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LIGHT SCATTERING AND BIREFRINGENCE CHANGES DURING ACTIVITY IN THE ELECTRIC ORGAN OF *ELECTROPHORUS ELECTRICUS*

BY L. B. COHEN,* B. HILLE† AND R. D. KEYNES
From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

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SUMMARY

1. In an attempt to obtain information about structural changes related to electrical activity in *Electrophorus* electroplates, we have determined the size and time course of the changes in light scattering and in birefringence that occur during and after the discharge of the electric organ.

2. The changes in light intensity detected with a photomultiplier were never greater than 0·2% for a single discharge, and were often much smaller than this, but records with an acceptable ratio of signal to noise could be obtained by signal-averaging techniques.

3. A single stimulus led to a decrease, then an increase, and finally another decrease in the light scattered by slices of the main electric organ. These three phases were designated E1, E2 and E3.

4. E1 started at the beginning of the action potential, and its peak was reached at the same time as the completion of repolarization, even when the repolarization was delayed by cooling or hastened by drawing larger currents from the tissue.

5. E2 was proportional to the integral of the current flowing through the slice of electric organ, and may arise from the swelling and shrinking of the tubules that stud the faces of the electroplates. It developed within a millisecond or two of the start of an applied current, and lasted for about 100 msec.

6. E3 was a variable decrease in scattering that lasted for some seconds.

7. A stimulus also led to a transient increase in the birefringence of the electric organ. The optical change followed the change in electrical potential across the innervated faces of the electroplates with a delay of somewhat under 50 μsec.

* National Science Foundation post-doctoral fellow. Present address: Department of Physiology, Yale University School of Medicine, New Haven, Connecticut.

† Helen Hay Whitney post-doctoral fellow. Present address: Department of Physiology and Biophysics, University of Washington School of Medicine, Seattle, Washington.

8. This voltage-dependent change in birefringence may arise from a Kerr effect (electric birefringence) in the membrane or from compression of the membrane.

INTRODUCTION

This paper describes the effects of excitation on the optical properties of the electric organ of the electric eel. Since the structure of the tissue and its optical properties are closely related, the experiments offer some direct information about the structural changes associated with electrical activity.

Light scattering and birefringence changes have been observed during the action potential in crab leg nerves and squid giant axons (Cohen & Keynes, 1968; Cohen, Keynes & Hille, 1968; Tasaki, Watanabe, Sandlin & Carnay, 1968). The time course of the birefringence change in nerve followed that of the membrane potential change with a short delay and some non-linearity; the birefringence change probably arose in the excitable membrane. The light scattering changes in nerve were divisible into two components. The first appeared transiently during the action potential, while the second followed immediately and persisted long after the action potential. Only the second component was affected by the difference in refractive index between the inside and the outside of the nerve. It was not determined whether the light scattering changes took place in the axon, in the Schwann cells, or in the extracellular spaces. Nor were they shown to be related to any specific process in the excitation.

One aim of the present experiments on the electric organ was to see if similar, rapid, optical changes would occur in this rather different tissue. The electroplates of the electric organ lack Schwann cells, and the membranes of the active and passive faces are invaginated by numerous tubules opening to the external solution (Luft, 1958). We hoped to utilize these properties of the electric organ as an aid to identifying the origins of the optical changes. Besides differing in its microscopical structure, the electric organ had the advantage of having many cells with nearly synchronous activity. This meant that it gave larger optical signals than a single squid axon, with much better synchrony than a whole crab nerve.

Relatively slow increases in the light transmission of the electric organ were observed by Aubert, Chance & Keynes (1964) to follow trains of 100 to 1000 stimuli. They also obtained indications that this was preceded by a transient decrease in transparency. We have found that during and after electrical activity the electric organ exhibits a sequence of rapid changes both in its birefringence and in its light scattering properties. The two slowest changes in light scattering are probably those seen by Aubert *et al.* (1964).

METHODS

The techniques used for keeping the electric eels and for dissecting and stimulating slices of the electric organ have been described by Aubert & Keynes (1968). The fifteen eels had been kept in an aquarium for periods between 4 weeks and 5 years. The fish ranged from 0·45 to 1·8 m in length. Most of the experiments were done on 5 mm thick transverse sections of the main electric organ placed between large chlorided silver plates in air. With some eels, slices giving good electrical responses could be cut from large pieces of the electric organ stored in *Electrophorus* Ringer (Keynes & Martins-Ferreira, 1953) up to 9 hr after the eel was decapitated. However, other specimens seemed in much poorer condition, and the action potential was small even in the first slice. The variability in the condition of the eels may account for some of the variations found in the sizes of the optical changes. The temperature of the slices was usually not closely regulated, and was about 2° above the room temperature (20–28° C) because of heating by the light beam.

General considerations. The fundamental experimental procedure was to measure variations in the intensity of a beam of light that arose from variations in the light scattering or birefringence of the electric organ. As the signals to be observed were very small changes in the total light intensity, considerable care had to be taken to minimize all sources of noise. The most stable sources of light for the time intervals in which we were interested were tungsten filament bulbs connected to lead accumulators. When all extraneous noise from mechanical vibrations and electrical or magnetic pickup was abolished, there still remained the irreducible noise from the statistical fluctuation in the rate of arrival of photons. This was the major source of noise in most of our experiments.

The root mean square statistical noise in a light measurement made over a certain period is equal to the square root of the number of photons detected during that period (Braddick, 1960). Thus the percentage of noise is reduced by increasing the number of photons in each measurement. This can be achieved by increasing the incident intensity, by gathering and detecting the light more efficiently, by lengthening the sampling period and by using low pass RC filters. Even after considerable attention had been given to these factors, the signal-to-noise ratio was unacceptably small, so we had to use signal-averaging techniques. With a signal averager, the signal-to-noise ratio is increased by the square root of the number of sweeps averaged. Thus the general formula for the final signal-to-noise ratio in our experiments was

$$\mathrm{S/N} = (\text{sweeps} \times \text{sampling period} \times \text{photons/sec})^{\frac{1}{2}} \times \Delta I/I.$$

The sampling period is the time in seconds during which the signal is accumulated in each memory address in a single sweep. The sampling period should be replaced by twice the time constant of the low pass RC filter if this is longer than the sampling period. The number of photons in the formula refers only to photons actually detected. The quantity $\Delta I/I$ is the amplitude of the signal, that is to say the change in the light intensity per impulse divided by the resting intensity. For the experiments discussed in this paper $\Delta I/I$ ranged from less than 10^{-5} to 2×10^{-3}. The results were always normalized to one impulse. Each experiment involved a compromise between the three factors in parentheses in the formula, for it was frequently desired to let the preparation survive longer by using fewer sweeps, to refine the optical conditions by reducing the angle of the cones of incident and measured light or by using monochromatic light, or to improve temporal resolution by shortening the sampling period. We tried to keep the signal-to-noise ratio greater than 10:1.

