Calcium Measurement in the Periphery of an Axon

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ABSTRACT Aequorin was microinjected into squid giant axons, the axons were stimulated, and the change in light emission was followed. This response was compared with that found when the axon, in addition to being microinjected with aequorin, is also injected with the dye phenol red. Large concentrations of phenol red injected into axons result in a high probability that photons emitted by aequorin, when it reacts with Ca in the core of the axoplasm, will be absorbed before they escape from the axon; photons produced by the aequorin reaction at the periphery of the axoplasm are much less likely to be absorbed. This technique thus favors observing changes in Ca, taking place in the periphery of the axon. Stimulation in 50 mM Ca seawater of an aequorin-phenol red-injected axon at 180 s⁻¹ for 1 min produces a scarcely detectable change in Ca,; the addition of 2 mM cyanide (CN) to the seawater produces an easily measurable increase in Ca, suggesting that mitochondrial buffering in the periphery is substantial. Making the pH of the axoplasm of a normal axon alkaline with 30 mM NH₄⁺-50 mM Ca seawater, reduces the resting glow of the axon but results in an even more rapid increase in Ca, with stimulation. In a phenol red-injected axon, this treatment results in a measureable response to stimulation in the absence of CN.

INTRODUCTION

The measurement of ionized Ca intracellularly is fraught with many difficulties as several recent investigations have underlined. One can, with some precision, measure the Caᵢ of an axon using arsenazo III (DiPolo et al., 1976); this measurement allows one to deduce an "average" value for Caᵢ throughout the axoplasm. Such measurements may be of interest if one is trying to establish values for Caᵢ that involve no component of a radial concentration gradient.

Another method that has been used to measure Caᵢ is to confine aequorin to a dialysis capillary at the center of an axon (DiPolo et al., 1976; Requena et al., 1977). Under these circumstances, the aequorin equilibrates with the local Caᵢ in the core of the axoplasm and a value is obtained that represents a Caᵢ in a highly local region. This value of Caᵢ may represent that for the entirety of the axoplasm, if radial [Ca] gradients can be eliminated.
In principle, one would like the same sort of measurement as that obtained with a dialysis capillary but with the measuring region confined to the periphery of the axoplasm just under the membrane rather than the core. The introduction of a dialysis capillary with the requisite positional accuracy appears beyond present techniques. Therefore in the experiments to be described recourse has been found in the injection of aequorin into the axon followed by the use of a variety of techniques that maximize light emission from the more peripheral parts of the axoplasm.

One technique that, in principle, ought to increase the sensitivity by which aequorin detects incoming Ca is to poison mitochondria with cyanide (CN) which prevents Ca uptake even though it allows mitochondria to retain previously accumulated Ca as long as ATP is at normal levels (Brinley et al., 1977 a). If buffering of Ca by mitochondria were an appreciable factor in attenuating the change in \( \text{Ca}^{2+} \) produced by, for example, Ca entry from bioelectric activity, then CN would be expected to enhance the response of aequorin to stimulation of the fiber.

A second factor identified as buffering Ca entering the axon is a nonmitochondrial buffer (Brinley et al., 1977 b). If this could be inhibited, then again the response of aequorin to entering Ca would be enhanced.

Finally, as regards stimulated Ca entry, the most interesting region is a thin section of axoplasm immediately under the excitable membrane, but most of the light in an aequorin-injected axon comes from the bulk of the axoplasm; if light emission in the bulk of the nerve fiber could be suppressed, measurements would then take place in the more peripheral parts of the axoplasm. One method of bringing about this differential sensitivity of light measurement is to inject a dye along with the aequorin. If the dye is selected such that it absorbs aequorin-emitted photons, then aequorin molecules in the center of the axon that emit photons subsequent to their reaction with Ca will have a much higher probability that the emitted photon is absorbed by the dye before it escapes from the axon than a photon from an aequorin molecule just under the membrane. Phenol red has been used as such a dye, both because it is known to be nontoxic and because it absorbs strongly at the aequorin emission peak. The results to be reported suggest that it confines the region in an axon where Ca is being measured to the more peripheral parts of the axoplasm.

**METHODS**

**Experimental Animals**

The squid used were collected and studied at the Marine Biological Laboratory, Woods Hole, Massachusetts, during May–June, 1978.

**Aequorin**

This material was a gift from Dr. O. Shimomura and Dr. F. Johnson and was prepared as previously described (DiPolo et al., 1976). Spectrophotometric measurements of the stock solution showed a protein concentration of 200 \( \mu \text{M} \).
Microinjection

A microinjector described previously (Brinley and Mullins, 1965) was modified so that it operated horizontally rather than vertically. Microinjection thus could take place in a dialysis-type chamber with a light pipe array as previously described by Requena et al. (1977). Glass capillaries used for the microinjection of aequorin were stored in 10 mM K₂EGTA, pH 7, and just prior to use were extensively rinsed with 1 μM K₂EGTA.

Solutions of apyrase (Sigma Chemical Co., St. Louis, Mo.) were made in 1 M potassium N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (KTES), pH 7.3, at a concentration of 100 mg/ml. These were passed through a Chelex column (Bio-Rad Laboratories, Richmond, Calif.), the pH was readjusted to 7.3 with KOH, and the solution was stored at −20°C. Aliquots of this solution were thawed and injected over a length such as to overlap the aequorin injection by 3 mm at each end of the path. The usual lengths of injection were aequorin 15 mm and apyrase 21 mm.

Light Measurement

Light was measured as previously described (Requena et al., 1977), with the single exception that the 10⁴ μF capacitor at the output of the amplifier was removed so that the time constant for the light response was limited by the speed of the chart recorder pen (0.5 s). In addition, when very low light levels were to be measured, photon counting was sometimes employed.

The light output from the optical guides was fed into a housing (Pacific Photometric Instruments, model 3262/F-AD4, Emeryville, Calif.) which contained a specially selected photomultiplier tube for photon counting (EMI Gencom Inc., model 9524A, Plainview, N.Y.) excited at −900 volts DC. Model 3262/F-AD is a self-contained photon-counting instrument that detects photon by AC amplification of a photomultiplier output signal and converts the photon-derived pulses into standard output pulses for accumulation for 10 or 15 s by a high speed counter (John Fluke Mfg. Co., Inc., model 1953A, Mountlake Terrace, Wash.). The counts per second were displayed in a digital printout format (Fluke model 2010A) and plotted later.

