J. Physiol. (1971), **218**, *pp.* 205–237
With 17 *text-figures*
Printed in Great Britain

ANALYSIS OF THE POTENTIAL-DEPENDENT CHANGES IN OPTICAL RETARDATION IN THE SQUID GIANT AXON

By L. B. COHEN,* B. HILLE,† R. D. KEYNES, D. LANDOWNE‡ and E. ROJAS§

From the Department of Physiology, Yale University School of Medicine, New Haven, Connecticut 06510, U.S.A.; the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge; the Laboratory of the Marine Biological Association, Plymouth; and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543, U.S.A.

(*Received 1 June 1971*)

SUMMARY

1. An analysis has been made of the change in optical retardation of the membrane elicited by the application of voltage-clamp pulses in squid giant axons.

2. The retardation response consists of three separate voltage-dependent components. For freshly mounted axons, defined as being in state 1, hyperpolarizing pulses give a rapid increase in the light intensity measured with crossed polarizers which has been termed the fast phase. This is followed by a rather slow return towards the base line termed the rebound. On treatment of the axon with certain agents that include tetrodotoxin, high calcium and terbium, the rebound disappears and the fast phase slows down, increases in size, and has a new slow component added to it. This transition from state 1 to a second state, 2, appears to be irreversible.

3. In state 1, the time constant of the fast phase is 20–40 μsec at 13° C; it has a very large negative temperature coefficient ($Q_{10} = Ca.\frac{1}{3}$). The size of the retardation change is independent of temperature and

* National Science Foundation post-doctoral fellow, 1966–68.

† Helen Hay Whitney post-doctoral fellow, 1967–68. Present address: Department of Physiology and Biophysics, University of Washington School of Medicine, Seattle, Washington 98105, U.S.A.

‡ Present address: Department of Zoology and Comparative Physiology, Queen Mary College, London E.1.

§ Overseas Fellow of Churchill College, 1969–70. Present address: Faculty of Sciences, University of Chile, Santiago, Chile.

varies as the square of the applied voltage, but the voltage–retardation curve is symmetrical about a point well beyond zero membrane potential, at an internal potential of around +70 mV. In state 2, the time constant is about five times larger, and varies much less markedly with temperature; the apex of the voltage–retardation curve is shifted to +200 mV.

4. The rebound has a time constant of the order of 20 msec at 13° C. A 10° rise in temperature more than halves the time constant and roughly doubles the amplitude of the rebound. The voltage dependence of the rebound differed from that of the fast phase.

5. The slow component of state 2 has a time constant of about 2 msec which does not change noticeably between 10 and 25° C. The size of this component seems to be linearly dependent on the applied voltage, rather than obeying a square law.

6. A tenfold increase in external calcium concentration had no discernible effect on the fast and slow phases, but reversibly reduced the amplitude of the rebound nearly to half.

7. In experiments on perfused axons, the retardation response was not measurably altered by any of the modifications made to the composition of the perfusing fluid.

8. There was some indication of the possible existence of a small current- or conductance-dependent component of the retardation response.

9. These phenomena seem likely to originate either from molecular relaxation processes analogous with the Kerr effect, or from changes in membrane thickness under the influence of the pressure exerted by the electric field. However, the specific molecules involved in the retardation response cannot yet be identified.

INTRODUCTION

We have discussed in previous papers (Cohen, Hille & Keynes, 1969, 1970) the evidence that electrical activity in non-myelinated nerve fibres and the electric organ is accompanied by a small change in the optical retardation of the membrane. The time courses of the changes in retardation and in membrane potential were closely similar, which suggested that the optical effect was primarily a potential-dependent phenomenon; but there might also have been smaller current-dependent or even conductance-linked components. From experiments in which only the effects of action potentials were observed, it would have been rather difficult to sort out the possible contributions of these different components. We therefore made a number of measurements of the retardation change produced by steps in membrane potential of pre-determined size and duration, applied under voltage–clamp conditions to the squid giant axon. This enabled us to

examine in some detail such features of the optical response as its time constant and voltage dependence, and the effects on them of varying the ambient temperature and the composition of the surrounding medium. We report here the results of our analysis of the characteristics of the changes in optical retardation of the membrane in squid axons.

METHODS

Most of the experiments were performed with giant axons 500–1000 μm in diameter dissected from the hindmost stellar nerve in refrigerated mantles from *Loligo forbesi* (Caldwell, Hodgkin, Keynes & Shaw, 1960). In some cases axons from *L. vulgaris* or *L. pealii* were used, and occasionally living squid were available in the laboratory. The results were not obviously affected either by species differences or by the length of time for which the mantle had been stored before dissection.

The optical equipment and the chamber in which intact axons were mounted have already been described by Cohen *et al.* (1969, 1970). In most of the experiments done at Woods Hole a simplified apparatus was used in which axons were mounted vertically in the chamber designed for light scattering measurements (Cohen, Keynes & Landowne, in preparation), and sheets of Polaroid with their planes of polarization at 45° and 135° to the vertical were inserted into the chamber on either side of the axon. Signal averaging was done with a TMC Computer of Average Transients (CAT 400C), a Princeton Waveform Eductor (type TDH-9), or a Biomac 1000.

Axons were internally perfused both by the 'garden roller' technique of Baker, Hodgkin & Shaw (1962) and by the suction technique of Tasaki (1963) and Rojas & Ehrenstein (1965). In order to apply the latter method, a perfusion chamber and three micromanipulators were mounted on a platform in such a way that after the pipettes and electrodes had been manoeuvred into position, the whole assembly could then be transferred from the dissecting stand to the stage of the polarizing microscope. The perfusion fluid contained 570 mM potassium fluoride and 35 mM potassium phosphate buffer pH 7·4 (Tasaki, Singer & Takenaka, 1965), or 550 mM potassium fluoride and 5 mM Tris buffer at various pH's between 6·6 and 7·6.

Two voltage–clamp systems were employed. One was very similar in design to that of Hodgkin, Huxley & Katz (1952), using operational amplifiers for voltage and current amplification. The internal electrode was a double spiral like that of Hodgkin *et al.* (1952), wound with 20 μm platinum wire instead of silver, and platinized according to the recipe given by Moore & Cole (1963). The exposed lengths were 2 mm for the voltage wire and 10 mm for the current wire; the other parts of the spirals were insulated with shellac or epoxy resin. The total membrane current was recorded as the voltage across a 10 ohm resistor connected between a large platinized bath electrode and ground. Compensation for the voltage drop across the Schwann cell series resistance was provided for in essentially the manner described by Hodgkin *et al.* (1952), the potentiometer being set just below the point at which oscillation occurred.

For much of the work the slightly different system devised by Moore & Cole (1963) and used by Rojas & Ehrenstein (1965) and others was employed, again incorporating Philbrick operational amplifiers. The internal electrode was once more a platinized double spiral, but in this case the external platinum electrode was divided into three sections, of which the outer two were grounded while the central section was used for current measurement. Again, electrical compensation was applied, the average value of the resistance across which the voltage drop was cancelled out being 9·2 $\Omega.cm^2$. In seven experiments on perfused axons in which the Schwann cell series

resistance was measured directly by Armstrong's (1969) current clamp method, the mean value obtained was $7{\cdot}8 \pm 0{\cdot}8\ \Omega.\text{cm}^2$.

The axons were bathed either in filtered sea water, or in an artificial sea water (a.s.w.) whose composition was: 460 mM-NaCl, 10 mM-KCl, 11 mM-$CaCl_2$, 55 mM-$MgCl_2$ and either 2·5 mM-$NaHCO_3$ or 5 mM-Tris chloride buffer, pH 7·4. In experiments on the effect of external calcium concentration the low-calcium solution contained: 394 mM-NaCl, 10 mM-KCl, 5·5 mM-$CaCl_2$ and 162 mM-Tris chloride buffer. The high-calcium solution contained: 394 mM-NaCl, 10 mM-KCl, 110 mM-$CaCl_2$ and 5 mM-Tris buffer.

The temperature of the optical recording chamber was controlled by circulating water from a refrigerated water-bath through it. Unless otherwise stated, all the experiments were performed at $13 \pm 1°$ C. It was advantageous to work at as low a temperature as possible, both in order to slow down the optical responses and to preserve the axons in good physiological condition; but below 10° C, fogging of the chamber windows gave trouble.

RESULTS

Changes in retardation on hyper- and depolarization

As may be seen in Fig. 1*C* and in Fig. 5 of Cohen, Keynes & Hille (1968), a rectangular voltage–clamp pulse gave an approximately rectangular retardation response when the membrane was hyperpolarized, but for potential changes in the other direction the optical response was rather far from being flat-topped. The simplest explanation for this behaviour would be that the voltage–clamp pulse was not corrected for the potential drop arising from the radial flow of current through the Schwann cell series resistance. The application of electrical compensation usually improved the appearance of the responses obtained with depolarizing pulses, as in the case shown in Fig. 1*D*, but sometimes even the introduction of the maximum amount of compensation that was compatible with the stability of the feed-back circuit had an incomplete squaring effect, especially for fresh axons exhibiting an extra large inward sodium current. Owing to the difficulty of adjusting the compensation to account for exactly 100% of the series resistance (see Hodgkin *et al.* 1952), it was uncertain whether the residual irregularities arose from imperfect compensation or should be partly attributed to the occurrence of a small retardation change dependent on current or conductance. As discussed on p. 230, there were other reasons for suspecting that the potential-dependent response could not always account for the whole of the observed retardation change; but further studies are needed before any definite conclusion can be reached on the size and characteristics of the possible current-dependent retardation response. Doubt on this point did not seriously affect experiments in which hyperpolarizing pulses were applied, nor those with short depolarizing pulses, which were the conditions under which all the results considered in this paper were obtained. It is clear that the retardation response is primarily voltage-dependent.

The components of the retardation response

The different states of the response. The four records traced in Fig. 2 show the characteristic sequence of changes undergone by the retardation response elicited by a long 50 mV hyperpolarizing pulse. When the axon was first mounted in the chamber for observation under the microscope,

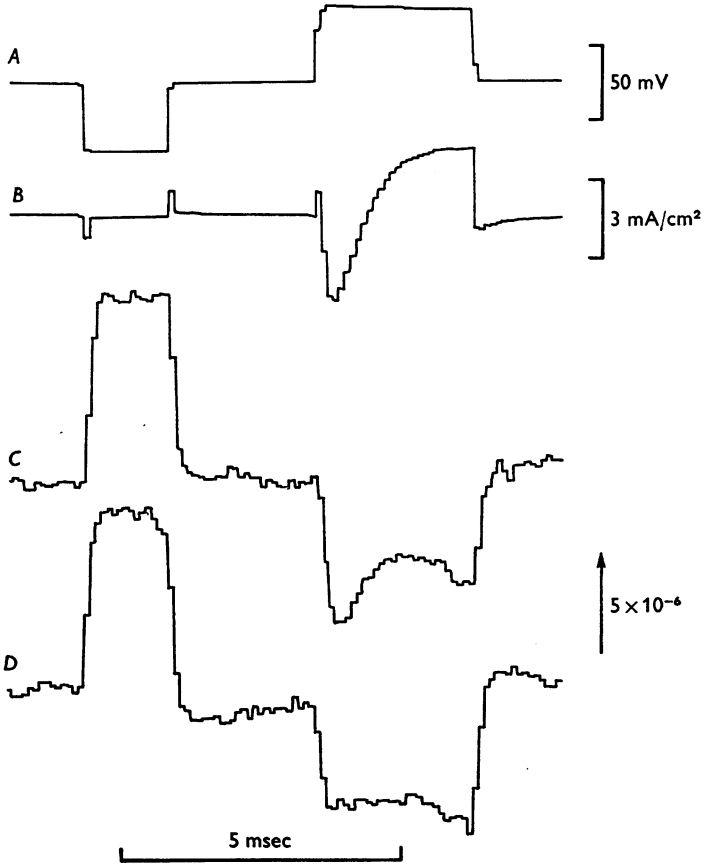

Fig. 1. Optical and voltage–clamp tracings for a freshly dissected axon, recorded with the Princeton Waveform Eductor set at a sweep duration of 10 msec. *A*: applied voltage pulses, 48 mV 1·5 msec hyperpolarizing followed by 52 mV 3·0 msec depolarizing. *B*: corresponding records of membrane current, no electrical compensation for series resistance. *C*: retardation response with no electrical compensation *D*: retardation response with greatest permissible electrical compensation. For *C* and *D* 1000 sweeps were averaged. In this and subsequent Figures the direction of the arrows to the right of the tracings indicates an increase in light intensity, and their length corresponds to the stated value of $\Delta I/I_r$ for a single pulse. Axon from *L. forbesi*, diameter 900 μm. Temperature 13° C. Optical recording time constant 68 μsec.

