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Photodynamic Alteration of Lobster Giant Axons in Calcium-Free and Calcium-Rich Media

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Summary. Photodynamic alteration of Eosin Y-sensitized lobster giant axons was compared under conditions of calcium-rich and calcium-free media, to see whether the conflicting reported descriptions of the photodynamic effect can be resolved in terms of variations in calcium content of the bathing medium. Both a voltage clamp analysis (sucrose gap technique) and a microelectrode analysis (nonclamped axons) were used. In calcium-free media the characteristic photodynamic alterations of sodium channels (reduction in maximum conductance; increase in time to peak current) occurred at 40% slower rates than in calcium-rich media, while the characteristic decrease in potassium current was unaffected by the calcium content. Photodynamic alteration depolarized nonvoltage clamped axons in both media, and there was an initial rapid depolarization phase in calcium-free media. All of these changes in both media were irreversible. In calcium-free media photodynamic alteration usually induced repetitive firing. The firing showed *apparent* reversibility, since it stopped after a period of time and could usually be reinitiated upon further illumination. It is proposed that decalcification does not alter the basic nature of the photodynamic effect, but complicates the axon response by its own independent effects.

Dye-sensitized photodynamic alteration of excitable tissues has been observed as a phenomenon for over 50 years, as reviewed by Blum (1941). At the cellular level, considerable conflicts exist as to the essential nature of the photodynamic effect. On the one hand, Chalazonitis and co-workers (Chalazonitis, 1954; Chalazonitis & Chagneux, 1961) and Lyudkovskaya (1961) have described the process as excitatory and reversible. In studies on dye-treated *Sepia* giant axons illumination induced depolarization, oscillations in membrane potential, and repetitive firing, leading Chalazonitis and co-workers to view sensitized axons as model photoreceptor cells. On the other hand, voltage clamp studies carried out on lobster giant axons (Pooler, 1968, 1972) revealed irreversible decreases in sodium conductance and prolongation of the sodium conductance time course. No signs of light-induced firing were observed in nonclamped axons. The conflicts could

either be attributed to species differences or to procedural differences. Part of the contradictions may lie in a complicating hyperexcitability induced by decalcification, since the reversible excitatory results on *Sepia* were obtained only when the bathing medium was calcium free or when it contained calcium chelating agents (Chalazonitis, 1954; Lyudkovskaya, 1961). *Irreversible* prolongation of electrically stimulated action potentials of *Sepia* axons was described by Lyudkovskaya in experiments with normal calcium levels (Lyudkovskaya & Kayushin, 1960). It seemed useful, therefore, to explore the role of decalcification in photodynamic alteration of lobster axons, to see whether decalcification would unmask effects not seen in calcium-rich media and to see whether excitatory effects could be obtained under non-voltage clamp conditions. To carry this out both a voltage clamp analysis and a microelectrode penetration technique were employed on axons in calcium-rich and calcium-free media. The voltage clamp allowed measurement of small changes in ionic currents, while the microelectrode experiments allowed measurement of resting potential changes and observation of light-induced firing.

Materials and Methods

Voltage Clamp Experiments

Axons were obtained from the lobster (*Homarus americanus*) circumesophageal connective. For voltage clamp experiments the double sucrose gap method was used (Julian, Moore & Goldman, 1962*a*, *b*). The nerve chamber, illumination system, recording technique and method of data analysis were the same as employed previously (Pooler, 1972). A constant maximum light intensity was used throughout. Measurement of the rate constant for increase in time to peak sodium current during illumination utilized the same assay as that for the fall in sodium current (Pooler, 1972).

Eosin Y (Fisher Scientific) was the sensitizer for all experiments. Dye solutions were prepared before each experiment by dissolving powdered dye in a few drops of distilled water and adding artificial seawater (ASW) to make a final dye concentration of 0.01 %. The ASW contained ions in the following millimolar concentrations: Na^+ 428, K^+ 10, Mg^{++} 8, Ca^{++} 50, Cl^- 546, SO_4^{--} 4, Tris (hydroxymethyl) aminomethane 4. Calcium-free seawater (Ca^{++}-free SW) had a chloride concentration of 446 mM, with sucrose added to maintain isosmolarity.

Each area of membrane (artificial node) to be studied in voltage clamp was bathed for 2 min in ASW containing dye, rinsed of dye in ASW or Ca^{++}-free SW for 2 min, and illuminated. Heating effects during illumination were negligible, as judged by lack of temperature rise (<1 °C change) in the chamber central pool and by lack of effect on sodium current kinetics for non-dye treated control areas. The normal temperature was 4(±1) °C.

