Ionized Calcium Concentrations in Squid Axons

R. DIPOLIO, J. REQUENA, F. J. BRINLEY, JR.,
L. J. MULLINS, A. SCARPA, and T. TIFFERT

From the Centro de Biofisica y Bioquimica, Instituto Venezolano de Investigaciones
Cientificas, Caracas, Venezuela, the Department of Physiology, Johns Hopkins University
School of Medicine, Baltimore, Maryland 21205, the Department of Biophysics, University of
Maryland School of Medicine, Baltimore, Maryland 21201, and the Johnson Foundation,
University of Pennsylvania, Philadelphia, Pennsylvania 19174.

ABSTRACT Values for ionized [Ca] in squid axons were obtained by measuring
the light emission from a 0.1-μl drop of aequorin confined to a plastic dialysis tube
of 140-μm diameter located axially. Ionized Ca had a mean value of 20 × 10⁻⁹ M as
judged by the subsequent introduction of CaEGTA/EGTA buffer (ratio ca. 0.1)
into the axoplasm, and light measurement on a second aequorin drop. Ionized Ca
in axoplasm was also measured by introducing arsenazo dye into an axon by
injection and measuring the Ca complex of such a dye by multichannel spectropho-
tometry. Values so obtained were ca. 50 × 10⁻⁹ M as calibrated against CaEGTA/
EGTA buffer mixtures. With a freshly isolated axon in 10 mM Ca seawater, the
aequorin glow invariably increased with time; a seawater [Ca] of 2-3 mM allowed a
steady state with respect to [Ca]. Replacement of Na⁺ in seawater with choline led
to a large increase in light emission from aequorin. Li seawater partially reversed
this change and the reintroduction of Na⁺ brought light levels back to their initial
value. Stimulation at 60/s for 2-5 min produced an increase in aequorin glow about
0.1% of that represented by the known Ca influx, suggesting operationally the
presence of substantial Ca buffering. Treatment of an axon with CN produced a
very large increase in aequorin glow and in Ca arsenazo formation only if the
external seawater contained Ca.

INTRODUCTION

It has been known for some time that the intracellular [Ca] in squid axoplasm is
much lower than the [Ca] in seawater, and that the fraction of intracellular Ca
which exists in ionized form is only a small fraction of the total. The existence of
an internal ionized Ca lower than that outside and a membrane potential inside
negative combine to produce a net inward directed driving force on Ca⁺. This
inward driving force implies that the direction of passive Ca leak will be inward
and that for a steady-state [Ca] to exist in the axoplasm, energy must be coupled
to the exit of Ca in some way. The factors regulating Ca transport across the
axolemma have been intensively studied in the past several years.

Information which is basic for any discussion of Ca transport either across the
axon membrane or within the cell, is the level of ionized Ca. The original
measurement of ionized Ca (Hodgkin and Keynes, 1956) indicated that it was in
the micromolar range. A subsequent investigation indicated it was somewhat
lower (Baker et al., 1971), about 350 nM. The present determinations set a range for ionized Ca of 20-50 nM.

In the present study two different techniques were used to estimate the internal ionized Ca \( \text{in situ} \). The first method made use of the Ca binding dye arsenazo III, in conjunction with dual wavelength absorption spectroscopy. In this method the dye was injected into the axon and allowed to reach diffusional equilibrium. In the range of ionized \([\text{Ca}]\) encountered in this study the absorbance change is strictly proportional to the free Ca. Since the dye is distributed uniformly throughout the axoplasm, measurements of ionized Ca with arsenazo reflect the average concentration of ionized Ca inside the fiber.

In the second method of determining ionized Ca, the photoprotein aequorin was used. In order to avoid some of the difficulties encountered with the injection of aequorin, the protein was confined inside a 140-\( \mu \)m dialysis tube located axially. Except when Ca fluxes are in a steady state, the ionized \([\text{Ca}]\) measured with this technique refers only to the core of the fiber, although it may be indirectly affected by local changes in ionized Ca at or near the membrane.

Both methods of determining ionized Ca were calibrated with reference to CaEGTA buffers; for aequorin such calibrations were in vivo, while for arsenazo they were in vitro. In order to express the data in terms of absolute \([\text{Ca}^{++}]\), it was necessary to have an accurate value for the effective dissociation constant for CaEGTA under conditions of pH, ionic strength, and temperature appropriate for squid axoplasm. In view of the report by Carini and Martell (1952) that the stability constant for CaEDTA varies significantly with ionic strength, a redetermination of the effective dissociation constant for CaEDTA and CaEGTA was done, including measurements at high ionic strength appropriate to squid axoplasm.

The major purpose of the present experiments was to obtain an accurate value for the concentration of ionized Ca in axoplasm under steady-state conditions. However, in order to check the validity of the methods used and to demonstrate their sensitivity to changes in ionized Ca, a variety of procedures designed to induce net Ca fluxes (electrical stimulation, high Ca seawater, Na-free seawater, application of CN) were employed. The general conclusions reached in these experiments are: (a) In an isolated squid axon intracellular Ca is in a steady state and concentration is the same throughout the axoplasm when the \([\text{Ca}]\) of the external solution is about 2-3 mM. (b) The internal ionized Ca is 20-50 nM. (c) The axoplasm effectively buffers against relatively small Ca loads of 5-40 \( \mu \)M induced by exposure to Na-free saline or stimulation. (d) CN treatment does not produce a significant increase in ionized Ca when axons are bathed in Ca-free seawater.

**METHODS**

*Experimental Material*

The hindmost giant axon was isolated from living specimens of *Loligo pealei* during May and June, 1975 at the Marine Biological Laboratory in Woods Hole, Massachusetts. Methods for isolating and cleaning the axons were standard.
Solutions
External and internal solutions used in these experiments are listed in Table I. The internal solution used in conjunction with arsenazo III for chelating Ca was 267 mM K$_2$EGTA and 79 mM potassium N-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (KTES), pH 7.3. The osmolarity of all solutions used was determined by comparison of the dewpoint of standard and unknown solutions using a commercial psychrometer (Wescor Inc., Logan, Utah). External solutions were adjusted to 900 mosmol/kg as measured against NaCl standards and were SO$_4^{2-}$-free to avoid complexing Ca. Internal dialysis solutions were adjusted to 810 mosmol/kg because, as previously noted (Brinley et al., 1975), this degree of hypotonicity improved survival of axons during dialysis.

Axon Chamber for Light Absorption Measurements
Cleaned squid axons were mounted in the dialysis chamber in the usual manner (Brinley and Mullins, 1967). The chamber was modified such that two light pipes provided a light source and a connection to the spectrophotometer. After the axon had been mounted, it was then microinjected over a 2-cm path with a solution of approximately 10 mM arsenazo III (2,2'-[1,8-dihydroxy-3,6-bisulfo-2,7-naphthalene-bis(azo)]-dibenzenearsonic acid) dissolved in a solution of 10 mM KTES, pH 7.3. Usually two injections of dye were made. The resulting concentration in the axoplasm, assuming diffusional equilibrium, was calculated to be 0.5-1.8 mM. This concentration of arsenazo III did not affect the excitability of the preparation. After injection of the dye, a porous plastic capillary was inserted inside the axon and located so that the porous region (~24 mm) extended beyond the injected region for about 2 mm on either end. Following these procedures the axon was maneuvered into the path of the light pipes and its position adjusted vertically for maximum absorbance. The light path had dimensions of 0.3 mm by 2 cm, and was positioned so that the 2-cm length of the light path was centered with respect to the injected region.

| TABLE I | COMPOSITION OF SOLUTIONS USED |
|---------------------------------|-----------------------------|
| **Constituent** | **Na seawater** | **0 Ca** | **0 Na** | **0 Na, 0 Ca** | **3 Ca** | **37 Ca** | **112 Ca** | **Internal solution (CTT)** |
|------------------|----------------|---------|---------|-------------|---------|---------|---------|-----------------------------|
| **K**            | 10            | 10      | 10      | 10          | 10      | 10      | 10      | 267                        |
| **Na**           | 452           | 462     | 2       | 2           | 459     | 417     | 400     | 30                         |
| **Li or choline**| 0             | 0       | 450     | 462         | 0       | 0       | 0       | 0                           |
| **Mg**           | 25            | 25      | 25      | 25          | 25      | 25      | 3       | 0                           |
| **Ca**           | 10            | 0       | 10      | 0           | 3       | 37      | 112     | 0                           |
| **Cl**           | 510           | 500     | 510     | 500         | 504     | 549     | 640     | 0                           |
| **Isethionate**  | 0             | 0       | 0       | 0           | 0       | 0       | 0       | 204                        |
| **D-Aspartate**  | 0             | 0       | 0       | 0           | 0       | 0       | 0       | 86                         |
| **EDTA**         | 0.1           | 0.1     | 0.1     | 0.1         | 0.1     | 0.1     | 0.1     | 0                           |
| **TES**          | 2             | 2       | 2       | 2           | 2       | 2       | 2       | 8                           |
| **Glycine**      | 0             | 0       | 0       | 0           | 0       | 0       | 0       | 204                        |
| **pH**           | 7.5           | 7.5     | 7.5     | 7.5         | 7.5     | 7.5     | 7.5     | 7.3                        |

CN seawater was made by removing 2 mmol/liter of NaCl, LiCl, or choline Cl and substituting 2 mmol/liter NaCN.
In order to avoid introduction of external Ca into axoplasm during the initial steps, the axons were mounted in Ca-free seawater. The duration of this exposure was about 30 min, after which the axon was returned to 10 mM Ca seawater before measurements were made. In addition, the tied ends were never closer than 6 mm from the edge of the injected dye region.