The phototube housing was fitted with a shutter and a 55% transmission narrow-band dielectric interference filter tuned to 470 nm with a half-width transmission of 30 nm (Barr and Stroud model MD6, Enniseland, Glasgow, Scotland).

External Solutions

The seawater used had the following composition (millimolar): Na 455, K 10, Mg 50, Ca 3, TES (pH 7.8) 10, EGTA 0.1, Cl 571. Solutions having 37 or 50 mM Ca were obtained by replacing Mg with Ca to reach the desired concentration. Cyanide seawater was obtained by adding sufficient 500 mM NaCN solution adjusted to pH 8 to seawater to produce a final concentration of 2 mM. Choline seawater was prepared by replacing the Na in the above formulation with choline. 30 mM NH₄⁺ seawater was prepared by replacing 30 mM of NaCl with NH₄Cl. Solutions were all adjusted to 1,000 ± 10 mosM using a Wescor dewpoint osmometer (Wescor, Inc., Logan, Utah), and to pH 7.8 ± 0.05 with a pH meter.

Phenol Red Solutions

Phenol red, 500 mg, was added to 5 ml of 1 M KTES buffer adjusted to pH 8. The solution was passed through a Chelex column and final adjustment of the phenol red solution pH was made by adding 1 M KOH or distilled water to bring the final
solution volume to 6.5 ml. Since the molecular weight of phenol red is 354, the nominal concentration was 220 mM. The solution was centrifuged and an aliquot was taken for spectrophotometric measurement. This solution was diluted 1:100 with 0.5 M KTES buffer and placed in a 1-mm path length cuvette. The absorbance was measured at 465 nm (the emission peak of aequorin) and a scan was made over 400–520 nm to examine the shape of the absorption peak. At 465 nm the absorbance was 1.85 at pH 8 and 2.25 at pH 7. The shape of the phenol red absorption peak over the range pH 7–8 reasonably approximates the emission peak of aequorin since peak absorption of phenol red at pH 7 is 430 nm and the half-band width is ±30 nm, while for aequorin (Shimomura and Johnson, 1970) the peak is 465 nm and half-band width is ±30 nm. Note that the isosbestic wavelength is 470 nm so that phenol red does not act as a pH indicator in this region of the spectrum.

We have taken a mean value for phenol red absorbance as 2.0 at the aequorin emission peak for a 1:100 dilution of the solution used and for a 0.1-cm path length. In a 600 μm axon the dilution of a phenol red solution is 28-fold so the absorbance of the solution upon dilution is 2.0 (100/28) = 7.14. Io/I is therefore 10^{7.14} = 1.38 × 10^7. For use in the equations that follow, (1/1.38 × 10^7) = exp − λ(0.1) where λ is extinction and 0.1 the path length in centimeters. Thus, λ = 164.

As an optical filter, phenol red has, as a first approximation, an absorption that superimposes on the light emission spectrum of aequorin. The analysis of how light emitted from any point inside the axon is likely to be measured can be understood by the reference to the diagram in Fig. 1. Here A is axon radius taken as 0.03 cm, x is the location of any arbitrary point in the axoplasm from which one wishes to evaluate the absorption of emitted photons by phenol red, and r, θ, define the path length and
direction of photons emitted from the point. Now
\[ r = x \cos \theta + \sqrt{A^2 - x^2 \sin^2 \theta} \]
\[ = A \left[ \frac{x}{A} \cos \theta + \sqrt{1 - \left( \frac{x}{A} \sin \theta \right)^2} \right]. \]

If \( I/I_0 \) is defined as the fraction of the light generated that escapes from the axon, then since \( I/I_0 = \exp - \lambda r \), where \( \lambda \) is the extinction for the concentration of phenol red used, then
\[ I = I(x) = I_0 \frac{1}{\pi} \int_0^\pi \left( \exp - A\lambda \left[ \frac{x}{A} \cos \theta + \sqrt{1 - \left( \frac{x}{A} \sin \theta \right)^2} \right] \right) d\theta \ dx. \]

If we assume that \([\text{Ca}]\) in axoplasm is everywhere uniform, then the same number of photons will be produced in each element of axoplasm of equal volume. If we are to evaluate all the photons escaping from a circle inside the axon with radius \( x \), then
\[ I_x = \int_{x=0}^x \int_{\phi=0}^{2\pi} I(x) \, d\phi \, dx = 2\pi \int_{x=0}^x xI(x) \, dx. \]

The relative number of photons escaping from a point can be expressed by dividing \( I_x \) by the total number of photons
\[ I_A = \pi A^2 I_0; \]
thus
\[ I_x | I_A = \frac{2}{A^2} \int_{x=0}^x \frac{1}{\pi} \int_0^\pi \left( \exp - A\lambda \left[ \frac{x}{A} \cos \theta + \sqrt{1 - \left( \frac{x}{A} \sin \theta \right)^2} \right] \right) d\theta \ dx, \]
for \( 0 \leq x \leq A \).

We may replace the variable \( x \) (or \( x/A \)) with a volume integration where the volume of a ring is \( 2\pi x dx = dv_x \) and a circle \( \pi x^2 = v_x \). We can also express the volume and the radius relative to \( A \),
\[ v = \frac{v_x}{\pi A^2} = \frac{x^2}{A^2}, \quad \frac{x}{A} = \sqrt{v}, \quad dv = \frac{2x \, dx}{A^2}; \]
and then rewrite the integral in terms of \( v \):
\[ I_v = I_x | I_A; (v_x = x^2/A^2) = \int_{v=0}^v \int_{\theta=0}^\pi \frac{1}{\pi} \exp - A\lambda[v^{1/2} \cos \theta + \sqrt{1 - v \sin^2 \theta}] \, d\theta \, dv, \]
for \( 0 \leq v_x \leq 1 \).