hyperpolarization of the membrane by 50 mV gave a rapid increase in light intensity such that the value of $\Delta I/I_r$ (the change in intensity for a single pulse divided by the resting intensity) was of the order of 1 part in 10^5; for nineteen axons the mean value was $1{\cdot}29 \pm 0{\cdot}14 \times 10^{-5}$. This initial increase in intensity, which will be referred to as the fast phase, had a time constant of around 30 μsec at 13° C; as will be seen later (p. 224) the time

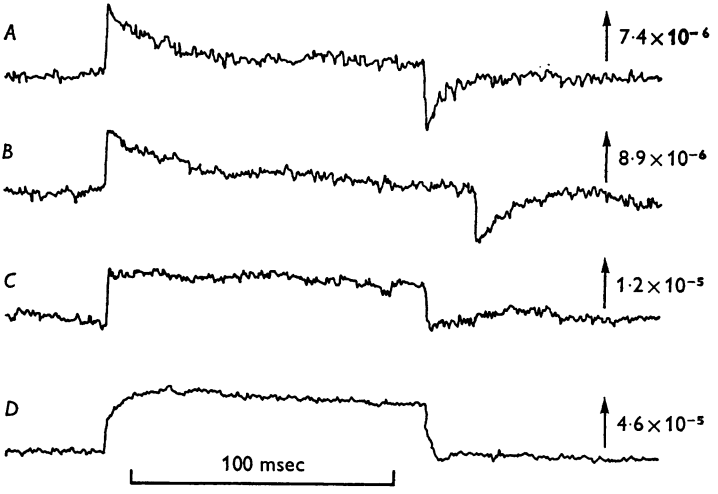

Fig. 2. The change in appearance of the retardation response during an experiment. All four records were made in normal a.s.w. with the same CAT sweep duration (250 msec) and amplifier gain. Amplitude of voltage-clamp pulse was 50 mV, hyperpolarizing; duration was 120 msec in *A*, *C* and *D*, 140 msec in *B*. *A*: soon after mounting the axon in the chamber; 259 sweeps averaged, $\Delta I/I_r = 1{\cdot}0 \times 10^{-5}$ impulse^{-1} at initial peak, rebound = 82%. *B*: 80 min later, after making some observations in 110 mM-Ca a.s.w. (see Fig. 16); 305 sweeps averaged, $\Delta I/I_r = 1{\cdot}1 \times 10^{-5}$ impulse^{-1} at initial peak, rebound 87%. *C*: 266 min after recording *A* the rebound has disappeared: 338 sweeps averaged, $\Delta I/I_r = 1{\cdot}2 \times 10^{-5}$ impulse^{-1}. *D*: 540 min after recording *A* the slow phase has appeared; 140 sweeps averaged, $\Delta I/I_r = 5{\cdot}5 \times 10^{-5}$ impulse^{-1}. Axon from *L. forbesi*, diameter 900 μm. Temperature 13–14° C. Optical recording time constant 130 μsec.

constant was much less at higher temperatures. Although the hyperpolarizing potential was then held constant at 50 mV above the resting potential, the increase in intensity was not maintained, but fell back with a time constant of about 25 msec to a level corresponding to only one fifth of the initial peak deflexion. This behaviour, which was typical of a fresh axon, may for convenience be described as a 'rebound'; but the use of this term is purely descriptive, and is not to be taken to imply that any particular mechanism is involved. As will become apparent when the properties

of the different components of the retardation response have been compared, the origin of the rebound appears to be quite distinct from that of the fast phase, and it should really be regarded as a separate change in retardation whose sign is the reverse of that of the fast (and slow) phases.

Examination of Fig. 2*C* will show that after some while the rebound was lost, leaving a still fast-rising but now flat-topped retardation response. The rebound was indeed a somewhat labile phenomenon, and experiments designed to explore quantitatively such features as its voltage dependence were apt to be spoiled by its abrupt disappearance before the measurements were complete. At about the same time, the retardation response became both much slower and appreciably larger; Fig. 2*D* shows how it looked several hours later when the transformation was complete. Axons giving responses of the type shown in Fig. 2*A* will from now on be described as being in state 1, while the condition illustrated in Fig. 2*D* will be termed state 2. In seven axons in state 2 the mean value of $\Delta I/I_r$ was $5{\cdot}4 \pm 0{\cdot}8 \times 10^{-5}$ for 50 mV hyperpolarization, so that on average the transition resulted in a fourfold increase in the total size of the retardation change.

Methods of inducing development of state 2. The first occasions on which the transition to state 2 were observed were, as described by Cohen *et al.* (1968), after treatment of the axons with tetrodotoxin (TTX) and saxitoxin (STX). It was clear from the outset that the change in retardation response was not directly related to the blocking of sodium conductance by TTX, since the sodium current always fell to zero within a minute or two when the TTX was applied, whereas the optical effect was often not apparent until a much longer period had elapsed. Conversely, the sodium conductance could quickly be restored by washing the TTX away, but the retardation response never reverted to state 1. There now seems no doubt that TTX is merely one of several agents that can hasten the transition from state 1 to state 2. With an irreversible change of this kind, which is somewhat variable in its readiness to take place, and may occur spontaneously, it is difficult to make a positive identification of all the effective triggering factors. However, we convinced ourselves that included among them were (1) repeated application of large voltage–clamp pulses, especially in the depolarizing direction, (2) raising the temperature for any length of time to 25° C or thereabouts, (3) TTX and STX, (4) high-calcium solutions, and most quickly and surely of all, (5) solutions containing 1 mM terbium (Tb^{3+}) or lanthanum (La^{3+}). A factor which did not seem to be of primary importance in this connexion was the passage of time. On a number of occasions, the second axon from a squid whose first axon had long since gone into state 2 was still found to be in state 1 when in its turn it was taken from the refrigerator and mounted in the optical chamber. There

was also some evidence that a release of ionized calcium inside the axon was not a triggering factor, since treatment of axons for several hours with either 2 mM cyanide or sodium-free lithium a.s.w., both of which should have brought about an appreciable rise in the internal [Ca^{2+}] (Blaustein & Hodgkin, 1969), left them still in state 1, but they soon went into state 2 when the external [Ca^{2+}] was raised. The importance of calcium in the external solution was also underlined by an experiment in which an axon was soaked in calcium-free a.s.w. containing 110 mM-magnesium and 0·5 mM-EGTA for 12 hr without any slowing of the retardation response, but immediately displayed the transition to state 2 when put back into normal a.s.w. Another agent which was ineffective when applied internally was TTX. An axon that was injected by the method of Hodgkin & Keynes (1956) with enough TTX to give an internal concentration of about 1 mM was still in state 1 almost 4 hr later, and in confirmation of Narahashi, Anderson & Moore (1967) the sodium conductance was unimpaired. Yet within 2 min of applying 300 nM-TTX outside, the sodium conductance had fallen to zero, and soon afterwards, as may be seen in Fig. 4, the transition to state 2 began.

As has already been mentioned, the transition to state 2 appeared to be irreversible. Attempts to restore the state 1 response were made on a number of occasions, both by altering the external pH in either direction, and by applying calcium-free solutions, but they were uniformly unsuccessful.

Analysis of the state 2 response. An important point in considering the state 2 retardation response was to know whether it simply represented a change in the speed and size of the state 1 response, or rather the development of an additional source of retardation. In order to examine this question, responses for an axon in state 2 recorded with adequate time resolution were replotted logarithmically as shown in Fig. 3. It was clear that both for the rising and for the falling phase of the response, the points could be perfectly fitted by the sum of two exponentials. Extrapolation of the slower phase to zero time gave a satisfactorily consistent estimate of its size, and indicated that both components generally had slightly larger (by up to 50 %) time constants during the falling phase than during the rising one. After a series of records had been analysed in this fashion, the relative sizes of the slow and fast components of the retardation response could be plotted against time, as in Fig. 4. The analysis showed that, in an experiment in which TTX was applied at zero time, the slow component did not become measurable until half an hour had elapsed. It then increased over the next 2 hr to about twice the size of the fast component, and finally began to decline. At the same time, the fast component also showed a definite increase in size before it too declined. In this particular case, no suitable records were made for comparison of the time constant

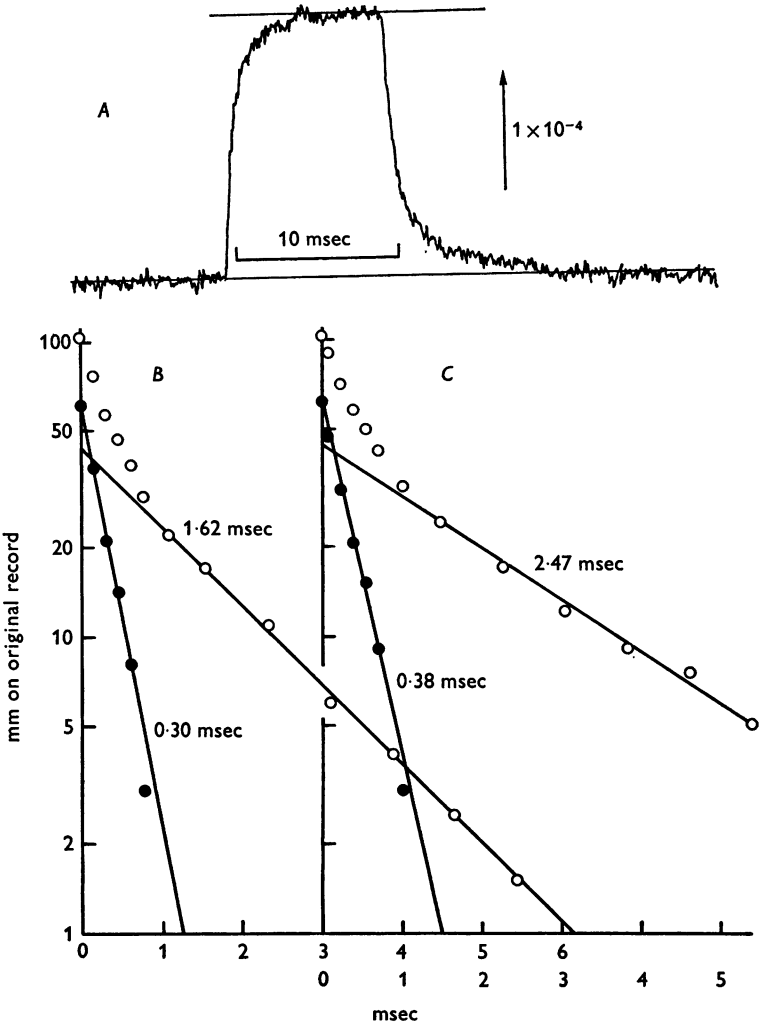

Fig. 3. Analysis of the time course of the state 2 retardation response for a large hyperpolarizing pulse. *A*: optical record of the retardation change in response to a 300 mV hyperpolarizing pulse lasting for 10 msec, applied to a Tb-treated axon; thirty-two sweeps averaged; $\Delta I/I_r = 12{\cdot}8 \times 10^{-5}$ impulse^{-1} for fast phase, $9{\cdot}2 \times 10^{-5}$ impulse^{-1} for slow phase. *B*: analysis of rising phase. ○, distance from plateau, measured in mm on original record, plotted on logarithmic scale against time. ●, fast phase after subtraction of values for extrapolated slow phase. *C*: analysis of falling phase. ○, distance from base line measured as before. ●, fast phase separated. The figures marked against each of the straight lines are time constants in msec. Axon from *L. pealii*, diameter 490 μm. Temperature 13° C.

of the fast phase of the retardation response before and after the transition to state 2; but in other experiments, such as that illustrated in Fig. 5, there was always found to be a slowing down of the fast phase by a factor of five or even more. There was thus no doubt that the development of the state 2 response involved both an appreciable modification of the fast phase of the state 1 response, and the addition of a second component which was always much slower and usually larger.

