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CHANGES IN AXON LIGHT SCATTERING THAT ACCOMPANY THE ACTION POTENTIAL: CURRENT-DEPENDENT COMPONENTS

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SUMMARY

1. When light scattering was measured during hyperpolarizing and depolarizing voltage-clamp steps, relatively large scattering changes were found during the depolarizing steps. These large changes were found to depend on the time integral of the ionic current and not on the changes in conductance or potential.

2. The current-dependent changes were examined at several scattering angles, and three distinct time courses were found. At 30–120°, the main change occurred after the current when steps of 2–5 msec duration were used. This change was called *I*–90°. At 15–30°, the change occurred with the same time course as the time integral of the current. This change was called *I*–25°. At 5–15° the scattering change occurred with a time course intermediate between that of *I*–90° and *I*–25°. This change was called *I*–10°.

3. In all experiments, outward potassium and outward sodium currents led to similar light scattering changes indicating that specific effects of the cation carrying the current across the membrane were not involved.

4. The size of *I*–90° was reduced by 29 % when an isethionate artificial sea water was substituted for the normal chloride artificial sea water. This reduction equalled the reduction predicted for a transport number effect at the membrane-solution interface. The time course of *I*–90° was similar to the predicted time course for a volume change in the periaxonal space, and such volume changes were tentatively identified as the origin of *I*–90°.

5. Because of difficulties in measuring the time course of *I*–25°, it was not possible to distinguish between a water of hydration effect and a transport number effect as the cause of this change. Similarly, the origins

of I–10° were not identified. Only I–10° was altered in size and time course when the external refractive index was increased with bovine albumin.

6. When the scattering changes during the action potential were examined in light of the voltage-clamp experiments, we concluded that the forward-angle change was potential-dependent and that the long-lasting change at right angles probably represented a swelling of the periaxonal space resulting from the fact that chloride carried a significant fraction of the outward current during the action potential.

INTRODUCTION

The change in right-angle light scattering that occurred during the action potential (Cohen, Keynes & Landowne, 1972) had a time course which indicated that not all of the change could be potential-dependent. Light-scattering measurements during voltage-clamp steps then showed that both at right angles and at forward angles there were relatively large changes which were either current or conductance-dependent. This paper is concerned with the discrimination between current and conductance-dependences, with the identification of the changes as a function of the scattering angle and with experiments designed to determine the structural origin of the changes.

METHODS

The axon dissection, experimental apparatus and procedures, and voltage-clamp system were the same as those used for the experiments discussed in the preceding paper (Cohen *et al.* 1972).

The scattering change shown in Fig. 6*B* was measured with a Spectra-Physics Model 125A 50 mW helium–neon laser used in place of the usual tungsten–halogen light source. Because the light output of the laser fluctuated considerably, a beam

TABLE 1. Composition of chloride and isethionate ASW

Chloride		Isethionate	
NaCl	497 mM	Na isethionate	497 mM
KCl	10 mM	K isethionate	10 mM
$CaCl_2$	20 mM	Ca glutamate	20 mM
$MgCl_2$	20 mM	Ma glutamate	20 mM
$NaHCO_3$	2·5 mM	$NaHCO_3$	2·5 mM

splitter was inserted to deflect a portion of the incident beam into a second photodiode, the signal from which was amplified, inverted and fed back to a Pockels cell (Lasermetrics Inc., model EOM 704) placed in the incident light path. Even though a five to tenfold decrease in the noise was achieved, inspection of Fig. 6*B* shows that the record was still noisier than those made with a tungsten–halogen light source.

The artificial sea water (ASW) sometimes used as the bathing solution was 10 K (Na) ASW (Baker, 1965). The compositions of the isethionate ASW and the chloride ASW used for comparison are given in Table 1. The pH of all artificial sea waters

was adjusted to pH 7·5–8·0. The sodium and potassium isethionates were obtained from Eastman Organic Chemicals; glutamic acid, ouabain (Strophanthin G), Dextran 60C and bovine albumin were obtained from Sigma Chemical Co. To prepare a 7 % solution of bovine albumin in sea water, 7 g powder was added to 80 ml. sea water, the pH adjusted to 7·5–8·0 with 0·5 M sodium hydroxide and sea water added to make a final volume of 100 ml. Dextran solutions were prepared similarly.

The chamber temperature was maintained constant at $12 \pm 1°$ C, except where specified otherwise.

RESULTS

When light scattering was measured during sweeps with both depolarizing and hyperpolarizing voltage-clamp steps, there were large changes during the depolarizing step (Fig. 1) (same as Fig. 9 in Cohen *et al.* 1972) at right angles (90°) and at forward angles (30°). There were also relatively smaller changes during the hyperpolarizing step, which were identified as potential-dependent (Cohen *et al.* 1972). Since these potential-dependent components were even smaller for depolarizing than for hyperpolarizing steps, such components could have made only a trivial contribution to the large changes during the depolarizing steps seen in Fig. 1. These relatively large changes must depend upon either membrane currents or membrane conductance, and the next section of the paper is concerned with discriminating between these two possibilities. In addition, Fig. 1 shows that the changes during the depolarizing step measured at the two angles differed in sign and time course. While postponing a discussion of the differences, we can label the type of change found at forward angles as I–25° and the type of change at right angles as I–90°.

Current or conductance dependence

One way of determining whether the changes depended on current or on conductance was to study situations where the conductance increases were uniformly large but the currents were either large or small.

One experiment compared the scattering changes resulting from depolarizing potential steps of two sizes. Fig. 2 illustrates the results of such an experiment, carried out on the right-angle scattering change, I–90°. Scattering changes were measured during sweeps with 50 and 92 mV depolarizing steps. Both steps resulted in the large early conductance increase, while only the 50 mV step resulted in a large inward current. The 92 mV step reached the reversal potential for sodium ions, and the current was small because the driving force was small. Even though the conductance increased in both instances, the light scattering increased only after the 50 mV step, when there was a large inward current; thus the scattering change depended on current. But because the main increase reached a peak later than the current flow, it did not appear to depend directly on the instantaneous current density.

A second experiment that distinguished between current and conductance-dependent changes involved replacement of the sodium in the sea water by choline, and thus a shift of the reversal potential for the early conductance increase. The same potential step resulted in similar mem-

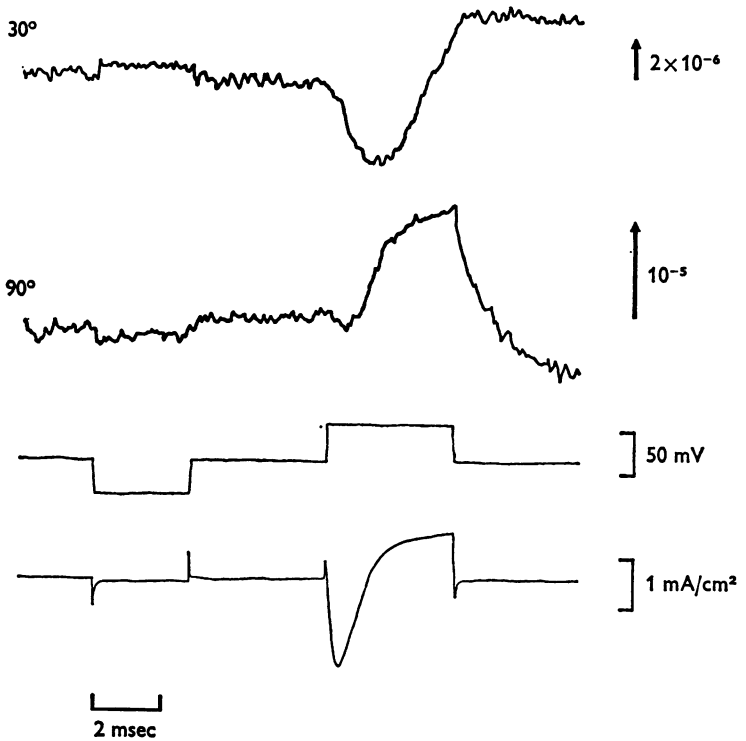

Fig. 1. Light scattering changes (heavy lines) at forward angles (30°) and at right angles (90°) during voltage-clamp steps. At both angles, there were large changes during the depolarizing step and only small changes during the hyperpolarizing step. Third trace, potential; bottom trace, current density. In this and subsequent Figures, the holding potential was the resting potential, hyperpolarizing was downward, inward current was downward. The light scattering traces were from different axons; the current trace shown was the actual trace for the experiment at forward angles. The currents for the experiment at 90° were similar in magnitude but had a more rapid time course because the chamber temperature was higher. The response time constant to a step change in light intensity was 70 μsec. *L. forbesi*; chamber temperature, forward angles, 13° C, right angles, 19° C; 900 sweeps averaged.

