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UPTAKE AND BINDING OF CALCIUM BY AXOPLASM ISOLATED FROM GIANT AXONS OF *LOLIGO* AND *MYXICOLA*

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SUMMARY

1. Axoplasm isolated from giant axons of the squid *Loligo* and of the polychaete worm *Myxicola* continues to bind Ca and maintain an ionized Ca concentration close to 0·1 μM which is similar to that seen in intact axons.

2. Injection of Ca into isolated axoplasm only produces a transient rise in ionized Ca showing that axoplasm can buffer a Ca challenge.

3. In order to characterize the Ca-binding systems isolated axoplasm was placed in small dialysis tubes and exposed to a variety of artificial axoplasms containing ^{45}Ca.

4. In the presence of ATP, orthophosphate and succinate, Ca uptake was appreciable and after 4 hr exposure of *Loligo* axoplasm to 0·1 μM-Ca, approximately 100 μmole Ca/kg axoplasm was bound. Binding could be divided operationally into two distinct processes, one that requires ATP or succinate together with orthophosphate and is blocked by cyanide and oligomyocin, and one that is unaffected by these reagents.

5. Energy-dependent binding has a large capacity, but a rather low affinity for Ca, being half-maximal between 20 and 60 μM-Ca. In *Loligo*, its properties closely parallel those of a crude mitochondrial preparation isolated from axoplasm; but there are some interesting differences in *Myxicola*. Energy-independent binding is half-maximal at ionized Ca concentrations between 80 and 160 nM but is readily saturated and has a capacity of 6–60 μmole/kg axoplasm.

6. Ca binding by *Loligo* is greatest in media containing roughly physiological concentrations of K and is reduced by isosmotic replacement of K by Na. This effect seems to be confined to the energy-dependent, presumed mitochondrial, component of binding.

7. Ca binding by *Loligo* axoplasm is reduced by both La and Mn ions.

INTRODUCTION

Less than 0·1 % of the total calcium inside squid axons is ionized (see Baker, 1972; Dipolo, Requena, Brinley, Mullins, Scarpa & Tiffert, 1976). A rather similar pattern of distribution is found in *Myxicola* axons and also in muscle and it seems probable that under normal circumstances the bulk of the calcium inside cells is bound. Although certain intracellular organelles, mitochondria and sarcoplasmic reticulum

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for example, are known to sequester Ca and a variety of Ca-binding proteins have been isolated, rather little is known about these systems in nerve cells where they might be expected amongst other things to contribute to the termination of transmitter release. We have examined this question using axoplasm isolated from giant axons of the squid, *Loligo*, and the polychaete worm, *Myxicola*. It has proved possible to characterize a number of Ca-binding systems in relatively undisturbed protoplasm and to relate some of these to the properties of partially purified subcellular fractions. The Ca-binding by axoplasm can be modified in a variety of ways which may be of physiological significance. Some of the results on squid axoplasm have been communicated to the Physiological Society (Baker & Schlaepfer, 1975) and the general significance of these observations has been mentioned in two recent reviews (Baker, 1976*a*, *b*).

METHODS

Material. The hindmost stellar giant axons from *Loligo forbesi* were used in all experiments on squid. Axons were normally dissected from refrigerated mantles, although live squid were used occasionally. The central end of the axon was freed of adhering small nerve fibres and washed thoroughly in Ca-free sea water for about 15 min. Axoplasm was obtained by extrusion through a cut in the cleaned end of the axon (Bear, Schmitt & Young, 1937; Baker & Shaw, 1965). Only excitable axons were used as a source of axoplasm.

Myxicola infundibulum was obtained from Salcombe Estuary and kept in a sea-water aquarium until required. For experiments on axoplasm, the anterior end of the giant axon was exposed, soaked for about 1 hr in cold Ca-free sea water and the axoplasm pulled out as described by Gilbert (1975*a*).

Measurements of ion mobility in isolated axoplasm. Axoplasm obtained from the polychaete worm *Myxicola* was held in cellulose acetate tubing (Fibre Spinning Services, F.R.L., Dedham, Mass. U.S.A., i.d. 800 μm, wall thickness 30 μm) and given a single axial injection over 2 mm of a solution containing a mixture of ^{24}Na and ^{45}Ca. The injector was left in place for 10 min and then withdrawn slowly. The exposed ends of the axoplasm were brought into contact with two pools of isotonic potassium glutamate, pH 7·2, containing 0·1 mM-EGTA and the preparation subjected to a longitudinal electric field. At the end of the experiment the tube was frozen on an aluminium block immersed in a mixture of 'dry ice' and acetone and cut into 1 or 2 mm sections each of which was counted first for ^{24}Na and again 2 weeks later for ^{45}Ca after the ^{24}Na had decayed away.

Measurement of ionized Ca. Aequorin was used to monitor intracellular ionized Ca and the methods were essentially the same as those previously described by Baker, Hodgkin & Ridgway (1971). For experiments on isolated axoplasm, the axoplasm was transferred to a glass or cellulose acetate capillary tube of diameter roughly equal to that of the original axon and handled as described previously (Baker *et al.* 1971). Isolated axoplasm can readily be drawn into a capillary by gentle suction and once inside the tube maintains levels of ionized Ca and ATP within the physiological range for many hours (Baker & Shaw, 1965; Baker *et al.* 1971).

Binding studies. For experiments on tracer uptake and loss, the isolated axoplasm was weighed and either (1) transferred to a small diameter dialysis tube (Visking size 1–8/32 in) the ends of which were brought together and closed with a paper clip. That portion of the sac which contained the axoplasm was immersed in the test solution ('artificial axoplasm'), held in a 5 ml. disposable beaker on a mechanical shaker. The half time for equilibration of ^{45}Ca with the contents of the dialysis sac averaged 3·1 min. or (2) placed on a piece of parafilm and floated, axoplasm side downwards, on the solution of 'artificial axoplasm' The parafilm technique minimized delays in equilibration because the axoplasm was exposed directly to the test solution, but had the disadvantage that the axoplasm often became detached either as a result of the mechanical agitation or because the axoplasm liquefied (Hodgkin & Katz, 1949). A modification of the dialysis technique was used in a few of the later experiments and seems to be the method of choice for future experiments. In this method axoplasm is drawn into a porous cellulose acetate capillary of diameter roughly equal to that of the axon (Baker, Knight & Pattni, 1977). After preincubation in non-radioactive test medium for 30–60 min and subsequent exposure to ^{45}Ca,

the dialysis sacs were rinsed in non-radioactive media and laid on filter paper. The bag was rapidly cut open and its contents removed and weighed. In most instances the axoplasm remained a cohesive gel, but if the axoplasm had liquefied, a weighed piece of Kleenex tissue was used to absorb the dispersed axoplasm in the dialysis sac and the weight of the axoplasm obtained by re-weighing the tissue. The dialysis sac which itself tended to bind ^{45}Ca was always separated from the sample of axoplasm. Binding of ^{45}Ca to the dialysis sacs was reduced by pre-soaking the sacs for 24 hr in 10 mM-EGTA, followed by boiling in this medium and subsequently washing thoroughly in distilled water.

Preparation of mitochondria from axoplasm. A crude preparation of mitochondria was prepared from isolated axoplasm by making use of the fact that the gel-like structure of axoplasm can be liquefied both by exposure to millimolar concentrations of Ca ions (Hodgkin & Katz, 1949) and by exposure to high concentrations of chloride (Baker, Ladd & Rubinson, 1977). According to Gilbert, Newby & Anderton (1975) Ca brings about liquefaction by activating a protease in the axoplasm. About 40 mg axoplasm was placed in a small disposable beaker to which was added 100 μl. of a solution containing 0·5 M-KCl and 1 mM-Ca acetate. Mixing was achieved by drawing the axoplasm and solution up and down in a disposable pipette tip. Once dispersed, the axoplasm was made up to 400 μl. by addition of the same KCl solution. A 0·22 μm Millipore filter was divided by pencil lines into sixteen segments and 25 μl. aliquots of the axoplasm suspension applied to each segment with gentle suction. The filter disk containing the crude mitochondrial preparation was removed, laid on a Whatman no. 1 filter paper soaked in 0·5 M-KCl and the individual segments rapidly cut out. For measurements of tracer uptake and release, each segment was floated on test solution in 5 ml disposable beakers as for whole axoplasm. Blank filters which had received no dispersed axoplasm were used as controls. Filters were normally soaked for 15 min in non-radioactive test solution, transferred for varying lengths of time to radioactive medium and uptake was terminated by a series of six rinses in ice-cold, non-radioactive medium, the filter being gently blotted on clean filter paper between each rinse. Tracer uptake was expressed in terms of the equivalent weight of dispersed axoplasm applied to the filter.

TABLE 1. Composition of basic 'artificial axoplasms' used. Concentrations in mM unless otherwise stated

	Solution A	Solution B	Solution C	Solution D
KCl	300	300	—	—
K glutamate	—	—	500	500
Sucrose	400	400	—	—
$MgCl_2$	10	10	10	10
Tris, pH 7·2	10	10	10	10
K_2 ATP	5	—	5	—
Sodium succinate	10	—	10	—
Potassium phosphate	10	—	10	—
Oligomycin (μg/ml.)	—	5	—	5
KCN	—	2	—	2

All solutions contained 0·1 % ethanol. After measurement of the total Ca by atomic absorption spectroscopy, Ca or EGTA was added to give a final Ca:EGTA appropriate for the experiment. The final pH was always adjusted to 7·2 by KOH. For some experiments KCl or K glutamate was replaced on a one for one basis by Na, Li or Tris salts and in some of the later experiments sucrose was replaced isosmotically by taurine in order to reduce changes in weight of the axoplasm during incubation.

