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# MOVEMENTS OF LABELLED CALCIUM IN SQUID GIANT AXONS

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A certain amount is known about the way in which calcium may affect the permeability of nerve membranes to sodium and potassium ions (see Brink, 1954; Frankenhaeuser & Hodgkin, 1957), but there is little information about the permeability to calcium itself. The main object of the experiments described in this paper was to measure the rate at which calcium labelled with the radioactive isotope $^{45}Ca$ crosses the surface membrane of resting squid axons, and to see whether calcium moves faster through the membrane during nervous activity. Investigations on the permeability of cell membranes to labelled substances are often complicated by uncertainty as to whether a genuine penetration of the intracellular region has taken place, or only an exchange with extracellular material. Such doubts tend to be especially acute with a substance like calcium whose internal concentration is very low (Keynes & Lewis, 1956). The squid giant axon is a good preparation for studying calcium permeability, because extruded axoplasm can be used for measurements of influx, while the micro-injection technique can be used to determine efflux. Partly because of the necessity of working with extruded axoplasm, and partly because of the softness of the $\beta$-radiation emitted by $^{45}Ca$, it was not feasible to make successive measurements of resting and stimulated influx, in the manner described by Keynes (1951) for sodium and potassium. However, by comparing influx figures for groups of axons treated differently it was possible to obtain reasonably consistent estimates of the extra entry of calcium associated with the conduction of impulses.

Radioactive calcium was also used in an attempt to throw some light on the state of calcium inside living cells. The calcium in a short length of axoplasm was labelled with the help of a microsyringe, and the subsequent movements of the radioactive patch were studied in the presence and absence of an applied longitudinal voltage gradient. In contrast to the behaviour of potassium under similar circumstances (Hodgkin & Keynes, 1953), the $^{45}Ca$ moved very little, suggesting that most of the calcium is in a relatively immobile form.

The permeability measurements were begun by one of us in the summer of 1954, in collaboration with Dr E. Flückiger (see Flückiger & Keynes, 1955), but the greater part of the work discussed here was done in the following year.

## METHODS

### *Materials*

Giant axons were dissected in the usual way from *Loligo forbesi*, cutting the branches as far as possible from their junction with the main axon. For experiments in which the axoplasm was to be extruded it was possible to work with smaller animals (which were often in more plentiful supply) than could conveniently be used when a 100 $\mu$ micro-electrode or microsyringe was to be inserted into the axon. A few experiments were done with second stellar nerves dissected from large specimens, whose innermost axons had been taken for other purposes. Some of the influx experiments, and all those on efflux and calcium mobility, were done with axons carefully cleaned from adherent tissue; but uncleaned axons, soaked in the radioactive solutions as intact nerve trunks, were used for most of the influx measurements.

### *Solutions*

Some experiments were done in an artificial sea water containing both calcium and magnesium, similar in composition to that used by Hodgkin & Keynes (1955), except that bicarbonate was substituted for phosphate. On other occasions the solutions were based on those used by Frankenhaeuser & Hodgkin (1957), magnesium and sulphate being omitted, and the amounts of $CaCl_2$ and NaCl varied in such a way as to maintain approximate isotonicity with sea water. The composition of the solutions in which the potassium concentration was normal is shown in Table 1; when the amount of KCl was raised to 52 or 208 mM, an equivalent quantity of NaCl was subtracted.

TABLE 1. Composition of solutions (mg-ions/l.)

| | (1) | (2) | (3) | (4) |
|---|---|---|---|---|
| $Na^+$ | 486 | 526 | 492 | 392 |
| $K^+$ | 10·4 | 10·4 | 10·4 | 10·4 |
| $Ca^{2+}$ | 10·7 | 22·4 | 44·8 | 112 |
| $Mg^{2+}$ | 55·2 | — | — | — |
| $Cl^-$ | 570 | 581 | 593 | 626 |
| $SO_4^{2-}$ | 29·2 | — | — | — |
| $HCO_3^-$ | 0·5 | 0·5 | 0·5 | 0·5 |

The radioactive calcium was supplied as a neutral solution of $^{45}CaCl_2$, about 0·03 M, specific activity 240 $\mu$c/mg Ca. The labelled sea water was made up by weighing out 100–300 mg of the $^{45}Ca$ sample, and adding appropriate weights of : (*a*) inactive $CaCl_2$, usually as a 0·5 M solution; (*b*) a solution containing NaCl, KCl, $NaHCO_3$, $MgCl_2$ and $MgSO_4$ at twice or four times their final desired concentration; (*c*) sufficient distilled water to make a total volume of about 1 ml. The pH was checked on a tile with bromthymol blue, and was never far from 7.

The specific activity of the calcium in each batch of labelled sea water was determined before and after using it, a portion being diluted by weight about 200 times with sea water, and 50 mg samples of the diluted solution being counted in the standard fashion (see below). Although none of the 1 ml. batches of radioactive sea water was used for more than seven influx determinations, some trouble was experienced from progressive evaporation of the sea water while axons were soaked in it at room temperature, and from condensation into it during the low temperature experiments. In the worst case the solution lost by evaporation about 37% of its initial volume, but usually the loss was under 15%. Checks showed that the final specific activity had changed much less than the tonicity, and was generally within 7% of the initial value. Only the last axons exposed to each radioactive sample could have been seriously affected by these changes, and

errors from differences in the solutions were in any case minimized by treating the axons in pairs. The results for one extreme case having been rejected, the remainder are unlikely to have been seriously in error because of evaporation.

*Radioactivity measurements*

Preliminary tests showed that adequately reproducible results could be obtained by putting 0·7 ml. of sea water containing some $^{45}Ca$ in a shallow flat nickel dish 25 mm in diameter, adding 2 drops of 0·2 M sodium oxalate to precipitate the calcium, and drying under a lamp. This treatment resulted in a uniform distribution of the calcium over the surface of the dish, and reduced the error from any slight crusting at the edges. The samples were counted under a mica end-window Geiger tube (G.E.C. Type EHM2S). In the course of standardizing a number of batches of radioactive sea water it was calculated that the standard deviation for a single sample was of the order of $\pm 3\%$. The $\beta$-radiation from $^{45}Ca$ has an energy of only 0·25 MeV; it was found that self-absorption by the solid matter in the samples (totalling about 5 mg/cm$^2$) reduced the counting rate to two-thirds of that obtained when the same trace quantity of $^{45}Ca$ was added to 0·7 ml. of distilled water and dried. However, since the standard volume of 0·7 ml. of sea water was added to all axoplasm samples and standards before precipitating the calcium and drying them, no correction had to be made for this self-absorption. It was also unnecessary in most of the experiments to correct for decay of the $^{45}Ca$, as the samples were generally counted within 2 days of counting the standards, and the half-life of the isotope is 152 days. In the cases where rather greater time intervals were involved, allowance was made for decay, and all counting rates quoted in the text or in figures have been suitably corrected.

*Influx determinations*

After the axons had been dissected, and again before the final extrusion, they were carefully examined for signs of deterioration at the bases of branches; this was visible as a brownish opacity in direct illumination, or as an area scattering more light when dark-ground illumination was used. Damaged extremities were tied off, and the excitability of the axon was tested with its two ends lifted in forceps just above the surface of the sea water, as described by Hodgkin & Keynes (1955). The axon was then immersed in a small rectangular glass vessel containing the $^{45}Ca$ sea water, either resting or stimulated at 25–156/sec, the ends being lowered beneath the surface every few minutes in order to prevent them from drying up. On completion of the period in radioactive sea water, the axon was transferred to a large dish of inactive sea water, in which its diameter was measured and a length of a few millimetres near one end quickly cleaned (where a whole nerve trunk was being used) under a dissecting microscope; this operation took 10–15 min. It was next detached from the forceps, cut through with scissors in the centre of the cleaned stretch, and laid on a piece of filter paper with the cleaned end overlapping on to a small weighed square of cellophane. The axoplasm was now extruded from the central part of the axon by gentle pressure from a small Perspex roller. After the cellophane and axoplasm had been reweighed on a torsion balance, they were put into 0·7 ml. of sea water on a nickel dish. In a minute or two the axoplasm had completely dispersed, owing to the action on it of the calcium in the sea water (Hodgkin & Katz, 1949*a*). The sodium oxalate was added, and the contents of the dish were now ready for drying. Since the cellophane square tended to curl up as it dried, it was removed at this stage; checks showed that the amount of radioactivity adhering to it was negligible. The dishes were counted in a conventional lead castle.

*Efflux determinations*

On two occasions axons were loaded with $^{45}Ca$ by soaking for 2 hr in radioactive sea water containing 10·7 mM-Ca* (the mixture of $^{40}Ca$ and $^{45}Ca$ in the labelled solution is designated Ca* throughout this paper) and 55 mM-Mg (solution (1), Table 1). The calcium in one axon was labelled by 20 min stimulation at 156/sec in sea water containing 112 mM-Ca* (solution (4), Table 1). For the other efflux experiments, the isotope was introduced into cleaned axons by the micro-injection of a small volume of the $^{45}CaCl_2$ sample diluted with 0·6 M-KCl. The procedure described by

Hodgkin & Keynes (1956) was simplified by omitting the initial insertion of a 100 $\mu$ microelectrode; the microsyringe was lowered into the axon until its tip was 20–30 mm below the cannula, and a column of fluid 15–20 mm in length was then injected. After the axons had been cleaned and suitably loaded with $^{45}Ca$, they were mounted on forceps, their condition being judged from the size of the externally recorded spike. The efflux was measured by determining the amount of radioactivity which emerged into a series of puddles of inactive sea water located in narrow grooves on a paraffin-wax block. The puddles had the standard volume of 0·7 ml., and were subsequently transferred to nickel dishes for the $^{45}Ca$ in them to be counted as before. During a few of the collecting periods the axons were stimulated at 50/sec. At the end of each experiment the whole axon was dried in a dish with 0·7 ml. sea water, in order to determine the amount of $^{45}Ca$ still remaining in it.

