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Tetracycline Fluorescence as Calcium-Probe for Nerve Membrane with Some Model Studies Using Erythrocyte Ghosts

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Summary. The tetracycline dyes, particularly chlorotetracycline, have been employed as probes of membrane-associated calcium during the excitation process of nerve. Both squid giant axons, stained internally, and lobster nerves, stained externally, show a small increase in fluorescent light during the action potential. Increasing the calcium concentration bathing a lobster nerve leads to a larger optical signal. Adding fluoride ion to the inside of a squid axon, which might be expected to influence the internal calcium-ion concentration, also leads to a larger optical signal. Squid axons have been studied under conditions of voltage clamp and the hyperpolarizing response. Model studies were done with erythrocyte ghosts to clarify the influence of membranes and calcium on the fluorescence of the tetracyclines. Chlorotetracycline may be monitoring calcium concentration associated with the inner surface of the nerve membrane.

The tetracycline antibiotics have been used for many years as labels for calcium deposition in bone [13] and have recently been introduced as divalent-cation probes for biological membranes by Caswell and Hutchison [2–4]. The fluorescence of the tetracyclines is enhanced when they have chelated divalent cations and the fluorescence of such complexes is markedly augmented in an apolar medium as when bound to microsomes or mitochondria [2–4]. Thus, it can be anticipated that these dyes can be used in physiologic experiments as probes of membrane-bound calcium. This paper presents our initial investigations using the tetracyclines to study squid giant axons and lobster nerves during excitation. We also report some model studies with human erythrocyte ghosts which have revealed important information about the physical chemistry of these molecules which is essential to interpret the physiological results. Some of these findings were discussed at a meeting of the Marine Biological Laboratory, Woods Hole, Massachusetts [11].

Materials and Methods

Dyes

Chlorotetracycline HCl (CTC) was obtained from K and K Laboratories and Nutritional Biochemical Corp.; Tetracycline HCl (TC) from Nutritional Biochemical Corp.; Demeclocycline HCl (DMC) from American Cyanamid Inc.; and Oxytetracycline HCl (OTC) was a gift from Pfizer Inc. The tetracyclines were used without further purification. CTC is unstable in alkaline media, decomposing to a divalent ion-insensitive compound with fluorescence excitation and emission peaks at 365 and 430 nm, respectively. Consequently, fresh solutions were prepared daily prior to use.

Fluorescence Studies of Nerve

Preparation of the axons, apparatus and technique are similar to previous studies and have been described in detail [17]. Squid giant axons were dissected, placed in a chamber for detection of fluorescence at 90° and injected or perfused with a solution of the tetracycline. Lobster nerve trunks were dissected from the walking legs, cleaned and soaked for 10 min in lobster fluid *(see below)* containing 2 mM dye before mounting in the chamber. Nerves were illuminated with quasi-monochromatic light of 400 nm (half-bandwidth 10 nm) by use of an interference filter from Infrared Industries, Inc. Emission was isolated with a Corning cut-off filter, CS 3-72, and detected with an RCA c70109E phototube. The light signal was amplified (as previously described) and led to a CAT 400 B (Mnemotron Corp.) computer for signal averaging.

For the squid work, dye concentration was 1 mM for injection and 0.1 mM for perfusion in various "internal solutions" all of which were 1.2 osmolar and pH 7.3. Internal solutions with 4% glycerol to maintain tonicity contained 400 mEquiv/liter of potassium (as the phosphate, fluoride or glutamate; the latter two were buffered with a small amount of phosphate). Another internal solution was just 12% glycerol with 2% K-phosphate buffer.

External solutions, bathing the axon, all of which were 1.2 osm and pH 8.0, are listed in Table 1.

Table 1. Composition of external solutions (mM)

Solution	Ca	Mg	Na	K	Glycerol
Ca seawaters	5	0	325	0	435
	10	0	325	0	420
	25	0	325	0	375
	75	0	325	0	225
	150	0	325	0	0
	200	0	300	0	0
Mg seawater with Ca	5	70	325	0	225
Lobster fluid [7]	26	8	480	16	0
No Ca	0	0	530	0	0

Voltage clamping of squid axons was done in the optical chamber using an apparatus previously described [17]. A correction for the series resistance of 6.2 Ω was employed.