Some filters were fixed in glutaraldehyde, osmicated, dehydrated and embedded in epoxy resin. Subsequent examination in the electron microscope of ultrathin sections revealed numerous organelles resembling fairly intact mitochondria in the interstices of the filter. The unfixed filters stained strongly for succinic dehydrogenase which is fully consistent with the electron microscopic observations.

Solutions. Table 1 lists the composition of the main solutions to which axoplasm was exposed.

Although no attempt was made to mimic precisely the complex composition of axoplasm, the solutions resembled axoplasm in a number of ways. Thus they were based on either K glutamate or KCl (Spec Pure, Johnson Matthey) and had concentrations of ionized Ca and pH in the physiological range. A mixture of KCl and sucrose (solution A) was used in all experiments on squid and axoplasm exposed to this medium remained in good condition for many hours. This contrasted with the behaviour of *Myxicola* axoplasm which liquefied in this solution and was only stable in K glutamate (solution C). The sensitivity of *Myxicola* axoplasm to chloride seems to be a specific anion effect which is also seen with squid axoplasm but at higher chloride concentrations (Baker *et al.* 1977).

Uptake studies were, in general, done under two sets of conditions: those that favoured mitochondrial function, i.e. addition of ATP, succinate and inorganic phosphate (solutions A, C) and those in which mitochondrial function should have been suppressed, i.e. lack of ATP, succinate and inorganic phosphate and addition of cyanide and oligomycin (solutions B, D). Oligomycin was dissolved in absolute ethanol (5 mg/ml.) and diluted 1:1000 into the experimental solution. At this dilution ethanol had no effect on Ca uptake. In some experiments KCl or K glutamate was replaced isosmotically by salts of Na, Li or Tris.

Total Ca in these solutions was measured by atomic absorption spectroscopy with suitable standards. The level of ionized calcium in the submicromolar range was controlled by addition of EGTA. As measurement of Ca binding requires a high specific activity, Ca-EGTA buffers were generated by addition of known amounts of EGTA to the contaminating levels of Ca in the test solutions. The ionized Ca was calculated from the measured Ca, Mg and pH of the solutions (Schwarzenbach, Senn & Anderegg, 1957). Higher Ca concentrations were obtained by addition of $CaCl_2$.

Measurement of potentials. Accumulation might result from the generation of a potential across the walls of the dialysis sac. The size of the potential set up was examined under a number of experimental conditions. It was never greater than 2 mV, axoplasm negative, and usually less than 1 mV.

Calculation of Ca binding. In order to calculate binding, it is necessary to know how much of the radioactivity in the dialysed axoplasm is unbound Ca resulting from the penetration of the test solution into the axoplasm. The extent of this penetration can be assessed by measurement of the distribution of a variety of marker substances that are not bound by axoplasm. The simplest routine measurement of the 'unbound Ca space' is to expose at least one sample of axoplasm in each series of determinations to nominally Ca-free artificial axoplasm containing 10 mM-EGTA. Ionized Ca in this medium is less than 10^{-9} M and the 'Ca space' measured in this way is similar to the 'Na space' estimated by inclusion of ^{22}Na in the artificial axoplasm. After correcting for unbound Ca, the bound Ca was expressed as μmole Ca bound/kg fresh axoplasm, thus avoiding errors due to changes in weight of the sample during exposure to some solutions.

RESULTS

Diffusion and mobility of ^{45}Ca in isolated axoplasm

Hodgkin & Keynes (1956) provided the first demonstration of Ca binding in squid axoplasm. They showed that a small patch of ^{45}Ca introduced axially into an axon remained close to the site of injection for many hours and failed to move in an imposed electric field. These results were confirmed by Baker & Crawford (1972), who also showed that, in contrast to Ca, a patch of ^{28}Mg injected into the axon broadened considerably and migrated towards the cathode when exposed to a longitudinal electric field.

Fig. 1 extends these observations in cephalopods to polychaete worms and shows that Ca is also bound powerfully in axoplasm isolated from giant axons of *Myxicola*. The behaviour of Ca in axoplasm differs markedly from that of Na which behaves as a freely mobile cation. (Gilbert, 1975*a*). Of a variety of ions examined in this way, only Ca and La were bound in axoplasm: Na, K, Rb and Cl all behaved as freely mobile ionic species (Baker & Schapira, 1977). The binding of Ca did not result from inter-

action with the walls of the tube because a patch of ^{45}Ca injected into the same tubes but filled with agar both broadened ($D = 10^{-5}$ cm^2/sec) and moved towards the cathode (mobility 2×10^{-4} cm/sec per V/cm). Experiments such as that shown in Fig. 1 enable an upper limit of 2×10^{-5} cm/sec per V/cm to be placed on the mobility of ^{45}Ca in *Myxicola* axoplasm. This is very close to the values of $0{\cdot}9 \times 10^{-5}$ cm/sec per V/cm and $< 10^{-5}$ cm/sec per V/cm obtained in intact squid axons (Hodgkin & Keynes, 1956; Baker & Crawford, 1972); but comparable measurements have not been made on axoplasm extruded from squid axons.

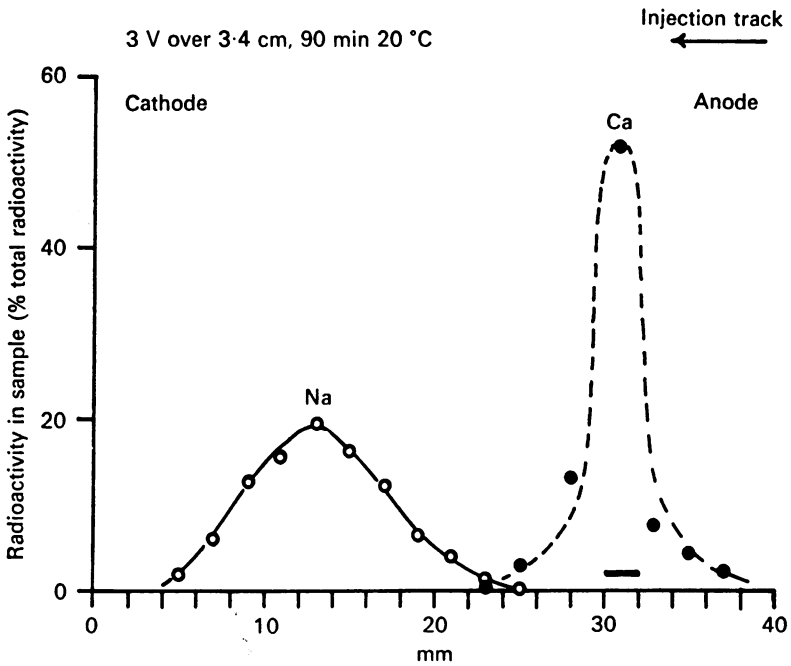

Fig. 1. Comparison of the mobility of ^{45}Ca and ^{22}Na in axoplasm isolated from the polychaete worm *Myxicola*. Axoplasm was held in cellulose acetate tubing and given a single axial injection over 2 mm of a solution containing ^{22}Na and ^{45}Ca. The position of the injection is shown with a solid black bar. The preparation was subjected to an electric field of 3 V over 3·4 cm for 90 min, with the side from which the injector entered being made the anode. Temperature 20 °C. The curves have been drawn by eye.

In two experiments, ^{45}Ca was injected into *Myxicola* axoplasm that had been dialysed against solution D for $\frac{3}{4}$ hr. This should have depleted the axoplasm of ATP and metabolic substrates and the presence of cyanide and oligomycin should have blocked Ca accumulation by mitochondria. The injected ^{45}Ca still remained close to its site of injection and failed to migrate in an electric field. This suggests that in *Myxicola* axoplasm Ca can be bound strongly by an energy-independent process.

Measurement of ionized Ca in isolated axoplasm

The Ca content of axoplasm isolated from giant axons of *Loligo forbesi* varies between 100 and 500 μmole/kg axoplasm. Values of 280 ± 200 μmole/kg axoplasm are quoted by Gilbert (1975*b*) for fresh *Myxicola infundibulum* axoplasm and our own

measurements, by atomic absorption spectroscopy, give values ranging from 107 to 507 μmole/kg axoplasm. In order to determine what fraction of axoplasmic Ca is ionized, Baker *et al.* (1971) injected aequorin to provide a reporter molecule for ionized Ca and by injecting various Ca-EGTA buffers estimated ionized Ca from the buffer that gave no change in light emission. Fig. 2 illustrates three experiments of this type. In Fig. 2*A* injection of EGTA into a squid axon reduced the resting glow and subsequent injection of a second buffer to give a final total Ca: total EGTA ratio

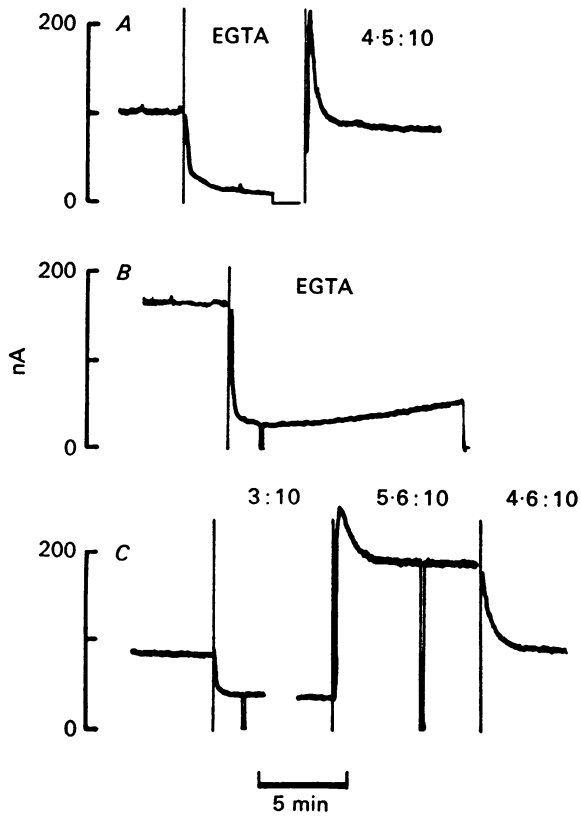

Fig. 2. Estimation of ionized Ca in aequorin injected squid axons and isolated axoplasm. Three separate experiments are illustrated. *A* and *B*, intact axons immersed in sea water containing 11 mM-Ca; *C*, axoplasm extruded into a Pyrex glass capillary tube 800 μm in diameter. Ordinate: light output (nA) and abscissa: time (min). In each experiment the light output is shown before injection of Ca buffer and after injection of buffers to give the ratio total Ca: total EGTA stated. Each injection added approximately 3 mM-EGTA except that in *B* which only added 1 mM-EGTA. Temperature 20–22 °C. Records traced from the original pen recordings to facilitate reproduction.

