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DEPOLARIZATION AND CALCIUM ENTRY IN SQUID GIANT AXONS

BY P. F. BAKER, A. L. HODGKIN AND E. B. RIDGWAY*

From the Laboratory of the Marine Biological Association, Plymouth and the Physiological Laboratory, University of Cambridge, Cambridge

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SUMMARY

1. Changes in ionized calcium in giant axons were followed by recording the light produced by injected aequorin.

2. From the effect of injecting calcium buffers the internal concentration of ionized calcium was found to be about the same as in a mixture of 45 Ca EGTA : 55 free EGTA, i.e. about 0·3 μM.

3. After an axon had been exposed to cyanide for 50–100 min the velocity of the aequorin reaction increased about 500 times. This effect, which could be reversed rapidly by removing cyanide, was probably brought about by release of calcium from an internal store.

4. Injecting 30 μmole ATP per litre of axoplasm into a cyanide-poisoned axon caused a transient lowering of light intensity; oligomycin blocked the effect.

5. Raising external calcium or replacing external sodium by choline or lithium reversibly increased the light produced by axons injected with aequorin.

6. Stimulation at 50–200 impulses/sec in a solution containing 112 mM-Ca caused the light intensity to increase to a new steady level; after stimulation the light intensity returned to its original level with a time constant of 10–30 sec. Similar but smaller effects were seen in solutions containing less external calcium. The recovery after stimulation is probably due to uptake of calcium by the internal store.

7. Injecting 3 m-mole EGTA per litre axoplasm lowered the resting glow and abolished the aequorin response to stimulation.

8. There was no light response to stimulation immediately after an axial injection of aequorin and the effect increased to a 'steady' level with a half-time of about 5 min. The conclusion is that the rise in calcium con-

* Present address: Friday Harbor Laboratories, University of Washington, Washington 98250, U.S.A.

centration resulting from stimulation is confined to the peripheral part of the axon and that the diffusion coefficient of aequorin in axoplasm is about 4×10^{-7} cm^2/sec.

9. The increment in light per impulse often increased markedly during the course of a long experiment and there was also considerable variation between axons.

10. If the light response to stimulation was small it was proportional to the frequency of stimulation; if large to the square of the frequency.

11. Voltage-clamp experiments showed that the calcium entry associated with a depolarizing pulse could be divided into an early component which was abolished by tetrodotoxin (TTX), and a late component which was unaffected by this inhibitor.

12. The time relations of the early calcium entry were consistent with its being a leak of calcium ions through the sodium channel; the permeability of the sodium channel to calcium was about 1% of the permeability to sodium.

13. The late entry of calcium was little changed by injecting enough tetraethylammonium (TEA) to block the outward potassium current; it was greatly reduced by external concentrations of manganese which had little effect on the maximum potassium conductance.

14. The voltage–response curve for the late entry of calcium had a well defined maximum and was similar in shape to the curve relating calcium entry to depolarization at the presynaptic ending (Katz & Miledi, 1969, 1970).

INTRODUCTION

It has been known for some time that propagation of impulses along giant nerve fibres is accompanied by a small inflow of calcium ions (Flückiger & Keynes, 1955; Hodgkin & Keynes, 1957). In sea water, which contains 11 mM-Ca, the calcium entry per impulse at 20° C was found to be about 0·006 p-mole/cm^2; that is, thirty-six calcium ions per square micron or 1/700 of the net entry of sodium. With 112 mM-Ca in the external solutions the entry was about 0·08 p-mole/cm^2 or 500 calcium ions per square micron. Even in high calcium the quantity of calcium which enters is far too small to carry appreciable current or to modify the shape of the action potential directly. Although the calcium entry is small it does represent a relatively large increase in permeability since stimulation at 150/sec increases the calcium influx about twentyfold. It is therefore important to know whether this change occurs during the early phase of increased sodium permeability or during the late phase of increased potassium permeability, or if it reflects some quite separate process. Provided a fairly rapid method of measuring calcium entry is available, this question

and others can be answered with the voltage-clamp technique. As will appear later, the calcium entry can be divided into an early phase which is abolished by TTX, and a late phase which is unaffected by it. At first it seemed that the early increase in calcium permeability preceded the rise in sodium permeability (Baker, Hodgkin & Ridgway, 1970), but later analysis showed that the two changes were similar in time course (Baker, Hodgkin & Ridgway, 1971). The simplest explanation of the early entry is that some calcium leaks through the sodium channel, the permeability ratio between the two ions being roughly $P_{Ca}/P_{Na} = 0{\cdot}01$. The timing of the delayed calcium entry was similar to that of the increase in potassium permeability, but the late entry was little changed by injecting enough TEA to block most of the potassium current. The channel involved in the delayed entry has several features in common with the calcium entry mechanism involved in transmitter release (Katz & Miledi, 1967, 1969). The latter is undoubtedly a highly specialized mechanism but investigating nerve may contribute to an understanding of events at synapses and junctions.

All the experiments were carried out by recording the light emitted from giant axons which had been injected with aequorin, a protein extracted from the jelly fish (*Aequorea forskalea*) which emits light in the presence of ionized calcium (Shimomura, Johnson & Saiga, 1962; Ridgway & Ashley, 1967; Ashley & Ridgway, 1970). The first part of the paper deals with resting axons and with the effect of solutions which might be expected to change the level of ionized calcium inside the axon. The second part is concerned with the effect of propagated impulses and the third with voltage-clamp measurements in the presence and absence of TTX.

METHODS

Material

Giant axons with diameters between 600 and 1000 μ were isolated from *Loligo forbesi*. Living squid were used in some of the experiments but as a rule axons from refrigerated mantles were employed.

Procedure

After the axon had been cleaned and cannulated 0·45 μl. of an aequorin solution was injected with an EGTA-filled Hamilton microsyringe over a length of 2·5 cm at a distance of 2·0–4·5 cm from the cannula. The axon was then suspended in front of a photomultiplier tube in the recording cell illustrated in Fig. 1. For voltage-clamp experiments a double spiral electrode was inserted, with the exposed part of the voltage wire near the middle of the aequorin patch and the exposed part of the current wire extending slightly beyond the length injected. In these experiments, membrane action potentials were elicited by applying a 100–500 μsec pulse of current between electrodes (1) and (4) and recording the voltage between electrodes (2) and (3). When studying propagated action potentials the stimulus was applied across the lower partitions and the spike was recorded from the cannula, or occasionally

from a 100 μ diameter capillary inside the axon. After setting up the axon, the recording cell was enclosed in a light-tight box and the shutter opened. In some experiments the axon was tied off and inserted in a tube of 3 mm internal diameter attached to a flow system. This enabled the solution bathing the axon to be changed rapidly without opening the box.

The photomultiplier tube was a specially selected EMI type 9635B with the following characteristics: cathode sensitivity (S) 86 μA/l.: over-all sensitivity (M) 2000 A/l. at 990 V HT; dark current 2 nA at 990 V. The tube was run with the

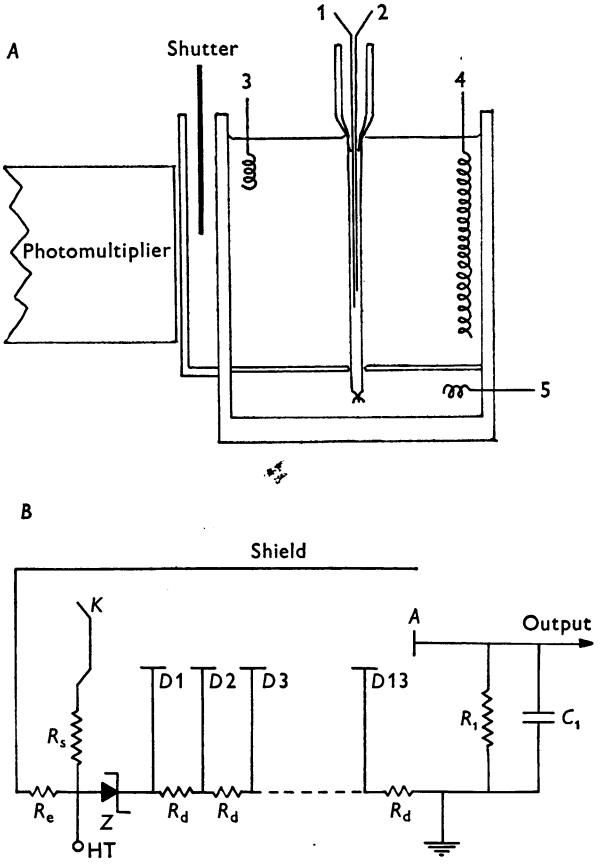

Fig. 1A. Diagram of the experimental arrangement for recording light output from an axon under voltage-clamp conditions. The whole apparatus was enclosed in a light-tight box.

B. Circuit of photomultiplier. K, cathode; A, anode; D1–13 dynodes; Z, zener diode (Texas type IS, 4150). An electrostatic shield maintained at cathode potential via a 10 MΩ resistor (R_e) surrounded the glass envelope of the tube. The cathode was protected by a 180 kΩ resistor (R_s) and the resistances in the linear dynode chain (R_d) were 100 kΩ. Negative high voltage was supplied to the cathode. This arrangement prevented the power supply ripple from being injected directly into the output through the anode load resistor.

cathode at -990 V and the anode near earth. The output current was recorded as the voltage across a resistance of 1 kΩ to 1 MΩ (R_1, Fig. 1*B*) and a variable capacity (C_1, Fig. 1*B*) with a minimum value of 150 pF (mainly cable capacity) and a maximum of 1 μF. In most experiments the light response to a train of spikes or pulses was recorded with a pen-recorder (Servoriter 2, Texas Instrument Company). In other instances the light was recorded with one beam of an oscilloscope (Tektronix 502A) or the average of 8–64 trains was obtained with a 'Biomac' computer of average transients. The response of the pen-recorder was 70 % complete in 0·15 sec; when using the pen-recorder the electrical smoothing time constant was commonly about 100 msec. When a light response was recorded on the oscilloscope or Biomac the recording time constant was between 1 and 10 msec.

Aequorin preparation

The aequorin used in these experiments was collected, extracted and partially purified according to the procedures described by Shimomura & Johnson (1969). Further purification was carried out at University College London, again according to the procedures of Shimomura & Johnson, except that the chromotography was only carried out twice on DEAE-cellulose and once on Sephadex. As a final step, a small portion of the ammonium sulphate precipitate from the second DEAE column was dissolved to saturation in 0·5 ml. 100 mM-EDTA pH 6·0 and then de-salted on a small (0·5 × 10 cm) column of Sephadex G-25 which had previously been equilibrated with 100 mM potassium phosphate at pH 6·0. This material constituted the injection solution; it was kept at $-20°$ C and thawed before use.

The aequorin solutions used in the experiments described here had no adverse effects on the axon and action potentials of normal amplitude were recorded from the injected region for 6–12 hr. It was important to make careful tests of toxicity since samples of aequorin prepared by a simpler method made the axon inexcitable in a few hours.

Calibration of light recording system

The experimental results will be given in terms of the output current of the photomultiplier when operated at 990 V. A factor which converts output current into the number of aequorin molecules reacting per second was obtained by a method suggested by Professor E. J. Denton.

The sensitivity of the photomultiplier was measured with a small radioactive light source (Betalight) which was placed in approximately the same position as the nerve and attenuated $10^{3\cdot05}$ times with neutral filters. The luminous intensity of the source was determined by the Electrical Quality Assurance Directorate (E.Q.A.D.) as $4\cdot2 \times 10^{-6}$ cd. From the energy distribution of the source, which was also determined by the E.Q.A.D., and similar curves for the photomultiplier, and for the aequorin emission, Professor E. J. Denton calculated that 1 μA of output current is equivalent to $1\cdot07 \times 10^{-12}$ W of light emitted by aequorin. In deriving this factor, Professor Denton used the formula

$$Q = \frac{4\pi n \int B(\lambda)S(\lambda)\,d\lambda \cdot \int A(\lambda)\,d\lambda}{\int A(\lambda)S(\lambda)\,d\lambda}, \qquad (1)$$

where Q is the power (W) emitted as light by aequorin over a sphere, n is the ratio of the output current produced by aequorin to the output current produced by the Betalight, $B(\lambda)\,d\lambda$ is the power in watts steradian^{-1} emitted by the Betalight between the wave-lengths λ and $\lambda + d\lambda$. $A(\lambda)$ is the emission spectrum of aequorin and $S(\lambda)$ is the spectral sensitivity of the photomultiplier. $A(\lambda)$ and $S(\lambda)$ need be

known only in relative terms since each appears in both the numerator and denominator of eqn. (1).

The rate at which photons are emitted, P, is given by

$$P = Q\bar{\lambda}/hc \tag{2}$$

when h is Planck's constant and c is the velocity of light, $\bar{\lambda}$ is the 'mean' wave-length of the light emitted by aequorin calculated by

$$\bar{\lambda} = \frac{\int \lambda A(\lambda)\,d\lambda}{\int A(\lambda)\,d\lambda}. \tag{3}$$

From the curve published by Shimomura & Johnson (1970), $\bar{\lambda}$ was calculated to be 474 nm, i.e. about 2 % greater than the peak of the emission spectrum of aequorin. Shimomura & Johnson (1969) give the quantum efficiency of the aequorin reaction as 0·23 photons per molecule aequorin reacting so the over-all calibration factors are as follows:

1 μA output current $\equiv 1{\cdot}07 \times 10^{-12}$ W of light
$\equiv 2{\cdot}5 \times 10^{6}$ photons/sec
$\equiv 1{\cdot}08 \times 10^{7}$ aequorin molecules reacting/sec.

Factors about twice as large as these were obtained from data provided by E.M.I. Ltd., the manufacturers of the phototube.

The total number of aequorin molecules injected could be calculated from the calibration factors. When 0·45 μl. aequorin solution was injected into 50 mM calcium acetate the total charge drawn from the photomultiplier was about 0·1 C. Similar values were obtained by injecting 1 M-$CaCl_2$ into axons previously injected with 0·45 μl. aequorin, or by treating axons with cyanide which liberates calcium from mitochondria and 'burns up' the aequorin (page 719). Since 0·1 C was equivalent to $1{\cdot}08 \times 10^{12}$ aequorin molecules it follows that the molarity of the injection solution was

$$\frac{1{\cdot}08 \times 10^{12}}{0{\cdot}45 \times 10^{-6} \times 6 \times 10^{23}} = 4\ \mu\text{M}.$$

The final concentration inside the axon of diameter 800 μ would then be about 0·14 μM which is too small to buffer the internal calcium or to interfere with calcium movements appreciably.

From the optical density of the injection solution and the extinction coefficient given by Shimomura & Johnson (1969) the concentration of aequorin was calculated as about 100 μM, on the assumption that no other substance was present. The discrepancy between this value and the previous estimate of 4 μM may be attributed to the presence of other proteins or to inactivation of aequorin during extraction and storage.

In vitro tests of aequorin

The relation between light intensity and the concentration of ionized calcium was tested by injecting 0·18 μl. aequorin solution into a 1·5 mm diameter glass tube containing mixtures of Ca-EGTA and EGTA. As can be seen from Fig. 2 the light intensity was approximately proportional to the square of the concentration of ionized calcium, as found by Shimomura, Johnson & Saiga (1963) and Ashley (1970). Magnesium inhibits the reaction, as do other substances, for example, glutamate (Shimomura *et al.* 1962). Fig. 2 also shows results obtained with axoplasm extruded into a tube. It suggests that axoplasm contains substances which inhibit the aequorin reaction.