Microelectrode Experiments

A multifiber preparation which contained the medial giant axon was transferred under ASW to a plexiglass experiment chamber of a design similar to that described by Dalton (1958). Recording employed a standard 3 M KCl-filled glass micropipette which

penetrated the axon and a Ag–AgCl reference ground in the outside bath. The potential between these was measured with an electrometer preamplifier and displayed on an oscilloscope and strip chart recorder (Bausch and Lomb VOM-5). A Grass camera photographed the oscilloscope display on 35 mm film. Axons were exposed to dye for about 7 min and rinsed in ASW or Ca^{++}-free SW for about 4 min prior to illumination.

While the voltage clamp system used a xenon arc lamp as a light source (Pooler, 1972), the microelectrode system used a 650 W quartz halogen incandescent lamp (General Electric model EKD). The lamp output was heat filtered with IR absorbing glass (Edmund Scientific #40,632) and focused onto the end of a $^1/_4$ inch diameter light guide. The other end of the light guide was positioned just above the chamber. Illumination raised the temperature of a thermistor located near the preparation by 3 to 4 °C. The normal temperature in the microelectrode chamber was $12(\pm 2)$ °C. The illumination reaching the chamber was about 3.5×10^3 $\mu W/cm^2$-nm in the wavelength region 555 nm to 625 nm, decreasing smoothly on either side of this range so that there was little UV or IR. At 545 nm, the peak of the action spectrum for Eosin Y (Pooler, 1972), the microelectrode system illumination was about 25% of that in the voltage clamp system.

Results

I. Voltage Clamp Experiments

Sodium Conductance Kinetics. Light decreases the maximum sodium conductance ($\bar{g}_{Na}$ of the Hodgkin-Huxley system) with an exponential time course, and simultaneously increases the time to peak sodium current (Pooler, 1972). These changes are seen in repetitive voltage-clamped step depolarizations to a given potential during illumination. In the present experiments this process was seen in both Ca^{++}-free SW and in ASW. The rate constants for light-induced change, however, were about 40% lower in Ca^{++}-free SW, so that a longer period of illumination was required in Ca^{++}-free SW to bring about a given amount of change. This data is presented in Fig. 1 and in Table 1, for 10 axons in Ca^{++}-free SW and 13 axons in ASW, during 5-sec illuminations. Since the rates of time to peak increase and sodium conductance fall are both lower in Ca^{++}-free SW, there is no *differential* effect induced by decalcification.

Reversibility. Changes in sodium conductance induced by light are not reversible, in either Ca^{++}-free SW or ASW. Reversibility was looked for by repetitively step depolarizing the membrane once per second, monitoring the sodium current during alternating periods of light and dark, and looking for recovery of sodium current during the dark periods. An example of this procedure in Ca^{++}-free SW is shown in Fig. 2. The figure shows that the sodium current does not recover in the dark periods. This procedure has been repeated in Ca^{++}-free SW on five axons without any sign of reversibility. In other experiments, a 10/sec repetition rate and short illumination periods were used to look for possible short-lived reversible changes. In still other

