Photodynamic Alteration of Sodium Currents in Lobster Axons

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ABSTRACT Photodynamic alteration of lobster giant axons drastically changed the magnitude and kinetics of sodium currents seen under voltage clamp using the sucrose gap technique. Illumination of axons following treatment with acridine orange or eosin Y decreased the maximum sodium conductance to a zero asymptote as an exponential function of illumination time. Normal sodium inactivation was slowed, with \( \tau_m \) more than doubled depending on experimental conditions. A second slower inactivation rate developed occasionally. \( \tau_m \) was altered little, if at all. Sodium current "tails" were not prolonged. At maximum light intensity and with eosin Y as sensitizer leakage current increased after 4–10 sec in light. These changes were irreversible. Decreases in maximum sodium conductance correlated highly with increases in time to peak sodium current. The magnitude of change varied linearly with light intensity. The action spectra for eosin Y and acridine orange peaked near 545 and 505 nm, respectively. The magnitude of change varied with preillumination dye exposure time in a quasi-exponential approach to a maximum effect. Sodium dithionite protected the axon from photodynamic change.

INTRODUCTION

Dye-sensitized photodynamic alteration of biological membranes, including excitable cell membranes, has been studied since the beginning of the century (Blum, 1964), but today remains poorly understood because the necessary membrane chemistry and photochemistry are still in developmental stages. With excitable cells progress has been additionally hampered because a description of the normal ionic events underlying excitation was not available until the publication of the Hodgkin-Huxley (H-H) model in 1952 (Hodgkin and Huxley, 1952). And today most of the photodynamic studies on excitable cells since 1952 have been performed without reference to the H-H model and without use of the powerful voltage clamp technique. The exception to this (Pooler, 1968) showed that acridine orange-sensitized photodynamic alteration of lobster giant axons resulted in irreversible decreases in the maximum sodium and potassium conductances, a shift of
some of the voltage dependent parameters for both sodium and potassium to more positive internal potentials, and a slowing of the kinetics for both sodium and potassium, including a pronounced prolongation of sodium current. Electrically stimulated action potentials were prolonged considerably while leakage was occasionally, but not usually, increased. These results answered some questions left by previous studies but raised new ones concerning critical details of the kinetic changes in the sodium system, and conflicted with the results of Chalazonitis (1954) and Lyudkovskaya (1961), who showed strictly reversible photodynamic alteration of *Sepia* giant axons.

In the first part of this study we attempted, therefore, to clear up the questions about reversibility and leakage, and to quantify the alterations in the sodium conductance system.

What is needed, and is still lacking, is a means to relate these particular excitation parameters to particular molecular events. The second part of this study lays the groundwork for an assault on this problem by measuring the dependence of photodynamic alteration on wavelength, intensity, and sensitizer exposure time.

**METHODS**

The experimental nerve chamber and apparatus for voltage clamping have been described previously (Julian et al., 1962a, b; Pooler, 1968). Isolated giant axons from the lobster circumesophageal connective were placed in a Lucite double sucrose gap chamber which connected via silver-silver chloride electrodes to the electrical system. The artificial seawater (ASW) bathing the axon contained 50 mM calcium and was buffered with bicarbonate to pH 7.85. The central pool input could be switched to solutions containing ASW, ASW with various concentrations of acridine orange (AO) or eosin Y (EY), or ASW with $10^{-6}$ M tetrodotoxin (TTX). Each artificial node of membrane brought into the central pool was normally exposed to dye for 2 min and illuminated without rinse. The central pool temperature was normally about 4°C. Illumination raised this by less than 1°C.

The optical system for illumination is illustrated schematically in Fig. 1. A 1000 w high pressure xenon arc lamp (Hanovia Lamp Div., Canrad Precision Industries, Newark, N.J.; 976-C), mounted in an air cooled housing (Electro Powerpac Corp., Cambridge, Mass., model 371), served as a light source. The quasi-collimated output beam from the housing passed through a UV- and IR-removing filter system and was focused to a small spot on the axon in the chamber central pool. Neutral density filters (Optics Technology, Inc., Palo Alto, Calif.; Set 12) or band-pass interference filters (Bausch and Lomb Inc., Rochester, N.Y.; series 44–78) could be placed within the beam when needed. The output spectrum of the optical system is shown in the inset of Fig. 1. Approximate illumination readings could be obtained by removing the nerve chamber and locating the sensing head of a spectroradiometer (Instrumentation Specialties Co., Inc., Lincoln, Neb., model SR) where the nerve normally was. This should give an upper limit to the actual illumination. The normal high peaks
around 470 nm in the xenon spectrum become smoothed over in Fig. 1 because of the rather wide (≈15 nm) bandwidth of the spectroradiometer.

