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CHANGES IN LIGHT SCATTERING ASSOCIATED WITH THE ACTION POTENTIAL IN CRAB NERVES

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SUMMARY

1. Changes in light scattering from stimulated crab leg nerves were measured in an effort to study changes in structure that occur during the action potential.

2. The peak changes in scattering after individual stimuli were 10^{-4}–10^{-5} of the resting scattering; signal averaging techniques were used to increase the signal-to-noise ratio.

3. During the compound action potential there was a transient increase in the 90° scattering which reached a peak about 20 msec after the stimulus. This increase was probably not due to a change in axon volume. Its time course was somewhat slower than that of birefringence changes measured at the same time.

4. In normal sea water the transient increase was followed by a longer lasting (persistent) decrease in scattering. This persistent decrease could be converted to an increase by reducing the tonicity of the sea water or by raising the external refractive index. This scattering change may represent a swelling of the axon resulting from the ionic exchanges that occur during the action potential.

INTRODUCTION

Since the light scattering from a nerve is related to its structure, we have studied changes in this scattering in an effort to discover something about the alterations in axon structure that accompany the action potential. Hill & Keynes (1949) and Hill (1950*a*) observed changes in the light scattering from crab leg nerves during and after a train of several hundred stimuli. These findings were confirmed and extended to other nerves (Bryant & Tobias, 1952, 1955; Shaw & Tobias, 1955; Solomon & Tobias,

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1960), but, with rare exceptions (Hill & Keynes, 1949; Tobias, 1952), scattering changes following individual stimuli could not be measured. This made it impossible to decide which of the several events taking place as a result of the action potential were giving rise to the scattering effects. With improved methods, including signal averaging, the possibility of detecting rapid changes in light intensity has been greatly increased.

With methods similar to Hill's (1950*a*), we again confirmed the existence of scattering changes resulting from trains of action potentials. Using signal averaging to measure the scattering change resulting from individual stimuli, we find two components, one a transient increase in scattering which occurs at the time of the action potential, and the second a persistent decrease which continues for several seconds after the action potential. It is the summation of this persistent component during a train of stimuli which appears to give rise to the kind of change described by Hill (1950*a*). Hill suggested that those changes might result from swelling of the axons, and we provide evidence to support this hypothesis.

Preliminary reports of these experiments have been published (Cohen & Keynes, 1968; Cohen, Keynes & Hille, 1968), and some of the results have been confirmed elsewhere (Tasaki, Watanabe, Sandlin & Carnay, 1968).

METHODS

Leg and claw nerves from the crab *Maia squinado* were dissected by Furusawa's 'pulling out' method (1929). In a few experiments leg nerves from the crabs *Carcinus maenas* or *Libinia emarginata* were used. The data reported are from *Maia*, but the light-scattering changes in nerves from the other two crabs were similar. The whole nerve trunk from one segment of a leg was tied at both ends and placed in a horizontal, temperature-regulated chamber that was divided into three sections. The middle section, about 2 cm long, was filled with sea water, the sections on either side with paraffin oil. The nerve was stimulated by passing current between an electrode on the nerve in the left section and an electrode in the middle section, using a Grass SD5 stimulator. The compound action potential was recorded between a second electrode in the middle section and an electrode on the nerve in the right section and displayed on a Tektronix 502 oscilloscope. Since this externally recorded action potential was probably a mixture of monophasic and diphasic signals and since the relative contributions of large and small fibres were indeterminate, the action potential measured in this way was used mainly as a qualitative indicator of the condition of the nerve.

A 3 mm length of nerve in the centre of the sea-water section was illuminated with a 12 V, 50 W tungsten-halogen lamp. Current for the lamp was supplied by three 12 V accumulators wired in parallel. The light was collimated by two lenses near the bulb, and heat was removed by two Balzers (Lichtenstein) B1/K1 interference filters. The light was focused on the nerve by a 16 mm microscope objective. Between 5 and 10 mW of light actually reached the nerve. This light was not parallel; the nerve was near the apex of a cone of light whose solid angle was about 0·23 steradian (Fig. 1). Since the direction of the incident light included angles within $\pm 15°$ of 0°, the nominal scattering angle was widened by the same $\pm 15°$. In addition, the photo-

detector accepted a solid angle of about 0·1 steradian, so that in the light scattering measurements nominally at 90°, angles of 65–115° were actually measured. Except where specifically indicated, the measurements in this paper were made at 90°. Optical phenomena other than light scattering, such as reflexion and refraction, may have contributed to the measurements. For the low-angle experiments (forward scattering) the solid angle accepted by the photodetector was less than 0·05 steradian, so that the scattering angles included in these measurements were about 20–50°.

The photodetector used for most of the experiments was a Mullard 53 CV phototube. Later, an SGD-100 photodiode (EG & G, Inc., Boston) was found preferable because of its higher quantum efficiency. The output of the phototube, developed across the anode load resistance, R_L, was directly coupled to the amplifier (Fig. 1).