A plot of \( I_v \) vs. both volume and radius of the fiber is shown in Fig. 1. One can note that half of the light being emitted from the fiber comes from 17% of the most peripheral volume of axon, and that this corresponds to a rim of axoplasm 25 \( \mu m \) thick. The plot also shows that the fraction of photons escaping from the axon is 0.13 of the number actually being produced.

This value of 0.13 can be compared with that in Table I, which shows that
measured light emission falls to 0.09 of its initial value when phenol red is injected into a fiber.

RESULTS

The presentation of experimental information is organized in the following way: first, the light emission from an aequorin-injected axon in response to stimulation is compared with that of an aequorin-phenol red-injected axon. Next, we have examined in detail the response measured as light emission to the transfer of both normal and phenol red axons from low Ca\(_{o}\) (1–3 mM) seawater to high (37–50 mM) Ca\(_{o}\) seawater. This study was necessary since the response to stimulation can only be observed with high Ca\(_{o}\) while the maintenance of a normal Ca content in an axon requires a low Ca\(_{o}\). Periods of low Ca\(_{o}\) serve, therefore, to allow an axon to recover from imposed Ca loads.

Since there was a possibility that metabolism might affect pH, and that this in turn might alter the measured responses of both normal and phenol red axons, measurements were made while a normal axon was treated with 30 mM NH\(_{4}\) seawater, a medium shown by Boron and DeWeer (1976) to increase axoplasmic pH to about 8.0. These measurements were repeated on phenol red-aequorin-injected axons.

Finally, since Requena et al. (1977) showed that the injection of apyrase altered the response of axons to low Na\(_{o}\) solutions, it seemed necessary to repeat these observations to see if buffering in the axon periphery was altered by apyrase.

Stimulation in the Presence of CN

When an aequorin-injected axon is treated with 50 mM Ca seawater, there is a step increase in resting glow as shown in Fig. 2, followed by a further increase in light emission as stimulation (120/s for 60 s) proceeds. Subthreshold stimuli are without effect on the aequorin glow of an axon. The application

| Axon reference | Diameter \(\mu m\) | Initial Glow | After phenol red Glow | Ratio: after/ initial |
|----------------|------------------|--------------|-----------------------|----------------------|
| 110578B        | 666              | 28           | 2.5                   | 0.09                 |
| 220578B        | 700              | 126          | 9.0                   | 0.07                 |
| 190578B        | 600              | 140          | 9.5                   | 0.07                 |
| 260578         | 500              | 10           | 1.0                   | 0.10                 |
| 200578         | 600              | 28           | 1.1                   | 0.04                 |
| 290578B        | 575              | 41           | 8.0                   | 0.20                 |
| 170578B        | 620              | 80           | 10.0                  | 0.13                 |
| 230578         | 666              | 38           | 1.2                   | 0.03                 |

0.09
of 2 mM CN in 50 Ca seawater is known to block mitochondrial uptake of Ca (Brinley et al., 1977a) but does not affect the Ca previously accumulated as long as ATP is present in the axon. The application of such a substance might be expected to enhance the sensitivity of axons to stimulation, if mitochondria buffer Ca entering the fiber, and provided that most of the light response in Fig. 2 originated in the periphery. It is clear from the record that CN has only a very modest effect, if any, in altering the response. A conclusion from this sort of finding might be that mitochondria are not buffering the Ca that enters during bioelectric activity. However, as will be shown below, this conclusion is unwarranted. A final feature of the record is the very slow decline of light following stimulation and transfer of the axon to 3 mM Ca seawater. The response to stimulation consists of an initially rapid rise in light emission that is followed by a slower rise. Emission ultimately reaches a plateau if stimulation is continued long enough. The decline in light emission after stimulation consists of a rapid (time constant ~ 1 min) and a slow (time constant ~ 20 min) phase that is not visible on the record shown in Fig. 2. It seems useful to define \( \Delta L \) (following Baker et al., 1971) as the initial increment in light emission per impulse. Table II gives the mean value for \( \Delta L \) for axons stimulated in 50 mM Ca seawater ± 2 mM CN. Clearly there is no difference in the mean response of the axons, whether or not CN is present.

**Stimulation in Phenol Red-Injected Axons**

If the experiment outlined above is performed on an axon injected with aequorin and a solution of 204 mM phenol red in 1 M KTES, pH 8, a record similar to that in Fig. 3 will be obtained. The resting glow is 1/20th that of the axon before phenol red; the response to the change from 3 Ca to 50 Ca seawater is a transient increase and decrease in glow rather than a step change; the response to stimulation (200/s) is virtually undetectable. The application of CN to the axon, however, yields a large increase in aequorin

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**Figure 2.** The response of an aequorin-injected axon to stimulation. Photo-multiplier current is plotted on the ordinate vs. time for an axon initially in 3 Ca seawater. Note large differences in initial and final resting glow levels. The notation of 3 Ca or 50 Ca means 3 mM Ca (Na) seawater or 50 mM Ca, and S-120-60 means stimulation, 120/s for 60 s.
light emission upon stimulation suggesting that buffering (of a CN-sensitive sort) is important for Ca entering during bioelectric activity. Recovery to resting glow levels is prompt. A summary of the response to stimulation in phenol red-treated axons is given in Table I, from which it can be concluded that in the presence of CN, the axons show as large an initial response to stimulation as do control axons. The question arises as to why there is not an enhancement in the aequorin response to stimulation in the presence of CN when one is observing light emission from the whole axon; the answer would appear to be that such an enhancement is masked by the diffusion of the entering Ca from the periphery to the core—unless such light emission can be masked, the response to stimulation does not accurately reflect changes in [Ca].

