J. Membrane Biol. 36, 159–173 (1977)

Internal and External Application of Photodynamic Sensitizers on Squid Giant Axons

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Received 2 December 1976; revised 3 March 1977

Summary. Squid giant axons were photosensitized by dyes applied internally or externally in air saturated solutions and photochemically modified by visible light. For most dyes the modifications included an irreversible block of sodium channels, a destruction of inactivation in some of the unblocked channels, and a slowing of inactivation. Internal application was up to 100-fold more effective in blocking sodium channels than external application, suggesting a site of block nearer the internal surface. Rose Bengal sensitized channel block and destruction of inactivation when applied internally, but sensitized only channel block when applied externally. In contrast, externally applied Eosin Y sensitized a clear slowing of inactivation plus channel block. Beta-carotene, an effective agent for quenching photochemically generated excited singlet oxygen, inhibited most of the modification sensitized by internally applied Methylene blue but not by Rose Bengal or Merocyanine 540.

Excitable cells can be photosensitized by the application of a variety of common dyes to exhibit an array of interesting complex electrical behaviors when exposed to white light (e.g., Lippay, 1929; Lillie, Hinrichs & Kosman, 1935; Arvanitaki & Chalazonitis, 1961; Pooler & Oxford, 1973). Initial voltage-clamp investigations of the sensitized changes in excitability properties of lobster axons (Pooler, 1968, 1972) have demonstrated the potential usefulness of sensitized photochemical modification as a probe of the molecular organization of membrane ionic channels. Work thus far has utilized two classical sensitizers (Eosin Y and Acridine orange) in a description of characteristic alterations of membrane properties of lobster axons. These modifications include decreases in membrane permeabilities to sodium and potassium and a slowing of the kinetics of the sodium inactivation process. The exact membrane loci

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for these effects remain unknown for lack of sufficient knowledge concerning accessibility of the inner and outer membrane surfaces to the dyes.

Several parallels can be drawn between sensitized modifications of excitable membranes and proteins, suggesting that successful use of photochemical modification in the study of ion channels may follow a path similar to that currently used to unravel the functional organization of proteins and enzymes (Jori, 1975; Spikes & MacNight, 1970). The modification of proteins and the decrease in membrane permeabilities are both first-order processes with a linear dependence on light intensity (Spikes & Straight, 1967; Pooler, 1972). Both require the presence of molecular oxygen and probably involve excited-state singlet oxygen in at least part of the modification process.

An alternative approach to the study of membrane excitability using several of the same dyes (Cohen, 1973; Tasaki, Watanabe & Hallett, 1972; Tasaki, Hallett & Carbone, 1973; Tasaki *et al.*, 1973) attempts to correlate extrinsic fluorescence signals with membrane permeability changes. This approach has also provided a promising new method of optically monitoring electrical activity in multi-component neural networks (Salzberg, Davila & Cohen, 1973) using voltage-dependent fluorescence changes. Large scale application of optical monitoring with fluorescent dyes is hampered, however, by the potent photodynamic activity of most of those dyes which provide adequate optical signals. This is of particular importance in cell systems which cannot be depleted of oxygen without affecting their viability. The discovery of a dye simultaneously possessing a good optical signal-to-noise ratio and low photodynamic activity would be of significant value. Recently a merocyanine-rhodanine dye has been found to be ~ 100 times less photodynamic than previous dyes while retaining a good optical signal-to-noise ratio (Salzberg *et al.*, 1975). Alternatively, an agent could be introduced which would inhibit photodynamic action, but be inert in other respects.

In this paper we describe photosensitization of squid giant axons with several dyes applied to both the outer and inner membrane surfaces. The work attempts (1) to describe photodynamic phenomena in the widely used squid axon preparation; (2) to distinguish possible differences between sensitization by internally and externally applied dyes; (3) to determine any dye-specific alterations which might be correlated with their known photochemistry; (4) to further explore the possible involvement of singlet oxygen by using the singlet oxygen quencher, β-carotene,

and (5) to examine the feasibility of utilizing singlet oxygen quenchers as protective agents against photodynamic effects in optical measurements of nerve activity.