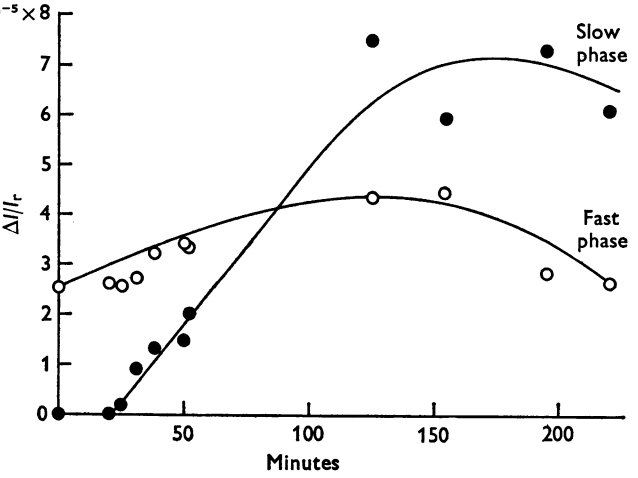

Fig. 4. The time course of development of the state 2 response in an axon from *L. forbesi* treated with 300 nM-TTX at zero time. Voltage-clamp pulses were 100 mV, hyperpolarizing. For the first five records the pulse and sweep durations were respectively 70 and 125 msec; then they were reduced to 15 and 31·25 msec. ○, $\Delta I/I_r$ for initial fast phase of retardation response; its time constant could not be measured in the early records, but in the later ones it averaged 0·25 msec. ●, $\Delta I/I_r$ for the subsequent slow phase of the response, whose time constant was 5·0 msec. Temperature 14° C.

A last feature of the way in which the state 2 response developed is illustrated in Fig. 5. In this and other experiments, the fast phase of the response slowed down from a time constant of 40–50 μsec to a value in the region of 300 μsec as soon as the transition began, but after this the time constant altered little. The time constant of the slow component, on the other hand, was definitely larger at the beginning of the series, when it was just under 10 msec, than at the end, when it had fallen to about 2 msec. The speeding-up was most marked for large hyperpolarizations (see Fig. 3); and the time constants for depolarization were always the larger, especially for the return to the base line. Because of this behaviour, the earliest sign of the impending transition was often the appearance of a seemingly linear upward creep superimposed on the response to a hyperpolarizing pulse. Usually the rebound had already vanished before this stage was reached, but some records were obtained whose time course suggested that all three components were present simultaneously and were being added together.

The nature of the optical signal

Experiments with optical compensation. The most severe test to verify that the optical signal under discussion represents a pure change in retardation consists in examining the effect of adding or subtracting a constant small retardation with some form of optical compensator. The retardation

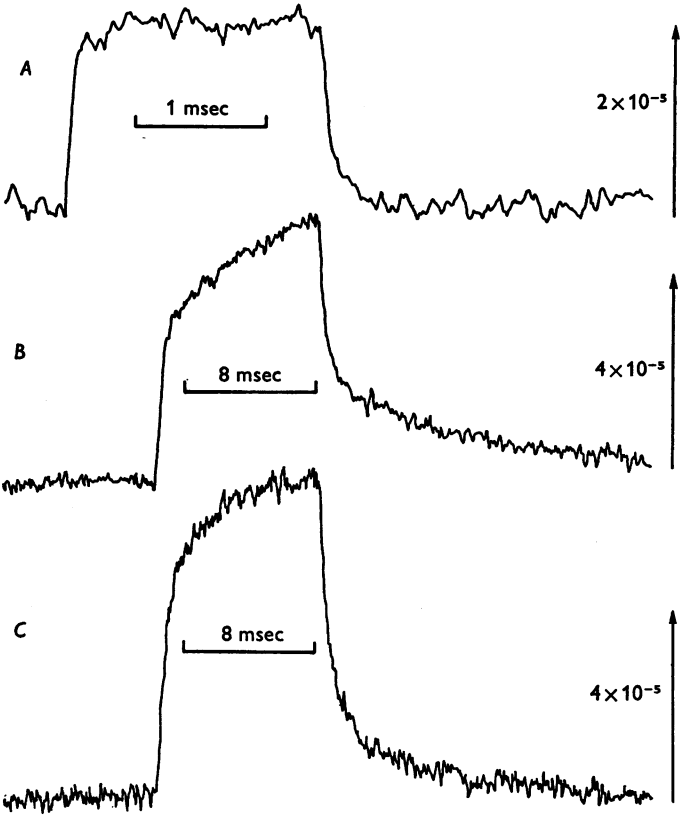

Fig. 5. Another example of the change in appearance of the retardation response on development of state 2. *A*: in normal a.s.w., 150 mV hyperpolarizing pulses, 1024 sweeps averaged, sweep duration 5 msec, $\Delta I/I_r = 1{\cdot}9 \times 10^{-5}$ impulse^{-1}, time constant 60 μsec up, 80 μsec down. *B*: after treatment with 1 mM-Tb for 10 min, 100 mV hyperpolarizing pulses, 256 sweeps averaged, sweep duration 40 msec, $\Delta I/I_r = 3{\cdot}1 \times 10^{-5}$ impulse^{-1} for fast phase, $3{\cdot}2 \times 10^{-5}$ impulse^{-1} for slow phase, time constant fast phase 300 μsec up, 380 μsec down, time constant slow phase 8·8 msec up, 7·6 msec down. *C*: 30 min later, still in Tb a.s.w., 100 mV hyperpolarizing pulses, 256 sweeps averaged, sweep duration 40 msec, $\Delta I/I_r = 3{\cdot}2 \times 10^{-5}$ impulse^{-1} for fast phase, $3{\cdot}4 \times 10^{-5}$ impulse^{-1} for slow phase, time constant fast phase 325 μsec up, 435 μsec down, time constant slow phase 2·8 msec up, 2·4 msec down. The next record taken in this series was that shown in Fig. 3. Axon from *L. pealii*, diameter 490 μm.

changes in a stimulated crab nerve have been investigated in this fashion by Cohen *et al.* (1970), who also showed that the signal observed during the spike in a squid axon could be more or less exactly inverted by over-compensation. This axon was in state 1, and it seemed desirable to make sure that in state 2 the conclusion that there was no serious interference from other optical effects was still valid. In the experiment shown in Fig. 6,

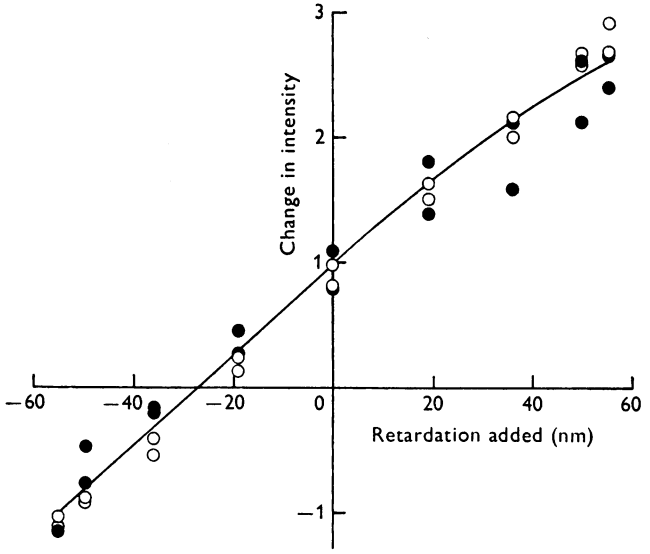

Fig. 6. The effect of adding or subtracting retardation by means of a Brace–Köhler compensator which gave a maximum retardation of 56·2 nm. Ordinate: change in intensity (ΔI) in arbitrary units. Abscissa: extra retardation in nm. The line is the value calculated from eqn. (6) of Cohen *et al.* (1970) for a constant small retardation change in an object whose resting retardation was 27 nm (in their Fig. 6*B* the corresponding line was drawn with insufficient curvature for added retardations). ○, compensator settings 5–85° and 185–265°; ●, 95–175° and 275–345°. Axon from *L. forbesi* which had been injected with 1 M-TEA and was in 110 mM-calcium a.s.w. With no retardation added the mean response was $8{\cdot}3 \times 10^{-4}$ for each 100 mV hyperpolarizing pulse. Pulse duration was 90 msec, optical response time constant was 2–5 msec. 9–150 sweeps averaged. Axon diameter 750 μm.

the resting retardation was reduced to a minimum by cutting down the width of the optical field so that the signal was derived only from the extreme edge of the axon, and a $\lambda/10$ Brace-Köhler rotating mica plate compensator could then provide the necessary range of added retardations. The axon had been treated with 110 mM-calcium a.s.w. and was giving a large and slow state 2 retardation response. The change in intensity (ΔI)

has been plotted against added retardation, calculated as $\sin 2(\psi - 45°) \times 56{\cdot}15$ nm, where ψ was the compensator setting. The points fit well with the theoretical relationship for an unadulterated retardation change, since as shown by Cohen *et al.* (1970) ΔI should be directly proportional to $\sin\theta$, where $\theta = 2\pi R/\lambda$ and R is the total resting retardation.

Interference from light scattering changes. On a few occasions, especially when attempts were made to apply long depolarizing pulses, records were obtained in which there did seem to be unwanted signals arising from light-scattering effects. However, attempts to modify the optical recording conditions so as to decide exactly how much contribution was being made by light scattering were only rarely successful. One method of measuring the 0° light-scattering changes possibly superimposed on the retardation response was to remove the polarizing prism but otherwise to leave the system undisturbed, apart from reducing the light intensity with a neutral filter in order not to saturate the photodiode. Probably because under these conditions the scattering angle was poorly defined, the light scattering response was apparently variable in direction; but except for long depolarizations, when the results were in any case rejected, the signals were always an order of magnitude smaller than the retardation response. It therefore seemed unlikely that the retardation response was ever seriously distorted by light scattering.