**TABLE II**

Mean Responses to Stimulation

| Condition               | ΔL pA/impulse | ΔL % Control |
|-------------------------|--------------|--------------|
| 50 Ca seawater          | 95 ± 17 (n=22) |
| 50 Ca seawater + 2 mM CN  | 21          | 100 ± 5 (n=7)  |
| 50 Ca seawater + 30 mM NH₄ | 382 ± 50 (n=11) |
| 50 Ca seawater + phenol red  | 5 ± 2 (n=5)   |
| 50 Ca seawater + phenol red + CN | 15          | 100 ± 30 (n=13) |
| 50 Ca seawater + phenol red + NH₄ | 376 (n=2)    |

The response of a phenol red-injected axon to high-Ca seawater is of some interest. An extreme example is shown in Fig. 4. This is a step change in light emission taking about 40 s to accomplish. In phenol red axons this response is always a transient that presumably relates to the speed of Ca buffering: Ca influx is increased almost 37-fold by the change from a Ca₀ 1–37 mM, and [Ca]ᵢ rises until mitochondrial buffering processes commence. There is then a rebound that brings [Ca]ᵢ to levels where fluxes balance. The phenomenon is not related to aequorin depletion since subsequent stimulation of this axon in CN seawater produced a clear response. Note the absence of a response to stimulation and the small decline in glow in going from 37 Ca → 1 Ca.

One of the points that seemed important to establish was whether the response to stimulation in a phenol red-injected axon without inhibitors such as CN was similar to that found in normal axons. An evaluation of this response was difficult using our conventional recording method since the response to stimulation was so small (see Fig. 3). Accordingly, we used photon counting and obtained the results shown in Fig. 5. The axon was first stimulated at 100 s⁻¹ for 1 min in 37 mM Ca seawater, and the response is shown as solid points. This gives a symmetrical rise and fall of [Ca]ᵢ with a time constant of about 30 s, a value similar to that found by Baker et al.
One also notes that the stimulation about doubles the resting glow. A second stimulation of the same axons at 100 s\(^{-1}\) for 120 s gave much the same result both in terms of the rise and fall of \([\text{Ca}]_i\) and in terms of time constants. The mean response of such axons to stimulation was, however, only 5\% of that of phenol red axons with CN as shown in Table II.

**The Effect of FCCP on Ca Release**

It has been established by Requena et al. (1977) that 10 \(\mu\text{M}\) FCCP produces an immediate release of Ca contained in the mitochondria of an axon when it is applied externally in seawater. In a freshly dissected axon, the magnitude of the change in \([\text{Ca}]_i\) measured at its center is a threefold one; this suggests that mitochondria have relatively little Ca to release.

![Figure 3](image)

**Figure 3.** An axon was injected with aequorin and its response to stimulation at 200 s\(^{-1}\) and 60 s\(^{-1}\) measured. It was then injected with phenol red (pH 8) and light output (as photomultiplier current) followed with time.

When FCCP is applied to an axon that had been injected with aequorin and phenol red, and kept in Ca-free seawater for 15 min, the result obtained is shown in Fig. 6. Photon counting was also used in this experiment because it was anticipated that a very large change in \([\text{Ca}]_i\) would be encountered and in fact the count went from a resting value of 3 to a peak value of 650 (both times \(10^5\) counts/s). This is over a 200-fold change and, if over most of the range a square-law relationship\(^1\) between \([\text{Ca}]_i\) and light obtained, then there would have been a 14-fold increase in \([\text{Ca}]_i\) at the peak. It is difficult to make a quantitative comparison between this sort of result and that found with dialysis capillaries on the axis of the axon (a threefold change in \([\text{Ca}]_i\) with FCCP), but it is clearly in the direction expected if the distribution of Ca in mitochondria is nonuniform and if, in fact, most of such Ca is held in the most peripheral mitochondria.

The recovery from the FCCP release is rapid, with a time constant of the order of 5 min. Most of this recovery results from the diffusion of Ca from the

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\(^1\) Blinks et al. (1976) have shown that, in the range of \([\text{Ca}]_i\) from \(10^{-8}\) to \(10^{-7}\) M, light emission increases roughly in a linear manner whereas, from \(10^{-8}\) to \(10^{-4}\) M, the light emitted is proportional to the square of \([\text{Ca}]_i\). We consider, therefore, that at physiological concentrations of \([\text{Ca}]_i\), aequorin light emission directly reflects \([\text{Ca}]_i\) changes whereas, for very large Ca releases such as that mentioned here, most of the light emission involves a square law relationship.
periphery to the "dark" part of the axon (since the time constant for Ca pumping is ~ 30 min), but part of the Ca is pumped out by the Na/Ca pump.

**Effect of NH₄ on Aequorin-Injected Axons**

Calculations given in Methods indicate that the injection of phenol red should reduce the resting glow of aequorin about eightfold, while the observed reduction (Table I) is 11-fold. It should be noted that the injection of a substance such as phenol red could, in addition to its purely optical effects (a) inhibit the aequorin reaction chemically, (b) interact with the buffering systems of axoplasm to change Caᵢ, and (c) have an effect that is dependent on the pH of the phenol red solution.

To examine some of these possibilities, a number of control experiments were carried out. Perhaps the simplest is to change pHᵢ in an aequorin-injected axon by the use of NH₄⁺ to produce an internal alkalinity (Boron and DeWeer, 1976). These authors found that with 50 mM NH₄⁺ a highly reproducible shift of pH in an alkaline direction of about 0.85 unit could be produced in squid axons with a time constant of about 6 min, whereas a shift of 0.45 unit was produced by 10 mM NH₄⁺. Interpolation between these values suggests that 30 mM NH₄⁺, the concentration we selected for use, would shift pHᵢ 0.7 unit, and since mean normal value for pHᵢ is 7.3, this would mean that our final pH would be 8.0. Higher concentrations of NH₄⁺ were not used because the
ion is depolarizing and much of the experimentation we carried out involved repetitive stimulation.

An experiment is shown in Fig. 7 where an aequorin-injected axon was tested for response to stimulation first in 50 mM Ca seawater (trace a), followed by treatment with 50 Ca seawater plus 30 mM NH₄⁺ for 10 min (trace b). This resulted in a fourfold decline in resting glow, and upon stimulation (trace b), a totally different shape of wave form (a square wave), and a response of substantially lower amplitude than that produced in the absence of NH₄⁺. Removal of NH₄⁺ resulted in a rise of resting glow, and when the axon was tested 6 min after NH₄⁺ removal, it gave the response in trace c. The response to NH₄⁺ seawater is always fully reversible and for this particular

axon there were three separate treatments with NH₄⁺ followed in each instance by complete reversal both of the resting glow and wave form of response to stimulation.