Fluorescence Studies with Red Cell Membranes

Red cell membranes were prepared from fresh human blood by hypotonic hemolysis in phosphate buffer, pH 7.3, as previously described [14]. Final washes of the ghosts were in 20 mM Tris-HCl buffer, pH 7.3.

Solution fluorescence measurements were made on an Aminco-Kiers Spectrofluorometer in 1-cm quartz fluorescence cuvettes. Excitation wavelength was 400 nm and emission spectra were scanned from 400 to 700 nm.

To determine the effects of Ca and red cell membrane on the fluorescence of CTC, all possible combinations of these components were run together with CTC in tris-buffered aqueous solutions, pH 7.3. The concentrations of the individual components were 0.1 mM CTC, 5 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.3) and a 30× dilution of erythrocyte ghosts pelleted at 30,000× g for 1 hr. In this situation, the dye-to-membrane ratio is comparable to that in the squid axon after internal staining: about 10^{-3} to 10^{-4} mg dye per cm^2 of membrane surface.

For comparison of the effects of Ca and Mg on enhancement of the fluorescence of CTC in aqueous media and in the presence of red cell membranes, the following solution concentrations were used: 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1 mM CTC, 10 mM Tris-HCl (pH 7.3), and a 30× dilution of erythrocyte ghosts pelleted as above.

The relative fluorescence intensity of the four tetracyclines, CTC, TC, DMC and OTC, were compared in aqueous solutions (0.13 mM) both with and without Ca (3.3 mM) and red cell membranes (30× pellet dilution).

Results

Fluorescence Studies of Nerve

Most studies were conducted with chlorotetracycline (CTC). Squid giant axons stained internally or lobster axons stained externally show an increase in fluorescent light during the passage of the action potential. Fig. 1 illustrates these transient fluorescent changes, referred to as optical signals, in relation to the electrical recording. Both signals are small and are of the order of 10^{-4} times the background light; averaging of about 2,600 runs for the squid and 330 runs for the lobster was needed to resolve the signal cleanly. For the squid axon, the optical signal follows the shape and time course of the action potential (recorded with an internal wire). For the lobster nerve the optical signal, obtained from the middle of the nerve, slightly precedes the compound action potential measured extracellularly at the side of the chamber. In this case, the optical signal was always a single curve and did not reflect the shape of the multi-phasic electric behavior which derives from populations of axons of differing diameters.

Using the squid axon preparation, three other tetracycline dyes were tested for optical signals. Under similar conditions, demeclocycline (DMC) gave a signal of about the same magnitude as CTC; tetracycline (TC) gave a signal about half that size and oxytetracycline (OTC) gave no signal (in two attempts). Thus, ordering the tetracyclines by signal size gives:

$$\text{CTC} \simeq \text{DMC} > \text{TC} > \text{OTC}.$$

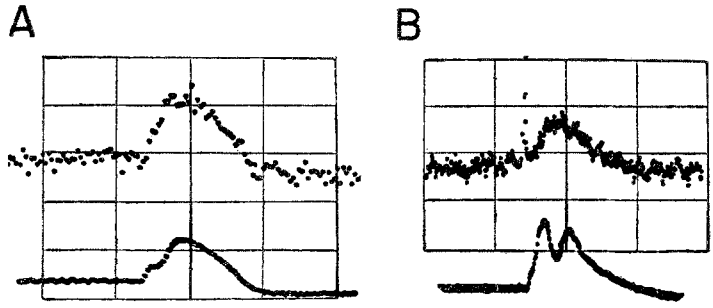

Fig. 1. Optical signals during the action potential with CTC. Upper traces record the fluorescent light and bottom traces record the electric activity. (*A*) Squid giant axon injected with about 1 μliter of 1 mM CTC in KF solution. The bath contains 150 mM Ca and is about 6.5 °C. The optical trace represents the average of about 2,600 runs. One vertical box corresponds to an increment in light of 7.1×10^{-5} times the background. One horizontal box is 4 msec. Electric stimulation and recording are accomplished by a pair of platinum wire electrodes inserted longitudinally into the axon. (*B*) Lobster nerve trunk stained externally for 10 min with 2 mM CTC in lobster fluid. The bath contains 75 mM Ca at about 20 °C. The optical trace is the average of about 330 runs. One vertical box represents a change of 4.8×10^{-5}; one horizontal box is 31 msec. Electrical activity is recorded with a pair of external electrodes at one end of the nerve trunk. The electrical trace in this part has been displaced slightly to the left

