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THE INTRACELLULAR CALCIUM CONTENTS OF SOME INVERTEBRATE NERVES

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Estimates of the calcium content of a variety of whole tissues have been reported, but there are indications that much of this calcium is extracellular, and there is little reliable information about the intracellular calcium concentration in living cells. In the course of an investigation on the role of calcium in invertebrate nerves (for preliminary accounts see Frankenhaeuser & Hodgkin, 1955; Flückiger & Keynes, 1955) a knowledge of the amount of calcium in nerve axoplasm became important. A micromethod for the determination of quantities of calcium of the order of 1 μg or less was therefore developed by one of us (P. R. L.), and its application to estimate the internal calcium of squid giant axons and of whole crab nerves is described here.

METHODS

Principle of the analytical technique

The analytical procedure was developed from the visual titration method due to Schwarzenbach and his collaborators (Schwarzenbach, Biedermann & Bangerter, 1946; Schwarzenbach & Gysling, 1949; see also Martell & Calvin, 1952). It consists in titrating calcium, in alkaline solution, against a solution of versene (the disodium salt of ethylenediamine tetracetic acid) in the presence of murexide (ammonium purpurate). Versene is a powerful chelating agent for many of the polyvalent metals, and combines quantitatively with the calcium; the murexide acts as an indicator, combining with any free calcium ions to form a red-coloured complex, which decomposes to liberate the purple-coloured purpurate ion when the concentration of ionic calcium falls on completing the titration.

The main problem in scaling down this technique lay in the detection of the end-point—when the last trace of red disappears from an otherwise purple solution. With the very small amount of indicator that could be tolerated in titrating, say 1 μg of calcium, the end-point cannot be distinguished visually. The micro-titration was therefore performed in a colorimeter cell, in position in the colorimeter, and the extinction of the solution was measured with a suitable blue filter after each addition of versene. The end-point was easily determined from a plot of extinction against volume of versene added. Fales (1953) has used a similar method on a somewhat larger scale for the determination of serum calcium, and Karsten, Kies, van Engelen & de Hoog (1955)

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have recently published a discussion of the theory of this type of titration. Hence only an outline of our procedure is given below, with those quantitative details dependent on the amount of calcium being determined.

Analytical procedure

The solutions used in the titration were:

(1) Disodium salt of ethylenediamine tetracetic acid, 2 mM (mol. wt. = 372).

(2) Ammonium purpurate, saturated solution in distilled water.

(3) Sodium hydroxide, free from calcium, 25%, w/v (British Drug Houses Ltd.), *or* potassium hydroxide, 6N, made up from solid KOH A.R.

The ammonium purpurate solution decomposes slowly; if kept in the dark at 1–4° C it can be used for several days, but not for more than a week.

Samples were incinerated in small platinum crucibles or in short tubes made from 8 mm silica tubing, at a temperature of 600–620° C, until completely reduced to a greyish white ash (large flecks of carbon interfere with the titration by causing fluctuations in the extinction when the solution is stirred). When cool, each sample was dissolved in 11 μl. of 2N-HCl, and put in an air oven at 105° C for a few minutes to decompose at least the major part of any pyrophosphate present (pyrophosphate interferes if present in large amounts, but its effect can easily be recognized, since it reduces the slope of the titration curve as shown in Fig. 1). The solution, plus several washings of distilled water, was transferred to the colorimeter cell with the aid of a small dropping tube; 11 μl. of the alkali and 20 μl. of the indicator solution were added; the total volume was made up to about 0·45 ml. with distilled water; and the titration was begun immediately.

A Spekker absorptiometer (Hilger Model H760) was used for the titration, with Ilford 621 filters. A micro-colorimeter cell (path length 1 cm, capacity 0·5 ml.) was supported in a special holder about 3 mm higher than usual, so that the meniscus was above the top of the light beam even when the cell was not quite full. The shutter in the comparison beam was adjusted until the drum reading on the logarithmic scale was about 0·15 (owing to the raised position of the cell, lower readings were not linearly related to the extinction of the solution). The precise initial drum reading was noted; 2·4 μl. of the versene solution was added from a micropipette; the solution was stirred by gentle blowing down the pipette; and, without altering the position of the comparison shutter, the drum was readjusted to the balance point and the new reading noted. Addition of versene was continued until the end-point was passed, as indicated by the fact that the extinction ceased to change appreciably. Typical titration curves are plotted in Fig. 1, from which it can be seen that the end-point can be determined graphically with fair precision. The micropipette used in the titration, and indeed all the pipettes used at other stages in the procedure, were of a self-filling type similar in principle to that described by Kirk (1950).