brane conductance increases in both sea waters (Hodgkin & Huxley, 1952*a*), but the current was much larger in sodium sea water than in choline sea water. Fig. 3 illustrates the results of such an experiment, studying the scattering change at forward angles, *I*–25°. In artificial sea

water, the 50 mV depolarizing potential step resulted in a large conductance increase and a large inward current; in choline artificial sea water, the step resulted in an equal conductance increase but a relatively small current. Even though the conductance increased in both instances, the light-scattering decrease occurred only when there was a large inward current.

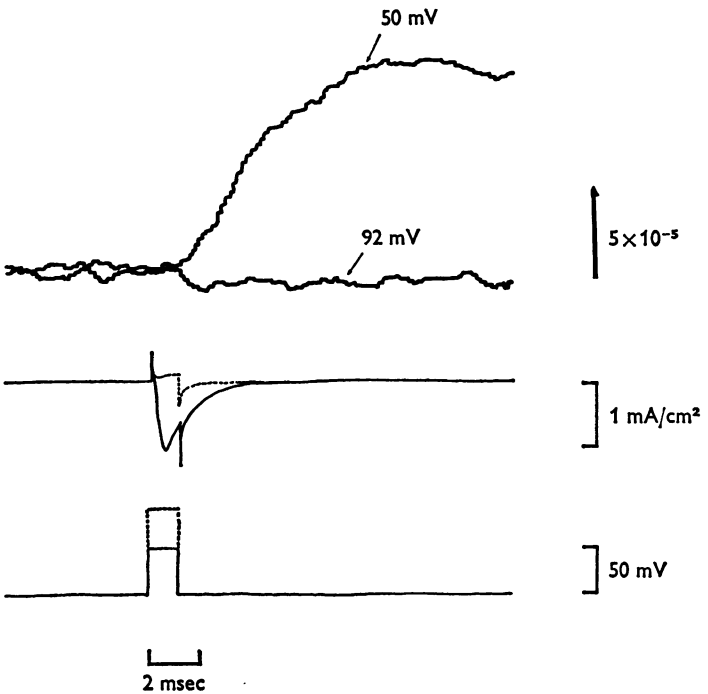

Fig. 2. Changes in 90° light scattering, *I*–90° (heavy lines), resulting from two different depolarizing steps (bottom trace). A 50 mV step (continuous curve, bottom trace) led to a large increase in conductance and a large inward current (continuous curve, middle trace). A large-scattering increase resulted. The larger, 92 mV, depolarizing step (dashed curve, bottom trace) was near the equilibrium potential, and thus the current (dashed curve, middle trace) was much smaller, even though the conductance increase was still large. When the current was reduced there was no clearly demonstrable scattering change. This axon had been micro-injected with tetraethylammonium bromide, final concentration 24 mM, to block delayed outward currents. *L. pealii*; time constant, 610 μsec; 256 sweeps averaged.

Therefore *I*–25° also appeared to depend upon current, not conductance. Both types of experiment, two depolarizing steps (Fig. 2) and choline (Fig. 3), were done on both *I*–25° and *I*–90°, with uniform results. The large changes during and after the depolarizing steps (Fig. 1) apparently depended in some manner on current, and not on conductance (or

potential). While both I–25° and I–90° appeared to depend upon current, their relationships to the current were different; for I–25° occurred during the current, with a time course similar to the time integral of the current (Fig. 3), but in the same situation I–90° reached a peak after the current

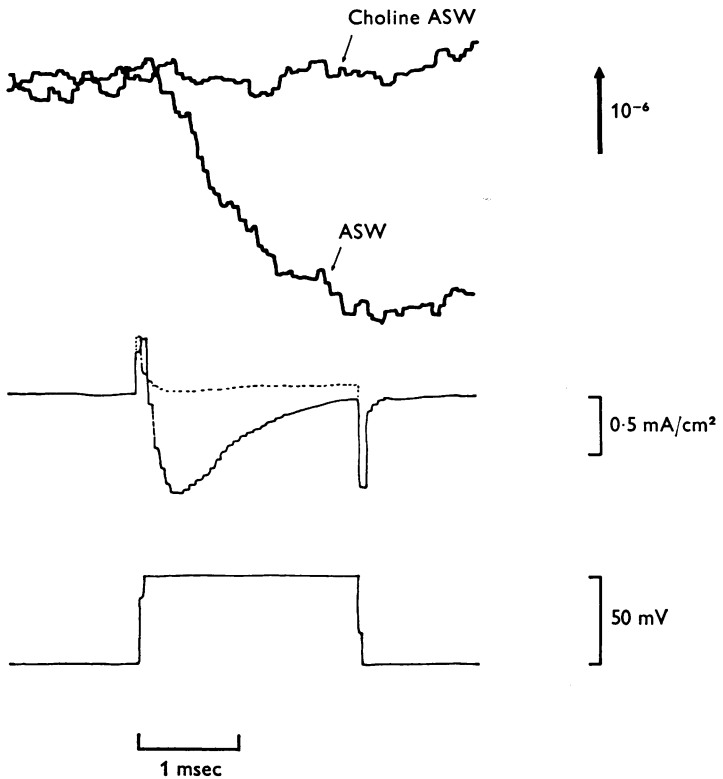

Fig. 3. Changes in forward scattering, I–25° (heavy lines), resulting from a 50 mV depolarizing potential step (bottom trace) in artificial sea water (ASW) and in choline artificial sea water (choline ASW). In ASW, the depolarizing step resulted in a large inward current (middle trace, continuous line) while in choline ASW the same step led to a much smaller current (middle trace, dashed curve). Even though the conductance increase was similar in both solutions, the scattering decrease only occurred when there was a large current. The axon was microinjected with tetraethylammonium bromide, final concentration 30 mM, to block the delayed outward currents. *L. forbesi*; time constant, 120 μsec; 1000 sweep averaged.

(Fig. 2). The axons used in Figs. 2 and 3 had been microinjected with tetraethylammonium bromide to block the delayed outward currents.

Further evidence to support the conclusion of current dependence was obtained by plotting the peak size of the scattering change against the

time integral of the current (Fig. 4). Depolarizing steps that induced both inward and outward currents were used along with those that resulted in unidirectional currents. For both I–25° and I–90°, the experimental points fell near a straight line which passed through the origin. While the results in Fig. 4 showed that I–25° and I–90° depend on current, they further indicated that I–25° and I–90° depended on the time integral of the current rather than instantaneous current density.