Voltage-clamp experiments

The internal electrode was similar to that described by Hodgkin, Huxley & Katz (1952) except that platinum wires coated with platinum black replaced the silver wires used previously. The voltage wire was exposed for about 1 cm and the current wire for 2·5–3·5 cm. Compensated feed-back was not employed and the total current rather than current density was measured. The feed-back amplifier which was lent to us by Professor E. Rojas was similar in design to that used by Atwater, Bezanilla & Rojas (1969).

Solutions

The composition of some of the external solutions employed is given in Table 1.

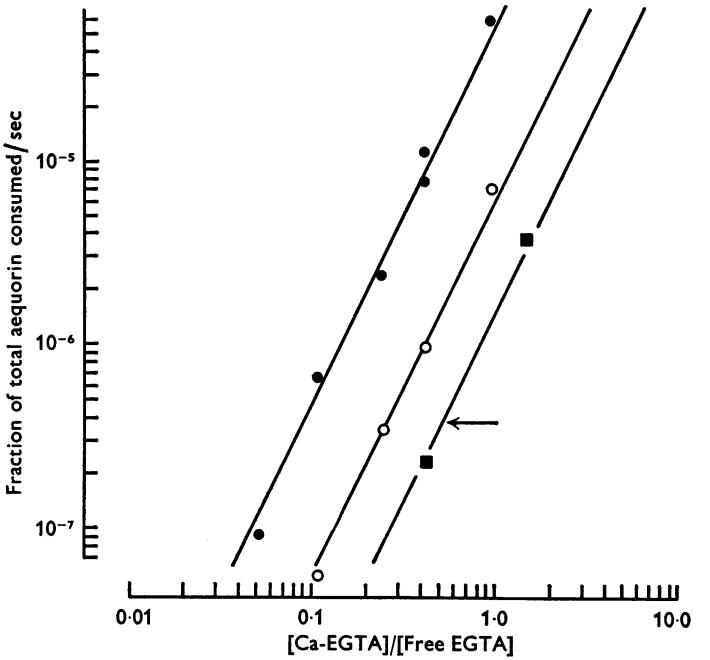

Fig. 2. Steady light production in the presence of Ca-EGTA buffers. Measurements were made by injecting 0·18 μl. aequorin into the test solution in a capillary tube of 1·5 mm diameter. The ordinate of the double logarithmic plot is the rate constant for aequorin consumption and the abscissa the ratio of [Ca-EGTA]/[free EGTA]. The parallel straight lines have a slope of 2 and were drawn through the points by eye. The total EGTA concentration was 6 mM. Three sets of determinations are shown (1) in the presence of KCl (0·55 M) and potassium phosphate buffer (16 mM) pH 7·3 (●); (2) as for (1) with the addition of 10 mM-$MgCl_2$ (○) and (3) in axoplasm extruded into a capillary (■). The arrow indicates the resting glow in the sample of axoplasm used for (3). Temp. 20° C. For line (1), 1·0 in the abscissa corresponds to a free calcium concentration of 6×10^{-7} M (Portzehl *et al.* 1964). The inhibitory action of Mg was half maximal at 1 mM.

TABLE 1. Composition of external solutions (mM)

	Solution	Ca	Mg	Na	K	Choline	Cl	HCO_3
A	Na-ASW	11	55	462·5	10	—	602	2·5
B	112 mM-Ca	112	0	402·5	10	—	634	2·5
C	44 mM-Ca	44	0	402·5	10	102	600	2·5
D	22 mM-Ca	22	0	402·5	10	135	589	2·5
E	11 mM-Ca, 90 mM-Mg	22	90	402·5	10	—	634	2·5
F	112 mM-Mg	0	112	402·5	10	—	634	2·5

Cyanide solutions were similar but contained 2 mM-CN. In Li-ASW, Li replaced Na in equimolar quantities. The pH of all solutions was near 7·8.

RESULTS

Resting axons

Level of internal ionized calcium with axons in artificial sea water (ASW). Two experiments with axons in ASW (11 mM-Ca) showed that injecting 100 mM-EGTA to give about 3 mM-EGTA in the axon reduced the resting glow to 0·17 and 0·13 of its previous value. A second injection of 90 mM-Ca–100 mM-EGTA (giving [^{45}Ca] EGTA) roughly restored the original level. In one experiment injection of 90 mM-Ca–100 mM-EGTA without previously injecting EGTA gave a transient increase to more than 4·5 times (off scale). In other experiments injection of buffers containing 10–30 mM-Ca and 100 mM-EGTA all reduced the resting glow. Although somewhat sparse this data is not inconsistent with the observation that the resting glow is roughly matched by a buffer containing 45 mM-Ca and 100 mM-EGTA. The ionized calcium stabilized by a buffer containing 45 mM-Ca and 100 mM-EGTA depends both on the pH and concentration of ionized magnesium inside the cell (Portzehl, Caldwell & Rüegg, 1964). The intracellular pH is about 7·1 but the concentration of ionized magnesium is not known. An upper limit of 0·3 μM for the concentration of ionized calcium is obtained if it is assumed that all the intracellular magnesium (10 mM) is ionized.

Effect of different external solutions

Changing the external solution from artificial sea water (ASW) with 11 mM-Ca and 55 mM-Mg to the standard high calcium solution containing 112 mM-Ca and 0-Mg increased the resting glow by an amount which varied between 1·2 and 3·0 in four axons. Replacing 11 mM-Ca ASW with a calcium-free solution containing magnesium reduced the resting glow by 40 % in one experiment.

Fig. 3 illustrates the effect of progressive replacement of external sodium by lithium and shows that total replacement of sodium increased the light production by a factor of about 3. Rather similar results were obtained

with choline. The effects were reversible (like that of high calcium) and took place with time constants of 1–3 min which was probably determined by the time required to change solutions. They fit qualitatively with the observations of Baker, Blaustein, Hodgkin & Steinhardt (1969) which showed that replacing external sodium by lithium caused a large increase in calcium influx and a decrease in calcium efflux.

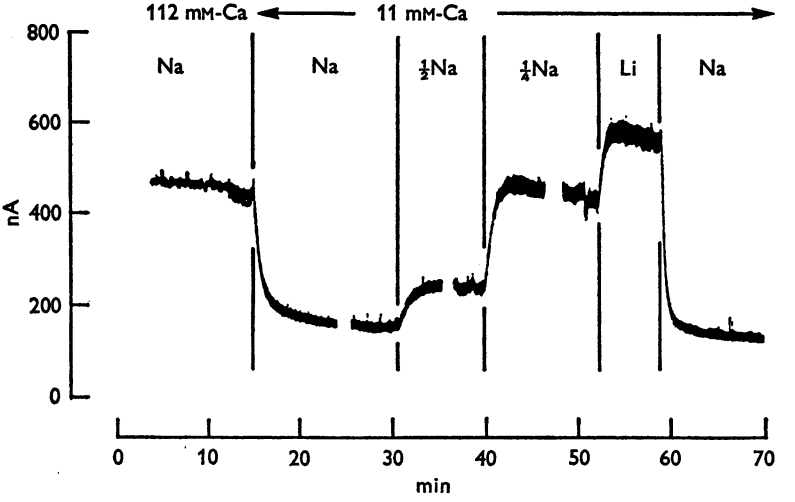

Fig. 3. Effect of ionic changes on light emitted by axon injected with aequorin. The ordinate is the output of the photomultiplier tube in nA. Axon 12; 700 μ diameter; temp. 22° C. The experiment was carried out in the flow tube apparatus described on page 712.

Effect of cyanide on resting light output

Blaustein & Hodgkin (1969) observed a large increase in calcium efflux after giant axons had been poisoned with cyanide for 1–2 hr. This effect was reversed rapidly when cyanide was removed. They also found that ^{45}Ca could be dialysed much more rapidly from cyanide-poisoned axons and concluded that poisoning with cyanide releases calcium from an internal store which was tentatively identified with mitochondria.

Fig. 4*A* illustrates the effect of 2 mM-CN on the light production by an aequorin-injected fibre. After about an hour the light output increased dramatically, and decreased promptly when cyanide was removed. A second application brought on the effect more quickly and the light output started to fall before removal of cyanide. The decline is attributed to loss of aequorin rather than loss of internal calcium since the rate constant for loss of aequorin continued to rise (Fig. 4*B*). At the end of the second exposure to cyanide the rate constant for loss of aequorin was about 1000 times greater than in the unpoisoned axon. The ordinate in Fig. 4*C*, which

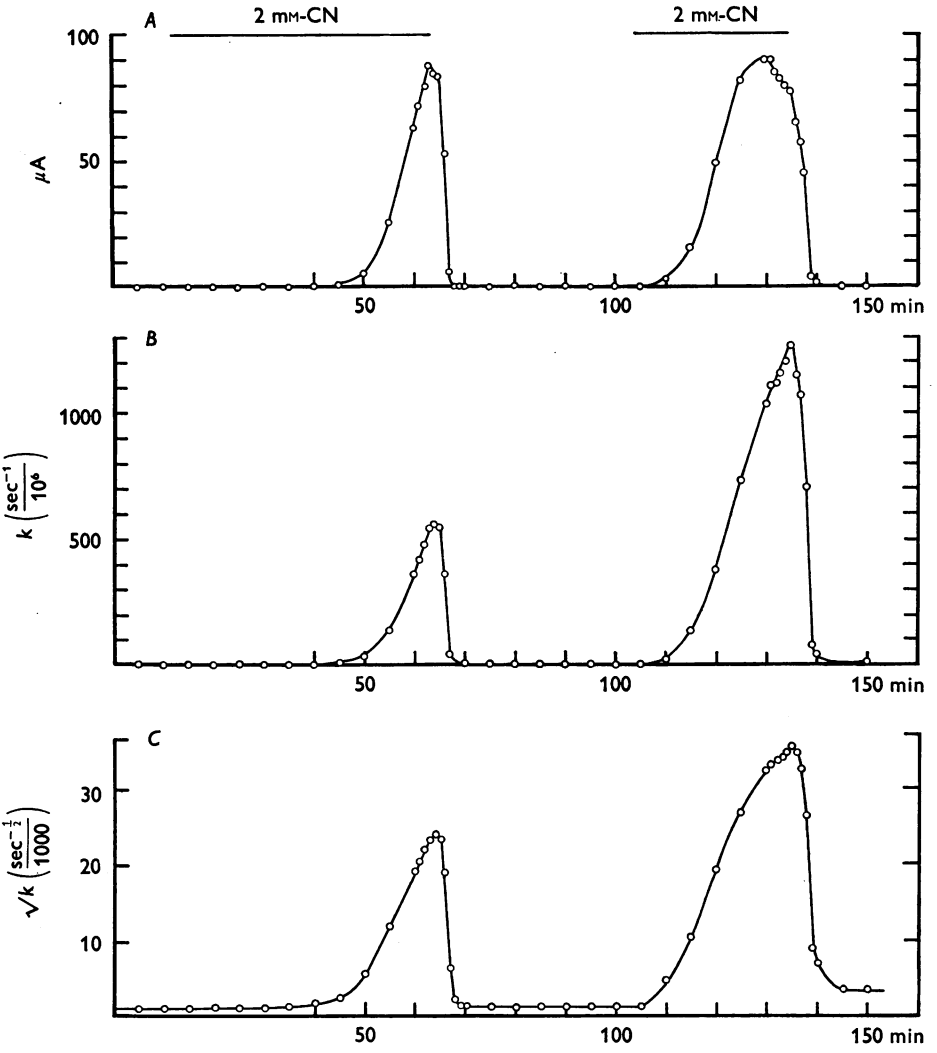

Fig. 4. Effect of cyanide on light emitted by axon injected with aequorin. Abscissa, time. Ordinate: *A*, photomultiplier output in μA; *B*, rate constant for loss of aequorin calculated as quantity of light per sec ÷ total quantity of light emitted by all aequorin present; *C*, square root of rate constant which may be proportional to concentration of ionized calcium. The amount of aequorin present at the end of the experiment was estimated by slowly injecting 1 M-$CaCl_2$ along the axis of the fibre and integrating the photomultiplier output. The points were measured from inkwriter records taken at different gains. In the upper curve the resting glow at the beginning of the experiment was 0·2 μA; in the lower curve the resting rate constant was $1{\cdot}1 \times 10^{-6}$ sec^{-1}, i.e. about 1/1000 of the peak rate constant in cyanide. Axon 2: diameter 750 μ; temp. 22° C. 2 cm of aequorin injected. Axon in artificial sea-water containing 11 mM-Ca, with 2 mM-CN present where shown.

gives the square root of the rate constant should be proportional to ionized calcium if the rate of the aequorin reaction is proportional to the first power of the aequorin concentration and the square of the ionized calcium concentration (Shimomura *et al.* 1963; Ashley, 1970 and Fig. 2 of this paper). On this basis the ionized calcium in a cyanide-poisoned axon is about 30 times greater than in an unpoisoned one.

In Fig. 5 the application of cyanide was continued for a longer period. Injection of 30 m-mole-Ca/l. axoplasm at the end of the experiment gave no light output, in contrast to the very large output seen when calcium is injected into a normal fibre. This showed that the aequorin had been used up completely during the period in cyanide, and provided further evidence

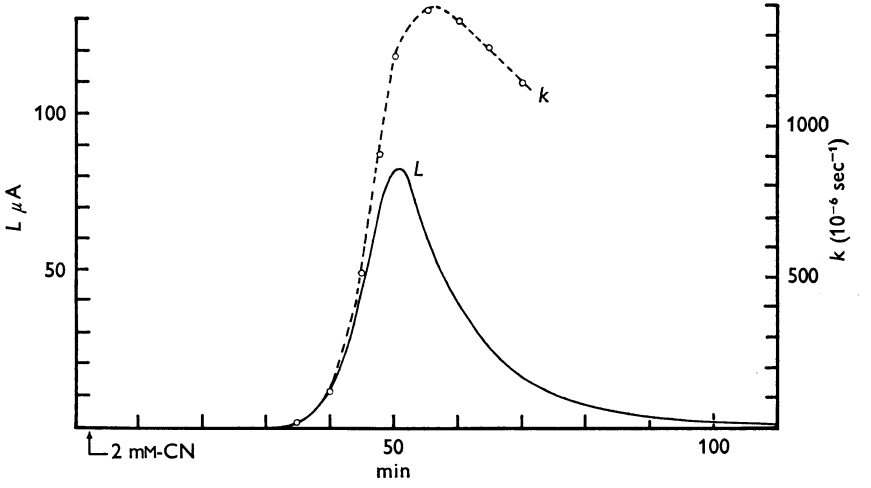

Fig. 5. Effect of cyanide. Similar to Fig. 4 but axon kept longer in cyanide. L is the output of the photomultiplier tube and k is the rate constant for loss of aequorin. The initial values of L and k were 0·15 μA and $1{\cdot}5 \times 10^{-6}$ sec^{-1} respectively.

that the falling part of the curve was mainly due to loss of aequorin. A single experiment showed that 0·2 mM-DNP at pH 7 had an effect similar to that of cyanide.

Injecting 1 m-mole ATP/l. axoplasm at the peak of the cyanide effect decreased the light intensity to approximately its resting level, and 30 μmole ATP/l. axoplasm reduced it to about one third (Fig. 6*A*). The decrease in light intensity was transient as would be expected since cyanide prevents resynthesis of ATP. Injecting 30 μmole ATP/l. axoplasm into an unpoisoned axon had no effect on light intensity.