of 4·5:10 brought the glow back close to its original value. Fig. 2*B* shows that the reduction in glow brought about by injection of small amounts of EGTA is not completely stable but tends to recover towards its initial level, possibly as the EGTA acquires Ca from both intra- and extracellular sources. Fig. 2*C* is a similar experiment to that shown in Fig. 2*A*, except that isolated axoplasm is used and not an intact axon. In both intact squid axons and isolated axoplasm, assuming an internal

pH of 7·2 (Caldwell, 1958) and an ionized Mg concentration of 2–3 m-mole/kg (Baker & Crawford, 1972; Brinley & Scarpa, 1975) the values obtained for ionized Ca were close to 0·1 μM.

Fig. 3 shows two similar measurements on *Myxicola* axoplasm. As Ca:EGTA buffers are very sensitive to pH, the pH of the injection solutions was stabilized at pH 7·2 by inclusion of 200 mM-HEPES buffer. In the experiment shown in Fig. 3*A* injection of a buffer containing 30 mM-Ca and 100 mM-EGTA reduced the resting glow and subsequent injection of a second buffer to give a final Ca:EGTA ratio of 6:10 brought the glow back above its original value. The order of buffer injections

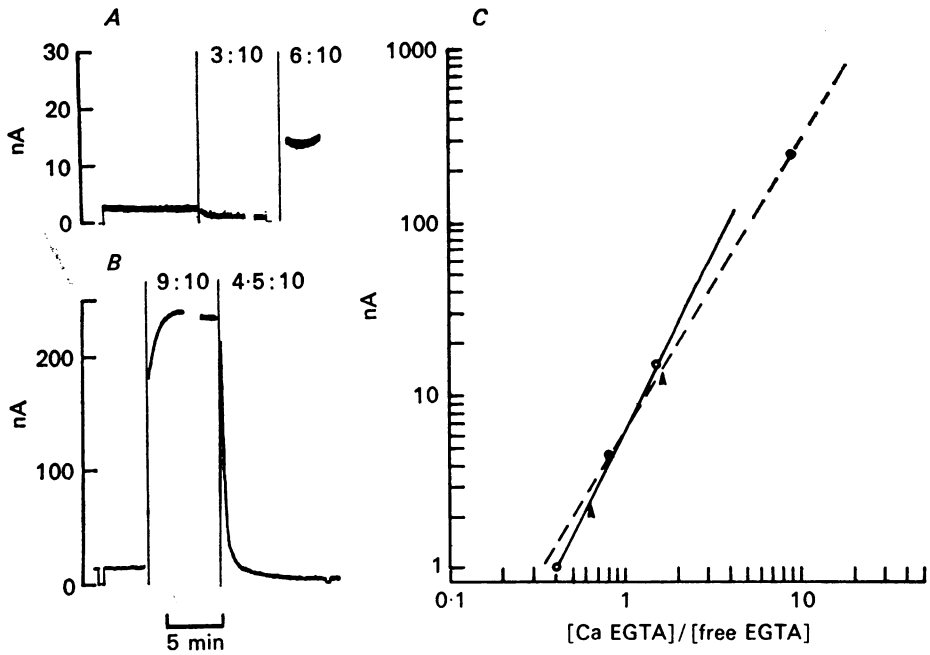

Fig. 3. Estimation of ionized Ca in axoplasm pulled from giant axons of *Myxicola*. Axoplasm was drawn into a glass capillary 800 μm in diameter and injected with aequorin. The injection of buffers was done about 1 hr later.

A and *B*, two separate experiments. Ordinate: light output (nA) and abscissa: time (min). The final ratio of total Ca: total EGTA injected into the axon is noted on the tracings. The absolute concentration of EGTA was raised by approximately 3 mM at each injection.

C shows a plot of the log of the light output (nA) from *A* and *B* *vs.* log [Ca EGTA]/[free EGTA]. The arrows mark the resting glow before injection of buffer. Temperature 22 °C.

was different in the experiment shown in Fig. 3*B*. Injection of a buffer containing 90 mM-Ca and 100 mM-EGTA markedly increased the glow and subsequent injection of EGTA to give a final Ca:EGTA ratio of 4·5:10 reduced the glow somewhat below its initial value. The data of Fig. 3*A* and 3*B* are replotted in Fig. 3*C*. The log of the light output increases steeply with the ratio Ca EGTA/free EGTA which is proportional to ionized Ca. For the two experiments the slopes of the log–log plots are 2·0 and 1·8 respectively, which is consistent with earlier calibrations of the aequorin

reaction both *in vitro* and in squid axoplasm (Baker *et al.* 1971). On the assumption that about half of the measured axoplasmic Mg content of 3·4 m-mole/kg axoplasm (Gilbert, 1975*b*) is ionized (as in squid (Baker & Crawford, 1972)) these results are consistent with an ionized Ca of 68–125 nM in axoplasm isolated from *Myxicola* axons.

Ca buffering properties of isolated axoplasm

Injection of micromolar amounts of Ca acetate into axoplasm isolated from giant axons of *Loligo* and *Myxicola* only results in a transient rise in axoplasmic ionized Ca. Thus Fig. 4 shows the response of aequorin-loaded axoplasm to the injection of

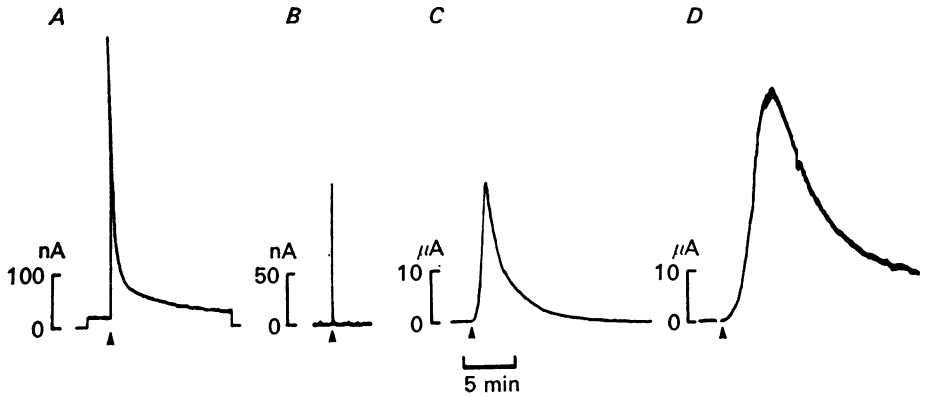

Fig. 4. Response of isolated axoplasm to a Ca challenge. Axoplasm isolated either from *Loligo* (*A*), or *Myxicola* (*B–D*). In each case the axoplasm was drawn into a Pyrex glass capillary of diameter 800 μm and injected with aequorin. Ordinate: light output (nA or μA as specified) and abscissa: time (min). Injections of calcium were made as Ca acetate in Tris-buffered isotonic potassium chloride, pH 7·2. In *A*, the resting glow was 200 nA before injection of 1 mM-Ca acetate and 280 nA 10 min after injection. In *B*, the resting glow was 5 nA before injection of 200 μM Ca acetate and 3 nA, 2 min later. In *C* the resting glow was 3 nA before injection of 1 mM-Ca acetate and 9 nA 10 min afterwards. Record *D* shows the effect of injecting 100 mM-DNP. Records *B–D* were obtained on the same sample of axoplasm in the order shown. Temperature: *A*, 18 °C; *B–D*, 20 °C. Different samples of aequorin were used for the two experiments.

enough Ca acetate to raise the total axoplasmic Ca by about 60 μM. It is clear that this Ca challenge only brings about a small maintained increase in ionized Ca and the bulk of the injected Ca seems to be sequestered and rapidly made unavailable to react with aequorin. This observation is fully consistent with the diffusion and mobility experiments described earlier. The fall in ionized Ca does not result from binding of Ca to the tube because when ^{45}Ca is injected the radioactivity can be recovered quantitatively in the axoplasm.

It is interesting that injection of 100 or 200 μM-Ca acetate to give a final concentration of 3–6 μM produces only a very transient increase in light which returns to its resting level within a minute (Fig. 4*B*) whereas the response to injection of larger amounts of calcium (1000–2000 μM) giving a final Ca concentration of 30–70 μM is more prolonged taking about 15 min to return close to its original level. (Fig. 4*A* and *C*). Fig. 4*D* shows that even after a large Ca challenge such as that shown in Fig. 4*C* there is still plenty of aequorin in the axon and injection of the mitochondrial

uncoupling agent DNP results in a rapid increase in light output. Taken together these data show that axoplasm can buffer up to 100 μM added Ca with rather little change in the level of ionized Ca. Recovery from a Ca challenge is very rapid for small increments in Ca up to 6 μM – and slower for larger additions, suggesting that more than one binding mechanism may be involved. The increase in light after addition of DNP suggests that some of the bound Ca may be in mitochondria.