A more detailed experiment is shown in Fig. 8. Here stimulation at a variety of frequencies produced a linear response of initial increase in light with frequency and the typical response of a continuously rising light emission with time of stimulation. The application of NH₄⁺ seawater reduced the resting glow fourfold with a time constant of 4 min, or about the time required for the pH change to take place in axoplasm. The response to stimulation is now a square wave, and although the amplitude is smaller, the rate of rise of the light signal is faster so that from Table II one can note that ΔL, the initial increase in light per impulse, is 3.8 times the control response. Recovery from this change in pH, is rapid, and Fig. 8 shows a partial recovery and a final stimulation test with CN seawater that apparently increases the response to stimulation.

A similar but smaller response to pH change can be obtained by the
injection of 1 M KTES, pH 8, into the axon. In three axons so examined, the mean decline in resting glow was fourfold and the response to stimulation was as in Fig. 8. The smaller response is presumably related to a smaller change in pH, produced by this treatment. A detailed summary of all experiments where stimulation was performed in alkalinized fibers is given in Table III.

**Effect of NH₄ on Phenol Red-Injected Axons**

An increase in pH, in an aequorin-injected axon appears to have the following effects: (a) it reduces the level of resting glow and (b) the shape of the response to stimulation appears to change such that there is a more rapid rise and fall of glow although the amplitude of the response is substantially smaller. It appeared useful therefore to examine the aequorin response in phenol red-injected axons with and without NH₄.

Fig. 9 shows the response of an axon to 50 Ca seawater (the usual transient increase and decrease of glow), the virtually unmeasurable response to 180 s⁻¹ stimulation, the increase in this response with CN and the further enhancement of the response when NH₄⁺ was added to the seawater. The response was independent of whether CN was present or not, but there was an enhanced response at the end of the experiment that was not seen at its start.

**Apyrase-Injected Axons**

The use of the hydrolytic enzyme apyrase has been shown by direct measurement of ATP in axoplasm to reduce (ATP) to 10–20 μM in three axons used in the present studies. The enzyme has been used previously (Requena et al.
1977) to find out whether a change in \([\text{Ca}^2+]\) occurred in axons with a dialysis capillary containing aequorin in their center. These results showed that apyrase-treated axons had a normal \([\text{Ca}^2+]\) and could recover from a Ca load imposed by Na-free seawater. These studies did not rule out the possibility that there might have been a change in the Ca buffering induced by the absence of ATP so it seemed useful to examine the behavior of apyrase-injected axons with phenol red.

A typical record is shown in Fig. 10; the axon was first injected with aequorin and apyrase, then placed in 50 mM Ca seawater, and stimulated. It gave a normal response and resting glow declined in 1 mM Ca seawater, but the decline of glow was halted if the seawater were made Na-free (choline). Phenol red was then injected and a test stimulation made in 50 Ca seawater—there was no response as was usual for this solution. The addition of CN to 50 Ca seawater resulted in an immediate increase in resting glow, a result that is expected since mitochondria cannot retain Ca when ATP is absent and electron transport is blocked. Stimulation did produce a large response but a change to 1 mM Ca seawater produced a rapid (time constant about 2 min)
decline in resting glow. This is much too rapid a change to be the result of Ca pumping and must be mainly the result of diffusion of Ca into the "dark" regions of the axon. The decline in resting glow could be reversed by 1 mM Ca choline seawater, and resting glow attained its initial level in a subsequent test with 1 mM Ca (Na) seawater. Other apyrase axons studied have their responses to stimulation shown in Table III; the results are that the values for responses are not different from control axons, implying strongly that apyrase does not change the measured buffering properties of the axon either with or without phenol red. The use of choline shows that Nao is essential to the holding of Ca at low values even though ATP is virtually absent.

Figure 8. This aequorin-injected axon was stimulated at frequencies from 30 to 180 s⁻¹ and this treatment was followed by exposure to 30 mM NH₄⁺-50 mM Ca seawater. After test stimulations, NH₄⁺ was removed and the record shows a partial recovery at 50 min and an overshoot in sensitivity at 60 min.

Discussion

It is important to recall that the light signal from aequorin depends, in addition to the Ca:aequorin stoichiometry, on the quantity of Ca present (QCa)/V, where V is the volume in which the Ca is dissolved, and this expression for [Ca] must be multiplied by f(B₁, B₂, B₃...), where B represents the buffering of Ca by some intracellular buffering system. If buffering were uniform throughout the axoplasm and were a linear function of [Ca], a quantitative description of axoplasmic [Ca] would be possible. As the results obtained in this study show, however, there is a large increase in aequorin glow when a phenol red-injected axon is treated with FCCP (Fig. 6) implying a highly nonuniform storage of Ca in mitochondria. There is also a requirement for the "priming" of mitochondria with Ca (Fig. 4) since large excursions in [Ca] at the periphery occur before buffering commences, yet buffering then continues at low levels of [Ca]. In addition to mitochondrial buffering, another buffer that is CN-insensitive was first described by DiPolo et al. (1976) and studied further by Brinley et al. (1977a, b) and by Baker and Schlaepfer (1978). In the present study it would appear that this buffer is responsible for
the pH effects that are observed—the results imply that at pH\textsubscript{i} = 8, the affinity of this buffer for Ca is greatly increased (and hence Ca\textsubscript{i} falls).