The reversal of the cyanide effect might be due either to combination of ATP with calcium or to utilization of ATP by the system which trans-

ports calcium into the internal store. The first alternative seems unlikely since 30 μM-ATP should have little direct effect on a total calcium concentration of 400 μM. Stronger evidence was obtained by repeating the whole experiment in the presence of internal and external oligomycin. According to Lehninger, Carafoli & Rossi (1967) oligomycin prevents ATP, but not electron transport, from concentrating calcium in mitochondria.

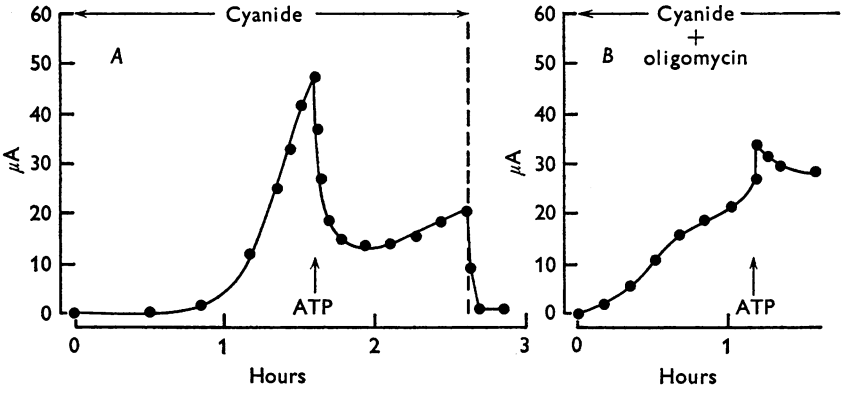

Fig. 6. Injection of ATP into cyanide-poisoned axons. In *A*, the axon was exposed to ASW containing 2 mM-CN at zero time and was returned to cyanide-free ASW at the time marked by a vertical dashed line. In *B*, the axon was pre-treated for 30 min with oligomycin both by injection to give a final concentration of 50 μg/ml. and also inclusion of 5 μg/ml. in the ASW. At zero time 2 mM-CN was added to the oligomycin-ASW bathing the axon. In both *A* and *B*, ATP was injected at the vertical arrows, to give a final concentration of 30 μM. Both axons contained 0·18 μl. aequorin. Diameter of axon *A*, 650 μ and *B* 850 μ. Temp. 21° C.

In the presence of oligomycin the light intensity should rise fairly promptly when cyanide is applied and injected ATP should no longer be effective. Fig. 6*B* shows that both predictions were verified. The light intensity started to increase within a few minutes of applying cyanide and was not reduced by a subsequent injection of ATP.

Evidence that the cyanide effect depends on a release of internal calcium was provided by the observation that the light intensity increased to a high level after removing calcium from the external solution. In this experiment calcium was removed about half way up the cyanide response and the fact that the light intensity continued to increase shows that the calcium involved near the peak of the aequorin reaction is not coming from the external solution. However, there may be conditions in which external calcium does affect the development of the cyanide response since the effect was small and prolonged in a calcium-free cyanide solution containing 112 mM-Mg and 400 mM-Na. In another experiment, replacing 112 mM-Ca–400 mM-Na with 112 mM-Mg–400 mM-Na checked the development of the cyanide response but the light intensity subsequently rose to a high level in a cyanide solution con-

taining only NaCl. The conclusion from this incomplete series of experiments is that the cyanide effect can take place in the absence of external calcium but it does so less regularly and repeatably than in the presence of external calcium.

Stimulated axons

Calcium entry during a train of action potentials. The effect of stimulation at various frequencies on the light produced by an axon in a solution containing 112 mM-Ca is shown in Fig. 7*A*. At the beginning of the train,

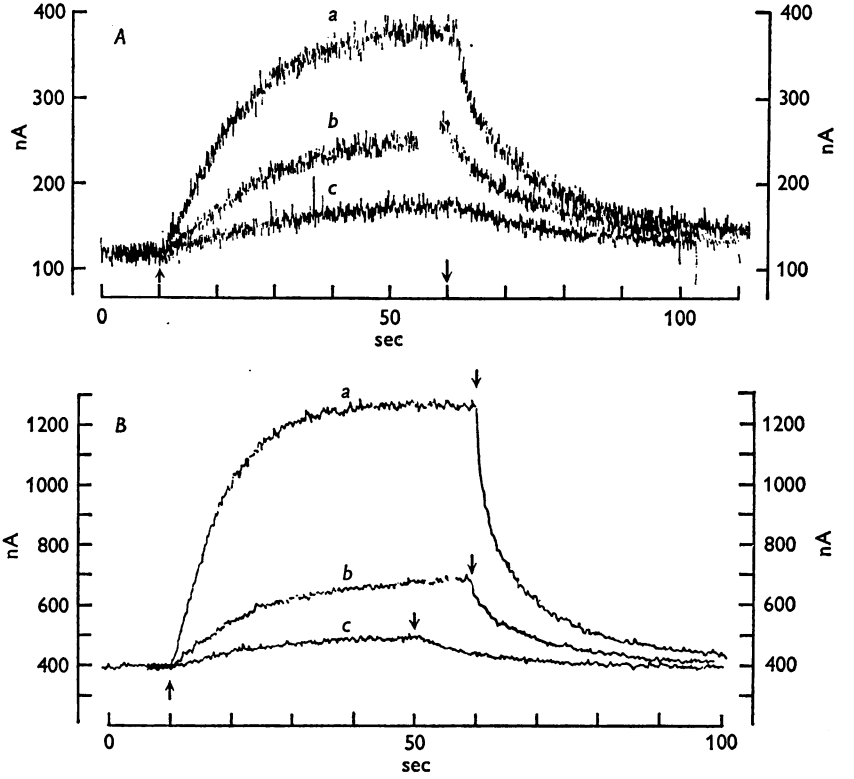

Fig. 7*A*. Inkwriter tracings of increase in light intensity produced by stimulating the fibre at the following frequencies: record *a*, 210/sec for 50 sec; *b*, 105/sec for 80 sec (30 sec gap in record); *c*, 53/sec for 50 sec. The beginning and end of the train are marked by arrows. The ordinate shows the photomultiplier output in nA (the resting glow was 115 nA). Axon 15; diameter, 690 μ; from live squid; records taken 3·5 hr after injecting 0·36 μl. aequorin over 2 cm; 22° C. The axon was stimulated at the gate and action potentials were recorded from the cannula. The external solution was 112 mM-Ca ASW.

B. Similar to Fig. 7*A* but using platinum spiral electrode and membrane action potentials rather than propagated action potentials. 10 sec gap in record *b*. Axon 43 from refrigerated mantle; records taken 4 hr after injecting 0·45 μl. aequorin over 2·5 cm; axon diameter 725 μ; 112 mM-Ca ASW. 18° C.

the light increased exponentially to a steady level which was maintained throughout the period of stimulation; it returned exponentially to its resting value with a time constant of 10–15 sec when the stimulus was switched off. Recovery was probably due to uptake by the internal store, and the steady level during the tetanus represents a balance between increased inflow at the surface and increased uptake by the internal store. The recovery time constant varied between 7 and 25 sec with a mean of 12 sec in eight axons in which the response was approximately proportional to frequency. Recovery was not strictly exponential and the return to the final level was often much slower than the initial fall.

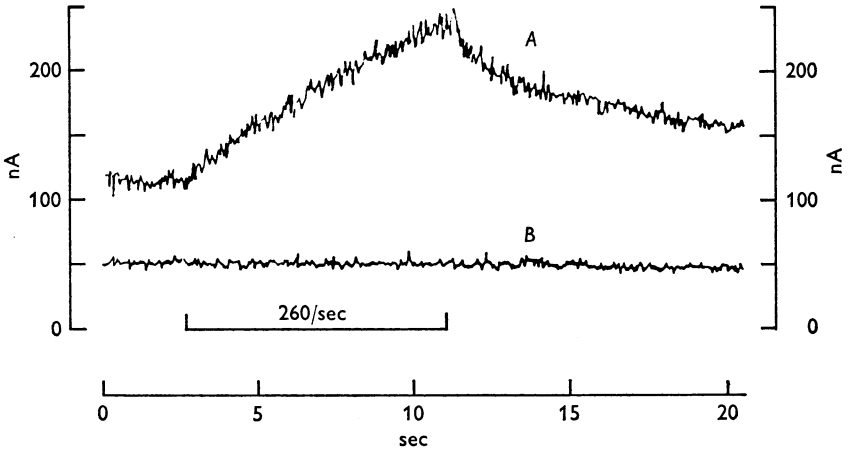

Fig. 8. Effect of injecting EGTA on light response to stimulation. *A*, before injecting EGTA; *B*, 30 min after injecting sufficient EGTA to raise the concentration in the axon by 5 mM. Electrical recording in the cannula showed that in both *A* and *B* action potentials were propagated without failure through the aequorin region at 260/sec. 0·36 μl. aequorin injected over 1 cm, 100 mM-EGTA injected over 3 cm overlapping aequorin region by 1 cm at either end. Axon 15; diameter 690 μ; from live squid. Record *A* taken 2·5 hr after injecting aequorin; record *B*, 1 hr later, and 30 min after injecting EGTA. Temp. 22° C; 112 mM-Ca ASW. Biomac records giving average response of sixteen sweeps spaced at 100 sec intervals.

In Fig. 7*A* the initial rate of rise and the steady displacement during the tetanus were roughly proportional to the frequency of stimulation. Many axons did not behave in this simple way and in several experiments, of which Fig. 7*B* is an example, the response was proportional to the square of the frequency. This variability, which complicated quantitative analysis, is considered further on page 726.

Fig. 8 shows that injecting sufficient EGTA to give a concentration of 5 mM in the axon abolished the light response to stimulation; the injection had no obvious effect on the action potentials. Presumably EGTA binds

any calcium that enters and prevents it affecting the aequorin. Replacement of external calcium by magnesium abolished the light response but not the action potential. (Axons sometimes become inexcitable in calcium-free magnesium solutions but the experiment was done in a fibre which retained excitability in the magnesium solution.) These experiments, together with others to be described later (Table 3), support the idea that the aequorin response of stimulated fibres is caused by entry of calcium.

It is important to realize that the aequorin response is probably caused by a change in ionized calcium in the peripheral regions of the axon. If calcium ions are taken up by mitochondria with a rate constant of 10^{-1} sec^{-1} they should not diffuse more than about 100 μ from the surface. As shown in the Appendix the steady rise in ionized calcium will be attenuated in an approximately exponential manner with a space constant of $\sqrt{(D/\kappa_1)}$ where D is the diffusion coefficient of free calcium, and κ_1 is the rate constant for uptake of calcium by the internal store. If D is taken as $6{\cdot}4 \times 10^{-6}$ cm^2/sec (cf. Blaustein & Hodgkin, 1969) and κ_1 as 0·1 sec^{-1} the space constant is 80 μ. The theoretical distribution of free calcium in the steady state and at various times after the beginning of the tetanus are shown in Fig. 22 (Appendix). With a brief train of action potentials the change is confined to the extreme periphery of the cell.

Radial diffusion of aequorin

Indirect evidence that the rise in ionized calcium is confined to the cortical regions of the cell is provided by the experiment of Fig. 9*A*. The axon was tested with a train of shocks of frequency 250/sec and duration 8 sec, at various times after an axial injection of aequorin. As can be seen from the Figure, there was no light response immediately after the injection and it took about 5 min for the response to reach half its final amplitude. The points are reasonably well fitted by the theoretical curve

$$\frac{y(a, t)}{y(a, \infty)} = 1 + \sum_{n=1}^{\infty} e^{-\alpha_n^2 D't/a^2}/J_0(\alpha_n) \qquad (4)$$

where $y(a, t)$ is the concentration of aequorin at time t at the edge of an insulated cylinder of radius a; α_n is a positive root (> 0) of $J_1(\alpha) = 0$, J_0 and J_1 are Bessel functions and D' is the diffusion coefficient which was taken as $6{\cdot}1 \times 10^{-7}$ cm^2/sec. This curve gives the concentration of a substance at $r = a$ as a function of the time after an instantaneous axial injection at $t = 0$ and $r = 0$ (Carslaw & Jaeger, 1959). The value for D' is likely to be an over-estimate since the initial column of aequorin had a radius of about 75 μ and the rise in calcium was not confined to the extreme periphery as assumed in the theory. Allowance for these factors might reduce D' to about 4×10^{-7} cm^2/sec. This seems a reasonable value since

Shimomura *et al.* (1969) give the diffusion coefficient of aequorin in free solution as $8{\cdot}7 \times 10^{-7}$ cm^2/sec and a lower value is to be expected in axoplasm.

Fig. 9*B* shows how the resting glow alters after an axial injection of aequorin. The first point to notice is that the glow immediately after the injection was less than that recorded with the column of aequorin still

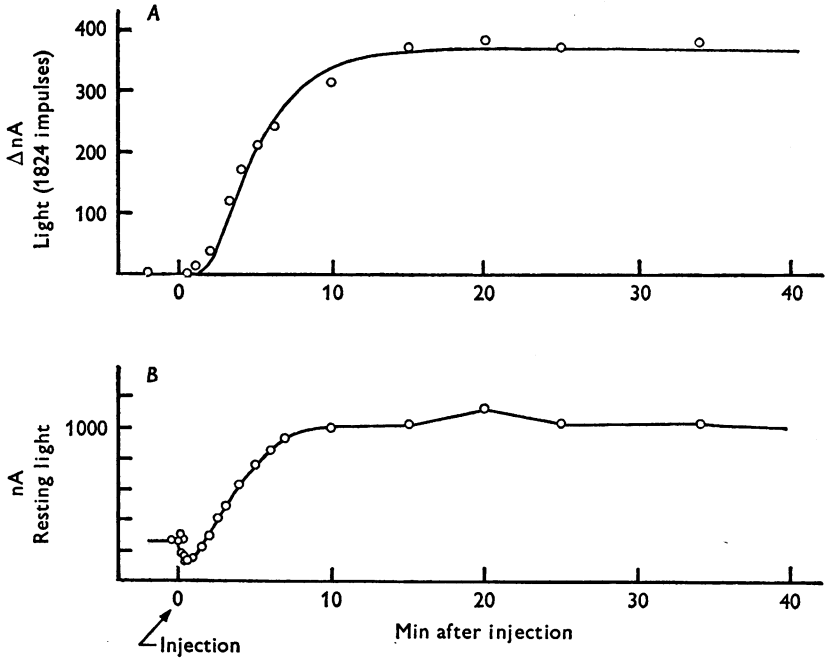

Fig. 9. Development of response to stimulation after axial injection of aequorin. *A*, increment in photomultiplier current produced by 1824 impulses (228/sec for 8 sec) at various times after injection. The smooth curve is drawn from eqn. (4) with $D'/a^2 = 0{\cdot}00036$ sec^{-1}.

B. resting photomultiplier current at various times after injection. Axon 38; diameter 825 μ; temp. 20° C. 112 mM-Ca ASW.

inside the glass capillary. Presumably, calcium contamination of the aequorin column gave a higher ionized calcium concentration than the very low concentration in axoplasm, so the initial effect of injection was to lower the light intensity. During the next 10 min the resting glow increased in much the same way as the response to stimulation. This slow rise was not observed in other experiments in which the axons were in artificial sea water (11 mM-Ca), and is explained by assuming that the axon in 112 mM-Ca was steadily gaining calcium. In that case the distribution of ionized calcium should be the same as the steady distribution

during a period of stimulation and the increase in resting glow can be attributed to diffusion of aequorin into the peripheral regions where the calcium concentration is higher than on the axis.