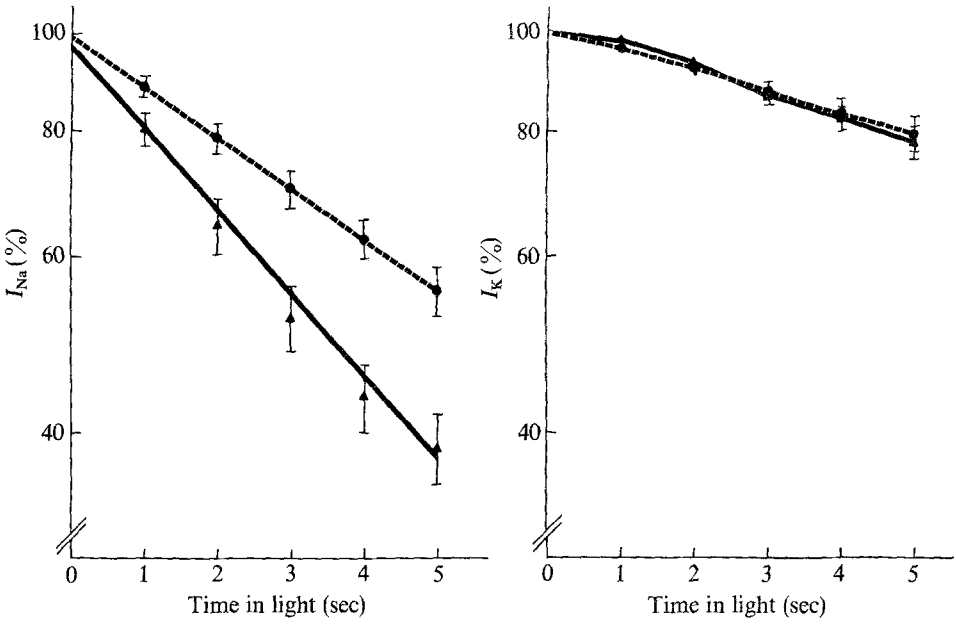

Fig. 1. *Left:* Fall in peak value of sodium current driven by constant step depolarizations during illumination. Solid curve in ASW, dashed curve in Ca^{++}-free SW. Points are means (±SE) of 10 experiments in ASW and 13 experiments in Ca^{++}-free SW. Straight lines calculated from a least-squares analysis. *Right:* Fall in peak value of potassium current driven by constant step depolarizations to the sodium reversal potential during illumination. Solid curve in ASW, dashed curve in Ca^{++}-free SW. Points are means (±SE) of four experiments in ASW and four experiments in Ca^{++}-free SW. Lines drawn segmentwise through means

Table 1. Rate constants for fall in maximum sodium conductance and increase in time to peak, in calcium-free SW and ASW

	Ca^{++}-free SW	ASW	Ca^{++}-free SW/ASW
$k_{\bar{g}_{Na}}$ (10^2 sec^{-1})	12.2 ±1.08 (13) [a]	20.47±1.38 (10)	0.59
k_{tp} (10^2 sec^{-1})	7.38±0.55 (13)	13.66±1.98 (10)	0.54

[a] Mean ± SE (number of experiments).

experiments, a step depolarization to a potential which just barely activated the sodium conductance was used to detect possible transient *increases* in current. In no case was a transient increase or transient recovery of sodium current detected in either Ca^{++}-free SW or ASW.

Potassium Conductance. Light lowers the maximum potassium conductance of dye-treated axons (Pooler, 1968), although the kinetics seem to be somewhat variable for extended periods of illumination. The time

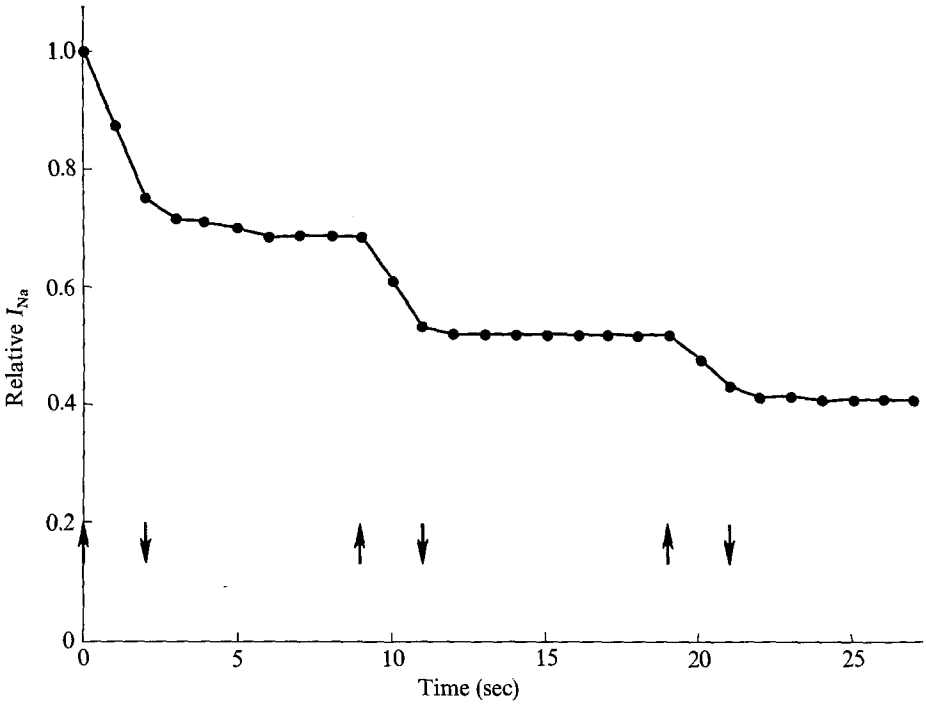

Fig. 2. Irreversibility of light-induced fall in peak value of sodium current driven by constant step depolarizations, during alternating periods of light and dark in Ca^{++}-free SW. Light on and off at up and down arrows, respectively