In the above experiments, we assumed that there were only two different light-scattering changes which depended on the integrated current. This assumption was examined by measuring scattering changes as a function

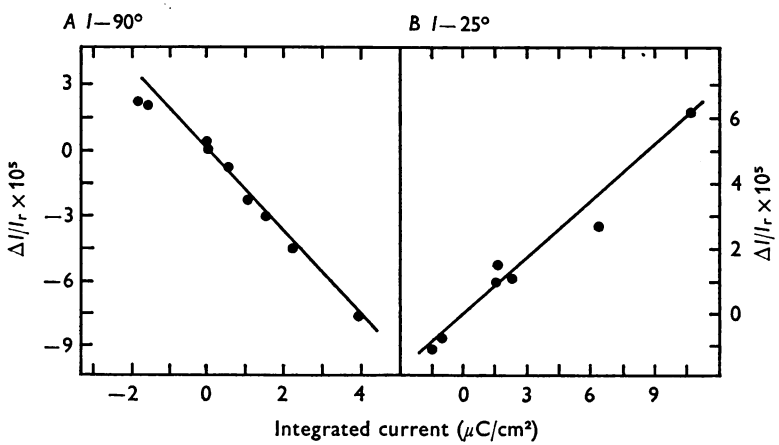

Fig. 4. The $\Delta I/I_r$-sweep *vs.* the integrated ionic current during depolarizing potential steps. For both I–25° and I–90°, the scattering change was linearly dependent on the mean current times the step duration. Lines drawn by eye. *A*, I–90°. The scattering change was measured 10 msec after the end of the potential step and the value for the time integral of the current was taken at that time. *B*, I–25°. The scattering change was measured at the end of the potential step, and the value for the time integral of the current was taken at that time.

of scattering angle. Fig. 5 illustrates the changes resulting from large outward currents measured at five angles from 10 to 90°. The time courses of these changes indicated that the assumption was false; three scattering changes were needed to explain the results. At 25°, there was a scattering increase during the outward current, which, like the change illustrated in Fig. 3 (ASW), had a time course similar to the time integral of the current; this was the change we labelled I–25°. The scattering returned to the base line with a time constant of some tens of milliseconds. At 90°, the major scattering change was a decrease reaching its maximum after the current and persisting for a relatively long time. This was the change we labelled

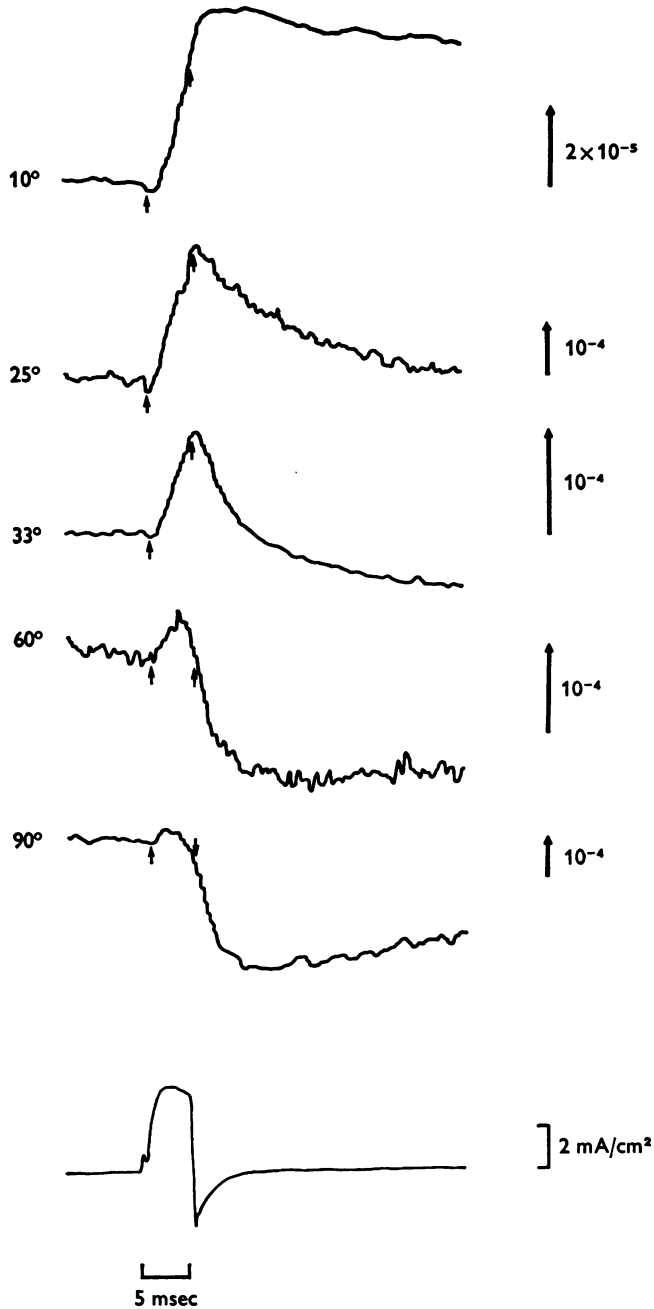

Fig. 5. Light-scattering changes at five angles (heavy lines) resulting from 110 mV depolarizing potential steps. The light-scattering changes at 33 and 60° can be described as mixtures of two different changes which occur at 25°, *I*–25°, and 90°, *I*–90°. The short arrows near the scattering traces indicate the beginning and end of the depolarizing step. Current density (thin line) is traced at the bottom. *L. pealii*; time constant, 190 μsec; 64 to 256 sweeps averaged.

I–90°. We interpret the scattering changes at 33 and 60° as mixtures of I–25° and I–90°, with 33° having relatively more I–25° and 60° relatively more I–90°.

But comparison of the results at 10 and 25° showed that the scattering change at 10° started with a delay, reached its peak later and returned toward the base line more slowly than I–25° (see also Fig. 10). The change at 10° started after a delay, and it continued to increase after the end of the outward current, even during the inward current tail. This difference

TABLE 2. Characteristics of the three current-dependent scattering changes

Change	Scattering angle	Time course	Sign of scattering change for an outward current
I–10°	5–15°	Intermediate between I–25° and I–90°	+
I–25°	15–30°	Occurs as time integral of current	+
I–90°	60–120°	Occurs after current	−

in time course between the changes at 10 and at 25° suggested that the change at 10° was different from I–25°, and we call that change I–10°. Further evidence for a difference between I–10° and I–25° came from experiments with increased external refractive indexes (see below). Experiments of the type illustrated in Figs. 2 and 4 showed that I–10° also depended on the integrated current. Thus, the data in Fig. 5 can be explained by three different light scattering changes which depend in some manner on the integrated ionic current (pertinent features are summarized in Table 2). Experiments designed to characterize the changes and to determine their origins are discussed below; first I–90°, then I–25°, and finally I–10°.

I–90°

The type of scattering change we have labelled I–90° was most often studied at 90°; but in some axons, the change was found at angles as low as 30°, and it was always found at 120°. *Inward* 'sodium' current resulted in a scattering increase, and outward 'potassium' current resulted in a scattering decrease (Fig. 6). When an *outward* 'sodium' current was obtained by using a 120 mV depolarizing step in an axon that had been micro-injected with tetraethylammonium chloride, the light-scattering change was similar to that occurring with outward 'potassium' currents. Thus I–90° did not depend on specific properties or effects of sodium or potassium ions.

The scattering changes for the different currents had similar time courses.

As the records in Figs. 2, 5 (90°) and 6 show, I–90° reached a peak after the current. The time course of the scattering increase resulting from an inward current (Figs. 2, 6B) could be approximated by a single exponential with a time constant that averaged 3 msec. The same measurement on the scattering decrease resulting from an outward current averaged 2 msec. In this analysis, we ignored the scattering during the depolarizing step as there might have been interference from I–25° at that time.

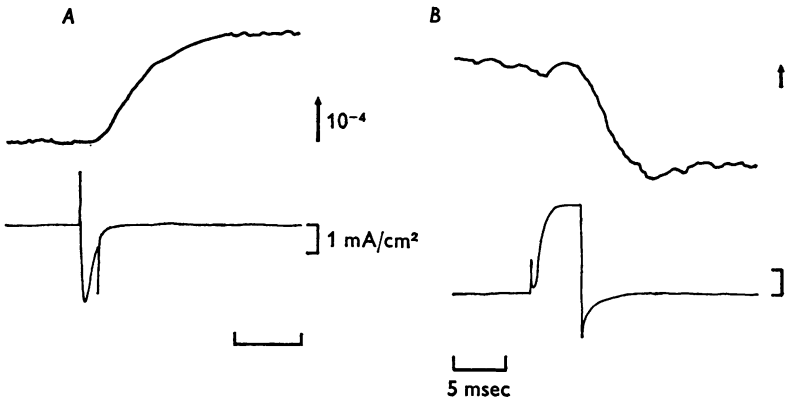

Fig. 6. Right-angle scattering changes, I–90° (heavy lines), for inward (A) and outward (B) currents (thin lines). Currents of opposite sign gave rise to scattering changes of opposite sign. A 50 mV depolarizing step was used in A, 110 mV in B. The light source for B was a 50 mW helium-neon laser, 632·8 nm. The results in A and B are from different axons. *L. pealii*; The sum of divergences was A, 18°, B, 6°; temperature, A, 13° C, B, 7° C; time constant, A, 600 μsec, B, 1800 μsec; 128 and 64 sweeps averaged.