The average standard error in estimating 1 μg samples of calcium was found to be $\pm 3\%$. Most of the samples of *Carcinus* nerve contained at least this quantity of calcium. The percentage error in the analysis of the squid axoplasm samples was certainly higher, since several of them contained under 0·2 μg of calcium; it may have been as high as $\pm 10\%$, but did not exceed $\pm 15\%$.

This method should be directly applicable to the accurate determination of calcium in most biological material. When larger amounts of calcium are available the whole procedure can be scaled up. The most convenient dilution to use is 2–3 μg Ca/ml. of titration fluid; with more concentrated solutions, accuracy may be improved, but the estimation is somewhat tedious unless a larger pipette is used for the initial part of the titration. The optimum amount of murexide to use is that which gives an extinction, relative to distilled water, of about 0·15 at the end-point.

Interference from other elements

In sufficiently alkaline solution magnesium does not interfere, nor do sulphate, carbonate or phosphate, unless present in large enough quantity to produce a visible precipitate. Pyrophosphate can interfere, but no error from this source was detectable in any of the analyses reported here. Invertebrate nerves, however, contain little phosphorus compared with their total sodium

and potassium. With tissues having a high ratio of phosphorus to total cations, it would probably be advisable to take special precautions to remove pyrophosphate. Certain heavy metals, such as iron, copper and zinc, could interfere if present in sufficient quantities; but such interference was negligible in our analyses of these invertebrate nerves.

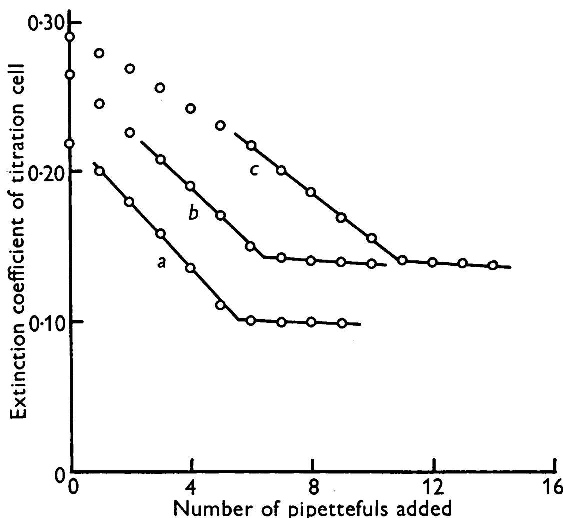

Fig. 1. Three typical titration curves showing the titration of (*a*) 1 μg calcium, (*b*) an incinerated sample of whole *Carcinus* nerve, (*c*) 2 μg calcium in the presence of a fourfold molar excess of pyrophosphate (35 μg as P_2O_7). The ordinates are the differences between the instrument readings and the reading when the cell contains only distilled water. A comparison of curves (*a*) and (*c*) shows that the presence of a moderate excess of pyrophosphate does not produce an appreciable error in the end-point, but does reduce the final slope of the curve, thereby reducing the accuracy with which the end-point can be assessed.

The collection and storage of squid axoplasm samples

The first axoplasm samples were extruded on to quartz threads, weighed on a torsion balance, dried, and stored in 3 mm quartz tubes as described by Keynes & Lewis (1951*b*). These gave unsatisfactory results because of the presence of traces of calcium on the walls of the quartz tubing. A second set of samples was therefore collected and stored in a slightly different fashion, with rigorous precautions against the possibility of contamination by extraneous calcium at any stage of the procedure. The first stellar nerve trunks were dissected from medium-sized specimens of *Loligo forbesi*, and the giant axons were examined critically for signs of damage. After their excitability had been tested, a short length of the distal part of the axon was cleaned from small fibres, and the axon was cut through in the centre of this stretch. The nerve was lightly blotted, and laid on filter-paper with its cleaned end just overlapping on to an underlying block of Perspex which was thoroughly washed with distilled water before each extrusion. The axoplasm was then extruded on to this block by gentle pressure from a small Perspex roller. It was quickly transferred with clean forceps to a cross of platinum wire fixed over the open end of a small specimen tube, and weighed with an air-damped reflecting balance accurate to 0·1 mg. The platinum wire had been cleaned by repeated dipping into concentrated HCl and flaming, and checks showed that there was no measurable quantity of calcium adhering to it. Each specimen tube was mounted inside a larger tube to avoid contamination by dust particles, and the samples were left to dry at room temperature. Before analysis the central part of the crossed wire was cut out with scissors, and sample and wire were incinerated together in small platinum crucibles.

