Mitochondria and Other Calcium Buffers of Squid Axon Studied *In Situ*

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ABSTRACT   Continuous nondestructive monitoring of intracellular ionized calcium in isolated squid axons by differential absorption spectroscopy (using arsenazo III and antipyrylazo III) was used to study uptake of calcium by carbonyl cyanide, p-trifluoromethoxy-phenylhydrazone (FCCP)- and (or) cyanide (CN)-sensitive and insensitive constituents of axoplasm. Known calcium loads imposed on the axon by stimulation produced proportional increments of free axoplasmic calcium. Measurement of increments in ionized calcium as a function of load confirmed earlier reports of buffering in normal and FCCP- and (or) CN-poisoned axons. Measurement of rates of calcium uptake by presumed mitochondria showed little uptake at ambient Ca below 200–400 nM, with sigmoidal rise to about 20–30 μmol/kg axoplasm per min (calculated to be about 200 mmol/kg mitochondrial protein per min) at 50 μM, indicating a functional threshold for presumed mitochondrial uptake well above physiological ionized calcium concentration. Treatment of stimulated axons with cyanide, to release calcium from presumed mitochondria, showed that the sensitivity to cyanide decreased progressively with time after stimulation (t_4 = 3–10 min) implying transfer of sequestered calcium into a less metabolically labile form.

INTRODUCTION

This paper is part of a continuing study of *in situ* calcium homeostasis in isolated giant axons of the squid with the intent of describing the role of mitochondria and other constituents of axoplasm in regulating physiological loads of calcium. A previous report (Brinley et al., 1977b) has described some aspects of the *in situ* calcium buffering capacity of squid axoplasm. It was found that the buffering capacity could be divided into at least two components, one presumably mitochondrial, and a second, poorly characterized, which persisted in the presence of carbonyl cyanide, p-trifluoromethoxy-phenylhydrazone (FCCP) or cyanide (CN) applied in the absence of endogenous ATP. Because these procedures are thought to inactivate completely the calcium-accumulating capacity of mitochondria, the presumption was that the residual buffering capacity resided in some soluble components or organelles other than mitochondria. In unpoisoned axons with active mitochondria, about 99.5% of a calculated imposed load was buffered, whereas in the absence of mitochondria no more than 95% of the load was buffered.

In this paper, as in the previous one, mitochondrial function was assessed solely by biochemical criteria, i.e., sensitivity to CN and (or) FCCP. Although these agents have been extensively used to measure function of isolated mitochondria, we have no direct evidence that they do not have other effects when applied to intact cells. Therefore, our statements regarding mitochondrial uptake and sequestration of calcium *in situ* are tentative until we confirm that mitochondria are the only organelles affected.

The present research extends the prior work in two areas: a study of rate of presumed mitochondrial uptake of axoplasmic calcium as a function of ambient-free calcium concentration, and an initial description of a process by which calcium taken up by the mitochondria passes into a state less dependent upon metabolism.

The results indicate that in squid mitochondria, the kinetic parameters of calcium uptake *in situ* are similar to those reported in vitro for many vertebrate systems, when allowance is made for the nearly 10°C lower temperature of the present experiments. The results also show that at physiological levels of free calcium, i.e., 20–50 nM, up to concentrations of 200–400 nM, the rate of mitochondrial uptake is very low, suggesting that the membrane calcium pump is more effective in regulating the internal ionized calcium. In addition, the present work suggests that the initial translocation of calcium within mitochondria involves a state in which continued retention is directly dependent upon respiration. This initial state is superseded, with a half time of a few minutes, by a state in which retention does not depend upon respiration, but is still sensitive to uncouplers such as FCCP.

METHODS

The methods used in this study have been described in two previous publications (DiPolo et al., 1976; Brinley et al., 1977b). Only important deviations from previous accounts are presented here.

Experimental Material

All experiments were performed during May and June of 1977 using living specimens of *Loligo pealei* supplied by the Marine Biological Laboratory in Woods Hole, Mass. After isolation from the mantle, axons were stored in 3 mM calcium seawater at 10°C. Unless otherwise specifically indicated, all experiments were performed on axons stored <4 h, and in most cases <2 h.

Apparatus

The apparatus used for mounting axons, microinjections, and optical measurements were essentially those described previously.

Metallochromic Indicators

Purified arsenazo III was obtained from Sigma Chemical Co. (St. Louis, Mo.). This material was stated to have 3.8% (mole fraction) calcium contamination. It was used for experiments in which the ionized calcium was in the range from 0 to 5 μM.

For very high ionized calcium concentrations, arsenazo III is not suitable as an indicator. In these circumstances another indicator was used: 3,6-bis(4-antipyrylazo)-4,5-dihydroxy-2,7-naphthalene-disulphonic acid, also named antipyrylazo III, diantipy-

rylazo, or compound "C" (Budĕšinský in Flashka and Barnard, 1969). This dye was supplied by ICN Pharmaceuticals Inc., (Cleveland, Ohio) under the name of "antipyrazolo III." It contained about 80% antipyrylazo III, and had a calcium contamination of <0.1% (mole fraction). The dissociation constant of the calcium complex at pH 7.3 and ionic strength 0.4 is 250 μM. A suitable wavelength pair for dual wavelength spectroscopy is 685–660 nm. A complete description of this dye and its use for determination of calcium in biological systems is published elsewhere (Scarpa et al., 1978).

Solutions and Reagents

The external solutions had the composition previously described (Table I in Requena et al., 1977). The solution used to calibrate the metallochromic indicators had an ionic strength and pH (7.3) similar to that of axoplasm and the following composition: potassium isethionate, 300 mM; sodium isethionate, 35 mM; $MgCl_2$, 3 mM; glycine, 235 mM; potassium N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (KTES), 20 mM. Carbonyl cyanide, p-trifluoromethoxy-phenylhydrazone (FCCP, Pierce Chemical Co., Rockford, Ill.) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2 mg/ml. Apyrase was obtained from Sigma Chemical Co. and purified of calcium by passage twice through a Chelex 100 column (Bio-Rad Laboratories, Richmond, Calif.). All prepared solutions used for injection were analyzed for calcium before use. Calcium contamination for reagents such as potassium isethionate, glycine, etc. was estimated to be 0.0001 mol fraction after purification.

Calculation of Exogenous Calcium Load

Axons were loaded with calcium as described previously either by electrical stimulation in high calcium solutions or by soaking in choline solutions containing calcium (Brinley et al., 1977b). The former method loads fibers at a rate of about 50 ± 4 SEM μM/axoplasm per min during stimulation at 100 impulses/s in 112 mM Ca saline. The soaking method loads fibers at about 4 μmol/kg axoplasm per min per mM extracellular calcium (Requena et al., 1977). These mean values were used for calculating imposed loads, because it is not feasible to make analytical measurements of the extent of calcium loading in each axon used. Based on a previous report (Brinley et al., 1977b, Table I) that compared measured and calculated loads, a variation as large as a factor of 2 could have occurred in the present experiments. However, this would not have affected any of the conclusions reached in this paper which are based upon levels of ionized calcium rather than extent of loading.

Critique of Spectrophotometric Measurement of Ionized Calcium Using Metallochromic Dyes

This section discusses several important aspects of absorption spectroscopy as applied to the measurement of intracellular free cation concentrations inside intact cells. Although the present work is concerned only with calcium, the discussion applies generally to the determination of other cations, e.g. magnesium and hydrogen.

ABSOLUTE VS. RELATIVE MEASUREMENTS Absorption spectroscopy is best suited for measurement of changes in ionized calcium from an initial level, rather than determination of an initial absolute level. In principle, the amount of free calcium could be calculated from the deviation of the observed spectrum, from that of the pure dye. However, this deviation amounts to only about 0.0005 A at the wavelengths employed, i.e. 685–660 nm for antipyrylazo III and 685–675 nm for arsenazo III, and would be unmeasurable in the presence of the absorbance of unbound dye (about 0.25 A) and the nonspecific absorbance of the axon (about 0.25 A). The only presently feasible method of detecting such small absorbance changes is by use of dual wavelength differential

spectroscopy to nullify the absolute absorbance. However, the initial absorbance in this method is arbitrary and does not reflect the absolute concentration of either the free or bound dye, and cannot be used to measure the initial level of free ion. Internal dialysis with high concentrations of EGTA of axons injected with arsenazo III has been used to establish a reference absorbance level corresponding to zero free calcium to calculate the initial free calcium in the fiber (DiPolo et al., 1976). However, this procedure distorts the buffer systems of the axon so that the fiber cannot be used subsequently for other purposes.

Although the aequorin resting glow has been used (Baker et al., 1971; DiPolo et al., 1976) to measure free calcium concentration in squid axons and does provide a qualitative measure of differences in initial free calcium level between different fibers, the complicated functional dependence of the aequorin light emission upon free calcium concentration (Allen et al., 1976) seems to preclude any simple *in situ* calibration of the luminescence. In particular, the presence of a substantial luminescence relatively unaffected by changes in free calcium at the low concentration obtained in biological systems would seem to limit greatly the usefulness of this material for quantitative measurement of free calcium in the biological range.

SELECTIVITY OF THE DYES   Neither of the dyes used in this study is inherently very selective for calcium over magnesium, which is the other common intracellular divalent cation. The ratio of the magnesium to calcium dissociation constants is about 50 for arsenazo III and about 10 for antipyrylazo III. Even though it is possible to find wavelength pairs (e.g. 685–675 nm for arsenazo III, and 685–660 nm for antipyrylazo III) at which the differential absorbance of the magnesium spectra is virtually zero, changes in magnesium concentration will still influence the differential absorbance by changing the free dye concentration upon which the differential absorbance depends. Magnesium interference is not thought to be a serious problem in these experiments, because, with one exception (Fig. 9), the measurements of ionized calcium were not done when the ionized magnesium was changing rapidly.