To compare the sizes of I–90° from different experiments, we calculated the peak change in scattering intensity, ΔI, per sweep, divided by the resting intensity, I_r, per coulomb, of charge that crossed the axon membrane in the 4 mm illuminated region. The mean for the large scattering changes in *L. pealii* axons was about $5 \times 10^{-4}/\mu$C (see Table 3). The sizes of the scattering changes resulting from inward and outward current were not significantly different. In one axon, the size of I–90° was measured five times and averaged $3{\cdot}8 \pm 0{\cdot}5 \times 10^{-4}$ (S.D. of an observation). I–90° was larger in *L. pealii* than in *L. forbesi* axons (Table 3), as were the scattering changes that occurred during the action potential or during hyperpolarizing potential steps (Cohen *et al.* 1972). But this difference may merely be related to the improved angular resolution in the experiments on *L. pealii* which would reduce interference from I–25°.

With longer sweeps, the duration of I–90° could be determined. Fig. 7 shows that with a sweep about twenty times longer than usual, the large

TABLE 3. The mean size, ΔI/sweep-I_r-μC, of the current-dependent scattering changes. The number of axons is in brackets

Change	Species	Direction of current	Mean ± S.D. of an observation ($\times 10^5$)
I–10°	*L. pealii*	Inward	−7·3 (1)
		Outward	+6·3 ± 3·9 (10)
I–25°	*L. pealii*	Outward	+9·4 ± 4·0 (8)
	L. forbesi	Inward	−6·2 ± 3·4 (10)
		Outward	+3·4 ± 1·2 (4)
I–90°	*L. pealii*	Inward	+72 ± 54 (6)
		Outward	−43 ± 30 (25)
	L. forbesi	Inward	+21 ± 7 (8)
		Outward	−15 ± 13 (6)

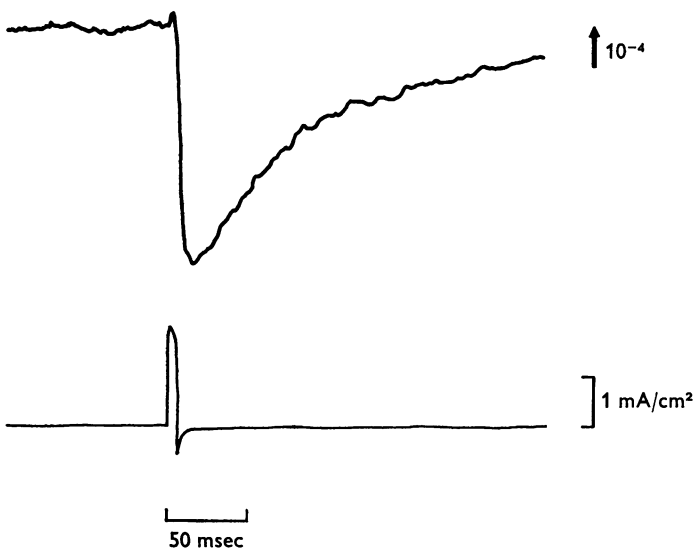

Fig. 7. Right-angle scattering change, *I*–90° (top trace), using a longer sweep to demonstrate the time course of the return of the scattering to the base line. The current density resulting from the 80 mV depolarizing step is drawn below the scattering trace. *L. pealii*; sum of divergences, 23°, time constant, 750 μsec; 64 sweeps averaged.

decrease following an outward current returned to the base line with a time course that could be approximated by a single exponential with a time constant of 65 msec. This time constant averaged 94 ± 26 msec (S.E. of mean) in measurements on thirteen axons. The time constant was thus longer than the Frankenhaeuser–Hodgkin time constant (Frankenhaeuser & Hodgkin, 1956) which we found to average 41 ± 12 msec (S.D. of an observation) in five *L. forbesi* and eight *L. pealii axons*.

One possible origin for all three scattering changes is a transport number effect (Barry & Hope, 1969*a*). This suggestion seems reasonable, because the size of the three changes was directly related to the time integral of the current (see Fig. 4) rather than its instantaneous value or the specific cation carrying the current across the membrane. While the current across the membrane is carried predominantly by cations, between the membrane and the electrode in the bath both cations and anions carry the current. For an outward potassium current, if 100 potassium ions crossed the membrane into the periaxonal space between it and the Schwann cell, the current flowing onward into the bath would be carried by approximately forty sodium ions transferred out of the space and sixty chloride ions moving into it. There would therefore be a net accumulation of potassium chloride in the periaxonal space. In a similar way an inward sodium current would deplete the space of NaCl. The change in salt concentration should occur with a time course identical to the time integral of the current. The speed of the subsequent restoration process would be restricted by the closely apposed Schwann cells. Frankenhaeuser & Hodgkin (1956) showed that accumulated potassium would diffuse away with a characteristic time constant (Frankenhaeuser–Hodgkin time constant) which we assume to apply in our experiments. Thus a light-scattering change that depended *directly* on a transport number effect would occur as the time integral of the current and would relax with the Frankenhaeuser–Hodgkin time constant.

With short depolarizing steps, I–90° occurred after the passage of the current and returned to the base line with a time constant longer than the Frankenhaeuser–Hodgkin time constant. Thus both the onset and the relaxation to the base line were delayed in comparison with the changes in salt concentration, so that I–90° cannot be a direct result of these changes. However, we could test the hypothesis that a transport-number effect was ultimately responsible for I–90° by changing the anion in the sea water from one of high mobility (chloride) to anions of lower mobility (isethionate and glutamate). With lower mobility anions, a smaller fraction of the current in the bathing solution would be carried by anions, and the changes in salt concentration in the periaxonal space should be correspondingly reduced. Fig. 8 shows that replacing chloride with anions of lower mobility reduced the size of I–90° for outward currents; in this experiment, the value of $\Delta I/I_r$-μC-sweep decreased by 23 % when chloride ASW was replaced by isethionate ASW. In twelve trials on four axons, measurements of I–90° in isethionate sea water were bracketed by measurements in chloride sea water. The average reduction of $\Delta I/I_r$-μC-sweep in isethionate was 29 ± 5 % (S.E. of mean). This result supports the hypothesis that I–90° resulted from a transport number effect.

Fig. 8 is drawn as if the only effect of isethionate ASW was to reduce the size of the scattering change ΔI. But, in addition, in these same experiments the outward currents were found to be reduced in isethionate sea waters by $15{\cdot}8 \pm 1{\cdot}8$ % (S.E. of mean). The smaller currents in isethionate ASW accounts for the fact that the reduction in ΔI in Fig. 8 is larger than the calculated reduction in $\Delta I/\mu C$. One possible explanation of the reduction in current in isethionate sea waters is that chloride moving inward might carry 10–20 % of the outward current found in normal sea waters. This conclusion would be consistent with the suggested explanation for the slow increase in scattering at right angles that follows the action potential.