**Determination of Intracellular [Ca] with Arsenazo III**

The use of dual wavelength absorption spectroscopy for determination of Ca and Mg in biological material has already been described (Scarpa, 1972). The application of these principles, in conjunction with the use of a microspectrophotometer, to permit nondestructive measurement of ionized Mg in single isolated squid giant axons has also been recently described (Brinley and Scarpa, 1975). Essentially the same apparatus was used in the present experiments, except that the phototube was an EMI 9592B operated at 350 V.

In the present series of experiments arsenazo III was used as a Ca\(^{++}\) indicator. This dye and related arylazo derivatives of 4,5-dihydroxynaphthalene-2,7-disulfonic acid, have been used for the photometric titration of numerous divalent and heavy metal cations (Burger, 1973; Pribil, 1972; Flashka and Barnard, 1969; Savvin, 1959). This dye has several advantages over murexide for the determination of low concentrations of ionized Ca in biological systems. (a) The molar extinction coefficients for the differential spectra maxima are approximately twofold greater than those for murexide. (b) Arsenazo III absorbs at longer wavelengths than murexide, which results in less interference from cytochrome absorption. (c) The apparent stability constant for arsenazo III at biological pH and ionic strength is about two orders of magnitude greater than that of murexide which greatly increases the differential absorbance change observed with a given [Ca].

The use of arsenazo III for the analytical determination of Ca at pH 8 has been described (Michaylova and Kouleva, 1972; Michaylova and Ilkova, 1971). For reference, the spectra of arsenazo III and Ca arsenazo III at \(\mu = 0.1\) are shown in Fig. 1. The difference spectra for the Ca and Mg chelates at \(\mu = 0.5\) are shown in Fig. 2. Although the apparent

![Figure 1](image-url)

**Figure 1.** Extinction coefficient as a function of wavelength for arsenazo III and Ca arsenazo III, ionic strength 0.1. Solution: 30 \(\mu\)M arsenazo III, 10 mM KH\(_2\)EDTA, KCl 100 mM, pH 7.0, temperature 18°C.
ionized Calcium Concentrations in Squid Axons

Dissociation constant for Mg is very much greater than that of Ca, the Mg interference is not negligible. It can be minimized by measuring the difference spectrum at 660-685 nm, a wavelength pair at which the difference signal for Mg is rather small. The extent of Mg interference on the Ca signal under the conditions of our experiments (pH 7.3, ionic strength 0.3) was investigated quantitatively in the experiment illustrated in fig. 3. In this experiment the measurement of absorbance was made as described in detail below except that a glass capillary with a lumen of 550 μm was substituted for a squid axon. Solutions of dialysis fluid (CTT) containing arsenazo III and the indicated amounts of EGTA, Ca, and Mg were flushed sequentially through the glass capillary and the resulting absorbance changes at 660-685 nm measured. The concentration of free Ca in the presence of 4 mM EGTA, without added divalent cations, was taken to be zero. The free Ca and Mg concentrations for the other solutions were calculated using an apparent dissociation constant of 0.15 μM for CaEGTA and 30 mM for MgEGTA. Using these calculated concentrations of free divalent cations, and the observed absorbance changes, the sensitivity of arsenazo III for Ca over Mg at the wavelength pair 660-685 nm (pH 7.3, ionic strength 0.4) was calculated to be 2950:1.

Arsenazo III was obtained from Sigma Chemical Company, St. Louis, Mo. This commercial product contained between 20 and 40% (mole fraction) Ca arsenazo III. Most of the Ca bound to arsenazo III was removed by ion exchange chromatography using Chelex 100 (Bio-rad Company, Richmond, Calif.) as a chelating resin. A solution of 5 mM arsenazo (20 ml) was passed three times through a column (4 × 45 cm) of Chelex 100 (100-300 mesh) prewashed with 0.1 M Na acetate, pH 6.5. The eluate of the third passage...
FIGURE 3. Calibration curve to show extent of Mg interference with Ca signal. A glass capillary of inside diameter 550 μm was mounted in the usual position and flushed with 780 μM arsenazo III in solution CTT containing the indicated amounts (mM) of EGTA, Ca, and Mg. Free Ca in first trace (4 mM EGTA) was taken as 0; in second trace calculated as 150 nM. In third trace free Ca was calculated to be 160 nM and the free Mg as 920 nM. From the observed absorbance changes, the sensitivity of Ca over Mg was calculated to be 2950:1.

through the column was concentrated to 10 ml in a rotating flask evaporator and the remaining water was removed by lyophilization. The arsenazo was then dissolved in deionized distilled water and contained approximately 2% Ca (mole fraction). This purified arsenazo was used throughout the experiments.

Estimation of [Mg]i
In a few experiments changes in the ionized [Mg] in squid axons were followed by the use of the Mg indicator Eriochrome Blue SE (3-[3-chloro-2-hydroxyphenylazo]-4,5-dihydroxy-2,7-naphthalene disulfonic acid; Plasmocorinth B; Mordant Blue B). The use of this material as a Mg indicator in the presence of small amounts of Ca has been described (Scarpa, 1974). Procedures for introducing the dye and mounting the axon were identical to those used for the Ca determinations. Eriochrome Blue SE was obtained from Sigma Chemical Company and twice recrystallized before use.

Determination of Effective Dissociation Constants for CaEDTA, CaEGTA, and Ca Arsenazo III
Arsenazo III was used as an indicator for Ca. Difference spectra of arsenazo III were determined using a split beam recording spectrophotometer (Aminco DW-2 American Instrument Company, Silver Spring, Md.). The [Ca] of all solutions used was determined on a Perkin-Elmer model 305B atomic absorption spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.) using commercially available Ca standards. The initial titration solution contained 50 μM arsenazo III, 10 mM potassium N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (KBES) adjusted to pH 7.0, 50 μM EDTA or EGTA, and either KCl, K isethionate, or K aspartate to give the desired ionic strength. Total Ca in the initial solution due to contamination from reagents (principally arsenazo III and KCl) was about 20 μM. CaCl₂ was added to this mixture to increase the total Ca in steps of approximately 5 μM. After each addition a difference spectrum was obtained. Representative spectra are shown at the left of Fig. 4 and the absorbance differences at 574–605 nm are plotted on the right. The first portion of the titration curve represents
titration of the principal chelator (EDTA or EGTA) by Ca and results in only a small absorbance change. After the first ligand has been completely chelated by Ca, the absorbance change becomes linear with added Ca, representing the initial stages of titration of the arsenazo III. As the arsenazo III becomes nearly saturated with Ca, the titration curve becomes curvilinear and approaches a plateau.

Since the Ca dissociation constants for EDTA or EGTA and arsenazo III are very different, it was possible to use the second part of the titration curve for measurement of

![Figure 4: Titration curves of arsenazo III in the presence of EDTA and EGTA at various ionic strengths. Top: 50 µM arsenazo III, 49 µM EGTA, KBES 10 mM, KCl 100 mM, pH 7.0, T = 16°C. Bottom: arsenazo III 50 µM, EDTA 49 µM, KBES 10 mM, KCl 500 mM, pH 7.0, T = 16°C. Left side of figure: Differential spectra of arsenazo III in the presence of varying amounts of total Ca. Calculated ionized [Ca] are shown above each curve. Right side of figure: Titration curve showing absorbance change as a function of total Ca. The initial part of the curve shows the titration in the presence of excess chelator, the final points in each graph show the titration in the presence of excess Ca. The linear portion of the curve can be extrapolated to give the concentration of chelator present.](image-url)
the dissociation constant and molar extinction coefficient for arsenazo III. These two pieces of data were obtained by a double reciprocal plot of absorbance and Ca, with extrapolation of the linear curve to infinite [Ca] (with appropriate correction for Ca bound to EGTA or EDTA). The apparent dissociation constants were calculated as follows. From the molar extinction coefficients, one could calculate the amount of Ca arsenazo III present after each addition of Ca in the initial part of the titration curve, and the free arsenazo III concentration obtained by subtracting this value from the total. Combining these two pieces of data with the dissociation constant for Ca arsenazo III allowed calculation of the ionized [Ca] present after each addition. The amount of either CaEDTA or CaEGTA present was then calculated as the difference between the total Ca present in the solution and that bound to arsenazo III or present as free Ca. Free EDTA or EGTA concentration was obtained by subtracting the total EDTA or EGTA present from the amount bound. Finally, the apparent dissociation constant for the chelator was calculated from the values for ionized Ca, bound ligand and free ligand. The steps in the calculation are listed below (A = arsenazo III).

\[
\begin{align*}
A_{\text{free}} &= A_{\text{total}} - CA, \\
CA &= \frac{CA}{K_{\text{Ca, A}}} \\
CaL &= CA_{\text{total}} - CA - Ca, \\
L_{\text{free}} &= L_{\text{total}} - CaL, \\
K_{\text{Ca, A, L}} &= \frac{CaL}{CaL}.
\end{align*}
\]

The calculated effective dissociation constants are shown in Fig. 5, as a function of ionic strength when the salt solution was either KCl or K isethionate. The values for EGTA and EDTA at 0.1 M ionic strength and pH 7.0 were 0.21 \( \mu \)M and 0.065 \( \mu \)M, respectively, which agree well with determinations by other methods previously reported in the literature.