COMMENT ON IN VITRO AND IN SITU CALIBRATIONS   Although absorption spectroscopy is not suited for absolute measurement of free calcium, the fact that the dye-calcium interaction is fast, reversible, and linear (for calcium concentrations small with respect to the dissociation constant of the dye) makes it convenient for measurement of relative changes in free calcium inside a single axon. In the absence of a suitable *in situ* calibration, we calibrated in vitro using a glass capillary with a diameter similar to that of axons (about 500 μm), a salt solution with a composition and pH similar to that of axoplasm, and a dye concentration similar to that inside the axon (about 500 μM).

The following manipulation of Beer's law permits explicit consideration of the variables determining the relation between absorbance and ionized calcium in vitro as well as in the axon.

For any wavelength, the total absorbance will be:

$$A_1 = (\epsilon^X[X]_1 + \epsilon^{CaX}[CaX]_1)L,$$

and

$$A_2 = (\epsilon^X[X]_2 + \epsilon^{CaX}[CaX]_2)L, \tag{1}$$

where the subscripts $_1$ and $_2$ refer to different concentrations of the absorbing species, X and CaX; ϵ is the specific absorbance; L is the path length. The change in absorbance in going from state 1 to state 2 is just the difference in the absorbance.

$$\Delta A = (\epsilon^X \Delta X + \epsilon^{CaX} \Delta CaX) \cdot L. \tag{2}$$

If we assume that Ca is the only binding species, then

$$\Delta X = -\Delta CaX$$

and

$$\Delta A = (\epsilon^{CaX} - \epsilon^X)(\Delta CaX)\cdot L. \tag{3}$$

If we make use of the defining relation for dissociation constant (K_D):

$$K_D = \frac{(Ca)[(X)_T - CaX]}{CaX}, \tag{4}$$

where $(X)_T$ is the total dye concentration in either free or bound form, we can write the following expression for the change in CaX concentration due to a change in ionized concentration from Ca_1 to Ca_2 (ΔCa), viz:

$$\Delta CaX = (X)_T \left(\frac{Ca_1}{K_D + Ca_1} - \frac{Ca_2}{K_D + Ca_2}\right). \tag{5}$$

This can be further simplified to

$$\Delta CaX = (X)_T \cdot K_D \frac{\Delta Ca}{(K_D + Ca_1)(K_D + Ca_2)}. \tag{6}$$

The complete expression for an absorbance change due to a change in free calcium is therefore:

$$\Delta A = (\epsilon^{CaX} - \epsilon^X)(X)_T K_D L \cdot \frac{\Delta Ca}{(K_D + Ca_1)(K_D + Ca_2)}. \tag{7}$$

From Eq. 7 it can be seen that using in vitro calibrations to convert *in situ* absorbance measurements to ionized calcium does not require that either the Ca-dye dissociation constant or the differential extinction coefficients be known accurately, but it does assume that they are identical in the calibrating solution and in axoplasm (see further comment in discussion of errors). The use of in vitro calibrations does require correction for differences in path length and dye concentration, but the differences between these parameters for axons and glass capillaries was never >30% and usually <20%.

CALIBRATION PROCEDURES Calibration procedures for arsenazo III and antipyrylazo III were different. An in vitro calibration for arsenazo III was performed as follows: three aliquots of dye were made up in the calibrating solution. Each aliquot had a free calcium concentration defined by the addition of small volumes of concentrated Ca-EGTA buffers. The three concentrations were 0 Ca:4 mM EGTA; 150 nM free Ca:2 mM Ca, 4 mM EGTA; and 680 nM free Ca:3.2 mM Ca, 4 mM EGTA. The contaminant calcium concentration was <1% of the total and was ignored. Magnesium (3 mM) was added to the calibrating solution on the basis of the estimated free magnesium concentration in axoplasm (Brinley and Scarpa, 1975; De Weer, 1976). The added magnesium had a small effect on the free calcium concentration for which correction was made using a value of 30 mM for the Mg-EGTA dissociation constant at pH 7.3 and ionic strength 0.4 (Brinley et al., 1977a). These solutions were flushed through the glass capillary and the measured absorbances used to construct a calibration curve.

Occasional checks of the performance of the microspectrophotometer were made by repeating the calibration measurements on a Perkin-Elmer dual wavelength spectrophotometer (model 571, Perkin-Elmer Corp., Norwalk, Conn.), using a 1-mm cuvette and

assuming that the mean path length of the capillary tubes was the same as that of right circular cylinder, i.e. 0.91 times the diameter. The absorbance changes measured on the two instruments agreed within 10–15%.

Antipyrylazo III was calibrated with unbuffered standards because the useful range of this dye (0–50 μM free Ca) is too high for the usual calcium buffers. The contaminant calcium concentration of all reagents, including common inorganic chemicals such as KCl, was taken into account in determining the concentration of added calcium. Analyses were done by the method of standard additions, because the large amount of broad band absorption generated by concentrated salt solutions precludes direct comparison of calcium in concentrated salt solutions with standards made up in distilled water.

ESTIMATION OF ERRORS   The dissociation constant of the Ca-arsenazo III or Ca-antipyrylazo III complexes is about 15% greater in a solution of ionic strength 0.4 compared to 0.3, although the absolute spectra of the free and calcium-bound complexes are little affected by ionic strength.

Because uncertainty in ionic strength in axoplasm is about 0.1, the absolute calibrations could be in error by this amount. A more serious error may occur due to pH mismatch, because the dissociation constant changes by a factor of about 1.2–1.3 for each 0.1 change in pH. The pH of the calibrating solution and injected dye was set equal to 7.3 by buffering with KTES on the basis of a recent determination of pH in axoplasm (Boron and De Weer, 1976). A reasonable estimate of the error due to pH mismatch would be no more than 0.2 pH U, or an error in dissociation constant of about 50%. Two other sources of error, nonuniform distribution of dye and binding of dye to intracellular organelles, seem not to occur (Scarpa et al., 1978; Brinley et al., 1977 b; Brinley, 1978). The noise in the spectrophotometer was equivalent to an absorbance change of about 0.00005 ΔA, corresponding to a nominal free calcium concentration change in most experiments of about 2 nM for arsenazo III and about 10–20 nM for antipyrylazo III.

Although Beer's law implies proportionality between absorbance and concentration of the absorbing species at any concentration, proportionality between free calcium and bound dye concentrations occurs only when the free calcium is low, compared to the dissociation constant of the calcium-dye complex (e.g. 50 μM for arsenazo III and 250 μM for antipyrylazo III at ionic strength 0.4). The maximum ionized calcium concentrations measured with arsenazo III and antipyrylazo III were 5 and 50 μM, respectively. At these concentrations, the calibration curve relating free calcium and absorbance deviates from linearity no more than 10 or 17%, respectively.

Combining the various sources of error, we estimate the absolute measurement of free calcium could be in error by as much as a factor of two, most of the uncertainty being in the possible existence of a pH mismatch. Expressed in logarithmic units, the error in free calcium concentration in a fresh axon (50 nM, pCa = 7.5) is no more than 0.2–0.3 pCa U.

RESULTS

1. Steady-State Calcium Buffering Capacity of Axoplasm

Previous work with both aequorin and arsenazo III has demonstrated that when a calcium load is imposed on an axon, only a fraction appears as ionized calcium within the axoplasm (DiPolo et al., 1976; Requena et al., 1977; Brinley et al., 1977 b). In the present research this buffering capacity was investigated further. Fig. 1 indicates that the previously reported degree of buffering (Brinley et al., 1977 b), using arsenazo III as a calcium monitor, during calcium loading of an intact axon by electrical stimulation (0.6 nM increment in ionized calcium per

micromolar load), and the extent of metabolic independent buffering (roughly 95% of an imposed load) do not depend upon the dye used. In this set of two experiments both axons had been injected with antipyrylazo III. The solid line indicates the level of ionized calcium in an axon stimulated at 100 pulses/s in 112 mM Ca ASW. Stimulation was begun at the first vertical arrow and continued for 29 min. After an initial rise of about 0.5 μM, the ionized calcium rose at a steady rate of about 0.1 μM/min of stimulation. On the basis of previous data (Brinley et al., 1977b) that stimulation of squid axons at 100 pulses/s in-
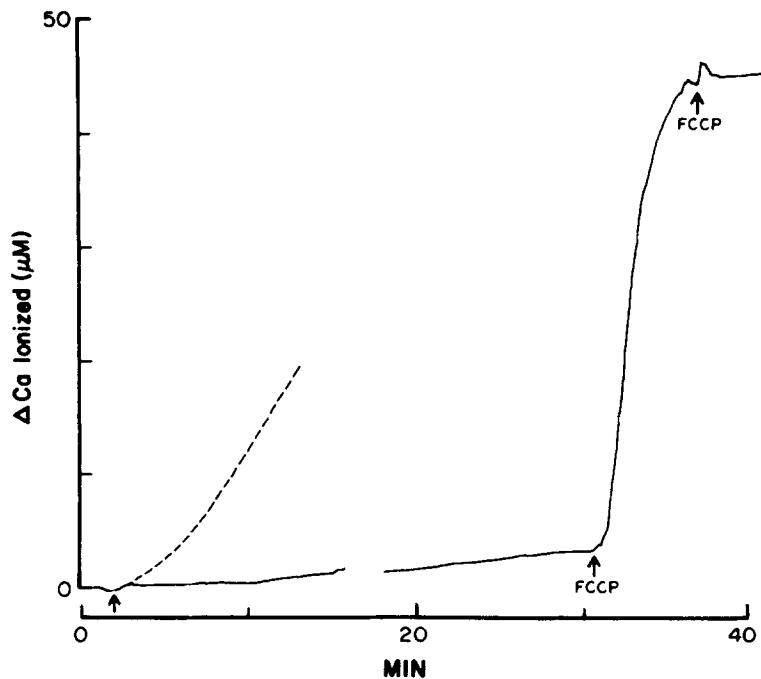
Figure 1. Rise in ionized calcium produced by stimulation at 100 pulses/s in 112 mM Ca seawater (ASW). Antipyrylazo III used as monitor of ionized calcium. First arrow indicates onset of stimulation in both experiments: axon in 2 mM CN-3 Ca ASW (- - -); axon in 3 Ca ASW (—). FCCP (5 μl of a solution of 2 mg FCCP/ml (DMSO) applied at second and third arrows.

creases the internal Ca approximately 50 μM/min, the observed rate of rise of free Ca in this experiment is approximately 2 nM/μM of internal load. At the end of the 29-min period of stimulation, at which time the axon contained a calculated calcium load of about 1,450 μM Ca, FCCP (5 μl of a 2 mg/ml DMSO solution) was placed on the surface of the axon with a microsyringe resulting in an abrupt rise in ionized calcium to a level of about 47 μM. This addition of FCCP was evidently maximal because a further addition had no effect other than to introduce a small optical artifact in the trace.