Lack of variation of the response with light wave-length. For a pure retardation change, the ratio $\Delta I/I_r$ would be independent of wave-length, as it turns out to be for light scattering (Cohen & Keynes, 1971). In order to check whether the spectra of the changing and fixed components of the axon retardation were indeed the same, we repeated the observations on the variation in the retardation response with wave-length mentioned in our earlier paper (Cohen *et al.* 1970). When interference filters were used to select different wave-bands, the reduction in light intensity resulted in an appreciable deterioration of the signal-to-noise ratio, but by applying 100 mV hyperpolarizing pulses to axons in state 2, reasonable records were obtained. It may be seen in Fig. 7, where the results of three experiments are plotted, that between 400 and 700 nm $\Delta I/I_r$ did not change significantly.

The voltage dependence of the retardation changes

Fast phase, state 1. When the value of $\Delta I/I_r$ during a rather brief voltage-clamp pulse applied to a fresh axon was plotted against the size of the potential as in Fig. 8, the points fitted well with a square law whose origin in this case was at 130 mV depolarization. Similar results were obtained for several other axons in state 1, the average amount of depolarization corresponding to the minimum of the curve being 130 mV (four experiments), and the value of ΔI relative to that for 50 mV hyperpolarization being 1·25 at the minimum and in the opposite direction. However, it was not easy to determine the position of the minimum in this way, particularly if one wished to follow the curve into the region where depolarization gave an intensity increase, because large potentials tended to damage the membrane and to cause an irreversible reduction in signal strength. Another way of locating the minimum was to fit a square law to the mean values of $\Delta I/I_r$ for 50 and 100 mV hyperpolarization. In six experiments where

duplicate determinations were made, the ratio $\Delta I_{100}/\Delta I_{50}$ was $2{\cdot}32 \pm 0{\cdot}05$. If the minimum corresponds to a depolarization V_0 and to an intensity change $-\Delta I_{V_0}$ then we can write

$$\Delta I_{V_0}/\Delta I_{50} = \alpha V_0^2$$

$$1 + \Delta I_{V_0}/\Delta I_{50} = \alpha(V_0 + 50)^2$$

$$2{\cdot}32 + \Delta I_{V_0}/\Delta I_{50} = \alpha(V_0 + 100)^2.$$

Eliminating the proportionality constant α, it follows that $V_0 = 131$ mV and $\Delta I_{V_0}/\Delta I_{50} = 1{\cdot}10$, in good agreement with the figures given above.

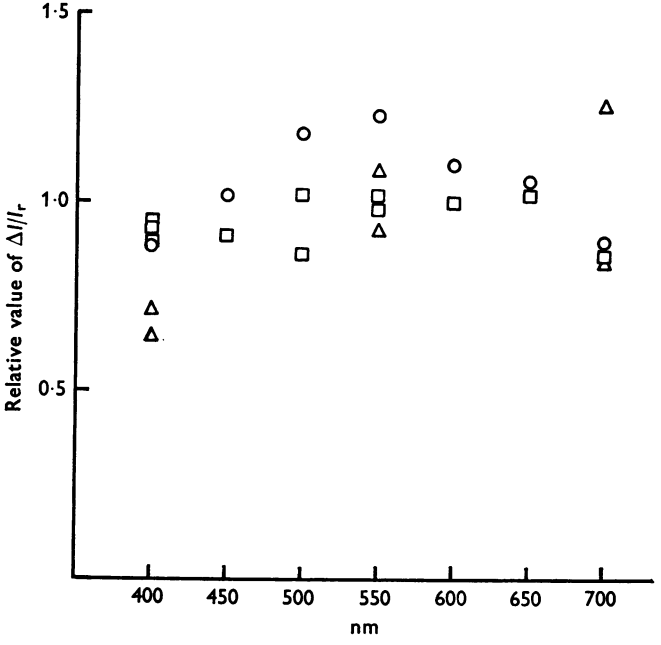

Fig. 7. Variation in the size of the retardation response when Balzers Type K wide-band interference filters were inserted in the light path. These measurements were all made on axons from *L. forbesi* immersed in 110 mM calcium a.s.w., after the transition to state 2 was complete. The voltage-clamp pulses were 100 mV, hyperpolarizing, duration 25 or 70 msec. ○, experiment of 15. xi. 69, values expressed relative to response of $2{\cdot}4 \pm 0{\cdot}7 \times 10^{-5}$ (S.D. of a single observation) per impulse in white light; axon had been injected with 1 M-TEA at start of experiment. Δ, experiment of 19. xi. 69, values expressed relative to mean response at 550 nm (absolute size not recorded). □, experiment of 20. xi. 69, values expressed relative to response of $5{\cdot}7 \pm 0{\cdot}14 \times 10^{-5}$ at 550 nm. In white light or at long wave-lengths about 250 sweeps were averaged; at short wave-lengths the decrease in sensitivity of the photodiode and of the light intensity made it necessary to average up to 3000 sweeps.

Rebound, state 1. It was of some interest to know whether the amplitude of the rebound for axons in state 1 also obeyed this square law relation. However, experimental difficulties prevented us from obtaining a proper answer to this question. One problem was the extreme lability of the rebound, which was apt to disappear as soon as the effect of at all large voltage–clamp pulses was examined. Another was that the rebound could not be measured satisfactorily when the membrane was depolarized, because interference apparently arising from outward current-dependent low-angle light scattering (Cohen *et al.* in preparation) became serious during voltage–clamp pulses of the necessary duration. Nevertheless, there seemed no doubt that the

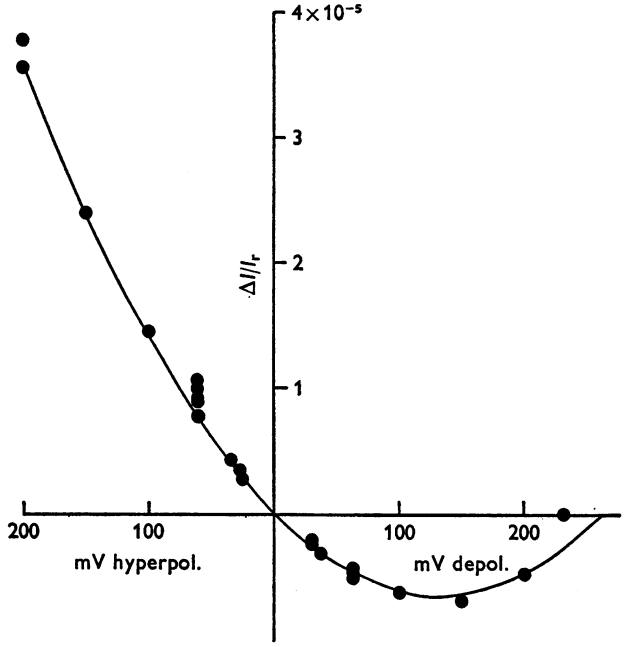

Fig. 8. The relationship between membrane potential and the retardation response for an axon from *L. forbesi* in state 1. Ordinate: value of $\Delta I/I_r$ for a single voltage–clamp pulse. Abscissa: size of applied potential measured relative to the resting potential, which was about 60 mV, inside negative. Duration of voltage–clamp pulses was 0·3 msec. 1000–2500 sweeps averaged. Axon diameter 900 μm. Temperature 12·5° C. The line was calculated as

$$\{3{\cdot}85 \times 10^{-5}\ (\text{hyperpolarization} + 130\ \text{mV})^2 - 0{\cdot}65\} \times 10^{-5}.$$

rebound did not have the same voltage-dependence as the fast phase of the retardation response. In Fig. 9 the responses for 50, 100 and 150 mV hyperpolarization have been traced, with a final record made at 50 mV to check that the rebound was still present after application of the 150 mV pulses. It will be noted that the rebound, expressed as a percentage of the initial deflexion, was smaller at 100 mV than at 50 mV, and that at 150 mV it had vanished. In six experiments in which measurements at 50 mV bracketed those at 100 mV, the ratio $\text{Rebound}_{100}/\text{Rebound}_{50}$ averaged $0{\cdot}68 \pm 0{\cdot}04$. Since in these same experiments the ratio of the initial deflexions

was 2·32 (see above), the absolute amplitude of the rebound was about 50 % greater at 100 mV than at 50 mV. Observations with 150 mV hyperpolarization gave less consistent results. In two cases the ratio $\text{Rebound}_{150}/\text{Rebound}_{50}$ was zero (Fig. 9) or rather small (0·17, expt. of 29. xi. 69); but in the experiment illustrated in Fig. 16 it was close to unity (0·97), although in the same axon R_{100}/R_{50} was 0·79. While, therefore, there does seem to be a definite change with potential in the relative sizes of the initial fast phase and the rebound, which reinforces the other evidence that they originate at different sites in the membrane, we cannot describe quantitatively the voltage-dependence of the rebound.

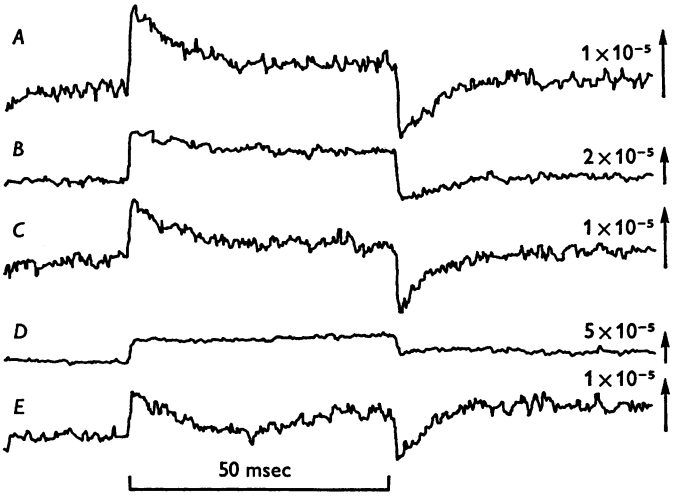

Fig. 9. Variation in the rebound for an axon in state 1 with different amounts of hyperpolarization. Duration of voltage-clamp pulse was 50 msec; sweep duration was 125 msec. *A*: 50 mV hyperpolarizing pulse, 597 sweeps averaged, $\Delta I/I_r = 1{\cdot}2 \times 10^{-5}$ impulse^{-1} for initial peak, amplitude of rebound = 72 % of initial peak. *B*: 100 mV hyperpolarizing pulse, 184 sweeps averaged, $\Delta I/I_r = 2{\cdot}4 \times 10^{-5}$ impulse^{-1}, rebound = 42 %. *C*: 50 mV again, 566 sweeps averaged, $\Delta I/I_r = 1{\cdot}0 \times 10^{-5}$ impulse^{-1}, rebound = 73 %. *D*: 150 mV hyperpolarizing pulse, 59 sweeps averaged, $\Delta I/I_r = 3{\cdot}5 \times 10^{-5}$ impulse^{-1}, no rebound. *E*: final control with 50 mV, 535 sweeps averaged, $\Delta I/I_r = 0{\cdot}8 \times 10^{-5}$ impulse^{-1}, rebound still about 80 %. Axon from *L. forbesi*, diameter 875 μm. Temperature 14° C.

