cohen *et al.* 1972) had a time course similar to the time integral of current or even slower; so it seemed quite unlikely that the changes illustrated in Fig. 10, which had a time course similar to that of the potential, could be current-dependent. Furthermore, when these relatively rapid changes were measured at various potentials, the size of the scattering change was proportional to potential squared, and not to current.

A systematic study of these potential-dependent changes as a function of scattering angle has not been carried out. The change labelled 'forward angle' was found at 10–30°, using narrow slits, and at 30–35°, with wide slits. The change labelled 'right angle' was always measured at 90°.

The size of the change in right-angle scattering, expressed as $\Delta I/I_r$ at the end of a single 2–5 msec hyperpolarizing step of 100 mV, averaged -2×10^{-5} (Table 1*d*). Similarly, the size of the first measurement of the change in forward scattering averaged $+2 \times 10^{-5}$ (Table 1*e*). In several axons, the increase at forward angles became much smaller with successive measurements and once even reversed in direction. In other axons, the size of the forward scattering change was constant in measurements made 300 min apart.

In experiments on three axons, the ratio of signal to noise was large enough to permit the measurement of the time course of the scattering

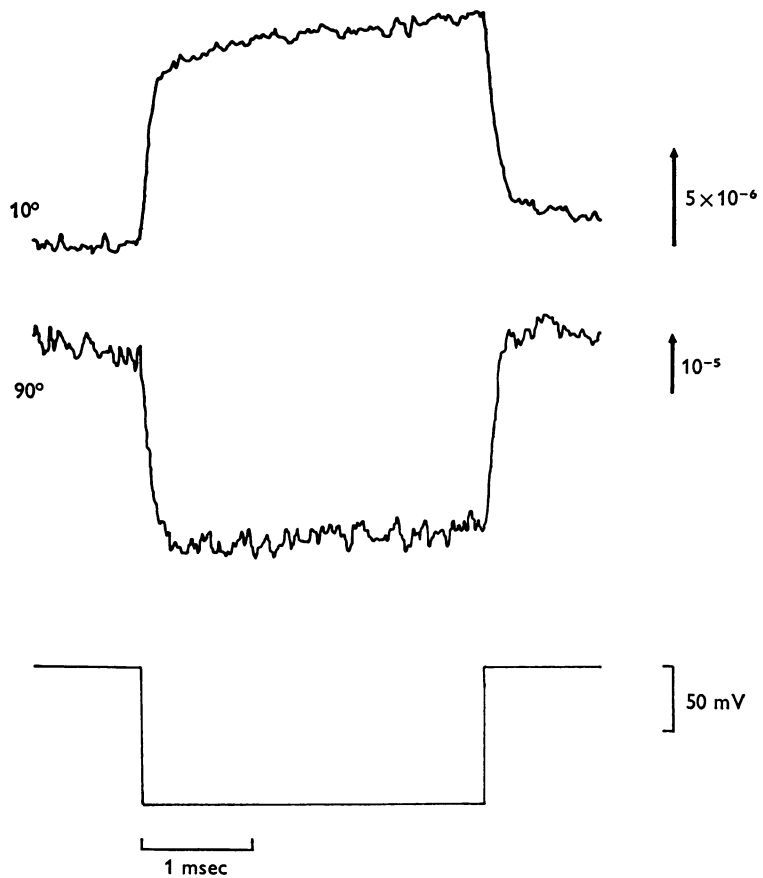

Fig. 10. Light-scattering changes (heavy lines) at forward angles (10°) and right angles (90°) during 107 mV hyperpolarizing potential steps (thin line). At both angles, scattering changes during the hyperpolarizing step had time courses similar to the time course of the potential. Inward current density at the end of the step was less than 0·1 mA/cm². *L. pealii*; time constant, 45 μsec; 8192 sweeps averaged.

change after a rapid step in potential. The experimentally determined time course was analysed as in Fig. 3 of Cohen *et al.* (1971), and corrected for the time constant of the light-recording system (4–45 μsec) and for the time constant of the change in membrane potential (8 μsec). The time course of the right-angle decrease could always be approximated by a single exponential, but two exponentials were needed to fit the time course of the increase at forward angles. At 13° C, the single time constant for the right-angle scattering decrease averaged 24 μsec, and the two time constants for