Using the lobster nerve preparation it was demonstrated that the concentration of Ca ion in the external bathing solution influenced the magnitude of the fluorescent change. Seawaters with Ca concentrations ranging from 5 mM to 150 mM with constant Na concentration were prepared using glycerol to maintain tonicity. The expectation with these solutions, which was satisfied at least with extracellular recording, is that the amplitude of the action potential will not be significantly changed, although it was noted that the conduction velocity decreased slightly in higher calcium solutions. Usually there were a few fibers in the trunk that repetitively fired in 5 mM Ca. Nerve trunks were studied first in a solution with low Ca concentration and then in a solution with higher Ca concentration. Since there is a general tendency for optical signals to become smaller with repeated experiments on the same nerve (partly caused by death of some fibers), the bias was against the higher Ca concentrations. The background light level dropped in successive experiments because of dye-washout while changing the bath.

An example of the best studied comparison between external Ca concentrations is illustrated in Fig. 2. The fluorescent increase in 5 mM Ca is about one-third the increase in 75 mM Ca. Above 75 mM Ca there was not much further enhancement, and the signal at 150 mM Ca is about the same as that at 75 mM. Optical signals obtained in 10 or 25 mM Ca were roughly one-half the size of those obtained in 75 or 150 mM Ca.

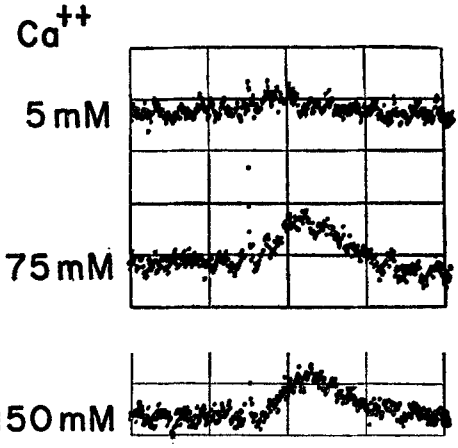

Fig. 2. Effect of external calcium concentration on CTC signals using lobster nerve. The traces are averaged records of fluorescent light during the passage of the action potential. All records are from the same axon and were obtained in the order of increasing Ca concentration. External Ca concentrations were 5 mM for the top record, 75 mM for the middle record and 150 mM for the bottom record. One vertical box represents a change in light intensity of 7.0×10^{-5}; one horizontal box corresponds to 31 msec

Attempts were made to study Ca–Mg difference in producing optical signals with lobster nerves. A solution containing 70 mM Mg and 5 mM Ca gave signals that appeared slightly smaller than those found with 75 mM Ca, but no significant difference could be proven.

In the squid preparation, some attempt was made to study the effect of internal environment on the fluorescent signal. A comparison was made between KF and plain glycerol as the medium for the injected CTC. The F ion has a high affinity for free Ca and might be expected to combine with and precipitate the Ca ion that it encounters. Based on two axons in each group there was indeed a slight decrement (factor of 1.2) in background fluorescent light emanating from the axons injected with KF rather than glycerol. There was a much larger difference in the optical signals (expressed as the change in light divided by the background light) between these two groups. The fluorescent change was about 4.5 times greater for the KF group (5 axons) as compared to the glycerol group (4 axons). It was also our impression (unproved) that the Ca chelator, EDTA, enhanced the optical signal when added to the internal solution.

Optical signals were obtained with CTC-injected squid axons under conditions of voltage clamp. Sample records and a summary of the data are presented in Fig. 3. In response to a square hyperpolarizing pulse there is a prompt and steady decrease in fluorescent light. For depolarizing pulses