Axon in state 2. In axons that had been treated with TTX or one of the other agents which facilitated the transition from state 1 to state 2, the relationship between retardation response and membrane potential was somewhat altered. As Fig. 10 shows, the curve no longer had a well defined minimum, and even with the largest depolarizations that could be applied $\Delta I/I_r$ was still slowly increasing. In this particular instance, a square law with its origin at 250 mV fitted with the results reasonably well, but in other cases it was impossible with any choice of constants to obtain a satisfactory fit. The response plotted in Fig. 10 was actually the sum of the

fast and slow phases, and it seemed worth enquiring whether if measured separately they would display the same voltage dependence. Records with the necessary good time resolution were made using two Tb-treated axons, and the results of analysing one set are shown in Fig. 11. It turned out that whereas the size of the slow component varied with potential in a roughly linear fashion, the fast component varied more nearly as (potential)^2, with a minimum at about 250 mV depolarization. The other experi-

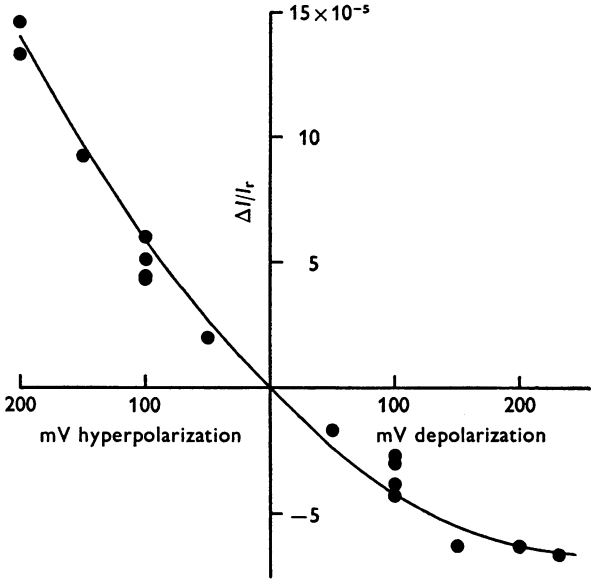

Fig. 10. Voltage–retardation curve for the axon used for Fig. 8 after transition to state 2 induced by treatment with 150 nM-TTX. Ordinate and abscissa as in Fig. 8. Duration of voltage-clamp pulses increased to 2 msec. 50–4000 sweeps averaged. The line was calculated as

$$\{10{\cdot}4 \times 10^{-5}\ (\text{hyperpolarization} + 250\ \text{mV})^2 - 6{\cdot}5\} \times 10^{-5}.$$

ment of this kind was marred by an appreciable drift with time of the relative sizes of the two components, but showed the same general tendencies. It would appear, then, that the membrane potential affects the slow and fast components in distinctly different ways, and that the transition to state 2 involves a shift to the right of the voltage–retardation curve for the fast component as well as the increase in size and time constant that have already been discussed (see p. 214).

The effect of ambient temperature on the retardation response

Fast phase, state 1. Although the size of the initial fast phase of the optical response in a fresh axon did not vary much with temperature, its

time constant had an extremely large negative temperature coefficient. As may be seen in Fig. 12*B*, at 12° C the retardation response had a time constant of about 45 μsec, of which 5·5 μsec could be attributed to the recording system. At 20° C the response time constant, corrected for the instrumental lag, was only 14·5 μsec. These figures assume that the rise time of the potential step applied to the membrane was zero, which in practice it could not have been. A reasonable estimate of the actual rise time with uncompensated feed-back could be obtained by integrating the

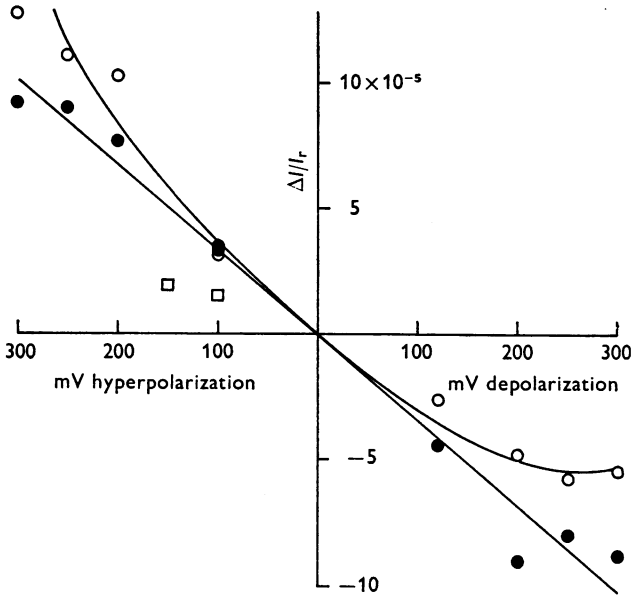

Fig. 11. The relationship between membrane potential and the sizes of the fast and slow components of the retardation change for an axon in state 2. Records were analysed as shown in Fig. 3; other examples of retardation responses from the same experiment can be seen in Fig. 5. □, preliminary observations in normal a.s.w. ○, fast phase, and ●, slow phase, after treatment of the axon with 1 mM-Tb. Axon from *L. pealii*, diameter 490 μm.

capacity transient in the voltage–clamp current records; it appeared to have a time constant of about 8 μsec, which is what would be expected for a membrane capacity of 1 μF/cm^2 and a Schwann cell series resistance of 8 Ω.cm^2 (Hodgkin *et al.* 1952). Allowing for this, the optical response time constants at 12 and 20° C were respectively 32 and 6·5 μsec, corresponding to a Q_{10} of 1/7·3. At the higher temperature, the accuracy with which the true time constant of the retardation response could be measured was obviously suspect. Superimposition of the integrated current record on the optical record, as in Fig. 12*A*, showed that the retardation response tailed

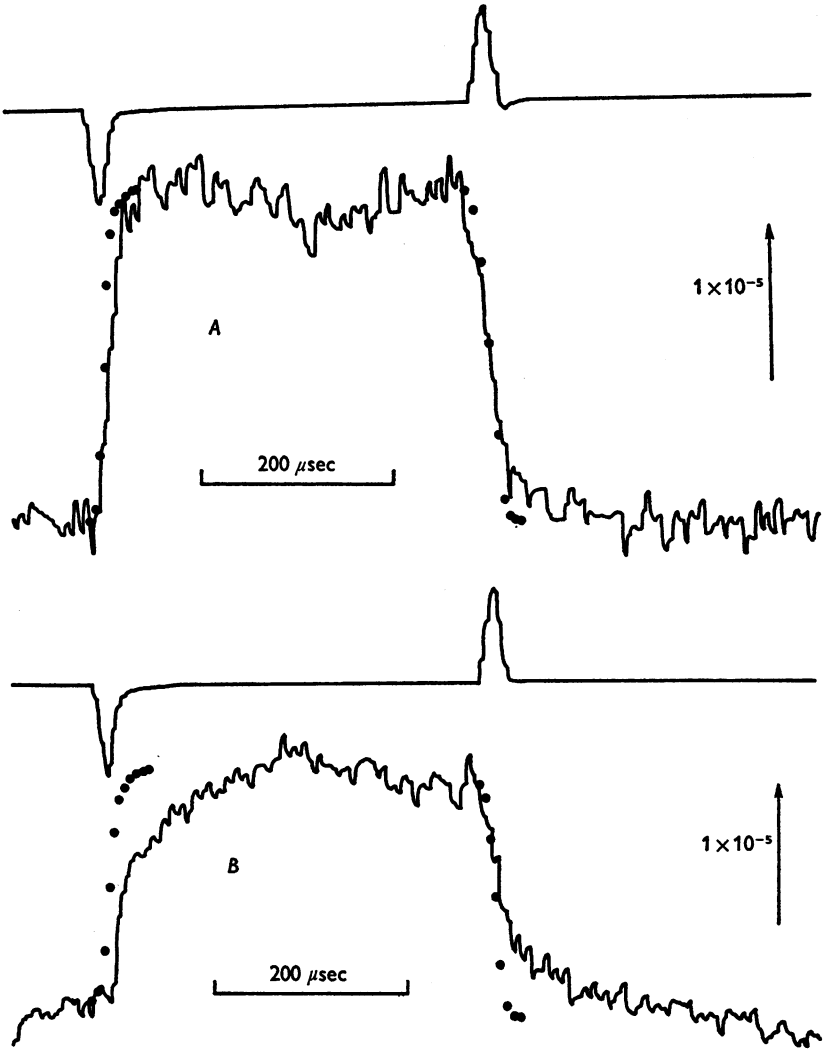

Fig. 12. The effect of ambient temperature on the initial fast phase of the retardation response. Voltage–clamp pulses were 130 mV, hyperpolarizing; their duration was 0·4 msec. Above each optical record the voltage–clamp current record has been traced; no electrical compensation was employed. The filled-in circles show the integrated current suitably scaled. *A*: at 20° C, 16384 sweeps averaged, $\Delta I/I_r = 2{\cdot}1 \times 10^{-5}$ impulse^{-1}. Time constants were 18·4 μsec (up), 19·0 μsec (down), 4·2 μsec (recording system). *B*: at 12° C, 17,600 sweeps averaged, $\Delta I/I_r = 1{\cdot}6 \times 10^{-5}$ impulse^{-1}. Time constants were 47·5 μsec (up), 42·5 μsec (down), 5·5 μsec (recording system). All records were written out on an expanded scale so that the signals in the individual memory addresses of the Biomac computer could be distinguished. The address time was 5 μsec. Axon from *L. pealii*, diameter 525 μm.

the voltage rise by only about one computer address (5 μsec), and clearly the instrumental limit had been reached as far as time resolution was concerned. In another experiment where the average response time constant calculated without a correction for the voltage rise time was 37 μsec at 14·5° C, warming up to 25° C reduced the uncorrected time constant to 8·3 μsec which was equal (within experimental error) to the voltage rise time.

In order to make a more reliable estimate of the temperature coefficient of the response time constant, several measurements were made on the same axon at various temperatures between 5 and 14° C. As shown in

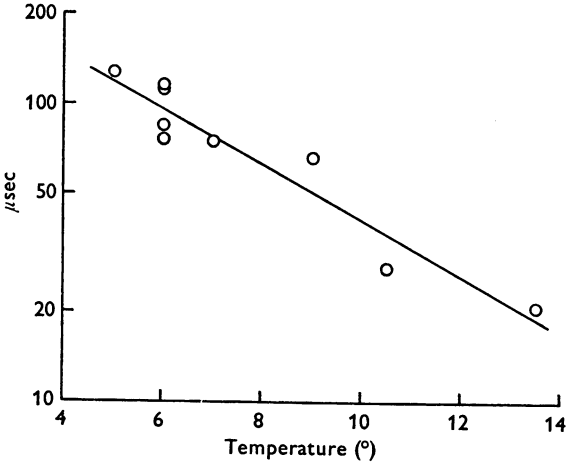

Fig. 13. The temperature dependence of the time constant of the initial fast phase of the retardation response. Voltage-clamp pulses were 120 mV hyperpolarizing, duration 400 μsec. Each value plotted is the mean for the on and off of the pulse, corrected for the instrumental time constant and the rise time of the voltage step. $\Delta I/I_r$ did not vary significantly with temperature, and averaged $2{\cdot}1 \times 10^{-5}$ impulse^{-1}. The slope of the line corresponds to a Q_{10} of 1/8·5. 4096–16,384 sweeps averaged. Axon from *L. pealii*, diameter 625 μm.

Fig. 13, the corrected time constant ranged from 20 to 140 μsec, with a Q_{10} estimated as 1/8·5. On this occasion, the value of $\Delta I/I_r$ remained roughly constant throughout, as it also did when all the results obtained in the experiment of Fig. 12 were averaged to eliminate drift with time. However, two other axons showed a consistent reduction of $\Delta I/I_r$ by nearly half when the temperature was raised from 11 to 22° C. It may be significant that in these two cases, but not in Figs. 12 and 13, the resting retardation appeared to be nearly 50% greater at the higher temperature, this being the effect reported by Forman (1966).