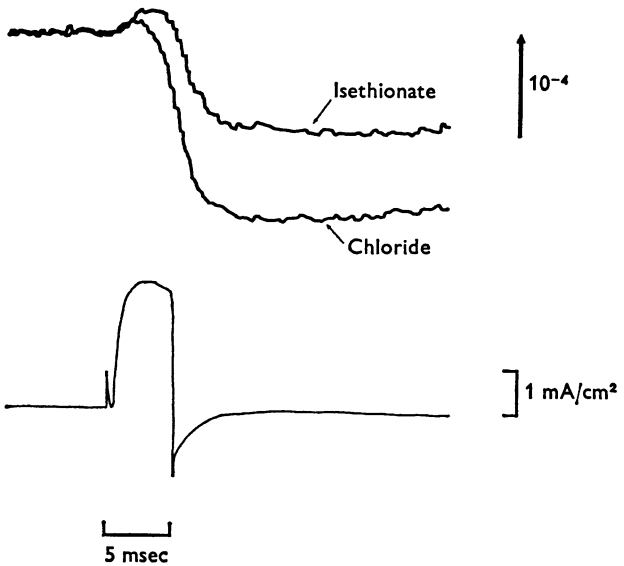

Fig. 8. The effect on I–90° (heavy lines) of replacing chloride with lower-mobility anions. I–90° was reduced in isethionate sea water. The compositions of the chloride and isethionate artificial sea waters are given in Table 1. The current density (bottom trace) resulted from a 120 mV depolarizing potential step. *L. pealii*; sum of divergences, 30°; time constant, 200 μsec; 64 sweeps averaged.

Since the persistent light-scattering change in *crab* nerve (measured at 90°) was inverted in sign by increasing the refractive index of the bathing medium (Cohen & Keynes, 1971), we looked to see whether the current-dependent changes in squid axons were similarly affected. For three axons the refractive index of the bathing solution was increased by the addition of bovine albumin, final concentration 7 %, or dextran, final concentration 10 %; but I–90° was unaffected by the increased refractive index.

The size and time course of I–90° appeared to be unchanged in axons internally perfused with fluoride solutions; and I–90° resulting from inward sodium currents was not affected by ouabain 3×10^{-5} g/ml. in the bathing solution.

The light scattered at 90° from a resting axon was preferentially polarized parallel to the axon axis (Cohen *et al.* 1972). In experiments at 90° on three axons, we tried to measure I–90° in the light polarized perpendicular to the axon, but no change could be detected. In one experiment, $\Delta I/I_r$-sweep for light perpendicular to the

axon was less than 10 % of that for light parallel to the axon. At 60°, the signal for light perpendicular to the axon was 40 % of that for light parallel; and at 120°, the signal for light perpendicular was 75 % of that for light parallel. Thus, at 90°, though

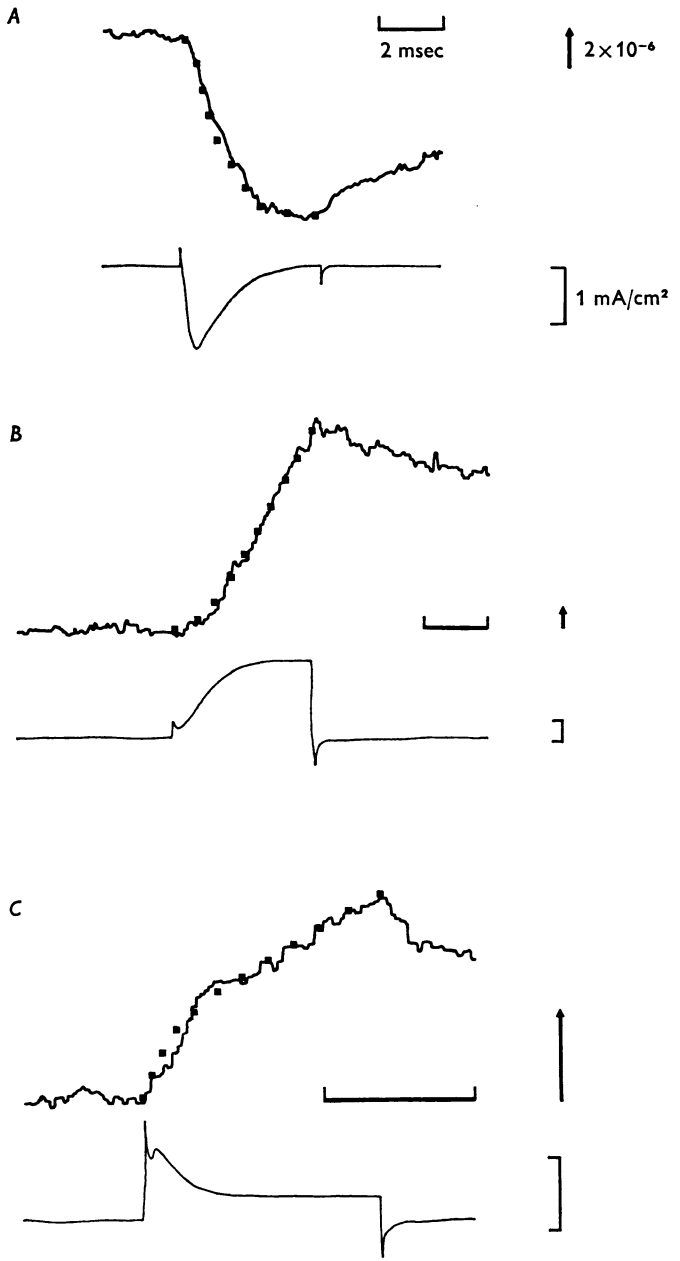

Fig. 9. For legend see foot of facing page.

not at 60 or 120°, I–90° was only detected in the scattered light which was parallel to the axis of the axon.

The record in Fig. 6B was made using a laser light source with a wave-length of 632·8 nm. Because the output of the laser was rather unstable, it could not be used routinely, but comparison of Fig. 6B with Fig. 5 (90°) shows that I–90° was essentially the same for both monochromatic and white light.

Often at 90°, small rapid scattering changes preceded the main change, e.g. the transient scattering increase during outward currents in Figs. 5 (90°) and 6B. These were larger in experiments on *L. forbesi* axons, averaging 14 % of the large change, than in *L. pealii* axons where they averaged less than 7 % of the large change. Because the angular resolution of the *L. forbesi* experiments was poorer (Cohen *et al.* 1972) it was possible that these small changes were caused by interference from I–25°. When I–90° was measured at 120° instead of the usual 90°, the small change was relatively smaller still. Although the small scattering increases in Figs. 5 (90°) and 6B were of the same sign as the potential-dependent change (Cohen *et al.* 1972), their time course was much slower than that of the potential-dependent change. In *L. forbesi* axons, where the small change was easier to measure, the decrease for an inward current averaged $-3{\cdot}6 \pm 0{\cdot}9 \times 10^{-5}/\mu$C (S.E. of mean) in six axons, while the increase for an outward current was only $1{\cdot}3 \pm 0{\cdot}3 \times 10^{-5}/\mu$C in six axons.

I–25°

Scattering changes of the I–25° type were generally found between 15 and 30°, although they were seen occasionally at smaller and larger angles.

Fig. 9 illustrates the time courses of I–25° that resulted from inward 'sodium', outward 'potassium' and outward 'sodium' currents. The appropriate current records are shown below the scattering traces. For each current, the time integral was calculated, adjusted in size so that its peak matched the peak of the scattering change and then shown as filled squares superimposed on the scattering records. In all three comparisons, the time course of I–25° and the time integral of current were identical. This correlation provides important limitations on possible explanations for I–25°. Outward 'potassium' and outward 'sodium' currents resulted in similar scattering changes (Fig. 9), so I–25° did not depend on specific properties of sodium or potassium ions.

The mean of $\Delta I/I_r$-μC-sweep for I–25° was about 7×10^{-5}, ten times

Fig. 9. Scattering changes, I–25° (heavy lines), measured at 35°, resulting from depolarizing steps which gave rise to inward 'sodium' (A), outward 'potassium', (B) and outward 'sodium', (C) currents. The time courses of the scattering changes and the time integrals (■) of the current were identical. The current densities (thin lines) resulting from depolarizing steps of 40 mV (A) 94 mV (B) and 96 mV (C) are drawn below the scattering traces. The axon used for A was internally injected with tetraethylammonium chloride, final concentration 12 mM. The axon used for C was internally injected with tetraethylammonium bromide, final concentration 29 mM. The bathing solution for C was choline ASW. The results in A, B and C were from three different axons. *L. forbesi*: time constant; 90 μsec; 120–1000 sweeps averaged.

smaller than the size of I–90° (Table 3). Inward and outward currents gave rise to scattering changes that were similar in size, although opposite in sign. The size of I–25° per μC often grew smaller during repeated measurements on the same axon and sometimes I–25° could not be found at all in a rundown axon. In other axons, the size of I–25° was relatively constant; in one case, $\Delta I/I_r$-μC-sweep was $7{\cdot}5 \times 10^{-5}$ in the first trial and $4{\cdot}3 \times 10^{-5}$ in the fifth.