Uptake of ^{45}Ca by isolated axoplasm

General features. In order to characterize the Ca-binding systems in isolated axoplasm, samples of axoplasm each about 10 mg in weight were placed in small dialysis sacs and exposed to a variety of artificial axoplasms (Table 1). Squid axoplasm took up an appreciable amount of Ca from both glutamate and chloride-

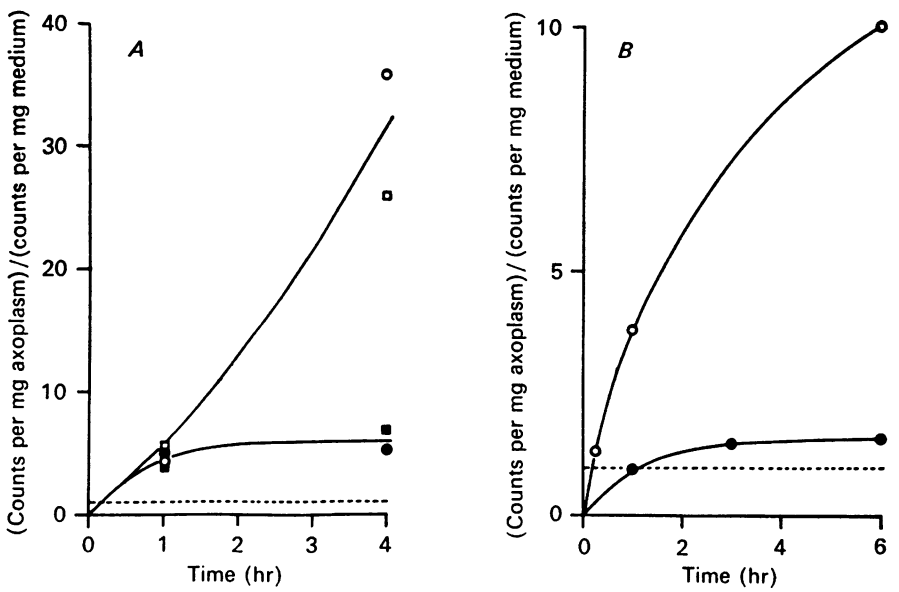

Fig. 5. Ca uptake by axoplasm contained in dialysis sacs. *A*, axoplasm from *Loligo*; *B*, axoplasm from *Myxicola*. In both graphs the ordinate is ^{45}Ca uptake expressed as (counts in axoplasm)/(counts in an equivalent weight of medium) and the abscissa is time in hr.

A, open circles, K glutamate axoplasm, solution C; filled circles, substrate-free K glutamate axoplasm containing cyanide and oligomycin, solution D; open squares, KCl-sucrose, solution A; filled squares, substrate-free KCl sucrose containing cyanide and oligomycin, solution B. Succinate was not present in either solution A or solution C.

B, open circles, K glutamate axoplasm, solution C; filled circles, K glutamate axoplasm, solution D but with 1 mM-DNP instead of cyanide and oligomycin.

Samples shaken gently throughout the experimental period. Temperature 22 °C. Curves drawn by eye. The horizontal interrupted dotted line represents simple equilibration between the contents of the dialysis sac and the bathing medium. The measured Ca content of the glutamate media was 10 μM and that of the KCl-sucrose media 14 μM.

sucrose media (solutions A, C; Table 1) but *Myxicola* axoplasm liquefied in KCl-sucrose and all uptake studies on *Myxicola* were done in glutamate media. Fig. 5 shows that axoplasm took up Ca from all these media and could accumulate Ca to levels in excess of thirtyfold. In the presence of ATP and orthophosphate, both

excellent substrates for mitochondria, uptake was roughly linear for many hours. In the absence of ATP and in the presence of cyanide or dinitrophenol, conditions that block Ca uptake by mitochondria, accumulation was still observed but to a smaller extent and the uptake differed from that in energized axoplasm in that a steady level of binding was reached in about 1 hr.

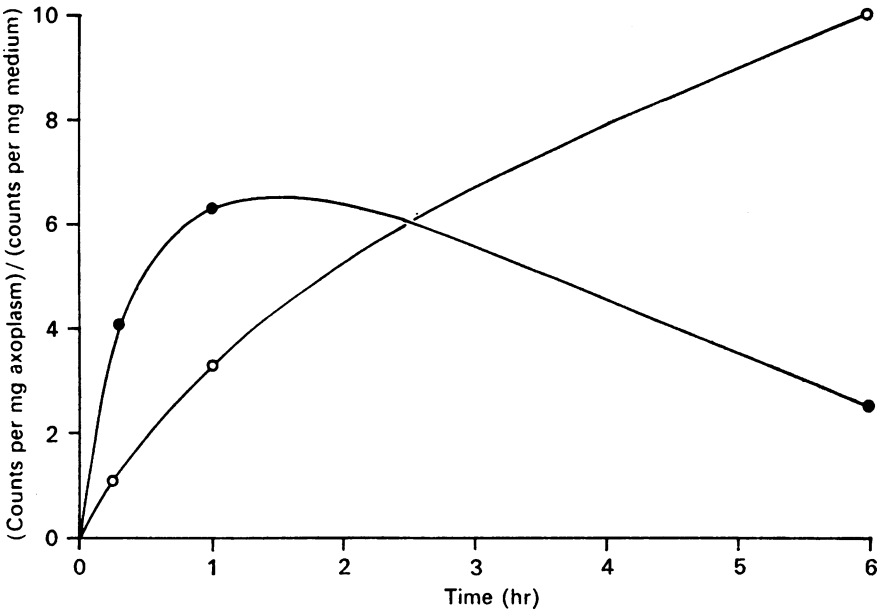

Fig. 6. Comparison of Ca uptake by 'naked' axoplasm (●) with that of axoplasm held in dialysis sacs (○). Axoplasm obtained from *Myxicola*, immersed in K glutamate medium (solution C) and shaken gently throughout the incubation period. Temperature 22 °C. The curves have been drawn through the points by eye. The measured Ca content of the medium was 10 μM.

In a few experiments axoplasm was exposed directly to the test solution. Fig. 6 shows that Ca binding to naked axoplasm was more rapid, as expected from its greater accessibility, but uptake was not maintained probably because of a slow loss of structure. Sensitivity to ATP and metabolic inhibitors was identical to that of axoplasm in dialysis sacs. Of particular relevance is the close similarity in energy-independent binding between naked axoplasm and axoplasm in dialysis sacs, an observation that rules out the possibility that this rather small component of binding may be an artifact created by the dialysis sacs used.

These observations suggest that Ca uptake by isolated axoplasm from both *Loligo* and *Myxicola* involves at least two operationally distinct processes, one that requires ATP and another that does not. In all subsequent experiments chloride-sucrose media were used for *Loligo*, glutamate for *Myxicola* and in all instances the axoplasm was enclosed in dialysis sacs.

A working hypothesis consistent with these results is that the energy-dependent component of Ca binding reflects uptake of Ca into mitochondria whereas the energy-independent component may be non-mitochondrial in origin. Mitochondrial uptake requires as a source of energy either electron transport or ATP and uptake is greater in the presence of a counter ion such as phosphate.

In the absence of these substrates, axoplasmic Ca binding was quite small and was not reduced by the further addition of cyanide or dinitrophenol. The large increase in Ca uptake seen in the presence of ATP and orthophosphate was not much increased by the addition of succinate, although this substrate was routinely included in all media in which energy-dependent uptake was under investigation because it seemed to increase greatly Ca uptake by isolated mitochondria (see Table 2). In order to block mitochondrial Ca uptake from media containing succinate and ATP, it is necessary to block the accumulation of Ca that depends on electron transport and also that which is directly dependent on ATP. This was found to be the case in axoplasm. The energy-dependent uptake of Ca in the presence of succinate and ATP required both cyanide and oligomycin to reduce uptake to its level in the absence of substrate. Conversely, in the presence of cyanide and oligomycin, removal of the succinate, ATP and orthophosphate produced only a very small further reduction in uptake, providing additional support for the view that the residual uptake under these conditions does not involve the mitochondria or other ATP-dependent processes. The residual uptake was also unaffected by the addition of 0·4 mM-DNP, although 2 mM-DNP did cause some reduction in binding.

Ca-dependence of binding

A very clear difference between the energy-dependent and energy-independent components of binding in squid axoplasm is seen in the form of their dependence on ionized Ca. Energy-independent binding can take place at much lower levels of ionized Ca than energy-dependent binding. This is illustrated in Figs. 7*A* and 8*A* for *Loligo* and *Myxicola* respectively. In both preparations there is appreciable Ca uptake at low levels of ionized Ca and this uptake persists in the absence of mitochondrial substrates and in the presence of cyanide or oligomycin. Raising the level of ionized Ca increases the energy-independent binding still further but now also activates an energy-dependent component as well. By subtracting binding in the non-energized state from that in the energized state, curves relating energy-dependent binding to ionized Ca can be constructed. These are shown for *Loligo* and *Myxicola* in Figs. 7*B* and 8*B* and will be discussed more fully in the next section (see p. 116).

Figs. 7*C* and 8*C* provide more detailed information on the Ca-dependence of the energy-independent component of Ca binding. The apparent K_m for Ca of the high affinity component of Ca binding in *Loligo* as determined from Scatchard plots of the data obtained in individual experiments varied from 80 to 130 nM and the Ca-binding capacity from 10 to 60 μmole/kg axoplasm. The data for *Myxicola* are less extensive but the apparent K_m for Ca of the high affinity component of binding varied from 100 to 160 nM and its capacity from 6 to 16 μmole/kg axoplasm. In both preparations there is a component of binding that increases roughly linearly up to the highest Ca concentrations examined (500 μM).

Three experiments were carried out on squid axoplasm to determine whether the high affinity component of binding is affected by colchicine. Axoplasm was pre-exposed to solution A containing colchicine (100 μM) and then to the same solution containing ^{45}Ca. No difference in high affinity Ca binding was detected. In a single experiment, *N*-ethyl maleimide (100 μM) had no effect on high affinity Ca binding.