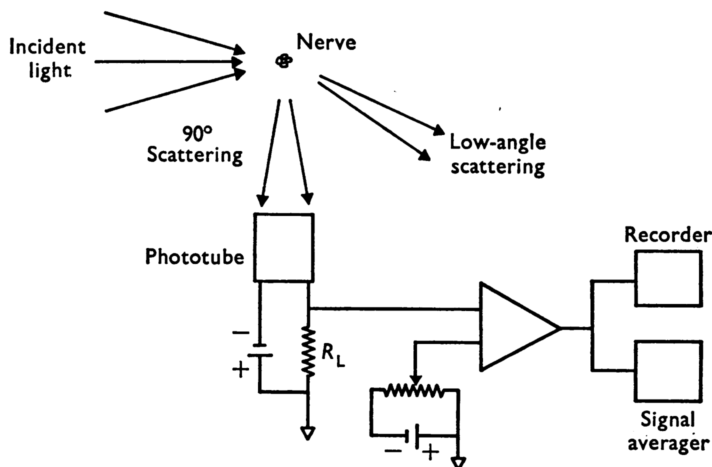

Fig. 1. Schematic diagram of the apparatus for light-scattering measurement. The nerve was perpendicular to the plane of the page, and was illuminated with convergent light. The intensity of the light scattered at 90° (actually 65–115°) was recorded as the voltage across the anode load resistance, R_L, of the phototube. For low-angle scattering the phototube was moved so that it measured light scattered between 20 and 50°.

To operate at high gain, a differential amplifier was used in which the steady component of the signal could be balanced against a DC potential. The output of the amplifier was fed either to a Texas Instruments chart recorder (signal *vs.* time) or to a TMC Computer of Average Transients (CAT 400C). Under optimal conditions the light scattering change following a single stimulation could just be detected. Most often, the signal-to-noise ratio was unsatisfactory, and the intensity changes resulting from a number of individual stimulations were averaged to improve the ratio (Cohen & Landowne, 1971, Fig. 1). The averaged intensities were drawn out on a chart recorder. When the amplifier output was fed directly to the chart recorder, the response time constant to an abrupt change in light intensity was limited by the pen speed ($t \simeq 0{\cdot}3$ sec). For experiments using the signal averager, the time constant was adjusted to the values given in the figure legends by placing capacitors in parallel with R_L.

The solution surrounding the nerve was usually sea water or an artificial sea water, 10K (Na) ASW (Baker, 1965). Tetrodotoxin was obtained from Sankyo Co.,

Ltd., Tokyo; choline chloride and ouabain (Strophanthin-G) were obtained from B.D.H. Ltd.; bovine albumin (Fraction V) and dextran (Type 60C, average molecular weight 78,200) were obtained from Sigma Chemical Co., St Louis. The inorganic chemicals were analytical grade reagents. The dextran and bovine albumin were dialysed against distilled water and added to a concentrate of artificial sea water so that the final solutions contained artificial sea water plus varying amounts of dextran or bovine albumin.

RESULTS

As Hill (1950*a*) had shown, the 90° light scattering, recorded on a relatively slowly running strip chart recorder, was found to decrease when the crab nerve was stimulated 10 times per second (Fig. 2*A*). Light-scattering changes measured in this way will be called integrated changes to distinguish them from the changes which follow individual stimuli. The average intensity change (ΔI) divided by the resting intensity (I_r) per stimulus in thirty-nine nerves was $-3{\cdot}0 \pm 1{\cdot}3 \times 10^{-5}$ (S.D. of an observation). In a few instances there appeared to be a small increase ($< 0{\cdot}2 \times 10^{-5}$) in scattering during the first second of stimulation.

To help determine how the integrated scattering change was related to the action potential, we attempted to measure light scattering changes after individual stimuli, using a faster time base and a signal averager. Fig. 2*B* shows that, rather surprisingly, the main scattering change immediately following the stimulus was a transient *increase*. The peak $\Delta I/I_r$ per sweep averaged $+4{\cdot}9 \pm 2{\cdot}1 \times 10^{-5}$ in experiments on fifty-eight nerves. Presumably it is this transient increase which was giving rise to the occasional small initial increase in scattering seen during measurements of the integrated scattering change.

Because the integrated scattering change was in the opposite direction, we suspected that the transient increase must be followed by a longer lasting decrease in scattering, and in three experiments where longer sweeps were used, such a persistent decrease was indeed found (Fig. 2*C*). The integrated scattering change was not recorded from any of the three nerves where longer sweeps were used to measure the persistent change, and therefore a direct comparison of the size of the persistent and integrated changes cannot be made.