*Mobility experiments*

Axons were cleaned and cannulated, and a point about 30 mm from the end of the cannula was marked by knotting a hair round a strand of connective tissue, care being taken not to harm the axon in the process. The microsyringe was loaded with a short column of $^{45}CaCl_2$, a very small air bubble being sucked in at the extreme tip in order to act as a guard against premature mixing with the axoplasm during the process of insertion, and was then lowered to a position about 1 mm beyond the knot. A 2 or 3 mm column of $^{45}CaCl_2$ was injected, the precise limits of the injected region being defined by the emergence into the axoplasm of two small air bubbles—the guard bubble at the lower end, and at the upper end part of the much larger bubble which routinely separated the $^{45}CaCl_2$ from the fluid filling the remainder of the shaft of the microsyringe. The location of these air bubbles relative to the knot and the exact length of the injected patch were observed with a dissecting microscope and micrometer eyepiece. The ends of the axon were identified by tying differently coloured threads round them, and the axon was then transferred to two pairs of forceps in which it was held horizontally in a dish of paraffin oil. A longitudinal voltage gradient could be applied by immersing the ends of the axon in two puddles of sea water under the oil, containing large Ag–AgCl electrodes with a 7·5 V dry battery and a 100 k$\Omega$ wire-wound resistor in series between them. The voltage drop along the 40 mm length of axon stretched between the puddles was measured by means of a voltmeter connected to the outputs of two cathode-followers. The wick leading to one of the cathode-followers could be slid along the axon during the experiment in order to measure the potential gradient, which was satisfactorily uniform over the central 30 mm. The other wick was dipped into the anodal puddle. The voltmeter was subsequently calibrated against a Weston standard cell. After varying periods in the oil, with or without electric current flowing along the axon, the excitability of the axon was checked and, having been lightly blotted, it was laid on a numbered glass slide with the knot close to a hair-line scratched across the glass. Some of the axons were allowed to dry at room temperature, and others were dried under a lamp.

The distribution of $^{45}Ca$ was determined by taking a series of counts with a mica end-window Geiger tube in a standard lead castle fitted with a special screening device. This consisted of a piece of 1 in. steel rule through which a short slot 1 mm wide was cut at right angles. Strips of cellophane tape were stuck along each side underneath the rule to act as spacers, and the glass slide was pressed against these by springs. The slide could thus be moved along beneath the slot, just far enough from it (0·4 mm) to avoid scraping the dried axon. The distance setting of the slide was done with the help of a microscope, which enabled the exact position of the knot on the axon to be observed relative to the hair-line on the slide and hence to the millimetre divisions engraved on the rule. The axons were counted (usually for 50 min in each position) at 0·5 or 1·0 mm intervals over a distance of about 6 mm on either side of the knot. Counting rates were small, and the background was therefore measured as accurately as possible by taking long counts throughout the night; it seemed to vary significantly from time to time, but was always between 6 and 7 counts/min. For two of the axons, preliminary counts were taken soon (1–9 days) after performing the experiment. The final counts for all the axons were taken in Cambridge, a month or two later. Apart from slight differences due to a change in the spacing between the slides and the steel rule,

the measured distribution of radioactivity in the two axons which were counted twice was unaltered by the lapse of about 8 weeks. This provided evidence that there was no appreciable movement of the $^{45}Ca$ once the axons had been dried.

In order to see how far the distribution patterns were likely to be distorted by the characteristics of the window, a set of counts was taken with a speck of dried $^{45}CaCl_2$ solution, 0·26 mm in diameter, on a slide. The cut-off was reasonably sharp, as Fig. 1 shows. This curve was used as described on p. 274 to correct theoretical distribution curves before fitting them to the observed curves.

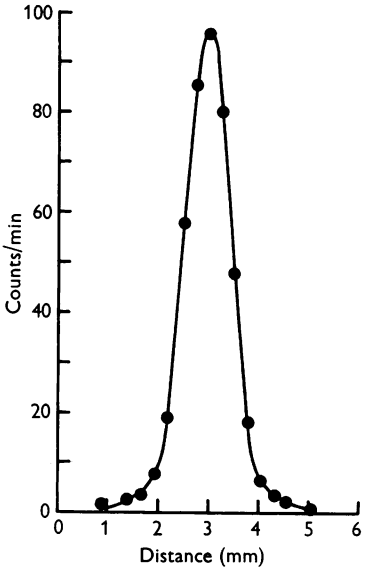


Fig. 1. The characteristic of the window used in counting the dried axons. For the purpose of correcting the theoretical distribution curves, the percentage count at different distances from the central position was taken from this curve as: 0, 100%; 0·4 mm, 70%; 0·8 mm, 19%; 1·2 mm, 5%; 1·6 mm, 2·5%; 2·0 mm, 1%.

## RESULTS

### *The time course of calcium exchange*

Before starting on the main series of influx determinations using extruded axoplasm, an experiment of the 'soak-in, soak-out' type was performed, with the object of obtaining a rough idea of the time constants involved in the exchange of calcium in a cleaned but intact axon. After the axon had spent 117 min in sea water containing 10·7 mM-Ca* and 55 mM-Mg (solution (1), Table 1), it was passed through a number of samples of inactive sea water with a similar composition, in most of which it remained for 20 or 30 min. These samples were treated in the usual way, dried and counted. The resulting counting rates, divided by the period spent in each sample so as to give the rate of loss of radioactivity from the axon, are plotted semi-logarithmically against time in Fig. 2*A*. At the end of the experiment the amount of $^{45}Ca$ still remaining in the axon was measured by counting some axoplasm extruded from

part of it. 3·5 mg of axoplasm gave 270 counts/min, that is to say 77 counts/min. mg, while the remaining 8·9 mg of axon and sheath gave 497 counts/min. As the total quantity of axoplasm calculated from the dimensions of the nerve (diameter 512 $\mu$, length 51 mm, density 1·05 g/cm$^3$) was 11·0 mg, the residue must have contained about 7·5 mg of axoplasm and 1·4 mg of sheath and connective tissue. Expressed in terms of axoplasm, its radioactivity was therefore 66 counts/min. mg. The discrepancy between this figure and that for the extruded axoplasm may have arisen partly from additional absorption of the radiation by the dried whole axon.

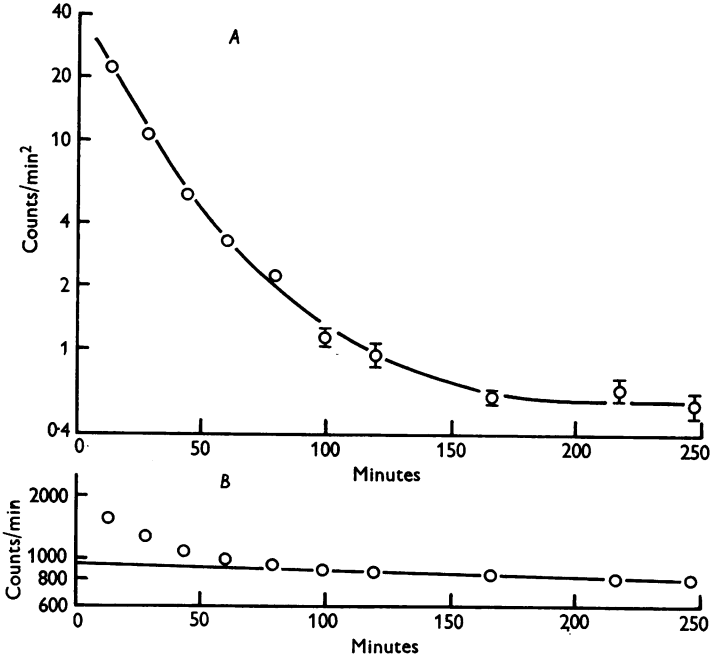


Fig. 2. The time course of calcium exchange in an isolated squid axon which first spent 117 min in $^{45}$Ca sea water and was then washed in a series of samples of inactive sea water. *A* shows how the rate of loss of radioactivity varied in successive samples. *B* shows the decline of the total $^{45}$Ca content, arrived at by adding the final count for the whole axon (767 counts/min) to the counts for the individual samples; the straight line was used to make a rough estimate of the initial intracellular radioactivity. Towards the end of the experiment errors from statistical fluctuations in the counting rates became appreciable; the vertical lines indicate $\pm 2 \times$ S.E. Axon diameter 512 $\mu$. Length 51 mm. Temperature 17° C. For calcium entry, 1 count/min was equivalent to 3·4 pmole.

One interpretation of the result illustrated in Fig. 2 is that during the first 90 min of exposure to inactive sea water the $^{45}$Ca lost is largely of extra-axonal origin, and that the final 0·6 counts/min$^2$ represents the rate of emergence of labelled calcium from the interior of the axon. The comparison made above between the activities of extruded axoplasm and of the residue at the end of

the experiment show that after 4 hr the sheath is unlikely to have retained an important fraction of the total radioactivity. Extrapolation of the supposed intracellular radioactivity to zero time, as in Fig. 2*B*, gives a figure of about 940 counts/min, and a corresponding extracellular radioactivity of well over 1000 counts/min. An entry of 940 counts/min in 117 min for a total membrane area of 0·82 $cm^2$ corresponds to a resting calcium influx of 0·56 pmole/$cm^2$.sec, since 1 count/min was equivalent to 3·4 pmoles of Ca*. This value is rather higher than those obtained later with extruded axoplasm (see Table 2), probably because the necessity for extreme vigilance in inspecting the axon for small injuries had not been fully appreciated at the time when the experiment was done, and the axon may have had a few slightly damaged branches where an abnormally fast inward leakage of calcium was occurring. However, its condition was certainly not bad, since at the end of the period in inactive sea water it was still giving a 70 mV spike (recorded externally) almost to its ends.

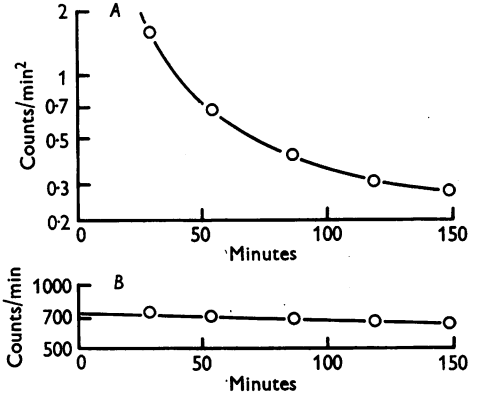


Fig. 3. The time course of calcium exchange in an isolated squid axon which was first stimulated at 156/sec for 20 min in a solution containing 112 mM-Ca* and was then washed in a series of samples of inactive sea water. *A* shows how the efflux of radioactivity (in counts/$min^2$) varied, while *B* shows the decline of the total amount of radioactivity (in counts/min) in the axon. Counted on a dish at the end of the experiment, the axon contained 654 counts/min. Axon diameter 469 $\mu$. The axon was still excitable at the end of the experiment, though its length was only 35 mm. Temperature 19° C. For calcium entry, 1 count/min was equivalent to 13·9 pmole.