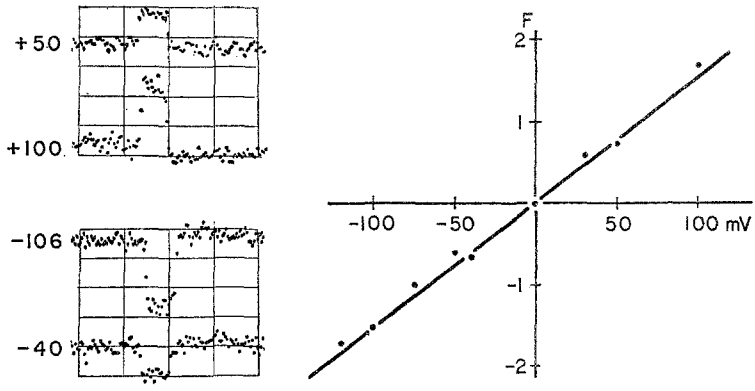

Fig. 3. Fluorescent change from CTC-stained squid axons with voltage clamp. Axons were perfused with CTC and placed in a bath containing 150 mM Ca. Two clamping pulses were studied simultaneously in each axon by means of the "alternation" technique. Eight axons were studied in all. *Left:* examples of the optical records. The top two records are from one axon and correspond to depolarizing pulses of 100 and 50 mV. One vertical box represents a change of 7.6×10^{-5}. The bottom records are from another axon and correspond to hyperpolarizing pulses of 40 and 106 mV. One vertical box represents a change of 5.2×10^{-5}. For both sets of records, one horizontal box is 7 msec. *Right:* summary of the data plotting fluorescence change (on an arbitrary scale) against magnitude of voltage clamp pulse. Each point is calculated from a single intraaxon comparison. The line through the points was the best fit by eye through zero

of less than about 50 mV there is a prompt and steady increase in light. With depolarization of greater than about 50 mV, the light increase is not steady for the duration of the pulse, but gradually decreases. The peak of optical response to the depolarizing pulse occurs early, but it cannot be said that its timing differs significantly from the time of the peak inward current. Comparative magnitude of voltage-clamp optical signals are determined by analyzing the relative difference between two clamping levels applied to each axon. (The details of this "alternation technique" are explained in ref. [17].) Using the magnitude of the peak of the response for depolarizing pulses and the plateau level of the response for hyperpolarizing pulses, it can be seen that the optical signal varies linearly with the voltage applied. Tetrodotoxin (TTX) applied to the outside of the axon eliminated inward current with depolarizing pulses, but the optical responses remained essentially unaltered.

Optical signals in CTC-stained squid axons were also produced during the hyperpolarizing response (a large negative swing of the membrane potential in KCl-depolarized axons in response to a pulse of inward current; *see* ref. [15]). Concomitant with the hyperpolarizing response there is a decrease in fluorescent light.

Emphasis has been placed on the changes in fluorescence, which presumably come from molecules in or near the membrane, rather than on the background fluorescence, which comes from a variety of sites. Even though the contribution of membrane sites to the background fluorescence is quantitatively unknown (and is probably small), some useful information can be obtained by studying the nature of this light. The influence of external Ca concentration on the background fluorescent light was studied with squid axons. Changing the bath of an axon injected with CTC from 200 mM Ca to 0 mM Ca (530 mM Na or choline) caused a drop in light by 10% and raising the Ca concentration from 0 to 200 mM usually caused an increase by the same factor. In one axon (which had also been perfused with 3 mM EDTA) changing the bath to 0 mM Ca with 20 mM EDTA did not cause a significantly larger decrement in light. Clearly, most of the Ca which is related to the background light is not free in the axon, but is quite strongly bound. The amount of this Ca can be altered, but only to a slight degree, by varying the external Ca concentration. In the context of these experiments, the fluorescent light from a CTC-perfused axon was compared to the fluorescent light from a glass capillary (similar in diameter to the axon) filled with the dye-containing perfusion fluid. The fluorescence of the axon was 3 to 4 times greater than that of the capillary, presumably from apolar binding sites of the axon including the axon membrane.

The spectrum of a CTC-stained lobster nerve was measured using a Perkin Elmer MPF-2A Spectrofluorometer. When the nerve is bathed in lobster fluid, which contains Ca and Mg, the emission peak is near 525 nm. In 75 mM Ca solution, which has no Mg, the peak is at 530 nm, indicating that at least in this circumstance a large majority of the background light is from CTC which has chelated Ca in an apolar environment *(see below)*.