Progressive changes in the response of aequorin to stimulation

One puzzling feature of the light response to stimulation is that it increased during the course of a long experiment. Fig. 10 is a continuation of Fig. 9 but covers a much longer period of time. Throughout the experi-

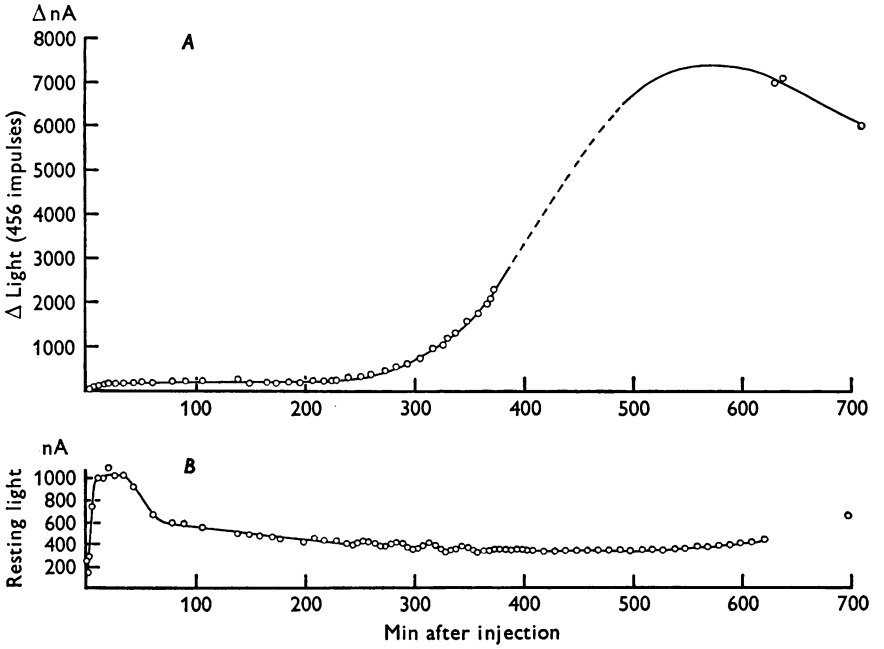

Fig. 10. Increase in light response during course of long experiment. *A*, increment in photomultiplier current produced by 456 impulses (228/sec for 2 sec) at different times after injection. The experiment was left running overnight and the gap between 380 and 620 min occurs because the response went off the scale on the recorder. *B*, resting photomultiplier current in the same experiment. The experiment is a continuation, on a slower time scale and reduced sensitivity (*A*), of that in Fig. 9.

ment the axon was stimulated with trains of frequency 228/sec and duration 2 sec; the interval between bursts was usually 12 min. The initial rise has already been considered in Fig. 9 and is attributed to diffusion of aequorin. There is then a period when the response remained relatively constant, but after several hours the response started to increase again and eventually reached a value which was 35 times greater than that observed 30–120 min after the injection. Similar effects were observed in other

experiments as may be seen from Table 2. The increase in the response happened without much change in the resting glow and did not depend on loss of excitability. It is not clear whether the effect depended on the time elapsed after injection or on the time for which the axon was kept at room temperature. Nor is it certain that the effect depended on a genuine increase in calcium inflow per impulse rather than on something which affects the over-all sensitivity of the aequorin to calcium entry at the surface. The majority of the experiments were carried out during the period between 1 and 4 hr after injection when the response was fairly constant, but occasionally it was convenient to use fibres in a very sensitive condition.

Magnitude and frequency dependence of the response of aequorin to stimulation

A convenient index of the aequorin response is the quantity $\Delta L/L_R$ which is tabulated in column 6 of Table 2. L_R is the resting light intensity and ΔL is the increment for one impulse in a train of frequency 200/sec, estimated by dividing the initial rate of rise by the frequency. As can be seen from the Table, $\Delta L/L_R$ varied from $0{\cdot}2 \times 10^{-3}$ to 400×10^{-3} in twenty-one experiments, in which the resting glow varied from 160 to 900 nA. The very high values listed in the right-hand part of Table 2 were observed at the end of experiments lasting 5–12 hr and provide further examples of the progressive change described in the previous section.

Fig. 11 is a double logarithmic plot of the relation between the initial rate of rise of the light response and the frequency of stimulation in five axons. The points are reasonably well fitted by straight lines but the slope of the lines varies between 1 and 2. From Table 2 it can be seen that fibres with large light responses gave slopes of about 2 whereas those with small light responses gave values close to unity. One possible explanation is that there is a genuine variation in calcium entry and that in all cases two calcium ions are required to trigger the aequorin reaction. When the calcium entry is high both calcium ions are provided by impulses, but when it is low, one is provided by an impulse and the other by the resting background inside the axon. In the first case the increment in light intensity after a given time might be proportional to the square of the frequency and in the second to the first power. To make this more quantitative, suppose that stimulation causes the concentration C of ionized calcium in some region to change from C_R to $C_R + \Delta C$ in one second. If the rate of the aequorin reaction is proportional to C^2, the increment in light emitted from that region will be proportional to $2C_R\Delta C + (\Delta C)^2$. A linear dependence on frequency is expected when $C_R \gg \Delta C$ and a square-law dependence when $\Delta C \gg C_R$.

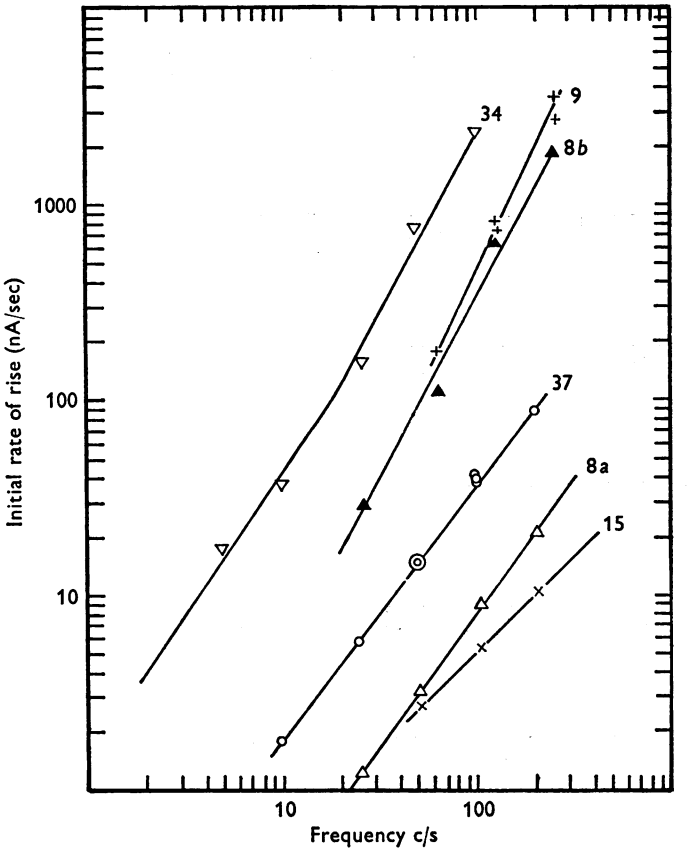

Fig. 11. Relation between initial rate of rise of light intensity and frequency of stimulation in five axons, with both scales logarithmic. Numbers are axon numbers and further details can be obtained from Table 2. Lines 8*a* and 8*b* are from the same axon at 1·5 hr after injection (8*a*) and 7 hr after injection (8*b*). The external medium was 112 mM-Ca ASW.

Comparison of effects of stimulation and axial injection of calcium

In Fig. 12 the aequorin response to an injection of 0·18 μl. 100 μM-$CaCl_2$ is compared with the responses to stimulation at 210 and 105/sec. The period of stimulation was 5 sec and the injection was carried out in approximately the same time. According to Hodgkin & Keynes (1957) the calcium entry per impulse from a solution containing 112 mM-Ca is 0·083 p-mole cm^{-2}. The quantity entering a 750 μ diameter axon in 1050 impulses should therefore be $2{\cdot}0 \times 10^{-11}$ mole/cm length of axon which is similar to the quantity injected $-1{\cdot}8 \times 10^{-11}$ mole/cm. However, as can be seen from Fig. 12 the injected calcium gave a deflexion about 200 times greater than that produced by 1050 impulses. Part of the discrepancy is explained by the fact that the injected calcium is confined in a relatively small area and should give more light if the aequorin reaction is proportional to the square of the calcium concentration. But it is difficult to attribute the whole difference to this cause. Since the diameter of the

TABLE 2. Effect of stimulation on light produced by axons injected with aequorin

1	2	3	4	5	6	7	8	9	10
Axon	Diameter (μ)	Time (hr)	L_R (nA)	n	$10^3 \Delta L/L_R$	Time (hr)	L_R (nA)	n	$10^3 \Delta L/L_R$
7	600	1·0	270	1·5	0·6	9	175	2·0	189
8	690	1·5	225	1·4	0·6	7	169	2·0	40
9	690	0·7	300	2·0	10·8	6	160	2·2	400
10	750	2·5	292	2·4	2·1	9	302	—	15
12	700	2·5	900	1·1	0·5	—	—	—	—
15	690	1·5	169	1·0	0·38	—	—	—	—
38	825	1·3	527	—	0·8	11	426	2·3	31
23	780	1·5	240	1·1	0·25	—	—	—	—
24	760	3·0	720	1·4	1·2	—	—	—	—
25	870	3·0	227	1·8	4·8	5	186	1·9	21
26	670	2·5	441	1·7	1·0	—	—	—	—
27	700	3·7	510	1·7	1·7	—	—	—	—
28	740	3·3	330	1·0	0·7	—	—	—	—
29	940	2·3	180	1·7	3·9	5·3	190	2·0	7·4
30	710	4·5	314	1·9	1·1	—	—	—	—
31	890	2·5	162	1·9	2·5	4·3	144	2·0	12·5
32	820	2·0	340	1·3	1·4	—	—	—	—
33	1030	2·7	300	1·6	1·4	—	—	—	—
34	860	2·7	182	2·1	11·5	7	780	2·0	62
35	850	2·5	333	1·3	1·2	—	—	—	—
43	714	3·8	400	2·0	5·0	—	—	—	—

Columns 3 and 7 give the time after injecting aequorin at which the measurements were made. n (columns 5 and 9) gives the frequency dependence of the light response and is defined by the equation, response $= K$ (frequency)^n where K is a constant. L_R is the resting light given as the output current of the photomultiplier and $\Delta L/L_R$ gives the increment in light per impulse relative to the resting light at a frequency of 200/sec; column 6 was obtained at the beginning of an experiment and column 10 at the end. Propagated action potentials were used in the first group (axons 7 to 38) and membrane action potentials in the second (axons 23 to 43). Membrane action potentials were started by applying a brief current pulse to the current wire in the double spiral electrode.

In most of the experiments 0·45 μl. aequorin was injected over 2·5 cm; in other cases L_R has been corrected for the amount injected.

The external solution was always 112 mM-Ca ASW; temp. 18–23° C.

injection capillary was 150 μ the radius of the region occupied by injected calcium is unlikely to be less than 75 μ. In the steady state, calcium entering at the surface gives a rise of concentration which falls off exponentially with a space constant of about 80 μ (page 723). In a fibre of diameter 750 μ the calcium entering at the surface might occupy about 8 times more area and on a square-law basis this should give one eighth as much light as injected calcium. Another possible explanation of

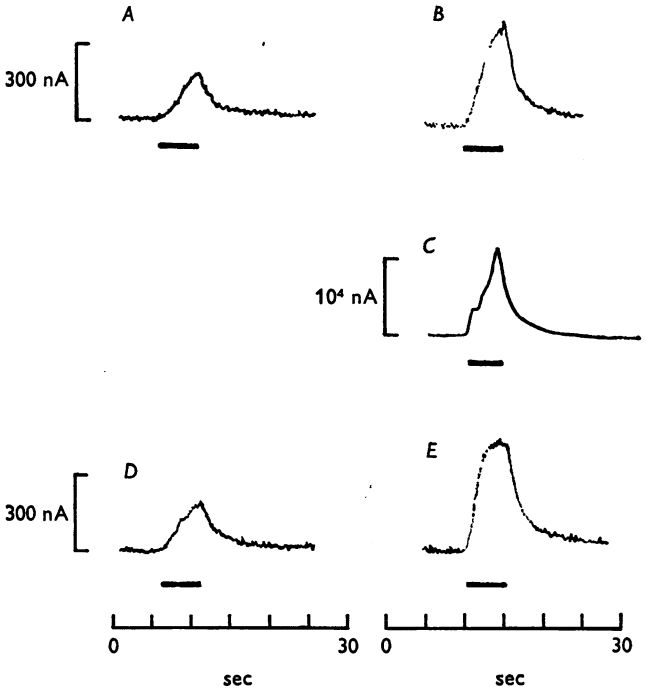

Fig. 12. Comparison of a tetanus with axial injection of $CaCl_2$. *A* and *D*, 100/sec; *B* and *E*, 200/sec; *C*, roughly axial injection of $1{\cdot}8 \times 10^{-11}$ mole $CaCl_2$/cm. The injection overlapped the patch of aequorin by about 1 cm at each end. Records were obtained in the order *A* to *E*. The horizontal bars represent the period of stimulation or in the case of *C* the time taken for the injection to be completed. Note the lower gain for record *C*. Axon diameter 750 μ. Temp. 20° C.

the discrepancy is that the axon might contain a limited amount of material which combines with calcium ions. This might have relatively less effect when the calcium ions were concentrated than when they were dilute. An alternative is that there might be more calcium combining groups at the surface, possibly on the membrane, than in the middle of the axon.

Effect of different external calcium concentrations on the aequorin response to stimulation

Hodgkin & Keynes (1957) found that the calcium entry per impulse varied with the external calcium concentration; at 20° C the entries were

0·083, 0·026 and 0·01 p-mole/cm^2 in 112, 44 and 22 mM-Ca (zero Mg in all cases); a different set of experiments gave the entry in artificial sea water (11 mM-Ca, 55 mM-Mg) as 0·006 p-mole/cm^2.

The experiments in Table 3 which were an attempt to check this result illustrate one of the difficulties inherent in the aequorin method. Axon 12 gave a light response which was proportional to frequency. The responses in 44 mM-Ca (0 Mg) and in 11 mM-Ca 55 mM-Mg were 0·33 and 0·02 of that in 112 mM-Ca–0 Mg. On a linear basis the result obtained with 44 mM-Ca agrees with that of Hodgkin & Keynes (1957) but the entry in 11 mM-Ca–55 mM-Mg is too small. However, this comparison may not be valid. If the rate of the aequorin reaction is proportional to the square of the ionized calcium concentration (C) the increment of light intensity (ΔL) from a given region is related to ΔC by

$$\Delta L = k(2C_R\Delta C + (\Delta C)^2) \tag{5}$$

where k is a constant and C_R is the resting calcium concentration. Linearity is then explained by assuming that $C_R \gg \Delta C$. The resting calcium concentration was almost certainly reduced in the low calcium solutions, but the change cannot be calculated from the reduction in resting glow since the concentration of ionized calcium is probably not uniform over the fibre. This makes it impossible to say whether or not the results in Table 3 are quantitatively consistent with those of Hodgkin & Keynes.