**Axon Chamber for Light Emission Measurements**

The apparatus is illustrated schematically in Fig. 6. A squid axon was mounted in a dialysis chamber similar to that described by Brinley and Mullins (1967) except that it was made shallower in depth so that a photomultiplier in a light-tight housing could be lowered to within 35 mm of the axon. A front surface mirror was placed immediately below the slot containing the axon to reflect light into the photomultiplier. Seawater flowing through the chamber at 1 ml/min passed through a heat exchanger just before entering the chamber in order to maintain the slot temperature at 16°C. An axon was cannulated at both ends, and an empty plastic dialysis tube 140-\( \mu \)m OD \( \times \) 90-\( \mu \)m ID was steered through the axon until the end of the dialysis tube emerged from the glass cannula at the far end of the axon. This part of the dialysis tube was washed repeatedly with 1 \( \mu \)M EGTA solution to remove traces of Ca. A sample of aequorin solution was brought into contact with the dialysis tube and 0.1 \( \mu \)l of aequorin aspirated into the dialysis tube. Using a microsyringe connected to the other end of the dialysis tube, this drop of aequorin was then sucked back along the dialysis tube until it reached the porous region of the dialysis tube which was centered under the photomultiplier. The photomultiplier was housed in a brass case with a rotatable shutter and was clamped to a vertical Palmer stand so that it could be positioned over the porous region in the dialysis chamber and removed when it was necessary for a microscope to be positioned over the dialysis chamber. After positioning the phototube, a light-tight cover was placed over the apparatus, the phototube shutter opened, and measurements were begun. Calibrating buffer drops, or additional aequorin drops were introduced in the same way.
DiPolo et al. Ionized Calcium Concentrations in Squid Axons

**Figure 5.** Apparent dissociation constants for CaEGTA, CaEDTA, and Ca arsenazo III as a function of medium composition and concentration. $T = 16^\circ C$, pH = 7.0.

**Figure 6.** Schematic diagram of apparatus (not to scale). (A) Arrangement of phototube over axon chamber. (B) Detail showing porous region containing aequorin drop. Illustrated are: axon (1), end cannula to support axon (2), porous capillary (3), chamber (4), front surface mirror (5), masks to limit light to porous region (6), photo cathode (7), support to Palmer stand (8), phototube connections (9).

**Light Emission Measurements**

The photomultiplier used was a 1P28 type (Aminco C58-6214) with the dynode resistor chain potted to minimize tube socket leakage. It was operated at an overall potential of 950 V and connected to a solid-state microphotometer (American Instrument Co., Silver Spring, Md.). This instrument supplied a stable adjustable high voltage supply, a current-subtracting network to balance dark current, an amplifier, and a network of variable anode load resistors and capacitors such that full scale output of the photomultiplier could be varied from 3 μA to 3 nA while the time constant of the circuit remained at 0.2 s. Amplifier output was recorded on a strip chart recorder.

The cathode of the photomultiplier had dimensions 24 mm long by 7.8 mm wide and it...
was positioned a distance of 35 mm from the axon. The length of the porous region of the capillary (and hence the length of the aequorin drop exposed to Ca) was 14 mm. From geometrical considerations, the photocathode should have directly intercepted 3.5% of the emitted light; the plane mirror below the chamber should have about doubled this so that 7% of the emitted light may have been collected. The photomultiplier had the following characteristics: cathode sensitivity 40 µA/L, anode sensitivity 100 A/L at 1,000 V. The tube we used was calibrated by Optronics Laboratories Inc. (Silver Spring, Md.) for absolute energy response at wavelengths between 400 and 500 nm. This range includes 95% of the energy emission of aequorin. Calculating both the quantum energy and the photocathode sensitivity as a function of wavelength gave a mean value of 2.2 × 10^4 A/W (1 µA = 4.5 × 10^{-11} W) when the tube was operated at a potential of 950 V. As the resting glow from aequorin inside an axon is typically 1 nA, this is equal to 4.5 × 10^{-11} W or 1.1 × 10^6 photons/s. As we estimate that only 7% of the light emitted was actually collected, total emission is 1.6 × 10^6 photons/s; Shimomura and Johnson (1969) gave 0.23 as the quantum efficiency so that is 7 × 10^6 aequorin molecules reacting per second.

The aequorin concentration was estimated to be 200 µM and 0.1 µl was used in an axon. This is 20 pmol or 1.2 × 10^{13} molecules of aequorin. The rate constant for molecules reacting is 7.6 × 10^6/1.2 × 10^{13} or 5.8 × 10^{-7} s^{-1} or a time constant for the decline of the aequorin glow of 480 h.

**Aequorin**

The aequorin used in this work was a gift of Drs. F. H. Johnson and O. Shimomura, and we are greatly indebted to them for making this work possible. The material was received as the purified NH₄SO₄ precipitated protein and desalted by methods of Shimomura et al. (1962). A Sephadex G-25 column 3-mm ID by 12 cm long was prepared. The Sephadex was repeatedly washed with 1 mM K₂EGTA adjusted to pH 7 followed by further washing with 1 µM K₂EGTA. The aequorin sample of about 1 mg of protein was dissolved in 30 µl of 1 µM EGTA solution and this placed on the column. Five-microliter samples of the column effluent were taken at regular time intervals. These were collected in plastic tubing and sealed at the ends before transferring them to storage at −65°C. The absorbance of a sample from this mixed batch was measured at 280 nm. From the measured value, the molecular weight of aequorin, and the specific absorbance, an aequorin concentration in the stock solution of 220 µM was estimated.

Aequorin solutions were never brought into contact with glass, and any plastic tube used to remove this protein from its stock plastic tube was washed with a 1 µM EGTA solution before use. The aequorin stock solution did have a resting glow, presumably indicating some [Ca] in spite of strenuous precautions against the introduction of Ca. However, this glow was of the order of 10% of that of an axon, and it did not change during the series of experiments.

**RESULTS**

**Determination of Ionized Ca in Axoplasm with Arsenazo**

**PRINCIPLE OF THE METHOD** The measurement of ionized Ca in the axoplasm of resting axons with arsenazo III depends upon measuring the differential absorbance change produced when the ionized Ca present in axoplasm is chelated by the sudden introduction of an excess of EGTA. In order to validate this technique, it is necessary to demonstrate that (a) the absorbance change is linear with ionized Ca in the range of interest, and (b) chelation of Ca by EGTA is complete under the experimental conditions.
A typical calibration experiment is shown in Fig. 7. In this experiment, a glass capillary with a lumen comparable to the usual diameter of a squid axon (500 μm) was positioned in the dialysis apparatus, and then flushed with calibrating solution (CTT) containing 500 μM arsenazo III dye and the indicated amounts of EGTA and CaEGTA. The lower part of the figure shows the absorbance changes produced at the wavelength pair 570–600 nm. The concentration of free Ca in the solution containing 2 mM EGTA but no added Ca was taken as zero. The insert shows the data replotted to demonstrate that the absorbance change is linear with ionized Ca. Using an apparent dissociation constant of 0.15 μM for CaEGTA (pH 7.3, ionic strength 0.3) gives a calculated ionized Ca of 0.15 μM for the middle point. Calibration for the differential absorbance measured at the wavelength pair 660–685 nm was also linear.

Fig. 8 illustrates a combined in vitro calibration and dialysis experiment to demonstrate that the differential absorbance change produced by chelation of Ca with EGTA can be used to measure the amount of ionized Ca present initially in the solution. A glass capillary was mounted in the apparatus and flushed with dialysis medium CTT containing 2 mM EGTA. The differential absorbance observed with this solution served as a reference for the 0 Ca base line. The capillary was then flushed with solutions containing the indicated ratios of CaEGTA and EGTA to establish a linear relation between ionized Ca and absorbance. The vertical arrow indicates the onset of dialysis with an isosmotic solution of EGTA and KTES (267 mM K₂EGTA and 79 mM KTES). As the figure illustrates, when the EGTA diffuses through the porous capillary into the lumen of the glass tube, the residual ionized Ca is chelated and the differential absorbance change plotted against ratio of free to bound Ca. Absorbance in 2 mM EGTA taken as 0 free Ca. Calibration curve for differential absorbance at 660–685 nm was similar.
Figure 8. In vitro calibration and dialysis. A dialysis capillary was placed inside a 550-μm ID glass capillary containing 500 μM arsenazo III in dialysis medium CTT. Left-hand portion of the figure shows absorbance changes produced when dye solutions containing EGTA buffer with various ratios of CaEGTA/EGTA were flushed through the glass capillary. Nominal concentrations are 0 and 150 nM, respectively. Right-hand side of figure shows the absorbance change when a pulse of EGTA solution (267 mM EGTA, 79 mM KTES, pH 7.3) was flushed through the porous dialysis capillary. The absorbance returns to the level of 0 Ca with a time-course appropriate for the dimensions of porous plastic capillary and glass capillary.

Absorbance returns to the base-line level corresponding to 0 Ca, with a time-course appropriate for the dimensions of the porous plastic and glass capillaries.