Ca uptake by a crude mitochondrial preparation from axoplasm

Perhaps the most direct way to separate mitochondrial from presumed non-mitochondrial Ca binding in axoplasm would be to centrifuge the axoplasm to effect physical separation of mitochondria from other components. Unfortunately, this proved impossible without first liquefying the axoplasm.

The method finally adopted is described in detail on page 105. Axoplasm was liquefied by exposure to 1 mM-Ca in 0·5 M-KCl and a crude mitochondrial preparation was obtained by passing the liquefied axoplasm through a suitable Millipore filter.

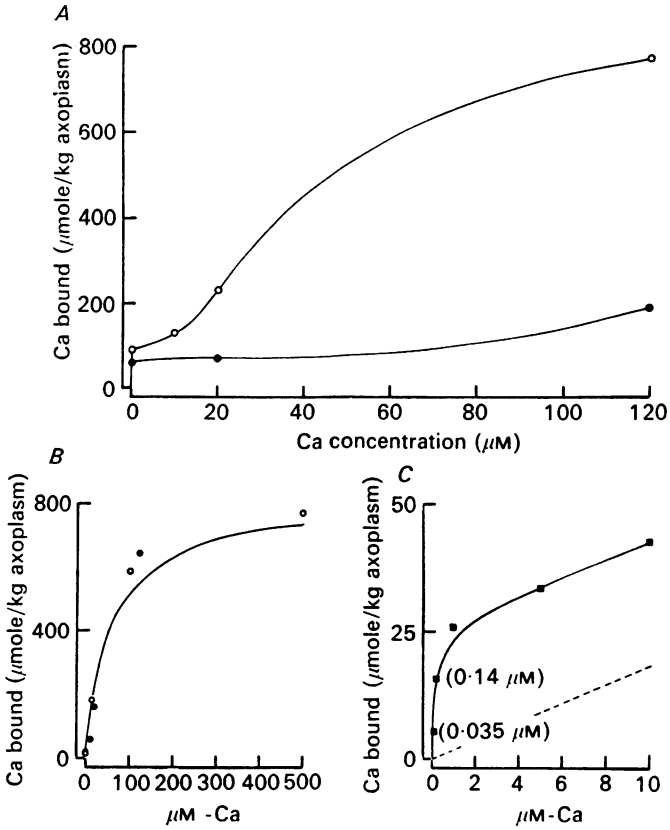

Fig. 7. Ca-dependence of Ca binding by axoplasm from *Loligo*.

A, Ca binding in the presence and absence of substrate at different Ca concentrations. The lowest Ca concentration used was 0·14 μM. ○, KCl-sucrose medium, solution A; ●, substrate-free KCl-sucrose medium, solution B. Uptake has been corrected for unbound Ca and is expressed as μmoles Ca bound/kg axoplasm. Each point is the mean of two separate determinations. Incubation time 4 hr. Axoplasm from four pairs of axons was used. The curves have been drawn through the points by eye. Temperature 20 °C.

B, energy-dependent, presumed mitochondrial, component of Ca uptake. ●, points obtained from Fig. 7*A* by subtracting the lower curve from the upper one. ○, points obtained on a crude mitochondrial preparation isolated from squid axoplasm. The continuous curve is a section of a rectangular hyperbola of the type: $V = V_{max}/(1+K_m/[Ca])$ with the apparent K_m = 50 μM and V_{max} = 800 μmole/kg axoplasm. Temperature 20 °C.

C, energy-independent component of Ca uptake. Pooled data from two separate experiments, different from the experiment shown in *A*. Uptake measured in solution B, over 2 hr. The numbers in parentheses refer to the ionized Ca present in the incubation medium. The data show a saturable and a linear component (interrupted line). The smooth curve has been drawn on the assumption that the saturable component can be described by a section of a rectangular hyperbola of the type $V = V_{max}/(1+K_m/[Ca])$ with the apparent K_m = 100 nM and V_{max} = 25 μmole/kg axoplasm. Temperature 20 °C.

Subsequent electron microscopic examination of the filters revealed the presence of numerous organelles resembling mitochondria in the interstices of the filter. Ca binding was expressed in terms of uptake by the equivalent volume of crude axoplasm from which the mitochondria had been isolated. Some of the properties of this crude mitochondrial preparation are shown in Figs. 7*B*, 8*B* and 9 and Table 2. Fig. 9 shows that in the presence of ATP, succinate and orthophosphate uptake was linear for at least 1 hr and could be completely abolished by removal of substrate and addition of

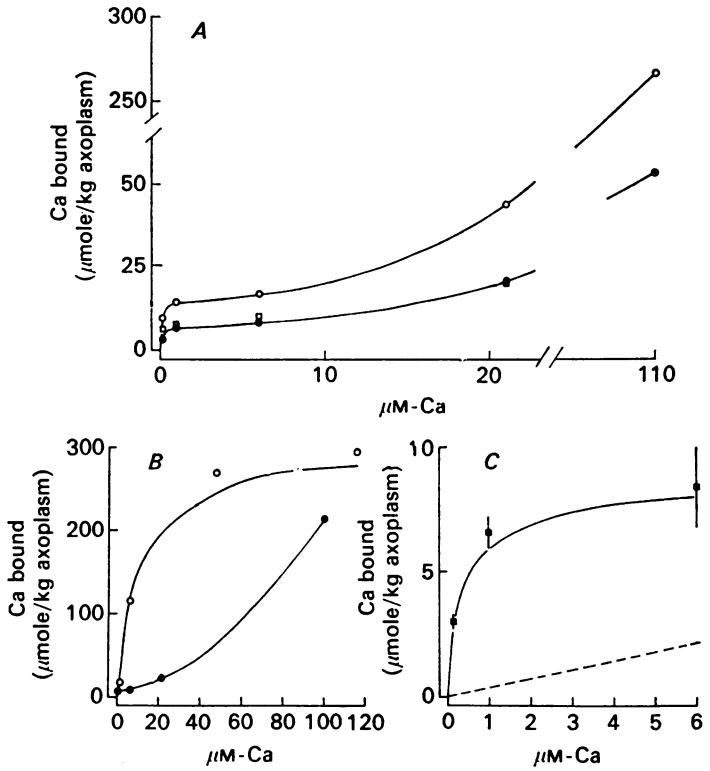

Fig. 8. Ca binding by *Myxicola* axoplasm at different Ca concentrations.

A, Ca binding in the presence and absence of substrate. ^{45}Ca uptake has been corrected for unbound Ca and is expressed as μmole Ca bound/kg axoplasm. Note the break in both ordinate and abscissa and the change in scale of the ordinate. ○, K glutamate medium containing substrate (solution C); ●, substrate-free K glutamate medium (solution D); □ solution D, with the addition of ATP (2·5 mM). Incubation time 1 hr. Temperature 22 °C. Curves drawn by eye.

B, substrate-dependent Ca binding. ●, difference between upper and lower curves of *A*; ○, energy-dependent Ca uptake by a crude preparation of mitochondria isolated from *Myxicola* axoplasm (obtained by difference in uptake in solutions C and D). Temperature 22 °C. The continuous curve through the open circles is a section of a rectangular hyperbola of the type $V = V_{max}/(1+K_m/[Ca])$ with the apparent K_m = 10 μM and V_{max}' = 300 μmole/kg axoplasm.

C, energy-independent Ca binding. Points determined in solution D. The vertical bars represent 2 × S.E. of mean. Temperature 22 °C. The continuous curve has been drawn on the assumption that uptake consists of two components, a linear component (shown dashed) and a saturable component which can be represented by a section of a rectangular hyperbola of the type $V = V_{max}/(1+K_m/[Ca])$ with the apparent K_m = 130 nM and V_{max}' = 6 μmole/kg axoplasm.

cyanide and oligomycin or dinitrophenol. Table 2 provides more detailed information on the relative effectiveness of various substrates in energizing uptake and Figs. 7*B* and 8*B* compare the Ca dependence of energized uptake with that of the presumed mitochondrial component of Ca uptake in whole axoplasm.

The properties of the crude mitochondrial preparation from squid axoplasm resemble very closely those of the presumed mitochondrial component of Ca uptake

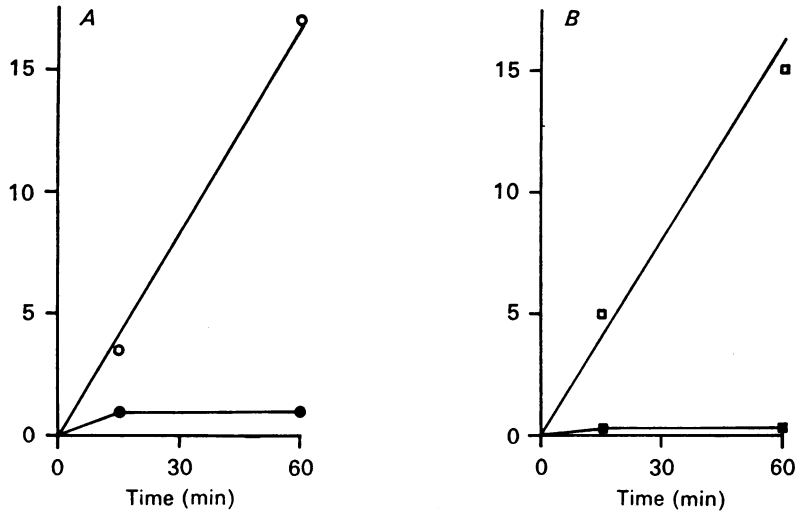

Fig. 9. Ca uptake by a crude preparation of mitochondria isolated from axoplasm (see Methods). *A*, crude mitochondrial preparation from *Loligo* axoplasm. *B*, a similar preparation from *Myxicola*. In both, the ordinate is the Ca bound in an equivalent volume of axoplasm expressed relative to the Ca in the same volume of medium and the abscissa is time (min). ○, solution A; ●, solution B; □, solution C; ■, solution D but with 1 mM-DNP in place of the cyanide and oligomycin. All solutions contained 10 μM-Ca. Temperature 20 °C.