Light scattering. Figure 1 is a diagram of the light scattering apparatus. A 50 or 100 W tungsten-halogen lamp in a water-cooled housing derived its power from four 12 V accumulators connected in parallel. The connexions to the base of the lamp were always screwed or soldered to avoid noise from fluctuations in contact resistance. The light was collected by a condenser lens, passed through two or three heat reflecting interference filters, and

focused by a 16 mm microscope objective on to the slice of electric organ. About 25 mW of light actually reached the tissue (7×10^{16} photons/sec). In some cases, one of several Balzers band pass interference filters was included after the heat filters. These filters transmitted a band of wave-lengths 30–60 nm wide, and are identified in the text by their wave-length of peak transmission.

Figure 2 shows in more detail the orientation of the stimulating plates and of the electric organ. Although only two electroplates are shown here in series and in parallel, the actual 5 mm slices had between twenty-seven and one hundred cells in series and at least six columns of cells in parallel. The light entered the tissue through a 3 mm hole in one stimulating plate

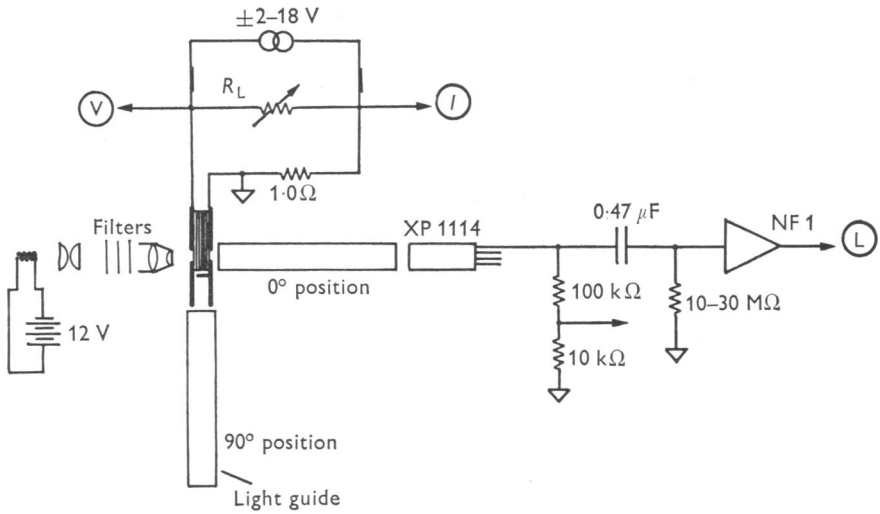

Fig. 1. Schematic diagram of the light scattering apparatus showing, from left to right, the lamp, condenser lenses, filters, focusing objective, slice of electric organ between stimulating plates with stop in place for 90° measurement, light guide, photomultiplier, coupling network, and preamplifier. The labels '*I*', 'V' and 'L' indicate where the current, voltage and light intensity signals are measured. The light guide is shown in the two possible positions.

and emerged in all directions because of scattering within the slice. The light leaving through a 3 mm hole in the second stimulating plate was called the light scattered at 0°. An alternative plate with an annular hole of 4 mm inside diameter and 8 mm outside diameter defined the light scattered at 30°. The light emerging laterally along the long axis of the electroplates was called the light scattered at 90°. When the light at 90° was to be measured, the slice was moved until the incident beam struck it at the lateral edge of the electroplates. In addition, an opaque stop was placed between the stimulator plates to mask out the light scattered from all but the first millimetre of the lateral edge of the tissue. In this way most of the measured light at 90° did not pass through unilluminated parts of the slice. Since almost all the light emerging under these conditions, or passing through the holes in the stimulating plates, was collected and measured, the angles of the scattered light would be more correctly termed $0 \pm 20°$, $30 \pm 20°$ and $90 \pm 20°$.

The light scattered from the electric organ was led to the photomultiplier through a light guide made from a Perspex rod 150 mm long, with polished ends 12 mm in diameter. It was not possible to place the photomultiplier near the stimulating plates without a light guide because of coupling artifacts from the 5–10 V electrical signals on the plates. With the light

guide these artifacts were eliminated. It was also necessary to place the photomultiplier in a mumetal shield to eliminate 50 and 100 Hz magnetic fields. The photomultiplier (Mullard XP1114) was a four-stage endwindow tube with a 14 mm type A (S11) photocathode. The cathode and dynodes were connected directly to a chain of five 90 V dry batteries. The anode was capacity coupled with a 5 to 14 sec time constant to a high impedance preamplifier (Bioelectronics NF1), and this time constant was generally 10 times greater than the sweep duration. Rapid interruption of the light beam by a rotating vane driven at 2000 rev/min showed that the system could follow step changes of light with a time constant of 6 μsec. Normally, however, the response time of the preamplifier was lengthened with a single time

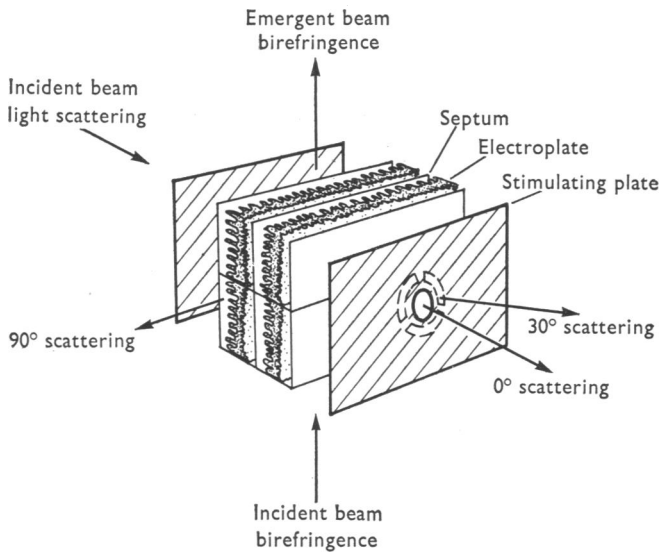

Fig. 2. Diagrammatic view of part of the slice of electric organ between the stimulating plates, showing the orientations of the incident and measured light beams. The near stimulating plate had either the round hole, the annular hole, or no hole, depending on what was to be measured. The electroplates are drawn to indicate the numerous macroscopic papillae that cover both faces but especially the non-innervated face. Not to scale.

constant filter to values that are mentioned where important. The input capacity neutralization of the preamplifier was not used. After each measurement the preamplifier was direct coupled to the 10 kΩ resistor in the anode load (Fig. 1) to obtain the total light intensity (I) for working out values of $\Delta I/I$.