Calibration of absorbance change At the end of an experiment, the axon was replaced by a glass capillary whose lumen was comparable in size to the diameter of the fiber. A sufficient amount of the dye previously used for injection into the axon was added to the calibrating solution (CTT) to give a dye concentration comparable to that in the axoplasm (0.5–1.5 mM). Aliquots of this dye solution were then treated with appropriate volumes of CaEGTA buffers to give solutions with free ionized Ca in the range 0–0.2 μM. These solutions were then flushed through the glass capillary in sequence and the differential absorbance changes noted. A linear dependence of absorbance upon path length and dye concentration was assumed in correcting these in vitro calibration factors for the exact diameter of the axon and in vivo concentration of arsenazo III. The correction factors were about 1.15–1.30.

Experimental results The result of dialysis in four axons is shown in Fig. 9. The vertical arrows indicate when the chelating solution was flushed through the capillary. In the top two experiments dialysis was not continuous, instead a 1–2-s pulse of solution was pushed through the capillary. In these experiments a subsequent short flush was given as indicated by the arrows to confirm that complete chelation of the free Ca had occurred.

The collected data for nine experiments are given in Table II. The average ionized Ca in these nine axons, which had been injected with arsenazo III but otherwise untreated, was 130 nM.
EFFECT OF Ca CHELATION BY ARSENazo UPON MEASURED LEVEL OF IONIZED Ca

Since these experiments involved the injection of millimolar amounts of arsenazo III, which is in effect a Ca chelator, it is necessary to consider the extent to which the true level of ionized Ca in the axoplasm is perturbed by the introduction of the dye. Using the following data, it is possible to calculate that the amount of Ca arsenazo III in equilibrium with the ionized Ca in the axoplasm is about 5 μM: \( \text{Ca}_0 = 130 \text{nM} \) (Table II); arsenazo = 1.0 mM (Table II); \( K_d \) for arsenazo = 40 μM (Fig. 5). The amount of Ca arsenazo present in the axoplasm at equilibration can also be calculated from the absorbance changes shown in the chelation experiments of Fig. 9 using molar extinction coefficients from Fig. 1. The values for the concentration of bound dye range from 5-9 μM and are in fair agreement with the value calculated from the dissociation constant.

Since the Ca contamination in the arsenazo III used in these experiments was approximately 2%, the injection of dye into the axons listed in Table II introduced between 10 and 40 μM of Ca as Ca arsenazo III. Since the final concentra-
Table II

IONIZED CALCIUM IN AXOPLASM, ARSENAZO METHOD
(10 mM Ca SEAWATER, 16°C)

| Axon reference | Axon diameter | Arsenazo III | [Ca]a M | [Ca]b M |
|----------------|---------------|--------------|--------|-------|
| 052975-3      | 425           | 1.6          | 140    |
| 053075        | 600           | 0.8          | 180    |
| 060275-2      | 420           | 1.6          | 90     |
| 060275-3      | 400           | 1.8          | 75     |
| 060375-2      | 450           | 1.4          | 100    |
| 060375-3      | 450           | 1.4          | 150    |
| 060375-4      | 500           | 1.1          | 130    |
| 060475-2      | 525           | 0.8          | 200*   |
| 060475-3      | 500           | 0.5          | 130    |
| 060475-4      | 500           | 0.5          | 180    |

Mean: 130
Mean (corrected for Mg): 50

* Treated with ca. 3 μM FCCP; excluded from mean.

...tion of bound dye was about 5-10 μM, in some cases as much as 90% of the injected Ca, i.e. 35 μM, must have been sequestered somewhere in the fiber. This point will be considered further in the Discussion. Of immediate relevance to the validity of the present results is whether the Ca injected with the dye has produced an artifically high level of ionized Ca. If this were so, then fibers injected with the largest amount of dye should have shown the largest apparent free Ca. The data in Table II have been plotted in Fig. 10 to show the variation of ionized Ca with concentration of injected dye. There is no marked relation between dye concentration and [Ca]. In fact, the two highest values for ionized Ca were obtained with the lowest dye concentration. We conclude therefore that if Ca in the injected dye contributed to the free Ca, the effect was maximal at the lowest dye concentration used. However, since we cannot exclude a contribution of injected Ca to the final level, we regard the mean concentration listed in Table II as an upper limit which is subject to a further correction for Mg interference as considered below.

**Correction for Chelated Mg**  During the dialysis with EGTA, some Mg will be chelated. Thus the observed absorbance change will reflect loss of the Mg arsenazo signal as well as that of Ca arsenazo. Since this factor has not been considered in the in vitro calibrations, the mean value for free Ca given in Table II is an overestimate. A correction factor for the Mg signal was obtained as follows. A rough calculation indicates that after 4 min the mean [EGTA] in the axoplasm is about 1% of the concentration initially in the lumen of the capillary (about 2.5 mM). No data are available on the dissociation constant for MgEGTA at high ionic strength, however if the dependence upon ionic strength is the same as it is for CaEGTA, the effective dissociation constant should be increased roughly three times and may therefore be taken as approximately 30 mM. Using this dissociation constant together with a value of 3 mM for the free Mg (Baker and Crawford, 1972; Brinley and Scarpa, 1975) and roughly
2.5 mM for free EGTA, allows one to calculate that about 250 μM MgEGTA is formed. Since the selectivity factor for Ca over Mg is about 3,000 (see Methods) the Mg signal is equivalent to approximately 80 nM of Ca. Making this correction reduces the mean ionized Ca in Table II to 50 nM.

**Experiments with Aequorin**

**RESTING AEQUORIN GLOW IN AXONS** When an aequorin droplet was introduced into the porous region of the plastic capillary, the aequorin luminescence usually increased gradually over a period of a few minutes to reach a steady level determined by the diffusion of Ca from the axoplasm across the wall of the porous capillary into the lumen of the porous capillary where it could react with aequorin. Records of light emission from 12 axons are shown in Fig. 11. The aequorin contained 1 μM EGTA to protect it during storage. Since the EGTA in the porous capillary was diluted approximately 25 times by diffusion within the axoplasm, this concentration of EGTA should have had a negligible effect upon the level of ionized Ca in the axoplasm even if all the EGTA were uncombined with Ca. The fact that the initial resting glow of a droplet of aequorin not exposed to axoplasm but positioned in the same way inside the axon in the chamber was 0.18 nA suggests that there was, in fact, some free Ca in the aequorin. Occasionally there was a drop in the resting glow, as can be seen in three of the experiments illustrated in Fig. 11, presumably due to some slight contamination of the aequorin as it was positioned in the porous region.

Fig. 11 also shows clearly that the level of resting glow depends upon the concentration of Ca in the external bathing medium. As can be seen from the first column of experiments in Fig. 11, the resting glow in 10 mM Ca seawater usually does not reach a steady level even after 20–30 min. In contrast, when the external medium contains 3 mM Ca, a plateau is reached. The sensitivity of the aequorin glow to [Ca]₀ can be seen even more dramatically in Fig. 12 where an axon was exposed sequentially to bathing solutions containing external Ca in concentrations 3, 11, 37, and finally 0 mM Ca. The rate of increase was most marked in 37 mM Ca. The ionized Ca was promptly reduced when the axon was bathed in Ca-free seawater.

![Figure 10](image_url)  
**Figure 10.** Steady level of ionized Ca in squid axons after injection of sufficient amounts of arsenazo III into axons to give the indicated concentrations in axoplasm.
Figure 11. Composite of 12 experiments showing the resting glow resulting from the introduction of the initial aequorin drop. Numbers above each trace indicate the [Ca] (millimolar) in the external seawater bathing the axon. Vertical arrows indicate change in external solutions. The vertical scale at lower left applies specifically to indicated experiment. The resting glows of other experiments were comparable.

Figure 12. Effect of changes in external Ca (3, 11, 37, and 0 mM) on resting glow of aequorin in squid axon. Resting glow increases about 10 times in 37 mM Ca during 30-min exposure (steady state was not reached) and recovers promptly in 0 Ca seawater.
CALIBRATING THE AEQUORIN GLOW  The aequorin luminescence from an axon was calibrated in terms of ionized Ca as follows. After obtaining a resting glow measurement for axoplasm, the aequorin was removed and replaced by a second drop of equal volume containing CaEGTA/EGTA buffer set to a ratio thought to give a free [Ca] close to that of axoplasm. The concentration of the buffer mixture was adjusted such that upon dilution by the axoplasm, the sum of EGTA + CaEGTA would be 0.5 -1.0 mM. This second Ca buffer drop was allowed to equilibrate with axoplasm for 30 min, a time selected because of experience with the equilibration of buffers with axoplasm in connection with efflux studies. After the equilibration of the buffer, the [Ca]$_i$ of axoplasm should now be determined by the CaEGTA/EGTA ratio rather than by physiological processes. After buffer equilibration, the drop was removed from the porous capillary and a new aequorin drop introduced and its light emission measured after a steady level had been reached.

Fig. 13 A shows an axon where the aequorin glow in 3 Ca seawater matched very closely the measured glow of a Ca buffer with a nominal value for a CaEGTA/EGTA of 0.15 or a calculated ionized Ca of 23 nM. In Fig. 13 B the
light output of another axon with an aequorin drop is shown in both 10 and 3 Ca seawater. Curve C is the calibration of the axon shown in B. In this case, the infinite time value of the calibration glow is about twice the initial value of resting glow for the axon.

In some cases two calibrations were obtained on the same axon by equilibrating the axoplasm sequentially with two buffers. Such droplet equilibration is analogous to microinjection in the sense that the two additions of buffer sum with those introduced later.