Time course of aequorin response in 'sensitive' axons

As mentioned previously axons which had been kept at room temperature for 5–10 hr gave large light responses which were proportional to the square of the frequency of stimulation. The responses of these 'sensitive' axons to short trains of impulses had a characteristic appearance which is illustrated by the Biomac records in Fig. 13. After an initial delay of about 0·05 sec the light intensity increased linearly with a slope proportional to the square of the frequency. At the end of the train the response declined rapidly with an initial half-time much shorter than that seen with a long train. This rapid decline is most easily explained by inward diffusion of calcium from the surface. If light intensity were proportional to the concentration (C) of ionized calcium, redistribution of ionized calcium would not cause any decline in light intensity. However, if the light intensity were proportional to the square of the concentration there should be a decline since the quantity $\int_0^a rC^2 dr$ decreases as diffusion proceeds.

TABLE 3. Effect of calcium concentration on calcium entries during stimulation

Axon	[Ca] (mM)	[Mg] (mM)	[Na] (mM)	L_R (nA)	ΔL (pA/impulse)	n
12	112	0	400	835	286	1·1
	11	55	460	440	13	—
	112	0	400	1020	450	—
	44	0	400	500	143	—
	112	0	400	825	411	—
25	112	0	400	227	1080	1·8
	11	55	460	126	24	—
	112	0	400	186	3900	1·9
28	112	0	400	330	230	1·0
	112	0	400	400	—	—
	22	0	400	230	66	—
	22	0	400	270	—	—
	22	90	400	280	26	—
29	112	0	400	180	710	1·7
	44	0	400	135	450	—
	22	0	400	130	220	—
	112	0	400	185	1160	—
31	112	0	400	162	400	1·9
	112	0	400	136	—	—
	0	112	400	64	0	—
	112	0	400	144	1800	2·0

L_R is the resting light and ΔL is the increment in light per impulse at a frequency of 200/sec. Propagated action potentials were used in axon 12 and membrane action potentials in the other cases; n is the frequency dependence defined as in Table 2; temp. 19–22° C.

Timing of aequorin responses in 'sensitive' axons

Although the axons which gave large light responses must be regarded as somewhat abnormal they were useful for studying the timing of the aequorin response. In Fig. 14, trace *A* shows the effect of 7 impulses and trace *B* of 13 impulses; the externally recorded diphasic action potentials recorded from the middle of the aequorin region are shown in *a* and *b*. As expected in a square-law fibre the effect of 13 impulses is more than twice that of 7.

The approximate effect of the last 6 impulses, obtained by subtraction and smoothing, is shown by the continuous curve in *C*. The broken curve was calculated by the subtangent method (Lucas, 1912) on the assumption that there is an exponential delay of 10 msec in the response of aequorin to a sudden change of calcium, as found by Hastings *et al.* (1969). The procedure is approximate, because of the non-linearity of the aequorin reaction, but it suggests that in this experiment the inflow of calcium did

not lag behind the action potentials by more than a few msec. More precise but less direct information about the timing of the inflow can be obtained by the voltage clamp experiments described in the next section.

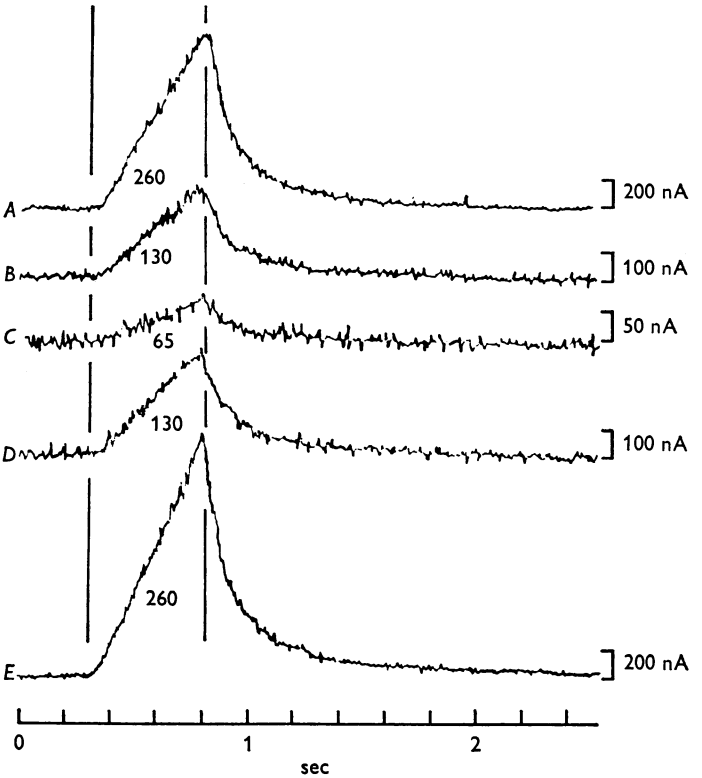

Fig. 13. Tracings of Biomac records showing light response to train of action potentials lasting 500 msec at frequency shown; sixteen trains were summed in *C* and eight in the other records, the calibrations apply to a single train. The spikes, which are not shown, were recorded from the cannula. Axon 9; diameter 690 μ; temp. 21° C; recording time constant 1 msec; 0·36 μl. aequorin injected over 2 cm; records taken 4·5 hr after injection. The resting light was 110 nA. The external solution was 112 mM-Ca ASW.

Voltage-clamp experiments

Evidence for two phases of calcium entry. Fig. 15 illustrates pen-recordings of the light responses produced by applying pulses of amplitude 80 mV and duration 20–900 μsec to a fibre which had been injected with aequorin. The frequency of the pulses was 200/sec, the duration of the train about 10 sec and the interval between trains about 2 min. Oscilloscope records of the current and voltage for single pulses were taken at each duration

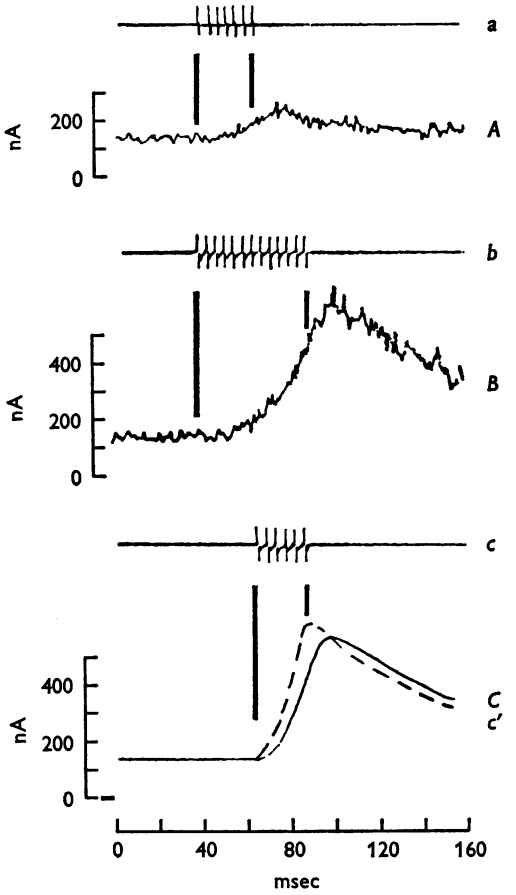

Fig. 14. Tracings of Biomac records showing light responses to short trains of action potentials at a frequency of 250/sec; eight trains were summed before plotting; the calibration is for a single train. The axon was in the 'flow tube' and the action potentials were recorded diphasically from electrodes spaced 3 mm apart in the middle of the aequorin region.

a, *b*, action potentials shown in the correct position but somewhat diagrammatically with shock artifacts omitted; *c*, last six action potentials in train *b*.

A, light response to 7 impulses.

B, light response to 13 impulses.

C, light response associated with last six impulses in *B* obtained by subtracting *A* from *B* (after smoothing).

c' (dashed curve), possible time course of calcium transient calculated for a total exponential lag of 10 msec in the aequorin reaction.

The smoothing time constant in the photomultiplier input was about 1 msec. Axon 7; diameter 600 μ. Records taken 6 hr after injection; temp. 23° C; 112 mM-Ca ASW. The light response per impulse was unusually large in this experiment.

and are illustrated in Fig. 16. Visual checks on the cathode ray tube indicated that there were no appreciable changes in current or voltage wave form during repetitive stimulation.

The middle column in Fig. 15 was obtained in the presence of 0·8 μM tetrodotoxin (TTX), the left- and right-hand columns show the responses before and after applying TTX. It can be seen that the aequorin response to a 100 μsec pulse is reversibly abolished by TTX but that an appreciable fraction of the response to pulses longer than 300 μsec persists in TTX.

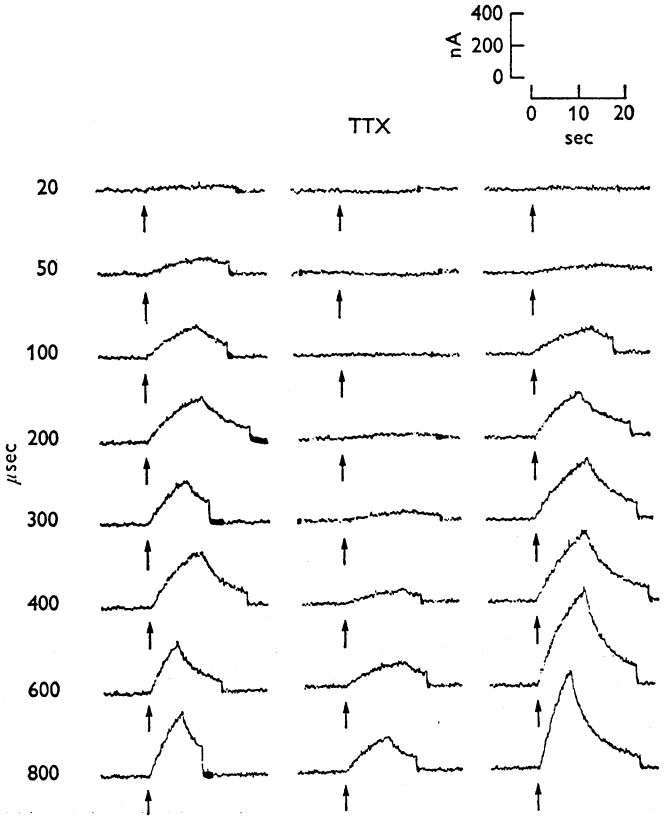

Fig. 15. Inkwriter records of aequorin responses to trains of pulses of different duration before (left), during (middle), and after (right) treatment with 0·8 μM-TTX. The duration of the pulse, in μ sec, is shown at the left; the amplitude was 80 mV throughout; the frequency was 200/sec and the duration of the train was 6–20 sec (usually 10 sec). The resting glow was 420 nA at the beginning of the experiment and remained at 360–420 nA during the whole experiment which lasted 4·5 hr. The experiment was started 2·5 hr after injecting 0·45 μl. aequorin over 2·5 cm. Axon 26; diameter 670 μ; external solution 112 mM-Ca ASW; temp. 22° C at beginning of experiment; electrical smoothing time constant 30 msec. The recorder was slowed by a factor of 60 during the recovery phase.

The relation between the light response and pulse duration is illustrated by Fig. 17. The left-hand set of curves is for an 80 mV pulse, which gave an inward sodium current, and the right-hand set is for a 120 mV pulse which gave an outward sodium current. The equilibrium potential for the early current (V'_{Na}) was about 110 mV in this experiment. The ordinate,

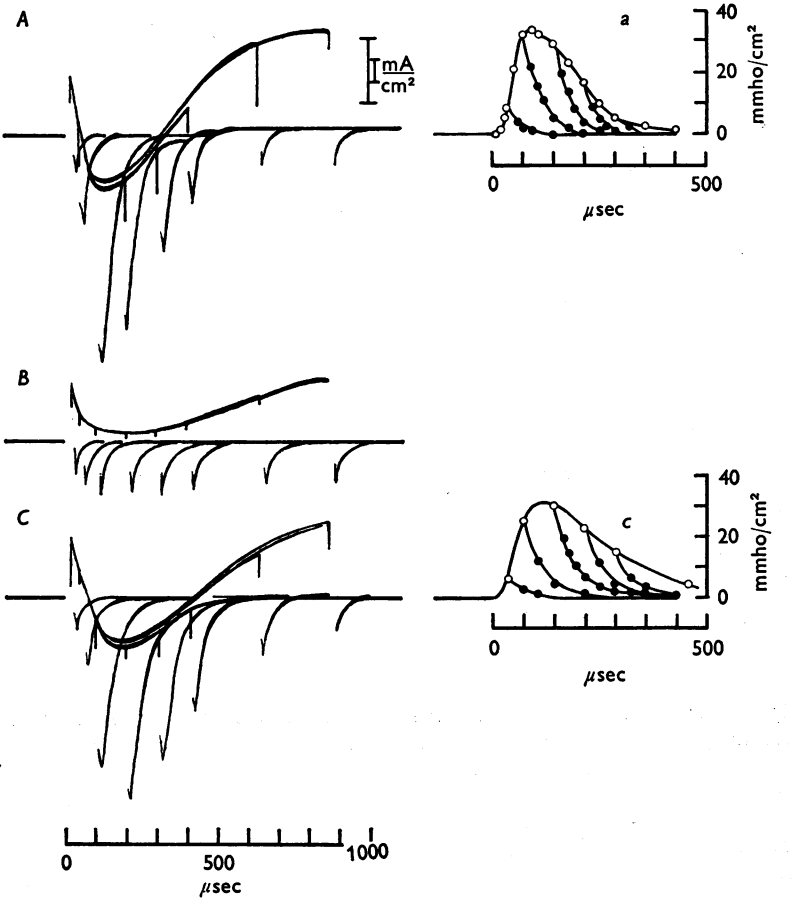

Fig. 16. Membrane currents produced by 80 mV pulses of different duration; *A*, normal; *B*, in presence of 0·8 μM-TTX; *C*, after removal of TTX. The time course of the sodium conductance is shown by curves *a* and *c*. From the experiment of Fig. 15. The temperature was 22° C at the beginning of the experiment but may have been 2–3° C lower at the end.

which gives the light per pulse relative to that produced by an action potential was defined as

$$\frac{\text{Initial rate of rise with duration } t' \text{ at 200/sec}}{\text{Initial rate of rise with action potentials at 200/sec}}.$$

Curves of similar shape were obtained if the increment in light intensity after 5 sec was taken as an index of the response.