course can be followed by plotting the maximum level of potassium current reached during depolarizations to the sodium reversal potential as a function of illumination time. Fig. 1 (right side) shows the data from four axons in ASW and four axons in Ca^{++}-free SW during 5-sec illuminations. In Ca^{++}-free SW the rate of decrease is not strikingly different from the rate in ASW, so that decalcification does not appear to interact in any major way with the photodynamic alteration of the potassium conductance system.

II. Microelectrode Experiments

Changes in Resting Potential. When nonvoltage clamped dye-treated lobster axons were illuminated they always depolarized irreversibly, at rather variable rates. Fig. 3 (right side) shows the time course of membrane potential during illumination for 12 axons bathed in ASW. The depolarization proceeded roughly linearly throughout the period of illumination. At cessation of illumination, the potential normally showed a slow meandering drift toward more depolarized levels which continued indefinitely until a state of inexcitability was reached. Fig. 3 (left side) shows similar experiments for 10 axons bathed in Ca^{++}-free SW.

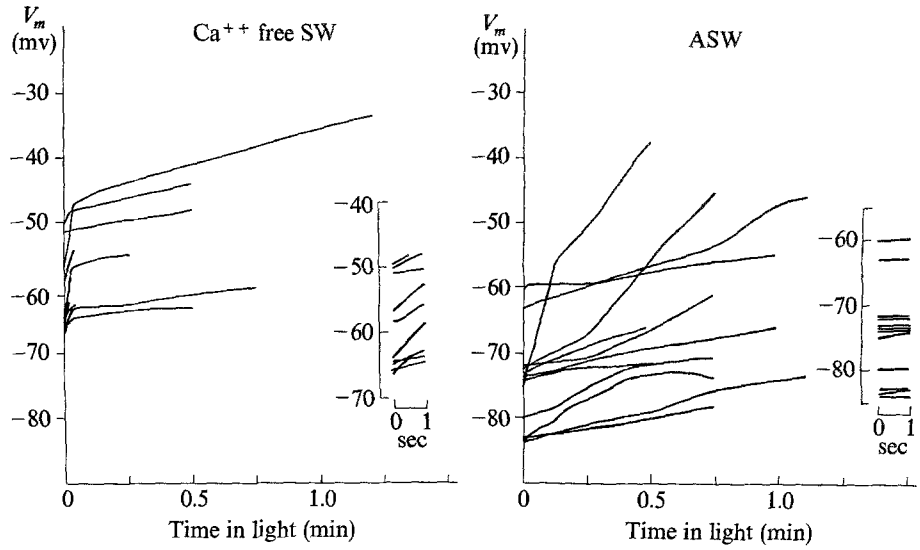

Fig. 3. Time course of membrane potential during illumination for 10 axons in Ca^{++}-free SW (left side) and 12 axons in ASW (right side). The insets are an expanded time scale of the first second of illumination

Decalcified axons show an initial rapid depolarization of several millivolts during the first few seconds of illumination which slows to a roughly linear rate after that. Since the rapid depolarization is not obviously attributable to the difference in preillumination potential, it is probably a direct effect of decalcification on the photodynamic process. Thus, a phase of rapid depolarization is one of the few differences seen between illumination in ASW and illumination in Ca^{++}-free SW. As in ASW, however, the membrane depolarization was irreversible in Ca^{++}-free SW, whether the illumination lasted a fraction of a second or as long as a minute. Following illumination, the potential meandered slowly in the depolarizing direction, as in ASW.

Light-Induced Firing. Illumination in Ca^{++}-free SW frequently resulted in repetitive firing, with trains of spikes continuing for seconds or even minutes. The firing was usually preceded by or was coincident with small oscillations in membrane potential. Fig. 4 shows several examples of this response to illumination. As was the case with *Sepia*, the effects were quite variable, probably due to varying interactions between the illuminated region and the surrounding normal regions.