The conclusion of this experiment with antipyrylazo III as the calcium monitor is the same as earlier ones (Brinley et al., 1977b) obtained with arsenazo

III, i.e. only a few percent of the calculated calcium load appears as free calcium after what is thought to be complete inactivation of the specific calcium-accumulating properties of the mitochondria.

As reported previously (Brinley et al., 1977b), and considered in greater detail later in this paper, cyanide treatment during active loading of a fiber interferes with calcium buffering. This effect is shown in Fig. 1 (dashed line) by the much more rapid rise in ionized calcium during loading, as compared with the unpoisoned fiber.

Because continuous stimulation sets up steady-state radial concentration gradients within the fiber (Baker et al., 1971), one cannot specify exactly the ionized calcium concentration in the region of axoplasm from which mitochon-
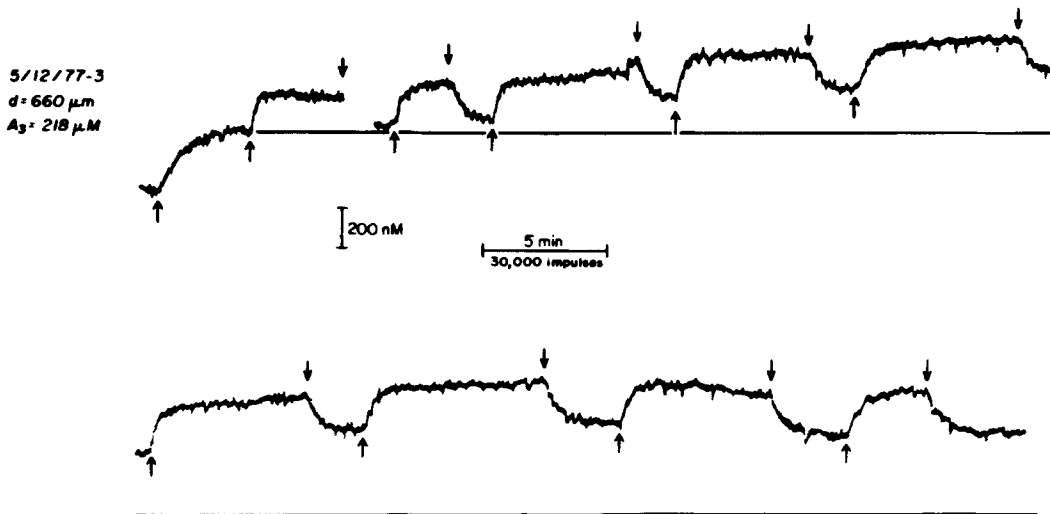
FIGURE 2.   Effect of intermittent stimulation on ionized calcium. Bottom trace is a continuation of top trace. Fiber immersed in 112 mM Ca ASW at first vertical arrow. Second and subsequent upward arrows indicate onset of stimulation at 100 pulses/s. Downward arrows indicate end of stimulation. Horizontal base line drawn to indicate level of ionized Ca in 112 mM Ca before stimulation. Axon was excitable throughout experiment but monophasic during last two periods of stimulation.

dria are taking up calcium. A variation of the above protocol is to interrupt stimulation at intervals, allow the calcium load to become uniformly distributed within the axoplasm, and then to measure steady-state levels of ionized calcium. Such a procedure not only allows calculation of the buffering capacity of axoplasm as a function of a quasi-steady-state internal ionized calcium, but also provides information on how the rate of mitochondrial calcium uptake varies with load, by looking at the time-course of recovery of ionized calcium after stimulation. Two experiments following this experimental strategy are shown in Figs. 2 and 3. The steady-state levels after each period of stimulation for these two and one additional experiment are plotted in Fig. 4 (bottom). The half time for recovery to the steady-state level also is plotted (Fig. 4, top). Because these fibers had been stimulated for variable periods before these records were taken,

the initial period of stimulation shown in Figs. 2 and 3 does not correspond to the first impulse that the fiber had conducted. The data show a rise of steady-state ionized calcium that is reasonably linear with the total number of impulses conducted. Although the load introduced into these fibers was near the maximum obtainable by the method of stimulation, because of ultimate conduction failure, i.e. 1.5–2.0 mmol/kg axoplasm, there is no indication that the buffering capacity of the intact axon was affected by the load. This conclusion is in accord with the result shown in Fig. 1 in which stimulation was continuous.

The slopes of the curves shown at the bottom of Fig. 4 afford another measure of the calculated buffering capacity of intact axoplasm. The slopes range from 0.06 to 0.2 nM increment in ionized calcium per micromolar load which is substantially less than that shown in Fig. 2 (2 nM) or that reported in an earlier paper (0.6 nM; Brinley et al., 1977 *b*). The difference might be due to the intermittent stimulation used in the present series. It is possible that during the
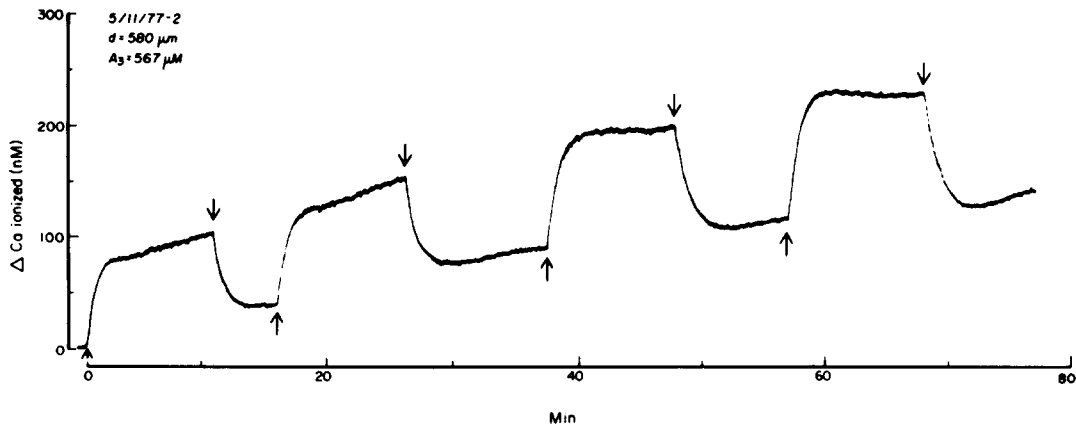

FIGURE 3. Experiment similar to Fig. 2, but showing continuous, slow upward drift in 112 mM Ca ASW during stimulation as well as interstimulation interval. Upward arrows indicate onset of stimulation at 100 pulses/s. Downward arrows indicate end of stimulation.

interstimulation intervals when calcium is being redistributed within the axon, the metabolically dependent buffer systems, presumably including mitochondria, may shift their load into a less labile compartment (see below, section 4). Such a circumstance could allow the sequestering machinery of the mitochondria to reprime itself, so that a subsequent period of stimulation occurs with the mitochondria in a less loaded condition than if the stimulation had been continued without interruption.

The fact that the buffering capacity of intact axons, whether measured by the intermittent or continuous stimulation method, is essentially constant for loads between 0–2 mM suggests that the buffering capacity of intact axoplasm is little affected by other changes such as those occurring in pH, sodium, or potassium content, which may be produced by stimulation.

The half time for recovery to the steady-state base line of ionized calcium after cessation of stimulation is plotted in the top part of Fig. 4, which shows that

the half time varies between 15–25 s, and is little affected by loads less than that equivalent to 150,000 impulses (approximately 1.2 mmol/kg axoplasm). Above this load, there is definite slowing of recovery in two experiments (open circles and x) and suggestion of an increase in half time for recovery in a third experiment (filled circles). This suggests that the sequestering process is less rapid as the axon approaches exhaustion, although this effect is not reflected in
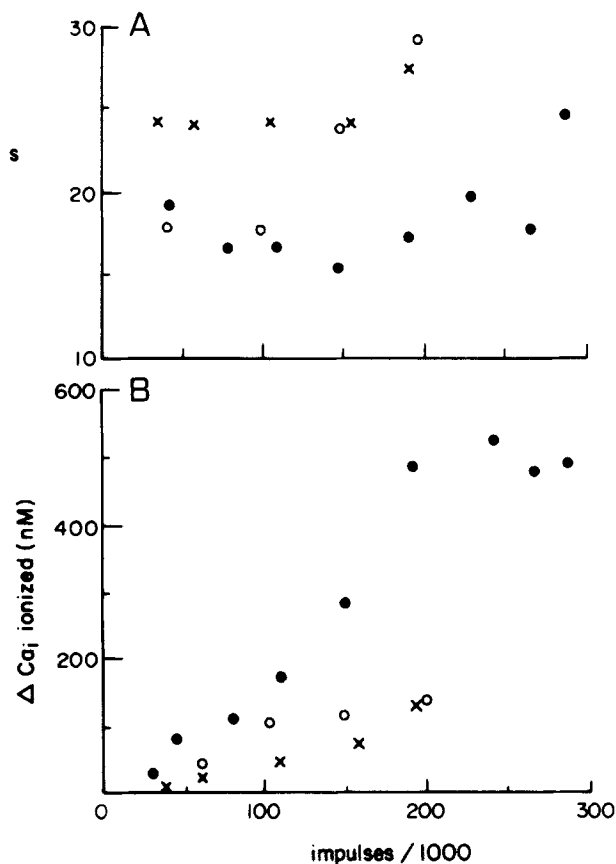

FIGURE 4. Collected data showing effect of intermittent stimulation on level of ionized calcium and half time for recovery from stimulation. Abscissa indicates the total number of impulses conducted by the fiber (irrespective of external solution). All axons were stimulated until they no longer responded 1:1 at 100 pulses/s. (A) Half time for recovery to base line after cessation of stimulation. (B) Level of ionized calcium after stimulation.

the steady-state level of ionized calcium that is ultimately reached after stimulation is stopped.