**The Response to Stimulation**

This is an initially rapid (20 pA/impulse) increase in light output that rapidly decreases in slope although the light output continues to increase slowly for some minutes. Recovery to initial resting glow levels is quite slow. This entry

![Figure 9](image-url)

**Figure 9.** This is an aequorin-phenol red-injected axon; a comparison is made of the light response (as photomultiplier current in microamperes) vs. time when the axon is stimulated in CN-free, CN, and NH\textsubscript{4}-containing seawater. The label "24 s" represents a portion of the recording taken at a fast chart speed to show that the \( \frac{1}{2} \) rise time with stimulation is \( \sim 6 \) s.

of Ca into a nerve fiber can be diagrammed as shown below:

\[
\frac{[\text{CaX}]_1}{k_i} \quad \frac{[\text{CaX}]_2}{k_{12}} \quad \frac{[\text{CaX}]_2}{k_{23}}
\]

\[
\frac{[\text{Ca}]_0}{k_0} \quad \frac{[\text{Ca}]_1}{k_{12}} \quad \frac{[\text{Ca}]_2}{k_{23}}
\]

\[
\frac{[\text{CaM}]_1}{k_m} \quad \frac{[\text{CaM}]_2}{k_{12}}
\]

Here [Ca]\textsubscript{1} in the first element of volume can be considered to include that volume of axoplasm seen in phenol red injected axons while [Ca]\textsubscript{2} and all further volume elements can be considered the axon "core." The above diagram shows that [Ca]\textsubscript{1} in the first element of volume is set by the relative values for the rate constants of Ca entry \( k_i \) less its exit \( k_0 \) and values for mitochondrial buffering, \( k_m, k_{-m}, k_{-x} \) and for diffusion \( k_{12}, k_{21} \). A quantitative treatment for a single buffer without a back reaction has been given by Baker et al. (1971).

In a normal axon there is no effect of CN on the response to stimulation, while in a phenol red axon the response to stimulation is scarcely visible without CN. These findings suggest that mitochondrial buffering in the first element of volume above is extremely active ([Ca] remains low) and that virtually all the observed responses of a normal axon occur in volume elements 2 and beyond, where the concentration of Ca does not rise to the levels...
necessary for the initiation of mitochondrial buffering. There is, of course, the normal signal obtained from volume 1, but this is recorded at a gain such that >90% of the light comes from the axon core and this resting glow dominates the measured output.

In a phenol red-injected axon, the maximum rate of increase of light per impulse in CN seawater is the same as in a normal axon. The amplitude of the signal is small, implying that a balance is rapidly struck between entering Ca, buffering via the X buffer, and diffusion of Ca into the next element of axoplasmic volume where it is effectively "invisible."

![Image]

**Figure 10.** This axon was injected with apyrase and aequorin and the light emission followed. It was then injected with phenol red at the point marked on the record and the light emission again followed. The external solution is Na seawater except where "Ch" appears on the record and then it is choline seawater. Note that the application of CN leads to an immediate increase in resting glow with time since, in the absence of ATP, mitochondria begin releasing Ca upon poisoning electron transport.

Changes in pH

In a normal axon the effect of making the axoplasm alkaline is twofold: it decreases the level of resting glow and it changes the shape of the curve for light response vs. time of the axon to stimulation. The finding of an apparent decrease in the level of resting glow with increase in pH complements the observations of Lea and Ashley (1978) that the application of CO$_2$ to barnacle fibers injected with aequorin increased the resting glow. Their experiments made it clear that changes in pH did not result from a change in the balance between Ca influx and efflux and they ascribe their result to a Ca release from an internal store, most likely either the sarcoplasmic reticulum or another Ca binding protein. Our observations do not agree with those of Baker and Honerjäger (1978) who found little effect of 10 mM NH$_4$Cl on the light emission from aequorin-injected squid axons but a decrease in light upon acidification of the axon with CO$_2$.

Now a change in pH could in principle (a) affect the light emission of aequorin by changing the sensitivity of aequorin to Ca, or by changing the pump-leak relationship of Ca fluxes across the axon membrane, (b) affect the
TABLE III
RESPONSES OF AXONS TO STIMULATION

| Axon reference | t min | Treatment | Resting glow nA | Frequency s⁻¹ | Time s | Peak glow nA | Slope nA/s | ΔL pA/impulse |
|----------------|-------|-----------|----------------|---------------|--------|-------------|------------|--------------|
| 190578B        | 0     | 3Ca       | 0.140          | 200           | 60     | 300         | 2.5        | 12.5         |
|                | 20    | 50Ca      | 2.40           | 200           | 60     | 300         | 2.5        | 12.5         |
|                | 60    | 50Ca + PR | 1.5            | 200           | 60     | 300         | 0.7        | (2.2)        |
|                | 90    | 50Ca      | 1.5            | 200           | 60     | 300         | 0.15       | (0.75)       |
| 260578A        | 0     | 3Ca       | 10             | 120           | 60     | 85          | 2.7        | 22.3         |
|                | 15    | 50Ca      | 160            | 120           | 90     | 480         | 2.0        | 16.6         |
| 02678B         | 0     | 3Ca       | 60             | 120           | 90     | 60          | 2.0        | 16.6         |
|                | 50    | 50Ca      | 400            | 120           | 90     | 480         | 2.0        | 16.6         |
|                | 100   | 50Ca + NH₄| 100            | 120           | 90     | 60          | 2.0        | 16.6         |
| 060678B        | 0     | 3Ca + PR  | 13             | 120           | 90     | 60          | 2.0        | 16.6         |
|                | 22    | 50Ca      | 15             | 120           | 90     | 60          | 2.0        | 16.6         |
|                | 40    | 50Ca + NH₄| 4              | 120           | 90     | 60          | 2.0        | 16.6         |
| 060678B        | 0     | 3Ca       | 24             | 120           | 60     | 220         | 1.3        | 10.8         |
|                | 30    | 50Ca      | 120            | 60            | 220    | 220         | 1.3        | 10.8         |
|                | 42    | 50Ca + CN | 200            | 60            | 220    | 220         | 1.3        | 10.8         |
|                | 60    | 50Ca      | 200            | 60            | 220    | 220         | 1.3        | 10.8         |
| 270578B        | 0     | 3Ca + PR  | 1.0            | 120           | 120    | 12          | 2.0        | 16.6         |
|                | 50    | 50Ca      | 0.0            | 120           | 120    | 12          | 2.0        | 16.6         |
|                | 50Ca + CN | 22         | 120           | 120          | 220    | 220         | 1.3        | 10.8         |
|                | 50Ca + CN | 22         | 120           | 120          | 220    | 220         | 1.3        | 10.8         |
| 200578A        | 0     | 3Ca       | 28             | 50            | 60     | 48          | 0.83       | 16.6         |
|                | 50    | 50Ca      | 50             | 60            | 50     | 50          | 0.83       | 16.6         |
|                | 50    | 50Ca      | 28             | 200           | 60     | 120         | 5.0        | 40           |
|                | 50    | 50Ca      | 52             | 100           | 60     | 148         | 5.0        | 40           |
|                | PR    | 1.1       |                |               |        |             |            |              |
|                | + 50Ca | 2.1       | 100           | 60            | 20     | 220         | 1.3        | 10.8         |
|                | CN    | 10.1      | 200           | 60            | 20     | 220         | 1.3        | 10.8         |
| 120578A        | 0     | 3Ca       | 120            | 1             | 25     | 25          | 1.0        | 25.0         |
|                | 50Ca + PR + CN | 2.5   | 100           | 180          | 0.27   | 2.5        |            |              |
|                | 50Ca-CN | 2.0       | 100           | 180          | 0      | 0          |            |              |
| 210578B        | 0     | 3Ca       | 25             | 120           | 60     | 240         | 2.7        | 22.5         |
|                | 20    | 50Ca      | 80             | 120           | 60     | 240         | 2.7        | 22.5         |
|                | 35    | 50Ca      | 240            | 60            | 300    | 2.3        | 23.0       |              |
|                | 40    | 50Ca      | 240            | 60            | 300    | 2.3        | 23.0       |              |
|                | 150   | 50Ca + CN | 200            | 60            | 280    | 1.3        | 21.7       |              |
|                | 175   | 50Ca + 1M | 200            | 60            | 280    | 1.3        | 21.7       |              |
|                | KTES  | 80        | 60             | 142           | 5.3    | 88          |            |              |
|                | pH 8  | 100       | 60             | 142           | 5.3    | 88          |            |              |
|                | 100   | 120       | 180            | 250           | 16.6   | 139         |            |              |