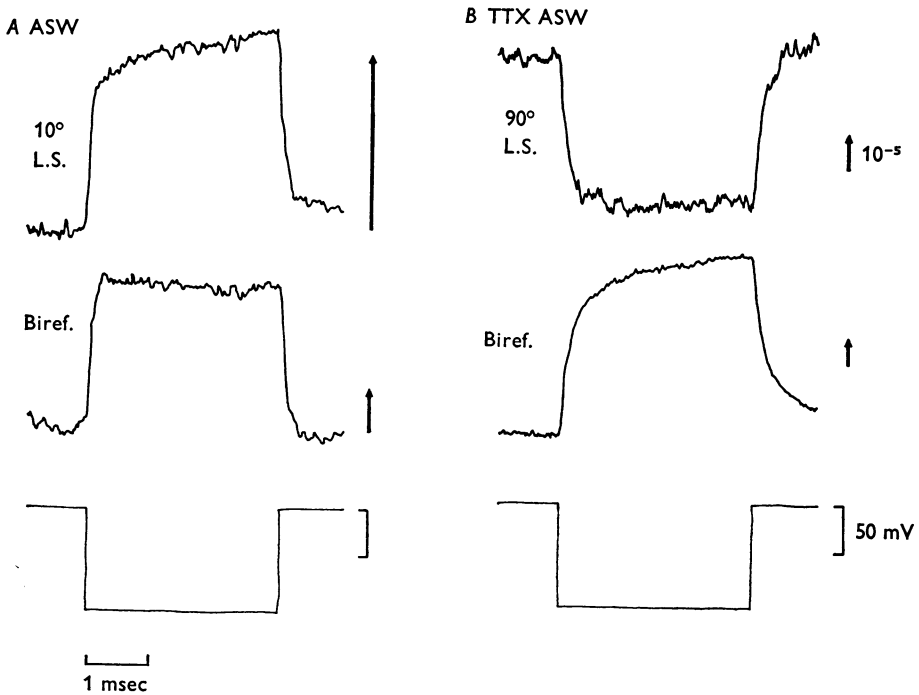

Fig. 11. Comparison of light scattering and birefringence changes (thick lines) during hyperpolarizing potential steps (thin lines). *A*, in artificial sea water (ASW), the light scattering change at 10° had both a fast and a slow phase while the birefringence change had only a fast phase. *B*, 150 min after adding 300 nM tetrodotoxin to the ASW (TTX ASW), the light scattering change at 90° had a single fast phase while the birefringence change had both a fast and a slow phase. Inward current density at the end of the steps was less than 0·1 mA/cm². *L. pealii*; time constant, 45 μsec; 8192 sweeps averaged. Same axon as Fig. 10.

the increase at forward angles averaged 23 and 900 μsec. The voltage steps used were not longer than 3 msec, so that the second time constant at forward angles was subject to some errors, as were the two fast time constants because of the relatively large instrumental corrections.

Since the potential-dependent scattering changes had different time courses at the two angles, they cannot have identical origins. This conclusion is supported by experiments on the effects of drugs and measurements of the changes at various potentials (see below). Furthermore, the differences in time course indicate that at least the slower component of the change measured at 10° was really scattered and not just transmitted light; for a change in intensity at 10° that was opposite in sign and of exactly the same time course as the change at right angles, could have arisen simply from transmitted light.

In addition to showing that the two light-scattering changes had at least partially independent origins we also wished to know whether either change was directly related to the potential-dependent birefringence changes (Cohen *et al.* 1971). In Fig. 11*A*, the forward-angle scattering is compared with birefringence measured at the same time. As usual, two time constants were needed to describe the time course of the forward-scattering change, while the birefringence change, in this fresh axon, had a single, fast time constant. In Fig. 11*B*, the right-angle scattering and birefringence are compared after the axon had been in a 300 nM tetrodotoxin sea water for 150 min. The birefringence change was now larger and needed at least two time constants to describe its time course, while the right-angle scattering change could still be described by a single time constant. While it was thus possible to distinguish both of the light-scattering changes and the birefringence change on the basis of their time courses, the individual time constants needed to describe the time courses were similar. The time constants for the right-angle scattering change, the fast birefringence change and the fast forward-angle scattering change were all about 25 μsec at 12° C. And the slow time constants for the forward-angle scattering change as well as the slow birefringence change were about 1 msec.

Effects of drugs. Several drugs known to affect electrical properties of the axon membrane were added to the bathing solution to see whether or not they modified these potential-dependent scattering changes. Because the changes at forward angles tended to be rather labile, most of these experiments were carried out on the right-angle change. As a comparison of Figs. 10 and 11*B* indicates, 300 nM tetrodotoxin did not seem to affect the size or the time course of the right-angle scattering change, when it did have marked effects on the birefringence change.