2. In Situ Kinetics of Calcium Uptake by Mitochondria: Physiological Levels of Ionized Calcium

The ability of the buffering systems in the axoplasm to respond to small calcium loads was tested as shown in Fig. 5. A fresh axon was given a series of small

calcium loads either by soaking or stimulating it in calcium containing seawaters. After loading, the bathing solution was changed to 0 Ca choline saline and the absorbance followed for about 10 min. The rationale behind the experiment is that the slow changes in absorbance after the loading period should reflect either mitochondrial uptake of calcium from the axoplasm or calcium extrusion by membrane pumps. Because the absorbance changes after loading are measured with the fiber in 0 Ca choline saline, the contribution of the calcium pump to the change in free calcium is minimized. However, the calcium pump is not completely blocked by sodium removal when the internal ionized calcium concentration is low (Blaustein and Russell, 1975; Brinley et al., 1975), therefore this method gives only an upper limit to the rate of mitochondrial uptake of calcium.
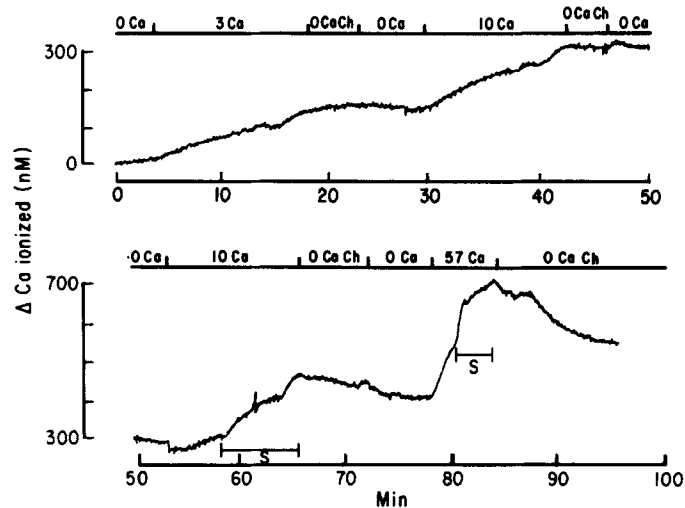

FIGURE 5. Continuous tracing, showing calcium buffering at small loads. Calcium loads were introduced by soaking fibers in either 3 or 10 mM Ca ASW or by stimulation in 10 or 57 mM Ca ASW. (Ordinate) Change of ionized calcium in axoplasm from the initial base line at beginning of experiment. Horizontal bars with "S" indicate stimulation at 100 impulses/s. Horizontal bars at top of trace indicate external solution; "Ch" indicates sodium replacement by choline.

The first loading period for the experiment shown in Fig. 5 occurred when the fiber was transferred from 0 to 3 Ca ASW. After the ionized calcium had increased about 150 nM, the fiber was transferred to 0 Ca choline, and then 0 Ca ASW. There was essentially no change in absorbance in these two solutions over the 10- to 15-min period in which absorbance was followed. From this finding it is concluded that the mitochondria were unable to buffer calcium (after the loading process ceased) at a detectable rate, at least over this time scale.

The fiber was then loaded with additional calcium by bathing the fiber in 10 Ca ASW until the free calcium rose to about 300 nM. The second recovery period in 0 Ca choline or 0 Ca ASW produced no clear change in absorbance. However, when the fiber was loaded to about 500 nM free Ca by stimulation in 10 Ca

ASW, there was a clear reduction of free calcium during the recovery period at a rate of about 4 nM/kg axoplasm per min. The final calcium load produced by stimulating the fiber in 57 mM Ca ASW raised the free calcium to about 650 nM, and resulted in reduction of free calcium at a rate of about 21 nM/kg axoplasm per min when the fiber was bathed in the recovery solution.

The results of this experiment and three others are collected in Fig. 6. The ordinate is the average rate at which ionized calcium is removed from the axoplasm during the period in 0 Ca choline saline. The abscissa represents the
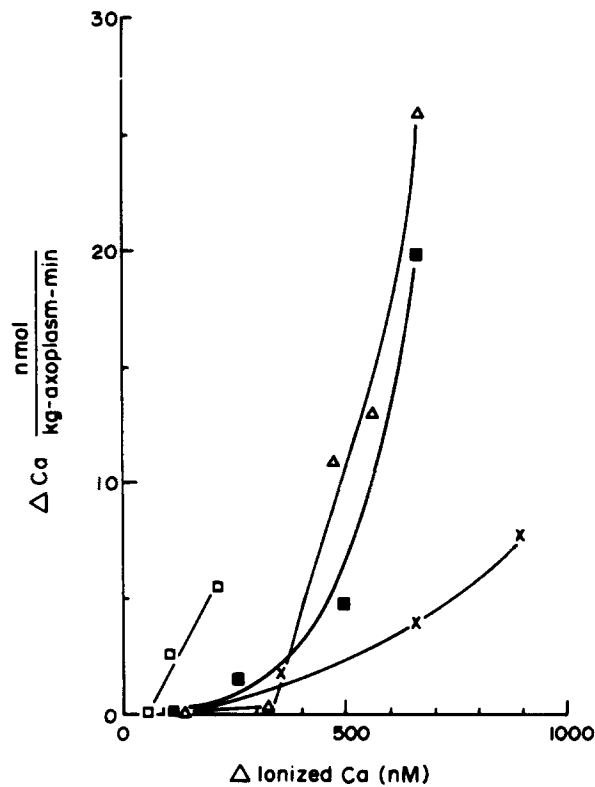
FIGURE 6. Collected results showing rate of uptake of free calcium from axoplasm of intact axons as a function of ionized concentration in axoplasm. Abscissa does not represent absolute level of ionized calcium but rather the increment in free calcium above initial level.

increment in ionized calcium, produced by the loading period, measured when the external solution was changed to 0 Ca choline. Several factors contribute to the considerable scatter in the rates. Because only the increment in calcium during the experiment was measured, and not the initial level, we cannot plot the experiments with respect to total axoplasmic ionized calcium, which is undoubtedly more important than the increment in determining buffering activity. Furthermore, as mentioned earlier, the observed rates of calcium buffering do not take account of extrusion by the membrane pump in 0 Ca choline, which can be variable at low ionized calcium concentrations. Despite

the ambiguity in the data, the rate of calcium buffering by presumed mitochondria is nearly zero until the ionized Ca concentration rises about 200–300 nM, at least over the time scale of these observations, i.e. 10 min. All the experiments show that the rate of uptake by the mitochondria increases rapidly with increase of ionized calcium above the threshold concentration.

3. In Situ Kinetics of Calcium Uptake by Mitochondria: High Concentrations of Free Calcium

The experiments described in the previous section are only feasible if the rate of calcium uptake by the presumed mitochondria is relatively slow compared with the rate of loading, or of calcium redistribution within the axoplasm after loading. To measure rates of uptake at high calcium concentrations, a different protocol was used based on the fact that in the absence of axoplasmic ATP, mitochondrial uptake of calcium depends mostly upon oxidation of endogenous substrate. Therefore, the ability of mitochondria to release or take up calcium could be controlled simply by adding or removing cyanide from the bathing solution surrounding the axon. Fig. 7 shows three experiments in which this was done. The axons were first injected with apyrase to give a final concentration of ~0.5 g/liter axoplasm. This enzyme, a nonspecific ATPase, produces a drastic reduction in ATP concentration of injected fibers. Judging from the results of DeWeer (1970), who measured the effect of injecting enzyme on sodium outflux of squid axons, the residual ATP concentration in treated fibers is around 50 μM, compared to an initial level of about 4 mM.

In the experiment shown in the top panel of Fig. 7, after 30 min of preincubation after apyrase injection, the fiber was exposed for a few minutes to 0 Ca ASW to give a base line from which increments in ionized calcium could be measured. The fiber was then exposed to 0 Ca ASW containing 2 mM CN. This procedure caused an immediate release of endogenous calcium from the mitochondria and the ionized Ca rose to about 0.8 μM. The fiber was then exposed to 10 Ca ASW-CN to load the fiber and raise the ionized Ca to about 1.3 μM. After the loading period in 10 Ca ASW-CN, the fiber was exposed to 0 Ca ASW-CN. The existence of a steady base line in this solution indicated that there had been internal equilibration of calcium throughout the cytoplasm and that there were no radial concentration gradients. The fiber was then exposed to 0 Ca ASW free of CN. The ionized calcium decreased abruptly as calcium was taken up, presumably by mitochondria. The maximum slope of the uptake curve was taken as the rate of mitochondrial sequestration of calcium at a particular initial uniform level of ionized calcium throughout the axoplasm. The slope should not reflect internal redistribution of calcium within the cytoplasm inasmuch as the mitochondria are uniformly distributed through the axoplasm, although local concentration gradients may develop around individual mitochondria.