Materials and Methods

Experiments were performed on giant axons from the squid *Loligo pealei* at the Marine Biological Laboratory, Woods Hole, Mass. Action potentials, resting potentials, and membrane conductances to sodium and potassium were measured using conventional current and voltage clamp techniques (Wang, Narahashi & Scuka, 1972; Wu & Narahashi, 1973). The internal axial electrode was of piggyback design with a platinized 75 μm Pt-Ir wire for passing current attached to a 75 μm glass capillary containing an electrically floating 25 μm Pt-Ir wire for monitoring the membrane potential.

Dyes were applied to the external membrane surface by dissolving in the artificial seawater (ASW) bathing the axons. Dyes were also applied to the inner surface of internally perfused axons prepared by the roller method originally developed by Baker, Hodgkin and Shaw (1961) by addition to a standard internal solution (SIS). The ASW was composed of (mM): Na^+ 449, K^+ 10, Ca^{++} 50, Cl^- 567, HEPES buffer 5 at pH 8.0. The SIS contained (mM): K^+ 350, Na^+ 50, glutamate 320, F^- 50, sucrose 333, phosphate buffer 15 at pH 7.3. Sensitizers used in these experiments included Eosin Y, Rose Bengal, Methylene blue, Crystal violet, Napthol green B, Bromothymol blue, Methyl orange, Hematoporphyrin and Merocyanine 540. All dyes were used as obtained from the manufacturers without further purification.

Axons were exposed externally to dyes for periods of 3–5 min and then rinsed briefly in ASW prior to illumination to reduce optical shielding artifacts by dye in the bathing medium. Some axons were, however, illuminated when dye was still present in the bath

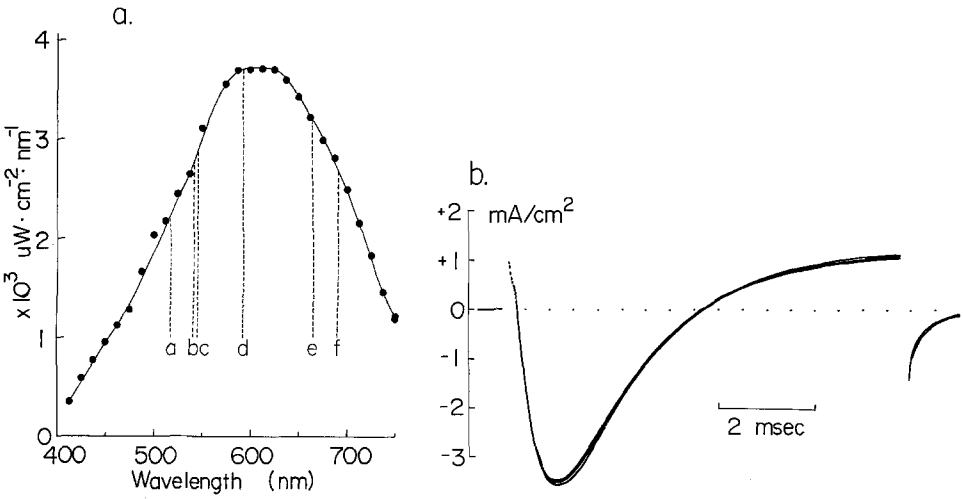

Fig. 1. (*a*): Output spectrum of the illumination system. Wavelengths of maximum absorption of dyes indicated as follows: *a.* Eosin Y; *b.* Merocyanine 540; *c.* Rose Bengal; *d.* Crystal Violet; *e.* Methylene Blue; and *f.* Napthol green B. (*b*): Superimposed voltage clamp currents at 0 mV recorded every 60 sec during illumination without dye for 3 min. The first trace was recorded immediately before illumination

with no observable difference in effect. Internally applied dyes remained in the axons during light exposure.

The optical apparatus for illuminating stained axons consisted of a 650-W quartz halogen lamp, heat filters and a fiber optics output (Pooler & Oxford, 1973). The output spectrum of the system (Fig. 1*a*) shows little contribution from the UV and IR regions. Absorption maxima for each dye are indicated on the spectrum. Control experiments without sensitizer show that light alone did not speed the kinetics of sodium currents (Fig. 1*b*), demonstrating that the results were not due to a heating artifact. All experiments were performed at 8–10 °C.