The signal from the preamplifier was further amplified in a Tektronix 565 oscilloscope and fed via a cathode follower and a single time constant low pass filter to a TMC Computer of Average Transients (CAT 400C). The output of the oscilloscope was also fed directly to a Princeton Waveform Eductor Type TDH-9, a fast analogue signal averaging device; the TDH-9 was always used with a 'characteristic time constant' of 5 sec. Thus the faster events in an experiment were analysed by the Waveform Eductor at a frequency response set by the filter on the preamplifier, while the slower events were averaged simultaneously in the CAT computer at a lower frequency response. The records were drawn out on chart recorders.

Birefringence. An object shows birefringence if it has different values of refractive index depending on the direction of measurement. The highest and lowest values of the refractive

index are at right angles and define the principal axes. On entering a birefringent medium, linearly polarized light whose electric vector is not coincident with one of the principal axes is split into two components propagating at different velocities and with perpendicular electric vectors. On emerging, one component is retarded with respect to the other, so that they recombine to form elliptically, rather than linearly, polarized light. Some of this light will pass through an analyser oriented at 90° to the polarizer. Thus birefringence is detected by looking for light from an object lying between a crossed polarizer and analyser. The emerging beam is brightest when the principal axes of the birefringent object are at 45° to the plane of polarization of the incident light. The retardation can be measured by finding an appropriately calibrated retarder that exactly compensates for the retardation in the test object.

The birefringence measurements were performed with a Reichert Zetopan polarizing microscope using methods similar to those for light scattering. The tungsten–halogen lamp was placed in the lamp housing of the microscope and shone through collector lenses and two heat reflecting filters on to the substage mirror. The light was reflected up through a Glan-Thompson prism polarizer and focused by a long working distance condenser on to the bottom of the slice of the electric organ (Fig. 2), with the direction of polarization at 45° to the electroplates. A better signal-to-noise ratio is obtained if the total retardation is less than a quarter of a wave-length, so the slices were cut down to two to four parallel columns of cells.

An image of the slice was formed at the top of the microscope by a $10\times$ (N.A. = 0·25) strain-free objective. Between the image and the objective was a rotatable polaroid analyser sometimes preceded by a quarter-wave plate. The combination of quarter-wave plate and rotatable analyser was used as a variable retarder according to the method of de Sénarmont-Friedel (Bennett, 1950) to compensate the birefringence of the slice. The photomultiplier and recording apparatus were the same as in the light scattering experiments. White light was used in all of the birefringence measurements. Retardations were calculated assuming a wave-length of 550 nm.

Electrical stimulation and recording. In order to provide the large current necessary to stimulate the electric organ without loading it electrically during the response, the stimulus source impedance was lowered to 1 Ω during the shock and raised to 500 kΩ afterwards by the circuit described by Aubert & Keynes (1968). Stimuli of 4–8 V lasting 50 μsec at 0·5–2/sec were normally used, corresponding to 100–150 mV/cell in the slice. The stimulus was always kept supramaximal so that all parts of the electric organ would fire in synchrony. At times, a resistive load placed across the stimulating plates drew extra current from the slice during the action potential (Fig. 1). The current was measured as the voltage drop across a 1·00 Ω resistor.

The signs given to the measured currents and voltages are appropriate to the innervated, excitable faces of the electroplates. Thus a positive current is an outward current that flows through the innervated face and depolarizes it when an external cathode is applied. With this convention the current generated by the electroplate itself during its discharge flows inwards and is therefore negative.

The action potential of the electric organ was measured by connecting the stimulating plates directly to the input of an oscilloscope. It was recorded on the signal averager for comparison with the optical records, using no low pass filters. This record was sufficiently accurate for dealing with light scattering, but not for interpreting the birefringence experiments. The problem was that the recorded potential was the sum of the voltage across many series elements in addition to that across the innervated faces of the electroplates. In order to correct the record, we calculated and subtracted the voltage across the unwanted series elements, assuming that it corresponded to the voltage drop in a series resistor for a current equal to the externally measured current.

Independent electrical experiments were undertaken to determine the value of the series

resistance. The slice of electric organ was treated as a two terminal network containing a series resistor and a parallel RC repeated a known number of times (Fessard, 1946). The parallel RC was assumed to represent the membrane of the innervated face, and the series resistor to represent the other elements. A long hyperpolarizing voltage pulse was applied to the stimulating plates, and the values of the three components of the equivalent circuit could be calculated several times from the following measurements: the steady-state current, the time constant and amplitude of the relaxation of the transient charging current (low stimulator impedance) and the time constant and amplitude of the relaxation of the voltage at the end of the pulse (high stimulator impedance). The equivalent circuit was a fair representation of the observations, but, presumably because of variation among cells in the slice a distribution of the resistance and capacitance values would have given a better fit. The mean results of these measurements on the main organ of the three eels were: series resistance 3·8 Ω cm^2/cell; innervated faces, resting resistance 19·1 Ω cm^2/cell and capacity 14·4 μF/cm^2 cell. Apparently the innervated faces in the main organ do not differ greatly from those in the organ of Sachs, for which Keynes & Martins-Ferreira (1953) found 7·4 Ω cm^2 and 15·6 μF/cm^2 with micro-electrode techniques.

RESULTS

Light scattering

A slice of electric organ was an effective diffuser of light, and, although it was illuminated at only one spot, the entire slice became luminous. The intensity of the light transmitted by a slice (0° or 30° position) varied in at least three phases after an electrical stimulus: first an increase of intensity, then a decrease, followed by a second increase (Fig. 3*a*). The changes at 0° and 30° were indistinguishable. Two controls showed that the light variations were not artifacts attributable to electrical pickup. When the earth was moved from one silver stimulating plate to the other, the electrical potential variation on the unearthed plate was reversed in sign, but the recorded light varied as before. In another control, the light source was moved to shine directly on to the photomultiplier and not on to the tissue. The light intensity was adjusted to approximate the intensity recorded before. Stimulation of the electric organ no longer elicited any variations in the optical record.

The amplitude ($\Delta I/I$) and time course of the light variations were nearly the same at wave-lengths of 415, 450, 505, 550 and 600 nm, suggesting that specific absorption changes were not significant. A triphasic variation of light intensity whose sign was the opposite to that seen at 0° or 30° could be recorded at 90° (Fig. 3*b*). As these signals were the inverse of the signals at 0°, the variations of light intensity at all angles must have been primarily due to light scattering changes rather than to absorption changes.

The amplitudes of the intensity changes were larger at 0° than at 90°, and most experiments were therefore done at 0°. All of the results presented below are from experiments at 0°. They are similar to conventional measurements of light transmission, where a decrease of light means an

increase in scattering. The three phases of the scattering changes responded differently to certain experimental manipulations, so we propose to treat them as three independent components, which we shall refer to as E1, E2 and E3.

*The rapid scattering decrease, E*1. Within 100 μsec of a stimulus, the light passing through the electric organ began to increase, reaching a peak when the action potential had fallen nearly back to the base line (Fig. 4). The light increase ($\Delta I/I$) ranged from 3·3 to 13·7 $\times 10^{-5}$.