Fluorescence Studies with Red Cell Membranes

The effects of Ca ion and red cell membranes on the fluorescence of CTC are illustrated in Fig. 4. When 5 mM $CaCl_2$ is added to an aqueous solution of 0.1 mM CTC, the fluorescence intensity is increased about 7 times. The effect of adding red cell membranes without Ca ion is a smaller enhancement. Ca ion and red cell membranes together are synergistic giving rise to a fluorescence intensity almost two orders of magnitude larger than CTC alone. This effect has been observed with lysolecithin as well as with red cell membranes.

The sensitivity of the fluorescence emission to addition of Ca ion, or membranes, or both, was compared for a series of tetracyclines. The relative

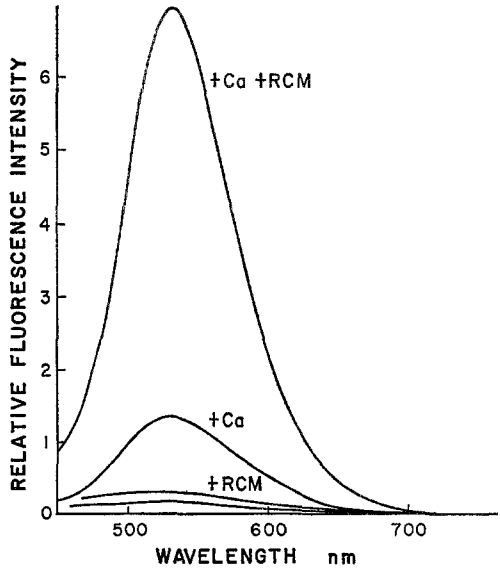

Fig. 4. Effects of calcium and red cell membranes on CTC fluorescence. The four curves were generated by adding to the CTC solutions (from bottom to top): nothing, red cell membranes (RCM), $CaCl_2$, and RCM plus Ca. Excitation wavelength was 400 nm

fluorescence increments of the four dyes upon addition of red cell membranes to the dye-Ca complex was

$$\text{CTC} \simeq \text{DMC} > \text{TC} > \text{OTC}.$$

The same sequence was obtained upon addition of Ca ion to a mixture of dye and membranes. It should be noted that the above sequence of dye fluorescence is quite different from that observed for the dye-membrane mixtures in the absence of divalent cations. The latter case gives a sequence of fluorescence intensities such that the CTC is now lowest:

$$\text{DMC} > \text{OTC} > \text{TC} > \text{CTC}.$$

Thus, the particular sequence of relative fluorescence increase of the dyes seems to depend on the presence of Ca ion and membranes. Similar results were found in experiments using lysolecithin rather than red cell membranes.

Fig. 5 compares the fluorescence spectra of CTC with Ca *vs.* Mg both in aqueous media and with red cell membranes. From the two lower curves we see that CTC gives a fluorescence about 3 times brighter in aqueous $MgCl_2$ solutions (5 mM) than in similar $CaCl_2$ solutions. In the presence of red cell membranes, however, the Ca-CTC complex undergoes a sixfold increase in peak emission intensity while the Mg-CTC complex increases

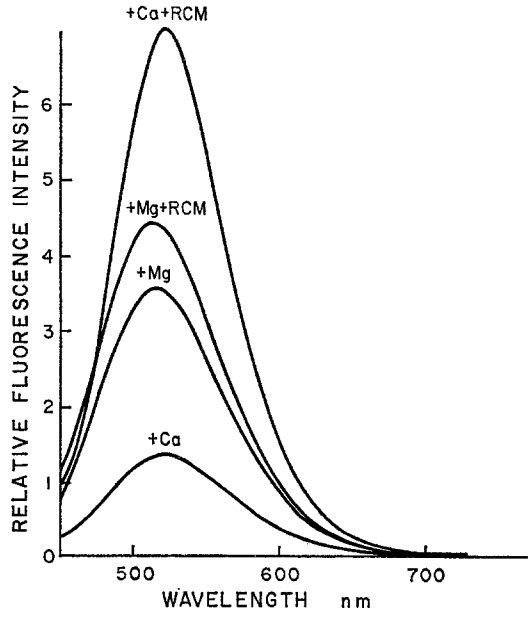

Fig. 5. Differential effect of Ca and Mg on CTC fluorescence. Illustrated are fluorescence spectra for Ca and Mg-CTC complexes with and without red cell membranes (RCM). Excitation wavelength was 400 nm

only about 20%. It is thus clear that CTC fluorescence is more sensitive to membrane-associated Ca than Mg. The Ca and Mg complexes may be distinguished from each other by the location of their emission peaks, which are at 530 nm for the Ca complex and 520 nm for the Mg complex.