The shape of the curves in Fig. 17 and the effects of TTX imply that calcium entry may be divided into an early component which is abolished by TTX and a late component which is unaffected by it. An approximate

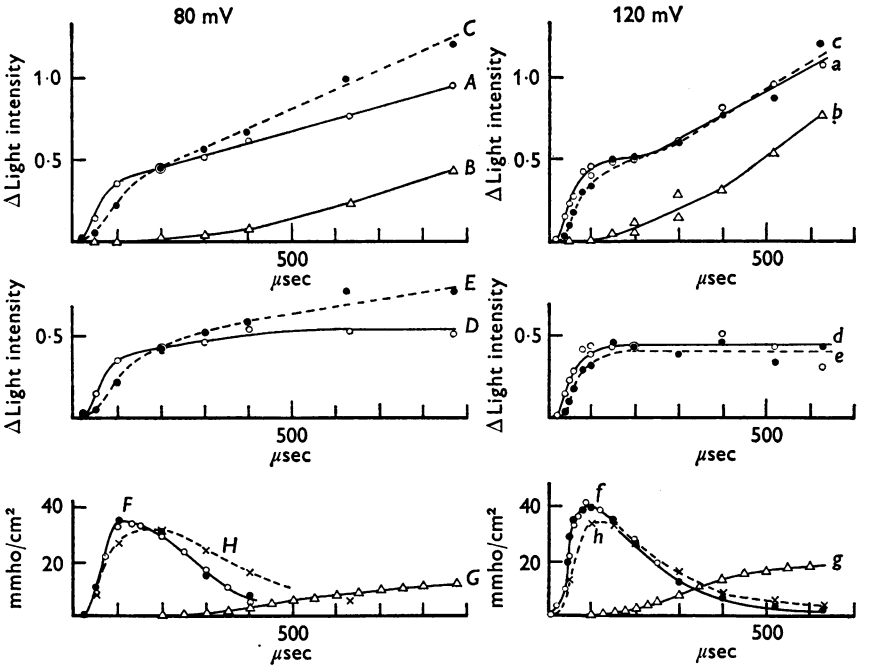

Fig. 17. The upper curves show the relation between the pulse duration (abscissa) and the increment in light intensity per pulse; *A*, *a* before TTX; *B*, *b* in TTX; *C*, *c* after removal of TTX; *A*, *B*, *C* 80 mV; *a*, *b*, *c* 120 mV; the ordinate was measured as the initial rate of rise of light intensity at 200 pulses/sec ÷ the initial rate of rise of light intensity at 200 action potentials/sec. For the middle curves the ordinate is the TTX-sensitive component of the calcium entry obtained as $D = A - B$ or $d = a - b$ before TTX and $E = C - B$ or $e = c - b$ after TTX. The bottom curves give the sodium conductance as a function of time before (*F*, *f*) and after (*H*, *h*) treatment with TTX; (○) by subtraction; (●, ×) from amplitude of tails. Curves *G* and *g* give the time course of the potassium conductance estimated from the records in TTX. *F*, *H*, *G* 80 mV; *f*, *h*, *g* 120 mV. From the experiment of Fig. 15.

estimate of the shape of the early component may be obtained by subtracting the response curve in TTX (*B*) from the normal response curve (*A* or *C*). This procedure depends on the assumption that the light per pulse is proportional to the rise in calcium concentration. The assumption

was probably valid at times less than 200 μsec but may introduce some error at longer times. Tests made at the beginning of the experiment showed that the light per pulse was independent of frequency when tested with an 80 mV pulse of duration 200 μsec at frequencies of 50, 100 and 200/sec. The frequency dependence of longer pulses was not tested but the light per action potential at 200/sec was twice that at 50/sec. At 200/sec an action potential gave about the same response as an 80 mV rectangular pulse of duration 1 msec.

The subtraction procedure shows that at 80 mV the early TTX-sensitive component (curve *D* and *d* of Fig. 17) was half maximal at 50–100 μsec whereas the TTX-insensitive component became appreciable at 200–300 μsec and continued to grow with pulse lengths up to durations of 900 μsec. Other experiments indicated that the late component increased with pulse length up to durations of at least 1 sec. In Fig. 17 the asymptote of curve *D*, which gives the early component, is 0·5. This suggests that not more than about 50 % of the calcium entry in an action potential in this fibre was in the early channel and the remainder was in the late channel. In sensitive axons the proportion contributed by the late component was much greater.

It was not always possible to obtain curves as complete and consistent as those in Fig. 17. However, the main features of that experiment were seen in all the axons examined. Table 4 shows that the response to a train of 100 μsec pulses of amplitude 80–130 mV was virtually abolished by TTX, whereas the increment brought in by longer pulses was much less affected by TTX. Progressive change in the delayed response and uncertainties about linearity make it difficult to determine whether or not TTX has any action on the delayed component but it is clear that it has no large effect.

Additional experiments which are not included in Table 4 showed that the early entry of calcium was not abolished by injection of sufficient TEA to block the potassium conductance, and that TTX had its usual effect in removing the early entry in axons injected with TEA ions.

One interpretation of the early calcium entry is that calcium ions are swept into the axon by the inward sodium current. This is made unlikely by the observation that an early entry is present when the potential is driven beyond the sodium equilibrium potential with depolarizing pulses of 120 or 130 mV. However, the observation is not decisive because calcium might enter after the pulse during the brief tail of inward current. Stronger evidence was provided by two experiments which showed that replacing sodium by choline did not change the entry observed with 100 or 200 μsec pulses. In one of the experiments application of TTX blocked the early calcium entry in choline sea-water. The conclusion is that the early

TABLE 4. Effect of tetrodotoxin (TTX) on aequorin responses with different pulse durations

Axon	Response to spike pA/impulse	Pulse amplitude (mV)	Response to 100 μsec pulse			pA/pulse	Response to 200 μsec pulse		
			Normal	TTX	Normal		Normal	TTX	Normal
25	1080	100	480	0	144		810	30	990
26	450	80	158	0	102		200	12	200
26	450	120	192	3	135		225	36	231
28	230	120	120	2	—		126	12	—
32	460	80	110	5	—		270	60	—
32	460	120	220	40	—		310	120	—
37	400	80	100	6	—		210	30	—
37	400	130	190	35	—		270	180	—
Mean	490	104	196	11	127		303	60	473
			630 μsec				Δ (630–200)		
25	1080	100	—	900	1800		—	870	810
26	450	80	342	105	450		142	93	250
26	450	120	480	345	540		255	309	310
28	230	120	160	36	—		34	24	—
32	460	80	640	380	—		370	340	—
32	460	120	700	680	—		390	560	—
37	400	80	440	380	—		230	350	—
37	400	130	800	840	—		530	660	—
Mean	490	104	509	458	930		279	401	457

Aequorin responses are given in terms of the increment in photomultiplier current per pulse or action potential. The entry Δ (630–200) in the last block means that the response at 200 μsec was subtracted from the response at 630 μsec to give the delayed component. All axons were injected with 0·45 μl. of the same aequorin sample. Five less complete experiments gave similar results, the mean effect of TTX being to reduce the response to a 100 μsec 100 mV pulse to about 7 %.

Temp. 18–21° C; TTX concentration 0·8–1·6 μM; axon diameters in Table 2. The figures for the action potential were obtained at the beginning of the experiment.

calcium entry is related to the change in sodium permeability and not to the direction of the sodium current.

Lowering the external calcium concentration to 11 or 22 mM-Ca reduced the magnitude of both early and late components of calcium entry but did not have any clear effect on the time course of either.

In prolonged experiments the delayed component of calcium entry increased, whereas the early entry remained more nearly constant. It seems probable, though not definitely proved, that the large aequorin responses in sensitive axons are caused by an increase in the delayed component.

Calculation of the early calcium entry from the change in sodium conductance

The most obvious explanation of the early calcium entry is that some calcium leaks through the sodium channel. At first we rejected this simple idea because the first derivative of the calcium entry–duration curve reached a maximum before the peak of the sodium conductance (Baker *et al.* 1970). However, it is only legitimate to identify the derivative of an entry–duration curve with a flow if all the entry occurs during the pulse. Later work indicated that a substantial fraction of the calcium entry probably occurred after the pulse and when this was allowed for, the observed relation between calcium entry and pulse duration could be reconstructed satisfactorily from the time course of the sodium conductance.

The quantitative assumptions made in testing the hypothesis were that the calcium conductance g_{Ca} had exactly the same time course as the sodium conductance, g_{Na}, and that the calcium current, I_{Ca}, could be calculated from

$$I_{Ca} = (V - V_{Ca})\alpha g_{Na} \tag{6}$$

when $\alpha = g_{Ca}/g_{Na}$, V is the membrane potential and V_{Ca} is the calcium equilibrium potential; both V and V_{Ca} are defined as displacements from the resting potential. From this the total calcium entry (Q_{Ca}) associated with a pulse of duration t' and amplitude V' is obtained as

$$Q_{Ca} = B\rho \int_0^{t'} g_{Na}\,dt + B\int_{t'}^{\infty} g_{Na}\,dt, \tag{7}$$

where

$$B = \frac{\alpha V_{Ca}}{2F} \quad \text{and} \quad \rho = \frac{V_{Ca} - V'}{V_{Ca}},$$

F being the Faraday. The first term on the right gives the entry during the pulse and the second gives the entry during the time when the sodium conductance is shutting off. The two integrals were obtained from g_{Na}–t curves derived from families of curves such as those in Fig. 16.

In principle g_{Na} can be found by subtracting a TTX record from a normal record taken at the same voltage (cf. Hille, 1967). This method worked well up to the maximum of g_{Na} but was unsatisfactory at long times since compensated feed-back was not employed, and with large currents the voltage across the membrane differed from the recorded voltage (Hodgkin *et al.* 1952). However, by measuring the amplitude of the tail currents after the pulse which are proportional to g_{Na} it was possible to obtain the time course of g_{Na} with sufficient accuracy for the present purpose. Fig. 16 illustrates the time course of g_{Na} with pulses of different durations.

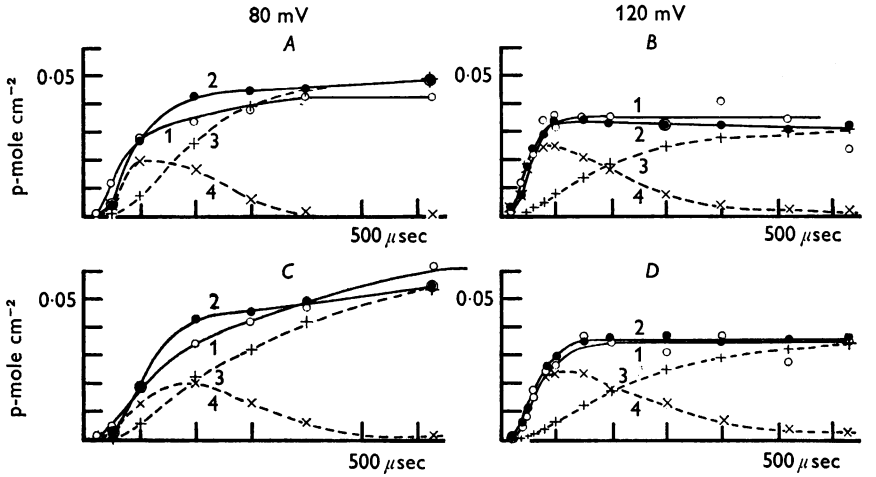

Fig. 18. Reconstruction of response–duration curves for TTX-sensitive component of calcium entry; *A*, *B*, before TTX; *C*, *D*, after TTX; *A*, *C*, with 80 mV pulses; *B*, *D*, with 120 mV pulses. Curve 1, TTX-sensitive calcium entry obtained from Fig. 17 by assuming that the entry per action potential was 0·08 p-mole/cm² (Hodgkin & Keynes, 1957). Curve 2, quantity of calcium entering calculated from eqn. (7) with V_{Ca} = 180 mV and α = 0·0107. Curve 3, calcium entry during pulse; curve 4, calcium entering after pulse.

In Fig. 18*A* the open circles (curve 1) give the observed calcium entries as a function of pulse duration. The absolute scale was obtained by assuming that the entry associated with an action potential was 0·08 p-mole/cm² as found by Hodgkin & Keynes (1957) for an external solution of the same composition and temperature. The filled circles (curve 2) which agree reasonably with curve 1 were calculated by eqn. (7) with α = 0·0107 and V_{Ca} = 180 mV. Curve 3 shows the entry during the pulse and curve 4 (which is nearly proportional to $g_{Na}(t')$) the entry after the pulse. The same values for α and V_{Ca} work equally well for the 120 mV pulse as can be seen from Fig. 18*B*. This provides strong evidence that the permeabilities to

sodium and calcium have similar time courses and invalidates our earlier suggestion that the calcium influx reached a peak before the sodium conductance. Curves *C* and *D* which are given for completeness show that the analysis applies to the calcium entry after recovery from TTX as well as at the beginning of the experiment.

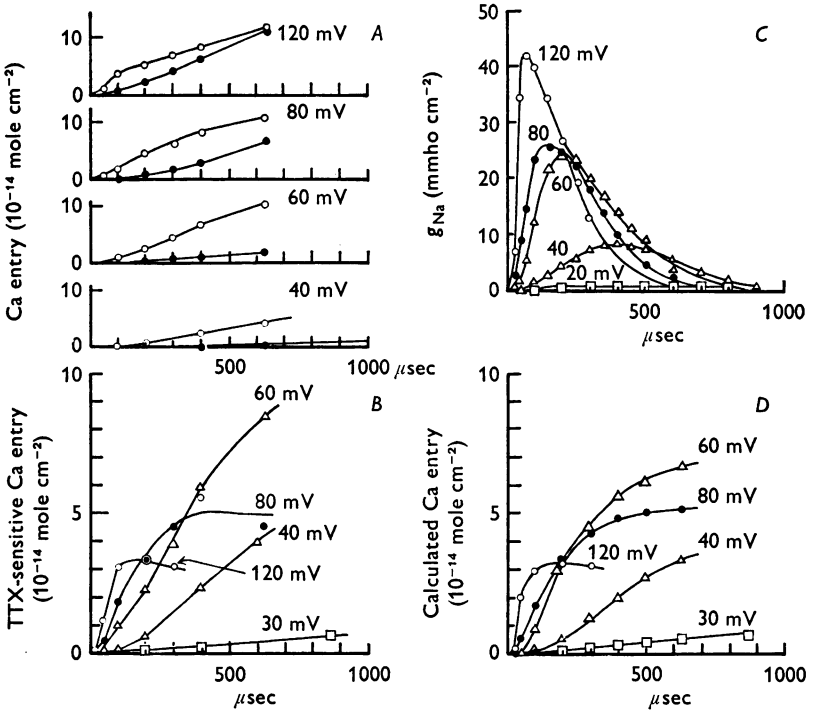

Fig. 19. Time relations of early calcium entry and sodium conductance at different membrane potentials. Abscissa, pulse duration (*A*, *B*, *D*) or time (*C*). Ordinate: *A*, calcium entry before TTX (○) and in 0·8 μM-TTX (●); *B*, TTX-sensitive component obtained by subtraction; *C*, sodium conductance; *D*, calcium entry calculated by eqn. (7) with V_{Ca} = 160 mV and α = 0·0145. In *A* and *B* the ordinate scale was obtained by assuming that the calcium entry per action potential was 0·08 p-mole/cm² as found by Hodgkin & Keynes (1957) for 112 mM-Ca. The pulse amplitude is shown by the number against each curve. Axon 32 in 112 mM-Ca, 400 mM-Na at 20° C.

In Fig. 19 the analysis is extended to a wide range of voltages, the values chosen for V_{Ca} and α being 160 mV and 0·0145. It is satisfactory that the rather complicated family of curves in *B* are reconstructed reasonably accurately in *D* from the time course of the sodium conductance. In this experiment the sodium conductance declined exponentially at the end of the pulse with a time constant (τ) of 40–45 μsec. The second integral in

eqn. (7) was therefore obtained as $\tau g_{Na}(t')$ and it was unnecessary to integrate each tail separately.