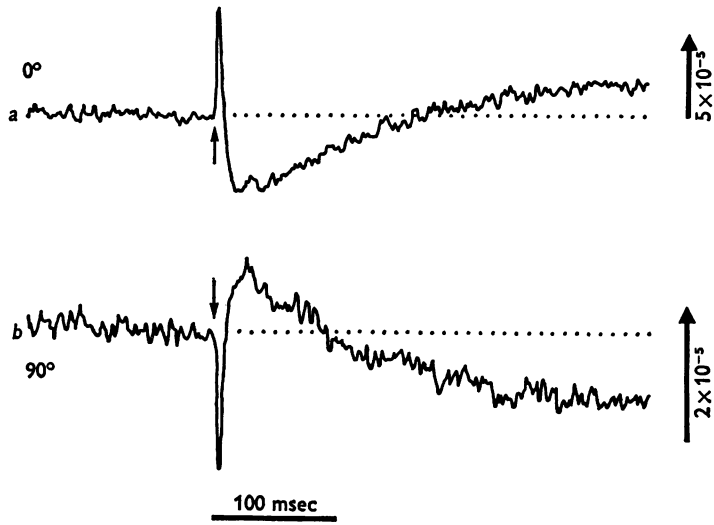

Fig. 3. The time course of the change in light intensity in the 0° position (*a*) and in the 90° position (*b*) following an action potential. In this and the remaining figures the arrows to the right indicate the direction and size of a light intensity increase of the indicated magnitude, for a single impulse. The smaller arrows show the moment of stimulation. All records are traced from the originals. Average of thirty sweeps. Temperature 26° C.

The light scattering decrease during E1 usually developed steadily from the beginning of the action potential and began to reverse only when the repolarization was almost complete. The relationship between the peak of E1 and the repolarization was not affected by several manipulations which altered the time course of the action potential. If the tissue was electrically loaded with a resistor, the action potential was shortened, and the time to the peak of E1 was correspondingly shortened (Fig. 9*a*). In four experiments the stimulating plates were cooled to 13° C, or warmed to 32° C by a jacket with circulating water. The duration of the action potential changed fourfold, but still the peak of E1 coincided with the repolarization (Fig. 5). The size of E1 was also not affected by temperature. The action potentials in the organ of Sachs are longer than those in the main

organ (Keynes & Martins-Ferreira, 1953) but again in two experiments with the organ of Sachs we found a decrease in light scattering with a peak just at the end of the action potential.

In an attempt to get a better understanding of the relationship of E 1 to the events occurring during the action potential, we depolarized the electroplates by about 90 mV for 4 msec by a long pulse from the low

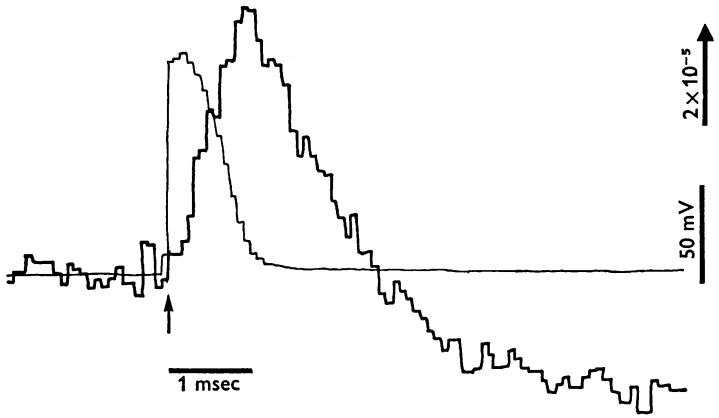

Fig. 4. Time course of the early light scattering change measured in the 30° position (heavy line) compared with the action potential (thin line). Average of 500 sweeps. Filtering time constant 55 μsec. Temperature 23° C.

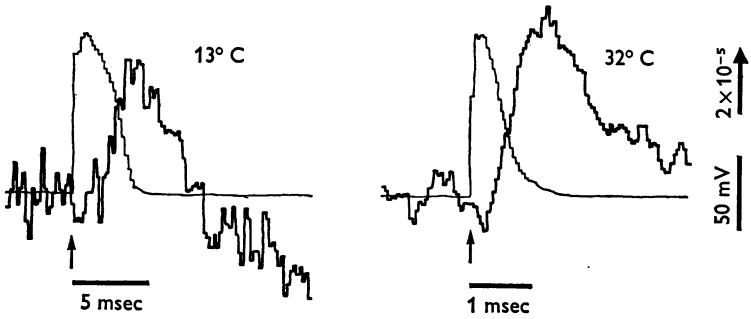

Fig. 5. The 0° light scattering change (heavy line) and the action potential (thin line) at two temperatures. Average of forty-two sweeps (13° C) and 100 sweeps (32° C). Filtering time constant 140 μsec.

impedance stimulator. The light scattering decreased throughout the pulse and began to rise just when repolarization was complete. The peak of the change seemed to depend more on the completion of repolarization of the electroplates than on some particular step in the conductance changes during the depolarization. Unfortunately, we could not be certain about the exact time course of E 1 in this experiment because it was overlapped by the time course of two other phenomena, E 0 and E 2.

When slices were taken from the electric organ several hours after the eel was killed, there appeared an additional light scattering increase which obscured the beginning of E 1. This increase, designated E 0, followed the time course of the potential changes with less than 50 μsec delay. We studied E 0 very little, as it was not characteristic of the freshest slices of electric organ. It often appeared gradually after several thousand stimulations. There is a small indication of E 0 in the light scattering records of Fig. 5.

The current-dependent scattering, E 2. In our early experiments the E 2 component of the light scattering was more variable than E 1. After finding values of $\Delta I/I$ ranging from $+0{\cdot}5 \times 10^{-5}$ to $-6{\cdot}5 \times 10^{-5}$, we observed that E 2 depended upon the strength of the stimulus. By raising the stimulus

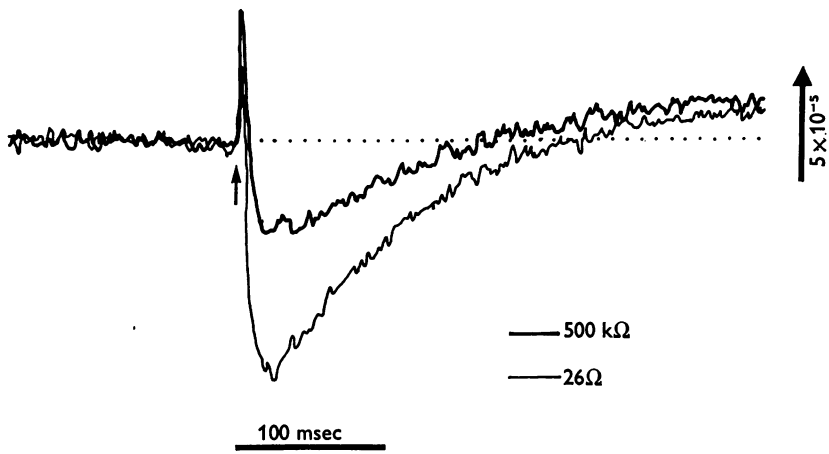

Fig. 6. The effect of a resistive load on the 0° light scattering change E 2. Average of thirty sweeps. Filtering time constant 1·5 msec. Temperature 26° C. Thick line: resistive load 500 kΩ. Thin line: resistive load 26 Ω, resulting in extra net charge movement of $-7{\cdot}4$ μC/cm^2.

from 6 to 18 V, we could convert a $\Delta I/I$ of $-1{\cdot}7 \times 10^{-5}$ during E 2 to a value of $+1{\cdot}3 \times 10^{-5}$, although the action potential was already maximal and the size of E 1 was not changed. This result suggested that E 2 might depend upon the current flowing through the electroplates. Accordingly, we carried out two other experiments designed to test this possibility directly.