This identification of slope with the rate of mitochondrial uptake takes no account of efflux of calcium across the axolemma energized by ATP generated when cyanide is removed (DiPolo, 1977; Blaustein, 1977). Such net extrusion is probably rather small for two reasons: (a) the injected apyrase keeps the mean concentration of ATP in the cytoplasm low; (b) there is some evidence that ATP

5/28/77-2
d=490 μm
A₃=387 μM

6/1/77-1
d=530 μm
Aₚ=354 μM

5/19/77-2B
d=650 μm
A₃=320 μM
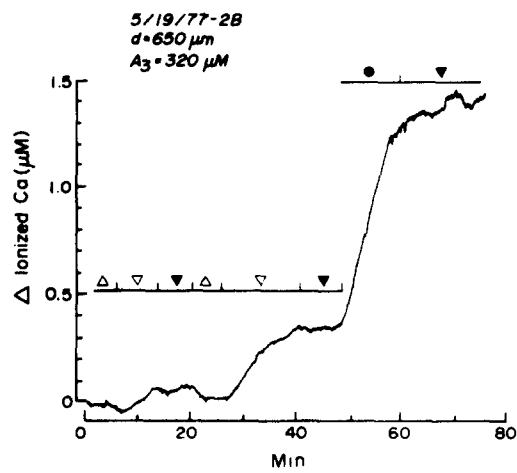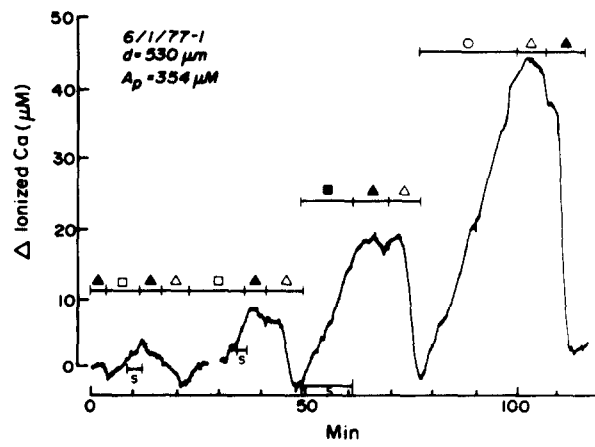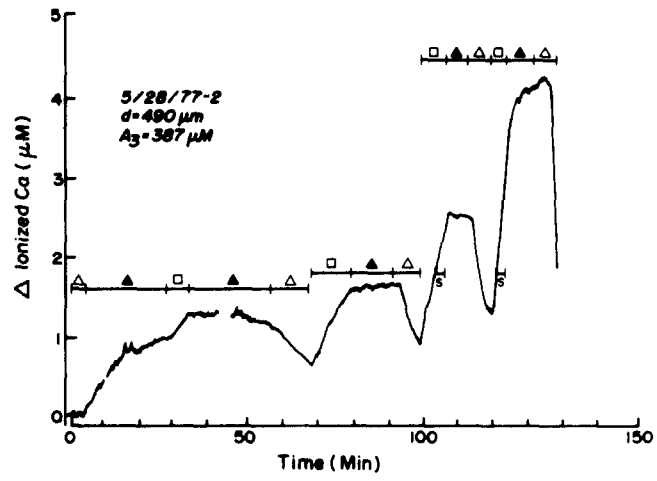
increases calcium influx as well as efflux[1] so the effect of ATP upon net extrusion could be less than the effect upon efflux alone.

The loading procedure was repeated by subjecting the fiber to a second exposure to 10 Ca ASW-CN, which released the calcium taken up by the mitochondria in the first loading and also added an additional load, resulting in a new level of ionized calcium higher than the initial one. The external solution was then changed once again to 0 Ca ASW-CN to obtain steady base line and the fiber exposed to 0 Ca ASW without CN, allowing uptake of calcium by the presumed mitochondria. To obtain even higher loading within a reasonable amount of time, during the third and fourth loading periods, the fiber was stimulated at 100 impulses/s for several minutes. This procedure resulted in a substantial increase in ionized calcium and is reflected in a progressive increase in the maximum slope of uptake during each recovery period in 0 Ca ASW without CN.

The middle panel in Fig. 7 illustrates a protocol similar to that shown in the top except that higher loads were imposed upon the axon with a subsequently greater rise in ionized calcium. In this fiber, because of the high ionized calcium concentrations, antipyrylazo III was used to monitor free calcium. The first and second loading periods were in 10 mM Ca ASW-CN with stimulation at 100/s. The third loading period was in 50 mM Ca ASW-CN with stimulation and the final period was in 112 mM Ca ASW-CN. Increasing the load upon the fiber obviously increased the rate of subsequent mitochondrial calcium uptake.

To confirm that the results shown in the top and middle panels of Fig. 7 resulted from mitochondrial action, the experiment shown in the bottom of Fig. 7 was performed. In this experiment the fiber not only was preinjected with apyrase, but also treated with a slow superfusion of FCCP during the entire experiment to inhibit calcium uptake by mitochondria. The experiment shows there was no reduction of ionized calcium after a change in bathing solution to 0 Ca choline without CN, even though the ionized Ca rose as high as 1.5 μM. In contrast (Fig. 7, top), when mitochondrial inhibition was released by removing cyanide, there was an abrupt drop in free calcium. The conclusion from these experiments is that the decrease in ionized Ca seen in 0 Ca choline ASW, after a fiber had received an exogenous load, was due to sequestration by cellular constituents that are sensitive to either cyanide or FCCP, and are presumably mitochondria.

The rates obtained directly from data such as that shown in Fig. 7 represent uptake of ionized calcium from the axoplasm. The following considerations allow these rates to be converted into uptake per kilogram of mitochondrial protein assuming that only mitochondria are responsible for calcium removal

[1] Mullins, L. J., and R. F. Abercrombie. Unpublished observations.

FIGURE 7 (*opposite*). Buffering of exogenous calcium load in apyrase-injected axons. (Ordinate) Increment in ionized calcium produced by loading. Stimulation at 100 pulses/s indicated by horizontal bars labeled "S." Symbols for external solutions: ($\triangle$) 0 Ca ASW; (∇) 3 Ca ASW; ($\blacktriangle$) 0 Ca ASW-2 mM CN; ($\square$) 10 Ca ASW-2 mM CN; ($\blacksquare$) 50 Ca ASW-2 mM CN; ($\bigcirc$) 100 Ca ASW-2 mM CN; ($\bullet$) 10 Ca ASW; ($\blacktriangledown$) 0 Ca choline ASW.

from axoplasm. In an earlier paper (Brinley et al., 1977b), it was estimated that in FCCP-treated axons, the ionized Ca was about 5% of the total load, but when mitochondria were active, only 0.5% or less appeared as ionized (see also Figs. 1–3). We assume that when the mitochondria are suddenly activated, they pull calcium not only out of the ionized component in the axoplasm, but also out of other buffers which, in the poisoned state, take up about 95% of an imposed load. Therefore, the rate of uptake in Fig. 7 represents roughly 20 times more calcium being extracted from axoplasm than appears from the reduction in ionized calcium. Inasmuch as mitochondria are only 1% of axoplasm volume (DiPolo, 1977), the uptake expressed in kilograms of mitochondria is 20 × 100 = 2,000 times the observed rate. An additional multiplicative factor of 3.43 converts kilograms of mitochondria to kilograms of mitochondrial protein (Brinley et al., 1977b). The overall multiplier to convert observed rates of disappearance from the axoplasm into uptake per kilogram mitochondrial protein is, therefore, 6,860. The rate of calcium uptake by presumed mitochondria shown in Fig. 7, together with two other experiments, is replotted in Fig. 8, showing rate as a function of increment in ionized calcium. Because the initial ionized calcium was only of the order of 50 nM (DiPolo et al., 1976), the increments plotted on the abscissa are essentially the ionized calcium concentration in the axoplasm. The data, therefore, can be taken as indicating the dependence of mitochondrial uptake on ambient ionized calcium in the axoplasm from 100 nM to 50 μM. The rates are plotted as observed rate of uptake of ionized calcium from the axoplasma (left ordinate) or as calculated uptake by mitochondria (right ordinate). Greater loading to achieve ionized calcium concentrations above 50 μM was not attempted, because axons tended to become inexcitable when loaded heavily with calcium in the presence of apyrase and cyanide. The rate of calcium uptake does not show a plateau at the highest
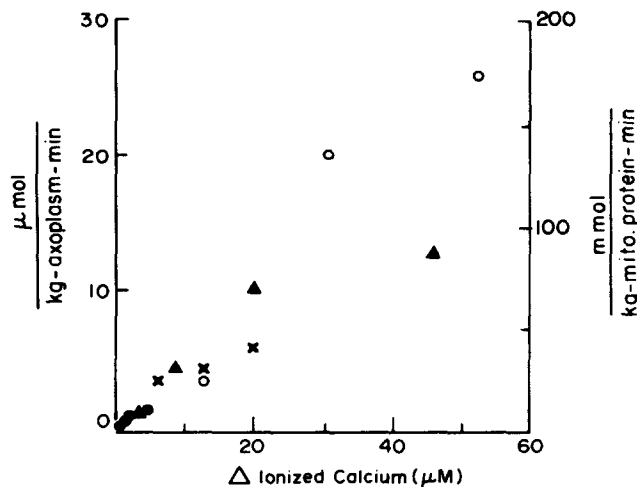

FIGURE 8. Collected results showing rate of uptake of calcium from axoplasm as a function of increment of free calcium from initial level. (Left ordinate) Measured uptake of ionized calcium from axoplasm. (Right ordinate) Uptake of calcium by presumed mitochondria calculated as described in text.

ionized calcium concentrations used, although the two experiments with the highest loading show that the rate of uptake increases less than proportionally above 10–20 μM ionized Ca. The significance of the results are considered in the Discussion.