The addition of either 100 mM butanol or 1 mM octanol to the bathing solution resulted in an increase in the size of the right-angle scattering change. Fig. 12 shows that the addition of 1 mM octanol resulted in an increase of about 60%. (These concentrations of butanol and octanol reduced the early conductance increase by more than 90% and the delayed conductance increase by more than 70%.) When the scattering decrease

in the presence of octanol or butanol was compared with the average of the decrease in their absence beforehand and afterwards, the decrease was 60% larger for butanol (three axons) and 50% larger for octanol (two axons). In two experiments, 1 mM octanol was added to the bathing solution while the forward scattering increase was being measured, but this change was apparently unaltered. Octanol and butanol did not markedly affect the birefringence change (L. B. Cohen, R. D. Keynes & D. Landowne, unpublished observations).

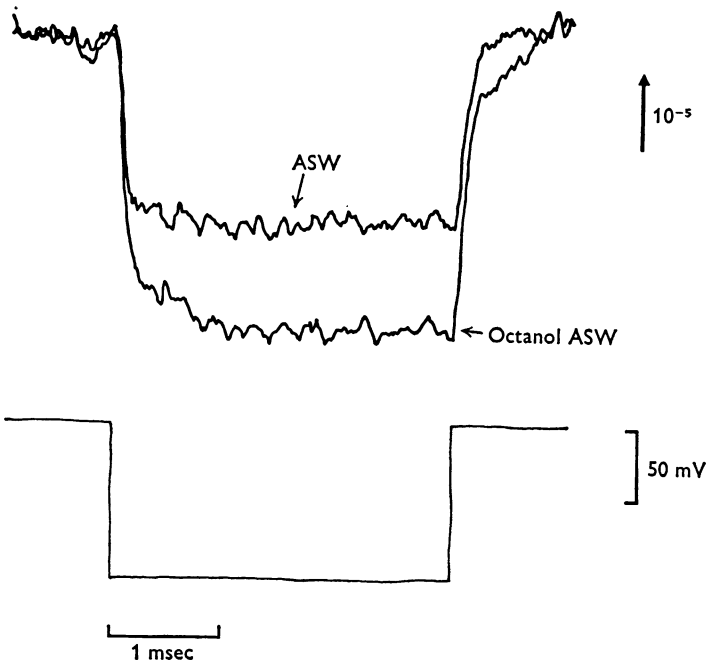

Fig. 12. Right-angle light scattering changes (heavy lines) in artificial sea water (ASW) and in artificial sea water with 1 mM octanol (octanol ASW) during a hyperpolarizing voltage step (thin line). In octanol, the scattering change was larger. The inward current at the end of the step was less than 0·1 mA/cm². *L. pealii*; time constant, 60 μsec; the sum of divergences was 25°; 8192 sweeps averaged.

Perfusion. Removal of the axoplasm and perfusion with fluoride solution did not seem to affect the size or time course of either potential-dependent change.

Temperature. The decrease in right-angle scattering in response to a hyperpolarizing step was more rapid at higher temperatures. When the chamber temperature was increased from 10 to 19° C in one experiment, the time constant of the scattering change was reduced from 120 to 50 μsec. The size of the scattering decrease did not seem to be affected by the change in temperature.

Both the size and the time course of the forward scattering increase were temperature dependent. In two axons, increasing the temperature from 14 to 24° C led to a 30–50 % reduction in the size of the change. In one axon, a similar temperature change reduced the time constant of the scattering change by about 40 %.

Polarization of incident light. In one experiment, the right-angle scattering was measured when the incident light was plane-polarized either perpendicular or parallel to the plane of observation. No change could be measured for light polarized parallel to the plane. But the resting intensity for light of this polarization was about five times less than that for light polarized perpendicular to the plane, so that when the value of $\Delta I/I_r$ for one sweep was calculated, we could only say that the change for light parallel to the plane of observation was less than 50 % of the change for light perpendicular to the plane.