First, extra current was drawn from the slice during the action potential by connecting a resistor between the stimulating plates. In every case, the scattering increase was greater ($\Delta I/I$ was increasingly negative) with the extra negative current (Fig. 6). Secondly, the size of E 2 was varied by externally applied currents. Positive, depolarizing currents always led to a decrease in scattering, and negative hyperpolarizing currents led to an increase (Fig. 7). In the bottom three curves of Fig. 7, current density and applied voltage were kept constant, while the duration of a hyperpolarizing

pulse was varied. These and other results showed that E2 depended primarily on the integral of the current (= net charge movement) and not on current density or applied voltage.

In Fig. 8 the size of E2 is plotted against the total transfer of charge for two experiments in which external currents were applied and one in which an action potential was elicited with and without a resistive load.

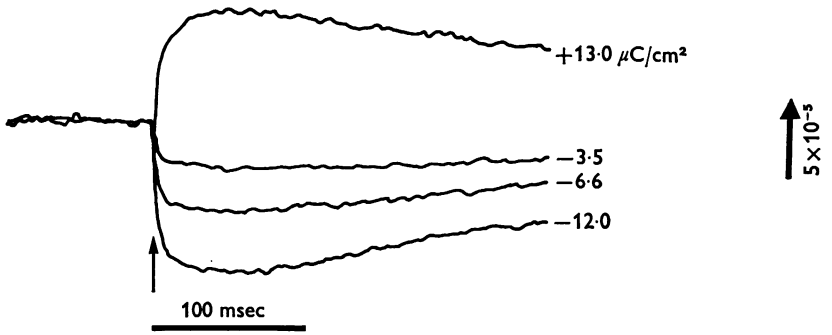

Fig. 7. The change in 0° light scattering following short applied currents. Hyperpolarizing pulses of −2 V lasting 0·5, 1·0 and 2·0 msec and depolarizing pulses of +6 V lasting 2·0 msec were applied to the slice of electric organ. Net charge movements are indicated beside each record. Average of 100 sweeps. Filtering time constant 3·5 msec. Temperature 26° C.

For the applied currents, the points fell fairly well on a straight line passing through the origin. For the action potentials, after taking into account the net charge movements of $+2\,\mu C/cm^2$ contributed by the stimulus and $-8\,\mu C/cm^2$ from the current generated by the tissue when it was electrically loaded, the points lay somewhat below this line. The discrepancy seemed likely to have arisen from current circulating in internal shunts within the electric organ when it was nominally loaded with 500 kΩ. Keynes & Martins-Ferreira (1953) measured the peak current flowing through the internal shunts in slices of the organ of Sachs during an action potential; by using this value of the current, and integrating over the time course of a typical action potential in the main organ, we estimated that the net charge movement from internal shunting effects could have been -1 to $-10\,\mu C/cm^2$. We therefore attribute E2 to net charge movements due to the stimulating current and to the current flowing through the shunting and loading resistances.

The time courses of the changes in E2 resulting from loading the slice and from hyperpolarizing currents are compared in Fig. 9. The arrows indicate the period of extra current flow. Both in the loaded slice (Fig. 9*a*) and in the hyperpolarized slice (Fig. 9*b*) most of the extra decrease in light occurred within a millisecond of the current flow. Presumably both kinds of current produce scattering changes by the same mechanism.

The slow scattering decrease, E3. The size ($\Delta I/I$) of E3 at 0° and 30° ranged up to $+18 \times 10^{-5}$. In many cases there was no positive E3 component at the end of a 250 or 500 msec sweep, while in others E3 seemed to have risen to a steady state within 100–200 msec of the stimulus. We were

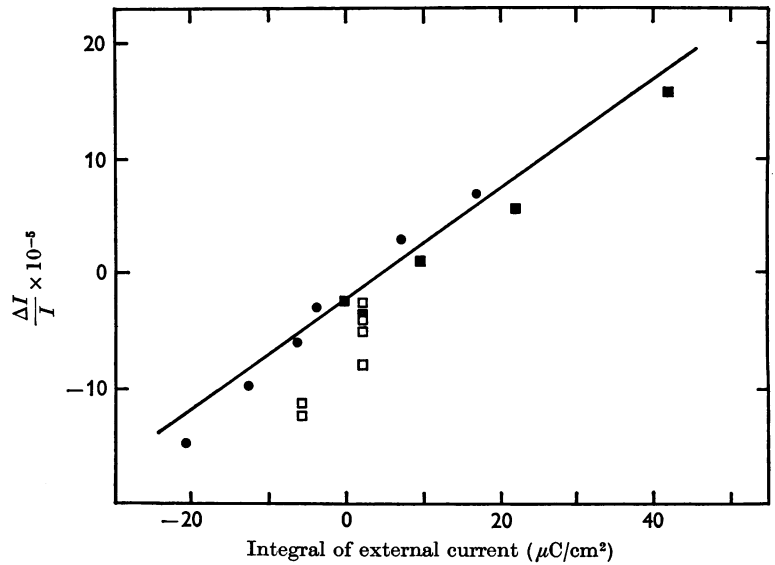

Fig. 8. The size of the peak change in light scattering during E2 as a function of the integral of the current flowing in the external circuit. The open symbols are for action potentials elicited by a brief stimulus either in the presence or absence of a resistive load. The filled symbols are for longer voltage pulses from the low impedance stimulator. Slice 41 ●; slice 54 ■ and □.