RESULTS

Analysis of the calcium in squid axoplasm

The first four samples of axoplasm, which had been stored in silica tubes, were found to contain very little calcium. However, the true calcium concentration in the axoplasm could not be calculated reliably, because the silica tubes were appreciably contaminated (washings of unused tubes contained about 0·2 μg Ca). After making an approximate correction for this contamination, the average calcium content of the axoplasm appeared to lie in the range 0·6–1·1 m-mole/kg, but the only certain conclusion that could be reached was that there must be less than about 1 m-mole/kg.

TABLE 1. The calcium contents of squid axoplasm

Sample no.	Time from decapitation (min)	Axon diameter (μ)	Weight of axoplasm (mg)	Total calcium content (mole $\times 10^{-9}$)	Calcium concentration (m-mole/kg)
1	80	—	8·3	4·6	0·55
2	115	612	12·7	4·6	0·36
3	*ca.* 80	490	7·9	3·95	0·50
4	67	566	12·4	4·7	0·38
5	85	596	9·0	3·0	0·33
6	125	582	11·6	20·2	1·74

Samples 1–5 were extruded from unstimulated axons, dissected in sea water. Sample 6 was extruded from an axon which was dissected in sea water, and then stimulated for 30 min at a rate of 156 impulses/sec in an artificial sea water containing 112 mM-$CaCl_2$, 10 mM-KCl and 392 mM-NaCl; temperature 16° C.

The second group of samples, collected at Plymouth a year later, was handled in such a way as to eliminate as far as possible any uncertainty of this kind. As may be seen from the results listed in Table 1, the calcium concentration in the axoplasm of unstimulated axons was usually less than 0·5 m-mole/kg, the average value for 5 axons being 0·42 m-mole/kg. Axon 1 was taken from a squid which had died a few minutes before the dissection was begun, while the others were from living squid; but there was no marked correlation between the calcium concentration and the condition of the squid, or the time taken to dissect out the axon and extrude its axoplasm. Our result is much lower than that recently reported by Koechlin (1955), who found $7 \pm 5\,\mu$equiv Ca/g (i.e. 3·5 m-mole/kg) in pooled axoplasm from a large number of squid. However, Koechlin did not regard this figure as being more than an upper limit to the amount of calcium in squid axoplasm, because it was obtained by flame photometry, which may not have given reliable values for a small quantity of Ca in the presence of much larger concentrations of Na and K. The tracer experiments of Rothenberg (1950) would suggest the presence in squid axoplasm of even higher concentrations of calcium, since he noted an exchange of about 8 m-mole ^{45}Ca/kg axoplasm. But it seems likely that he was working with damaged axons into which there was an

abnormally high net influx of calcium (Flückiger & Keynes, 1955), whereas we were careful to exclude any axons with the least signs of deterioration at cut branches.

The last axon in Table 1 was treated differently from the others, having been stimulated (in collaboration with Professor Hodgkin) for some while in a medium containing ten times the normal amount of calcium. Taking the initial calcium level as 0·42 m-mole/kg, the passage of about 280,000 impulses appears to have resulted in a net gain of 1·3 m-mole Ca/kg. The axon diameter was 582 μ, so that this corresponds to a net calcium entry of some 0·07 pmole/cm^2 impulse. This figure agrees well with the results of ^{45}Ca experiments on stimulated squid axons, and provides useful confirmation for the occurrence of a net inward movement of calcium during nervous activity. Flückiger & Keynes (1955) found an extra influx of 0·015 pmole Ca/cm^2 impulse in sea water containing 10·7 mM of labelled calcium, and the influx is appreciably larger when the external calcium concentration is raised (Hodgkin & Keynes, in preparation).

The intracellular calcium content of crab nerve

Any attempt to estimate intracellular concentrations in multifibre preparations is complicated by the need to correct the analytical values for the content of the extracellular space. This correction is particularly large, of course, for constituents whose concentration inside the fibres is much smaller than outside. To determine the calcium content of crab nerve fibres, therefore, whole nerves were analysed after soaking for short periods in Ringer's solutions (for basic composition see Keynes & Lewis, 1951 *a*) in which various proportions of the normal amount of calcium chloride had been replaced by an osmotically equivalent amount of sodium chloride. Each nerve was soaked for 10–15 min, a period long enough for most of the extracellular calcium to have diffused away, but insufficient for much of the intracellular calcium to have escaped. The results of this series of analyses are summarized in Fig. 2, where the individual analytical values (in m-mole/kg wet weight of whole nerve) are plotted against the calcium concentration in the Ringer's solution.