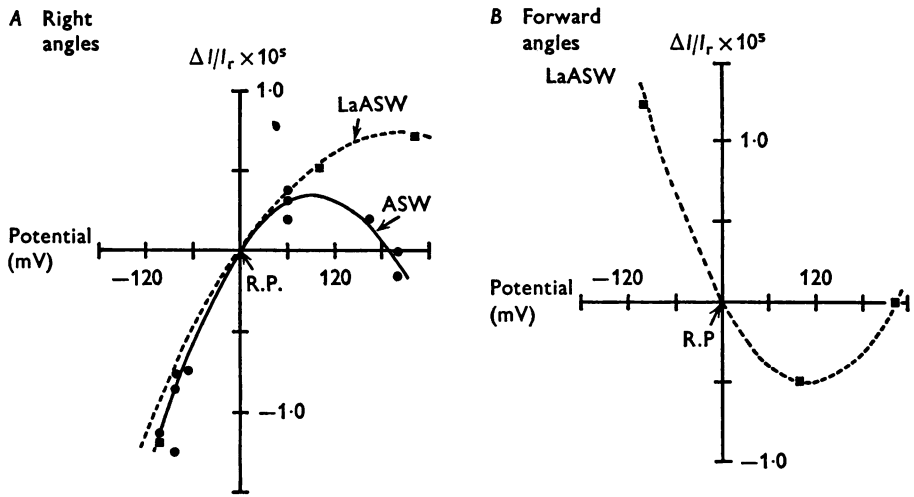

Fig. 13. *A*, the right-angle scattering change that depended upon potential plotted as a function of potential in artificial sea water (●) (ASW) or in artificial sea water plus 10 mM $LaCl_3$ (■) (LaASW). In both media, the experimental points fell near the curves which represented potential squared. The experimental points in ASW were multiplied by 0·6 to make them fit on the same scale with the results in LaASW. *B*, the forward-angle scattering change that depended upon potential plotted as a function of potential; in LaASW. The experimental points fell near the curve which represented potential squared. The same axon was used for LaASW in *A*. The scattering was measured at 30° instead of the more commonly used 10°. The voltage–clamp amplifier was set at 90 % compensation. *L. pealii*.

Scattering changes vs. potential. In several axons, we tried to determine how the two scattering changes depended upon potential. These experiments were especially difficult in the depolarizing direction because of the large scattering changes which accompany either current or conductance (Fig. 9). To overcome this problem, the experiments were done either with short (0·3 msec) depolarizing steps or in axons whose conductance changes

had been blocked by the addition of $LaCl_3$ to the external solution (Takata, Pickard, Lettvin & Moore, 1966).

Reasonably successful experiments at right angles were carried out in three axons in artificial sea water (ASW) and in three other axons with $LaCl_3$ added to the sea water (LaASW). The results in ASW were different from those in LaASW, and typical experiments are illustrated in Fig. 13*A*. In both cases, the experimental points fell reasonably close to curves which represented potential squared. However, in ASW, the curve was symmetrical about a potential 95 mV positive to the resting potential, while in LaASW the points could be fitted by a curve that was symmetrical about a potential 200 mV positive to the resting potential. In the presence of $LaCl_3$, $\Delta I/I_r$ for a single 100 mV hyperpolarizing step averaged $-17{\cdot}1 \pm 5{\cdot}1 \times 10^{-6}$ (S.E. of mean) in three *L. pealii* axons, a value similar to that obtained for axons in artificial sea water (Table 1*d*).

We did not succeed in measuring the potential dependence of the forward scattering change in artificial sea water, but Fig. 13*B* shows the results of a measurement in LaASW. Again, the experimental points fell near a curve which represented potential squared. The experiments in LaASW in Fig. 13*A* and *B* were successive measurements on the same axon. Since the forward-angle change was best fitted by a curve symmetrical about +110 mV while the right-angle change was symmetrical about +200 mV, the experiment provided further evidence that the two changes had different origins. These results would be more convincing if there were more experimental points.

Controls

Since all of the scattering changes were small, 10^{-5} of the resting intensity or less, control experiments were done to show that the changes were not artifacts.

Not all possible controls were done for the two changes that occurred during the action potential, but those we did indicated that the changes were not artifacts. Two controls were done to rule out artifacts resulting from the stimulus current. First, the action potential was blocked either by adding 150 nM tetrodotoxin, by replacing the sodium in the sea water and with choline or by increasing the potassium chloride concentration to 100 mM; each procedure also blocked the scattering change, even though the stimulus voltage remained the same. Secondly, we varied the stimulus voltage from just subthreshold to four times threshold; the scattering change was the same size at all suprathreshold stimuli and was not detected with subthreshold stimuli. These controls were carried out only for the forward-angle change, but two other arguments indicate that the right-angle change was not a stimulus artifact, either. First, the right-angle scattering change did not occur at the time of the stimulus, as would be expected if it were related to the stimulus current, but occurred, as did the action potential, after a delay of 1 msec (Fig. 7). Secondly, when the axon was stimulated to exhaustion and action potentials were no longer propagated, the scattering change disappeared, even though the stimulus voltage

was unchanged. Thus it seemed quite unlikely that either change that occurred during the action potential was a stimulus artifact.