ability of mitochondria to buffer Ca levels in the fiber, (c) change the amount of Ca entering per impulse, or (d) affect other buffers of intracellular Ca. Point
TABLE III (continued)

| Axon reference | t (min) | Treatment | Resting glow (nA) | Frequency (s⁻¹) | Time (s) | Peak glow (nA) | Slope (nA/s) | ΔL (pA/impulse) |
|----------------|--------|-----------|------------------|-----------------|---------|---------------|-------------|----------------|
| 290578B (Apyrase) | 0 | 3Ca | 40 | 120 | 60 | 280 | 5.3 | 44 |
| | 60 | 50Ca | 160 | 60 | 60 | 280 | 33 |
| | | + PR | | | | | | |
| | 95 | 3Ca | 8 | | | | | |
| | | 50Ca | 50 | 120 | 60 | 0.06 | (0.05) |
| | | 50Ca + CN | 50 | 69 | 69 | 0.55 | (9) |
| 170578B | 0 | 3Ca | 80 | | | | | |
| | 18 | 50Ca | 440 | 100 | 60 | 600 | 2.7 | 27 |
| | | 3Ca + PR | 5 | | | | | |
| | 42 | 50Ca + PR + CN | 10 | 100 | 60 | 1.0 | (10) |
| 050678B | 0 | 3Ca + PR | 3.2 | | | | | |
| | 22 | 50Ca + CN | 3.2 | 180 | 60 | 0.5 | (2.8) |
| | | 50Ca + NH₄ | | 180 | 60 | 3.3 | (18.3) |
| | 50 | 50Ca + NH₄ | 3 | 180 | 60 | 10.0 | 56.0 |
| 040678 | 0 | 3Ca | 40 | | | | | |
| | | 50Ca | 80 | 120 | 90 | 160 | 0.73 | 6.1 |
| | | 50Ca + CN | 122 | 120 | 120 | 240 | 0.83 | 6.9 |
| | | 50Ca + NH₄ | 80 | 120 | 90 | 200 | 2.0 | 16.7 |
| | | 50Ca + NH₄ | 40 | 120 | 150 | 80 | 4.2 | 24.7 |
| | | 50Ca | 80 | 120 | 120 | 200 | 0.83 | 6.9 |
| 310578B | 0 | 3Ca | 12 | | | | | |
| | | 50Ca | 12 | 280 | 60 | 120 | 2.7 | 14.8 |
| | | 50Ca | 60 | 60 | 60 | 116 | 1.5 | 25.0 |
| | | 50Ca | 88 | 120 | 60 | 210 | 2.5 | 20.8 |
| | | 50Ca | 120 | 30 | 60 | 160 | 0.7 | 22.3 |
| | | 50Ca | 130 | 180 | 60 | 320 | 4.0 | 22.2 |
| | | 50Ca + NH₄ | 24 | 180 | 60 | 64 | 18.7 | 104 |
| | | 50Ca + NH₄ | 24 | 60 | 60 | 32 | 3.3 | 55.6 |
| | | 50Ca + NH₄ | 24 | 120 | 60 | 48 | 3.3 | 55.6 |
| 110578B | 0 | 37Ca | 28 | 100 | 180 | 160 | 0.03 | 0.3 |
| | 30 | 37Ca + PR | 2.5 | 100 | 180 | 0.05 | 0.5 |
| 220578B | 0 | 3Ca | 16 | | | | | |
| | 13 | 50Ca | 128 | | | | | |
| | | 50Ca | 128 | 60 | 60 | 270 | 4.0 | 66.7 |
| | | 50Ca + CN | 128 | 60 | 66 | 11 | 0.08 | (1.3) |
| | | 48 | 50Ca + CN | 9 | 60 | 60 | 50 | 0.25 | (4.1) |
| | | 50Ca + PR | 40 | 60 | 60 | 75 | 0.83 | (6.9) |
| 030678A | 0 | 3Ca | 40 | | | | | |
| | | 50Ca | 80 | 120 | 90 | 320 | 1.33 | 11.1 |
| | | + CN | 120 | 120 | 30 | 320 | 1.33 | 11.1 |
| | | + NH₄ | 56 | 120 | 60 | 128 | 5.66 | 47.2 |