Data Analysis Oscilloscope traces of current and voltage were photographed on 35 mm film and analyzed by hand with the aid of a programmable desk calculator. Time constants for $m$ and $h$ in the H-H system were obtained at five values of membrane potential over a span of 40 mV centered at the potential of maximum preillumination sodium current. This potential was chosen by repetitively step depolarizing the membrane while continuously varying the step size, and observing the resulting currents on an oscilloscope. From axon to axon the potential of maximum sodium current varied from $-20$ to $+10$ mV, with an average near $-10$ mV. To calculate $\tau_m$ and $\tau_h$ each axon area was posttreated with $10^{-6}$ M TTX to close the sodium channels. Currents before and after TTX treatment at each potential were
subtracted to give the net sodium current, which was then analyzed for the time constants. The sodium currents normally obeyed $m^3h$ kinetics of the H-H system quite well. For the studies not involving measurement of time constants the total membrane current at the time of peak sodium current was leakage corrected by linearly extrapolating the current measured during a standard 20 mv depolarizing prepulse. The normal holding potential was $-100$ mv.

RESULTS

Part I

Previous work showed that illumination of dye-sensitized axons decreased the maximum sodium conductance, $g_{Na}$.

The present results indicate that the decrease proceeds as an exponential fall to a zero or near zero asymptote. Fig. 2 shows the exponential time-course of maximum sodium current decline. In this experiment three different EY-treated artificial nodes from one axon were illuminated at three different light intensities, resulting in different rates of fall for each area.

TIME CONSTANTS For light exposures which only partially lower the maximum sodium conductance one can study the alterations in kinetics of the sodium system. Measurement of the inactivation time constant, $\tau_h$, following illumination revealed the increase produced by photodynamic alteration which was expected from previous results. For the standard 8 sec

![Figure 2](image)

**Figure 2.** Time-course of fall of maximum sodium current driven by constant step potential changes during illumination, for three different artificial nodes illuminated at three different light intensities. Each node was bathed in 0.01% eosin Y for 2 min and illuminated without rinse. Further details are given in the text.
of illumination with AO as sensitizer, the average increase in $\tau_h$ was 66% at 20 mv less than the potential of maximum $I_{Na}$ and 135% at 20 mv more positive than the maximum $I_{Na}$ potential. These results, averaged from 20 treated areas and a separate 20 control areas, are shown in Fig. 3 (left side). The magnitude of photodynamic effect was quite variable and is reflected in Fig. 3 by the larger standard errors for the treated axons. Despite the scatter it is clear that photodynamic alteration considerably increases $\tau_h$. Longer illumination times yield a still further prolongation of the inactivation time constant, but the decrease in current due to the decrease in maximum sodium conductance makes reliable measurement impossible.

On several nodes the already slow inactivation gave way to a second very much slower time constant. An example of this is given in Fig. 4. Shown here are the total membrane currents at a depolarization to 0 mv after photodynamic alteration, and again following TTX treatment. The difference between the curves indicated by the shaded area is the net sodium current. The sodium current clearly inactivates part way and then remains at a near steady value. To ensure that this phenomenon was not just an artifact resulting from a change in membrane area the figure also shows the currents at a potential of +60 mv, which was close to the sodium reversal potential. The currents with and without TTX superimpose at this potential, which can happen only if the area remains constant.

Photodynamic alteration prolongs electrically stimulated action potentials also (Pooler, 1968; Lyudkovskaya and Kayushin, 1959, 1960), usually by a few milliseconds, and occasionally by several hundred milliseconds. This observation is consistent with the $\tau_h$ measurements, since increases in $\tau_h$
lead to prolonged action potentials. The very long action potentials observed occasionally are to be expected also if the sodium inactivation system gives way to a very slow second time constant occasionally.