If each whole nerve is assumed to consist of two separate compartments—an intracellular one containing calcium at the unaltered, *in vivo*, concentration, and an extracellular one with the concentration in the Ringer's solution used for soaking—then the slope of the regression line in Fig. 2 gives the mean extracellular space as a fraction, and its intercept on the y axis gives the calcium content of the intracellular space. The values so obtained, together with their S.E., are: volume of extracellular space = $0{\cdot}216 \pm 0{\cdot}013$ l./kg whole nerve, calcium content of intracellular space = $0{\cdot}95 \pm 0{\cdot}10$ m-mole/kg whole nerve. Now several lines of evidence suggest that the true extracellular space in these nerves lies between 0·24 and 0·27 (Keynes & Lewis, 1951 *a*; Lewis,

1952), with 0·25 as the most probable value. The figure deduced from Fig. 2 is therefore slightly low. Such a discrepancy is, however, to be expected if the calcium in the extracellular space does not reach quite complete diffusion equilibrium with that in the Ringer's solution in the time allowed for soaking; incompleteness of extracellular exchange would also make the apparent intracellular calcium content too high. It is not unreasonable to suggest that diffusion equilibrium might not have been fully achieved in 10 or 15 min, because the half-times for exchange of extracellular sodium and amino-acids have been found to be of the order of 3–5 min (Keynes & Lewis, 1951*a*; Lewis, 1952). By assuming that the disagreement in extracellular space (taking the correct value as $0{\cdot}25 \pm 0{\cdot}01$) arose wholly from this cause, a corrected figure for the intracellular calcium could be worked out, this being $0{\cdot}54 \pm 0{\cdot}17$ m-mole/kg. Although the basis for the correction cannot be regarded as entirely reliable, this value agrees satisfactorily with that for squid axoplasm, where the analyses were not complicated by the presence of any extracellular calcium.

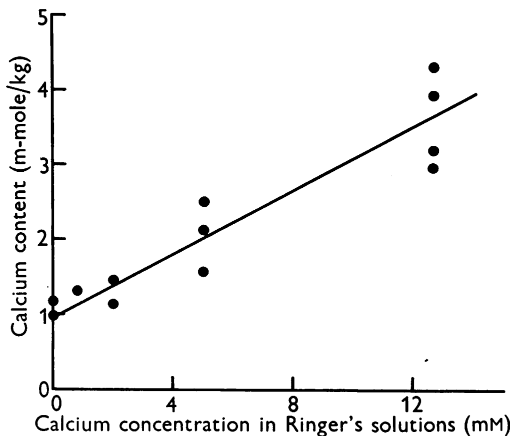

Fig. 2. The calcium contents of whole *Carcinus* nerves, after soaking for 10–15 min in solutions containing various concentrations of calcium.

The high calcium content of nerves dissected in sea water

Several *Carcinus* nerves dissected out in sea water instead of Ringer's solution contained surprisingly large amounts of calcium, up to 50% more than would be expected from the results shown in Fig. 2. The only obvious differences between the Ringer's solution and the sea water were that the former, like the animal's blood, contained rather more calcium, but much less magnesium and sulphate, and was less alkaline. The high values were not due to precipitation of calcium sulphate, since several nerves dissected in Ringer's

solution containing extra sulphate were found to have a normal calcium content. Dissection in alkaline Ringer's solution did, however, lead to some high calcium values, and during the dissection of two such nerves transient formation of a white precipitate was seen. The blood of these marine invertebrates is almost certainly saturated, if not actually supersaturated, with respect to calcium carbonate (see Potts, 1954), which is liable to be precipitated in the interfibre spaces of the nerve if the dissecting medium is more alkaline than the animal's body fluids.