TABLE 2. Ca uptake by a crude preparation of mitochondria isolated from squid axoplasm. Effect of varying concentrations of ATP, orthophosphate and succinate in solution A. The mitochondria were isolated from a single batch of axoplasm pooled from four axons

ATP (mM)	Orthophosphate (mM)	Succinate (mM)	Mean Ca uptake (μmole/kg axoplasm)*
0	0	0	0
0	10	0	4
0	0	10	15·0
0	10	10	164·7
0·5	0	0	26·9
0·5	10	0	31·5
0·5	10	10	271·3
5·0	0	0	35·3
5·0	10	0	57·0
5·0	10	10	222·8

* Ca uptake has been expressed in terms of the volume of axoplasm from which the mitochondria were obtained. Incubation was for 1 hr at 20 °C. All solutions contained 10 μM Ca.

in whole axoplasm. The Ca concentration curve for mitochondria *in situ* was slightly sigmoidal. In the presence of ATP, succinate, orthophosphate and Mg (10 mM), the Ca concentration giving half maximal Ca uptake by squid mitochondria, both isolated and *in situ* varied between 20 and 60 μM and in both preparations, after 1 hr the maximum amount of Ca bound approached 1000 μmole/kg axoplasm. The *Myxicola* data, however, show a very interesting difference: the apparent affinity of the presumed mitochondrial component of Ca binding in whole axoplasm is much lower than that of the crude isolated mitochondrial fraction (see Fig. 8*B*). This observation suggests either that the energy-dependent component of Ca uptake in *Myxicola* axoplasm involves something other than mitochondria or that isolation of mitochondria from axoplasm may free their Ca uptake system from some sort of inhibition. If such an inhibitor exists in whole axoplasm, it must be non-dialysable. There is some suggestion of a similar effect in *Loligo* axoplasm (Fig. 7*B*) but it is much less marked.

The ability of mitochondria isolated from both *Loligo* and *Myxicola* to bind Ca is reduced by inclusion of the ionophore A23187 in the medium. Thus Ca uptake by a crude preparation of mitochondria from *Myxicola* was drastically reduced by inclusion of A23187 (10 μg/ml.); but when axoplasm was exposed to a large excess of the same concentration of ionophore the energy-dependent and presumed mitochondrial component of Ca uptake was unaffected. Either the ionophore failed to penetrate the dialysis sac and intervening axoplasm or mitochondria in axoplasm are less sensitive than isolated organelles.

Non-mitochondrial ATP-dependent Ca binding

The possibility that axoplasm might contain an ATP-dependent but non-mitochondrial component of Ca binding was investigated in a few experiments on *Myxicola*. Mitochondrial uptake was blocked by absence of substrate and addition of cyanide and oligomycin (solution D) and uptake in this solution with or without the addition of 2·5 mM-ATP was compared. The results are shown in Fig. 8*A*. The addition of ATP did not cause any significant increase in Ca binding.

The influence of monovalent cations on Ca binding

Complete replacement of KCl in solution A (Table 1) by NaCl or LiCl resulted in a reduced uptake of ^{45}Ca by energized squid axoplasm. Comparable experiments were not done on *Myxicola* axoplasm. After replacement of KCl by NaCl or LiCl total uptake of Ca was reduced by $42{\cdot}9 \pm 4{\cdot}8$ and $26{\cdot}6 \pm 7{\cdot}9$ % respectively. Intermediate Na and K concentrations were examined in only a few experiments, but in these the effect of cation replacement on Ca uptake was half maximal in media containing 50 mM-NaCl and 300 mM-KCl, which suggests that Ca uptake by axoplasm may be sensitive to alterations in the Na/K ratio in the physiological range.

These results were obtained on whole axoplasm in the presence of a source of energy. Experiments were also done on whole axoplasm in the absence of energy sources and on isolated mitochondria. Replacement of K by Na had no significant effect on the energy-independent component of Ca binding in whole axoplasm, but produced a marked reduction in Ca uptake by a crude preparation of mitochondria from squid axoplasm (Table 3). These observations suggest that the response of whole axoplasm to alterations in monovalent cation concentration results from an effect primarily on mitochondria. It should, however, be noted that the observations were

only made at one Ca concentration, 10 μM, which is close to the apparent K_m for the presumed mitochondrial component of binding and it is possible that monovalent cations might also affect the energy-independent component of binding if experiments were conducted close to the apparent K_m for Ca of this system.

In some experiments KCl was replaced by Tris-Cl in an attempt to decide whether the rise in Na or fall in K was responsible for the observed alterations in binding. No conclusion could be drawn because the results on both axoplasm and mitochondria were very variable: in some Tris-Cl behaved like KCl and in others like NaCl.

Results not immediately consistent with these observations were obtained in two experiments on aequorin injected squid axoplasm. In these experiments either 2 M-NaCl (Spec pure) or 2 M-LiCl (Fisher) was injected into the axoplasm and failed to produce any sustained change in light output. This point clearly needs investigating further.

TABLE 3. Effect of monovalent cations on Ca uptake by a crude preparation of mitochondria from squid axoplasm. Uptake measured in solution A containing 10 μM Ca with the major cation either K, Na or Li

	Ca bound (μmole/kg axoplasm)*		
	Na	*K*	*Li*
5. xii. 74	65·0	172·7	50·0
	39·9	117·5	50·6
9. xii. 74	165·5	269·1	212·9
	172·8	267·0	226·5
12. xii. 74	233·4	272·9	228·0
	210·4	319·2	166·6
10. i. 75	177·0	222·1	133·4
	170·0	201·9	130·6
Mean ± S.E.	154·3 ± 23·5	230·3 ± 17·6	149·8 ± 25·2

* For each experiment the mitochondria were isolated from a single sample of axoplasm and the Ca uptake by the crude mitochondrial preparation has been expressed in terms of the volume of axoplasm from which the mitochondria were obtained. Incubation for 1 hr at 20 °C.

Ca binding in the presence of La and Mn

The influence on Ca binding of the heavy metals La and Mn was investigated in a few experiments. Because of interaction between these ions and phosphate compounds most of the experiments were done on the energy-independent component of binding with whole axoplasm exposed to substrate-free media in the presence of cyanide and oligomycin. Fig. 10 shows that both La^{3+} and Mn^{2+} reduced Ca binding substantially. The concentrations needed were quite high; but the experiments were all made in the presence of 10 μM-Ca which is 100 times higher than is usually found in axoplasm. The Ca buffer systems available did not permit examination of Ca binding in the presence of La^{3+} or Mn^{2+} at physiological levels of Ca.

Taking the data of Fig. 10 and assuming simple competition for the energy-independent high-affinity Ca binding site, the true inhibitor constants for La and Mn are about 5 and 30 μM respectively.

The effects of Mn and La on *Myxicola* axoplasm were not examined. Exposure of this preparation to ruthenium red (3 mM) failed to reduce energy-independent Ca binding whereas the same concentration inhibited Ca uptake by a crude preparation of mitochondria isolated from *Myxicola* axoplasm.

The effects of injecting heavy metals on the resting light output from aequorin-injected axoplasm of both *Loligo* and *Myxicola* were examined in a few experiments. Mn, Co and La all tended to produce a slow rise in light output and a slowing of recovery after injection of Ca; but the effects were not large, required mM final concentrations of heavy metal and their interpretation may be complicated by the possibility of direct interaction between aequorin and the heavy metals (see Blinks, Prendergast & Allen, 1976). A possible reason for the rather high concentrations required is that both Mn and La are bound strongly in axoplasm and it may be necessary to inject millimolar amounts to ensure that the heavy metal becomes distributed throughout the preparation. Because of this binding, the final free concentration of heavy metal may be in the micromolar range or lower.

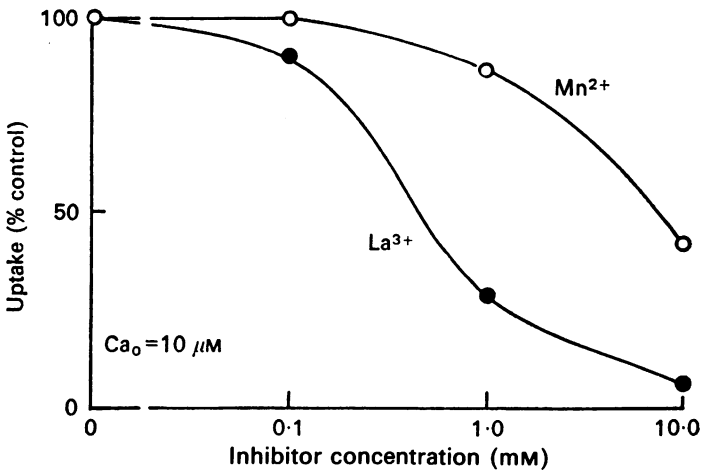

Fig. 10. Inhibition of energy-independent Ca binding by La (●) and Mn (○). Experiments on squid axoplasm in solution B containing 10 μM-Ca. Note the logarithmic scale of the abscissa. Temperature 20 °C.