Several axons were calibrated with two different CaEGTA/EGTA ratios. While results between axons cannot be expected to be comparable because aequorin light emission depends on pH, [Mg], ionic strength, as well as on some unknown factors that may be present in axoplasm, the collected results of double calibrations are shown in Fig. 14 from which it is clear that the relationship between ionized [Ca] and light emission is linear over the range studied.

As a check on the linearity of the in vivo double calibration, measurements were made with a glass tube with an internal diameter of about 500 μm. The tube contained a CaEGTA/EGTA buffer mixture with various ratios, and light emission was measured with the glass tube in the position usually occupied by an axon in the light-measuring apparatus. A porous plastic capillary was inserted in the glass tube, an aequorin drop positioned in the usual way and light emissions measured. The results are shown in Fig. 15 where it is clear that light emission is reasonably linear with [Ca] when these extend from about 8–80 nM (CaEGTA/EGTA).
EGTA = 0.05-0.50). Some physiological studies have assumed a square-law relationship between light emission and [Ca] based on in vitro calibrations, but such calibrations have usually been done in dilute solutions of aequorin and in vitro. A study by Azzi and Chance (1969, fig. 1 b) showed that aequorin gave a linear increase in light production for 50-150 nM Ca (with a signal/noise ratio of 10) and that sensitivity was 5 nM. At micromolar concentrations of Ca, Azzi and Chance noted a slope of 2 for the curve on a log-log plot relating light output to [Ca]. A similar finding, linearity at low [Ca] and a square law at higher [Ca], has been described in situ for barnacle muscle fibers (Ashley, 1970). Our conditions of calibration were such that resting glow was closely matched by the glow of aequorin in calibration buffer mixtures. This obviated a requirement for knowing the exact relationship between light emission and [Ca].

An unexplained feature of the in vivo method of calibrating the aequorin response is shown in Fig. 16. In A the resting glow of an axon was measured for the transition 10 Ca to 3 Ca seawater and it was then calibrated with a buffer whose total [EGTA + CaEGTA] was 6 mM. The light emission from this buffer mixture (with a nominal Ca of 40 nM) was much higher than expected. A second axon (Fig. 16 B) was stimulated, leading to an increased aequorin glow, and then calibrated with a buffer that was 12.5 mM total EGTA in axoplasm. This gave an even higher light emission although the nominal [Ca] remained the same, 40 nM. It is possible that these higher EGTA concentrations reduce free Mg in the axoplasm by chelation. Since Mg is inhibitory to the aequorin light reaction, a reduction in Mg would increase aequorin luminescence at constant Ca.

IONIZED [Ca] IN AXOPLASM All measurements of ionized Ca in axoplasm are shown in Table III which gives the initial value of the resting glow (RG) of an aequorin drop measured within a few minutes of its introduction into the porous region of a dialysis tube and with the axon in 3-10 Ca, Na seawater. The table also gives a subsequently measured glow (CG) from a second aequorin drop after a calibrating buffer mixture had been equilibrated with axoplasm for 30 min along with the nominal [Ca] of the calibrating buffer, its [EGTA], and the ionized [Ca] of the axoplasm. The mean ionized [Ca] of axoplasm in fresh squid axons was 20 nM.

[Ca] as a function of [Ca]. Fig. 17 shows the slope of the linear portion of the resting glow curve plotted against extracellular [Ca] for 11 axons. A slope of zero, implying no net gain or loss of ionized Ca, can be interpolated from these data and occurs at about 2.8 mM. This interpolated concentration at which the net change in ionized Ca in central axoplasm is zero is considerably lower than the [Ca] of seawater, the composition of which forms the basis for most artificial salines. However, it is not far from the figure of 4 mM of free ionized Ca in squid hemolymph calculated by Blaustein (1974).

For 10 mM Ca seawater the increase in light is approximately +0.011 nA/min. On the basis of calibration data obtained on other axons and discussed earlier, this rate of increase of light emission corresponds to a net increase of approximately 0.4 nM/min in the axoplasm surrounding the porous capillary. It should
be emphasized that this result only applies to the central core of axoplasm and only to axons about 60 min after isolation from living squid. The effect of external Ca on ionized Ca in the peripheral axoplasm could be quite different.

**EFFECT OF STIMULATION ON [Ca]$_{i}$**  It has been known for some time that during stimulation of an axon Ca influx increases substantially (Hodgkin and Keynes, 1957; Meves and Vogel, 1973) and that part of this extra Ca influx is a result of Ca going through the Na channel. For an axon in normal seawater, the electrical effects of this Ca entry are negligible as compared with the Na current, but in terms of the Ca flux balance of the axon, stimulation can produce large changes in internal [Ca]. Baker et al. (1971) showed that stimulation produces an immediate substantial increase in the resting glow of an aequorin-injected axon and that this increased light emission subsides after stimulation with a time constant of 15 s.

In the present experiments the aequorin response to stimulation was much
delayed as shown in Fig. 18 where the axon was in 10 Ca seawater and gave the usual increase in resting glow with time, a change to 3 Ca seawater substantially reduced this slope, while a change to 37 Ca seawater increased it again. The period of stimulation (60/s for 150 s) is indicated on the tracing. Light emission began to rise only after a substantial time delay and it continued to rise after stimulation ceased. Recovery to the 37 Ca seawater base line (extrapolated from before the period of stimulation) took at least 10 min.

TABLE III
IONIZED CALCIUM IN AXOPLASM, AEQUORIN METHOD
(3–10 mM Ca SEAWATER, 16°C)

| Axon reference | Axon diameter | Axon RG* | Buffer CG | Calibrating buffer | Axon ionized [Ca] |
|----------------|---------------|----------|-----------|--------------------|------------------|
|                | μm            | nA       | nA        | [Ca] μM           | [EGTA] nM        | [Ca] nM         |
| 280575A        | 650           | 0.36     | 1.4       | 38                | 800              | 12              |
| 280575B        | 625           | 0.57     | 0.9       | 38                | 470              | 23              |
| 240575A        | 550           | 0.54     | 2.9       | 67                | 1200             | 13              |
| 270575A        | 475           | 0.66     | 1.9       | 28                | 1600             | 10              |
| 290575C        | 625           | 0.39     | 0.7       | 62                | 650              | 37              |
| 300575A        | 475           | 0.45     | 0.3       | 38                | 500              | 57              |
| 020675A        | 550           | 0.54     | 4.2       | 68                | 500              | 9§              |
| 020675B        | 415           | 0.72     | 2.1       | 68                | 100              | 23              |
| 030675A        | 475           | 1.1      | 4.3       | 38                | 770              | 9§              |
| 030675B        | 610           | 1.0      | 1.9       | 28                | 600              | 15§             |
| 040675A        | 450           | 0.45     | 1.0       | 17                | 450              | 8               |

* Resting glow.
§ Calibrating glow.
§ 3 Ca seawater; others 10 Ca.

FIGURE 17. Change in light emission for axons immersed in artificial seawater of various [Ca]. Slope was measured from the linearly changing portion of the light emission curve approximately 15 min after a solution change. Rate of increase of light emission in 10 mM Ca seawater corresponds to about 0.4 nM/min increase in free Ca (measured on axis of fiber).
FIGURE 18. Effect of external Ca and stimulation upon resting glow. The upper
legend indicates the external [Ca] in the bathing media. The horizontal bar labeled
"S" indicates the period during which the fiber was stimulated at 60 impulses/s for
150 s. Note the abrupt increase in level of resting glow is maintained after stimula-
tion ended. Ordinate, phototube current in nanoamperes.

The net Ca entry found by Hodgkin and Keynes (1957) for stimulation in 44
mM Ca seawater was 26 fmol/cm² impulse (and was linear with [Ca]₀) so that for
37 mM Ca seawater one may use an entry of 20 fmol/cm² impulse. The axon in
Fig. 18 conducted 9,000 impulses so that the total entry per square centimeter
would be 180 pmol of Ca. An axon with 1 cm² of membrane area and a diameter
of 500 µm has 12.5 µl of axoplasm so that in the absence of intracellular buffer-
ing, the expected change in [Ca] as a result of stimulation is 180 pmol/12.5 µl
or 14.4 µM. The actual change in [Ca]ᵢ at the center of the axon was close to
14 nM or 0.1% of the expected change. This result emphasizes the capacity of
Ca buffering systems in the axoplasm to control [Ca]ᵢ within quite narrow limits.

Experimental results with four axons, including one using arsenazo III as a Ca
indicator, are listed in Table IV. In all cases stimulation produced a definite but
small increase in ionized Ca.

EFFECTS OF Na-FREE SOLUTIONS The work of Baker et al. (1969) showed
that the removal of Na from seawater bathing an axon reduced Ca efflux and
increased influx. One would predict, therefore, that exposing an axon to Na-
free solutions should result in substantial net entry of Ca.