When the time course of the transient increase (Fig. 2*B*) was compared with that of the action potential measured in a sucrose-gap experiment, the scattering increase reached its peak later and lasted longer than the action potential. When the light scattering and the birefringence change (Cohen *et al.* 1968) were measured simultaneously, the peak of the change in light scattering occurred slightly later than that in the birefringence and the width of the light scattering change at half height was about 50% greater than the width of the birefringence change at half height. Since

the birefringence change provides a good index of the action potential in the giant axon of the squid and in the electric organ (Cohen *et al.* 1968; Cohen, Hille & Keynes, 1969), the results suggest that the transient in-

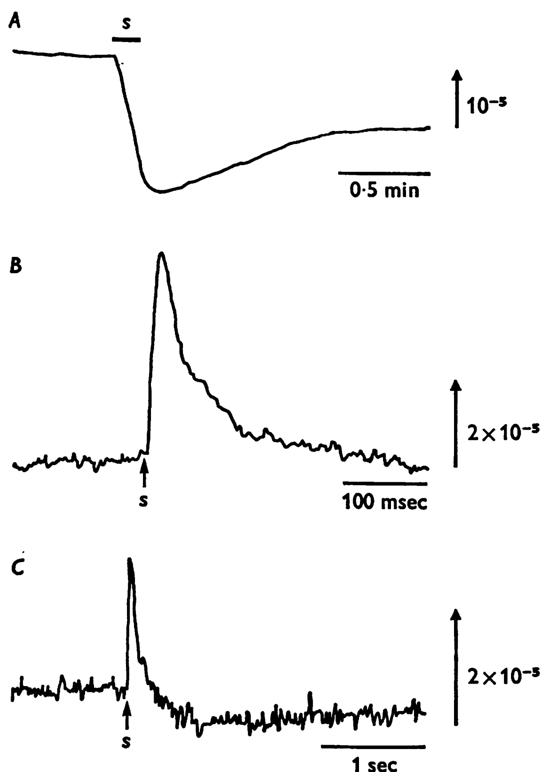

Fig 2. Light-scattering changes at 90°, white light; artificial sea water bathing the nerve. *A*. Integrated light-scattering change measured on strip chart recorder (amplifier output *vs.* time). When the nerve was stimulated 10 times per sec during the time interval indicated by the bar labelled *s*, the light scattering decreased. In all Figures the direction of the vertical arrow to the right of the traces represents an increase in intensity. Where integrated changes are measured, the size of the arrow represents the stated value of $\Delta I/I_r$ per stimulus. All records are traced from the originals. Temperature, 15° C. *B*. The transient increase in light scattering which followed an individual stimulus. Here and in other Figures the arrow labelled *s* indicates moment of stimulus. Where the results of individual stimuli are measured, the vertical arrow to the right represents the stated value of $\Delta I/I_r$ per sweep. Temperature, 15° C; 105 sweeps averaged. The response time constant to a step change in light was 6 msec. *C*. Light scattering changes following individual stimuli using a longer sweep, different nerve. Following the transient increase there was a smaller, longer-lasting decrease in scattering. Temperature, 15° C; 107 sweeps averaged; time constant, 2 msec.

crease in light scattering occurs more slowly and is more prolonged than the action potential. The persistent decrease following individual stimulation was still present long after the action potential had passed.

The percentage of the incident light transmitted by crab nerve averaged 39 % in measurements on six nerves. Van de Hulst (1957) states that corrections for multiple scattering are necessary if the percentage transmission is less than 75 %, and we must assume that, on average, the light reaching the photodetector has been scattered more than once. Thus, at a nominal angle of 90°, the measured light would include some contribution from scattering at angles even lower than 60°: i.e. light scattered twice, once at 10° and a second time at 50°, would be measured. Additionally, the scattering centres in the crab nerve are packed close together, giving rise to non-independent scattering (Van de Hulst, 1957).

The scattering changes illustrated in Fig. 2 occurred when the nominal scattering angle was 90°. In experiments on thirteen nerves, scattering changes were measured at low angles, and in six of these the light-scattering changes were consistently reversed in direction. The integrated change was an increase, and the transient change was a decrease. Because of multiple scattering, poor angular resolution, and the possibility that low-angle scattering is partly a transmission measurement, we cannot tell whether the reversed changes at low angles have different origins from those found at 90°.

To determine whether the scattering changes varied with the plane of polarization of the incident light, a polarizer (Polaroid, HN32) was placed between the light source and nerve. The resting scattering intensity with the electric vector of the incident light polarized parallel to the nerve axis (perpendicular to the plane of observation) was 1·5–2·5 times greater than the scattering intensity with the incident light polarized perpendicular to the nerve axis. However, when the $\Delta I/I_r$ per sweep for light polarized parallel and perpendicular to the nerve axis was compared, the two were the same within experimental error. The identity of the $\Delta I/I_r$ for both planes of polarization was true of both the transient increase in 90° scattering and the transient decreases sometimes seen in the low-angle scattering.