Pooler (1968) also reported that the sodium inactivation curve \( h_o \) was shifted to more positive internal potentials and that the slope factor was decreased. The occasional observations in the present experiments of a second very slow inactivation time constant may be cases in which the inactivation curve was shifted so far that \( h_o \) during the test pulse was not zero.

Somewhat to our surprise the sodium activation time constant, \( \tau_m \), was changed very little, if at all, by photodynamic alteration. \( \tau_m \) was measured on the same axon areas as was \( \tau_h \). The results are given in Fig. 3 (right side).

**Figure 4.** Currents in voltage clamp (tracings of oscilloscope photographs) after photodynamic treatment (as in Fig. 3), illustrating noninactivated component of sodium current. The net sodium current is given by the shaded region. The lower pair of curves is at 0 mv and the upper pair is at +60 mv, a potential close to the sodium reversal potential. The solid curves are before and the dashed curves are after 1 min in \( 10^{-6} \) M TTX.

Inspection of the figure indicates a possible potential dependence of the photodynamic effect. Accurate determination of changes this small are probably beyond the capability of the sucrose gap system, however. The change in \( \tau_m \) is certainly small and stands in obvious contrast to the large increase in \( \tau_h \).

As a check on the accuracy of the time constant determinations, the measured time to peak sodium current \( (t_p) \) for each area was always compared with the time to peak calculated from the time constants using equation 1 (Binstock and Lecar, 1969). In every case the difference was within the uncertainty in \( t_p \) measurement, for both control and experimental areas.

\[
t_p = \tau_m \ln (1 + 3 \tau_h/\tau_m).
\]  

(1)

As described previously (Pooler, 1968), the photodynamic effect increases
the time to peak sodium current. This was confirmed in the present experiments, as shown in Fig. 5, which is a plot of mean measured $t_p$'s calculated from the mean values of the time constants using equation 1. The agreement is quite good and shows, since the $\tau_m$'s are not changed very much, that the light induced increase in $t_p$ can be accounted for entirely by the increase in $\tau_h$.

The slowing of inactivation without apparent changes in activation is reminiscent of the effect of dichlorodiphenyltrichloroethane (DDT) on frog nodes (Hille, 1968) and lobster axons (Narahashi and Haas, 1968). Hille has described the DDT effect as "holding open sodium channels." Is the photodynamically prolonged sodium current a similar holding open of channels or is it an effect strictly on inactivation? We attempted to answer this by observing the "sodium tails" obtained when the membrane is repolarized quickly at a time when depolarization has opened the sodium channels maximally. If the channels are being held open by photodynamic effects a pronounced slowly decaying current tail should be seen rather than the normal rapidly decaying tail. Technically this is a somewhat difficult experiment to perform because the initial portions of the tails merge with the capacitive transient and leakage transient. By repolarizing to a potential where $\tau_m$ is large and $m_\infty$ is zero, however, the ends of the tails can be seen.
clearly and measured. Fig. 6 illustrates one such experiment performed during 8 sec of illumination. The photodynamic process decreased the amplitude of sodium current during the depolarizing prepulse and during the tails, but in each case the tail fell with the same time-course. There is no sign of tail prolongation. Thus it seems that there is no DDT-like holding open of channels. Apparently the photodynamic alteration of sodium current kinetics in lobster axons is an effect mainly, if not strictly, on the inactivation system.

**Figure 6**. Membrane current showing sodium "tails" (upper) and membrane potential (lower) during 8 sec illumination (retouched oscilloscope photograph). The scales are current (5 ma/cm$^2$), potential (50 mv), and time (0.5 msec). The prepulse was to $-20$ mv and the test pulse was to $-55$ mv. The holding potential was $-100$ mv. The axon was bathed in 0.01% acridine orange for 2 min and illuminated without rinse. The current patterns change characteristically during illumination, but the tail time-course is not altered.

**Figure 7**. Outward sodium currents during illumination (retouched oscilloscope photograph). The maximum current declines during illumination but the outward currents at times past the peak are increased because of time-course prolongation. The scales are current (2 ma/cm$^2$) and time (0.5 msec). The zero current level is off the scale.