4. Respiration-Dependent Uptake of Calcium by Mitochondria

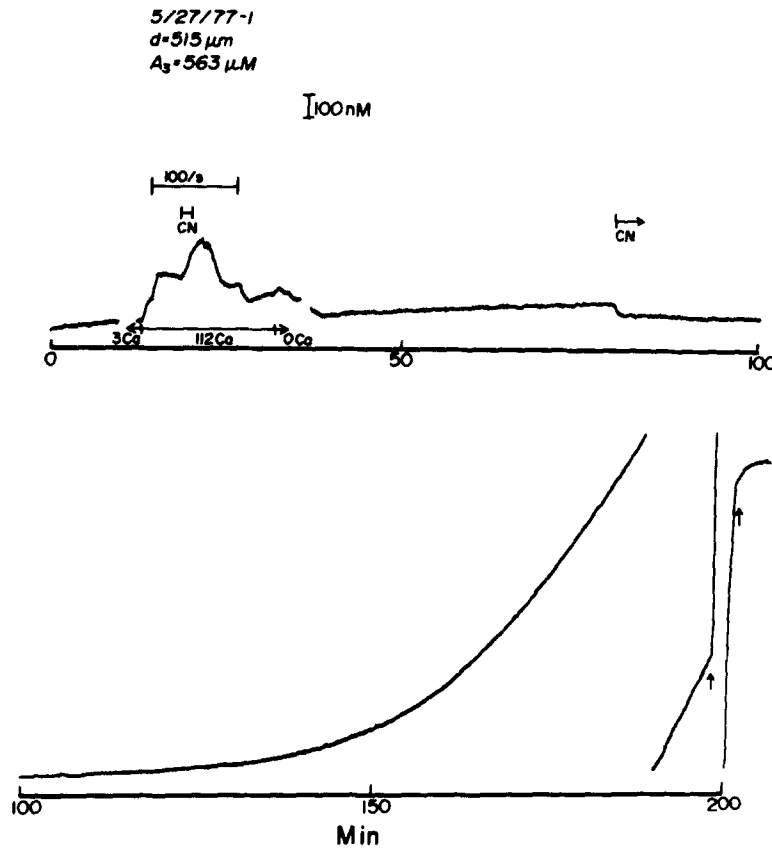This section can be introduced by considering the experiment in Fig. 9. This

FIGURE 9.   Effect of cyanide upon ionized calcium in an axon at rest and during stimulation. Traces are continuous. Application of cyanide during stimulation (top trace) produces immediate increment in ionized calcium. Application of cyanide after stimulation (end of top trace and entire bottom trace) produces only a delayed increase. FCCP applied at vertical arrows to effect complete release. Discontinuities at end of bottom trace result from resetting pen recorder.

experiment shows that the effect of cyanide upon the free ion concentration depends upon whether or not the axon is taking up calcium rapidly. The experiment began with the fiber in 3 mM Ca ASW to obtain a base line. The fiber was then transferred to 112 mM Ca ASW and shortly thereafter loading by stimulation at 100 pulses/s was begun. After a new steady base line had been reached, the fiber was exposed for approximately 1 min to a saline containing 112 mM Ca plus 2 mM CN while stimulation continued. The ionized calcium rose abruptly

when the axon was in this solution and returned to base line after cyanide was removed. The cyanide was not applied long enough for the ionized calcium to reach a new steady state because the purpose of the experiment was simply to show that the level of ionized calcium was immediately sensitive to cyanide under these conditions.

When the fiber had recovered from the brief exposure to cyanide, stimulation was ended and the 112 mM Ca ASW replaced by 0 Ca ASW. The fiber remained in 0 Ca for about 40 min at which time the solution was replaced by one containing 2 mM CN. In contrast to the first exposure and in accordance with previous reports (Baker et al., 1971; DiPolo et al., 1976), the second application of cyanide produced no immediate effect on ionized calcium. However, after 30–40 min, the level of ionized calcium began to rise. The vertical arrows at the end of the trace indicate the application of FCCP which produced a large increase in free ion.

Because the metallochromic indicators are not perfectly selective for calcium, the rise in ionized calcium during prolonged cyanide poisoning might be distorted by a rise in ionized magnesium which occurs during the initial stages of cyanide poisoning. However, this effect is probably small. An earlier report (DiPolo et al., 1976, Fig. 25) showed that the release of magnesium (as measured with Eriochrome blue SE) was nearly complete 70–80 min after onset of cyanide poisoning, in accordance with direct analysis of ATP content of poisoned axons by Caldwell (1960). Release of magnesium, therefore, should have been virtually complete by 160 min after the start of the experiment, although, as Fig. 9 shows, the rise in ionized calcium was just beginning.

The simplest conclusion to draw from the two different effects of CN shown in this experiment is that the process of sequestering a load as it is being applied requires respiration, but that the retention of a previously administered load requires only the presence of axoplasmic ATP. This conclusion suggests two possible consequences: (a) there may be some maximum rate of loading of the axon at which the mitochondria can just maintain the process of sequestration without respiration; (b) after a load has been applied to the mitochondria during stimulation, a finite amount of time may be required for the mitochondria to transform the sequestered calcium into a form in which it can be retained within the mitochondria by ATP alone. These considerations formed the rationale for experiments described in the following paragraphs.

RESPIRATION-INDEPENDENT CALCIUM UPTAKE   Fig. 10 illustrates an experiment to determine the maximum rate at which calcium can be sequestered in the absence of respiration. The fiber was exposed successively to ASW containing increasing amounts of calcium, as indicated by the numbers on the left side of the traces. After a steady base line had been reached in each of the saline solutions, the fiber was exposed to 2 mM CN during the period indicated by the horizontal bars over the traces. The dotted line above the 3 mM Ca ASW trace shows the effect upon the base line of simply switching the inflow lines without introducing cyanide. The apparent rise seen in the traces for 3 and 10 mM Ca ASW during cyanide treatment is probably artifactual due to switching transients in the flow lines. However, brief exposure to cyanide when the fiber is

immersed in 20 mM Ca ASW or 37 mM Ca ASW definitely produces an immediate rise in ionized calcium.

The conclusion from the experiment is that a load imposed by soaking fibers in 10 Ca ASW can probably be buffered by the axon in the absence of respiration. Inasmuch as direct analytical measurement of the calcium content of fibers soaked in 10 Ca ASW gave an uptake of 0.3 μmol/kg per min (Requena et al., 1977), the traces in Fig. 10 show that this rate is nearly the maximum at which calcium can be sequestered *in situ* without functioning mitochondria. Two additional experiments gave the same result. Longer exposure to cyanide might have revealed respiration-independent buffering at a lower loading rate, but the interpretation of the absorbance traces of such experiments would have
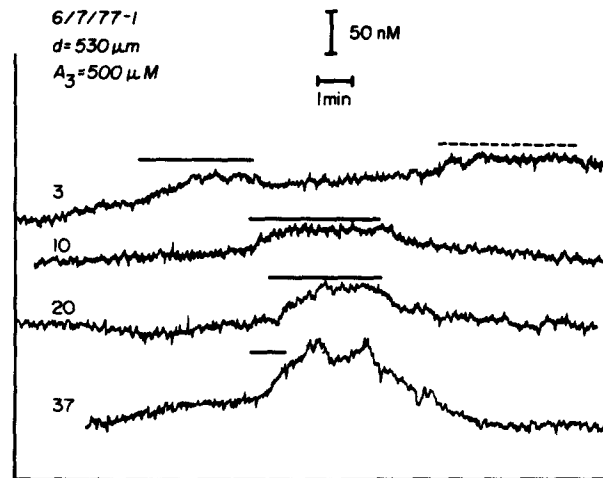

FIGURE 10. Effect of cyanide pulses on ionized calcium concentration. Solid horizontal bars indicate application of 2 mM CN to fiber. Dotted horizontal bar indicates change of flow system from one flow channel to a second flow channel to evaluate "switching" artifact. Numbers on left side indicate calcium concentration in the ASW (mM).

been complicated by the fact that exposure of fibers to cyanide longer than a few minutes is associated with some ATP breakdown and changes in free magnesium and pH.

The experiment described in this section used arsenazo III as a null point indicator and relied upon analytical measurements of changes in total calcium content to give the rate of uptake. The result, therefore, is not influenced by uncertainties which may arise when calibrating absorbance changes in terms of ionized calcium.

The probable maximum rate of calcium buffering in the absence of functioning mitochondria (about 0.3 μmol/kg per min) explains why, in experiments such as those illustrated in Fig. 9, a load of 50 μM/min being imposed by stimulation (in the presence of cyanide) cannot be buffered. This result is

consistent with data presented earlier (Fig. 6) indicating that the threshold concentration for calcium uptake by mitochondria was 200–300 nM. In the experiments shown in Fig. 10, the small loads produced by soaking axons in 10 mM Ca ASW incremented the free Ca by only 50–100 nM, which is less than the threshold concentration, and therefore should not have triggered any mitochondrial (i.e. cyanide-sensitive) uptake of calcium.