The duration of I–25° could be estimated from records like that for 25° in Fig. 5. When the return to the base line was treated as a single exponential, the time constant ranged from 10 to 40 msec in seven axons. However, because of interference from I–90° or I–10° the significance of the measurement was uncertain. To test the possibility that I–25 resulted from a transport-number effect, the chloride bathing solution was replaced with isethionate artificial sea water. The size of I–25° was not changed by more than 10 %. While this negative result suggested that a transport-number effect was not involved, other possible explanations are considered in the Discussion section.

In experiments on four axons, we increased the refractive index of the bathing solution by adding 7 % dextran or 7 % bovine albumin, but no change was found in I–25°. In one experiment, I–25° resulting from an inward current was the same before and after the addition of 4×10^{-5} M ouabain. I–25° was the same size for light polarized parallel and perpendicular to the axon.

I–10°

Scattering changes of the type I–10° were found between 5 and 15°. Fig. 10 illustrates the scattering changes which resulted from an outward current, (A), and an inward one (B). Again, inward and outward currents gave rise to scattering changes that were opposite in sign. The delay of the scattering change from the onset of the current seen in Fig. 10A was the longest observed in experiments on seven axons.

The value of $\Delta I/I_r$-μC-sweep for I–10° averaged 6×10^5 (Table 3), so that it was similar in size to I–25°; and, as with I–25°, I–10° decreased in size as the axon became fatigued. The records in Fig. 5 (10°) and Fig. 10 show that I–10° had a relatively long duration. In one experiment, a sweep of 80 msec was used, and at the end of this sweep I–10° had returned less than one third of the way to the base line.

A phenomenon that also distinguished I–10° from I–25° and I–90° was that, rather surprisingly, I–10° was sensitive to increased external refractive index (Fig. 11). In the bathing solution of higher refractive index, the scattering change was reversed in sign and had a more rapid time course. In four experiments where the external refractive index was increased with

bovine albumin, the sign of the change was always reversed, but the time course of the return to the base line was somewhat variable, ranging from a time constant of 2 msec to the time constant of 13 msec illustrated in Fig. 11. The light-scattering changes in crab nerve (Cohen & Keynes, 1971),

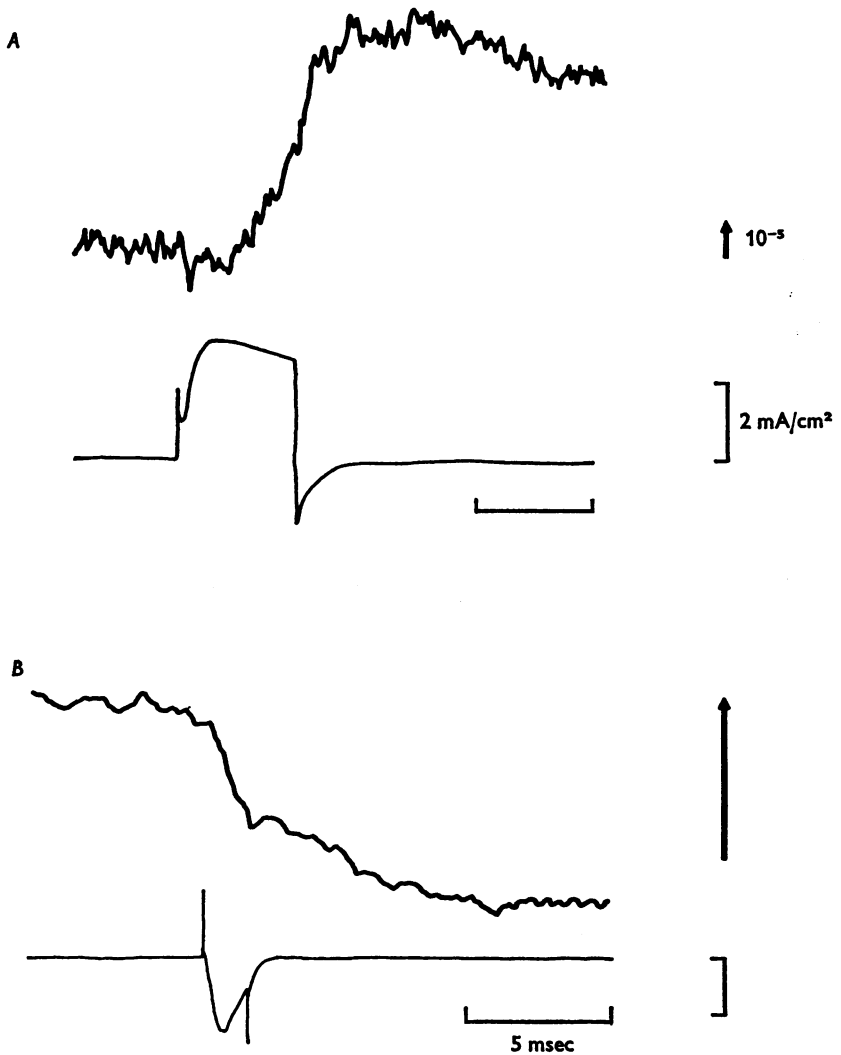

Fig. 10. Light scattering changes, I–10° (heavy lines), at 10° resulting from outward (A) and inward (B) currents. I–10° has a time course that is slower than the time integral of the current. The current densities (thin lines) that resulted from 110 mV (A) and 50 mV (B) depolarizing steps are drawn below the scattering traces. Records from two axons are illustrated. *L. pealii*: time constants, A, 200 μsec, B, 680 μsec; A, 128, and B, 512 sweeps averaged.

measured at 90°, were drastically altered by changes in refractive index. Even though in the squid only *I*–10° was affected, the results in both preparations may be comparable because, in the crab experiments, multiple scattering obtained, which meant that light measured at 90° would include scattering from all forward angles.

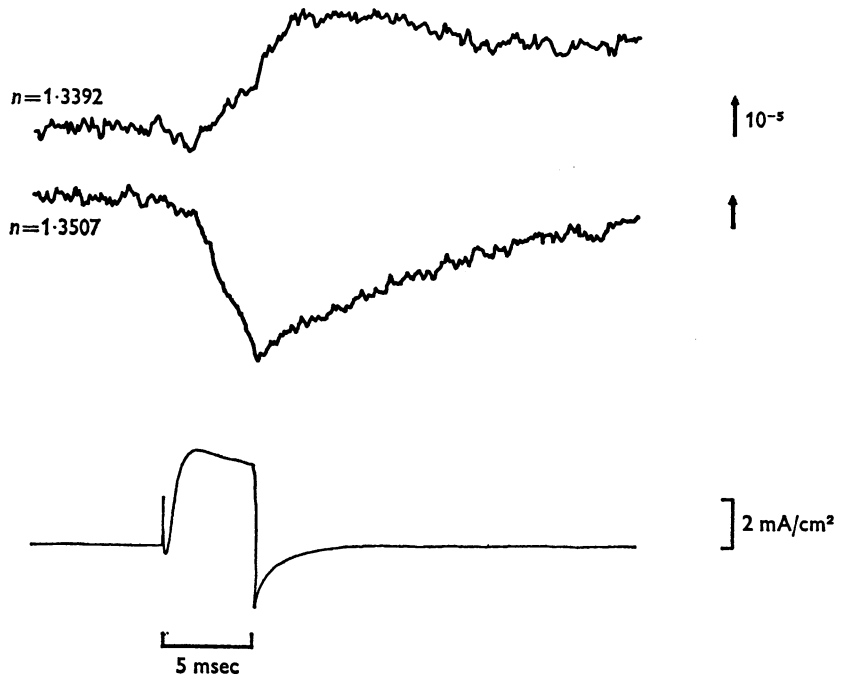

Fig. 11. Light scattering changes, *I*–10° (heavy lines), at 10° in sea water, external refractive index of 1·3392, and in sea water with 7% bovine albumin, refractive index of 1·3507. Increasing the refractive index resulted in a change in sign and time course of *I*–10°. The current density (thin line) resulting from a 116 mV depolarizing step is drawn below the scattering changes. *L. pealii*; time constant, 200 μsec; 64 sweeps averaged.