One type of artifact that was encountered in preliminary experiments was electrical coupling between the action potential or the system measuring the action potential and the system measuring light. To show that this coupling did not exist during the experiments reported here, we adjusted the light source so that no light reached the axon but the light intensity measured by the photodetector was unchanged. Now, during action potential propagation, or during voltage–clamp sweeps, no intensity changes were found. Thus, the changes reported above were not artifacts resulting from electrical coupling. This control against electrical coupling was the only one we did (and the only one we thought of) for the two potential-dependent changes. However, some characteristics of the changes provided further evidence against the possibility of artifact. The 90° change could not be measured in light polarized perpendicular to the long axis of the axon; the 90° change was increased in size in the presence of octanol or butanol; and both the 90° and the 10° change were dependent on potential squared, not directly on potential.

DISCUSSION

We have presented three different potential-dependent light-scattering changes: a change seen at right angles which occurred with a time constant of about 25 μsec and two changes which were seen at forward angles, a fast change with a time constant of about 25 μsec and a slower change with a time constant of about 1 msec. Our studies of the birefringence changes in squid axons (Cohen *et al.* 1971) revealed the existence of three potential-dependent components whose origins were apparently distinct from one another. We now have to ask whether the potential-dependent light-scattering changes are manifestations of the same three molecular conformation changes, or must be considered to have different origins.

The potential-dependent effect most likely to be common to birefringence and light scattering at both angles is the fast change which in each case had a time constant of about 25 μsec. But the fast time constants did not seem to have similar temperature coefficients, those for light scattering having a Q_{10} of the order of $\frac{1}{2}$ as compared with $\frac{1}{3}$ for birefringence (Cohen *et al.* 1971). Experiments on the factors influencing the size of the changes also helped to differentiate the three changes. Thus a rise in temperature had no effect for birefringence or right-angle scattering, but appreciably reduced the change in forward scattering. And, right-angle scattering was increased by about 50% in the presence of butanol or octanol, whereas forward-angle scattering and birefringence were unaffected. On the other hand, the potential dependence of right-angle scattering and birefringence were similar and were similarly affected by lanthanum. The slow component of the forward scattering and the birefringence change following tetrodotoxin addition both had time constants of about 1 msec. But, in a fresh axon only the forward scattering had a slow phase, while

the birefringence change developed a slow phase only after the addition of tetrodotoxin suggesting separate origins.

But, in the absence of any information about the precise nature of the molecular reorientation responsible for the scattering and birefringence changes, it is hard to know what weight to attach to these findings. It is not impossible to envisage molecular events that would show up as a birefringence change without greatly altering the amount of light scattered in one plane, or vice versa; or whose several optical consequences would not all be affected to the same extent by a given change in the experimental conditions. Hence a difference in the time constant may be a more reliable criterion than a difference in the size for deciding whether or not any of the optical changes have a common origin. So even though we found experimental means of differentiating the changes from each other suggesting that their origins were different, this conclusion must be tentative.

The maximum number of independently observable potential-dependent optical changes thus far is therefore six, that is three components of the retardation response, two of forward scattering, and one of right-angle scattering. To these might be added the changes in fluorescence in appropriately stained axons that were first described by Tasaki, Watanabe, Sandlin & Carnay (1968). But although these are potential-dependent (Cohen, Davila & Waggoner, 1971) they may arise from changes in dye location and thus are not necessarily related to changes in membrane structure.

Two further questions may be asked about the potential-dependent changes. Precisely where do they arise, and what mechanism is responsible? It seems reasonable to assume that they are always closely associated with the membrane, since the voltage gradient across other parts of the axon is several orders of magnitude smaller. The experiments with perfused axons support this conclusion. Of those changes tested, only the right-angle scattering was affected by the addition of butanol or octanol to the bathing solution. Both alcohols penetrate into nerve lipid monolayers (Skou, 1954, 1958) and cause increases in the ionic permeability of liposomes (Johnson & Bangham, 1969). This might suggest that the right-angle scattering involves properties of the lipid portion of the membrane, and that because they are unchanged by alcohols the other changes are associated with the non-lipid portions. One of the mechanisms likely to give rise to potential-dependent optical effects is a thinning of the membrane dielectric caused by electrostriction. For the birefringence change, we have shown that this is quantitatively plausible (Cohen *et al.* 1971), and we cannot for the present rule out the possibility that a change in membrane thickness underlies the fast phases of forward and right-angle scattering as well. The other kind of mechanism that is probably operative

is a reorientation of dipoles analogous with the Kerr effect. Stoylov & Sokerov (1967) have described potential-dependent changes in light scattering in solutions that evidently involve molecular relaxation processes of this general type. But we cannot yet distinguish between these alternative physical origins for the optical effects that we have observed, nor can we identify the specific components of the membrane in which the effects arise.

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