The inference that immediately after removal from the radioactive solution there was more $^{45}Ca$ outside the membrane than inside was borne out by some subsequent experiments in which the sheath was counted as well as the extruded axoplasm. Even after a prolonged exposure to $^{45}Ca$ sea water, and despite a 10 min rinse in inactive sea water before extrusion, the count per mg axoplasm was sometimes less than one-fifth of the count per mg residue.

Fig. 3 shows the results of another 'soak-out' experiment, in which the axon was stimulated at a high rate in the radioactive solution instead of being

left at rest, and in which the external [Ca*] was ten times higher. It is clear from Fig. 3*B* that this treatment gave a slowly exchanging fraction which formed a larger proportion of the total radioactivity taken up than in Fig. 2*B*, i.e. that the amount of $^{45}Ca$ which had apparently entered the axoplasm was appreciably greater in comparison with that which had exchanged with external calcium. This conclusion was not checked by separate counts of axoplasm and sheath, but agrees with the results for extruded axoplasm (see Table 4), which showed that stimulation with raised external calcium greatly accelerated the calcium influx. The extrapolated count for intracellular $^{45}Ca$ was 730 counts/min. Since the axon had a membrane area of 0·52 cm$^2$ and had conducted 187,200 impulses, and 1 count/min being equivalent to 13·9 pmole Ca*, the estimated calcium entry per impulse was about 0·10 pmole/cm$^2$, which fits well with the figures in Table 4 and with an analysis of the intracellular calcium content of a squid axon treated similarly (Keynes & Lewis, 1956).

In Fig. 2 the rate constant for loss of radioactivity during the last 2 hr of the experiment was about $0{\cdot}6 \times 60/800 = 0{\cdot}045$ hr$^{-1}$. In Fig. 3 it was 0·025 hr$^{-1}$. These figures are within the range found for the extrusion of $^{45}Ca$ introduced into axons by micro-injection (see Table 6). The intracellular calcium was therefore exchanged with a rather long half-period, of the order of 20 hr. Although the extracellular calcium was exchanged with substantially greater rapidity than this, the half-period of the initial phase in Fig. 2 was not less than 15 min. This is about double the figure reported by Soloway, Welsh & Solomon (1953) for the first phase of exchange of $^{45}Ca$ in crayfish nerve, and is long enough to make it difficult to derive accurate values for the calcium influx from measurements on intact axons, particularly at low external calcium concentrations. After repeating the experiment of Fig. 2 once, with results which were very similar except that the slowly exchanging fraction was somewhat smaller (700 counts/min gained in 123 min) and the faster fraction larger (*ca.* 2000 counts/min), influxes were therefore measured by counting extruded axoplasm.

### *The calcium influx into squid axons*

Those who work with squid axons are apt to become familiar with the brownish patches which appear in the axoplasm close to the bases of branches which have inadvertently been damaged, or which have been cut off too short in the course of dissection. In the giant axons of *Sepia*, too, similar patches are often observed at places where the fibre has been pinched, and their occurrence is usually accompanied by a partial or total loss of excitability over the affected region. There is reason for thinking that these patches are probably caused by an unduly fast penetration of calcium at the injured point. Thus they are generally associated with a greater liquidity of the axoplasm,

which might well result from a calcium entry (Hodgkin & Katz, 1949*a*), while Hodgkin & Keynes (1956) noticed that injection of calcium into squid axons brought about a definite increase in the opacity of the axoplasm as well as the liquefaction observed by Chambers & Kao (1952).

In order to make a direct test of the causal connexion between an entry of calcium and discoloration of the axoplasm, some cleaned squid axons were deliberately maltreated both in normal sea water and in an artificial sea water containing no calcium. With calcium present, when branches were torn off altogether so as to leave a visible hole, axoplasm flowed out, and although browning developed in the vicinity, the whole axon tended to collapse. Typical brown patches could best be produced by nipping branches at their bases with fine forceps: opacity was first visible after about 10 min, and the characteristic appearance was fully developed in 30 min. Similar treatment in a calcium-free medium never gave rise to any opaque patches, even after 24 hr. Taken with the earlier evidence, this suggested that the appearance of a brown patch on a squid axon almost certainly indicated an abnormally rapid entry of calcium in its immediate locality, so that axoplasm extruded from this part of the axon was unlikely to give a reliable figure for the calcium influx. The calcium-free solution contained the usual 55 mM magnesium, so that the effect on the axoplasm is evidently specific for calcium; this agrees with the findings of Hodgkin & Katz (1949*a*).

The importance of rejecting blemished axons was emphasized by the results of some of the first extrusions when, for lack of better material, axoplasm from some imperfectly dissected specimens was examined. One axon, which was inexcitable and whose axoplasm was recorded as being liquid and brownish, contained 3480 pmole Ca*/mg axoplasm after 132 min in sea water containing 10·7 mM-Ca*, corresponding to a calcium influx of 6·6 pmole/cm$^2$.sec. Calcium was thus entering very much faster than into any of the axons in good condition exposed to this medium, for which figures are given in Table 2. All other axons which were rejected as being visibly defective were found to give influxes intermediate between this exceptionally large value and the highest of the results for resting axons listed in Table 2. Perhaps the clearest instance of the effect of damage was provided by an axon which was extruded in two parts because there were two small brown patches towards one end. The 'bad' end contained nearly three times as much labelled calcium as the 'good' one. In view of these observations, particular care was taken to inspect all axons thoroughly before extruding the axoplasm, and the extrusion procedure was designed so that the axoplasm came only from the centre of the axon, about 15 mm at either end being left unextruded.

The results of measuring the calcium influx in uncleaned resting squid axons with an external calcium concentration of 10·7 mM (together with 55 mM-Mg, solution (1), Table 1) are given in Table 2. Before these experiments were done, a number of observations were made with cleaned axons, but it was finally thought best to exclude all the results for cleaned axons from Table 2, because their reliability seemed somewhat doubtful. The resting calcium influx into four apparently undamaged cleaned axons ranged from 0·048 to 0·36 pmole/cm$^2$.sec, and the mean ($0{\cdot}16 \pm 0{\cdot}07$ pmole/cm$^2$.sec) did not differ significantly from that in Table 2(*a*); but one or two of the large values were

probably spuriously high because of undetected injuries in the part of the axon from which axoplasm was extruded. The danger of accidentally damaging the axon could be appreciably lessened by omitting the cleaning process, and most of the work was therefore done with uncleaned axons. The main group of figures in Table 2 is for axons which were soaked in the radioactive solution as intact nerve trunks, and which had not been stimulated more than was necessary in order to test their excitability. They gave an average calcium influx of 0·076 pmole/cm$^2$.sec. The two values in group (*b*) were obtained with axons which had been subjected to prolonged stimulation (one rather more than the other) in inactive sea water before exposure to $^{45}$Ca. They were within the range for unstimulated axons, which serves to show that the effects of stimulation on the calcium influx discussed below did not consist simply in an irreversibly increased leakiness.

TABLE 2. The calcium influx into resting squid axons at room temperature (17–19° C) from a solution containing 10·7 mM-Ca* and 55 mM-Mg

| Axon diam. ($\mu$) | Time in Ca* (min) | Amount of labelled calcium in axoplasm (pmole/mg) | Calcium influx (pmole/cm$^2$.sec) |
|---|---|---|---|
| | (*a*) Uncleaned axons, unstimulated | | |
| 531 | 78 | 18·4 | 0·055 |
| 593 | 108 | 60·6 | 0·146 |
| 535 | 60 | 23·4 | 0·091 |
| 720 | 84·5 | 13·4 | 0·050 |
| 500 | 517 | 95·4 | 0·040 |
| Mean and S.E. | — | — | 0·076±0·019 |
| (*b*) Uncleaned axons, previously stimulated 20 and 33 min at 50/sec | | | |
| 524 | 57 | 23·4 | 0·094 |
| 425 | 61 | 43·4 | 0·132 |

A striking feature of the experiments listed in Table 2 is the smallness of the quantity of calcium exchanged. Even in an axon which was left during the night (8·6 hr) in the radioactive solution, the Ca* content of the axoplasm was only 95 pmole/mg, that is to say just under 0·1 m-mole/kg of labelled calcium had entered the axon. These results are therefore wholly at variance with those of Rothenberg (1950), who reported Ca* concentrations up to 10 m-mole/kg in axoplasm extruded from *Loligo pealii* axons after soaking for 50 min in $^{45}$Ca sea water. The discrepancy suggests that the axons studied by Rothenberg may have been in poor physiological condition. The total calcium content of axoplasm from freshly dissected squid axons is under 0·5 m-mole/kg (Keynes & Lewis, 1956), and figures so much higher for labelled calcium can only mean that a rapid net inward movement of calcium was taking place. This suggestion is supported by Rothenberg's values for the sodium contents of another series of axons, which were very much higher than the analyses of Steinbach & Spiegelman (1943) and Keynes & Lewis (1951), sodium being another ion which would be expected to leak into a damaged fibre.