It must be emphasized that the separation of the current-dependent light scattering changes into three types, *I*–90°, *I*–25° and *I*–10° is partly a matter of convenience for it is possible to synthesize a scattering change with a time course similar to *I*–10° out of combinations of *I*–90° and *I*–25°, although it takes some special pleading to fit the very slow return to the base line and the refractive index effect found with *I*–10°.

DISCUSSION

Origins of the three changes

We feel that the experiments illustrated in Figs. 2 and 3 provide strong evidence that the large light-scattering changes resulting from depolarizing voltage steps depend on current, not on potential or conductance. The further results shown in Fig. 4 and elsewhere demonstrated that the changes depend on the time integral of the current, rather than the instantaneous current or the specific cation carrying the current across the membrane.

The explanations for the three scattering changes that we have considered most seriously involve changes in salt concentration or changes in volume in the periaxonal space, the 100 Å space between the axon and Schwann cell membranes (Geren & Schmitt, 1954; Golfand, Komissarchik, Levin, Rosenthal & Troshin, 1966; Villegas & Villegas, 1968). Water moving with ions as water of hydration (Washburn, 1909) could cause the space to swell or shrink. A second mechanism that could give rise to such changes is a transport-number effect. Both mechanisms (Barry & Hope, 1969*a*, *b*) were previously considered by Girardier, Reuben, Brandt & Grundfest (1963) in order to explain swelling of the crayfish transverse tubular system after applying currents for 5–10 min.

Both mechanisms operate in situations where membrane current is carried by ions of only positive charge. Such currents crossing ion exchange or biological membranes can cause a concomitant transferance of solvent in the direction of the movement of charge. Stallworthy (1970) estimated that about 16 moles of water accompany each Faraday of current crossing the axon membrane. An outward current would lead to a decrease in axon volume and extra water would be added to the periaxonal space. The opposite changes would occur for an inward current. If the periaxonal space is 100 Å thick, then for an axon 1000 μm in diameter the relative volume change would be about 10^4 times greater for the periaxonal space than for the axon itself.

The water of hydration is transferred at the same time as the current and thus the volume of the space would change with a time course identical to the time integral of the current. Ignoring any transport-number effect, the space would then return to its original size with a time constant dependent on the rate of diffusion of the excess water out of the space. This time constant was estimated to be 10 msec by the following considerations.

Water may leave or enter the space via three paths: first, across the axon membrane; secondly, by crossing the Schwann cell membrane; and thirdly, by travelling out in the channels between or through Schwann cells (Holtzman, Freeman & Kashner, 1970). The experiments of Nevis (1958)

indicated a permeability coefficient of 10^{-4} cm/sec for the axon membrane (Villegas & Villegas, 1960). We can assume the same permeability for the Schwann cell. Frankenhaeuser & Hodgkin (1956) calculated a potassium permeability for an unspecified outer layer of 6×10^{-5} cm/sec. If we accept that potassium leaves via water-filled spaces between Schwann cells, and allow for the difference in diffusion constants between water and potassium, then the water permeability of this third path would also be about

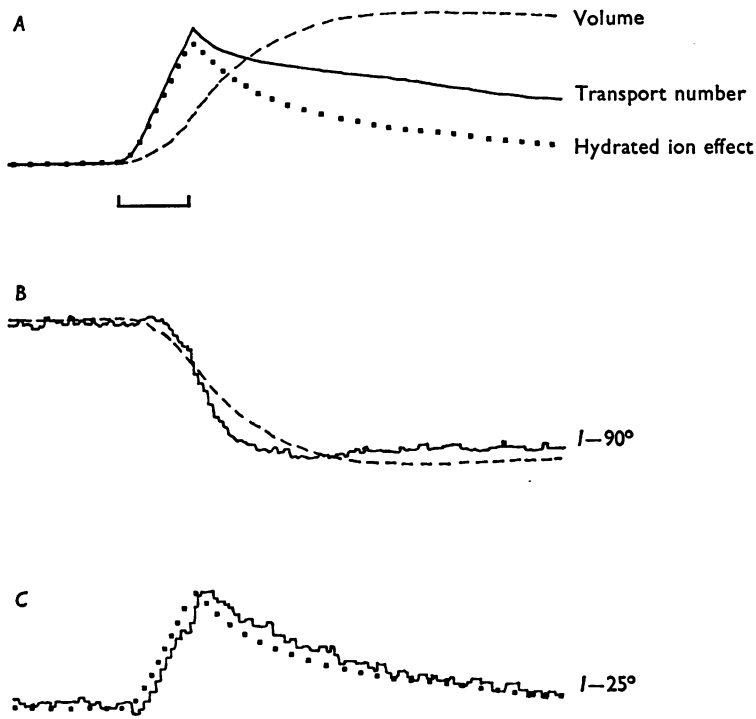

Fig. 12*A*. Predicted time course for a light-scattering change resulting from hydrated ion effects (dotted line), from a transport-number effect (continuous line) or from a volume change in the periaxonal space that would result from a transport number effect (dashed line). *B*, Comparison of the time course of *I*–90° and the predicted time course for a volume change. *C*, Comparison of the time course of *I*–25° and the predicted time course for a hydrated ion effect. See text for further details. The horizontal bar indicates the time of a 100 mV depolarizing potential step which was 4 msec in duration.

10^{-4} cm/sec. Thus there are three parallel paths with a total water permeability of about 3×10^{-4} cm/sec. Since the time constant for diffusion from the space is inversely proportional to the permeability coefficient (Frankenhaeuser & Hodgkin, 1956) we estimate that the time constant for

water diffusion was about 10 msec. The predicted time course for a swelling due to the water of hydration is indicated as the dotted line in Fig. 12*A*.

There is a second consequence of current being carried across the membrane exclusively by cations. For every 100 potassium ions crossing the membrane into the periaxonal space, approximately forty sodium ions would leave the space and sixty chloride ions would enter it, and thus sixty molecules of salt would be added to the space when 100 potassium ions crossed the membrane. We can term such a change in salt concentration a transport-number effect. For an outward potassium current the extra salt would be potassium chloride, for an outward sodium current it would be sodium chloride and for an inward sodium current the sodium chloride would be depleted (ignoring divalent ions). A transport-number effect would occur with a time course identical to the time integral of the current, and the concentration in the periaxonal space would presumably return to its original level with a time constant similar to the Frankenhaeuser–Hodgkin time constant (40 msec). The time course of the change in salt concentration in the periaxonal space is shown as the solid line in Fig. 12*A*.

This curve is also the time course for the occurrence of an osmotic gradient, and water will flow into or out of the space, tending to neutralize that gradient. Again assuming that water moved into or out of the space with a time constant of 10 msec, we calculated the time course for swelling or shrinkage of the space, and this is shown as the dashed line in Fig. 12*A*.

These calculated curves indicate that one way of distinguishing between water movements caused by hydrated ions and transport-number effects would be by comparison of time courses. But the two effects can also be distinguished by a further experiment. Since the transport-number effect depends on an anion carrying part of the current in the artificial sea water (but not across the membrane), the size of a transport-number effect can be changed by varying the mobility of the anion. In our experiments, we compared an artificial sea water containing chloride as the anion with one containing isethionate and glutamate. Using the list of ion mobilities provided by MacInnes (1939), we calculate that the chloride carried 60·5 % of the current in chloride artificial sea water and that the anion carried 38·5 % of the current in isethionate sea water. To make this calculation, isethionate and glutamate were estimated to have mobilities of 0·42 times that of chloride. Conductivity measurements on chloride and isethionate artificial sea waters showed that this estimate was reasonable. Thus it was predicted that a transport-number effect would be reduced by 36 % when chloride artificial sea water was replaced by isethionate artificial sea water. To a first approximation, the change in anions would not alter the size of a water of hydration effect.