Discussion

There are two basic questions to which this work is addressed. Can the apparent differences in the nature of the photodynamic effect as revealed on

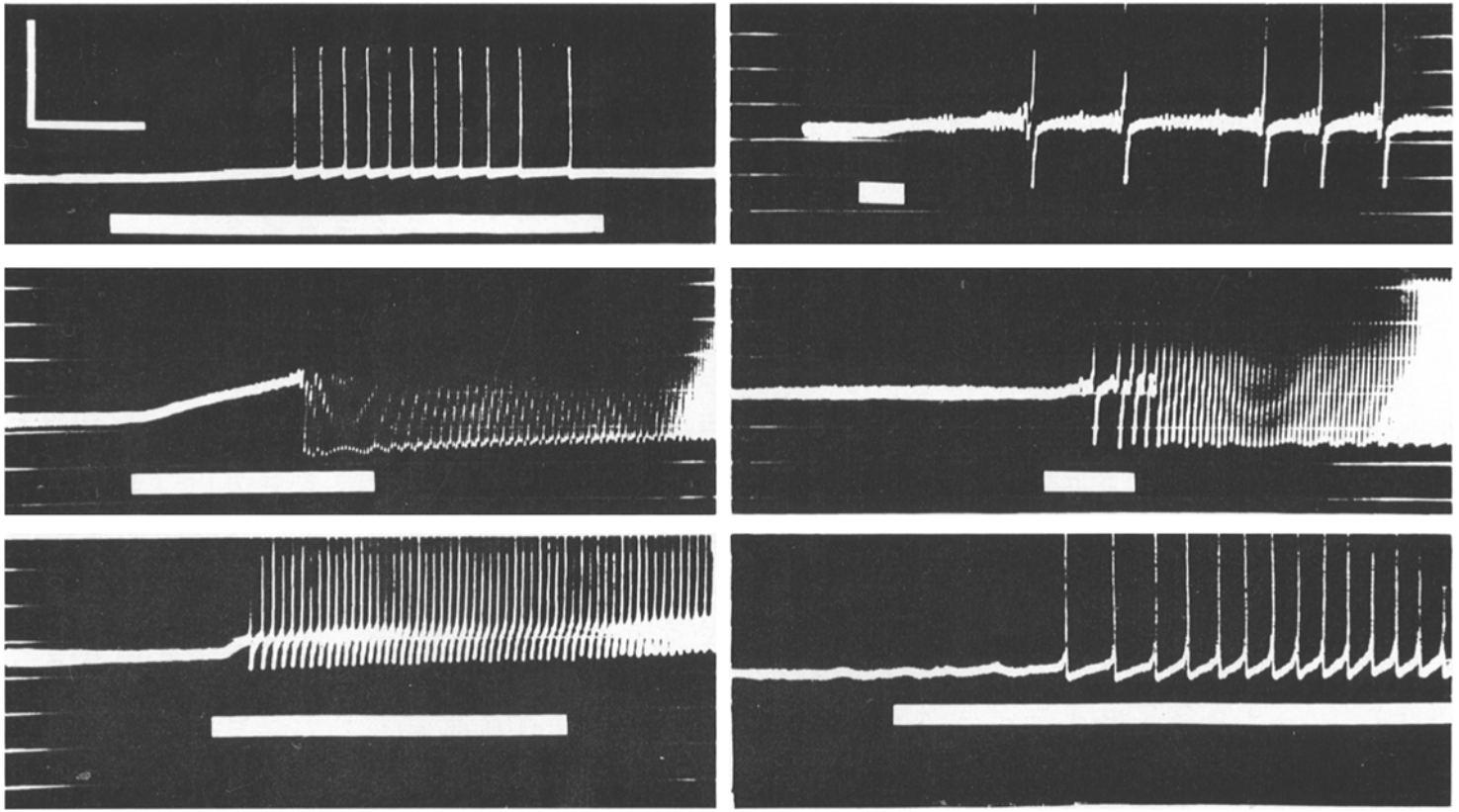

Fig. 4. Light-induced firing in Ca^{++}-free SW. Illumination duration indicated by solid bar in each case. The scale factors are: upper left 50 mV, 0.4 sec; middle and lower left, upper and middle right 10 mV, 1 sec; lower right 10 mV, 0.4 sec. Middle left, upper and middle right are from same axon. Other records are each from separate axons

Sepia and lobster axons be reconciled by taking into account the complication of decalcification? And does decalcification unmask reversible phenomena not seen in calcium-rich media?

Excitation or Prolongation?