Table 3 gives figures for the calcium influx into squid axons which were stimulated at 50 or 156 impulses/sec while exposed to a solution containing 10·7 mM-Ca* and 55 mM-Mg. In every case a substantial increase in the influx was observed, the average extra entry of calcium into the uncleaned axons amounting to 0·0062 pmole/cm².impulse; this corresponds to the passage of 38 calcium ions across 1 $\mu^2$ of surface membrane during each impulse. Preliminary experiments with cleaned axons gave extra entries between 0·0040 and 0·043 pmole/cm².impulse, the mean for six axons being 0·016 ± 0·006 pmole/cm².impulse, but again several of the axons gave influxes so high as to raise suspicion that some damage to them had passed unnoticed, and it appeared safest to reject all these results from the table. However, the somewhat higher average entry found in cleaned axons did suggest that the small fibres surrounding the large one might hinder the influx of calcium into an uncleaned axon, and two experiments were undertaken at a later stage in the work to test this point. The two values in group (*b*) were for axons from the

TABLE 3. The entry of calcium into stimulated squid axons from a solution containing 10·7 mM-Ca* and 55 mM-Mg

| Axon diam. ($\mu$) | Time in Ca* (min) | Amount of Ca* in axoplasm (pmole/mg) | Total calcium influx (pmole/cm².sec) | Rate of stimulation (impulses/sec) | Extra entry of calcium per impulse (pmole/cm²) |
|---|---|---|---|---|---|
| | | | (*a*) Uncleaned axons | | |
| 585 | 42 | 81·6 | 0·50 | 50 | 0·0085 |
| 516 | 30 | 54·3 | 0·41 | 50 | 0·0067 |
| 409 | 40 | 116·2 | 0·52 | 50 | 0·0089 |
| 612 | 21·5 | 32·7 | 0·41† | 156 | 0·0023 |
| 501 | 20·8 | 100·0 | 1·06 | 156 | 0·0065 |
| 628 | 20·8 | 74·0 | 0·74 | 156 | 0·0044 |
| Mean and S.E. | | — | — | — | 0·0062 ± 0·0010 |
| | | | (*b*) Cleaned axons | | |
| 460 | 20·6 | 102 | 1·00 | 156 | 0·0061 |
| 465 | 20·8 | 95·0 | 0·93 | 156 | 0·0057 |

In order to calculate the extra entry of calcium, the resting influx was taken as 0·076 pmole / cm².sec (from Table 2), except for the case marked †, where a paired axon from the same squid gave 0·050 pmole/cm².sec. In some cases allowance has been made for the fact that the axon was not stimulated for quite the whole of the period in Ca*. Temperature 17–19° C.

same squid as provided the last two values in group (*a*), dissected and cleaned with especial care. The stimulated influxes into the cleaned and uncleaned members of each pair of axons did not differ appreciably, showing that any effect of cleaning on the entry of calcium must have been a small one.

Having established that there was an increased entry of calcium during nervous activity, we investigated the effect on it of varying the external calcium concentration and the temperature. The results given in Table 4 were obtained with pairs of uncleaned axons, soaked for 20 or 30 min in radioactive solutions made up according to formulae (2), (3) or (4) in Table 1, one axon resting and the other stimulated at 156 or 25/sec. Only one axon at a time

could be exposed to $^{45}Ca$; sometimes the resting influx was measured first, and sometimes the stimulated influx. In working out the extra entry of calcium per impulse, the value of the resting influx for the corresponding control axon was subtracted from the total influx into the stimulated axon, and allowance was made for the amount by which the period in $^{45}Ca$ (usually 20 min 30 sec) exceeded the actual duration of stimulation (usually 20 min exactly). A plot of the resting calcium influx against calcium concentration (Fig. 4) suggests that in the Mg-free solutions the resting influx at first rises with concentration,

TABLE 4. The effect of stimulation on the calcium influx into pairs of uncleaned squid axons from solutions containing various amounts of calcium and no magnesium

| Axon 1 diam. ($\mu$) | Resting calcium influx (pmole/cm$^2$.sec) | Temp. (° C) | Axon 2 diam. ($\mu$) | Total calcium influx during stimulation (pmole/cm$^2$.sec) | Stimulation rate (impulses/sec) | Extra entry of calcium per impulse (pmole/cm$^2$) |
|---|---|---|---|---|---|---|
| | | | (*a*) In 22 mM-Ca* | | | |
| 494 | 0·25 | 21 | 532 | 2·24 | 156 | 0·0130 |
| 553 | 0·43 | 20·5 | 574 | 1·81 | 156 | 0·0091 |
| 634 | 0·44 | 21·5 | 602 | 1·57 | 156 | 0·0074 |
| Mean and S.E. | 0·37±0·06 | — | — | — | — | 0·0098±0·0017 |
| | | | (*b*) In 44 mM-Ca* | | | |
| 561 | 0·58 | 24 | 534 | 4·06 | 156 | 0·023 |
| 566 | 0·80 | 24 | 561 | 4·31 | 156 | 0·023 |
| 522 | 0·35 | 22 | 514 | 5·1 | 156 | 0·031 |
| Mean and S.E. | 0·58±0·13 | — | — | — | — | 0·026±0·003 |
| | | | (*c*) In 112 mM-Ca* | | | |
| 515 | 0·73 | 22 | 563 | 12·0 | 156 | 0·074 |
| 577 | 0·52 | 22 | 591 | 12·9 | 156 | 0·083 |
| 567 | 0·57 | 22 | 602 | 2·81 | 25 | 0·093 |
| Mean and S.E. | 0·61±0·06 | 22 | — | — | — | 0·083±0·005 |
| 469 | 0·62 | 8 | 476 | 3·78 | 25 | 0·129 |
| 507 | 0·34 | 8 | 490 | 4·81 | 25 | 0·182 |
| 504 | 0·46 | 8 | 504 | 3·72 | 25 | 0·132 |
| Mean and S.E. | 0·47±0·08 | 8 | — | — | — | 0·148±0·017 |

but then reaches a limiting value of about 0·6 pmole/cm$^2$.sec. The influx of about 0·1 pmole/cm$^2$.sec from artificial sea water containing 10·7 mM-Ca and 55 mM-Mg (see Table 2) would be consistent with this figure if magnesium competes, with approximately the same effectiveness as calcium, in the process which limits calcium entry at high concentrations. The extra entry of calcium during stimulation behaves somewhat differently, and can be seen from Fig. 4 to rise roughly in proportion to external concentration over the range between 22 and 112 mM-Ca ([Mg] = 0). The value from Table 3 for the extra entry from 10·7 mM-Ca and 55 mM-Mg is also plotted in Fig. 4, and lies on the same straight line as the other points. The slope of this line is the constant of proportionality relating extra entry to calcium concentration, and a value of $6{\cdot}6 \times 10^{-4}$ pmole/cm$^2$.impulse per mM-Ca fits the data reasonably well.

Since the entry of calcium into resting axons appears to reach saturation

level at high external [Ca], whereas the entry into stimulated axons does not, the effect of stimulation was most striking for 112 mM-Ca, which was the highest concentration used. Here the total influx at 156 impulses/sec was about 20 times greater than in a resting axon. One experiment at a lower rate of stimulation (25/sec) showed, as might be expected, a smaller rise in total influx, but gave about the same value for the extra entry per impulse.

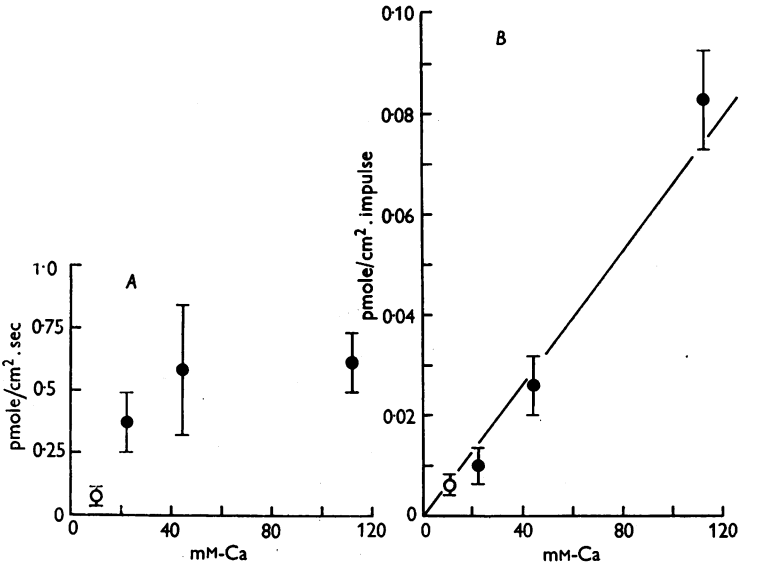


Fig. 4. Average values for calcium influxes into uncleaned squid axons, taken from Tables 2–4, plotted against external calcium concentration. *A*, resting influx, *B*, extra influx during stimulation. Vertical bars indicate $\pm 2 \times$ S.E. of mean influx. For the solution containing 10·7 mM-Ca, ○, 55 mM-Mg was also present; the other solutions, ●, contained no magnesium.

The observations just described were made at a room temperature of about 22° C. Three experiments at a lower temperature (8° C) showed that the effect of cooling was to decrease the resting influx slightly, but nearly to double the extra entry per impulse. The increase in the extra entry is a change in the same direction as that undergone by the duration of the spike (Hodgkin & Katz, 1949*c*), though it is somewhat smaller. It is to be expected (see Hodgkin & Huxley, 1952) that the amounts of sodium and potassium transferred through the membrane during the spike will also increase at low temperatures, and in the case of potassium an increase has been demonstrated experimentally by Shanes (1954).

Finally, the effect of depolarizing the membrane by raising the external potassium concentration was investigated. The values in Table 5 show that the resting calcium influx from a solution containing 112 mM-Ca* and 52 or 208 mM-K was appreciably greater than the average of 0·61 pmole/cm².sec

obtained for the same calcium concentration with only 10·4 mM-K (see Table 4). Nevertheless, the sustained depolarization brought about by 208 mM-K raised the influx much less than stimulation at 156 impulses/sec, as may be seen by comparing the high-K values in Table 5 with those for paired axons stimulated in 112 mM-Ca, or with those for stimulated axons in Table 4.

TABLE 5. The effect on the calcium influx of raising the external potassium concentration; the solutions contained 112 mM-Ca*

| Axon diam. ($\mu$) | Temp. (° C) | Potassium concentration (mM) | Resting calcium influx (pmole/cm$^2$.sec) | Total influx into paired axon stim at 156/sec in 10 mM-K (pmole/cm$^2$.sec) |
|---|---|---|---|---|
| 515 | 20 | 52 | 2·74 | — |
| 480 | 20 | 208 | 1·58 | — |
| 413 | 20 | 208 | 4·83 | 17·9 |
| 462 | 19 | 208 | 2·28 | 12·5 |
| Mean and S.E. | — | 208 | 2·9±1·0 | — |

*The calcium efflux*

It was clear from the experiment illustrated in Fig. 2 that it would be difficult to make reliable measurements of the calcium efflux after labelling the internal calcium by soaking axons in a radioactive solution, because it would be necessary to wash the axons for an excessively lengthy period in inactive sea water before the loss of $^{45}$Ca could safely be assumed to represent a pure efflux from within the cell, unmixed with an exchange of extra-axonal calcium. However, the calcium efflux could be determined without interference from extracellular calcium by introducing the $^{45}$Ca directly into the interior of the axon by means of the microsyringe described by Hodgkin & Keynes (1956). The range of experiments that could be performed was somewhat restricted, since large amounts of calcium could not be injected for fear of damaging the axon, while the injection of quantities of labelled calcium much smaller than the original calcium content of the axoplasm was not feasible because the counting rates would not have been adequate.