An experiment to test the behavior of our method is shown in Fig. 19 where an
axon was kept in 3 mM Ca seawater and showed a steady resting glow corre-
sponding to a [Ca]ᵢ of about 15 nM. A replacement of all the Na in seawater
(holding Ca₀ constant) resulted in a large increase in aequorin glow. The initial
slope of the rise in light output in choline seawater corresponds to a change in
[Ca]ᵢ with time of the order of 3 nM/min. This is only a small fraction (0.1%) of
TABLE IV
INCREASES IN [Ca], WITH STIMULATION

| Axon reference | Axon diameter | Frequency | Duration | Impulses | [Ca], | Δ[Ca], | Apparent net Ca influx | Normalized Ca influx if [Ca], = 10 mM |
|----------------|---------------|-----------|----------|----------|-------|--------|------------------------|----------------------------------|
|                | μm            | s⁻¹       | s        | x 10⁶    | mM    | nA     | nM⁻¹                  | 10⁻¹⁸ mol/cm² impulse            |
| 150575A‡       | 625           | 50        | 600      | 30       | 2     | 0.12   | 5                     | 2.6                              |
|                |               | 50        | 600      | 30       | 10    | 0.37   | 24                    | 12                               |
| 210575B‡       | 600           | 60        | 180      | 10.8     | 10    | 0.18   | 8                     | 11                               |
| 050675B‡       | 500           | 60        | 150      | 9        | 37    | 0.42   | 18                    | 25                               |
| 060575-1§      | 550           | 50        | 300      | 15       | 10    | 0.10   | 9                     | 9                                |
* From columns 3 and 7, Table III; 1 nA = 42 nM.
‡ Aequorin.
§ Arsenazo III.

**FIGURE 19.** Effect of external Na replacement upon resting glow. The legend at the top of the figure indicates when external Na was replaced, first by choline and then by Li. The resting glow increased steadily when external Na was replaced by choline, indicating a steady increase in internal ionized Ca in the center of the fiber. The effect was reversed by external Li, indicating that some Na-Ca exchange was possible under these circumstances. A final solution change returned the fiber to Na containing saline, which allowed the internal Na to be pumped down to near the initial resting level. External saline contained 3 mM Ca during the entire experiment. Ordinate, photomultiplier current in nanoamperes.
the increase to be expected from the net influx of Ca as the following rough
calculation shows: Net flux in 3 mM Ca (Na) seawater = 0; influx in 3 mM Ca
(choline) seawater = 1 pmol/cm²s; efflux in 3 mM Ca (choline) seawater = 0; net
flux in 3 mM Ca (choline) seawater = 1 pmol/cm²s; \([\text{Ca}]_t\) change in 3 mM Ca
(choline) seawater = 2.4 \(\mu\text{M/min.}\)

It was of interest to compare the effects of choline and Li seawater because
previous work (Brinley et al., 1975) showed that Ca efflux from squid axons at
\([\text{Ca}]_i\) of 100 nM or less was the same for Na or Li seawater. A change from
choline to Li seawater clearly reduced the aequorin glow and since Ca influx is
either the same or even a little higher in Li than in choline seawater (cf. Baker et
al., 1969) it is clear that the effect of a change from choline to Li on the net Ca
flux is a result of increasing Ca efflux. A change to Na seawater decreased the
\([\text{Ca}]_t\) still further. This finding might be thought to be the result of a larger Ca
efflux in Na as compared to Li seawater. However, Li is known to allow a Ca
influx that is 10–50 times as great as that from Na seawater, while Ca efflux at a
\([\text{Ca}]_i\) of 20 nM is the same in both Li and Na seawater. Thus the change from
Li to Na seawater can be expected to reduce \([\text{Ca}]_t\) principally by reducing Ca
influx.

EFFECT OF CN ON AQUEORIN GLOW Treatment of squid axons with CN is
known to result in a decrease in [ATP] as a result of the block of electron flow in
mitochondria. After 1–2 h in CN-containing seawater, ATP levels in axoplasm
are reduced from the normal value of 4 mM to approximately 100 \(\mu\text{M}\) (Caldwell,
1960; Mullins and Brinley, 1967). Another effect of stopping electron flow in
mitochondria is that the ability of these organelles to retain the Ca that they
normally accumulate is impaired. In aequorin-injected axons, Baker et al. (1971)
found nearly a 1,000-fold increase in light emission during CN treatment. We
undertook similar experiments to examine effects of CN treatment when the
dialysis capillary containing aequorin was near the axis of the fiber.

Fig. 20 (insert) shows an experiment where an axon was first treated with
choline seawater, producing a modest rise in light emission. With a more
condensed time scale it also shows the effect of this choline substitution followed
by a period of CN treatment of the axon. The axon was in 10 Ca seawater
throughout these treatments. Since prolonged CN treatment allows \([\text{Ca}]_i\) to rise
to levels that consume all of the aequorin, the CN exposure was brief and full
recovery of the resting level of light emission was obtained. This result confirms
those of Baker et al. (1971). The conclusion that the increase in light emission
results from an increase in \([\text{Ca}]_i\) is supported by the experiment in Fig. 21. In
this experiment the axon was microinjected with sufficient EGTA to make the
final concentration in axoplasm 0.47 mM after dilution. Subsequent exposure to
CN failed to produce any increase in light emission, as would be expected given
the large quantity of free EGTA present in axoplasm. In fact, a slight decrease is
seen in the figure starting about 10–15 min after exposure to CN. This decrease
roughly parallels the increase in ionized Mg which occurs in CN (see Fig. 25) and
probably represents Mg inhibition of aequorin luminescence.
Figure 20. Effect of CN upon resting glow. The first 160 min of this experiment shown in insert at an expanded vertical scale. Dashed line indicates period of exposure to 2 mM CN. Axon bathed in 10 mM Ca seawater throughout experiment. Exposure to test solutions was not long enough to establish new steady state.

Figure 21. Lack of effect of CN upon resting aequorin glow in axon preinjected with sufficient EGTA to make final concentration in axoplasm 470 μM. Dashed line indicates period of exposure to 2 mM CN. External [Ca] indicated at top of figure.
In Fig. 22 the experimental protocol was varied by placing the axon in 0 Ca seawater simultaneously with the addition of external CN. In contrast to the marked rise seen in Fig. 20 when the external solution contained CN plus Ca, the aequorin glow in this experiment increased only very slightly during a period of almost 2 h in CN. However, when CN plus 3 mM Ca was placed outside, there was a pronounced increase in the aequorin glow to a level almost 100 times that of the resting glow. The light level would undoubtedly have risen higher had not the CN been removed to prevent burn-up of the aequorin.

It could be argued that the failure to observe a rise in aequorin glow in the experiment shown in Fig. 22 was due to the fact that the axon was losing Ca as fast as it was being released from internal stores. To investigate this possibility, the experiment shown in Fig. 23 was done. Here, the fiber was kept in a Ca-free, Na-free solution during the entire period of exposure to CN seawater. In these circumstances the Na-Ca coupling mechanism cannot function and as Blaustein and Hodgkin (1969) have shown, Ca efflux falls to very low levels. Despite the fact that under these circumstances the axon presumably could lose little Ca, there was essentially no increase in the level of aequorin glow until, after almost 2 h of CN treatment, 0.1 mM external Ca was applied to the fiber. The aequorin glow began to rise directly after this application was made, and the aequorin...
Figure 23. Effect of 2 mM external CN on level of ionized [Ca] in squid axons. Periods of exposure to CN indicated by the dashed lines. Ordinate, light emission plotted relative to the resting level after 20 min in 3 mM Ca saline solution. The ionized [Ca] did not rise appreciably until the Ca-free external solution was replaced by one containing Ca.

glow increased still further when 1 mM Ca was placed outside the fiber.

This result cannot be ascribed to peculiar behavior of aequorin. Fig. 24 shows that essentially the same result was obtained when arszenazo III was used as a Ca indicator. It can be seen that there was very little rise in ionized Ca during the period of almost 2 h in CN, until the external solution was replaced with one containing 10 mM Ca.

It is possible that the failure to observe an increase in aequorin glow was due to the fact that for some reason CN treatment did not reduce the ATP levels in these experiments. To check on this possibility, the experiment in Fig. 25 was done. In this experiment an axon was injected with Eriochrome Blue SE to serve as an indicator for ionized Mg (Scarpa, 1974). In this experiment it can be seen that when the fiber was treated with CN the ionized Mg began to increase after a delay of some 10 min; it reached its maximal level after about 90 min of exposure to CN. Since the only significant reservoir of bound Mg, releasable by CN, in squid axons is in the phosphonucleotides, this experiment indicates that the breakdown of nucleotides was essentially complete at the end of 90 min of exposure to CN, a result fully in agreement with the original experiments by Caldwell (1960).

The actual increase in ionized Mg resulting from CN treatment is probably greater than that shown in Fig. 25 (about 1 mM). The breakdown of phosphonucleotides associated with CN treatment of course releases considerable phos-
FIGURE 24. Effect of 2 mM external CN on the concentration of ionized Ca in an axon injected with arsenazo III to give a final concentration of dye in axoplasm of 1.28 mM. Dashed line indicates period of exposure to CN. Ordinate, change in internal ionized Ca. Absolute level not measured in this experiment. Note that ionized Ca increases no more than 100 nM during 90 min in CN.

FIGURE 25. Effect of 2 mM external CN on ionized [Mg] in an axon injected with sufficient Eriochrome Blue SE to give a final concentration of 1.08 mM in axoplasm. After an initial delay of about 10 min, the ionized [Mg] rises steadily and reaches a plateau after 90 min of exposure to CN. Vertical bar gives differential absorbance change corresponding to change in ionized Mg as determined in subsequent in vitro calibration.

phate and this circumstance can result in significant lowering of pH in axoplasm (about 0.3 pH units. Boron and De Weer, 1976). The effective dissociation constant for Eriochrome blue SE is educed substantially by pH in the range 7.0-7.5, consequently acidification of the axoplasm reduces the differential
absorbance change for Mg and leads to an underestimate for the actual increase in Mg associated with CN treatment.