Although the sodium conductance vs. voltage curve is altered during light, it is not quite accurate to describe these changes as a simple shift to more positive internal potentials along the voltage axis, as was implied previously (Pooler, 1968). Such a shift suggests changes in the $m_\infty$ vs. voltage curve. After seeing that $r_m$ was not altered very much by light, we performed a few preliminary experiments to see whether $m_\infty$ was shifted. This was done by observing the fall of sodium current during light at two potentials: a medium depolarization where sodium is barely activated, and a larger depolarization where it is fully activated. A shift of the $m_\infty$ curve to more posi-
tive internal potentials would decrease the sodium current at the smaller depolarization proportionally more than at the larger depolarization. No such differential decrease was found. The current at both potentials appeared to fall with the same time-course, suggesting that $m_\infty$ is not altered by light. A full set of experiments is needed, however, to confirm the point.

OUTWARD SODIUM CURRENT The photodynamic alteration of the sodium system appears to affect outward sodium in the same way as inward sodium. When the membrane is successively depolarized beyond the sodium reversal potential during illumination, the maximum outward current declines with time and the inactivation kinetics are slowed. An example of this experiment is shown in Fig. 7. The current patterns are a mirror image of those when depolarization is to a potential less than the sodium reversal potential during illumination. There seems to be no directional effect on the sodium system induced by light. Even though the maximum obtainable outward current declines during illumination, the slowing in the rate of inactivation actually increases the outward current at times beyond the peak outward current. Because this noninactivated current reverses direction at the normal sodium reversal potential, it is clear that it is carried by sodium and not by an ion with an anomolous conductance induced by light.

LEAKAGE CURRENT Previous attempts to determine possible changes in leakage conductance during illumination of acridine orange treated axons were equivocal. Normally leakage was unaffected, but occasionally an increase was seen which correlated with illumination (Pooler, 1971). We have repeated these experiments using the more potent sensitizing dye, EY. It is now quite clear that photodynamic alteration, with EY as sensitizer and at the highest light intensities possible with our apparatus, induces a large increase in leakage conductance, but only after 4–10 sec in light. To measure leakage changes with EY as sensitizer the membrane was alternately depolarized and hyperpolarized by symmetrical steps about the holding potential during illumination. Leakage current declines with time following a step potential change so that one must specify a time as well as a magnitude for its value (see Moore et al., 1966). We arbitrarily chose 1 msec after the step change. A reading at times different from this does not alter our findings. In Fig. 8 the leakage currents at 1 msec are plotted as a function of illumination time for a typical experiment in which the step potential changes were ±50 mv about a −120 mv holding potential. The inset shows tracings of the complete current vs. time for the first two step changes. Fig. 8 illustrates several major findings. (a) Leakage conductance rises precipitously during illumination after several seconds in light. (b) Before light-induced effects the leakage currents rectify, with depolarization driving a larger current. (c) Hyperpolarizing currents increase more than depolarizing currents during light, so that the preillumination rectification is reversed. The rectification
reported here may ultimately be related to the potassium conductance system, but it is not normal delayed rectification because it does not appear with a delay.

These experiments have been repeated at holding potentials from $-130$ to $-60$ mV and step sizes from 10 to 50 mV. The results are qualitatively always the same. The delay in leakage change is fortunate in that sodium and potassium currents can be measured during illumination before the complicating effects of a later change in leakage. When the light intensity is lowered by the use of neutral density or band-pass filters the delay period is evidently increased to more than 10 sec because no leakage change has been observed at anything but maximum light intensities.

![Figure 8](image)

**Figure 8.** Leakage current for 50-mv depolarizing and hyperpolarizing steps about a $-120$ mV holding potential before and during illumination. Currents were measured 1 msec after step change. The inset shows the complete sweeps (tracings of oscilloscope photographs) for the preillumination steps. The axon was bathed in 0.01% eosin Y for 2 min and illuminated without rinse.