Definite evidence that some calcium entry occurred after the end of the pulse was provided by the experiment of Table 5. Here the fibre was repolarized either to the resting potential or to -40 mV, i.e. to a hyperpolarized level; repolarization to $+20$ mV was also used in one instance. The time constants with which g_{Na} declined were approximately 30, 55

TABLE 5. Effect of altering potential to which membrane is repolarized on calculated and observed calcium entries

				5	6	7	8
				Ca entry from aequorin response			
1	2	3	4			Δ (Normal	Calculated
t_1	V_1	V_2	τ	Normal	TTX	−TTX)	Ca entry
(μsec)	(mV)	(mV)	(μsec)			10^{-14} mole cm^{-2}	
100	80	0	58	1·92	0·12	1·80	2·00
100	80	−40	26	1·30	—	1·18	1·28
150	80	0	70	3·25	0·18	3·07	3·10
150	80	−40	35	2·10	—	1·92	2·26
200	80	0	53	4·25	0·60	3·65	3·30
200	80	−40	30	3·10	—	2·50	2·92
200	80	0	55	3·80	0·60	3·20	3·42
200	80	+20	95	5·30	—	4·70	4·02
50	130	0	32	0·50	0	0·50	1·05
50	130	−40	25	0·44	0	0·44	0·88
100	130	0	70	3·78	0·70	3·08	2·67
100	130	−40	40	2·52	—	1·82	1·96

t_1 is the duration of the first pulse; the duration of the second pulse was 300 μsec. V_1 and V_2 are the amplitudes of the first and second pulse respectively, relative to the resting potential. Calcium entries in columns 5, 6 and 7 were calculated from the aequorin response on the basis that the calcium entry in one action potential is 0·08 p-mole cm^{-2} as found by Hodgkin & Keynes (1957) for this temperature and external solution. Column 8 was calculated from g_{Na} by eqn. (7) with $\alpha = 0{\cdot}0075$ and $V_{Ca} = 180$ mV. In calculating column 7 it was assumed that the small delayed component (column 6) was unaffected by the level of repolarization.

Temp., *c*, 18° C; axon diameter 850 μ; axon 37 external solution 112 mM-Ca, 400 mM-Na.

and 95 μsec at -40, 0 and $+20$ mV respectively. Although the driving force on the calcium ions, $V - V_{Ca}$, is 20 % greater at $V = -40$ mV than at $V = 0$, the effect is much less than on the time constant and a reduction of calcium entry is expected when V_2 is changed from zero to -40 mV. For the first pair in Table 5, eqn. (6) predicts that the calcium entry should decrease from 2·00 to $1{\cdot}28 \times 10^{-14}$ mole cm^{-2} when V_2 is changed from zero to -40 mV. The observed entries of 1·8 and $1{\cdot}18 \times 10^{-14}$ mole cm^{-2} are in good agreement with this prediction.

In the three fully analysed experiments, the values found for g_{Ca}/g_{Na} were 0·0107, 0·0075 and 0·0145. Since sodium was about four times more concentrated than calcium in the 112 mM-Ca solution but calcium carries twice the charge, a conductance ratio of 1:100 indicates that the permeability of the early channel to sodium is about 100 times greater than that to calcium. The values chosen for V_{Ca} were 180, 180 and 160 mV but the true calcium equilibrium potential might be higher if there were any constant field type of curvature in the instantaneous relation between calcium current and voltage.

Properties of the delayed entry of calcium

Since the early component of the aequorin response is probably caused by an inward movement of calcium ions through the sodium channel, it is natural to attribute the delayed component to leakage through the potassium channel. The timing and voltage dependence of the late component, as well as its insensitivity to TTX, are consistent with this idea, but there is other evidence that points fairly strongly in the opposite direction. A delayed component of the usual magnitude was present in axons which had been injected with sufficient TEA to block most of the outward potassium current (15 m-mole TEA/l. axoplasm). It was also found that inclusion of manganese in the external solution blocked the late response but had little effect on potassium conductance. Manganese concentrations up to 50 mM which completely blocked the late light response, did not abolish the early TTX-sensitive component (these results will be described fully in a later paper, Baker, Meves & Ridgway, 1971). A further difference is that the aequorin response to a long pulse increased during the course of a long experiment whereas the potassium conductance remained constant or decreased.

The insensitivity of the late component of the aequorin response to TTX and TEA enabled its properties to be examined with single pulses of long duration and large amplitude. In an untreated axon a 100 mV pulse is associated with an outward potassium current of several milliamps per square centimetre and it is difficult to make internal electrodes which will pass such large currents for more than a few milliseconds. After treatment with external TTX and internal TEA, experiments with single pulses can be carried out without much difficulty, and provide interesting information about the voltage dependence of the late component. Fig. 20 illustrates the results of an experiment in which the aequorin response was recorded on the oscilloscope with single 0·1 sec pulses of amplitude up to 180 mV. The external solution contained 22 mM-Ca, but essentially similar results were obtained with 112 mM-Ca except that the maximum response was larger and the response voltage curve shifted in the positive

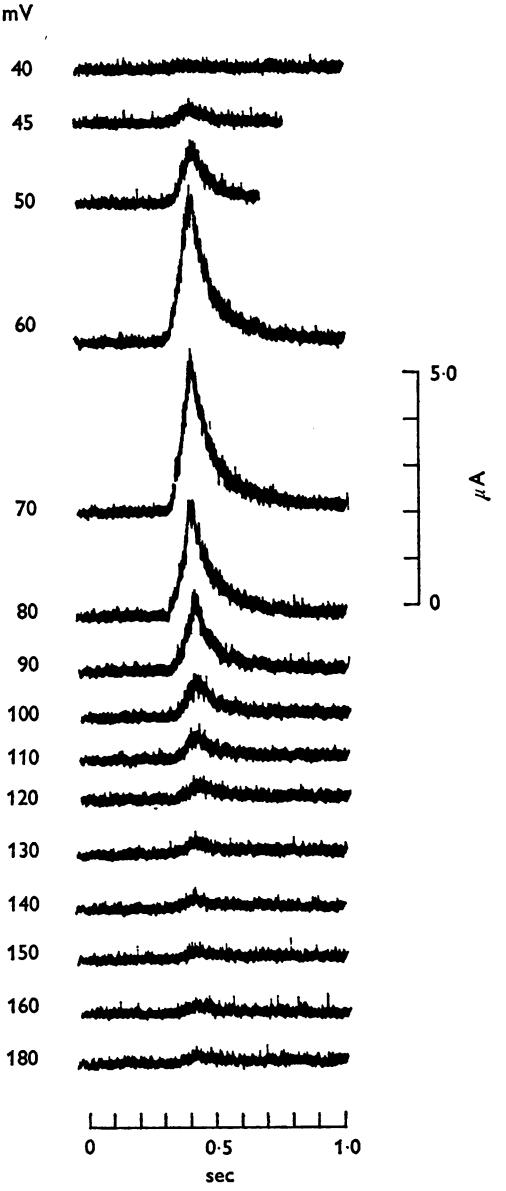

Fig. 20. Aequorin responses to 100 msec pulses of different amplitudes in an artificial sea-water containing 22 mM-Ca, 0-Mg, 400 mM-Na, with 1·6 μM-TTX; the axon had been injected with 15 m-mole tetraethylammonium chloride/l. axoplasm; temp. 22–25° C. The numbers on the left give the amplitude of the depolarizing pulse.

direction (Fig. 21). The records in Fig. 20 were obtained 12 hr after injecting aequorin and the delayed response in 112 mM-Ca was then about 60 times greater than at the beginning of the experiment. They show that the response increased steeply between 35 and 50 mV, reached a maximum

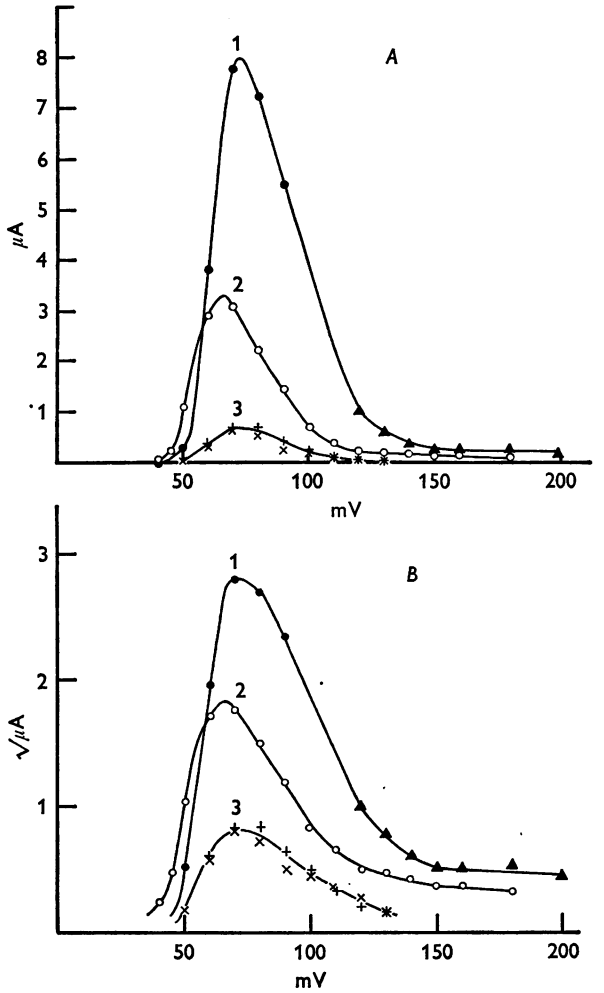

Fig. 21. Effect of pulse amplitude on the aequorin response to single pulses of duration 100 msec. The abscissa is the amplitude of the pulse and the ordinate is the peak of the aequorin response (*A*) or the square root of the peak response (*B*). The composition of the external solution was: Curve 1, 112 mM-Ca, 0-Mg; 2, 22 mM-Ca, 0-Mg; 3, 22 mM-Ca, 90 mM-Mg. The order in which the points were determined was ▲ (112-Ca); + (22-Ca, 90-Mg); ○ (22-Ca); × (22-Ca, 90-Mg); ● (112-Ca). Measurements with a 40 msec pulse made before ▲ gave a curve of the same shape as 1. From the experiment of Fig. 20.

at a depolarization of 70 mV and declined to a low value with a further increase from 80 to 120 mV.

In the upper part of Fig. 21 the amplitude of the response is plotted against the applied voltage with three different external solutions, namely: 112 mM-Ca (curve 1), 22 mM-Ca (curve 2) and 22 mM-Ca, 90 mM-Mg (curve 3). In the lower part of the Figure the ordinate is the square root of the light intensity which is probably a better measure of ionized calcium than the first power of the response. The conclusions from the experiment are: (1) that external magnesium ions inhibit the response; (2) changing from 112 mM-Ca to 22 mM-Ca decreased the amplitude at 70 mV, but increased it at 40 mV, presumably because high calcium shifts the curve to more positive voltage; (3) the aequorin response increased exponentially with pulse amplitude between 35 and 45 mV. Measurements with a pulse of duration 1 sec showed that an e-fold increase in response occurred in 3·15 mV; the square root of the response which is a better indication of calcium entry increased e-fold in 6·3 mV; (4) the decline in amplitude with increasing voltage occurs at potentials that are smaller than the likely value of the calcium equilibrium potential. If the resting potential is taken as -60 mV and the internal ionized calcium as 0·5 μM, the calcium equilibrium potential in 112 mM-Ca should be about 215 mV positive to the resting potential. Given such a high value it is rather surprising that the light response declines when V exceeds 80 mV. A possible explanation is that the effective calcium permeability is maximal when the absolute membrane potential is near zero and is reduced by an increase of membrane potential in either positive or negative direction; (5) records of membrane current gave no indication of any inward current corresponding to the calcium influx in Fig. 21.

DISCUSSION

Resting axons

The experiments in the first part of the paper provide clear evidence that treatment with cyanide leads to a marked rise in the concentration of ionized calcium in axoplasm. Some of this calcium must come from an internal store, since the cyanide effect can take place in the absence of external calcium. From the effects of injecting ATP and of oligomycin it seems that calcium can be concentrated in the internal store either by electron transport or by ATP. This makes it likely that the internal store may be identified, at least in part, with mitochondria, as suggested by Blaustein & Hodgkin (1969). The timing and reversibility of the effect of cyanide on the aequorin response also agree well with previous studies on calcium efflux and on the ease with which calcium can be dialysed from

axoplasm (Blaustein & Hodgkin, 1969). This is satisfactory, but there is still some doubt about the way in which cell calcium should be divided quantitatively. On general grounds one might expect that the total calcium in axoplasm, which amounts to about 400 μM (Keynes & Lewis, 1956; Blaustein & Hodgkin, 1969) should be divisible into the following three fractions: (1) calcium which cannot be dialysed from axoplasm and which is mainly in an internal store where it is maintained by ATP or electron transport; (2) a fraction of un-ionized but diffusible calcium, consisting of calcium combined with small molecules like ATP, citrate or glutamate; (3) ionized calcium. Since Blaustein & Hodgkin concluded that approximately 1/40 of the calcium in normal axoplasm was diffusible whereas the present estimate of ionized calcium is about 0·3 μM the tentative conclusion is that the concentrations of calcium in the three states are approximately 400 μM in the internal store, 10 μM diffusible but un-ionized, and 0·3 μM ionized. After poisoning with cyanide, most of the calcium is lost from the store and can be dialysed from axoplasm (Blaustein & Hodgkin, 1969). If there was a large excess of diffusible buffering material, one would expect both un-ionized and ionized components of the diffusible fraction to increase in proportion giving about 390 μM un-ionized and 12 μM ionized for the cyanide-poisoned axon. The fortyfold increase in ionized calcium is of the same order as that calculated on p. 719 from the effect of cyanide on the light output from axons injected with aequorin.

For fibres in which the critical rate of rise of light intensity is proportional to the frequency of stimulation it may be permissible to calculate the resting level of rapidly exchangeable calcium from the ratio of the increment in light per impulse to the resting glow. On a linear basis

$$\frac{2\Delta Q_{Ca}}{a[Ca]_R} = \frac{\Delta L}{L_R}, \tag{8}$$

where ΔQ_{Ca} is the calcium entry per impulse which may be taken as 0·083 p-mole/cm^2 in 112 mM-Ca (Hodgkin & Keynes, 1957), $[Ca]_R$ is the resting level of rapidly exchangeable calcium, a is the radius, ΔL is the increment in light per impulse and L_R is the resting glow. In the four axons in Table 2 in which $n = 1{\cdot}0$ or 1·1, $\Delta L/L_R$ averaged $0{\cdot}46 \times 10^{-3}$, the mean diameter was 728 μ and the value of $[Ca]_R$ calculated by eqn. (8) was 9·6 μM. This is much higher than the estimate of ionized calcium but similar to the tentative figure for diffusible calcium. It therefore seems possible that most of the calcium which enters during the impulse is immediately converted into the diffusible but un-ionized form.

The finding that replacing external sodium by lithium increases the rate of the aequorin reaction agrees with tracer measurements which show a large increase in calcium influx under these conditions (Baker *et al.* 1969).

It is difficult to make a quantitative comparison because calcium influx increases with internal sodium (Baker *et al.* 1969) and because we have found no satisfactory method of calibrating the aequorin reaction inside the axon. The increment in light intensity on replacing sodium sea water with lithium sea water was about the same as the increment produced by stimulating a fibre in 112 mM-Ca at 100/sec. From Hodgkin & Keynes (1957) the influx of calcium under these conditions should be about 8 p-mole cm^{-2} sec^{-1} which is just within the range found by Baker *et al.* for the difference between calcium influx from lithium and sodium sea waters.

Depolarization and calcium entry

The results described in the second part of the paper provide clear evidence that the calcium entry associated with depolarization can be divided into an early component which is blocked by TTX and a late component which is unaffected by this inhibitor. Another pharmacological difference is that the delayed component is largely removed by manganese at a concentration of 50 mM in the external solution whereas the early component is much less affected. Both early and late components are present in fibres into which enough TEA has been injected to block most of the increase in potassium conductance. In fresh axons about 40 % of the calcium entry during a propagated action potential is probably in the early channel and the remainder in the late channel. The proportion in the late channel may be larger in old fibres which gave large light responses.