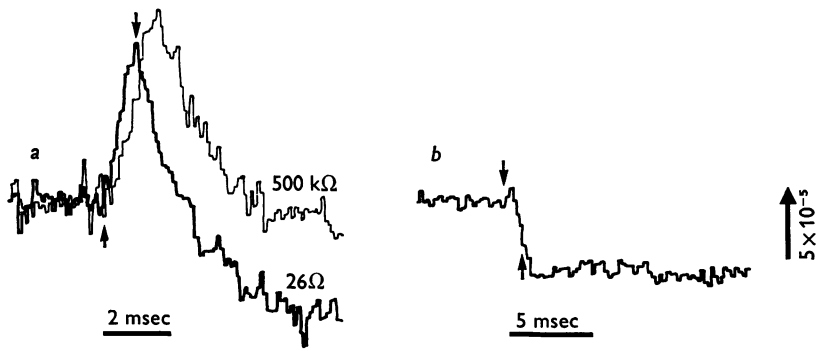

Fig. 9. A comparison between the extra light scattering produced (*a*) when a stimulated slice is loaded with a resistor and (*b*) when a slice is hyperpolarized by a 1 msec 2 V pulse. The arrows in (*a*) give the duration of the action potential (in the loaded condition), and the arrows in (*b*) give the duration of the external current. Same experiments as Figs. 6 and 7. Average of thirty sweeps (*a*) and 100 sweeps (*b*). Filtering time constant 140 μsec. Temperature 26° C.

unable to discover the cause of this variability. There could have been a prolonged negative E2 or a delayed onset of E3, or E3 may actually have been negative. In one case where the signal averager sweep continued for 5 sec after the stimulus, E3 reached a maximum 350 msec after the action potential and was still present after the 5 sec.

E3 was distinguished from E2 by its relative insensitivity to the total movement of charge. When a positive E3 was seen, it was only slightly reduced by a resistive load on the stimulating plates (Fig. 6). Moreover, externally applied currents always gave a monophasic change in light scattering which has already been seen to be analogous with E2, and not a diphasic change which could have been identified with E2 and E3 together. The size of E3 for separate impulses was not much changed by varying the stimulus frequency from 3 to 60/min, apart from a slight reduction which may have been due to a prolonged E2. As this list shows, we did not discover any experimental parameter that would bring about consistent changes in E3.

Transmission changes resulting from multiple stimulation. Using direct coupling between the photomultiplier and the amplifier we have observed long lasting increases in transmission similar to those found by Aubert *et al.* (1964). These changes began during a train of 30–1000 stimuli and lasted more than 30 sec after the last stimulus. At a stimulus frequency of 100/sec the value of $\Delta I/I$ following the first train of stimuli applied ranged from $0{\cdot}7 \times 10^{-5}$ to 600×10^{-5} per impulse, with most of the results between 170 and 600×10^{-5}; in Fig. 19 of Aubert *et al.* (1964) the value was $3{\cdot}3 \times 10^{-5}$ per impulse. These long lasting changes seem to be much too large to be accounted for by a simple accumulation of the E3 from each impulse. However, the conditions of the two sets of measurements were not strictly comparable, for the stimulus frequency used to determine the size of E3 had to be 1/sec or slower, because otherwise the next action potential came before the E3 of the previous one had fully developed. When the stimulus frequency in the direct coupled experiments was lowered from 100 to 20/sec, the long lasting change for a given number of impulses was reduced by 93–99%. The changes at the lower stimulation frequency could then be explained as the sum of the E3 components from each impulse. These experiments suggest that E3 may increase greatly with stimulation rate at the higher frequencies, but other explanations cannot be ruled out.

In all our experiments the size of the long lasting changes declined rapidly with repeated application of trains of stimuli, although there was no concomitant decline in the action potential.

The resting scattering of the electric organ. Under most circumstances the light scattered from the electric organ was multiply scattered. Van de Hulst (1957) states that corrections for multiple scattering are necessary if the object transmits undeviated less than 75 % of the incident light for some orientation of the incident beam. We measured the undeviated light (550 nm) reaching a photomultiplier tube placed 60 cm from the stimulating plates in the 0° position. A 5 mm slice of the electric organ from a large eel (thirty-five cells thick) decreased the light to 0·6%, and a slice from a small eel (eighty cells) decreased it to 0·2%. Thus Van de Hulst's criterion for single scattering was far from satisfied.

When the amount of transmitted light (0° position) was measured at various wave-lengths, a 5 mm slice of main organ was found to transmit orange (600 nm) light 5–20 times more than blue (415 nm) light. This suggested that the scattering increased with decreasing wavelength as is typical for particles smaller than the wave-length of light. The wave-length

dependence of the laterally scattered light (90° scattering) was also measured. Contrary to our expectations, there was again more orange light than blue, although in this case only 1·5–4 times as much orange than blue. This was true even under the conditions designed to reduce the contribution of multiple scattering (see Methods). Thus the light measured at 90° had a wave-length dependence more appropriate to transmitted light than to scattered light. On the other hand, the intensity changes at 90° during the action potential are reversed from those at 0° (Fig. 3), showing that this component of the light at 90° is certainly scattered. We do not know the reason for the discrepancy. Possibly there are some absorbing groups in the tissue that absorb broadly in the short wave-length end of the visible spectrum and the scattering itself is nearly independent of wave-length.

Birefringence

Birefringence and the membrane potential. The entire slice of electric organ appeared birefringent when it was illuminated from below as in Fig. 2. Experiments designed to measure birefringence changes were undertaken on slices from five eels. Stimulation of the electroplates produced a transient increase in light passing the crossed polarizer and analyser of $2\text{–}80 \times 10^{-5}$. When the changes were small, experiments with the rotating compensator showed that only a part of this increase resulted from a change in birefringence; and the remainder of the intensity change had the same time course as the light scattering changes. This interference from light scattering changes was severe in the birefringence experiments on three of the eels, and the most useful results were obtained with the remaining two eels. An additional problem was the poor survival of the thin (two to four cells thick) pieces of electric organ used for these experiments. Many slices had to be rejected because the action potential declined too rapidly during the optical measurements. For these reasons our results are not extensive.

Figure 10*a* shows the changes in the intensity of light passing through the analyser during an action potential. In one record the analyser was in the crossed position (thick line) and in the other (thin line) it was rotated by 50° to subtract 150 nm of retardation from the 75 nm resting retardation of the tissue (Bennett, 1950). To facilitate comparison the record with compensation has been inverted. The similarity of the two records show that essentially all of the optical signals in this experiment arises from a transient increase of the birefringence of the tissue. Signals from light scattering and light absorption would not have been inverted by compensation.