**Reversibility** The question of whether or not the changes reported here are reversible seems particularly important to any future discussion of the molecular events underlying changes in electrical properties. The original work by Chalazonitis (1954) on photodynamic alteration of isolated giant axons from *Sepia* described effects which were reversible and which were rather different from those described here. Many of those differences may be more apparent than real, as discussed before (Pooler, 1968), but the question of reversibility remains. Since it may be that there was partial reversibility in our previous experiments which went undetected, a new and closer look was taken. The parameter most sensitive to light-induced change, and therefore the one we guess to be most likely to reveal reversibility, is the maximum sodium conductance. By repetitively depolarizing the membrane to a given potential during alternating periods of light and dark, any recovery of
sodium current during the dark period can be seen. The results of such an experiment are shown in Fig. 9, which is a plot of peak sodium current during step depolarizations to 0 mv, as a function of time during alternating periods of light and dark. It seems quite clear that there is no recovery of sodium current during the dark periods. In fact, what is revealed is a possible continuance of the light-induced decrease of sodium current into the initial portion of the dark periods. Little can be said about this latter phenomenon as yet, considering the lack of time resolution in our experiment and the uncertainty in light period duration introduced by the use of a hand-held card for a shutter. But there is sufficient time to detect reversibility and it is not seen. Increasing the duration of the dark periods does not change this.

It should be pointed out that Chalazonitis (1954) reported reversibility of light-induced decreases in the resting potential rather than sodium current (which his technique could not measure). Since a decrease in sodium conductance should have essentially no effect on the resting potential perhaps he was observing a reversible phenomenon unrelated to the irreversible changes measured in our experiments. We have performed a number of experiments measuring resting potential changes with microelectrode penetration of lobster axons not in sucrose gap. We detect a fall in resting potential during illumination, but no recovery.
ASSAY

In order to quantify the magnitude of photodynamic effect under different conditions it was necessary to develop an assay. We chose the decay rate of $g_{Na}$, expressed as a reciprocal time constant, as the assay. To measure the reciprocal time constant the following procedure was followed. Before and during illumination the membrane was step depolarized in voltage clamp at a normal repetition rate of once per second to a potential approximately 15 mV more positive than the potential of preillumination maximum sodium current. This potential was chosen so that the fall in current during light would reflect changes in $g_{Na}$ only, and not a possible shift of the sodium conductance curve along the voltage axis. The illumination period varied from 5 to 20 sec depending on whether a large or small light-induced change was expected. In the postexperiment data analysis the logarithm of the peak sodium current was plotted as a function of illumination time. This was then subjected to a least squares analysis and the slope of the resulting line was converted to the reciprocal time constant. The straight lines and numbers to the right of each curve in Fig. 2 illustrate the calculation.

PARALLEL CHANGES

Since several excitation parameters are altered by photodynamic effects it seemed important to know whether they always occur in parallel. We compared two of these effects—the decay rate of maximum sodium conductance and the increase in time to peak sodium current ($t_p$). Fig. 10 is a scatterplot of the per cent increase in $t_p$ versus the reciprocal time constant, under experimental conditions which allowed for varying amounts of light-induced change. The two measures are highly correlated ($r = 0.85$). The correlation would probably be much higher if it were not for errors due to a 5–10% uncertainty in the ability to measure $t_p$ and fluctuations in sodium current not compensated by time to peak change, resulting from minor fluctuations in membrane gap area. The increase in time to peak sodium current reflects changes in the inactivation time constant, $r_h$, as discussed previously, while the decay of $g_{Na}$ reflects the closing of sodium channels.

LIGHT INTENSITY

The magnitude of the light-induced effect varied more or less linearly with light intensity. This is shown in Fig. 11 where EY was the sensitizer. The intensity was controlled with neutral density filters, so that the spectrum and axon illumination in each case was that shown in Fig. 1 modified by the filter attenuation factor. There is no obvious sign of either a threshold intensity at the low end or a saturation at the high end. Sensitizers other than EY with different efficiencies, however, may show different behavior. We have not yet examined this point.

ACTION SPECTRA

A point of obvious interest and key importance in a consideration of molecular events is the wavelength dependence for the photon-
Figure 10. Scatterplot of per cent change in time to peak sodium current versus reciprocal time constant, illustrating correlation between the two measures \( r = 0.85; n = 50 \). Variations in magnitude of photodynamic effect were produced by interposing various band-pass filters in the light path. Each axon area was bathed in 0.01% eosin Y for 2 min and illuminated without rinse.