Armed with the predicted effects of anion substitution and the pre-

dicted time courses (Fig. 12*A*) we examined each of the current-dependent changes in an attempt to identify their origins.

I–90°. This scattering change was reduced in size by 29 ± 5% when isethionate artificial sea water was substituted for chloride artificial sea water (Fig. 8). This was close to the predicted reduction for a transport-number effect, and we can conclude that *I*–90° resulted from such an effect. The time course of *I*–90° was then compared with the predicted time course for a volume change in the periaxonal space induced by a transport-number effect. In Fig. 12*B*, the predicted time course (smooth curve) has been inverted and adjusted in size to match the observed light-scattering change (noisy trace). It did not seem unreasonable that a volume *increase* could result in a scattering *decrease*, for this relationship between volume and scattering holds for suspensions of erythrocytes and mitochondria (Orskov, 1935; Tedeschi & Harris, 1955). The predicted and experimental time courses were reasonably close, confirming that *I*–90° could well have resulted from volume changes in the periaxonal space.

Since the resting isethionate permeability is low (Brinley & Mullins, 1965), short-term changes in external anions should have very little effect on the internal anion composition. Therefore, the reduction of *I*–90° in isethionate sea water made it reasonable to ignore the transport-number effect occurring on the internal surface of the axon membrane and supported the suggestion that *I*–90° occurred in the periaxonal space.

I–25°. The change from chloride to isethionate artificial sea waters did not affect the size of *I*–25°. One might suppose that this would rule out the possibility of a transport-number effect in the periaxonal space. If, however, *I*–25° arose from changes in refractive index in the space resulting from changes in salt concentration, then the anion substitution might not affect the size of *I*–25°. Even though the changes in salt concentration would be smaller, one mole of isethionate or glutamate would increase the refractive index of the solution more than does one mole of chloride. An estimate of the contributions of the anion to refractive index was obtained by examining the data of de Garcia (1920) for the refractive indices of solutions of sodium salts of several organic anions (not, however, including isethionate or glutamate) and comparing his values with the refractive index of sodium chloride. This supported the possibility that a reduced concentration change in isethionate artificial sea water might just be counterbalanced by the increased refractive index of the larger anions, so that the refractive index change in the space could remain the same in the two artificial sea waters. Thus a transport-number effect cannot be ruled out, even though isethionate replacement did not cause a reduction in the size of *I*–25°. Fig. 12*C* shows a comparison of the predicted time course for the water of hydration effect and *I*–25°. Here, the two had similar time courses, but as was indicated in the results, in other instances *I*–25°

returned to the base line more slowly and then had a time course similar to that predicted for a transport-number effect. Thus our data on the time course of I–25° were also inadequate to allow a decision to be made about the origin of this change.

I–10°. The anion replacement experiment was not done for I–10°. Its time course was not similar to any of the predicted time courses, and at this time, little can be said about its origin.

In spite of the fact that identification of the origins of I–25° and I–10° was not possible, we were pleased to be able to make at least a tentative identification for I–90°. This is the first optical change that has been identified structurally and mechanistically, and we hope that the same can be done for other changes.

Identification of the changes occurring during action potentials

Forward angles. From its time course, we suspected that the forward angle light scattering change seen during the action potential (Fig. 4, Cohen *et al.* 1972) was potential dependent. The following calculation indicates that the potential-dependent component found during voltage-clamp steps at forward angles might be large enough to account for the change during the spike. A 100 mV *hyperpolarizing* voltage–clamp step gave a scattering increase which averaged $+19{\cdot}3 \times 10^{-6}$. Using the curve in Fig. 13*B* (Cohen *et al.* 1972) to predict the corresponding change for a 100 mV *depolarizing* step, we obtain a value of -8×10^{-6}, essentially equal to the peak decrease of $-7{\cdot}5 \times 10^{-6}$ measured during the action potential. So the potential component was large enough. A further calculation indicates that current-dependent components would be too small to make an appreciable change during the action potential. The current through the membrane during a propagated impulse is proportional to the second derivative of the potential, thus the time integral of the current varies as the first derivative of the potential. Using the data of Hodgkin & Huxley (1952*b*), Fig. 18, the maximum value of the integral of the current is approximately 5 n-coul. of charge in a 4 mm length of axon. The data in Table 3 indicate a forward-angle scattering change of about 3×10^{-7} from such a current. This change was only one tenth the change measured during the spike, so that any current-dependent change would have been much smaller than the change resulting from the potential. The results of voltage-clamp experiments were thus in agreement with the notion that the forward-angle change occurring during the action potential was mainly potential dependent.

Right-angle changes. The current-dependent changes at 90° were 10 times larger than at 30°; therefore, we expected current-dependent changes to be more prominent in the right-angle action potential experiments.

We presume that the scattering increase that occurred long after the action potential (Fig. 7, Cohen *et al.* 1972) was current dependent because the only long-lasting changes we found during voltage-clamp experiments depended on current. The increase following the action potential was in the same direction as I–90° for an inward current. Its peak size, 5×10^{-6}, corresponded to a net inward cation flux during the action potential of 7 nC/4 mm length or 0·8 p-mole/cm^2. Hill (1950) measured an increase in the radius in the *Sepia* giant axon following tetanic stimulation and concluded that the increase could be accounted for by the transfer of 1·7 p-mole/cm^2-impulse of sodium chloride, the rest of the sodium ions during the action potential being exchanged for potassium ions. This much chloride movement is more than adequate to account for the long-lasting light scattering changes which accompany the action potential. Furthermore, our current records from voltage–clamp experiments in chloride and isethionate sea water suggested that 15% of the outward current might be carried by chloride moving inward. If the same was true during the action potential there would be a net chloride influx of 0·8 p-mole/cm^2-impulse.

Keynes & Lewis (1951) reported a net chloride influx of $0{\cdot}55 \pm 0{\cdot}15$ p-mole/cm^2-impulse which is also the appropriate magnitude to account for the effect we have described. However Caldwell & Keynes (1960) found only $0{\cdot}046 \pm 0{\cdot}015$ p-mole/cm^2-impulse. In both flux measurements it was necessary to stimulate at high repetition rates for a long period of time. Such stimulation leads to a substantial reduction in the long-lasting light-scattering increase (Fig. 8, Cohen *et al.* 1972). This, combined with the difference in stimulation frequency and temperature between our experiments and the ion flux measurements makes it difficult to compare these results quantitatively and we can only say that all four measurements suggest there is a small influx of chloride ions during the nerve impulse.

The right-angle change that occurred during the spike itself may have both potential- and current-dependent components. A 100 mV hyperpolarizing step gave rise to a scattering change of $-8{\cdot}7 \times 10^{-6}$. Again, using Fig. 13*A* in Cohen *et al.* (1972) to estimate the size of the potential component during a depolarizing step, we obtained a value of $+2{\cdot}2 \times 10^{-6}$. This may be compared to the value of $+1{\cdot}3 \times 10^{-6}$ for the peak change during the spike. The evaluation of current-dependent components is difficult because the main change, I–90°, occurred too slowly to contribute to the scattering change during the spike, and only the small change mentioned in fine print on p. 741 would contribute at this time. From the values given on p. 741, a inward flux of 5 nC would contribute a scattering change of $-0{\cdot}2 \times 10^{-6}$, so that the potential- and current-dependent components would subtract, as was necessary to explain the negative slope in Fig. 8 of Cohen *et al.* (1972). The voltage–clamp experiments are thus in reasonable

agreement with the results from action potential measurements. This agreement further reduces the possibility that the voltage-clamp results were due to electrode artifacts.

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