Oscilloscope records of membrane currents were analyzed within the framework of the Hodgkin-Huxley model of excitability (Hodgkin & Huxley, 1952*b*). In sodium inactivation experiments tetraethylammonium ions (20 mM) were perfused internally to eliminate delayed potassium currents (Armstrong & Binstock, 1965), permitting more accurate records of sodium current.

Results

Internal Application of Dyes

The present results show that squid axons, like many other excitable cell preparations, may be photodynamically altered when sensitized with appropriate dyes. Internal application of dye, which heretofore has not been attempted for photosensitization purposes, is extremely effective. Figure 2 illustrates characteristic progressive changes in action potential configuration during illumination with internally applied Eosin Y as

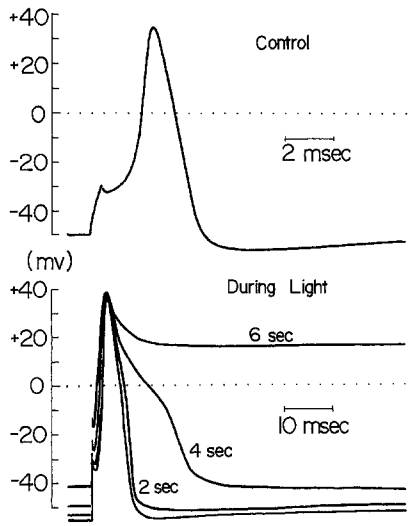

Fig. 2. Action potentials during a control period (upper) and during illumination when Eosin Y (0.005%) was being internally perfused (lower). Note the difference in time scales. The times by each trace indicate the duration of illumination

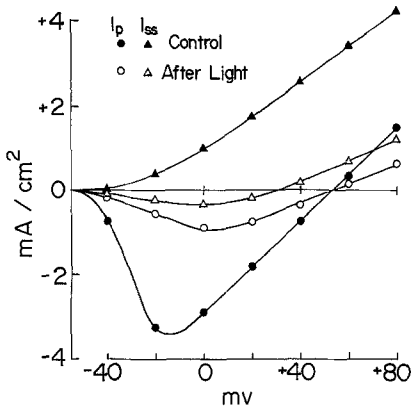

Fig. 3. Current-voltage curves for peak transient and maximum steady-state currents (8.2 msec) obtained from the axon in Fig. 2 after 6 sec of illumination during internal perfusion with Eosin Y (0.005%). All points have been corrected for leakage

sensitizer. The action potential duration becomes greatly prolonged, with a long plateau phase forming after 6 sec of illumination. There is a simultaneous decline in resting potential. Continued illumination leads to eventual inexcitability. A voltage clamp analysis shows a decrease in amplitude of both peak transient current and maximum steady-state current (Fig. 3). The reversal potential for sodium is not changed by illumination (Fig. 3) suggesting that the selectivity properties of sodium channels remain intact (Hille, 1972).

Over most of the potential range investigated an inward steady-state current develops. In this particular case the total current (nonleakage corrected) reversed direction at +18 mV, corresponding to the plateau level of the prolonged action potential. The inward steady-state current has been found to result from a persistent movement of ions through sodium channels which remain open during depolarization following photodynamic treatment and do not inactivate completely. Upon addition of 1×10^{-6} M tetrodotoxin (TTX), which specifically interacts with normal membranes to block sodium channels (Narahashi, Moore & Scott, 1964), both the transient and residual inward currents were eliminated (Fig. 4). Incomplete sodium inactivation was induced by illumination of axons internally perfused with Rose Bengal, Napthol green B, Methylene blue, Methyl orange, and Merocyanine 540, as well as Eosin Y. These dyes exerted generally similar effects when applied internally. Eosin Y and Rose Bengal were apparently the most potent sensitizers and Methyl orange the least.