*a* has been specifically ruled out by experiment (Lea and Ashley, 1978) as has point b (Chance, 1965). Point c seems unlikely since Ca entry, at least via Na.
channels, may be expected to be inhibited by an acid, rather than an alkaline pH. A nonmitochondrial buffer in squid axons has been described by DiPolo et al. (1976) and Brinley et al. (1977a, b) and further characterized (Brinley et al., 1978). This seems likely to be the endoplasmic reticulum of squid axoplasm (see Henkart et al., 1978; Blaustein et al., 1978). Given the fact that saturation of this buffer has never been observed, it is not possible to characterize its binding more than to say that at physiological values of [Ca], most of the analytical Ca of the nerve fiber appears to be complexed with this material. If [Ca], is taken as 30 nM (DiPolo et al., 1976) and total analytical Ca as 50 µM (Requena et al., 1979), then the ratio, ionized Ca: total Ca, appears to remain constant over a wide range of total Ca. For example, Baker et al. (1971) report an ionized Ca of 300 nM in axons where the analytical Ca can be expected to be 400 µmol/kg axoplasm. Since this is eight times the content of fresh axons, we expect ionized Ca to be (8 × 30) or 240 nM. Since much of intracellular buffering is nonmitochondrial, if Ca++ and H+ competed for a buffer binding site, then a decrease in [H]i would allow more Ca to bind, hence a change in pH would effectively alter the [Ca]. Such an effect does not rule out a possible effect of pH on Ca fluxes, but the fact that the time constant of a change in [Ca], with pH is of the order of 5 min while the time constant for a change in Ca content produced by pumping is of the order of 30 min makes it clear that we are dealing with an altered ability of an internal store to bind Ca.

A second observation is that an aequorin-injected (but phenol red-free) axon shows a markedly smaller response to stimulation when the axoplasm is alkaline. The response observed in fact approaches that observed in a phenol red-injected axon plus CN in terms both of rate of rise of light per impulse (Table II) and in size of the response. This response is to be expected from the preceding assumptions which were: (a) that the response of an aequorin-injected axon occurs mainly in the core or less peripheral parts of axoplasm because mitochondrial buffering prevents a peripheral response, (b) that alkalinizing the axoplasm with NH4 increases the affinity of the X buffer and lowers Cai sufficiently such that mitochondrial buffering in the periphery cannot take place since the [Ca] does not reach threshold, and (c) the core response seen in axons with normal pH is eliminated by the enhanced X buffering.

In a phenol red-injected axon, we cannot observe a large decline in resting glow upon alkalinizing the axoplasm since the signal:noise ratio is so poor, but there is no reason not to suppose that this reduction in resting glow actually occurs. What is observed is that the response to stimulation in a NH4-treated fiber is independent of whether CN is present or not. This suggests that [Ca], remains below the level necessary to trigger mitochondrial buffering.

It might be suggested that since phenol red is injected in KTES buffer, pH 8, the effects ascribed to this dye are in reality pH effects such as those demonstrated for NH4 seawater. The following considerations argue against such a suggestion: (a) phenol red solutions are about 250 mM dye acid and 1,000 mM buffer and some further acid is contributed by the Chelex column used to purify the dye, (b) the dye solution is diluted 30-fold upon injection in
axons so buffer is now 750/30 = 25 mM or less than axoplasm buffer capacity 30 mM/pH, and (c) metabolism can be expected to produce acid continuously to overcome the added buffer.

Additional evidence for a real role of phenol red in absorbing aequorin photons comes from: (a) FCCP applied to aequorin-injected axons produces a threefold increase in glow; in phenol red axons the increase is 200-fold. Since alkalization of an axon reduces resting glow fivefold, a pH change could only increase the FCCP effect by 5 × 3, or 15-fold or more than an order of magnitude less than the observed effect. (b) Alkalinizing axoplasm reduces the height of the response to stimulation from 200 to 50 nA, or fourfold, but adding phenol red to an axon reduces the response from 200 to 1-5 nA or a 40-200-fold change (i.e., an order of magnitude larger effect). (c) The response of the aequorin reaction to NH₄ treatment in normal and phenol red axons is opposite; NH₄ decreases the normal axon response to stimulation, and it increases the response by a phenol red axon.

For these and other reasons it is clear that principal effect of phenol red is that of limiting the region of the fiber from which light is collected while alkalinizing the axoplasm alters Ca buffering.

The Effect of Changes in [Ca]₀
The way that light emission from aequorin changes with changes in Ca₀ enables one to tell something about both the mechanism of the aequorin reaction and about buffering. In aequorin-injected axons, Baker et al. (1971) found about a 2.5-fold increase in light emission in changing Ca₀ 10-fold. This change occurred with a time constant of 1-2 min. In the present study we find that with a similar time constant a 17-fold change in Ca₀ can change the aequorin glow by 10-12-fold but the response can also be much less than this. In addition, in phenol red axons there is a transient light response to altered Ca₀ which in Fig. 4 is a 40-fold increase in light emission. If the Ca₀ was 30 nM in 1 Ca seawater, and the increase in light emission was linear with Ca₀, then Ca₀ increased to 1.2 µM before the signal began to fall. The steady-state light emission was four times the initial resting glow or a Ca₀ of 120 nM. Such measurements imply that Caᵢ must rise to the range of 1 µM before mitochondrial buffering can commence, but that once started, it is capable of operating at levels only 1/10th as high. We have no systematic information that would indicate how long "priming" lasts or just how low Caᵢ can become and still have a component of mitochondrial buffering. These results are consistent with the measurements of Brinley et al. (1978) who show that measurable CN-sensitive Ca buffering in axons injected with arsenazo III begins only when Caᵢ is in the range of 1 µM. They are also consistent with the finding in this study that FCCP-releasable Ca is very large even though the axon is in Ca-free seawater.

Apyrase-Injected Axons
Experiments with apyrase injected into phenol red axons were done to see if axoplasmic buffering were changed by the loss of ATP; that follows apyrase treatment of axoplasm. The finding that there is no obvious difference in the response of the axon to stimulation and a variety of other treatments suggest
that neither the $M$ nor $X$ buffering depends on ATP. Apyrase axons do have ATP that by analysis is in the range 10–30 μM, so that Ca buffering that requires ATP at lower levels of concentration could not be expected to be much affected by the treatment employed (see Blaustein et al., 1978).

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