Rebound, state 1. The tracings in Fig. 14 show the effect on the rebound

of raising the temperature by 11° C. Not only was the time constant reduced to about half, but the amplitude was increased roughly threefold, so that by the end of the pulse the intensity had actually overshot the original base line. In this experiment, the average value of the time constant in three records made at a mean temperature of 13·5° was 19·0 msec,

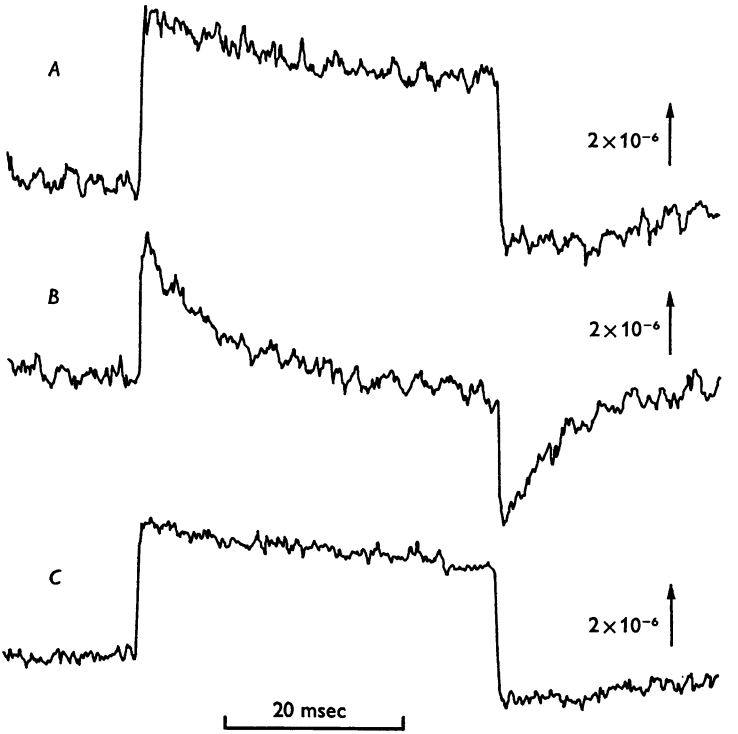

Fig. 14. The effect of ambient temperature on the rebound of the retardation response in an axon from *L. pealii* in state 1. Voltage–clamp pulses were 55 mV, hyperpolarizing; pulse duration was 40 msec, sweep duration 80 msec. 2048 sweeps averaged in each case. *A*: at 13° C, $\Delta I/I_r = 5{\cdot}3 \times 10^{-6}$ impulse^{-1}, amplitude of rebound = 36%, time constant = 15 msec. *B*: at 24° C, $\Delta I/I_r = 4{\cdot}3 \times 10^{-6}$ impulse^{-1}; amplitude of rebound = 105%, time constant = 9·4 msec. *C*: at 13·5° C again, $\Delta I/I_r = 4{\cdot}2 \times 10^{-6}$ impulse^{-1}; amplitude of rebound = 29%, time constant = 26 msec. Axon diameter 525 μm.

while in two runs at 24·5° it was 7·8 msec. The Q_{10} was therefore 1/2·3, while for the size of rebound it was 2·7. The effect of temperature on the rebound was satisfactorily reproducible, for in two further experiments in which measurements at 21·5° were bracketed at 11·5° the Q_{10}'s were: time constant 1/2·4 and 1/3·2, amplitude of rebound 2·2 and 1·5. Thus the rebound was speeded up appreciably less than the fast phase of the retardation response, but its size was much more temperature-sensitive.

Axon in state 2. Two experiments were performed to examine the effect of temperature on TTX-treated axons which were in state 2 and were exhibiting typically large and slow biphasic retardation responses. In the first one the time constant of the fast phase measured at 13, 24 and 13° again was 119, 105 and 109 μsec; for the slow phase the figures were 10·2, 14·4 and 14·9 msec. On the second occasion the measurements were made at 13·5, 26 and 14°, giving for the fast phase 42, 21 and 40 μsec, and for the slow phase 0·64, 0·60 and 0·60 msec. The Q_{10}'s were therefore 1/1·1 and 1/1·7 for the fast phase, and 1·1 and 1·0 for the slow phase. Neither component varied significantly in size on warming up.

The effect of calcium

Since a twenty-fold change in external calcium concentration is known to shift the curves relating ionic conductance to membrane potential by 18–27 mV (Frankenhaeuser & Hodgkin, 1957), possibly by altering the electric field in the outer region of the membrane, it seemed that an investigation of the effect of changing $[Ca^{2+}]_0$ might throw some light on the location of any molecular rearrangement underlying the retardation response. However, several experiments like that illustrated in Fig. 15 gave uniformly negative results. As may be seen, raising the calcium from 5·5 to 110 mM had no significant effect on the normalized retardation-voltage relationship. Another way of treating the results was to calculate the ratio ($\Delta I/I_r$ in 5·5 mM calcium)/($\Delta I/I_r$ in 110 mM calcium) for the four pulse sizes where the measurements in 110 mM calcium a.s.w. were bracketed in 5·5 mM calcium a.s.w. Its mean value was $0{\cdot}96 \pm 0{\cdot}035$, which is not significantly different from unity. This particular axon was in state 1, but the conclusion was the same for others that were in state 2. The records for axons in state 1 were not made at a high enough sweep speed for the time constants to be measured satisfactorily, but for those in state 2 the half-times were certainly the same at both calcium concentrations. While it would therefore appear that the fast and slow phases of the retardation response were not affected by calcium, it must be appreciated that if in fact the sole consequence of a change in $[Ca^{2+}]$ was a shift of the retardation–voltage curve sideways without a change in its shape, such a displacement would have been detected in our measurements only if it had been greater than about 40 mV.

The rebound was distinctly more sensitive to calcium. As is shown in Fig. 16, its amplitude was appreciably reduced by raising $[Ca^{2+}]_0$. In three experiments in which the retardation response in 110 mM calcium a.s.w. was satisfactorily bracketed in 11 mM calcium – an essential precaution since application of high calcium was one of the ways of promoting the transition to state 2 – the mean value of the ratio $\text{Rebound}_{110}/\text{Rebound}_{11}$

was $0{\cdot}64 \pm 0{\cdot}04$, while once again the size of the initial fast phase remained constant, as did the half-time of the rebound. The same effect was observed qualitatively on several other occasions. This result suggests that the conformational change responsible for the rebound may take place in a part of the membrane that is readily accessible to external calcium.

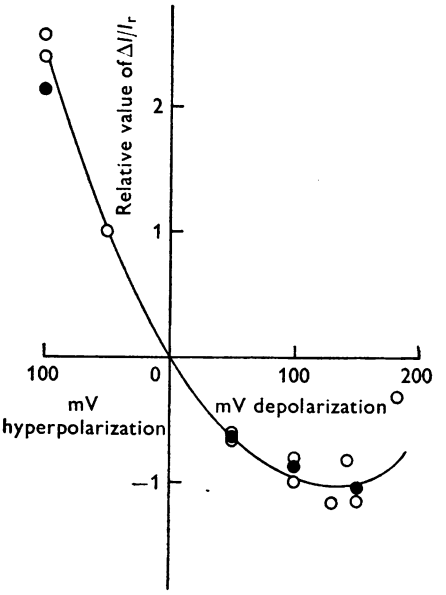

Fig. 15. The lack of effect of calcium on the fast phase of the retardation response of an axon from *L. forbesi* in state 1. For each point, the value of $\Delta I/I_r$ is expressed relative to that for a 50 mV hyperpolarizing pulse applied during the same sweep. ●, in 110 mM calcium a.s.w. ○, in 5·5 mM calcium a.s.w. before and after making the measurements in high calcium. Mean values of $\Delta I/I_r$ for 50 mV hyperpolarization were 1·45, 1·48 and $1{\cdot}36 \times 10^{-5}$ impulse^{-1} in 5·5, 110 and 5·5 mM calcium respectively. 500–1000 sweeps averaged. Axon diameter 850 μm. Temperature 13·5–15° C.

Observations on perfused axons

The process of replacing the axoplasm with a fluoride perfusion medium should perhaps be added to the list of ways in which the transition from state 1 to state 2 can be facilitated. In five of the seven successful perfusions that were performed, the axon was initially in state 1, giving a rapid optical response to 50 mV hyperpolarization for which $\Delta I/I_r$ averaged $1{\cdot}4 \pm 0{\cdot}1 \times 10^{-5}$ impulse^{-1}, which was close to the mean value for intact axons (see p. 210). There was no obvious rebound in any of these experiments. However, within an hour the response had always slowed down and increased in size, so that, with the inclusion of the results for two axons which were in state 2 from the start, the average value of $\Delta I/I_r$ for 50 mV hyperpolarization became $5{\cdot}5 \pm 3{\cdot}4 \times 10^{-5}$ impulse^{-1}, again much as in the intact axons. But of course it is impossible to say whether the causative agent was the change in composition of

the internal medium or the mechanical trauma of coring out the axoplasm. The retardation responses were identical in appearance to those recorded in intact axons, except that interference possibly arising from current-dependent light scattering effects was observed during depolarization in four of the seven axons. On two occasions voltage–retardation curves were plotted; they did not differ noticeably from that shown in Fig. 10.

The results of changing the composition of the perfusing fluid were disappointing in that no clear-cut effects were found. The changes that were tested were as follows: (1) variation in pH between 6·4 and 7·6 (low pHs had an adverse effect on the

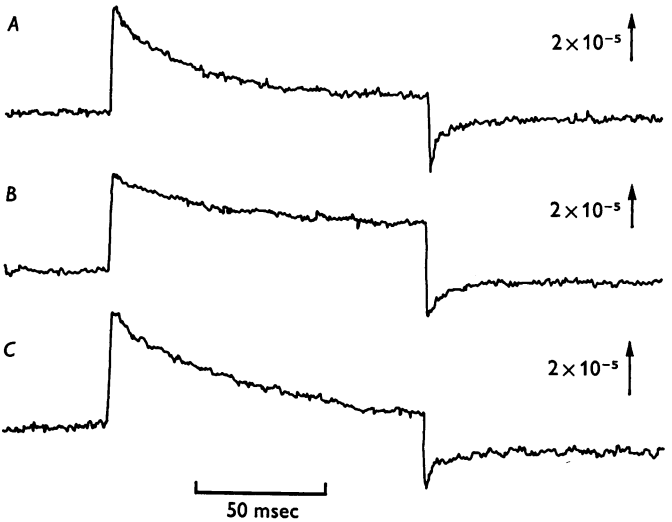

Fig. 16. The effect of external [Ca] on the rebound of the retardation response of an axon from *L. forbesi* in state 1. All three records were made with the same sweep duration (250 msec) and amplifier gain. Amplitude of voltage–clamp pulse was 150 mV, hyperpolarizing; duration was 120 msec. *A*: in normal a.s.w. with 11 mM calcium, 105 sweeps averaged, $\Delta I/I_r$ = $4{\cdot}4 \times 10^{-5}$ impulse^{-1} at initial peak, rebound = 81 %. *B*: in 110 mM calcium a.s.w., 105 sweeps averaged, $\Delta I/I_r = 4{\cdot}4 \times 10^{-5}$ impulse^{-1} at initial peak, rebound = 47 %. *C*: in normal a.s.w. again, 172 sweeps averaged, $\Delta I/I_r$ = $3{\cdot}7 \times 10^{-5}$ impulse^{-1} at initial peak, rebound = 87 %. The length of the arrow to the right of each record represents a change in intensity of 2×10^{-5} for a single impulse. Axon diameter 900 μm.

electrical properties of the membrane, but not on the optical ones); (2) addition of 2 mM-EGTA to reduce the ionized calcium level; (3) employment of Tris buffer instead of potassium phosphate; (4) substitution of chloride for the usual fluoride; (5) a low ionic strength medium that contained 55 mM potassium fluoride, 3 mM potassium phosphate and 940 mM-sucrose; (6) a hypertonic medium that contained 625 mM potassium fluoride and 3 mM potassium phosphate, pH 6·7. In no case was there any indication that the retardation response was affected by the change in the internal medium. It had been hoped that if there was a fixed surface charge effect of the type discussed by Chandler, Hodgkin & Meves (1965) when the internal ionic strength was changed, a retardation response originating at the inner face of the

membrane might prove to be sensitive to ionic strength. The voltage–retardation curve was not in fact noticeably shifted, but once more it may be that the displacement was too small to be detected by the relatively crude type of measurement that was made.