The wave-length dependence of light scattering can often provide useful information about the size of the scattering particles. Using wide-band interference filters, we determined the wave-length dependence of the resting scattering, I_r (Fig. 3, top), and the transient increase, $\Delta I/I_r$ per sweep (Fig. 3, bottom). To determine the relative I_r, the measured scattering intensity was corrected for the wave-length dependence of the light source and the phototube sensitivity using the intensities measured by shining the light directly at the phototube through a calibrated neutral density filter. The experimental points are near the continuous line which is proportional to $1/\lambda^2$, and clearly they do not coincide with the dashed curve, which is proportional to $1/\lambda^4$. As the $\Delta I/I_r$ (Fig. 3, bottom) was roughly constant at all wave-lengths, ΔI was also proportional to $1/\lambda^2$. The light scattering change which occurred when the external osmotic pressure was reduced by dilution was also measured as a function of wave-length. This change was similarly proportional to $1/\lambda^2$, so that the resting scattering, the transient increase in scattering resulting from stimulation

and the scattering change resulting from swelling all had the same wave-length dependence.

Attempts to modify the light scattering changes

Osmotic pressure. Confirming Hill (1950*a*) and Bryant & Tobias (1952), we found that when the tonicity of the bathing solution was reduced the integrated light scattering change reversed in sign to be an increase (Fig. 4*A*). The scattering change resulting from individual stimulation was

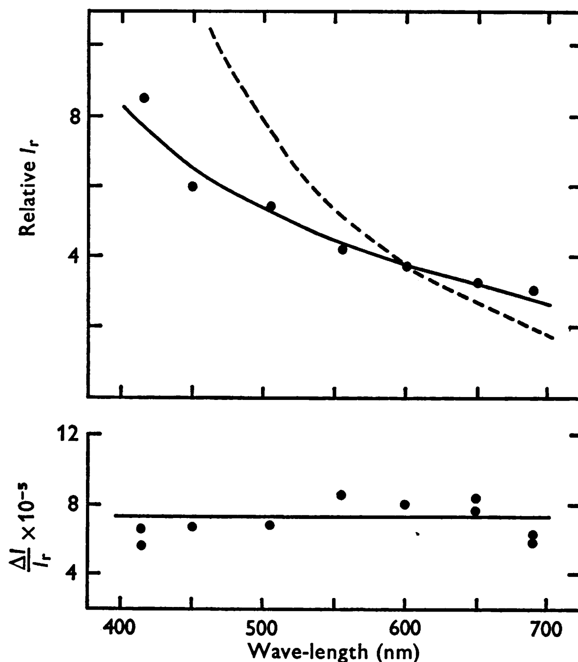

Fig. 3. Wave-length dependence of the resting scattering I_r (top) and of the change in scattering divided by I_r, per sweep (bottom). The continuous line in the upper graph represents $1/\lambda^2$; the dashed line represents $1/\lambda^4$. The value of $\Delta I/I_r$ per sweep was independent of wave-length in the visible range. Temperature, 14°; 90–280 sweeps averaged for $\Delta I/I_r$; 90° scattering.

measured and the transient increase was relatively unaffected, while the persistent component did reverse in direction and was now an increase (Fig. 4*B*). It seemed likely that the reversal in sign of the persistent component accounted for the reversal in sign of the integrated change. In hypotonic sea waters the size of the integrated change averaged $1 \cdot 3 \pm 0 \cdot 4$ times the size of the persistent change measured in the same nerve (three experiments). Sea waters made hypertonic by the addition of 50 mM-NaCl did not affect any of the light-scattering changes. The integrated scattering

increase gradually reverted to a decrease during a series of measurements in hypotonic sea water. After the nerve had been in hypotonic solution about 30 min, the integrated change was usually a much smaller increase or a small decrease. Since dilution of the sea water by 10–15 % caused a reversal in the sign of the integrated scattering change, a smaller dilution was sought in which there was no integrated scattering change following stimulation. This dilution was estimated in four experiments and averaged 3·5 %.

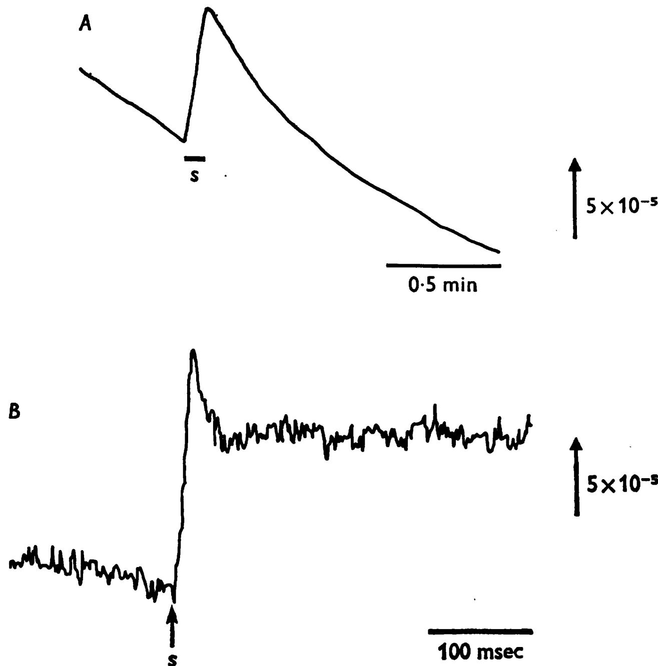

Fig. 4. Light-scattering changes in hypotonic artificial sea water (85 % artificial sea water, 15 % water); 90°; white light. *A*. The integrated light scattering change was reversed in direction (cf. Fig. 2*A*). The nerve was stimulated 5 times per second during the interval indicated by the bar. Temperature, 15° C. *B*. Light-scattering changes resulting from individual stimuli (same nerve), 5 min earlier. The persistent change was now an increase. Temperature, 15° C; 140 sweeps averaged; time constant, 2 msec.