Figure 11. Magnitude of light-induced effect (means of reciprocal time constant ± se) versus light intensity. Intensity was controlled with neutral density filters. Each axon area was bathed in 0.01% eosin Y for 2 min and illuminated for 5 sec without rinse. From low to high intensity, \( n \) was 6, 10, 8, and 7.
dynamic process. This was determined by placing band-pass interference filters in the optical pathway. The filters isolated narrow wavelength bands (half-width = 8 nm), and also attenuated the light at the center of the pass band by 70%. The action spectrum for EY shown in Fig. 12 (top) is a plot of the reciprocal time constant for the fall in maximum sodium conductance versus the nominal peak transmission wavelength of the filters. The process peaks at a wavelength between 540 and 550 nm. Since the data are not corrected for intensity differences at different wavelengths the smaller secondary peak between 460 and 470 nm in all likelihood reflects the intensity peak in the xenon spectrum in this region (see Fig. 1). The peak of the action spectrum is somewhat red shifted compared with the absorption spectrum, since in free solution EY absorbs maximally near 518 nm. Fig. 12 (bottom) shows the action spectrum for AO, determined on a more limited number of axons than for EY. The peak here occurs at a wavelength between 500 and 510 nm. The peak of the action spectrum for AO shows a red shift also.

OXYGEN Because oxygen is clearly implicated in the photodynamic process, we performed some preliminary qualitative experiments to see whether oxygen was important here also. We used sodium dithionite, a potent reducing agent, as a means to lower the oxygen tension. The experimental procedure was to (a) treat the axon with 0.1% AO in artificial seawater (ASW), (b) rinse the axon in ASW containing 1 mM dithionite, (c) illuminate in the presence of dithionite, (d) rinse the axon with normal ASW, and (e) illuminate again in normal ASW. Illumination in the presence of the dithionite did not visibly alter electrically stimulated action potentials. The dithionite, presumably by lowering the oxygen tension, protected the axon against photodynamic alteration. Illumination again, following dithionite washout, produced the typical prolongation of electrically stimulated action potentials that are normally seen. Thus the dye, which had been applied before either illumination period, was now able to sensitize photodynamic change. This experiment is illustrated in Fig. 13. The upper part of the figure shows electrically stimulated action potentials during light in the presence of dithionite, and the lower part of the figure shows the same thing following rinseout of the dithionite. A voltage clamp analysis, which might have revealed small light-induced effects obscured in the action potentials, was not carried out. We detected no changes in electrical properties induced by the dithionite itself, nor did the dithionite alter the visible region absorption spectrum of the dye in free solution. The dithionite, because of its potency as a reducing agent, may have protected the axon by means other than lowering the oxygen tension, but oxygen tension lowering seems to be the most likely reason.

DYE EXPOSURE TIME Large light-induced changes may be seen following dye exposure of just a few seconds, or many minutes (Pooler, 1971). The
FIGURE 12. Action spectrum for eosin Y (top) and acridine orange (bottom). Means of the reciprocal time constant (±SE) are plotted versus the nominal center wavelength of band-pass filters. The curves are not normalized for intensity. For eosin Y each node was bathed in 0.01% eosin Y for 2 min and illuminated without rinse for 10 sec. From short to long wavelengths, n was 4, 4, 5, 2, 3, 4, 4, 5, 5, 7, 7, 5, 4, and 4. For acridine orange each node was bathed in 0.03% acridine orange for 2 min, rinsed in ASW for 2 min, and illuminated for 10 sec. Individual data points are shown, except at 500 and 510 nm, where n was 6 and 5.

Magnitude of change is quite variable, however, and an extensive series of experiments has revealed a clear relation between magnitude of light-induced change and dye exposure time. This is given in Fig. 14, which shows the
FIGURE 13. Protection of axon from photodynamic effect with sodium dithionite. Retouched oscilloscope photographs of electrically stimulated action potentials, during light in the presence of sodium dithionite (upper) and during light after rinse (lower). See text for details of procedure. The scales are voltage (50 mv) and time (2 msec).