Reconstruction of the response during the action potential

As a further check on the assumption that the retardation response was primarily a voltage–dependent phenomenon, with little or no contribution from current- or conductance-dependent effects, the retardation response during a conducted action potential was computed from the state 1

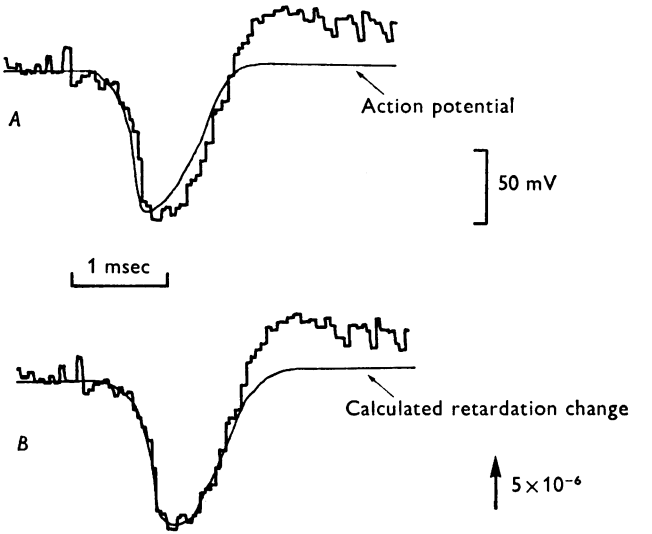

Fig. 17. Comparison of the retardation change recorded during a propagated spike (heavy lines, *A* and *B*) with the electrical potential change (thin line, *A*) and with the calculated retardation change (thin line, *B*). Temperature 14·5° C; 2000 sweeps averaged; optical recording time constant 24 μsec. Axon from *L. forbesi*, diameter 800 μm.

voltage–retardation curve (Fig. 8), the time constant of the fast phase in state 1, and an electrical recording of the spike made with an internal electrode. In Fig. 17*A* the observed retardation response has been superimposed on a suitably scaled and inverted record of the action potential; as noted by Cohen *et al.* (1970), the optical record was slightly delayed relative to the electrical one. In Fig. 17*B* the computed and experimental retardation responses are similarly presented. Both in amplitude and in time course there was an excellent match over the main part of the spike, but during the undershoot or positive phase the retardation change was appreciably larger in practice than in theory. Thus in four experiments the peak size of $\Delta I/I_r$ during the action potential, whose amplitude averaged 100 mV, was $99 \pm 10\%$ of its value during application of a 100

mV depolarizing voltage-clamp pulse, whereas during the 12 mV reversal of potential during the positive phase $\Delta I/I_r$ corresponded to a hyperpolarization of roughly 25 mV.

The reason for the discrepancy at the foot of the spike is not entirely clear. One possibility is that it might be due to interference from low-angle scattering changes. Attempts to make direct measurements of the contribution from light scattering for the optical conditions under which retardation was recorded were unsuccessful, but there are two arguments which suggest that it was probably rather small. One is the absence of a detectable change in light intensity at the centre of the axon (see Cohen *et al.* 1970, Fig. 10), though of course the light-scattering change might be distributed across the image of the axon in the same way as the retardation change. The other is the fact that the reversal of the intensity change during the positive phase was if anything slightly increased by overcompensation, as may be seen in Fig. 5 of Cohen *et al.* (1970). A light-scattering response would not have been reversed in direction by this procedure, so that normally it would have made the discrepancy less rather than more prominent. It therefore seems that the extra increase in intensity after the action potential must arise from a genuine retardation effect. Recalling the difficulties encountered in connexion with the shape of the optical response for a 50 mV depolarizing pulse (see p. 208), it seems possible that there is a small current- or conductance-dependent retardation response which shows up after the passage of ionic current through the membrane. However, nothing can yet be said about the mechanisms involved.

DISCUSSION

The first conclusion that emerges from these experiments is that we are concerned with more than one source of retardation change. Table 1 summarizes the properties of the fast and slow phases of the retardation response, and of the rebound. Since the three components have widely different time constants, and are quite differently affected by voltage and temperature, they seem rather unlikely to have a common origin. There may, however, be a fairly close connexion between the fast and slow phases, because the first appearance of the slow phase when the transition to state 2 takes place is always accompanied by a simultaneous modification of the characteristics of the fast phase. In addition to the retardation changes, there seem to be other voltage-dependent changes in the unstained membrane that can be detected optically, since the time constants of the light-scattering changes at large and small angles turn out to be different again when they are measured at the same time as those of the retardation response (Cohen *et al.* in preparation). It would also appear that

under some conditions a small conductance- or current-dependent retardation change can be recorded. Further voltage-dependent changes are seen in axons stained with fluorescent dyes (Cohen, Landowne, Shrivastav & Ritchie, 1970; Conti & Tasaki, 1970).

Two explanations for the retardation changes may be suggested. First, the attractive force between the ionic double layers on either side of the membrane will vary with the potential, thus compressing and decompressing the membrane, and so altering its thickness and hence its intrinsic and form birefringence. Secondly, the electric field may polarize and align anisotropic molecules in the membrane as in the Kerr effect, or electric

TABLE 1. Properties of the three components of the retardation response

	Voltage dependence	Time constant at 13° C	Q_{10} of size	Q_{10} of time constant	Effect of calcium
Fast phase	$\propto V^2$ Origin at +70 mV internal potential; shifts to +200 mV in state 2	40 μsec Increases 5× in state 2	1	1/8·5 in state 1 1/1·4 in state 2	Nil
Slow phase	$\propto V^1$	2 msec	1	1	Nil
Rebound	Different from fast phase, but exact relationship not yet determined	20 msec	2·1	1/2·6	High [Ca] reduces size

double refraction (for reviews of this subject see Le Fèvre & Le Fèvre, 1960 and Powers & Peticolas, 1967). Let us next consider the possible order of magnitude of the changes that might be produced by these effects.

The force exerted across a membrane of thickness t and capacity C by a potential difference V can be shown to be $-CV^2/2t$. If C is 1 μF/cm^2 (Hodgkin *et al.* 1952), or 9×10^5 e.s.u./cm^2, the thickness for a dielectric constant of 2·14 may be taken as 20 Å (Fettiplace, Andrews & Haydon, 1971, Table 7), or 2×10^{-7} cm. For a potential of 50 mV ($=\frac{50}{3} \times 10^{-5}$ e.s.u.) the force then works out as $6{\cdot}25 \times 10^4$ dyne/cm^2. No direct measurements of the compressibility of natural membranes have yet been reported, but Haydon (1970) has calculated that it is unlikely to be less than 5×10^{-9} cm^2/dyne. Measurements for artificial phospholipid bilayers are of limited relevance, because the presence of any solvent markedly increases their compressibility; the smallest value so far obtained experimentally by D.A. Haydon (personal communication, 26. i. 71) is 8×10^{-8} cm^2/dyne.

Using these two figures as upper and lower limits, the fractional increase in membrane thickness on abolition of a 50 mV potential would lie between 5×10^{-3} and 3×10^{-4}; Berestovsky, Frank, Liberman, Lunevsky & Razhin (1970) calculated the change in thickness during the action potential as 3×10^{-2} to 2×10^{-3} Å, which is in rough agreement. From the results discussed here and in our earlier paper (Cohen *et al.* 1970), the appropriate value of $\Delta I/I_r$ for purposes of comparison would be 1×10^{-5}, which on the same basis as before corresponds to a change in the product of birefringence and membrane thickness of 7×10^{-5} nm. If the product for the resting membrane is 0·085 nm (Cohen *et al.* 1970), the fractional change is 8×10^{-4}, which falls comfortably inside the range calculated above. Obviously no great reliance can be placed on these figures, but it seems reasonable to conclude that electrostriction could produce effects as large as the observed retardation change.

The relationship between induced birefringence and electric field strength established by Kerr can be written (see Le Fèvre & Le Fèvre, 1960) as

$$\frac{l(n_e - n_0)}{\lambda} = BlE^2$$

where l is the light path length, $(n_e - n_0)$ is the birefringence, λ is the wave-length, E is the field strength, and B is the Kerr constant. For 50 mV potential difference across a membrane of thickness 2×10^{-7} cm, the value of lE^2 in appropriate units is $(\frac{25}{9} \times 10^{-8}) \div (2 \times 10^{-7}) = 0{\cdot}14$. If the product of birefringence and membrane thickness is changed by 7×10^{-5} nm at a wave-length of 550 nm, the induced retardation expressed as a fraction of the wave-length is $1{\cdot}27 \times 10^{-7}$. The Kerr constant would therefore be $1{\cdot}27 \times 10^{-7} \div 0{\cdot}14 = 9 \times 10^{-7}$. As has been pointed out in our previous paper (Cohen *et al.* 1970) the increase in retardation referred to the longitudinal axis of the axon that is produced by a hyperpolarizing potential would correspond to a decrease in the intrinsic positive birefringence of the membrane, so that the Kerr constant would need to be negative. This raises no particular difficulty, because as may be seen in Table III of Le Fèvre & Le Fèvre (1960), certain non-polar liquids such as cyclohexanol do have a negative Kerr constant of just about this size. For molecules with polar side-chains such as proteins the Kerr constant may be several orders of magnitude larger (Tinoco, 1955). It would therefore be plausible to attribute the observed retardation change to a Kerr-type effect even if the molecules thought to be responsible were located at the surface of the membrane and had only a fraction of the electric field acting on them.

Consideration of the time constants of the components of the retardation response suggests that a simple reorientation of polarized side-groups will not suffice to explain our observations. Intramolecular relaxation times or

Kerr effect time constants in organic liquids are likely to be in the picosecond region (Smyth, 1966). The relaxation of bound water and of protein side-chains takes no more than a few nanoseconds, and the time constants for rotation of small proteins in solution are not much greater (Hendrickx, Verbruggen, Rosseneau-Motreff & Peters, 1969). Even a molecule as large as haemocyanin only has a birefringence relaxation time of 12 μsec for the monomer and 55 μsec for the dimer (Pytkowicz & O'Konski, 1959). It seems unlikely that the structural change does involve the rotation of a whole protein molecule, and the motion responsible for the retardation response must therefore take place in surroundings whose effective viscosity is relatively high. From the list given by Hammes (1968), conformational changes in proteins appear to provide the best precedents for time constants of the sort of size encountered in the retardation response.