The units in which the effluxes were measured were counts/min$^2$. Knowing the area of membrane around the injected region (0·25–0·46 cm$^2$), and the specific activity of the radioactive solution (after correcting for decay, 1 count/min was equivalent in every case to 1·4 pmoles Ca, since the activities of the $^{45}$Ca samples happened to be the same in 1954 and 1955), the effluxes of labelled calcium could be expressed in pmole/cm$^2$.sec. The values obtained in five experiments are plotted in Fig. 5, and further details for these and four other experiments are given in Table 6. The absolute size of the calcium efflux was, in all probability, somewhat larger than the efflux of Ca*, owing to dilution of the injected calcium by the unlabelled calcium originally present in the axoplasm. On the assumptions that the labelled and unlabelled calcium

became uniformly mixed within a few minutes of the injection, and that the initial calcium content was 0·42 mM (from the analyses of Keynes & Lewis, 1956), the possible extent of the dilution could be worked out. As may be seen from column 6 of Table 6, the mean Ca* concentrations in the axoplasm ranged from 0·15 to 0·87 mM, giving dilution factors between (0·15+0·42)/0·15=3·8 (Expt. 4) and (0·87+0·42)/0·87=1·5 (Expt. 2). Multiplication of the effluxes of labelled calcium by these factors gave absolute effluxes ranging from 0·075 to 0·50 pmole/cm$^2$.sec.

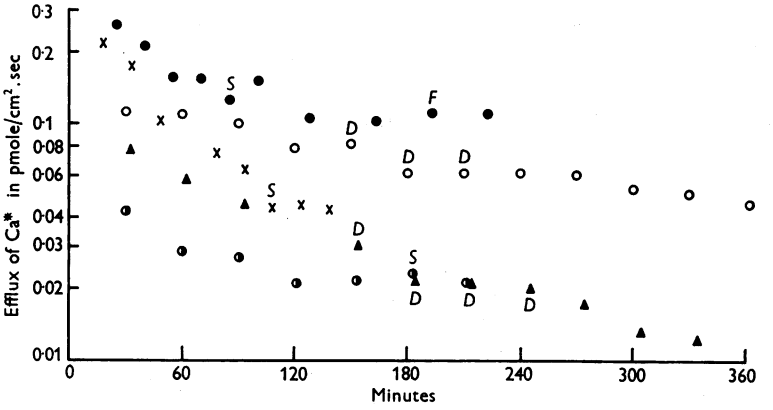


Fig. 5. The efflux of labelled calcium introduced into cleaned squid axons by micro-injection. Experimental details are given in Table 6, the symbols being as follows: ×, Expt. 1; ●, Expt. 3; ◑, Expt. 6; ○, Expt. 7; ▲, Expt. 8. *S*, axon stimulated at 50 impulses/sec. *F*, axon in calcium-free sea water. *D*, axon treated with 0·2 mM-DNP.

TABLE 6. Effluxes of labelled calcium from injected squid axons

| Expt. no. | Axon diam. ($\mu$) | Length injected (mm) | Volume injected ($10^{-6}$ ml./mm) | [Ca*] injected (mM) | Internal [Ca*] immediately after injection (mM) | Initial efflux of labelled calcium (pmole/cm$^2$.sec) | Rate constant for loss of $^{45}$Ca: Initial ($hr^{-1}$) | Rate constant for loss of $^{45}$Ca: Final ($hr^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| 1 | 585 | 15 | 8·5 | 18·8 | 0·60 | 0·221 | 0·125 | 0·027 |
| 2 | 524 | 20 | 9·9 | 18·8 | 0·87 | 0·336 | 0·111 | 0·044 |
| 3 | 528 | 15 | 9·9 | 18·8 | 0·85 | 0·264 | 0·087 | 0·043 |
| 4 | 546 | 17 | 9·9 | 3·6 | 0·15 | 0·083 | 0·139 | 0·092 |
| 5 | 612 | 20 | 9·75 | 10·4 | 0·34 | 0·034 | 0·027 | 0·019 |
| 6 | 610 | 17 | 9·75 | 10·4 | 0·35 | 0·042 | 0·030 | 0·015 |
| 7 | 637 | 17 | 9·75 | 10·4 | 0·32 | 0·111 | 0·080 | 0·048 |
| 8 | 620 | 17 | 9·75 | 10·4 | 0·34 | 0·076 | 0·058 | 0·011 |
| 9 | 735 | 20 | 9·75 | 10·4 | 0·24 | 0·063 | 0·058 | 0·050 |

The injected solutions contained about 0·57 M-KCl as well as the concentrations of Ca* shown in column 5 of the table. The duration of the experiments was about 100 min (Expts. 2 and 9), 150 min (1, 4 and 5), 220 min (3 and 6), and 350 min (7 and 8). Temperature 18–20° C. The external medium was artificial sea water containing 10·7 mM-Ca and 55 mM-Mg (formula (1) in Table 1), except for Expt. 5 when it had 44 mM-Ca (formula (3) in Table 1). All the axons were cleaned from adherent small fibres before introducing the labelled calcium into them.

It will be observed in Fig. 5 that on every occasion the efflux of Ca* declined with time. Since the Ca* content of the axoplasm was falling throughout the experiment, some reduction in Ca* efflux was to be expected. But the total loss of Ca* was never greater than one-third (in Expt. 7 altogether 32 % of the injected Ca* emerged from the axon, leaving 68 % inside it), and in most cases about 85 % of the injected Ca* still remained in the axon at the end of the last collecting period. The drop in efflux was therefore appreciably greater than could be accounted for by disappearance of the internal Ca*. The magnitude of the effect can be seen most clearly by comparing the figures in the last two columns of Table 6, which show the initial and final rate constants for loss of Ca*. These were obtained by dividing the efflux (in counts/min$^2$) by the total internal radioactivity (in counts/min), so that they give an idea of the extra amount by which the efflux fell in addition to the reduction from loss of Ca*. In some experiments the decline was not very great—in Expt. 7, for example, the rate constant was still at 60 % of its initial value after about 6 hr—but in others the final value was only 20 % of the initial one.

Several reasons for the decline can be suggested. One possibility is that the efficacy of the mechanism responsible for the outward transport of calcium through the membrane falls off in the same sort of fashion in dissected squid axons as does that of the sodium pump (Hodgkin & Keynes, 1956). Another is that the labelled calcium is gradually diluted by progressive mixing with bound calcium in the axoplasm, resulting in a gradual reduction of the specific activity of the calcium available for extrusion from the axon. However, an effect of this kind could not explain the whole of the changes in the rate constants, since it may be seen from Table 6 that in some experiments the rate constant changed by more than twice the dilution factor for complete mixing (e.g. in Expts. 1 and 8, dilution factors of 1·7 and 2·2 could hardly account for reduction in rate constants by factors of 4·6 and 5·3). Moreover, this explanation would predict the greatest effect in the experiment when least Ca* was injected, which does not seem to fit the relatively small change in rate constant found in Expt. 4. A possibility considered (and rejected) in the case of sodium (Hodgkin & Keynes, 1956) was that the efflux was saturated, and that the influx of unlabelled ions greatly exceeded the efflux, so that the internal specific activity fell faster than would otherwise be expected; but this also seems inapplicable for calcium, since it would require the influxes in the experiments of Table 6 to have been substantially greater than the values given in Table 2 for comparable conditions. An interesting way of explaining the fall in rate constant would be as follows: suppose that the efflux is proportional to the internal concentration of ionized calcium, and that there are calcium-binding substances in the axoplasm with a high affinity for Ca. The relationship between efflux and total internal calcium would then be non-linear, the efflux changing rather slowly at low intracellular concentrations, but rising much more steeply when there was sufficient calcium in the axoplasm to saturate the calcium-binders. If the normal calcium content of the axoplasm were roughly at the inflexion of the efflux-[Ca] curve, the efflux would start off at a high level after injecting extra calcium, but would decline rapidly as the total calcium inside the axon dropped back towards its original value. This suggestion seems a plausible one, and may account for some part of the observed changes in rate constant, but it does not appear to be entirely consistent with the experimental results. Thus it would lead one to expect the smallest change in rate constant when the least quantity of Ca* was injected, and although the figures in Table 6 show some tendency in this direction, the trend is not at all definite. A test of the hypothesis is to examine the relationship between total calcium efflux (calculated as described on p. 267) and total intracellular calcium concentration, but unfortunately the scatter of the values which can be derived from Table 6 is too great for a decisive conclusion to be reached ,

and the points would fit no better on to a curve of the suggested type than on to a straight line drawn to pass through the origin. Although it would not be difficult to devise experiments that would help to discriminate between these various possibilities, we did not have an opportunity of doing so, and hence can only conclude tentatively that any or all of them may contribute in some degree to the decline in efflux.

In a steady state the influx and efflux of calcium should be equal, and it is therefore interesting to see whether the estimates of efflux are reasonably consistent with those of influx. If the efflux from a freshly dissected axon 600 $\mu$ in diameter containing 0·4 mM-Ca were equal to the influx from a solution with 10·7 mM-Ca and 55 mM-Mg, which was (from Table 2) 0·08 pmole/cm$^2$.sec, the rate constant for exchange of internal calcium would be 0·048 hr$^{-1}$. This figure lies in the middle of the range of values given in Table 6, and is close to that calculated for Fig. 2, whence it may be concluded that the influxes and effluxes are in tolerable agreement.

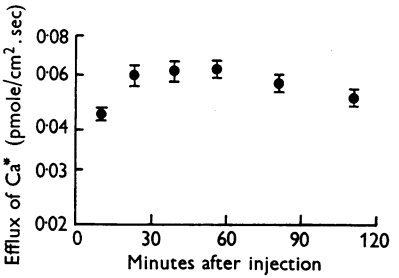


Fig. 6. The efflux of labelled calcium from a cleaned squid axon. Collection of the first sample was begun 5 min after injecting the isotope solution. For further experimental details see Expt. 9, Table 6. The vertical bars show $\pm 2 \times$ S.E. of the counts.