Whether in calcium-free or calcium-rich media, the essential nature of the photodynamic effect on lobster axons appears to be: (*i*) a progressive closure of sodium channels; (*ii*) a progressive prolongation of sodium currents; (*iii*) a progressive closure of potassium channels; and (*iv*) depolarization. In calcium-rich media one would expect several seconds illumination to prolong electrically stimulated action potentials, with extended illumination leading to complete inexcitability. This is what is observed, on lobster axons (Pooler, 1968), *Sepia* axons (Chalazonitis & Chagneux, 1961), and Pacific squid (*Ommatostrephes*) (Lyudkovskaya & Kayushin, 1959). In calcium-free media one would expect these results also, but obscured by periods of repetitive firing in cases where decalcification has lowered the threshold close to the resting potential. Termination of illumination prior to significant prolongation would reveal only the depolarization and repetitive firing. This is what is observed, on lobster axons and *Sepia* axons (Chalazonitis, 1954; Lyudkovskaya, 1961). (No calcium-free experiments have been reported for squid.) Intermediate illumination periods in calcium-free media lead to long depolarized plateaus on lobster axons and *Sepia*. It would seem then, that there is qualitatively only one kind of photodynamic effect and that it occurs on both lobster and *Sepia*, regardless of the calcium content of the bathing medium.

To picture how the effect can appear excitatory, even though sodium channels are being closed, one could say that the membrane potential "chases" the threshold, with repetitive firing occurring if the threshold is caught. The phase of rapid depolarization and the lower rate of sodium channel closure in calcium-free media mean that depolarization will be greater relative to the rise in threshold potential, thus increasing the probability of light-induced firing in low calcium solutions. Excitable cells with thresholds close to the resting potential might easily be excited by light without decalcification, while others, like the lobster or *Sepia* normally require decalcification.

Are Photodynamic Effects Reversible?

The sum total of experiments on lobster axons, in both calcium-free and calcium-rich media have not revealed any reversible changes. Some of the microelectrode experiments yielded examples of what might be called

apparent reversibility, however. On one axon spontaneous firing was initiated a total of seven times, with firing ceasing after some tens of seconds in each case. The reason for calling this behavior apparent reversibility is that decalcification, while initially causing a lowering of threshold (toward the resting potential) also induces a slow upward drift of the threshold (away from the resting potential) as shown by Adelman (1956). In calcium-free solution, axons are not really in a steady state and the calcium concentration at the outside of the membrane cannot be precisely defined at any point in time. As axons approach a state of complete decalcification they also approach a state of complete inexcitability (Adelman, 1956; Adelman & Adams, 1959). When light-induced firing stops after a period of time in calcium-free media, it is probably a reflection of an upward threshold drift rather than a reversal of the membrane changes which originally led to excitation. A crossing and uncrossing of potential and threshold during intermittent illumination would lead to alternating periods of activity and quiescence.

The several degree temperature rise seen in the microelectrode experiments is probably not important. Photodynamic processes do not seem to be highly temperature dependent (Spikes & Livingston, 1969), and increases in temperature raise the resting potential and decrease spike duration (Dalton & Hendrix, 1962). Both of these effects are in the opposite direction to the results obtained here.

It is difficult to tell whether all of the reversible phenomena seen on *Sepia* are examples of apparent reversibility. Recent work on *Helix* indicates truly reversible photodepolarizations (Arvanitaki, Romey & Chalazonitis, 1968). Thus, there still may be some species differences between lobster, *Sepia*, and other preparations.

The whole question of the photodynamic effect is difficult to resolve in nonvoltage clamp experiments because of interactions between photodynamically treated membrane areas and surrounding normal areas. Most of the variability seen on lobster and *Sepia* probably arises from varying local eddy currents and slow drift effects. The observed axon behavior in these conditions is not amenable to a simple description. The reversibility question on *Sepia* and other preparations could best be settled with a voltage clamp analysis.

Conclusions

The effects of decalcification on photodynamic alteration of lobster axons can best be characterized as a summation of the independent effects of these two procedures. The exceptions are that the rate at which sodium

channels are characteristically altered photodynamically is slowed by 40% in calcium-free media and that photodynamic depolarization has an initial rapid phase in calcium-free media. Photodynamic alteration is irreversible, even though the upward drift of threshold in calcium-free media may lead to apparent reversibility of spontaneous firing. There is nothing in the results obtained here to encourage the view advanced by Chalazonitis and co-workers that sensitized axons are model photoreceptor cells. The remaining discrepancies between the results obtained on lobster and other preparations may be due to species differences.

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