Efflux of ^{45}Ca from axoplasm

The loss of Ca from axoplasm was studied in a few experiments. The results were similar to those previously reported by Luxoro & Yanez (1968) & Blaustein & Hodgkin (1969). ^{45}Ca was introduced either by injection into intact axons before extrusion or directly into isolated axoplasm held in a capillary tube. The injection raised the Ca content of the axoplasm by about 100 μM, the axoplasm was left for varying periods of time after injection before transfer to a dialysis sac or porous capillary for measurement of Ca efflux into artificial axoplasm (solution A for *Loligo* and solution C for *Myxicola*). Only two experiments were done on *Loligo* and eight on *Myxicola*. In *Myxicola* the Ca efflux into nominally Ca-free solutions containing ATP could be described by two exponentials: a fast phase ($\tau_{\frac{1}{2}}$ 2–4 min) which averaged 4·7 ± 2·2 % of the total radioactivity in the axon and a much slower phase ($\tau_{\frac{1}{2}}$ 2–6 hr). The size of these phases was similar in experiments begun 15 min and 6 hr after injection of ^{45}Ca. The fast phase may reflect loss of Ca in association with low-molecular-weight components of axoplasm and the slow phase release of Ca from binding sites in axoplasm. On the assumption that the total Ca in axoplasm is 500 μM, the amount of Ca in a freely dialysable form is 25 μM and in many axons because of their lower Ca content is probably very much less than this. The rate constant for the slow phase of Ca loss in the presence of ATP averaged 0·001 min^{-1} in *Loligo* and

0·0037 min^{-1} in *Myxicola*. It was increased three to tenfold by addition to the bathing medium of EGTA (5 mM) and up to tenfold by removal of ATP and addition of cyanide (2 mM) or DNP (1 mM). The effect of metabolic poisons was, however, rather variable and virtually no increase in efflux was seen in some experiments on *Myxicola*. These experiments suggest that in *Myxicola* much of the Ca in axoplasm is bound to non-mitochondrial sites and transfer of ^{45}Ca from these sites to the external medium is facilitated by a mobile calcium binder such as EGTA. If the loss of Ca from axoplasm is slowed by the presence of Ca-binding sites, saturation of these sites with cold Ca or calcium analogues should also increase the rate of loss of ^{45}Ca. After addition of 1 mM-Ca or 1 mM-Mn to the bathing solution an increase in Ca efflux was seen; but only two experiments were done and in these the efflux increased twofold which is less than the increase seen with EGTA on different samples of axoplasm.

At first sight, these results do not seem quantitatively consistent with the measurements of ionized Ca described earlier (p.107). As pointed out by Blaustein & Hodgkin (1969), provided bound and free Ca in axoplasm are in equilibrium and diffusion is not rate limiting, the rate constant for loss of Ca should be given by

$$k = fP/L,$$

where f is the fraction of Ca in diffusible form, P is the permeability of the dialysis membrane and L the volume/surface area ratio. Taking $P = 1{\cdot}6 \times 10^{-5}$ cm/sec and $L = 0{\cdot}55$ mm (Blaustein & Hodgkin, 1969) values of f from the present experiments are 0·043 for *Loligo* and 0·16 for *Myxicola*, indicating that 4·3 and 16% respectively of the total Ca is diffusible. It is natural to enquire the nature of this apparently constant supply of diffusible Ca. Any diffusible endogenous Ca chelators should be washed out of the axoplasm quite rapidly and their loss probably contributes to the fast phase of Ca efflux. In the absence of diffusible Ca chelators in the axoplasm and in the bathing medium, it seems most likely that the diffusible Ca is free calcium. If this is the case, there is a clear conflict between the measured ionized Ca of 0·1 μM in undialysed axoplasm and values in excess of 5 μM estimated from the efflux experiments. The most likely explanation is the presence in the nominally Ca-free artificial axoplasm used for the efflux studies of appreciable amounts of Ca. The measured Ca contamination varied from batch to batch but was usually between 5 and 20 μM. It seems likely that it is the level of this contaminating Ca that determines the efflux rate constant and if a more physiological level of Ca could be stabilized in the bathing medium without the use of mobile Ca chelators, the efflux rate constant would be much lower and should approach 10^{-5} min^{-1} in a medium containing 0·1 μM unbuffered Ca.

DISCUSSION

The experiments described in this paper provide clear evidence for the ability of isolated axoplasm to bind Ca and to maintain a concentration of ionized Ca close to that which is found inside intact axons. More than one mechanism contributes to axoplasmic Ca binding. These can be separated operationally into energy-dependent and energy-independent components and tentatively identified with mitochondrial and non-mitochondrial processes. There was no evidence that uptake results from the generation of a membrane potential across the walls of the dialysis sac.

The energy-dependent component requires either succinate and orthophosphate or ATP and orthophosphate and is inhibited by cyanide and oligomycin. No evidence was found for an ATP-dependent but cyanide and oligomycin-insensitive component of binding. When fuelled with succinate or ATP and orthophosphate, Ca uptake by a crude preparation of mitochondria isolated from axoplasm is also sensitive to cyanide and oligomycin. This similarity is strengthened further in *Loligo* axoplasm where the Ca-dependence of Ca binding is roughly the same for the energy-dependent com-

ponent of Ca binding in axoplasm and in a crude mitochondrial preparation obtained from axoplasm. For both, Ca uptake is half-maximal at 20–60 μM which is close to that found in mitochondria isolated from a variety of other cells (see Carafoli & Crompton, 1976). Such a close similarity was not found in *Myxicola* where the apparent affinity for Ca of presumed mitochondrial binding in axoplasm is much lower than in isolated mitochondria. Either the energy-dependent component of binding in *Myxicola* reflects another ATP-dependent and oligomycin-sensitive process or isolation of mitochondria from axoplasm releases them from some sort of inhibition of Ca uptake. This latter possibility is particularly interesting and merits further study.

The component of Ca uptake that persists in the absence of ATP and presence of cyanide and oligomycin has more than one Ca binding site. A component of binding that is half-maximal at about 0·1 μM can be detected at low Ca concentrations; but as the Ca concentration is increased, Ca continues to be bound up to 500 μM, the highest concentration examined. The high-affinity component of binding has a relatively small capacity (6–60 μmole/kg axoplasm) but seems ideally placed to buffer Ca in the physiological range. It is not clear whether this component is totally separate from mitochondria. Some high-affinity binding can always be detected in crude mitochondrial preparations exposed to cyanide and oligomycin; but whereas the bulk of the presumed mitochondrial binding can be recovered in these preparations, the amount of high-affinity binding is always less than that in intact axoplasm. A clear answer requires isolation of the high-affinity component which has not yet been attempted. It should, however, be pointed out that a number of workers have purified Ca-binding proteins from nerve cells (Calissano, Moore & Friesen, 1969; Kuo & Coffee, 1976) including squid axons (Alema, Calissano, Rusca & Guiditta, 1973) and the high affinity Ca binding system in intact squid axoplasm may be related to these although it seems to have a higher affinity for Ca than any of the proteins so far isolated and in this respect resembles the parvalbumins and troponins. The protein isolated by Alema *et al.* (1973) from the optic lobes of the squid had a molecular weight of 13,500–15,000 and two to three Ca binding sites per molecule. Its apparent dissociation constant for Ca was 25 μM. Additional studies showed that a molecule very similar to this protein is also present in squid axoplasm where its Ca-binding capacity was estimated at about 300 μmole/kg. These observations suggest that the protein of Alema *et al.* may be responsible for one of the low affinity components of Ca binding in squid axoplasm; but the possibility cannot be excluded that under normal circumstances in axoplasm a small fraction of this protein is present in a form with high affinity and this is altered or lost on isolation.

The present results have all been obtained on isolated axoplasm which lacks protoplasm from the extreme periphery of the axon. The method of extrusion always leaves a thin layer (*ca.* 10 μm) of axoplasm immediately under the axolemma (Baker, Hodgkin & Shaw, 1962) and the Ca binding properties of this layer might be different from those of the bulk of the axoplasm.

The existence of a number of Ca binding systems in axoplasm may help explain why exposure of aequorin-loaded axons to cyanide never releases as ionized Ca the total Ca measured by atomic absorption spectroscopy (Baker *et al.* 1971; DiPolo *et al.* 1976). Thus, as Ca is released from the mitochondria, it will be bound by the cyanide-

insensitive binding processes and not all the Ca released from energy-dependent reservoirs will become available as ionized Ca.

Axoplasm isolated from squid giant axons normally contains 100–500 μmole Ca/kg of which about 0·1 μM is ionized. It follows that provided all the Ca-binding systems are functioning normally, isolated axoplasm exposed to 0·1 μM-Ca should accumulate ^{45}Ca to a level similar to that found in fresh axoplasm. The maximum binding observed in isolated squid axoplasm was 96 μmole/kg axoplasm after 4 hr exposure to an ionized Ca concentration of 0·1 μM and this could be divided into 60 μmole/kg of presumed non-mitochondrial binding and 36 μmole/kg of presumed mitochondrial binding. At longer times binding did not increase further and often decreased, suggesting that the preparation was deteriorating. It seems likely that such deterioration limits the performance of the isolated axoplasm preparation and it is quite possible that *in vivo* mitochondria exposed to 0·1 μM ionized Ca may continue to take up Ca to much higher levels than can be obtained in the isolated axoplasm preparation. Indeed, if the driving force for mitochondrial uptake is solely the potential across the inner mitochondrial membrane, it is hard to see why mitochondria do not accumulate Ca to levels even higher than is usually associated with them and it seems necessary to invoke the existence of some sort of pump expelling Ca from the mitochondrion (see Baker, 1976; Carafoli & Crompton, 1976). The large capacity of the mitochondrion may be particularly important under conditions where calcium entry into cells is increased (see Schlaepfer, 1974).

It is possible only to speculate about the possible physiological significance of these intracellular Ca-binding processes. The presence of an axoplasmic component with exceptionally high affinity for Ca might support the view that intra-axonal Ca concentration is very important in axoplasmic functions such as axonal transport. A popular model accounting for the rapid saltatory movements of particulates in rapid axonal flow has, in fact, drawn close analogy to the actin–myosin interaction of striated muscle (Schmitt, 1968). Both actin (Fine & Bray, 1971; Chang & Goldman, 1973) and myosin-like protein (Hoffman & Lasek, 1975) are present in mammalian peripheral nerve tissues. Some experiments have also shown a reduction of *in vitro* axonal transport in Ca-free media containing EGTA (Dravid & Hammerschlag, 1975).