Refractive index. One result of swelling the nerve in hypotonic sea waters is a reduction in the difference, Δn, between the refractive indices inside, n_i, and outside, n_e, of the axon, since the axoplasm is diluted. Another way of reducing Δn is to increase n_e. When the size of n_e was increased beyond that of sea water (1·339) with dextran or bovine albumin, there were marked effects on the persistent and integrated scattering changes, and these effects were similar to those resulting from hypotonic sea waters. In two

experiments, when n_e was increased by an average of 0·0013, the integrated light-scattering change was zero. When the external refractive index was further increased, the integrated change reversed to become an increase in

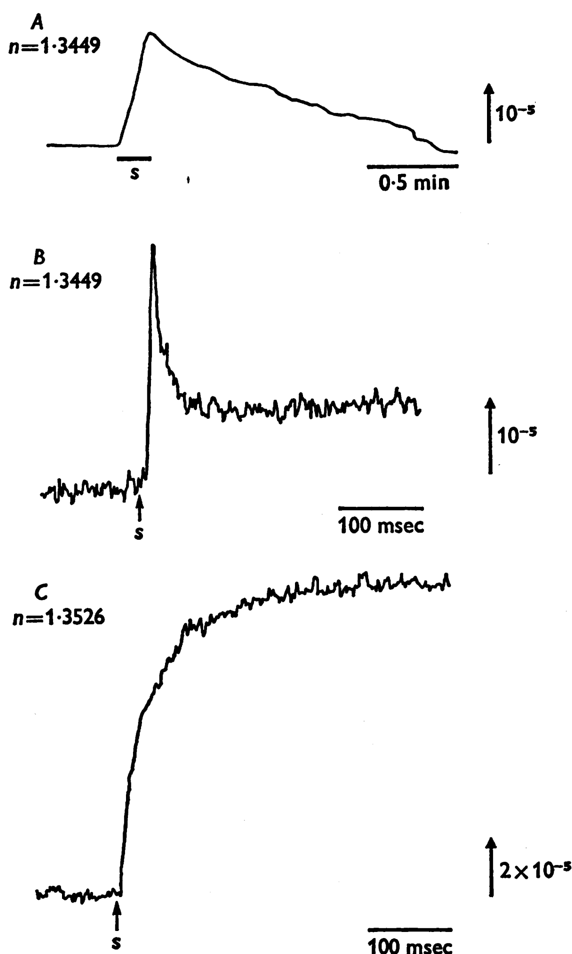

Fig. 5. Light-scattering changes in artificial sea waters modified to have increased refractive indices; 90°; white light. The records in *A* and *B* are from a nerve in artificial sea water plus 4 % dextran; in *C* the dextran concentration was 9·7 %. *A*. Stimulation 9·5 times per second during the interval indicated by the bar resulted in an increase in light scattering. Temperature, 5° C. *B*. Correspondingly, the persistent component of the light scattering changes following individual stimuli became an increase. Temperature, 5° C; 120 sweeps averaged; time constant, 2 msec. *C*. At a higher dextran concentration the persistent component became so large that the initial transient component could not be separately discerned. Temperature, 19° C; 77 sweeps averaged; time constant 2 msec. The experiments were started 20 min after changing the bathing solution.

scattering (Fig. 5*A*), and concomitantly the persistent component following a single stimulus was now an increase (Fig. 5*B*). The increased external refractive index did not obviously affect the transient increase (Fig. 5*B*). However, when the external refractive index was increased still further, the persistent component was larger and the transient component following individual stimuli could no longer be distinguished (Fig. 5*C*). With n_e increased by 0·011 with dextran, the value of $\Delta I/I_r$ per sweep for the persistent change was $+5{\cdot}7 \pm 3{\cdot}2 \times 10^{-5}$ measured 350 msec after stimulation (eight nerves). In the same nerves the value of $\Delta I/I_r$ per stimulus of the integrated change was $+10{\cdot}2 \pm 5{\cdot}1 \times 10^{-5}$. Assuming that the integrated change is merely the sum of the persistent changes, the fact that the integrated change was larger suggests that the persistent component continues to increase slowly after the measurement at 350 msec.

Experiments using bovine albumin to raise the external refractive index, gave similar results, except that the external refractive index had to be increased about 3 times as much to achieve the same effects. This difference may reflect differences in penetrability of the periaxonal spaces.