TRANSFORMATION OF SEQUESTERED CALCIUM TO RETAINED FORM   To investigate whether calcium can be converted from the form in which it is initially sequestered to a form less sensitive to respiration, experiments such as that illustrated in Fig. 11 were performed. The fiber was loaded with approximately 1,100 μmol Ca/kg axoplasm by stimulation at 100 pulses/s in 112 mM Ca ASW. The fiber was then transferred to a 0 Ca-choline ASW to minimize calcium extrusion by the fiber. At intervals thereafter the sensitivity of the buffer systems to respiration was tested by exposing the fiber, at the times indicated by the vertical arrows, to 10-s pulses of 0 Ca-choline ASW containing 2 mM CN. Sensitivity was high for several minutes after the end of the loading process and
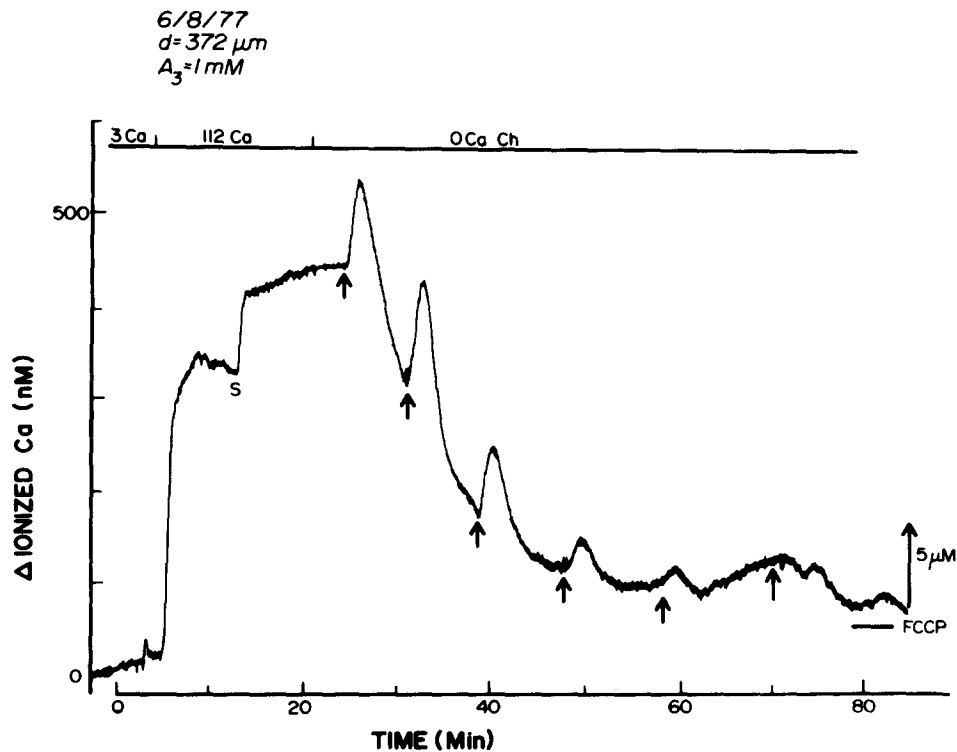

FIGURE 11. Effect upon ionized calcium of cyanide pulses applied after stimulation. "S" indicates onset of stimulation at 100 pulses/s. Stimulation terminated approximately when solution changed to 0 Ca choline ASW. Vertical arrows indicate application of 10-s pulses of CN. Horizontal bar at end of experiment indicates 4-min application of CN. FCCP, applied at last arrow, increased ionized Ca about 5 μM.

then declined progressively until it virtually disappeared at the end of some 40 min. Even though the sequestered calcium had evidently been transformed into a state not sensitive to 10-s pulses of CN, some cyanide sensitivity did persist as indicated by the horizontal bar which marks the slight rise in ionized calcium after a 4-min exposure to CN. It is doubtful that this release was secondary to a significant decrease in ATP concentration in the axoplasm, since breakdown begins about 10–20 min after exposure to CN (Caldwell, 1960; DiPolo et al., 1976). It may be, however, that the retained form of mitochondrial calcium is sensitive to the axoplasmic ATP/ADP ratio because this is affected by cyanide before the absolute level of ATP drops.

Retention of the calcium, in the cyanide-insensitive form, still requires metabolic energy as shown by the final procedure in the experiment of Fig. 11. The fiber was tested with FCCP, producing a rise in ionized calcium to about 5 μM. Although the existence of a hypothetical nonmitochondrial compartment sensitive to FCCP but not to cyanide has not been ruled out, the data suggest that the calcium sequestered in the cyanide-insensitive form is held within mitochondria.

The results of this experiment and three others with varying amounts of initial calcium load are shown in Fig. 12. The half time for conversion of calcium from the sequestered to retained form is about 3–10 min. There is some suggestion from the data that heavier loads are converted more slowly. The half time is probably faster than illustrated in Fig. 12, because the pulses of cyanide produced an increase in ionized calcium which persisted far longer than the pulse itself, presumably because of slow diffusion of cyanide through the connective tissue and axoplasm.

The effect of cyanide in the early stages of the sequestration to retention process must be very rapid because clear interference with the reaction could be
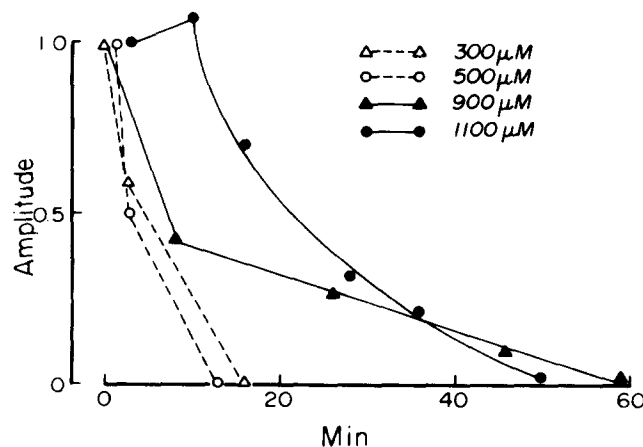

FIGURE 12. Composite of four experiments showing time-course of peak response to cyanide pulses applied after cessation of stimulation. (Abscissa) Time after end of stimulation. (Ordinate) Normalized peak response. Numbers beside symbols indicate the calculated load in axon at end of stimulation.

seen after a 5-s exposure to cyanide. Calculations indicate that diffusion of cyanide from the surface of the fiber to the interior would raise the mean axoplasmic concentration above the minimum required for complete inhibition of the cytochrome reactions (100 μM; Doane, 1967), within 2–3 s. Even allowing for a generous delay because of slow mixing in the flow stream and diffusion through connective tissue around the fiber, it is difficult to escape the conclusion that loss of sequestering ability follows interference with respiration with a delay of no more than 1–2 s.

DISCUSSION

1. Comparison of Rates of Calcium Uptake by Mitochondria

Kinetic measurements of calcium uptake by FCCP- or cyanide-sensitive structures, e.g. Fig. 8, indicate a value of approximately 1 μmol free Ca/kg axoplasm per min removed from axoplasm, or 20 μmol/kg axoplasm of total Ca, taken up by presumed mitochondria at an ambient ionized Ca of about 1–3 μM. This result implies that the metabolically dependent buffers in intact axon should be able to buffer effectively a load of this magnitude if it is imposed upon an intracellular free Ca concentration of 1–3 μM.

Such a circumstance is approximately realized when a squid axon is stimulated for long periods in 112 mM Ca ASW. After an initial rise of 1–3 μM (Brinley et al., 1977b; Fig. 2), the ionized calcium is effectively buffered for the rest of the period of stimulation, implying that the mitochondrial and nonmitochondrial buffers are absorbing virtually the entire load. The rate of axoplasm loading under these circumstances has been estimated from direct analysis and tracer measurements to be 50 μmol/kg axoplasm per min. Because previous estimates of partitioning of calcium into the mitochondrial and nonmitochondrial buffers (Brinley et al., 1977b) indicate that only about one-third goes into the mitochondrial buffer, the amount entering this organelle is about 15 μmol/kg axoplasm per min, compared to the rate estimated above at which structures presumed to be mitochondria take up calcium at a comparable level of ionized calcium, i.e. 20 μmol/kg axoplasm per min.

2. Comparison of Kinetic Parameters for Calcium Uptake by Squid Mitochondria with Other Tissues

The data from the eight experiments shown in Figs. 6 and 8 are replotted on a double logarithmic scale in Fig. 13 to show the relation between calculated rate of calcium uptake by mitochondria (expressed as millimoles per kilogram mitochondrial protein per minute) and ambient ionized calcium, over the range 100 nM to 50 μM. Below 200 nM, the mitochondria take up calcium at a rate too low for us to measure and the rate is given as zero.

Despite the fact that the absolute rates and ionized calcium concentrations are subject to considerable error, and the methods of determining rates at high and low ionized calcium were completely different, the points from the two sets of experiments are consistent with a smooth increase in rate of calcium uptake over a range of nearly 3 log units, with indications of sigmoidal relations at either end of the concentration range.

Comparing the *in situ* rates of calcium uptake by squid mitochondria with the results of in vitro experiments using mitochondria from other species is somewhat difficult, because squid axoplasm has a higher ionic strength (about 0.4 M) and a higher free magnesium concentration (2-3 mM) (Brinley and Scarpa, 1975; DeWeer, 1976) compared to vertebrate cytoplasm. Furthermore, the physiological temperature for these experiments (17°C) is considerably lower than the temperature of most in vitro experiments, i.e. about 26°C.

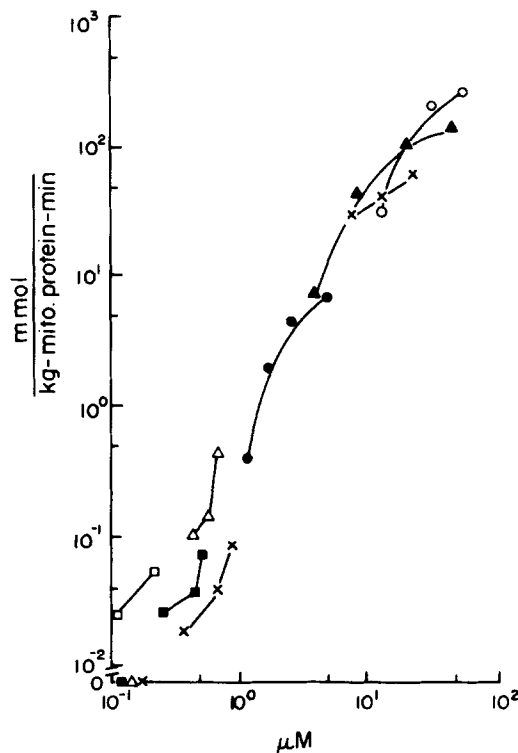However, to the extent that comparison can be made, mitochondrial calcium

FIGURE 13.   Double logarithmic plot of data from Figs. 6 and 8 showing calculated rate of calcium uptake by presumed mitochondria as a function of initial ionized calcium. Logarithmic compression makes sigmoidicity of the curve less obvious than linear plot.

uptake at high ionized calcium in squid axons, *in situ*, appears to be about the same as or somewhat higher than in vitro uptake by mitochondria isolated from other species. The *in situ* rate for squid at 17°C is about 200 mmol of Ca/kg mitochondrial protein per min at an ambient ionized Ca of 50 μM. Assuming a Q_{10} of 3, this corresponds to an uptake of about 500 mmol/kg mitochondrial protein per min at 26°C, the temperature in which in vitro studies have been done. Measurement of in vitro rates at 50 μM ionized Ca range from 180 mmol/kg protein per min in rat heart or liver (Scarpa and Graziotti, 1973; Vinogradov and Scarpa, 1973) to a low of 40 mmol/kg protein per min, calculated from the

data of Sordahl (1974) using his figures for the effect of magnesium on maximum rate.