The birefringence change seemed to depend closely on the membrane potential of the innervated face of the electroplates. The potential recorded from the stimulating plates and the estimated action potential across the innervated face of the electroplates (see Methods) are given in Fig. 10*b*. As is shown in Fig. 10*c* the birefringence changed more slowly and reached a peak later than the action potential of the innervated face. Part, but not all, of this delay in the optical record came from the 18 μsec recording time

constant of the photomultiplier–preamplifier combination. By redrawing the action potential as it would appear if recorded by a system with a 55 μsec exponential time constant, we obtained a curve (dots in Fig. 10*c*) that matched the optical record more closely. Thus, to a first approximation,

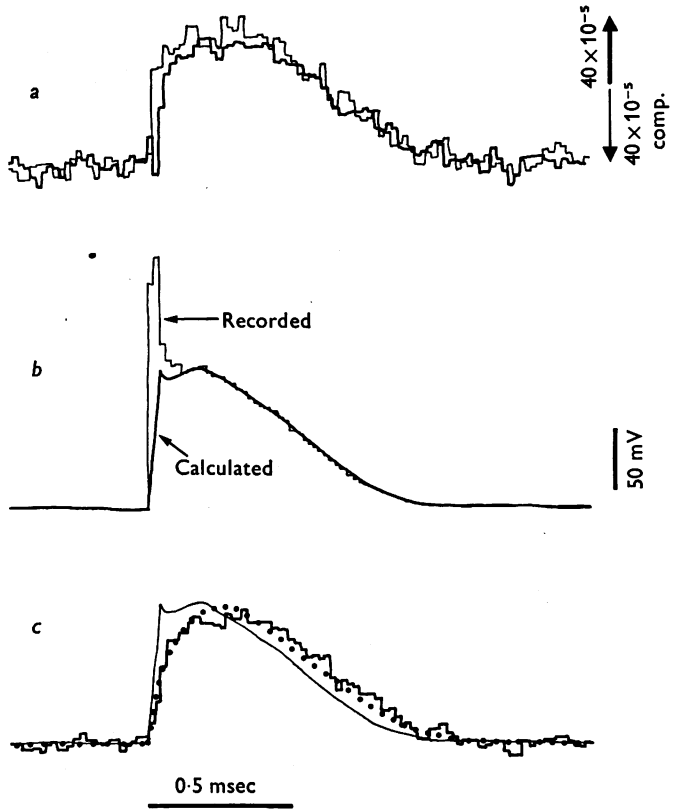

Fig. 10. Birefringence changes during the action potential. (*a*) shows the time course of the light intensity change without (thick line) and with (thin line) 150 nm of compensation. The record with compensation (comp.) has been inverted. Averages of sixty sweeps. Recording time constant 18 μsec. (*b*) shows the action potential recorded on the stimulating plates (thin line) and as estimated for the innervated faces by subtracting the voltage drop in the series resistance (thick line). (*c*) shows the action potential as estimated for the innervated faces (thin line) and after imposing an exponential delay of 55 μsec (dots) compared with the average of the light intensity records in (*a*) (thick line). Temperature 26° C.

the birefringence of the tissue followed the membrane potential of the innervated face with an exponential delay of about 37 μsec (55 μsec minus the 18 μsec instrumental delay). This fit would be improved if a somewhat longer time constant were used for the falling phase of the action potential.

Even in experiments with contamination from light scattering in the

optical record, we could demonstrate a delay between the rise of the action potential and the increase of birefringence. This was possible because the light scattering changed appreciably more slowly than the birefringence. The estimated delay was always less than 100 μsec.

When the electroplates were hyperpolarized by 55 mV/cell, the light passing through the crossed polarizer and analyser decreased. Again the optical signal could be reversed with the compensator. The birefringence decrease was about one third as big as the increase during an action potential, and it was also slightly delayed.

Slow birefringence changes. As with light scattering, there may be more than one component of the birefringence change. During a long applied hyperpolarization, the birefringence continued to decrease with a slow steady 'creep' after the initial rapid decrease, and at the end of the hyperpolarization there was a rapid and a slow phase in the restoration of the birefringence; the slow phase lasted 100 msec. Similarly an applied depolarization produced a rapid and slow increase of birefringence. After generation of an action potential there was also a slowly disappearing decrease of birefringence. These slow changes were reminiscent of the current-dependent component of light scattering, E2, and might well have had a different origin from the rapid birefringence changes.

The resting birefringence. We tried to identify the major sources of the resting birefringence of fresh 0·5 mm sections of the electric organ by examining them under the microscope between crossed polarizers and analysers and a first order red plate. No change of the red tint, and hence no birefringence, could be detected in the electroplates themselves, whether they were viewed from the face, the top or the side. However, as the microscope stage was rotated, those connective tissue septa (Fig. 2) that were seen edge on turned alternately yellow and blue. The fast axis of this birefringence was normal to the plane of each septum. Septa seen face on showed little birefringence. Myelinated nerve fibres and criss-crossing ribbons of what appeared to be collagen were also birefringent (de Almeida, de Oliveira Castro, Miranda & Chagas, 1963).

The slices used for the study of the fast birefringence changes were sufficiently thick for every optical path to include several septa as well as electroplates. Because of the septa, the whole slice appeared weakly birefringent with the fast axis normal to the plane of the electroplates. The slices appeared to have an average retardation of 10 and 20 nm/mm tissue, but different areas of the slices had different retardations and no area could be completely darkened by compensation. It seemed that the light had been partially depolarized by passage through the tissue.

DISCUSSION

Light scattering

Our experiments revealed at least three changes in the light scattering properties of the electric organ during activity. Each of these must arise through some structural modification such as the redistribution of ions and water, conformational changes in macromolecules, the formation and break-down of macromolecular aggregates, or the swelling and shrinking of cellular organelles or even of the electroplates themselves. Although we are unable to reach any final conclusion as to the precise mechanism of the light scattering changes in the electric organ, we can consider the relative merits of some hypotheses.

E 1. The E 1 component of scattering appears to start as soon as the membrane is depolarized, and to stop when repolarization is complete. It does not arise when the membrane is hyperpolarized. Its total size remains the same whether the duration of the action potential is reduced by loading the tissue electrically or increased by lowering the temperature. In both situations the amounts of sodium and potassium crossing the innervated membranes would be raised appreciably. The time course of E 1 does not follow the time course of the sodium or potassium conductance changes measured by Nakamura, Nakajima & Grundfest (1965) in electroplates from the organ of Sachs. The experiments with long depolarizing pulses appeared to rule out any correlation with the inactivation of the sodium conductance mechanism. At present we can only suggest that E 1 somehow involves a combination of voltage- and current-dependent changes which might be similar to effects observed in squid axons (L. B. Cohen & R. D. Keynes, unpublished observations).

E 2. As we have already shown, the light scattering increase during E 2 probably arises from a net movement of ions into the electroplates. The results of applying currents from an external source are consistent with this view. A light scattering change that may be analogous follows the application of current to crayfish muscle fibres (Girardier, Reuben, Brandt & Grundfest, 1963); an applied inward current increased the opacity of the muscle fibres by causing the transverse tubular system to swell. After passage of 60 μC from a micro-electrode, the change was readily visible on inspection through a microscope. They pointed out that if the tubular membranes were selectively permeable to chloride, an inward current would tend to increase the salt concentration in the tubules, thus raising the osmotic pressure and drawing water into the tubule. Such an effect might exist wherever an ionic current flows across a selectively permeable membrane that encloses a restricted space.