To summarize the experimental findings with CN⁻, [Ca]ᵢ increases to high levels when an axon is treated with CN in 10 Ca seawater (Fig. 20) and this increase in [Ca]ᵢ is abolished if an axon is injected with EGTA before CN treatment (Fig. 21). If an axon is kept in Ca-free seawater during exposure to CN⁻, there is only a modest increase in [Ca]ᵢ (a doubling) but upon application of 3 mM Ca seawater there is a 75-fold increase in light emission (Fig. 22). If the seawater is Na free, then a [Ca]ᵢ of only 0.1 mM is sufficient to induce a large increase in [Ca]ᵢ (Fig. 23). A similar result is seen with the arsenazo method (Fig. 24). As to whether the exposures to CN⁻ were long enough to deplete ATP, an experiment where free Mg was measured spectrophotometrically, showed that in 90 min, free Mg (released from complexation with ATP) was maximal. The increases in [Ca]ᵢ observed with CN in Ca containing seawater can be quickly reversed by removing CN⁻, hence the [Ca]ᵢ increases were not an irreversible change such as the development of a membrane leak to Ca.

The conclusion from these experiments is that CN treatment does not produce a marked increase in internal ionized Ca in the absence of extracellular Ca. This point will be considered further in the Discussion.

**DISCUSSION**

**Concentration of Ionized Ca in Resting Squid Axons**

Although the estimates of internal ionized [Ca] as determined by aequorin (20 nM) (equivalent to a CaEGTA/EGTA ratio of 0.13) and arsenazo III (50 nM) (equivalent to a CaEGTA/EGTA ratio of 0.33) differ by a factor of 2.5, they are reasonably concordant considering the extremely low level of ionized Ca measured and the fact that the two techniques are subject to entirely different sorts of error. With the aequorin method, a possible source of artifact arises from the fact that the ionized Ca is so low that in order to calibrate with EGTA buffers which produce light emission comparable to the resting glow of the axon, it was necessary to use ratios of CaEGTA/EGTA that were approximately 0.1. At this ratio EGTA buffers the internal axoplasm rather poorly.

For arsenazo, since the differential absorbance measured was strictly proportional to the concentration of Ca arsenazo III, it was not necessary to calibrate the reaction at low ratios of CaEGTA/EGTA. However, the method did depend upon the comparison of in vitro calibrations with in vivo measurement, which necessitated corrections, albeit small ones, for differences in dye concentration and optical path length. The method also involved injection of micromolar concentrations of Ca into the fiber (present as contamination of the arsenazo). However, reasons have been given in the Results section for believing that if this injected Ca load raised the steady state of ionized Ca, the effect was not dependent upon the amount introduced but was maximal at the lowest Ca loads injected. The final result also depends upon a large correction for chelated Mg.

Because the arsenazo was distributed throughout the axoplasm, it measures a mean concentration rather than just a concentration at the central core. In
the presence of a significant inward leak of ionized Ca sufficient to generate a quasi steady-state gradient of Ca from axolemma to axis of the fiber, the mean concentration would be expected to be higher than the axial concentration.

Both methods depend on CaEGTA/EGTA ratios for calibration. In order to convert these ratios into concentrations, it is necessary to decide upon the appropriate apparent dissociation constant for CaEGTA in the axoplasm. The ionic constituents of axoplasm as reported by Defner (1961) are roughly equivalent to an ionic strength of approximately 0.3. The data in Fig. 5 indicate that for mixtures of KCl and K isethionate at that ionic strength, the apparent dissociation constant for CaEGTA in axoplasm is 0.5 μM. This value should be reduced to 0.15 to allow for the difference in pH between these measurements (7.0) and the pH of axoplasm (7.3, Boron and De Weer, 1976). Using this value, the ionized [Ca] of axoplasm at flux balance (3 mM Ca0) is estimated to lie between 20 and 50 nM.

Under conditions of flux balance at the plasma membrane, we have been unable to construct any model system of buffers and sinks for Ca that will give a gradient in [Ca] from the membrane to the core. We believe, therefore, that aequorin or arsenazo measurements made at flux balance actually specify the [Ca] everywhere in the axoplasm. However, when there is a net flux of Ca, the aequorin capillary only measures the [Ca] in the immediate surroundings, and arsenazo a mean [Ca] throughout the axoplasm.

Other Estimates of Ionized Ca

Baker et al. (1971) measured the resting ionized Ca using aequorin-injected axons. Their results also depended upon matching the resting glow with a “calibration glow” resulting from the injection of EGTA solutions containing various ratios of free and bound EGTA. They reported an upper limit of 350 nM assuming an effective dissociation constant of CaEGTA appropriate for an internal ionized Mg of 10 mM. Subsequent determination of the ionized [Mg] indicated it to be about 3 mM (Baker and Crawford, 1972; Brinley and Scarpa, 1975). Therefore their resting glows actually represented an ionized Ca which was lower than 350 nM.

Ionized Ca can also be estimated by determining the internal Ca level necessary for influx and efflux to balance. Ca influx from 10 Ca seawater as measured either with the extrusion or dialysis techniques amounts to about 0.1–0.2 pmol/cm²s. Ca efflux studies in which efflux was measured as a function of internal ionized Ca (Brinley et al., 1975) suggest that an ionized Ca of about 100 nM would be required to generate an efflux sufficient to balance this influx. However, the present result that ionized Ca is in a steady state at 2–3 mM Ca0 rather than 10 mM requires that the ionized Ca in axoplasm be even lower than 100 nM.

[Ca]i can also be calculated from the equilibrium potential for Ca. Baker et al. (1971), in analyzing Ca movement through the Na channel, found that an $E_{\text{Ca}}$ of 180 mV in 112 mM Ca seawater was necessary to fit their curves. Since an equilibrium potential of 180 mV represents a concentration ratio of $1.6 \times 10^6$, the internal ionized Ca was therefore $112 \times 10^{-3}/1.6 \times 10^6 = 70$ nM.
Meech and Standen (1975) have shown in voltage clamped Helix neurons that it is possible to obtain a reversal of the Ca current and that the reversal potential corresponds to a [Ca] of between 30 and 80 nM. Their values are in good agreement with other estimates for the [Ca] of squid axons.

Effects of [Ca] on [Ca]i

The present results demonstrate that the axoplasm in fibers bathed in artificial salines containing 10 mM Ca increased in ionized Ca at a slow but significant rate. The increase in light emission of a fiber in 10 mM Ca seawater is, from Fig. 17, +0.01 nA/min. From the collected data in Table III, 1 nA is taken as equivalent to 42 nM of ionized Ca. Therefore, the rate of increase of ionized Ca in resting fibers in 10 mM Ca is about 0.4 nM/min, or roughly 1%/min. The net influx calculated from this rate of increase is about 10^-15 mol/cm^2s, but the true net influx across the axolemma could be much higher if the buffering capacity of axoplasm absorbs most of the net influx.

One obvious explanation for the observed increase is that the trauma of dissecting and mounting the axon has increased the Ca permeability of the membrane allowing the inward driving force to move Ca inward at a greater rate.

An alternative explanation is that an external Ca of 10 mM is unphysiologically high. Blaustein (1974) estimated that the [Ca] in squid hemolymph is 7 mM, of which 3 mM is complexed to sulfate, leaving an effective [Ca] of about 4 mM which is reasonably close to the extracellular Ca of 2.8 mM at which the axoplasmic Ca does not change.

Implications for Na-Ca Exchange

Our finding that [Ca], is in the range 20-50 nM and that an axon appears to be in a steady state when the [Ca] of seawater is 3 mM means that the concentration ratio for Ca across the membrane is about 10^5. The Na+ concentration ratio is about 10 in fresh axons so that, with an assumed membrane potential of -60 mV \((E_{ca} - E) = 205\) mV and \((E_{na} - E) = 120\) mV. If the Na gradient across the membrane is to supply the energy for Ca extrusion then \(zFE_{na} - E)n\) must be greater than \(zFE_{ca} - E)\), where \(n\) is the number of Na+ coupled to the extrusion of 1 Ca++. Since the energy required for Ca extrusion is 0.41 × 10^6 joule/mol and the energy in the Na gradient is only 0.12 × 10^6 joule/mol, it is clear that \(n\) must be about 4 if the efficiency of coupling were 100%.

A previous study of the effects of membrane potential on Ca efflux from dialyzed axons (Mullins and Brinley, 1975) showed that when [Ca], was 100 nM or less, Ca efflux increased with membrane potential as if the rate-limiting step in the transport reaction were the movement of a single charge (such as the movement of 3 Na inward coupled to the movement of 1 Ca outward) while at [Ca], of 200 μM, Ca efflux was relatively insensitive to membrane potential. These results are compatible with the calculations in the foregoing paragraph and suggest that Ca extrusion may have a variable coupling ratio depending on the level of [Ca].
Buffering Capacity of Axoplasm

There are two lines of evidence to support the conclusion that the axoplasm can effectively buffer against changes in ionized Ca. From tracer measurements Hodgkin and Keynes (1957) concluded that Ca entered the axon during an action potential. By demonstrating an increase in light emission from aequorin-injected axons, Baker et al. (1971) verified that Ca actually appeared inside the axon. The present measurements of aequorin glow made during stimulation also confirm that Ca enters during the action potential, but indicate that the increase in ionized Ca produced by such activity is only a very small fraction of that which crossed the membrane, implying that most of the entering Ca was sequestered.