FIGURE 14. Magnitude of light-induced effect expressed as means of reciprocal time constant (±se) versus preillumination dye exposure time. Each node was bathed in 0.01% eosin Y for the time indicated on the abscissa and then illuminated for 5 sec without rinse. From short to long times, n was 12, 22, 24, 24, and 10.

reciprocal time constant for $g_{Na}$ decrease during 5 sec illumination, as a function of preillumination dye exposure time. In this experiment the dye flow in the chamber central pool was kept constant. There was no rinse before illumination. Time zero in each case was the moment when each area of membrane was pulled from the left chamber pool, containing no dye, into the central chamber pool, which contained dye. Thus there was a step rise in dye concentration surrounding the membrane without a complication introduced by chamber washout time. The measurement of light-induced change
took 5 sec, however, which is a significant fraction of the preillumination dye exposure time for the 10- and 20-sec periods.

The data indicate a quasi-exponential time relation between the magnitude of light-induced effect and dye exposure time, with an asymptotic approach to maximum effect at infinite dye exposure times. The half time for maximum effect is about 40 sec. If one assumes that the dye must reach some key site in order to photosensitize, and that the magnitude of effect is proportional to dye concentration at the key site, then the dye exposure time-course may represent the time-course of dye concentration rise at the key site, as it crosses a diffusion barrier from the bathing medium.

**DISCUSSION**

Experimental prolongation of sodium currents in giant axons with pharmacological agents is now a commonplace procedure. (See reviews by Hille, 1970 and in Adelman, 1971.) However, only treatments with *Condylactis* toxin (Narahashi et al., 1969) or scorpion venom (Köppenhöfer and Schmidt, 1968 a, b, c; Narahashi et al., 1972) appear to act similarly to photodynamic treatment in that they do not exert a simultaneous effect on the rate of rise and fall of sodium activation. (An increase in time to peak sodium due to a slowing of inactivation, incidentally, can mislead one into thinking that $\tau_m$ has changed, although it does not have to.) The lack of tail prolongation during repolarization indicates that all channels remaining open during depolarization were capable of closing normally. Photodynamic treatment did not prevent channels from being closed. We also looked for, and failed to see, any staircase phenomenon, where successive depolarizations after light might have revealed successive prolongations. If the axon was kept polarized during light, the first depolarization after light yielded the full light-induced effect.

The apparent delay in light-induced increase in leakage may be a true delay, during which a reactant is building to a critical concentration, for example, or it may simply be the time taken for the "real" leakage to rise a sufficient amount to overwhelm the parallel current component which exists in the sucrose gap system. The meaning of leakage current changes in sucrose gap is difficult to interpret, due to the recent finding that the vast majority of measured leakage current flows in parallel with the membrane in the gap (Pooler and Oxford, 1972). We should emphasize that leakage change has been observed so far only at the highest light intensity. With eosin Y as sensitizer a leakage change is normally seen; with acridine orange a leakage change is rarely seen. When increases in leakage are seen there is a reversal of the normal preillumination rectification about the holding potential. Hyperpolarization now drives more current than an equivalent depolarization. It also appears that the membrane may be more susceptible to dielec-
tric breakdown. Sometimes holding potentials of \(-120\) mV lead to a steadily increasing inward holding current during and after light. The reversal of rectification and the apparent dielectric breakdown are probably manifestations of the same process in the membrane. In this sense photodynamic effects with high intensity light may be described as “damaging,” as they have been for other systems (see Blum, 1964), although the use of the word damage to characterize the other changes described here may not be appropriate.

There are still unresolved differences between these results and those obtained by Chalazonitis on \textit{Sepia} and Lyudkovskaya on \textit{Sepia} or pacific squid. Lyudkovskaya found on both \textit{Sepia} and pacific squid that light produced irreversible prolongation of electrically stimulated action potentials (Lyudkovskaya and Kaiushin, 1959, 1960). This is consistent with our findings on lobster. But in a later experiment Lyudkovskaya (1961) obtained conflicting results which, however, with the findings of Chalazonitis (Chalazonitis, 1954; Arvanitaki and Chalazonitis, 1961; Chalazonitis and Chagnneux, 1961). Both Chalazonitis and Lyudkovskaya, in her later paper, described reversible oscillations in membrane potential and repetitive firing without electrical stimulation. It now appears that decalcification probably hypersensitized the axons in this case so that they were on the verge of oscillatory activity even without light (see Pooler, 1968, for a discussion of this point). Yet decalcification does not explain why depolarization was reversible on \textit{Sepia}. It may be a simple species difference, where the \textit{Sepia} axon can sustain reversible changes for a short time before irreversibility sets in, while the lobster cannot. Judging from all of the results so far, the \textit{Sepia} axon appears to be far more susceptible to photodynamic change than the lobster axon. The rather slow rate of change in resting potential seen when lobster axons not in sucrose gap are illuminated almost leads one to conclude falsely that the lobster axon is not very photosensitizable.