The resting scattering of the nerve changes when the osmotic pressure of the bathing medium is changed. In normal sea water when the salt concentration was reduced the resting scattering decreased (cf. Hill, 1950*a*). However, when the refractive index of the sea water was raised by 0·0064 to 1·3454 (two nerves) with dextran, the resting scattering was unchanged when the concentration was decreased; and when the n_e was raised further (five nerves), then the resting scattering increased when the concentration was decreased. This result suggests that if activity leads to a simple volume increase in all of the cellular components of the nerve, then a resulting light scattering change should be zero at an external refractive index of about 1·3454 and should be negative at refractive indices lower and positive at refractive indices higher than this value.

Ouabain. The persistent and integrated changes continued long after the action potential; therefore the possibility that they were related to recovery processes was investigated. In one experiment the sodium in the artificial sea water was replaced by lithium. The lithium sea water did not obviously affect the transient increase, but did seem to make the persistent decrease more pronounced. The addition of 1·4 mM ouabain (Baker & Blaustein, 1968) to the sea water had similar results, and therefore we measured the integrated scattering changes before and after the addition of ouabain. Preliminary results showed that ouabain did not affect the scattering decrease in sea water (one experiment, Fig. 6*A*), or the scattering increase obtained in sea waters with an increased external refractive index (one experiment, Fig. 6*B*). These results indicate that those recovery processes immediately associated with the maintenance of

the ionic gradients are not giving rise to the light scattering changes. Bryant & Tobias (1955) found that azide blocked the return of the integrated scattering change to the resting level. In our experiments the return to the resting level was not readily recorded, but the results do not indicate any marked effects of ouabain.

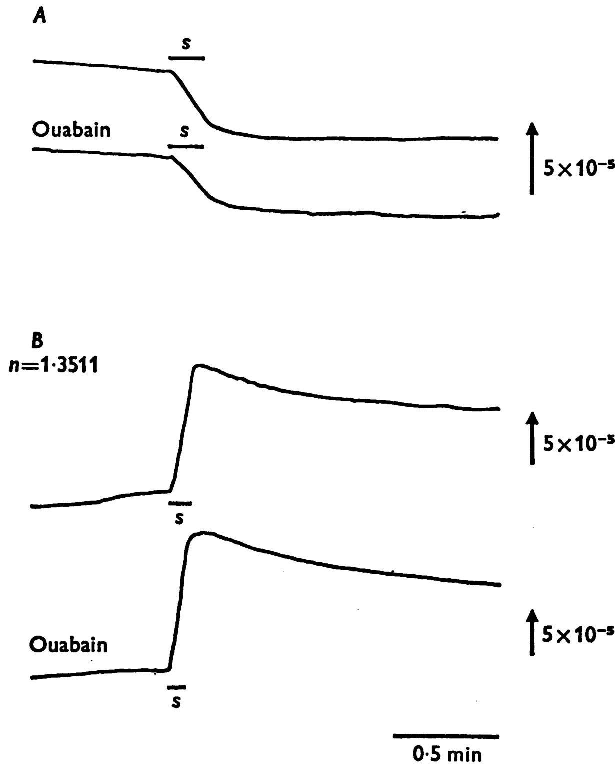

Fig. 6. The effect of 1·4 mM ouabain on the integrated light-scattering changes at 90°, white light, in sea water (*A*), and in sea water plus 7·9 % dextran (*B*). Ouabain did not affect the light scattering changes in either situation. *A*. Temperature, 9° C; stimulated 10/sec during the intervals indicated by the bars. *B*. Temperature, 15° C; stimulated at 15/sec during the intervals indicated by the bars. The scattering changes in the presence of ouabain were measured 15 min after its addition. Longer times in the presence of ouabain resulted in a decline of excitability.

Controls

Both the transient and the persistent changes in light scattering are very small in comparison with the resting light scattering; we therefore wished to eliminate the possibility that the changes were artifacts. Two types of experiment showed that the light scattering changes were not stimulus artifacts. When the action potential was blocked with 3 μM tetrodotoxin (Fig. 7*A*) the light scattering changes were elimi-

nated even though the stimulus voltage was unchanged. Similarly, when the action potential was blocked by raising the KCl concentration of the sea water to 60 mM or by replacing the sodium with choline, the light scattering change failed to appear. The second type of control against a stimulus artifact is illustrated in Fig. 7*B*, where the change in scattering is plotted against the stimulus voltage. It is seen that the effects reach a plateau. If the scattering change were a stimulus artifact, it would presumably have increased indefinitely as the voltage was raised.

As a control against electrical coupling between the circuit measuring action potentials and light, or a possible change in nerve luminescence, the light was turned off. There was then no apparent intensity change when the nerve was stimulated.