It seems likely that intracellular Ca-binding processes may play an important part in lowering intracellular ionized Ca after a period of nervous activity, and, as such, could be concerned in the termination of transmitter release. A proper examination of this possibility requires more information on the rate of reaction of the various binding systems with Ca. In addition, with such a large fraction of the total Ca bound, small changes in the rate constants governing binding and release could produce large changes in ionized Ca. Two possible examples of this form of control have been noted: alterations in monovalent cation concentrations and addition of heavy metals.

Of potential physiological significance is the observation that progressive replacement of K by Na or Li impairs the ability of axoplasm to bind Ca. This seems to result primarily from an action on the energy-dependent, presumed mitochondrial, component of binding and similar observations have been made by Carafoli *et al.* (1974) on isolated cardiac mitochondria. The precise mechanism of this Na-dependent reduction in mitochondrial binding is not settled although it might result from a fall in mitochondrial membrane potential or activation by Na and Li of an outwardly

directed Ca pump in the mitochondrial membrane (see Baker, 1976; Carafoli & Crompton, 1976). That a similar impairment of Ca-binding might occur in some intact nerve terminals is suggested by the observation that the spontaneous release of transmitter from the frog neuromuscular junction is increased under conditions where the Na pump is inhibited (Birks & Cohen, 1968; Baker & Crawford, 1975) or after replacement of extracellular Na by Li (Crawford, 1975). In both instances the increase is independent of an external source of Ca.

Although perhaps of less physiological significance but certainly of pharmacological importance is the finding that La and Mn, the only two heavy metals tested, both impair Ca binding by axoplasm apparently through an action on the presumed non-mitochondrial component of binding. Unfortunately the action of these agents on the presumed mitochondrial component of binding was not investigated; but if La inhibits Ca binding by mitochondria *in situ* as it does in isolated mitochondria, the net effect of an influx of La into a cell will be to raise the level of ionized Ca in the cytosol. This may contribute to the dramatic increase in transmitter release which often follows application of La to nerve terminals.

The experiments described in this paper confirm that the isolated axoplasm preparation may be particularly valuable for studying the processes responsible for binding Ca inside cells. They provide quantitative information on the relative affinities and capacities of presumed mitochondrial and non-mitochondrial Ca binding systems but do not help decide on the relative importance of these systems in lowering cytoplasmic Ca after a period of nervous activity. Despite their relatively low affinity for Ca, mitochondria have a very large capacity and the crucial factor contributing to a reduction in ionized Ca under physiological conditions is the relative rates of action of the various Ca-binding systems and this information is not yet available. The aequorin data of Fig. 4. indicate that small quantities of added Ca can be sequestered very rapidly whereas larger additions are only removed over many minutes. It is tempting to ascribe the rapid Ca binding to the high affinity system and the slower binding to mitochondria, but definitive proof must await more experiments.

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REFERENCES

ALEMA, S., CALISSANO, P., RUSCA, G. & GUIDITTA, A. (1973). Identification of a calcium-binding brain specific protein in the axoplasm of squid giant axons. *J. Neurochem.* **20**, 681–689.

BAKER, P. F (1972). Transport and metabolism of calcium ions in nerve. *Prog. Biophys. molec. Biol.* **24**, 177–223.

BAKER, P. F. (1976*a*). The regulation of intracellular calcium. *Symp. Soc. exp. Biol.* no. XXX, pp. 67–88.

BAKER, P. F. (1976*b*). Regulation of intracellular Ca and Mg in squid axons. *Fedn Proc.* **35**, 2589–2596.

BAKER, P. F. & CRAWFORD, A. C. (1972). Mobility and transport of magnesium in squid giant axons. *J. Physiol.* **227**, 855–874.

BAKER, P. F. & CRAWFORD, A. C. (1975). A note on the mechanism by which inhibitors of the sodium pump accelerate spontaneous release of transmitter from motor nerve terminals. *J. Physiol.* **247**, 209–226.

BAKER, P. F., HODGKIN, A. L. & RIDGWAY, E. B. (1971). Depolarization and calcium entry in squid giant axons. *J. Physiol.* **218**, 709–755.

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

BAKER, P. F., KNIGHT, D. E. & PATTNI, R. D. D. (1977). Porous cellulose acetate tubing provides a suitable support for isolated protoplasm during studies under controlled conditions. *J. Physiol.* **266**, 6*P*.

BAKER, P. F., LADD, M. & RUBINSON, K. A. (1977). Measurement of the flow properties of isolated axoplasm in a defined chemical environment. *J. Physiol.* **269**, 10–11*P*.

BAKER, P. F. & SCHAPIRA, A. H. V. (1977). Measurement of ionic diffusion and mobility in axoplasm isolated from giant axons of *Myxicola*. *J. Physiol.* **266**, 5*P*.

BAKER, P. F. & SCHLAEPFER, W. (1975). Calcium uptake by axoplasm extruded from giant axons of *Loligo*. *J. Physiol.* **249**, 37–38*P*.

BAKER, P. F. & SHAW, T. I. (1965). A comparison of the phosphorus metabolism of intact squid nerve with that of the isolated axoplasm and sheath. *J. Physiol.* **180**, 424–438.

BEAR, R. S., SCHMITT, F. O. & YOUNG, J. Z. (1937). Investigations on the protein constituents of nerve axoplasm. *Proc. R. Soc.* B, **123**, 520–529.

BIRKS, R. I. & COHEN, M. W. (1968). The action of sodium pump inhibitors on neuromuscular transmission. *Proc. R. Soc.* B, **170**, 381–399.

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

BLINKS, J. R., PRENDERGAST, F. G. & ALLEN, D. G. (1976). Photoproteins as biological calcium indicators. *Pharmac. Rev.* **28**, 1–93.

BRINLEY, F. J. & SCARPA, A. (1975). Ionized magnesium concentration in axoplasm of dialysed squid axons. *FEBS Lett.* **50**, 82–85.

CALDWELL, P. C. (1958). Studies on the internal pH of large muscle and nerve fibres. *J. Physiol.* **142**, 22–62.

CALISSANO, P., MOORE, B. W. & FRIESEN, A. (1969). Effect of calcium ion on S-100, a protein of the nervous system. *Biochemistry, N.Y.* **8**, 4318–4326.

CARAFOLI, E. & CROMPTON, M. (1976). Calcium ions and mitochondria. *Symp. Soc. exp. Biol.* no. XXX, pp. 89–115.

CARAFOLI, E., TIOZZO, R., LUGLI, G., CROVETTI, F. & KRATZING, C. (1974). The release of Ca^{2+} from heart mitochondria by sodium. *J. molec. cell. Cardiol.* **6**, 361–371.

CHANG, C. M. & GOLDMAN, R. D. (1973). The location of actin-like fibres in cultured neuroblastoma cells as revealed by heavy meromyosin binding. *J. cell Biol.* **57**, 867–874.

CRAWFORD, A. C. (1975). Lithium ions and the release of transmitter at the frog neuromuscular junction. *J. Physiol.* **246**, 109–142.

DIPOLO, R., REQUENA, J., BRINLEY, F. J., MULLINS, L. J., SCARPA, A. & TIFFERT, T. (1976). Ionized calcium concentrations in squid axons. *J. gen. Physiol.* **67**, 433–467.

DRAVID, A. R. & HAMMERSCHLAG, R. (1975). Axoplasmic transport of proteins *in vitro* in primary afferent neurons of frog spinal cord: Effect of Ca^{2+}-free incubation conditions. *J. Neurochem.* **24**, 711–718.

FINE, R. E. & BRAY, D. (1971). Actin in growing nerve cells. *Nature, New Biol.* **234**, 115–118.

GILBERT, D. S. (1975*a*). Axoplasm architecture and physical properties as seen in the *Myxicola* giant axon. *J. Physiol.* **253**, 257–301.

GILBERT, D. S. (1975*b*). Axoplasm chemical composition in *Myxicola* and solubility properties of its structural proteins. *J. Physiol.* **253**, 303–319.

GILBERT, D. S., NEWBY, B. & ANDERTON, B. (1975). Neurofilament disguise, destruction and discipline. *Nature, Lond.* **256**, 586–589.

HODGKIN, A. L. & KATZ, B. (1949). The effect of calcium on the axoplasm of giant nerve fibres. *J. exp. Biol.* **26**, 292–294.

HODGKIN, A. L. & KEYNES, R. D. (1956). Movement of labelled calcium in squid giant axons. *J. Physiol.* **138**, 253–281.

HOFFMAN, P. W. & LASEK, R. J. (1975). The slow component of axonal transport. Identification of major structural polypeptides of the axon and their generality among mammalian neurones. *J. cell Biol.* **66**, 351–366.

KUO, I. C. Y. & COFFEE, S. J. (1976). Purification and characterization of a troponin-C-like protein from bovine adrenal medulla. *J. biol. Chem.* **251**, 1603–1609.

LUXORO, M. & YANEZ, E. (1968). Permeability of the giant axon of *Dosidicus gigas* to calcium ions. *J. gen. Physiol.* **51**, 115*s*.

SCHLAEPFER, W. W. (1974). Calcium-induced degeneration of axoplasm in isolated segments of rat peripheral nerve. *Brain Res.* **69**, 203–215.

SCHMITT, F. O. (1968). Fibrous proteins-neuronal organelles. *Proc. natn. Acad. Sci. U.S.A.* **60**, 1092-1101.

SCHWARZENBACH, G., SENN, H. & ANDEREGG, G. (1957). Komplexone XXIV. Ein grosser Chelateffekt besonderer Art. *Helv. chim. Acta* **40**, 1186–1900.