DISCUSSION

Our analyses show that the intracellular calcium concentration in both squid and *Carcinus* nerve fibres is of the order of 0·5 m-mole/kg axoplasm. At first sight this value may appear surprisingly low, but its essential correctness is confirmed by other evidence. Flückiger & Keynes (1955) and Hodgkin & Keynes (unpublished) have used ^{45}Ca to study the calcium fluxes in squid axons, and the tracer results are consistent with the chemical analyses in that they are compatible with the presence of only small amounts of exchangeable calcium inside axons in good condition. The observations by Hodgkin & Katz (1949) on the dispersive effects on extruded squid axoplasm of solutions containing small quantities of calcium also suggest that the internal concentration of ionized calcium is unlikely to be higher than about 1 m-mole/kg. It is probable that many other tissues have a similarly low intracellular calcium content. Some samples of 5- and 6-day-old rabbit blastocytes were analysed and found to contain about 1 m-mole Ca/kg (P. R. Lewis & C. Lutwak-Mann, unpublished), while values of the same order have been obtained for muscle (Fenn, Cobb, Manery & Bloor, 1938; Slater & Cleland, 1953). Tipton (1934) reported rather higher values for frog nerve, but here much of the calcium is probably associated with the phospholipids of the myelin sheath.

The tracer experiments mentioned above have shown that most of the calcium in squid axoplasm is not in a freely ionized form. This is presumably also the case in crab nerve, which is known to contain a number of substances which have a tendency to combine with calcium ions, particularly phosphorus compounds such as ATP, phospholipids and nucleic acids. Moreover, Rudenberg (1954) has reported that nearly half the calcium in homogenates of whole lobster nerve is only slowly removed by dialysis against distilled water. Further evidence was provided by examining the calcium content of *Carcinus* nerves whose membranes had been rendered freely permeable, if not actually destroyed. Thus several crab nerves were first soaked for 20 min in a solution containing 2 mM-$Ca(NO_3)_2$ and no other salts, were then transferred for 12 min to *Carcinus* Ringer's solution also containing only 2 mM-Ca, and finally were analysed; in the first solution they swelled rapidly, and their membranes were probably ruptured, since under these conditions small intracellular solutes

such as K^+ ions and amino-acids are soon lost. These nerves contained over 4 m-mole/kg of calcium—more than double the concentration of ionic calcium in the soaking fluids, and over three times the content of intact nerves treated with similar Ringer's solution (see Fig. 2). A similar result was obtained with nerves soaked in Ringer's solution containing 2 mM-Ca and saturated with chloroform to destroy their membranes. Over half the calcium in these nerve residues must have been in a combined form, and on any reasonable assumption concerning the type of binding in the axoplasm, the bound fraction would have been an even larger proportion of the whole had a lower concentration of ionic calcium been used. Although the state of the axoplasm in the residues may well be rather different from that in an intact nerve, these observations suggest that the concentration of ionized calcium in the axoplasm of living nerves is probably not higher than one- or two-tenths of a millimole per kg.

The concentration of ionic calcium inside these invertebrate nerve fibres is therefore much lower than in the surrounding medium—by a factor of 10 or even 100 times. The nerve membrane is clearly not completely impermeable to calcium ions (Flückiger & Keynes, 1955), so that some form of active transport mechanism must exist to transfer calcium from inside to outside, against both the potential and concentration gradients. The evolution of such a mechanism could be a biological accident, but it would seem more plausible to suppose that a low internal calcium concentration is functionally advantageous. This proposition is, perhaps, supported by the fact that the intracellular calcium concentration is no higher in invertebrates than in vertebrates, in spite of the much higher level of ionized calcium in the blood of the former. The particular cell function which requires low internal calcium cannot be identified with certainty, but calcium ions undoubtedly have a profound influence on many cellular constituents (see Heilbrunn, 1952, for an extensive review). Thus they affect the physical state of many proteins, and injected calcium has been shown to liquefy the axoplasm of squid axons (Chambers & Kao, 1952; Hodgkin & Keynes, 1956); they have a marked tendency to form co-valent complexes with many organic phosphates; they rapidly deactivate respiratory granules such as the sarcosomes of mammalian heart muscles (Slater & Cleland, 1953); and they are intimately concerned in the working of excitable membranes (Brink, 1954; Weidmann, 1955; Frankenhaeuser & Hodgkin, 1955). The role of calcium in one or more of these respects may be such that an unduly high internal concentration would seriously interfere with the well-being of the cell.

SUMMARY

1. A method is described for the determination in biological samples of 1 μg or less of calcium.

2. Analyses of (*a*) axoplasm extruded from freshly dissected squid axons, and (*b*) whole *Carcinus* nerves which had been soaked in Ringer's solution

containing various amounts of calcium, showed that in both types of invertebrate nerve the internal calcium concentration is of the order of 0·5 m-mole/kg wet weight.

Some of the squid axons were dissected by Professor A. L. Hodgkin, to whom we are also indebted for reading the manuscript. The expenses of this work were met by grants from the Rockefeller and Nuffield Foundations.

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