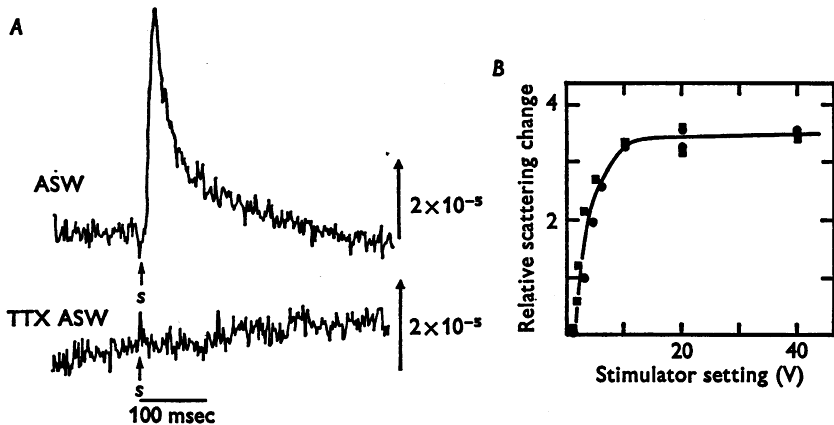

Fig. 7. Control experiments for 90° light-scattering changes (*A*). The transient increase in light-scattering found in artificial sea water (ASW) was blocked when the sea water contained 3 μM tetrodotoxin (TTX ASW). Temperature, 15° C; 115 and 170 sweeps averaged; time constant, 2 msec. (*B*). Relative $\Delta I/I_r$ per sweep of the transient increase in ASW (○) and of the persistent increase in 9·7 % dextran (■) as a function of stimulator setting. The scattering changes reached a maximum size when the stimulator was set at about 10 V. Temperature, 10° C; 70–110 sweeps averaged.

Bryant & Tobias (1955) reported that a crab nerve shortened by one part in 30,000 per 1000 stimuli when given a train of stimuli at 30/sec. Since the scattering from a nerve was not homogeneous along its length, movement might give rise to a scattering change. To show that shortening was unlikely to account for the scattering changes, we compared the changes which resulted from stimulating opposite ends of the nerve. If the scattering resulted from movement along the nerve axis, then the changes should have been of opposite sign when the nerve was stimulated at opposite ends, but they were not. A further possibility was that stimulation might have caused a movement perpendicular to the nerve axis and to the incident light. This possibility was eliminated by showing that the changes in light scattered from the two sides of the nerve were similar.

DISCUSSION

Persistent and integrated changes. Several experiments suggest that the integrated light-scattering changes during trains of action potentials are simply the sum of the persistent changes that follow individual stimulations. Both the persistent and the integrated changes were reversed in sign when the bathing medium was made hypotonic or its refractive index was increased. The size of the integrated and the persistent changes were usually the same (within a factor of two) when they were compared in the same axon under identical conditions. In experiments where several external refractive indices were tested, the size of the integrated change was closely correlated with the size of the persistent change. Thus, in crab nerve, the persistent and integrated changes appear to have the same origin. This was not true of similarly measured light scattering changes in eel electric organ (Cohen *et al.* 1969).

In the crab nerve the integrated scattering changes may be the more reliable indication of the size and duration of the slow events. Our measurements of the persistent change following individual stimuli were made at a repetition rate of 1/10 sec in the experiment illustrated in Fig. 2*C*. Since the integrated change continues for at least 50 sec, attempts to record the persistent change at 10 sec intervals will falsely register the scattering level at the end of the sweep equal to the scattering level at the beginning of the sweep. With noisy records this kind of distortion may go unnoticed and lead to an underestimation of the size and the duration of the persistent scattering change.

Hypotonic solutions and increased external refractive index, n_e, may act by the same mechanism. The internal refractive index of the axons, n_i, was calculated to be 1·360 by assuming that the axoplasm protein concentration in *Maia* nerves was 0·11 g/ml. (Bear, Schmitt & Young, 1937), and using a refractive index increment of 0·19 ml./g (Perlmann & Longsworth, 1948). This refractive index is 0·021 greater than that of sea water. Using dextran to increase n_e, the integrated change was zero when n_e was increased by 0·0013. Thus, a reduction in the difference between the internal and external refractive indices (Δn) by about 6 % caused the integrated scattering change to go to zero. If the hypotonic sea waters were acting by the same mechanism, then a 6 % swelling of the cell should similarly reduce Δn and lead to a zero integrated change. The actual dilution needed to produce a zero integrated change was somewhat less, 3·5 %. It is possible that hypotonic solutions were more effective than increasing n_e in reducing Δn, because the swelling of both axons and Schwann cell might bring them closer together and thus increase the contribution of the Schwann cell refractive index to the effective n_e.