In most of the experiments some 15 min elapsed between injecting the $^{45}$Ca and starting the first collecting period, and the results gave no useful information about the rate of diffusion of the labelled calcium from the centre of the axon to the surface. On one occasion, however, the collection was begun within 5 min of completing the injection, and it was then found that the first sample contained significantly less $^{45}$Ca than the subsequent ones. The counts shown in Fig. 6 suggest that the mean efflux of $^{45}$Ca ($y$) during the period 5–15 min after the injection was roughly 70 % of the steady-state value ($y_\infty$). This observation can be used to derive a value for the effective diffusion constant ($D$) of calcium in the axoplasm, by applying the argument used for the radial diffusion of sodium in an earlier paper on micro-injection (Hodgkin & Keynes, 1956). For $y/y_\infty = 0{\cdot}7$, the value of $Dt/a^2$ given by equation (14) in that paper is 0·14 (for sodium, the curve was summated before being applied to the results, but in the present case it is more appropriate to use the equation unmodified, since the collection of $^{45}$Ca did not include the first 5 min after the injection). The radius of the axon ($a$) was 368 $\mu$, and the mid time ($t$) was

600 sec, so that $D$ was about $0{\cdot}3 \times 10^{-6}$ cm$^2$/sec. The uncertainty in this calculation arising from the appreciable standard errors of the counts and other sources is of the order of $\pm 0{\cdot}1 \times 10^{-6}$ cm$^2$/sec, so that although $D$ is undoubtedly much less than the self-diffusion coefficient of calcium ions in free solution (about $6 \times 10^{-6}$ cm$^2$/sec, see p. 275), it is probably not significantly different from the values estimated for the longitudinal diffusion of calcium in the axoplasm (see Table 7).

In view of the marked effect of stimulation on the calcium influx, it was important to see whether there was a similar rise in the efflux, i.e. to decide whether there was a virtually unidirectional movement of calcium through the membrane during nervous activity, or whether there was an accelerated turnover in both directions. During the whole of one collecting period in each of three experiments (points marked $S$ in Fig. 5) the axon was therefore stimulated at 50 impulses/sec. In no case did stimulation cause a noticeable change in efflux. This observation indicates that the extra calcium movement during activity is almost wholly inward. It is confirmed by the agreement already mentioned between the entry of labelled calcium into stimulated squid axons and the net gain of calcium under similar circumstances (Keynes & Lewis, 1956).

Two other variables were investigated in the course of the efflux measurements. During one collecting period (point marked $F$ in Fig. 5) the axon was exposed to an artificial sea water from which all the calcium was omitted (leaving, however, 55 mM-Mg). This treatment did not obviously alter the calcium efflux. During two other experiments, the axons were treated with 0·2 mM-DNP for 90 and 120 min respectively (points marked $D$ in Fig. 5). Again there was no clear alteration in efflux, or at least none that was comparable with the effect of metabolic inhibitors on the sodium efflux (Hodgkin & Keynes, 1955). A reservation must, however, be made about this observation. It has recently been found (P. C. Caldwell & R. D. Keynes, unpublished) that the action of DNP on squid axons is very sensitive to external pH, so that although the effect described previously is readily obtained at pH 7 or below, 0·2 mM-DNP reduces the sodium efflux very little at pH's much above 8. Checks with a glass electrode assembly and pH meter made some time after completing the experimental work gave a pH of 6·8–6·9 for solutions made up according to the formulae in Table 1, but the pH was not measured accurately at the time when the experiments were being done. Although it appears likely that the DNP solutions used in the $^{45}$Ca experiments were sufficiently acid to be effective in blocking metabolism, it is impossible to be absolutely certain that they were.

*The mobility of calcium in the axoplasm*

The extent to which radioactive calcium was free to move longitudinally in the axoplasm was investigated by labelling a short patch and observing the

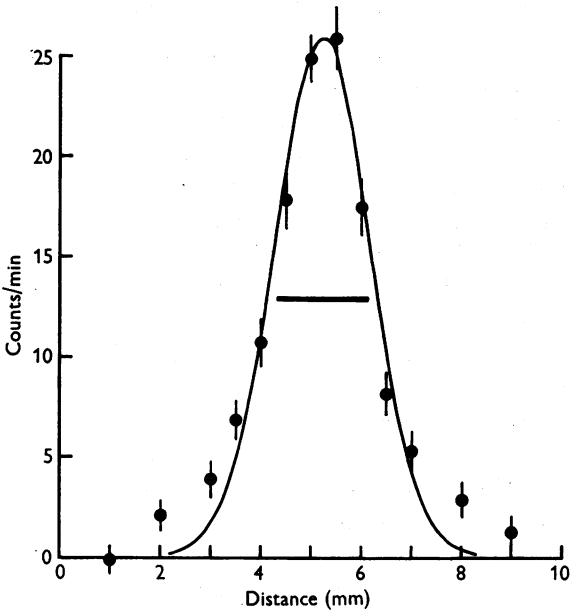


Fig. 7. The distribution of radioactivity in an axon which was mounted on its glass slide and left to dry at room temperature 14 min after injecting a column of radioactive calcium 1·82 mm in length (Expt. 8, Table 7). The nominal position of the injected column is indicated by the horizontal bar. The vertical lines show $\pm 2 \times$ S.E. of the counts. The curve was drawn by taking $h = 0{\cdot}091$, $2\sqrt{Dt} = 0{\cdot}083$ cm in equation (1), and correcting for the window characteristic.

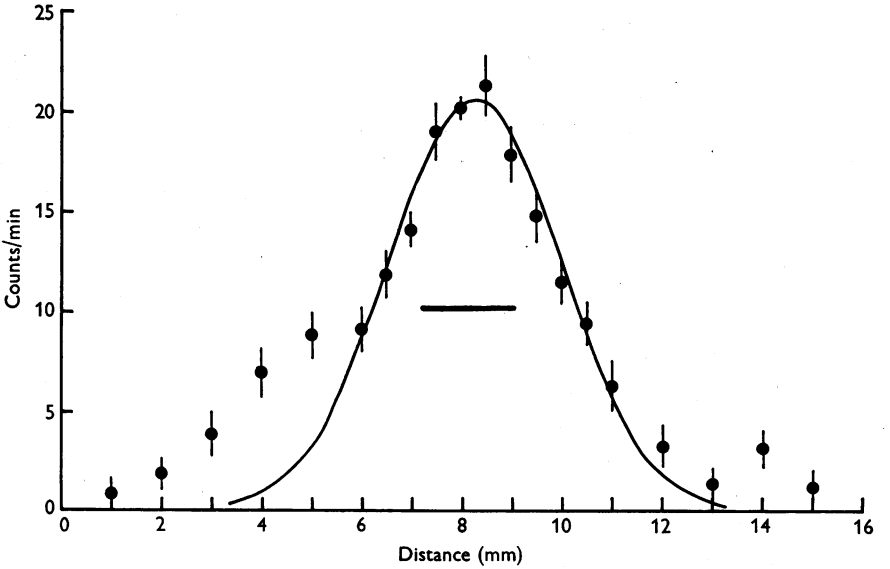


Fig. 8. The distribution of radioactivity in an axon which was mounted on its glass slide and dried under a lamp 478 min after injecting a column of $^{45}CaCl_2$ (Expt. 4, Table 7). The cannulated end of the axon was to the left of the injected patch, both here and in Figs. 7 and 9. The vertical lines show $\pm 2 \times$ S.E. of the counts. The curve was drawn by taking $h = 0{\cdot}092$, $2\sqrt{Dt} = 0{\cdot}22$ cm in equation (1), and correcting for the window characteristic. Its lateral position was chosen to fit the counts, not the nominal centre of the injected patch, shown as a thick horizontal bar.

subsequent movements of the $^{45}Ca$. A 1·8–3·6 mm column of a solution containing 10·4 mM-Ca*$Cl_2$ and 560 mM-KCl was injected into the axon, which was then mounted on forceps and transferred to a dish of sea water or paraffin oil, in which a longitudinal voltage gradient could be applied. After diffusion of calcium within the axon had been allowed to continue for periods up to 12 hr, either with or without applied current, the axon was mounted on a glass slide and dried. The procedure for locating the injected region and for measuring the final distribution of $^{45}Ca$ is described under 'Methods'. Fig. 7 shows the counts taken in an experiment where the axon was dried as soon as possible (14 min) after injecting the isotope solution, in order to test the sharpness of the initial distribution. The position at which the counting rate was greatest agreed satisfactorily with the nominal centre of the injected patch, indicated on the figure as a thick bar, and the total width of the distribution curve at half the height of its peak was very little greater than the over-all length of the injected patch. This demonstrates that the injection technique gave a labelled region which was initially well defined, and provides some evidence that its nominal centre could be localized with reasonable accuracy.

When the axon was left without current for some time after injecting the $^{45}Ca$ and before drying it, the patch of radioactivity became appreciably wider relative to the length of the injected region. Fig. 8 shows the measured distribution of radioactivity in such an axon left for 8 hr before drying, and it will be seen that the patch was broader than in Fig. 7 and had become slightly asymmetrical. The position of maximum radioactivity again coincided fairly well with the nominal centre. Fig. 9 is a similar curve for an experiment in which a voltage gradient of 0·51 V/cm was applied to the axon for 110 min, in order to see how fast the labelled calcium would move in an electric field. If the $^{45}Ca$ had had the same mobility in the axoplasm as in free solution (about $4 \times 10^{-4}$ cm/sec per V/cm, see p. 275), this procedure would have shifted the radioactive patch about 13 mm towards the cathode. The actual movement was estimated as only 0·31 mm towards the cathode, while in two further experiments of the same sort the movements were in the other direction, being 0·20 and 0·13 mm towards the anode (Table 7). It is doubtful if any of these small displacements can be attributed to the influence of the electric field, since it will be seen from Table 7 that not only were they all towards the cannulated end of the axon, suggesting that there was a systematic trend in this direction superimposed on any action of the field, but also that shifts of roughly equal size were recorded in all but one of the experiments in which no volts were applied. The upshot is that any movement of calcium in the axoplasm that did result from the voltage gradient was too small to be measured satisfactorily by this technique. In similar experiments on frog muscle, Harris (1957) also found that intracellular $^{45}Ca$ did not move

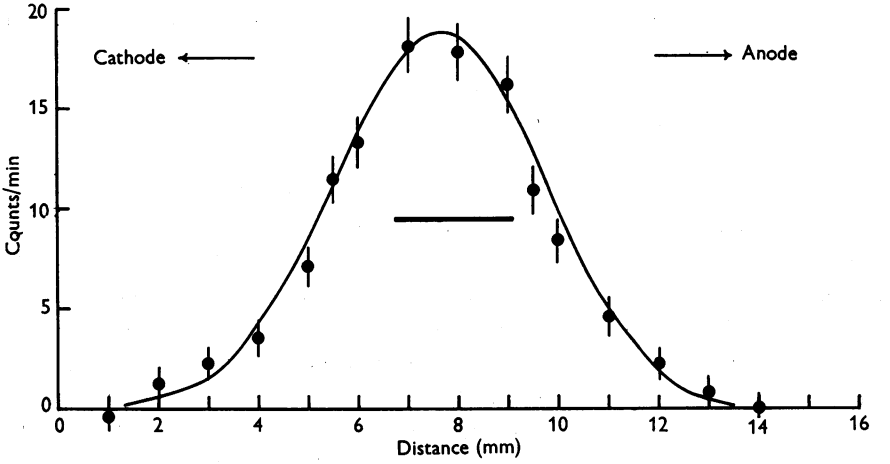


Fig. 9. The distribution of radioactivity in an axon which was mounted on its glass slide and left to dry at room temperature 159 min after injecting a column of $^{45}CaCl_2$ (Expt. 6, Table 7). Electric current flowed along the axon for 110 min, the voltage gradient in the injected region being 0·51 V/cm. The vertical lines show $\pm 2 \times$ S.E. of the counts. The curve was drawn by taking $h = 0{\cdot}116$, $2\sqrt{Dt} = 0{\cdot}262$ cm in equation (1), and correcting for the window characteristic. The nominal position of the injected patch is shown as a thick horizontal bar.