We suggest that swelling of tubules is the basis for the E 2 component of light scattering. As in the electroplates of other fishes, the surface area of the anterior and posterior faces of *Electrophorus* electroplates is greatly expanded by the presence of numerous closely spaced, narrow tubules that extend several microns into the cytoplasm (Luft, 1958; Bloom & Barrnett, 1966). The tubules of the non-innervated face are more numerous and longer than those of the innervated face and might be expected to make a greater contribution to any light scattering changes. If the tubular membranes were more permeable to cations than to anions, the tubules would swell after an applied outward current. Thus, a negative current through a slice of electric organ (inward at the innervated faces and outward at the non-innervated faces) would cause the tubules of the non-innervated faces to swell, and the light scattering would change. It would then be reasonable

to suppose that a positive current would cause the same set of tubules to shrink. We have not tested this type of explanation experimentally, but it seems to be a plausible way to account for a light scattering change that depends on the integral of the current.

E 3. The variability of E 3 from eel to eel, and its insensitivity to those experimental manipulations that were tried, makes it very hard to decide on its probable origin. The most obvious possibility was that it might be connected with metabolic reactions following the movement of ions, since the swelling and shrinking of mitochondria, or the break-down of glycogen particles, would be expected to alter the light scattering. However, when the slices were loaded so that the inward sodium current was increased, the size of E 3 was not much changed, and if anything, was reduced. This suggested that the metabolic events related to sodium extrusion were not involved in E 3. Moreover, an analysis by Dr J. C. Ellory of frozen samples from the main organ of each electric eel that we used showed that there was considerable variation in the relative amounts of creatine phosphate to free creatine, but no correlation with the presence or absence of E 3. More experiments are needed to explain the effect of stimulation frequency and fatigue on the slow changes in light scattering, and to test for any relationship between the full time courses of E 3 and of the recovery heat (Aubert *et al.* 1964).

Birefringence

Birefringence originates from the orientation of anisotropic molecules and from the organization of molecules into anisotropic structures. As with the squid axon (Cohen *et al.* 1968), most of the birefringence change during an action potential in the electric organ followed the time course of the membrane potential with only a short delay. In the squid axon this and several other observations showed that the birefringence change arose in the immediate vicinity of the excitable membrane. A similar conclusion seems warranted for the electric organ. The occurrence of the effect in this tissue also demonstrates that Schwann cells are not necessary for a voltage-dependent birefringence change.

The magnitude and sign. If the changes do arise in the cell membrane, much useful information could be gained from the sign, magnitude, and time course of the effects at the membrane level. We have already shown that there was an apparent increase in retardation that lagged about 40 μsec behind the voltage during an action potential.

It is possible to calculate a lower limit for the retardation increase during an action potential. In the experiment of Fig. 10, there was a light increase of about 8×10^{-4} arising from a tissue with 75 nm of retardation. For a homogeneous birefringent object, the light intensity passing crossed

polarizers and analysers varies as $\sin^2(\frac{1}{2}\delta)$, where δ is the retardation in radians. If we assume that the electric organ was homogeneous and that the change within it during the action potential was also homogeneous, it follows that the retardation increased by 30 pm during the action potential. An internal calibration with small rotations of the compensator suggested that the depolarization of the light by the tissue caused a 100 % error in the preceding calculation. A better estimate is, therefore, that the average retardation increases by 60 pm. This may be compared with a decrease of 0·2 pm seen during the action potential in a single squid giant axon and a decrease of 10 pm during the compound action potential in a crab leg nerve (Cohen *et al.* 1968).

We would like to calculate the birefringence change of a single membrane. Unfortunately, the surface of the innervated face of the electroplate is so crowded with papillae and fine tubules that the number of membranes in any optical path is not accurately known. Most of the membrane area is in the walls of the tubules. Furthermore, in a measurement of birefringence, the contribution from membranes perpendicular to the plane of the electroplates is of the opposite sign to that from membranes parallel to the electroplates. This subtraction of effects will reduce the observed signal. We cannot determine from the published electron micrographs whether the predominant orientation of the membranes of the innervated face is transverse or longitudinal. Hence until more information is available, we shall not know the sign or magnitude of the birefringence change at the membrane level. If the orientation of the membranes is effectively perpendicular to the plane of the electroplates, the sign of the change would be the same as it is in squid giant axons and in crab leg nerves.

The Kerr effect. From our experiments with the squid giant axon (Cohen *et al.* 1968) we suggested that the birefringence change might be a Kerr effect arising from the reorientation of polar molecules within the membrane under the influence of the intense electric field, or, alternatively, that it might involve a change in the compression of the membrane by the mutual attraction of the ionic double layers. Either mechanism might give an effect as large as that observed. It was shown that if the effect was a Kerr effect, the molecules responsible would have to have a Kerr constant at least as large as that for water. These conclusions apply to the electric organ as well. Additional limits may be placed on the possible Kerr effect. The time constant of the change (40 μsec) would require the turning molecules to be quite large or to lie in a very viscous environment. For example, Debye (1929, p. 85) used the Stokes formula for rotating spheres, which indicates a diameter of almost 50 nm for a sphere reorienting in water with a time constant of 40 μsec.

Pressure on the membrane. At a membrane potential of 100 mV the

attraction of the double layers (the changes across the membrane capacitance) exerts a pressure of the order of 10^5 dyn/cm^2 on the insulating part of the membrane. The release of this pressure during a depolarization could lead to a small thickening of the membrane. One way to monitor the thickness of the membrane is to measure its capacitance. Cole & Curtis (1939) noted a decrease in the capacitance of the axolemma of the squid giant axon during the passage of the action potential that was not greater than 2%. Somewhat larger changes have been reported in experiments with black lipid films, where the capacitance is said to increase several per cent with an applied voltage of 100 mV (Babakov, Ermishkin & Liberman, 1966; Rosen & Sutton, 1968).

Another effect of applied voltage is found in black lipid films. In unpublished experiments with Mr J. A. Bangham (using a suspension of phosphatidyl choline in *n*-decane) we observed that a 100 mV pulse produces rapid and reversible increases in the area of the black film as well as decreases in the radius of the lens-shaped islands of bulk material floating in the thin film (see Haydon & Taylor, 1968). In some cases these changes were half complete in 3 msec. The effects could be attributed to the decreased surface tension of the film caused by the storage of electrical energy in its capacitance. We conclude from these various studies that biological membranes must suffer *some* mechanical changes when the potential across them changes, but there is as yet little indication of the absolute magnitude of such effects. These mechanical changes might be sufficient to explain the rapid changes in birefringence during an action potential.

Both the suggestions of a Kerr effect and of a decreased electrostriction predict a reversal of the direction of the birefringence change when zero membrane potential is reached, so that the birefringence change during an action potential would look somewhat triphasic and not monophasic. This difficulty may be avoided by assuming that the membrane is asymmetrical. For example, a layer of fixed charge on one surface of the membrane could bias the reversal potential of either of these effects to beyond the physiological range.

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