Accepting our hypothesis that a reduction in Δn gave rise to the change in sign of the light scattering in both kinds of experiments, we may then

ask what kinds of structural modifications lead to light scattering changes that are sensitive to Δn. The light scattering from erythrocytes was found to be proportional to $(\Delta n)^2$ and only indirectly related to cell size (L. B. Cohen & D. L. Harris, unpublished results). This result suggested that the scattering change resulting from erythrocyte swelling (reducing n_i) would be a *decrease* when $n_e < n_i$ and would change sign to an *increase* when n_e was raised so that $n_e > n_i$; and this was confirmed experimentally. Since the persistent scattering decrease in the crab nerve changed sign to an increase when n_e was increased, this suggests that the persistent changes resulted from swelling of the axon following stimulation. While this hypothesis is in qualitative agreement with the experimental results, it predicts that the scattering changes would reverse direction when Δn was reduced to zero; but experimentally the scattering reversed when Δn was reduced by less than 10%. However, this calculation of the reduction in Δn assumed that the periaxonal space has a refractive index equal to that of the bathing medium, whereas mucopolysaccharides and other substances (Villegas & Villegas, 1968) in the space may give it a refractive index much closer to that of the inside of the axon. If this were true, then small increases in n_e might make $\Delta n = 0$. An increase in axon volume following stimulation is thus a possible explanation for the persistent and integrated scattering changes. Dr D. K. Hill has kindly called our attention to the fact that this argument assumes that the axon volume changes while the Schwann cell volume remains constant, and therefore, if the axon swells, then the volume of the periaxonal space would decrease. There are reasons to expect axon swelling resulting from the action potential (see below). Since the integrated scattering change in sea water is a decrease, we can, by analogy with erythrocytes, whose scattering decreases when their volume increases, suggest that it is the change in axon volume itself rather than the change in the volume of the periaxonal space which is the predominant factor in our scattering measurements.

Our rather indirect evidence for an increase in volume in crab nerve is strengthened by Hill's (1950*b*) direct measurement of a swelling during high frequency stimulation of giant axons from *Sepia officinalis*. Assuming that we have correctly identified the integrated and persistent changes as arising from axon swelling, the amount of swelling can be established using the procedure of Hill (1950*a*). With the $\Delta I/I_r$ per stimulus of 3×10^{-5}, the $\Delta I/I_r$ from a 1% change in tonicity of $1{\cdot}5\times10^{-2}$, and an osmotically inactive space of 25% (Hill, 1950*b*), we calculate that the axons swell by 15×10^{-6} of their original volume following a single action potential.

Hill (1950*a*) considered several mechanisms which might account for a swelling of axons. Since 1950 more information has been obtained about

the ionic fluxes that occur during the action potential and about the fibre diameters in crab nerve, which can be used to estimate the quantitative contribution of two of the possible mechanisms. Abbott, Hill & Howarth (1958) obtained a median diameter for axons of crab nerve of 1·2 μm. We measured fibre diameters from electron micrographs of transverse sections of crab nerve kindly provided by Dr P. F. Baker, and we found a considerable number of fibres in the range of 0·08–0·2 μm, a class of fibre diameter not described by Abbott *et al.* (1958). Therefore, we have used a somewhat smaller median diameter, 1·0 μm.

During the action potential in *Sepia* giant axons there is an exchange of internal potassium for sodium of about 5 p-mole/cm^2 (Keynes, 1951). On the assumption that the net potassium efflux exactly matches the net sodium influx, Hill (1950*b*) argued that the added sodium would exert an osmotic pressure of about 5 % larger than the potassium which it replaced. Applying these numbers to a 1 μm axon, this extra osmotic pressure would result in a swelling of about 8×10^{-6}, or about half of the swelling calculated from the light scattering data.

Hill (1950*a*) further suggested that any chloride influx during the action potential would result in swelling. Caldwell & Keynes (1960) have since found an excess chloride influx during the action potential of 0·046 p-mole/cm^2 in giant axons from *Loligo*. A similar influx in a 1 μm crab axon would result in a swelling of 4×10^{-6}, which provides another substantial fraction of the swelling estimated from the light scattering data. This postulated mechanism could be examined by replacing the chloride in the sea water by a less permeant anion, but this experiment has not been done. While the chloride influx of 0·046 p-mole/cm^2 was large enough to account for a substantial fraction of the swelling in crab nerves, as Caldwell & Keynes pointed out, the influx is more than 10 times too small to account for the swelling observed in *Sepia* giant axons (Hill, 1950*b*).

We conclude that there are several reasons for expecting the axon to swell slightly following an action potential. The predicted amount of swelling is close to that calculated from the light scattering change, and thus we feel that axon swelling is a likely explanation for the long term light scattering changes.

Transient increase. The transient increase in scattering following individual stimuli was not affected by hypotonic sea waters nor by increased n_e. Therefore it seemed unlikely that the transient increase resulted from a change in axon volume. The wave-length dependence of the change, $1/\lambda^2$, was less than the expected dependence ($1/\lambda^4$) for particles small compared to the wave-length of light, suggesting that the transient increase originated in a structure some of whose dimensions were large compared to the wave-length. Since the transient increase was somewhat slower than

the potential-dependent birefringence change it seems unlikely that all of the transient increase depended upon the potential across the axon membrane. We hope that further identification of the origins of this scattering change will come from experiments on single axon preparations.

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