The ionic basis for light-induced depolarization, whether reversible or irreversible, has not yet been elucidated. It is conceivable, as Chalazonitis suggests (Chalazonitis, 1964), that the reversible depolarization on \textit{Sepia} is a pure photovoltaic effect, unrelated to the normal ionic mechanism for membrane potentials, and that the later irreversible changes represent structural changes within the membrane affecting ionic permeabilities.

It is logical to expect correspondence between the measured action spectra for photodynamic effects and the normal absorption spectra for photosensitizing dyes. Yet in these experiments this was not strictly the case as the peak of the action spectra for the dyes used was somewhat red shifted. Dimerization tends to blue shift the absorption for both AO (Lamm and Neville, 1965; Zanker, 1952 \textit{a, b}) and EY (Rohatgi and Mukhopadhyay, 1971) while dye binding red shifts the absorption for AO (Armstrong et al., 1970) and EY (Youtsey and Grossweiner, 1971). The red shift may mean that the sensitizing molecules in these experiments were bound.
There are a number of striking parallels between photodynamic alteration of excitable membranes and photodynamic destruction of proteins, which suggests that the effects described here may result from destruction of proteins in the membrane, possibly via the singlet oxygen mechanism. This suggestion is supported by the following findings. (a) Both proteins and excitable membranes can be altered photodynamically under the same conditions. With proteins there is side chain destruction without damage to peptide bonds (Fowlks, 1959; Spikes and Livingston, 1969; Spikes and McNight, 1970). (b) A variety of sensitizers, with different structures and absorption spectra, can sensitize both proteins and excitable membranes. (c) For proteins, as well as for excitable membranes, the effect is linear with light intensity and follows a first-order decay during light (Spikes and Straight, 1967). (d) Molecular oxygen is required for photodynamic alteration of excitable membranes (Lillie et al., 1935; Chalazonitis, 1954; Sazonenko, 1963; Blum, 1964) and in the vast majority of cases for protein destruction (Spikes and Livingston, 1969). (e) Singlet oxygen is generated (by quenching excited dye triplets) by illumination of most of the sensitizers responsible for photodynamic action and is probably the agent which photoxidizes proteins (see Foote, 1968). The possibility that the photodynamic effect on cellular excitation works via membrane proteins receives independent support from experiments where other agents which alter proteins also alter electrical properties. Examples of this are internal perfusion with Pronase (Calbiochem, Los Angeles, Calif.) (Rojas and Armstrong, 1972), protein cross-link formation with aldehydes (Shrager et al., 1969), treatment with sulphhydryl reagents (Takahashi et al., 1958), and treatment with oxidizing agents (Huneeus-Cox et al., 1966). Prolongation of action potentials or sodium currents is a common feature of these treatments which alter membrane proteins and is a characteristic feature of photodynamic alteration.

Fox and Stämpfli (1971) have recently reported photoinactivation of the sodium conductance on frog nodes using UV light at 280 nm. Their voltage clamp analysis clears up some of the confusion about UV effects which has existed for quite some time (see the review by Lieberman, 1970). While the mechanism of UV effects is not understood any better than the photodynamic effect it seems fair to say that the mechanisms are distinctly different. Inactivation by UV involves direct light absorption by the membrane with photon energies exceeding 100 kcal/mole, which is sufficient to break some chemical bonds. Photodynamic effects, however, probably proceed from triplet sensitizers with much lower energies (e.g., 34 kcal/mole for the potent sensitizer methylene blue).

The present investigations are continuing with attempts to photooxidize selectively particular amino acid residues as a means of specifying the membrane components responsible for photodynamic alteration of electrical properties.
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