Studies of the fluorescence of the above CTC-divalent ion-membrane complexes as a function of divalent ion concentration indicated that for a 0.1 mM CTC concentration mixed with a 1/30 dilution of pelleted intact ghosts, the major portion of fluorescence increase occurred between 0 and 1.0 mM divalent ion concentrations. Further addition of divalent ions to concentrations of 5.0 mM caused relatively smaller increases in fluorescence. Thus, the fluorescence of CTC is most sensitive to changes in Ca concentration in the range of very low Ca concentrations.

Discussion

The essential factors which influence the fluorescence of CTC are clear. In the model studies here with red cell ghosts it has been shown that while Ca alone or membrane alone can increase the fluorescence, Ca plus mem-

brane produces a marked increase. In a biological system, then, a large contribution to the light will be derived from CTC which has chelated membrane-associated Ca (or other diamagnetic divalent cations). This is just one more example of the general rule proposed by Caswell and Hutchison that the fluorescence of CTC is greatly enhanced when the divalent cation-CTC chelate is in an apolar medium [3]. It should be noted, however, that the fluorescence of a CTC-membrane system is not very sensitive to a change in Ca concentration in all ranges of Ca; the major effect is at low Ca levels. Caswell and Hutchison also suggest that the Ca which is detected by the CTC is bound to the Stern layer of the membrane [2, 4]. In a mitochondrial system they suggest that most of the light comes from the inner side of the inner membrane [4].

Calcium plays an important role in nerve excitation. It is an essential external ion for the maintenance of excitability and the stabilization of the resting state of the membrane. It has been suggested that a fundamental event in excitation is an ion-exchange reaction of Ca for univalent cation at crucial membrane negative sites [16]. There is also an increase in the influx of Ca during the action potential, which has been monitored by radioactive tracers [12, 18] and by aequorin, a calcium-sensitive luminescent protein [1, 10]. Both of these techniques measure Ca flux, not concentration, and suffer from poor time resolution. CTC should be a probe of Ca concentration at the membrane and have adequate time resolution.

There are several lines of evidence which suggest the optical signals in nerve are related to Ca. The external Ca concentration has a clear effect on the size of the optical signal in lobster nerves. In the conditions of the experiments reported here, with a constant external Na concentration, the resistance of the membrane and the action potential amplitude are expected to remain unchanged (the somewhat indirect evidence of the extracellular recording seems to indicate that this is true). The effect of Ca is most apparent between 5 and 75 mM and seems to have reached saturation above 75 mM.

In the squid axon, internal F ion would be expected to reduce the internal free Ca-ion concentration. F ion does lead to a dramatic increase in the magnitude of the optical signal. Perhaps this is because F ion reduces the Ca-ion concentration to a level where CTC is more sensitive to changes in Ca.

Further evidence implicating Ca in the etiology of the fluorescent signals is the sequence of signal sizes observed for the different tetracyclines in squid axons. This sequence was the same as the sequence of fluorescence changes of a dye-red cell membrane mixture upon the addition of Ca. In

the absence of Ca, the sequence of fluorescence intensities of the tetracyclines was completely different.

Finally, although the evidence is weak, the Ca–Mg difference found in lobster nerve again implicates the influence of divalent cations on the optical signal. The comparison of a pure Ca with a pure Mg solution could not be done because a solution containing only Mg does not maintain the membrane resistance nor support the action potential amplitude. Thus, a difference in the physiology might overwhelm any difference in the chemistry. The comparison made here was between 75 mM Ca and 70 mM Mg with 5 mM Ca. There was not any obvious difference in the extracellularly recorded potentials with these two solutions. The Ca signal was probably slightly larger than the Mg signal. This difference can also be modeled by the red cell system; Ca causes a greater enhancement of fluorescence of a membrane-CTC system than does Mg.