TABLE 7. Longitudinal movements of labelled calcium in squid axons

| Expt. no. | Axon diam. ($\mu$) | ($=2h$) Length of patch injected (mm) | Measured width of radioactive patch (mm) | ($=t$) Total duration (min) | Voltage gradient (V/cm) | Duration of current flow (min) | Movement towards cannulated end (mm) | ($=D$) Calculated diffusion constant ($10^{-6}$ cm$^2$/sec) |
|---|---|---|---|---|---|---|---|---|
| 1 | 630 | 3·6 | 5·4 | 135 | 0·4 | 65 | 0·20 | 2·3 |
| 2 | 672 | 3·5 | 6·7 | 761 | — | — | 0·62 | 0·8 |
| 3 | 735 | 3·0 | 3·6 | 19 | — | — | −0·38 | 4·6 |
| 4 | 620 | 1·8 | 4·1 | 478 | — | — | 0·18 | 0·4 |
| 5 | 602 | 2·2 | 3·5 | 160 | 0·43 | 100 | 0·13 | 0·8 |
| 6 | 620 | 2·3 | 4·5 | 159 | 0·51 | 110 | 0·31 | 1·8 |
| 7 | 766 | 2·6 | 3·1 | 18·5 | — | — | 0·05 | 3·2 |
| 8 | 672 | 1·8 | 2·0 | 14 | — | — | 0·04 | 2·0 |

The final widths of the radioactive patches given in column 4 were measured at half the peak, and the values for $D$ were chosen to make the widths of the curves calculated from equation (1) equal to these after allowing for the window characteristic. The mean position of each distribution curve relative to the nominal centre of the injected patch was worked out by averaging the figures for the mid points at 75, 50 and 25% of the peak height, and the rather consistent shift towards the cannulated end resulted from the asymmetry of most of the curves. The cannulated end was anodal for Expts. 1 and 5, and cathodal for Expt. 6. Temperature 18–20° C. Except in Expt. 2, where there was only a local response, all axons gave a spike over the central 20 mm immediately before being mounted on their slides for drying. The labelled solution injected into the axons contained 10·4 mM-Ca*, so that the average concentration of Ca* in the axoplasm shortly after the injection was about 0·3 mM.

perceptibly in an electric field. The experiment of Fig. 9 will, however, serve to set an upper limit to the effective mobility of calcium in the axoplasm. The movement of 0·31 mm in 110 min with a gradient of 0·51 V/cm corresponds to a mobility of $0{\cdot}9 \times 10^{-5}$ cm/sec per V/cm, but the true value of the mobility may have been considerably less.

*The diffusion coefficient of calcium in the axoplasm*

The broadening of the patch of radioactivity noted in Fig. 8 should provide a basis for calculating the effective diffusion coefficient of calcium in the axoplasm. Such calculations are, however, subject to several sources of uncertainty. Thus: (1) There may be movements of the $^{45}Ca$ while the axon is being dried, whose extent may vary with the general condition of the axon or with the temperature at which it is dried. (2) Scattering of $\beta$-radiation within the dried axon may increase the apparent width of the patch (the window correction described below compensates partially for scattering effects, but there was probably more scattering for the axons than for the speck of $^{45}CaCl_2$ used to obtain Fig. 1). (3) There may sometimes have been some stirring at the edge of the injected patch during the withdrawal of the microsyringe. (4) As may be seen in Fig. 8 the patch tended to become slightly asymmetrical, with the radioactivity more spread out towards the cannulated end of the fibre. Apparently the $^{45}Ca$ was able to move faster through axoplasm which had been disturbed by the passage of the shaft of the microsyringe than through undisturbed axoplasm. In spite of these uncertainties, it seemed worth while to estimate diffusion constants from the measured distribution curves of radioactivity. The equation governing linear diffusion from an initial rectangular distribution is (see, for example, Crank (1956))

$$C = \tfrac{1}{2}C_0 \left\{ \operatorname{erf} \frac{h+x}{2\sqrt{Dt}} + \operatorname{erf} \frac{h-x}{2\sqrt{Dt}} \right\}, \qquad (1)$$

where $C$ is the concentration of the diffusing substance at time $t$ and distance $x$, $D$ is its diffusion coefficient, and the initial distribution at $t=0$ is $C=C_0$ for $-h<x<h$ and $C=0$ elsewhere. This equation could not be fitted directly to the results, since allowance had to be made for the characteristics of the window used in counting the axons (see Fig. 1). Its cut-off was fairly sharp, but calculation shows that it must, nevertheless, have made the measured distribution curves somewhat broader than the true ones. The smooth curves drawn in Figs. 7–9 were therefore obtained by substituting appropriate values for $h$ and $2\sqrt{Dt}$ in equation (1), working out $C/C_0$ at 0·4 mm intervals of $x$ with the help of a table of error functions, multiplying by the window characteristic displaced at 0·4 mm intervals, and scaling the resulting curve to fit the experimental curve at its peak. The values of $h$ and $t$ were known for each experiment, and those for $D$ were chosen by trial and error to make the

widths of the calculated and observed distributions the same at half their peaks. The lateral positions of the curves were decided by making them fit the counts at half the peak.

This procedure may be seen in Figs. 7–9 to give a reasonable though by no means perfect fit to the data, the chief discrepancy being a tendency for the theoretical curves to be too narrow at the base when they were made to fit halfway up. Table 7 gives the values of $D$ obtained in these and five other experiments. Since all the sources of error were likely to broaden the curves and none to sharpen them, most weight may be attached to the lower values of $D$. Thus the larger diffusion constants calculated for the experiments of less than 20 min duration may have arisen from spreading of the radioactive patch during drying or from mixing during the process of injection. Excluding these results, the average value of $D$ was $1{\cdot}2 \times 10^{-6}$ cm$^2$/sec; for the two longest experiments, which probably give the most reliable information, it was $0{\cdot}6 \times 10^{-6}$ cm$^2$/sec.

## DISCUSSION

### *The state of calcium in the axoplasm*

A comparison of the values obtained for the diffusion coefficient and mobility of calcium inside the axon with those for calcium ions in free solution gives useful information about the state of calcium in the axoplasm. It is first of all necessary to decide what values would apply if all the calcium were freely ionized. The limiting values for the mobility and self-diffusion coefficient at infinite dilution are given by

$$u = \frac{\lambda^0}{F} \text{ cm/sec per V/cm} \tag{2}$$

and

$$D = \frac{RT}{zF} u \text{ cm}^2\text{/sec} \tag{3}$$

(see Robinson & Stokes, 1955), where $\lambda^0$ is the limiting equivalent conductivity, $z$ is the valency, and $R$, $T$ and $F$ have their usual meaning. At 18° C, Robinson & Stokes give the limiting equivalent conductivity of $Ca^{2+}$ as 50·7 mho.cm$^2$/g equiv, while $RT/F = 25$ mV and $F = 96{,}500$ coulombs/g equiv. Making a small allowance for the presence in the axoplasm of an appreciable concentration of $K^+$, $Cl^-$ and other ions, equations (2) and (3) lead to an expected free solution mobility of about $4 \times 10^{-4}$ cm/sec per V/cm, and to a self-diffusion coefficient of about $6 \times 10^{-6}$ cm$^2$/sec.

It is immediately obvious that the experimental values for $u$ and $D$ in the axoplasm are much less than the free solution values. The mobility was so small that it could not be measured precisely, but even the estimated upper limit of $0{\cdot}9 \times 10^{-5}$ cm/sec per V/cm is only about 1/45 of the free solution value. This suggests that the ratio of ionized to total calcium cannot be

greater than about 0·02; and since the total concentration of calcium is about 0·4 mM (Keynes & Lewis, 1956), it follows that the concentration of ionized calcium in the axoplasm is probably less than 0·01 mM. There are three possible ways of accounting for the apparent failure of the remaining 98% of the calcium, which must be present in the form of a complex, to move in an electric field. One would be that the complexing molecules are structurally fixed, but this is at least partly ruled out by the fact that the difference between the observed and free solution values for $D$ was much less than that for $u$. The calculated values for $D$ were of the same order as those estimated for the diffusion of large dye molecules in the axoplasm (Hodgkin & Keynes, 1956), which suggests that the complexing molecules may all be mobile, and may be roughly of the same size as eosin and methylene blue. In this case, however, they must either carry a net charge of zero, so that the electric field exerts no force on them, or there must be approximately equal numbers of molecules with net positive and net negative charges. The latter possibility should presumably result in apparently greater values for $D$ during current flow, and there was no clear indication that this happened. But unless the calcium complexes were multiply charged, one would expect them to have rather low mobilities, so that it cannot legitimately be concluded that they are necessarily all uncharged. The results of measuring $u$ and $D$ may therefore be summarized by saying that the great bulk of the calcium in the axoplasm is in the form of a complex, leaving under 0·01 mM of ionized calcium; that most of the complexing agents are apparently free to move within the axon; and that the net charge on the complexes is preponderantly neither negative nor positive.