In suggesting that the retardation response may arise because of conformational changes in the membrane, we do not wish to subscribe to the view of Tasaki, Barry & Carnay (1970) that the changes observed by optical techniques are the ones which underlie the conduction mechanism. It certainly seems probable that the alterations in the ionic permeability of excitable membranes that are responsible for electrical activity do involve large conformational changes at certain points in the membrane, but none of the experimental evidence supports the idea that these changes have yet been detected optically. One of the most disappointing features of our work, both on nerve and on the electric organ (Cohen *et al.* 1969) was the absence of any component that could easily be linked through similarity of time course or voltage dependence with the sodium or potassium conductance of the membrane. It is true that we have considered here the possibility that there is a small current-dependent component of the retardation response, but it might well be like the current-dependent light scattering response (Cohen *et al.*; in preparation) in arising because of changes in the ionic content of the space between the Schwann cell and the axon membrane, which would affect the form birefringence of the Schwann cell layer; it is thus connected only indirectly with membrane conductance. When we first observed the effect of TTX on the retardation response, we momentarily had high hopes that this discovery would reveal something useful about the nature of the sodium channels, but they were soon dashed when it became clear (see p. 211) that the highly selective blocking action of TTX on sodium conductance could be entirely dissociated from its slowing and magnifying action on the retardation response, which it appeared to share with various other agents. In view of recent evidence (Moore, Narahashi & Shaw, 1967; Keynes, Ritchie & Rojas, 1971) that in 1 μm^2 of nerve membrane there are only some fifty TTX-binding sodium sites, as compared with several million phospholipid and other

molecules, the prospects of picking out for investigation any conductance-dependent retardation changes seem rather poor, since they are more than likely to be altogether swamped by unspecific effects. It also seems fair to comment that if the conformational changes responsible for excitation are intimately connected with movements of calcium in the membrane, as proposed by Tasaki *et al.* (1970), they should surely be measurably affected by changing the external calcium concentration. In fact, the only component of the retardation response which seemed to be at all calcium sensitive was one which on several grounds cannot be supposed to be directly related to membrane conductance.

We do not feel, therefore, that there is a good prospect of obtaining by this particular type of optical study much direct information about the nature, at the molecular level, of the conformational changes involved in the activation and inactivation of the sodium and potassium channels. However, a proper identification of the molecules responsible for the retardation changes would clearly provide a valuable insight into the general problem of membrane structure and its interaction with the electric field. Although we have described a number of new findings that will ultimately require an explanation, none of them seems to provide any really specific clue as to what might be happening in the membrane. Among these findings, one of the most interesting is the displacement, well beyond zero potential difference between the bulk phases, of the origin of the square law relating the size of the fast component of the retardation response to membrane potential. Apparently the membrane possesses substantial asymmetries.

Although on grounds neither of order of size nor of relaxation time is it possible to identify any one of the components of the retardation response with the electrocompression rather than a Kerr-type effect, two of them seem unlikely to arise directly from a change in membrane thickness. If one were to select either the rebound or the slow phase of the response as candidates for the optical manifestation of a thickness increase, one would have to explain why the compressibility of the membrane should on the one hand drop to zero or on the other only assume a finite value when the transition from state 1 to state 2 takes place. It would seem more plausible to suppose that the fast phase is connected with membrane thickness, and that the transition involves a subtle change in membrane structure in the course of which the compressibility is slightly increased and the temperature dependence of the relaxation time is reduced. At the same time a fixed dipole is freed from previous constraints so that it can be reorientated in the electric field.

One line of attack on the molecular origin of the retardation response would be to see which, if any, of the phenomena that have been described

here can also be observed in artificial bilayers of known chemical composition. As mentioned in an earlier paper (Cohen *et al.* 1969), preliminary trials were undertaken using membranes of phosphatidylcholine in *n*-decane; but it soon became clear that the presence of the solvent would greatly complicate interpretation of the results. Further studies of this kind with solvent-free systems would be of considerable interest, although the failure of Berestovsky *et al.* (1970) to obtain any measurable retardation changes in brain phospholipid bilayers indicates that the results may be negative. Another line worth pursuing would be to look for changes in membrane capacity under conditions now known to affect the retardation response. If the thickness of the membrane does indeed alter with electric potential, the capacity should change at the same time. Since the original report of Cole & Curtis (1939) that if there was a capacitance decrease during the action potential it did not exceed 2%, this subject has been curiously neglected. Furthermore, any Kerr effects in the membrane would constitute a type of dielectric relaxation, so that these too might show up if appropriate measurements were made, though it would be hard to predict what the quantitative relationship would be between capacitance and birefringence. However, there might be a detectable change in the dielectric dispersion of the membrane accompanying the transition from state 1 to state 2, when its optical properties are quite markedly altered.

We are particularly indebted to the Director and Staff of the Laboratory of the Marine Biological Association at Plymouth, where most of the work was done, for their hospitality. We thank Messrs S. B. Cross and D. V. Barker for building special apparatus, and Professor A. von Muralt and Dr D. A. Haydon for their advice and help. These experiments were supported in part by U.S. Public Health Service grant number NB 08437 from the National Institute of Neurological Diseases and Stroke.

REFERENCES

ARMSTRONG, C. M. (1969). Inactivation of the potassium conductance and related phenomena caused by quaternary ammonium ion injection in squid axons. *J. gen. Physiol.* **54**, 553–575.

BAKER, P. F., HODGKIN, A. L. & SHAW, T. I. (1962). Replacement of the axoplasm of giant nerve fibres with artificial solutions. *J. Physiol.* **164**, 330–354.

BERESTOVSKY, G. N., FRANK, G. M., LIBERMAN, E. A., LUNEVSKY, V. Z. & RAZHIN, V. D. (1970). Electrooptical phenomena in bimolecular phospholipid membranes. *Biochim. biophys. Acta* **219**, 263–275.

BLAUSTEIN, M. P. & HODGKIN, A. L. (1969). The effect of cyanide on the efflux of calcium from squid axons. *J. Physiol.* **200**, 497–527.

CALDWELL, P. C., HODGKIN, A. L., KEYNES, R. D. & SHAW, T. I. (1960). The effects of injecting 'energy-rich' phosphate compounds on the active transport of ions in the giant axons of *Loligo*. *J. Physiol.* **152**, 561–590.

CHANDLER, W. K., HODGKIN, A. L. & MEVES, H. (1965). The effect of changing the internal solution on sodium inactivation and related phenomena in giant axons. *J. Physiol.* **180**, 821–836.

COHEN, L. B., HILLE, B. & KEYNES, R. D. (1969). Light scattering and birefringence changes during activity in the electric organ of *Electrophorus electricus*. *J. Physiol.* **203**, 489–509.

COHEN, L. B., HILLE, B. & KEYNES, R. D. (1970). Changes in axon birefringence during the action potential. *J. Physiol.* **211**, 495–515.

COHEN, L. B., KEYNES, R. D. & HILLE, B. (1968). Light scattering and birefringence changes during nerve activity. *Nature, Lond.* **218**, 438–441.

COHEN, L. B. & KEYNES, R. D. (1971). Changes in light scattering associated with the action potential in crab nerves. *J. Physiol.* **212**, 259–275.

COHEN, L. B., LANDOWNE, D., SHRIVASTAV, B. B. & RITCHIE, J. M. (1970). Changes in fluorescence of squid axons during activity. *Biol. Bull. mar. biol. Lab. Woods Hole* **139**, 418–419.

COLE, K. S. & CURTIS, H. J. (1939). Electric impedance of squid giant axon during activity. *J. gen. Physiol.* **22**, 649–670.

CONTI, F. & TASAKI, I. (1970). Changes in extrinsic fluorescence in squid axons during voltage-clamp. *Science, N.Y.* **169**, 1322–1324.

FETTIPLACE, R., ANDREWS, D. M. & HAYDON, D. A. (1971). The thickness, composition and structure of some lipid bilayers and natural membranes. *J. membrane Biol.* **5**, 277–296.

FORMAN, D. B. (1966). Reversible changes in the birefringence of the squid giant axon with temperature. *Biol. Bull. mar. biol. Lab. Woods Hole* **131**, 390–391.

FRANKENHAEUSER, B. & HODGKIN, A. L. (1957). The action of calcium on the electrical properties of squid axons. *J. Physiol.* **137**, 218–244.

HAMMES, G. G. (1968). Relaxation spectrometry of biological systems. *Adv. Protein Chem.* **23**, 1–57.

HAYDON, D. A. (1970). A critique of the black lipid film as a membrane model. In *Permeability and Function of Biological Membranes: Proceedings of the* 1969 *meeting of the International Conference on Biological Membranes*, pp. 185–194. Amsterdam: North Holland.

HENDRICKX, H., VERBRUGGEN, R., ROSSENEAU-MOTREFF, M. Y. & PETERS, H. (1969). The dipolar origin of protein relaxation. *Biochem. J.* **110**, 419–424.

HODGKIN, A. L., HUXLEY, A. F. & KATZ, B. (1952). Measurement of current–voltage relations in the membrane of the giant axons of *Loligo*. *J. Physiol.* **116**, 424–448.

HODGKIN, A. L. & KEYNES, R. D. (1956). Experiments on the injection of substances into squid giant axons by means of a microsyringe. *J. Physiol.* **131**, 592–616.

KEYNES, R. D., RITCHIE, J. M. & ROJAS, E. (1971). The binding of tetrodotoxin to nerve membranes. *J. Physiol.* **213**, 235–254.

LE FÈVRE, C. G. & LE FÈVRE, R. J. W. (1960). The Kerr effect. Chap. XXXVI in 3rd edition of *Physical Methods of Organic Chemistry*, part III, ed. A. WEISSBERGER. New York: Interscience.

MOORE, J. W. & COLE, K. S. (1963). Voltage clamp technique. In *Physical Techniques in Biological Research, Vol. VI, Electrophysiological Methods*, part B, ed. NASTUK, W. L. New York: Academic Press.

MOORE, J. W., NARAHASHI, T. & SHAW, T. I. (1967). An upper limit to the number of sodium channels in nerve membrane? *J. Physiol.* **188**, 99–105.

NARAHASHI, T., ANDERSON, N. C. & MOORE, J. W. (1967). Comparison of tetrodotoxin and procaine in internally perfused squid giant axons. *J. gen. Physiol.* **50**, 1413–1428.

POWERS, J. C. & PETICOLAS, W. L. (1967). Electrical ordering in polypeptide solutions. *Adv. Chem.* **63**, 217–231.

PYTKOWICZ, R. M. & O'KONSKI, C. T. (1959). Characterization of Helix pomatia hemocyanin by transient electric birefringence. *Biochim. biophys. Acta* **36**, 466–470.

ROJAS, E. & EHRENSTEIN, G. (1965). Voltage clamp experiments on axons with potassium as the only internal and external cation. *J. cell. comp. Physiol.* **66**, suppl., 71–75.

SMYTH, C. P. (1966). Dielectric relaxation times. In *Molecular Relaxation Processes: Chemical Society Special Publication* No. 20, pp. 1–13. London: Chemical Society and Academic Press.

TASAKI, I. (1963). Permeability of squid axon membrane to various ions. *J. gen. Physiol.* **46**, 755–772.

TASAKI, I., BARRY, W. & CARNAY, L. (1970). Optical and electro-physiological evidence for conformational changes in membrane macromolecules during nerve excitation. In *Physical Principles of Biological Membranes*, ed. SNELL, F. and others, pp. 17–31. New York: Gordon and Breach.

TASAKI, I., SINGER, I. & TAKENAKA, T. (1965). Effects of internal and external ionic environment on excitability of squid giant axon. A macromolecular approach. *J. gen. Physiol.* **48**, 1095–1123.

TINOCO, I. (1955). The conversion of fibrinogen to fibrin. XVI. Electrical birefringence of fibrinogen and activated fibrinogen. *J. Am. chem. Soc.* **77**, 3476–3480.