Given then that the optical signals are related to Ca, the next – and more difficult – question is where is the Ca being monitored. The fact that the optical signals follow the electrical activity quite promptly suggest that the dye molecules of interest are located in or near the membrane. In the two situations reported here, squid axons stained internally and lobster nerves stained externally, the optical signals show an increase during excitation. A positive signal is likely to derive from a site where the concentration of the Ca-dye complex transiently increases during excitation. It may not be unreasonable to believe that the signals derived from similar places in the two situations. This site may be the inner side of the nerve membrane. In both circumstances, the external surface is rinsed and bathed in a dye-free solution. In the case of squid axons, the dye is placed on the inside and in the case of lobster nerves, the dye may penetrate into the inside during the staining procedure. There is a resting influx of Ca into nerve and the CTC may travel chelated to this Ca or the CTC may have an influx of its own. It has been shown that CTC can penetrate membranes [4, 9].

The counter-ions for the membrane negative sites, during the resting state of the membrane are thought to be Ca. During the action potential this Ca is exchanged for univalent cations *(see above)*. If CTC were monitoring this Ca, there would be a decrease in light during the action potential. Thus, this is not the phenomenon revealed by the tetracyclines. There probably are also Ca ions associated with the inner side of the membrane. The significant increase in the influx of Ca which occurs with the action potential might increase the Ca concentration at this side of the membrane during this time. (There does not seem to be any prior suggestion from electrophysiology to support this notion.) CTC may be monitoring such an in-

crease and, in fact, as mentioned above, CTC has been believed to be a monitor of divalent cations associated with membrane Stern layers [2, 4]. The Ca-ion concentration in the axon interior is quite low which means that the CTC would be in an environment where it should be quite sensitive to changes in Ca concentration.

The hyperpolarizing response represents a transition of the membrane from a low resistance state to a high resistance state. Presumably, the Ca influx is reduced at this time. This would explain the decrease in fluorescent light seen during the hyperpolarizing response.

The fluorescent changes with voltage clamping can also be explained, at least qualitatively, by changes in Ca influx. Depolarization enhances and hyperpolarization might be expected to reduce the Ca influx. Quantitative analysis of these signals, however, suggests an alternate explanation for the origin of tetracycline fluorescence changes. The magnitude of the fluorescent change is not directly related to the conductance state of the membrane as has been shown for 2-*p*-toluidinylnaphthalene-6-sulfonate with a parallel polarizer [17]. On the depolarizing side, at least, the signal does not follow closely the membrane currents and thus, presumably, the etiology of the signal is not from these effects. There is a good correlation, however, between the voltage applied and magnitude of the optical signal, as has been found for some other dyes [5, 6, 8]. For reasons previously discussed [17], voltage is not expected to directly affect the fluorescence.

Voltage can, on the other hand, affect fluorescence by inducing an alteration in the partition of charged particles between the axoplasm and the inner membrane surface. There are three species in the axon whose distribution could be affected by voltage that would cause a fluorescence change: CTC, Ca ion and CTC-Ca chelate. Ca ion and Ca-CTC chelate would both carry a net positive charge. The charge on CTC at pH 7.3, however, is not clear. We have found the charge to be positive by electrophoresis in agar. Deductions from the pK values suggest a net negative charge [4], although uncertainties exist because of the ambiguity in assigning pK values to specific groups on the molecule. The species with a positive charge would tend to be attracted toward the membrane during a depolarizing pulse and excluded from it during a hyperpolarizing pulse. This would lead to fluorescent changes of the correct sign. One fact which probably supports this idea is that TTX has essentially no effect on the voltage clamp optical signals.

For hyperpolarizing voltage clamp pulses and depolarizing pulses less than 50 mV the shape of the optical signal is roughly rectilinear, similar to the shape of the clamping pulse. This is, of course, expected if the signals are simply linearly correlated with the voltage. For depolarizing pulses

greater than 50 mV, however, the signal is not rectilinear. Thus, the voltage theory does not provide a complete explanation. No good explanation for the shape of the latter part of the optical signal has been made for any probe, no doubt because of the mostly undefined gradual changes in the state of the membrane and the cation interdiffusion [17].

The expectation that the tetracyclines would be probes for the interaction of calcium with the nerve cell membrane seems to be satisfied. Both of the possible underlying mechanisms proposed here involve an interaction at the inner surface of the membrane. Further elucidation of mechanism, however, is required before the tetracyclines can be used as tools to learn more about the excitation process.

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