At present the nature of the calcium-binding substances in axoplasm is unknown. Brink (1954) has given a useful list of the dissociation constants of a number of proteins and other organic compounds such as citrate or ATP, which form complexes with calcium. One of the lowest dissociation constants is that for the Ca–ATP complex, which has $K = 8{\cdot}7 \times 10^{-5}$ mole/l., and it is interesting to see how far ATP could account for the observed binding of calcium. According to Caldwell (1956) the ATP content of squid axoplasm is 58–135 $\mu$g P/g, which corresponds to a concentration of the order of 1 mM. If the concentration of $Ca^{2+}$ is less than 0·01 mM it follows that the concentration of the Ca–ATP complex must be less than 0·1 mM. In measuring the calcium mobility, an extra 0·3 mM-Ca was added to the 0·4 mM initially present without apparently saturating the calcium-binding agents, so that it would seem that formation of a Ca–ATP complex cannot account for more than about a seventh of the total amount of calcium which may be present in bound form.

*Calcium movements through the resting membrane*

It is clear from the measurements of calcium influx that the permeability of the resting membrane to calcium ions must be very low. The calcium influx from a solution resembling normal sea water in its composition and containing 10·7 mM-Ca* was about 0·08 pmole/cm$^2$.sec, that is about 0·007 pmole/cm$^2$.sec per mM-Ca outside. This may be compared with a resting sodium influx of about 50 pmole/cm$^2$.sec from a similar medium (Hodgkin & Keynes, 1955; Shanes & Berman, 1955), corresponding to about 0·1 pmole/cm$^2$.sec per mM-Na outside. According to Shanes & Berman the potassium influx from artificial sea water containing 10 mM-K* is just over 40 pmole/cm$^2$.sec, so that the flux per mM-K outside is of the order of 4 pmole/cm$^2$.sec. On this basis the inward transfer constants for K, Na and Ca are in the ratios of 1:0·025:0·00175. In making this comparison no account has been taken of the fact that the inward force exerted by the resting potential on a $Ca^{2+}$ ion is twice as great as it is for a $K^+$ or $Na^+$ ion. If this is allowed for by means of the constant field equation (Goldman, 1943; Hodgkin & Katz, 1949*b*), the apparent permeability coefficients (defined as the product of the diffusion coefficient and partition coefficient in the membrane, divided by the thickness of the membrane) are found to be in the ratios $P_K : P_{Na} : P_{Ca} = 1 : 0{\cdot}025 : 0{\cdot}001$. (In order to make this calculation, the resting potential was taken as 60 mV.) In view of the complexity of the membrane, and of the fact that some potassium is probably transported inwards by a secretary process, such calculations are of limited value. However, although there is no satisfactory way of making a quantitative comparison, there seems little doubt about the qualitative conclusion that the resting permeability to calcium is less than that to sodium and very much less than that to potassium.

If calcium ions diffuse passively into and out of the nerve, the ratio of the amounts of ionized calcium on either side of the membrane at equilibrium should be given by the Nernst equation, i.e.

$$[Ca^{2+}]_i = [Ca^{2+}]_o \exp -2VF/RT. \qquad (4)$$

Here $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$ are the concentrations (or, strictly, activities) of ionized calcium inside and outside the axon, and $V$ is the potential difference across the membrane (inside minus outside). $V$ is about $-60$ mV, so that $[Ca^{2+}]_i/[Ca^{2+}]_o$ ought to be over 100 for a system in passive equilibrium with respect to calcium. Since the concentration of ionized calcium in axoplasm was estimated as 0·01 mM, as against about 11 mM in sea water or squid blood (Robertson, 1949), it follows that the system is actually very far from equilibrium, and that $[Ca^{2+}]_i$ is less than $10^{-5}$ of the value which would be in equilibrium with a resting potential of 60 mV. This indicates that the observed outward flux of labelled calcium cannot be a simple diffusion, and that calcium

ions must either cross the membrane in close association with an anion, or must be extruded by some other kind of active transport mechanism (see Gilbert & Fenn, 1957).

*Calcium movements in stimulated axons*

Although stimulation at 156 impulses/sec may increase the rate of entry of calcium 20-fold, the absolute quantity of calcium which enters the axon during activity is very much less than the corresponding figure for sodium. Thus in sea water containing 10·7 mM-Ca the entry of calcium is only about 0·006 pmole/cm$^2$.impulse, whereas the net entry of sodium is about 4 pmole/cm$^2$.impulse and the extra influx of labelled sodium about 12 pmole/cm$^2$.impulse (Keynes & Lewis, 1951; Hodgkin & Keynes, 1955). In the squid axon it therefore appears that the movements of calcium are too small to carry more than a minute fraction of the total inward membrane current. This conclusion does not necessarily hold for other excitable tissues, and it would be interesting to make similar measurements with crab muscle, where Fatt & Katz (1953) have suggested that an influx of calcium or magnesium may, under some circumstances, be responsible for transporting much of the charge through the membrane. The fact that during stimulation of squid axons no extra efflux of calcium was observed, fits with the conclusion that the concentration of ionized calcium in the axoplasm is exceedingly low. For if $[Ca^{2+}]_i$ is taken as 0·01 mM and $[Ca^{2+}]_o$ as 11 mM, then equation (4) implies that the passive efflux of calcium ions does not become equal to the influx until the internal potential reaches a value of +88 mV relative to the outside. Since in practice $V$ does not exceed about +40 mV at the crest of the spike, the passive efflux must remain small compared to the influx throughout the spike.

The simplest explanation of the calcium entry associated with nervous activity is that calcium ions cross the membrane through the channels which allow sodium ions to enter the fibre during the rising phase of the spike. It seems less likely that calcium entry is associated with the increased potassium permeability during the falling phase of the spike, since this hypothesis predicts that a sustained depolarization by raising the external potassium should give a much greater entry of calcium than the intermittent depolarization that occurs during a train of impulses at 156/sec. Taking the average calcium influx from a solution containing 112 mM-Ca* and 10·4 mM-K as 0·6 pmole/cm$^2$.sec (Table 4), Table 5 shows that treatment with 208 mM-K increased the influx about 5 times, whereas stimulation at 156/sec increased it 20–30 times. The fivefold increase with 208 mM-K is not inconsistent with the idea that calcium ions move inwards through the sodium channel, since, although during a maintained depolarization of 50 mV the sodium conductance declines after its initial rise, it may not fall back as far as the resting value.

Since there is evidence that external calcium ions stabilize the membrane by reducing sodium permeability (Frankenhaeuser & Hodgkin, 1957), it is attractive to suppose that the calcium entry during the spike may not be an accidental accompaniment of activity, but is somehow connected with the development of the state of increased sodium permeability. Thus it might be imagined that when the fibre is suddenly depolarized, calcium ions are enabled to discharge from the membrane into the axoplasm and that this liberates carriers which are then able to transport sodium for a limited period. In a system of this kind one might expect that the calcium entry would be independent of the external calcium concentration when the latter was high enough to saturate the carriers in the resting state. However, our experiments provide no evidence for the occurrence of such saturation, since the entry of calcium per impulse was roughly proportional to calcium concentration outside the axon over the range 22–112 mM.

The finding of a greater uptake of calcium by stimulated nerve may be relevant in considering the mode of release of chemical transmitters by nerve endings. In addition to its action in dispersing squid axoplasm (Hodgkin & Katz, 1949*a*), calcium has been found to have a disruptive effect on other intracellular structures, such as the sarcosomes of heart muscle (Cleland & Slater, 1953), and it is interesting to speculate whether a penetration of calcium at the nerve ending might not be one of the factors involved in breaking up the intracellular vesicles near the membrane (del Castillo & Katz, 1956) and releasing acetylcholine from them. There is some indication that $Mg^{2+}$ ions do not have the same action as $Ca^{2+}$ on intracellular structures, so that a competition between them to enter the nerve terminals, together with a failure of $Mg^{2+}$ to disrupt the vesicles, might help to explain the inhibitory effect of magnesium.

SUMMARY

1. Cleaned squid axons soaked for 2 hr in $^{45}Ca$ sea water and then washed in inactive sea water showed an initial rapid phase (half-period about 15 min) of loss of radioactivity, followed by a slow phase (half-period about 20 hr) shown to correspond to an exchange of intracellular calcium.

2. Measurements with extruded axoplasm showed that the resting calcium influx from a solution containing 10·7 mM-Ca* and 55 mM-Mg was somewhat less than 0·1 pmole/cm$^2$.sec. Stimulation in this medium caused an additional calcium entry of the order of 0·006 pmole/cm$^2$.impulse (38 ions/$\mu^2$. impulse).

3. The resting calcium influx from a magnesium-free solution containing 22 mM-Ca* was 0·4 pmole/cm$^2$.sec. Stimulation in this medium caused an additional calcium entry of 0·01 pmole/cm$^2$.impulse.

4. Changing the external calcium concentration to 112 mM increased the resting influx slightly, and raised the extra calcium entry to 0·08 pmole/cm$^2$.

impulse. In this solution, stimulation at 156 impulses/sec thus gave rise to a 20-fold increase in total calcium influx.

5. On cooling from room temperature to 8° C, the resting calcium influx from 112 mM-Ca* was slightly reduced, but the additional entry during stimulation was nearly doubled.

6. Depolarization of the membrane by raising the external potassium concentration caused a fivefold increase in the calcium influx, but had a much smaller effect than stimulation at 156 impulses/sec.

7. Measurements of the calcium efflux from axons loaded with $^{45}Ca$ by micro-injection showed that intracellular radioactivity was lost with a half-period of the order of 20 hr. The rate constant for disappearance of labelled calcium showed a tendency to decline in the course of the experiments.

8. No change in calcium efflux could be detected during stimulation at 50 impulses/sec. The movement during nervous activity thus seemed to be wholly inward.

9. The mobility and diffusion constant of the calcium in the axoplasm were determined by measuring the distribution after varying periods in the presence and absence of an applied voltage gradient, of short patches of $^{45}Ca$ introduced into axons by micro-injection. The patches did not move measurably in an electric field, and the mobility of the calcium was estimated to be less than 1/45 of the free solution value. From the broadening of the radioactive patches the diffusion constant was estimated to be about a tenth of the free solution value.

10. It was concluded that almost all the calcium in the axoplasm is in bound form, and that the intracellular concentration of ionized calcium may be less than 0·01 mM.

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