Another example of the buffering capacity of axoplasm is seen when axons are bathed in Na-free solutions (e.g., Fig. 19). Na-free solutions increase net Ca influx into axons substantially (Baker et al., 1969). The exact increment depends upon the storage condition for the axons, but in fresh fibers the net influx amounts to about 1.0 pmol/cm²s. For the axon illustrated in Fig. 19, this would amount to an increase of about 150 µM during the 50 min it was exposed to choline seawater. As in the case of Ca entry with stimulation, Baker et al. (1971) demonstrated that free Ca actually appeared at the inner surface of the membrane during exposure to Na-free solutions. In our experiments the ionized Ca rose only fivefold to a level no more than 100–200 nM, or about 0.1% of the expected amount, implying that the buffering capacity of the axoplasm must have absorbed essentially all the Ca which entered.

Effect of CN on Ionized Ca

The present results confirm the earlier observations of Blaustein and Hodgkin (1969) and Baker et al. (1971) that axons immersed in 10 mM Ca seawater and exposed to CN undergo a large increase in internal ionized Ca, but indicate that this increase does not occur in the absence of external Ca.

Since treatment with 2 mM CN should have blocked mitochondrial respiration (Doane, 1967), the simplest interpretation of our experiments is that the mitochondria contained insufficient releasable Ca to saturate the CN-insensitive Ca buffers in the axoplasm.

The magnitude of such buffering capacity was not measured directly in the present experiments. However, we have collected in Table V estimates of the Ca

---

**Table V**

**EFFECT OF Ca LOADING UPON IONIZED Ca IN AXOPLASM**

| Procedure                        | Ca load | Δ Ca | Ca load/Δ Ca |
|----------------------------------|---------|------|--------------|
| A Arsenazo injection             | 10-40 µM| 50 nM (max)| 200-800     |
| B Na-free solution (Fig. 19)     | 5 µM/min| 3 nM/min| 1,670        |
| C Electrical stimulation 9,000-30,000 impulses 10 mM [Ca]_o (Table IV) | 7 µM | 14 nM | 500 |
| D CN-treated axon, 0.1 mM [Ca]_o (Table IV) | 0.1 µM/min | 1 nM/min | 100 |

---
load imposed upon axons by several procedures used in this study. The data suggest that axoplasm can sequester 5-40 μM Ca with no more than a two- to threefold increase in ionized Ca. This conclusion is consistent with the recent observations of Baker and Schlæpfer (1975) of the existence of a Ca binding material in axoplasm with a capacity of around 30 μM. It is also consistent with some recent biochemical evidence (Rottenberg and Scarpa, 1974) that mitochondria can hold only about 1,000-5,000 times the external [Ca] in a form releasable by mitochondrial inhibitors.

If we take 5,000 as the maximum factor by which mitochondria concentrate Ca, then at 20 nM axoplasmic [Ca] the mitochondria have a [Ca] of 100 μM. This amount of Ca if released from the mitochondria, which occupy about 1% of the axon volume, would raise axoplasmic Ca about 1 μM, a change easily controlled by the axoplasmic buffers.

*Note Added in Proof*  Further consideration of the relationship between light output and ionized [Ca] is given in the following references: Moisescu, D. G., C. C. Ashley, and A. K. Campbell. 1975. Comparative aspects of the calcium-sensitive photoproteins aequorin and obelin. Biochim. Biophys. Acta. 396:133-140, and Blinks, J. R., F. G. Prendergast, and D. G. Allen. 1975. Photoproteins as biological calcium indicators. Pharmacol. Rev. 27:438.

We are indebted to Drs. L. Pinto and J. Cooper for helpful discussion concerning arsenazo III, to Dr. S. Spangler for computer programming, to Dr. P. De Weer for helpful suggestions concerning determination of dissociation constants, and to Dr. A. L. Lehninger for permitting us to use his recording spectrophotometer.

We thank the Director and Staff of the Marine Biological Laboratory, Woods Hole, Massachusetts for the facilities put at our disposal.

Aided by grants from the National Institutes of Health (NS05846, NS08386, HL15835) and the National Science Foundation (GB41593).

J. R. was a Fellow of the Grass Foundation; A. S. is an Established Investigator of the American Heart Association; R. D. was the recipient of a travel grant from the John Boulton Foundation, Caracas, Venezuela. T. T. was on leave of absence from the Universidad Peruana Cayetano Heredia, Lima, Peru.

*Received for publication 3 September 1975.*

**REFERENCES**

ASHLEY, C. C. 1970. An estimate of calcium concentration changes during the contraction of single muscle fibers. J. Physiol. (Lond.). 210:133P–134P.

AZZI, A., and B. CHANCE. 1969. The “energized state” of mitochondria: Lifetime and ATP equivalence. Biochim. Biophys. Acta. 189:141–151.

BAKER, P. F., M. P. BLAUSTEIN, A. L. HODGKIN, and R. A. STEINHARDT. 1969. The influence of calcium on sodium efflux in squid axons. J. Physiol. (Lond.). 200:431–458.

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

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

BAKER, P. F., and W. SCHLÆPFER. 1975. Calcium uptake by axoplasm extruded from...
giant axons of Loligo. J. Physiol. (Lond.). 249:37P–39P.

Blautstein, M. P. 1974. The interrelationship between sodium and calcium fluxes across cell membranes. Rev. Physiol. Biochem. Exp. Pharmacol. 70:33–82.

Blautstein, 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.

Boron, W. F., and P. De Weer. 1976. Intracellular pH transients in squid giant axons caused by CO₂, NH₃, and metabolic inhibitors. J. Gen. Physiol. 67:91–112.

Brinley, F. J., Jr., and L. J. Mullins. 1967. Sodium extrusion by internally dialyzed squid axons. J. Gen. Physiol. 50:2303–2331.

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.

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.

Burger, K. 1973. Organic Reagents in Metal Analysis. International Series of Monographs in Analytical Chemistry. Vol. 54. Pergamon Press, Oxford.

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.

Carini, F. F., and A. E. Martell. 1952. The effect of potassium chloride on the equilibrium between ethylenediaminetetraacetate and calcium ions. J. Am. Chem. Soc. 75:5745–5748.

Deffner, G. G. J. 1961. The dialyzable free organic constituents of squid blood; a comparison with nerve axoplasm. Biochim. Biophys. Acta. 47:378–388.

Doane, M. G. 1967. Fluorometric measurement of pyridine nucleotide reduction in the giant axon of the squid. J. Gen. Physiol. 50:2603–2652.

Flashka, H., and A. J. Barnard, Jr. 1969. Chelates in Analytical Chemistry. Vol. 2. Marcel Dekker, Inc., New York.

Hodgkin, A. L., and R. D. Keynes. 1956. Experiments on the injection of substances into squid giant axons by means of a microsyringe. J. Physiol. (Lond.). 131:592–616.

Hodgkin, A. L., and R. D. Keynes. 1957. Movements of labelled calcium in squid giant axons. J. Physiol. (Lond.). 138:253–281.

Meech, R. W., and N. B. Standen. 1975. Potassium activation in Helix Aspersa neurones under voltage clamp: A component mediated by Ca influx. J. Physiol. (Lond.). 249:211–239.

Meves, H., and W. Vogel. 1973. Calcium inward currents in internally perfused giant axons. J. Physiol. (Lond.). 235:225–265.

Michaylova, V., and P. Ilkova. 1971. Photometric determination of microscopic amounts of calcium with Arsenazo III. Chem. Acta. 53:194–198.

Michaylova, V. Y., and N. K. Kouliva. 1972. Complexometric determination of calcium with Arsenazo III as metallochromic indicator in a slightly alkaline medium. C. R. Acad. Bulg. Sci. 25:949–952.

Mullins, L. J., and F. J. Brinley, Jr. 1967. Some factors influencing sodium extrusion by internally dialyzed squid axons. J. Gen. Physiol. 50:2333–2355.

Mullins, L. S., and F. J. Brinley, Jr. 1975. The sensitivity of calcium efflux from squid axons to changes in membrane potential. J. Gen. Physiol. 65:135–152.

Pribil, R. 1972. Analytical Applications of EDTA and Related Compounds. International Series of Monographs in Analytical Chemistry. Vol. 52. Pergamon Press, Oxford.
Rottenberg, H., and A. Scarpa. 1974. Calcium uptake and membrane potential in mitochondria. *Biochemistry.* 13:4811–4817.

Savvin, S. B. 1959. Photometric determination of thorium and uranium with the reagent Arsenazo III. *Dokl. Akad. Nauk SSSR Biochem. Sect. (Engl. Transl.).* 127:1231–1234.

Scarpa, A. 1972. Spectrophotometric measurement of calcium by Murexide. *Methods Enzymol.* 24:343–351.

Scarpa, A. 1974. Indicators of free magnesium in biological systems. *Biochemistry.* 13:2789–2794.

Shimomura, O., and F. H. Johnson. 1969. Properties of the bioluminescent protein Aequorin. *Biochemistry.* 8:3991–3997.

Shimomura, O., F. H. Johnson, and Y. Saiga. 1962. Extraction, purification and properties of Aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. *J. Cell. Comp. Physiol.* 59:233–239.