Analysis of the relation between pulse duration and the TTX- sensitive component of the aequorin response indicates that the time course of the early calcium influx is close to that expected from the time course of the sodium conductance. The evidence is impressive because the only quantities available to fit a complicated family of experimental curves are the calcium equilibrium potential (V_{Ca}) and the ratio of calcium to sodium conductance, α. In Fig. 19, V_{Ca} and α were chosen for the data at 120 mV, and the remaining theoretical curves, which are obviously close to the experimental ones, were calculated without any arbitrary constants at all. Such experiments provide strong evidence that the early entry is a leak of calcium ions through the sodium channel and that the ratio of calcium to sodium conductance is approximately 0·01 with 112 mM-Ca and 400 mM-Na in the external solution.

An obvious explanation of the delayed component of calcium entry is that it represents an inward movement of calcium ions through the potassium channel. However, this does not fit with the observation that axons injected with sufficient TEA to block most of the outward potassium current have a delayed calcium entry of the usual magnitude. Nor is the idea consistent with the fact that external manganese blocked the delayed

calcium entry but had little effect on potassium conductance. In several respects the properties of the late component resemble those of the channel which is responsible for release of transmitter at the presynaptic nerve terminals in the stellate ganglion of *Loligo* (Katz & Miledi, 1967, 1969). In both cases, manganese and magnesium reduce calcium entry, and the curves relating aequorin and transmitter response to pulse amplitude both have a maximum at 70–80 mV. Between 35 and 45 mV the square root of the aequorin response increased *e*-fold in 6 mV, and a similar figure is obtained from Fig. 9 of Katz & Miledi (1970) which gives calcium permeability as a function of membrane potential. These similarities raise the possibility that calcium channels of the kind described by Katz & Miledi may be present all the way along the axon, though probably at greater density in the presynaptic terminal.

The most puzzling result encountered in the present investigation is the large increase in the light response which occurs when axons are kept for 6–12 hr after injecting aequorin. It is not clear whether this involves a genuine increase in calcium entry or whether it depends on a change in the sensitivity of the injected aequorin to a given rise in calcium concentration, resulting perhaps from the loss of a substance which interferes with the aequorin reaction. On the second hypothesis one might expect a change in sensitivity to have an equal effect both on the resting glow and on the increment in light per impulse, whereas the resting glow often declined during a long experiment in spite of a large increase in the light per impulse. An observation which is even more difficult to reconcile with any simple change in sensitivity is that the delayed calcium entry increased progressively during a long experiment whereas the early entry seemed to change much less. To explain a differential effect of this kind one must suppose either that there is a genuine increase in the amount of calcium entering through the late channel, or that the change in aequorin sensitivity is confined to the calcium entering through the late channel. Before attempting to decide between the two alternatives it is desirable to see whether tracer measurements provide evidence of a progressive increase in the amount of calcium which enters during a relatively long pulse of depolarization.

Advantages and disadvantages of aequorin as a calcium indicator

The most obvious advantage of the aequorin method is that it provides a rapid and continuous measurement of the concentration of ionized calcium inside a cell. Since the internal ionized calcium is maintained at a low level, one can use the method to make many measurements of calcium entry in one experiment without loss of sensitivity. However, although the method is very convenient in exploratory work it suffers from a number of

drawbacks. As pointed out by Hastings *et al.* (1969) the 10 msec lag in the aequorin response makes it difficult to resolve calcium movements on the time scale of an action potential. Indirect information can be obtained by the voltage-clamp method described in this paper but it is important to distinguish between entry during and after the voltage pulse. We have found no satisfactory way of calibrating the aequorin response, and the non-linearity of the reaction makes it hard to determine the relative magnitude of the entry under different conditions. It is also necessary to exercise caution in identifying an increase in the light emitted by aequorin with an entry of calcium at the surface. Alternative possibilities are that the stimulus may liberate calcium from an internal store or that it removes an inhibitor of the aequorin reaction. Finally, there is the awkward but perhaps unlikely possibility that the stimulus might liberate aequorin into the external solution where it would rapidly react with calcium. As with other new techniques it is clearly desirable to check results obtained with aequorin by other methods whenever possible.

We are greatly indebted to Dr R. H. Adrian for checking the Appendix and for writing the computer programme. Our thanks are also due to Professor E. Rojas for help with the voltage-clamp technique, to Professor E. J. Denton for help with the light calibrations and to Mr R. H. Cook for designing and building equipment. E.B.R. was supported by a post-doctoral fellowship from the U.S. Public Health Service and P.F.B. was in receipt of a grant from the Dale Fund of the Physiological Society.

APPENDIX

The object of this section is to give approximate equations for the distribution of ionized calcium at various times after the beginning and end of a period of increased calcium inflow. The model used is a simplified version of that described by Blaustein & Hodgkin (1969). As in their treatment it is assumed that calcium exists in free and bound forms and that only the free form is diffusible. The concentration of free calcium will be denoted by Y_1 and of calcium bound in the internal store by Y_2. It is assumed that the forward flux from the free to the bound form is given by $\kappa_1 Y_1$ but that the flux in the opposite direction is a constant denoted by M. This simplification is probably justified during a tetanus lasting 10–30 sec since there might be little change in Y_2 during that time. It might be true for a longer period if calcium inside the internal store was buffered or if the backward flux was saturated with respect to Y_2. On this basis the equation for free and bound calcium is

$$D\left(\frac{\partial^2 Y_1}{\partial r^2}+\frac{1}{r}\frac{\partial Y_1}{\partial r}\right) = \frac{\partial Y_1}{\partial t}+\kappa_1 Y_1 - M, \tag{1}$$

$$\frac{\partial Y_2}{\partial t} = \kappa_1 Y_1 - M \tag{2}$$

where D is the diffusion coefficient of free calcium, r is distance in the radial direction and t is time. When there is no gain or loss of total calcium, the steady level of ionized calcium is given by M/κ_1. If $U[= Y_1-(M/\kappa_1)]$ represents the displacement from this level eqn. (1) becomes

$$D\left(\frac{\partial^2 U}{\partial r^2}+\frac{1}{r}\frac{\partial U}{\partial r}\right) = \frac{\partial U}{\partial t}+\kappa_1 U. \tag{3}$$

This equation is put forward as an approximation but there is not necessarily any inconsistency between the present treatment and that used by Blaustein & Hodgkin (1969). In considering an exchange experiment, M is replaced by My_2/Y_2 where y_2 is the concentration of tracer in the bound form. Provided Y_2 remains constant M/Y_2 can be replaced by the rate constant κ_2 and one obtains the pair of equations used by Blaustein & Hodgkin, namely

$$D\left(\frac{\partial^2 y_1}{\partial r^2}+\frac{1}{r}\frac{\partial y_1}{\partial r}\right) = \frac{\partial y_1}{\partial t}+\kappa_1 y_1-\kappa_2 y_2 \tag{4}$$

$$\frac{\partial y_2}{\partial t} = \kappa_1 y_1-\kappa_2 y_2 \tag{5}$$

where y_1 is the concentration of tracer in the free form. In addition to its convenience the assumption that M is constant is supported by the observation that prolonged or repeated stimulation did not lead to any marked increase in the resting level of light production.

To illustrate what might be expected to happen during a short tetanus we shall suppose that the net flow of calcium into the fibre is zero for $t < 0$ and increases suddenly to a constant value F_0 at $t = 0$. The boundary conditions are

$$U = 0, \quad t < 0$$

$$\frac{\partial U}{\partial r} = \frac{F_0}{D}, \quad t > 0 \quad \text{at} \quad r = a$$

and the appropriate solution of eqn. (2) is

$$\frac{U}{F_0} = \frac{1}{\sqrt{(D\kappa_1)}}\frac{\mathrm{I}_0[r(\kappa_1/D)^{\frac{1}{2}}]}{\mathrm{I}_1[a(\kappa_1/D)^{\frac{1}{2}}]} - \frac{2}{a}\sum_{n=0}^{\infty}\frac{\exp-[(\kappa_1+D\alpha_n^2/a^2)t]}{(\kappa_1+D\alpha_n^2/a^2)}\frac{\mathrm{J}_0(r\alpha_n/a)}{\mathrm{J}_0(\alpha_n)}. \tag{6}$$

where $\alpha_0 \ldots \alpha_n$ are the roots ($\geqslant 0$) of $\mathrm{J}_1(\alpha) = 0$. I_0, I_1, J_0 and J_1 are Bessel functions. The first term in eqn. (6) gives the steady state and the second the transient.

Fig. 22*A* gives the distribution of calcium expected at various times after a step in calcium inflow at the surface. The diffusion coefficient (D) of free calcium was assumed to be $6{\cdot}4 \times 10^{-6}$ cm^2/sec which is close to the value adopted by Blaustein & Hodgkin (1969) or Hodgkin & Keynes

(1957); κ_1 was taken as 0·1 sec^{-1} which is similar to that observed in many experiments. These values give a space constant ($\sqrt{(D/\kappa_1)}$) of 80 μ so the change in free calcium is always confined to the cortical regions of the axon. This fits with the experimental observation (Fig. 9*A*) that there is

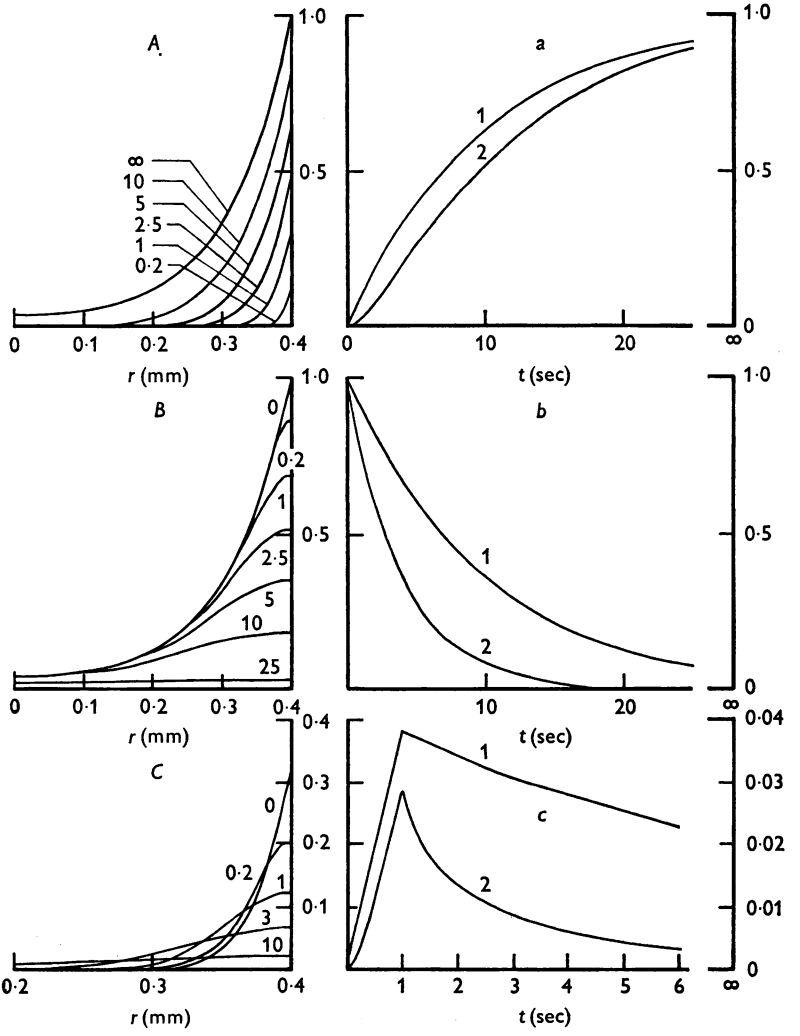

Fig. 22. For legend see opposite page.

no light response to stimulation immediately after the injection when the aequorin is still in the middle of the fibre.

Fig. 22*B* and *C* give respectively the distribution of calcium at various times after switching off a steady inflow of calcium and after a rectangular

pulse of inflow lasting 1 sec. The curves were computed from eqn. (6) using the superposition principle. Equation (6) was evaluated on the Titan computer (ICL Atlas 2) using a programme kindly written by Dr R. H. Adrian. In order to obtain the scales in Fig. 22 in non-dimensional form the units of time should be multiplied by 0·1 sec^{-1} to give $\kappa_1 t$ and the units of radial distance by 12·5 mm^{-1} to give $r(\kappa_1/D)^{\frac{1}{2}}$.

Aequorin response during stimulation

(*a*) *Fibres with a linear response.* The equation for the change in light intensity during a tetanus is simple if it can be assumed that there is a linear relation between the increase in light intensity and the rise in calcium concentration.

If Q is the increment in the light intensity per unit length we have

$$Q = 2\pi b \int_0^a Ur\,dr \tag{7}$$

where b is a constant relating the increment in light intensity per unit volume to the rise in calcium concentration. Eqn. (3) can be written in the form

$$D\frac{\partial}{\partial r}\left(r\frac{\partial U}{\partial r}\right) = r\frac{\partial U}{\partial t} + \kappa_1 rU \tag{8}$$

Legend to Fig. 22.

Fig. 22. *Left-hand curves: A.* Increase (U) in calcium concentration as a function of radial distance (abscissa) at various times after switching on a constant inflow of calcium at the surface of a fibre in which the radius (a) is 0·4 mm, $\kappa_1 = 0{\cdot}1\ sec^{-1}$ and $D = 6{\cdot}4 \times 10^{-6}\ cm^2/sec$. The time in seconds is shown against each curve. The ordinate is given as $U(r, t)/U(a, \infty)$ and was computed from eqn. (6). With the constants used a steady inflow of 10 p-mole $cm^{-2}\ sec^{-1}$ raises the calcium concentration by 14 μM at the surface.

B. Distribution of U at various times after switching off a steady surface inflow; details as in *A*.

C. Distribution of U at various times after an inflow lasting 1 sec; details as in *A*.

Right hand curves: Time course of $\int_0^a rU\,dr$ (curves 1) and of $\int_0^a rU^2\,dr$ (curves 2) computed from eqn. (6) for a, beginning, b end of long rectangular pulse of inflow, and c, for rectangular pulse lasting 1 sec. The ordinate of all curves except c 1 is given as a fraction of the steady-state value; in c 1 this fraction has been multiplied by 0·4.

Curves 1 may be appropriate to a linear fibre and curves 2 to a square-law fibre.

and on integrating between $r = 0$ where $\partial U/\partial r = 0$ and $r = a$ where $D(\partial U/\partial r) = F_0$ we obtain

$$2\pi abF_0 = \frac{dQ}{dt} + \kappa_1 Q,$$

since the order of the operations $\partial/\partial t$ and $\int dr$ can be reversed in a linear system. Hence the light emitted by the fibre should rise and fall in an exponential manner, and the steady displacement should be equal to $2\pi abF_0/\kappa_1$.

(*b*) *Fibres with a square-law response.* For fibres with a square-law response it is assumed that the increment in light intensity is proportional to $\int_0^a rU^2 dr$. In this case we have not been able to find any simple relations for the increment in light but the type of behaviour expected can be illustrated by two examples. Fig. 22*a* and *b* give the rise and fall of light intensity expected in a linear fibre (curves labelled 1) and in a square-law fibre (curves 2). The linear fibre is symmetrical but in the square-law fibre the rise and fall of light-intensity are highly asymmetrical with a slow 'on' and a rapid 'off'. Fig. 22*c* illustrates the effect of a short period of calcium inflow. The rapid decline in the ordinate of curve 2 at the end of the inflow period is not due to conversion of free to bound calcium but to inward diffusion of calcium. As calcium diffuses inwards the quantity $\int_0^a rU\,dr$ does not alter but $\int_0^a rU^2 dr$ declines rapidly. This effect probably accounts for the very rapid decline in light intensity seen after a brief tetanus in a square-law fibre (Fig. 13).

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