Rates of uptake at levels of 50 μM ionized Ca are of little physiological relevance, because the ionized calcium rises to such levels only under extreme conditions of loading. However, the reasonable concordance between *in situ* and in vitro rates of calcium uptake, at free calcium concentrations where the two sets of data can be directly compared, may provide some independent support for the reliability of the measurements at physiological concentrations of ionized calcium, i.e. 50 nM range, where there are no in vitro data for direct comparison.

The data reported in this paper indicate that uptake of calcium by mitochondria occurs very slowly below 200–300 nM. Because it is doubtful that physiological perturbations of calcium concentration, e.g. as might occur after nervous impulse conduction, ever raise the free calcium to that level in the center of the fiber, the clear implication of the present results is that mitochondria are not important buffers for rapid fluctuations in calcium concentration in the physiological range. Physiological loads of calcium can, however, be extruded by the axolemma which shows sensitivity to free calcium, both in the presence and absence of ATP, down to levels of 10 nM (Blaustein and Russell, 1975; Brinley et al., 1975; DiPolo, 1977).

Our conclusion regarding the limited ability of mitochondria to buffer physiological variations in free calcium, applies only to rapid changes which may occur with a time-course of seconds or minutes. It need not apply to long-term changes in free calcium which could result, for example, from aging of the fiber or changes in content of energy substrates, over a period of hours.

3. Sequestration — Retention Transient

Fig. 14 summarizes formation presented in this and previous studies illustrating some steps in the buffering of an exogenous calcium load. About $^2/_3$ of the calcium load is buffered by a process (the X buffer) that apparently does not involve directly metabolic energy, or at least is insensitive to combinations of FCCP, apyrase injection, and cyanide treatment. The remaining $^1/_3$ of such a load appears to enter a metabolically labile buffer (the M buffer) which is probably the mitochondria (Brinley et al., 1977 b). The process that moves calcium from the axoplasm into the M buffer system requires respiratory substrate or energy derived from the respiratory chain (i.e. it is cyanide sensitive). From this state the calcium moves, by a process with a half time of about 3–10 min, into another state in which it is no longer immediately sensitive to cyanide. In this intermediate state, the calcium is still retained within the mitochondria by metabolic energy, because uncoupling by FCCP produces an immediate massive release of calcium.

The diagram also indicates another step in the intramitochondrial metabolism of calcium in which the calcium is transformed from a state that is FCCP sensitive to an apparently metabolically stable state. The existence of such a state, after exogenous loading, is implicit in the data of an earlier paper (Brinley et al., 1977 b) in which it was noted that FCCP treatment of squid axons after long periods of stimulation seemed to release calcium in amounts (even after

correcting for the amount taken up by the X buffer system after release) that were substantially smaller than the calculated exogenous load introduced by such stimulation. The tentative conclusion reached in that paper was that there must be a substantial amount of calcium in a form that was not immediately or readily released by the FCCP treatment. There is presently no evidence to indicate the form of such calcium or the kinetics of transformation from the intermediate state to the stable state.
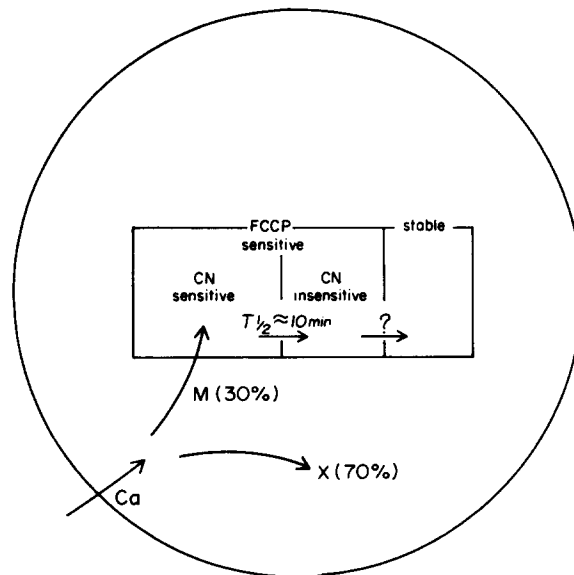

FIGURE 14. Diagram of calcium metabolism in squid mitochondria summarizing experimental findings reported in this and a previous paper (Brinley et al., 1977 *b*). Calcium entering the axoplasm either by stimulation or soaking in sodium-free solutions is distributed between a metabolic-insensitive buffer (X) and a metabolic-labile buffer (M) presumed to be contained in the mitochondria. Diagram illustrates successive states in the sequestration process as calcium moves first into a cyanide-sensitive state, then to a cyanide-insensitive state with half time of about 10 min, and finally into a state where retention does not depend directly upon metabolism.

REFERENCES

ALLEN, D. G., J. R. BLINKS, and F. G. PRENDERGAST. 1976. Aequorin luminescence: relation of light emission to calcium concentration—a calcium-independent compo-

nent. *Science* (*Wash. D. C.*). **195**:996–998.

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

BLAUSTEIN, M. P. 1977. Effects of internal and external cations and of ATP on Na-Ca and Ca-Ca exchange in squid axons. *Biophys. J.* **20**:79–111.

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

BLAUSTEIN, M. P., and J. M. RUSSELL. 1975. Sodium-calcium exchange and calcium-calcium exchange in internally dialyzed squid giant axons. *J. Membr. Biol.* **22**:285–312.

BORON, W. F., and P. DE WEER. 1976. Intracellular pH transients in squid giant axons caused by CO_2, NH_3 and metabolic inhibitors. *J. Gen. Physiol.* **67**:91–112.

BRINLEY, F. J., JR. 1978. Calcium buffering in squid axons. *Annu. Rev. Biophys. Bioeng.* **7**:363–392.

BRINLEY, F. J., JR., and A. SCARPA. 1975. Ionized magnesium concentration in axoplasm of dialyzed squid axons. *FEBS* (*Fed. Eur. Biochem. Soc.*) *Lett.* **50**:82–85.

BRINELY, F. J., JR., A. SCARPA, and T. TIFFERT. 1977a. The concentration of ionized magnesium in barnacle muscle fibers. *J. Physiol.* (*Lond.*). **266**:545–565.

BRINLEY, F. J., JR., S. G. SPANGLER, and L. J. MULLINS. 1975. Calcium and EDTA fluxes in dialyzed squid axons. *J. Gen. Physiol.* **66**:223–250.

BRINLEY, F. J., JR., T. TIFFERT, A. SCARPA, and L. J. MULLINS. 1977b. Intracellular calcium buffering capacity in isolated squid axons. *J. Gen. Physiol.* **70**:355–384.

CALDWELL, P. C. 1960. The phosphorus metabolism of squid axons and its relationship to the active transport of sodium. *J. Physiol.* (*Lond.*). **152**:545–560.

DE WEER, P. 1976. Axoplasmic free magnesium levels and magnesium extrusion from squid giant axons. *J. Gen. Physiol.* **68**:159–178.

DE WEER, P. 1970. Effects of intracellular adenosine-5'-diphosphate and orthophosphate on the sensitivity of sodium efflux from squid axons to external sodium and potassium. *J. Gen. Physiol.* **56**:583–620.

DIPOLO, R. 1973. Calcium efflux from internally dialyzed squid giant axons. *J. Gen. Physiol.* **62**:575–589.

DIPOLO, R. 1977. Characterization of the ATP-dependent calcium efflux in dialyzed squid axons. *J. Gen. Physiol.* **69**:795–813.

DIPOLO, R. J. REQUENA, F. J. BRINLEY, JR., L. J. MULLINS, A. SCARPA, and T. TIFFERT. 1976. Ionized calcium concentrations in squid axons. *J. Gen. Physiol.* **67**:433–467.

DOANE, M. G. 1967. Fluorometric measurement of pyridine nucleotide reduction in the giant axon of the squid. *J. Gen. Physiol.* **50**:2603–2632.

FLASHKA, H., and A. J. BARNARD, JR. 1969. Chelates in Analytical Chemistry. Vol. 2. Marcel Dekker, Inc., New York.

REQUENA, J., R. DIPOLO, F. J. BRINLEY, JR., and L. J. MULLINS. 1977. The control of ionized calcium in squid axons. *J. Gen. Physiol.* **70**:329–353.

SCARPA, A., F. J. BRINLEY, JR., and G. DUBYAK. 1978. Antipyrylazo III: A middle range calcium metallochromic indicator. *Biochemistry.* **17**:1378–1386.

SCARPA, A., and P. GRAZIOTTI. 1973. Mechanisms for intracellular calcium regulation in heart. I. Stopped-flow measurements of Ca^{++} uptake by cardiac mitochondria. *J. Gen. Physiol.* **62**:756–772.

SORDAHL, L. A. 1974. Effects of magnesium, ruthenium red and the antibiotic ionophore A-23187 on initial rates of calcium uptake and release by heart mitochondria. *Arch.*

Biochem. Biophys. **167**:104–115.

VINOGRADOV, A., and A. SCARPA. 1973. The initial velocities of calcium uptake by rat liver mitochondria. *J. Biol. Chem.* **248**:5527–5531.