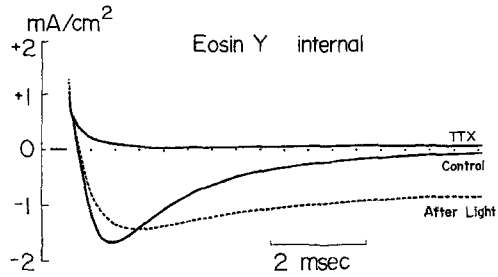

Fig. 4. Superimposed voltage clamp currents at 0 mV before illumination, after 10-sec illumination, and then after external application of TTX in an axon internally perfused with Eosin Y (0.001%). The elimination of the light-induced residual inward current by TTX indicates that it flows through sodium channels

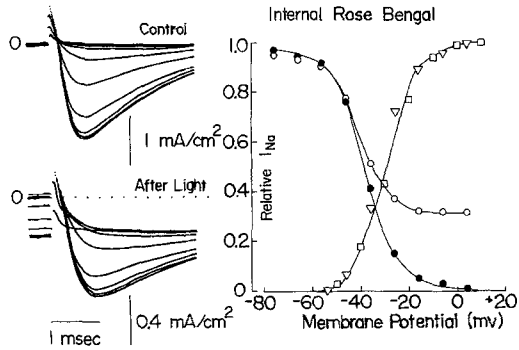

Fig. 5. Steady-state sodium inactivation *vs.* voltage before (filled circles) and after (open circles) 8 sec illumination of an axon internally perfused with Rose Bengal (100 nM). Raw current records during the last 0.5 msec of the prepulse and during the test pulse is shown to the left. The figure also illustrates the correspondence between the difference between inactivation curves (open triangles) and the peak sodium conductance (open squares) assessed from a standard current-voltage curve, both scaled to maximum values of one on the same voltage axis

The incomplete inactivation of sodium conductance induced by internally applied photosensitizers was examined further by measuring its voltage dependence. Steady-state sodium inactivation data were obtained by the standard double pulse technique (Hodgkin & Huxley, 1952*a*) using a conditioning pulse of 70-msec duration followed by a test depolarization to a region of the positive limb of the sodium current-voltage relation. The sodium steady-state inactivation is plotted as a function of membrane potential in Fig. 5 for an axon before (closed circles) and after (open circles) illumination with internally applied Rose Bengal as sensitizer. Superimposed current records during the test pulse preceded

by the final 0.5-msec portion of the prepulse are shown to the left of the curves. Following photochemical modification, which blocked many of the channels, the inactivation curve of the remaining channels failed to reach zero at more positive conditioning potentials, instead developing a plateau-like "foot" of constant value. The shape of the curve implies that there are two populations of unblocked channels: one which inactivates with a normal voltage dependence and one which fails to inactivate. This is in contrast to a situation where the unblocked channel population remains homogeneous but has an altered voltage dependence such as a simple shift along the voltage axis and/or a change in slope. Furthermore, the data suggest that it is only those channels opened by the conditioning pulse which fail to inactivate. The rest (which do not open during the conditioning pulse) apparently inactivate normally. This is demonstrated by comparing the voltage dependence for the opening of channels (peak g_{Na} *vs.* voltage) with the voltage dependence for the failure to inactivate (difference between before and after-light h_∞ curves *vs.* voltage). The comparison is shown in Fig. 5 where values of peak g_{Na} (open squares) and before and after-light h_∞ differences (open triangles) are plotted on the same voltage axis and normalized to a maximum value of one. The two sets of values agree reasonably well. The alternative possibility that some fraction of the channels cannot inactivate at all, regardless of whether they are opened by the conditioning pulse, leads to a derived h_∞ difference curve which does *not* match the peak g_{Na} curve.

If there are two populations of channels, the plateau level of 0.3 in the inactivation curve of Fig. 5 corresponds to the fraction of the remaining functional sodium channels in which the inactivation process is prevented. This fraction varied considerably between axons and dyes, ranging from 0.03 to 0.57. For those channels which did inactivate, it appeared that the kinetics were slowed. Because the currents were so small in magnitude, however, it was impossible to quantify this slowing or determine if it occurred with all dyes.

External Application of Dyes

With the exception of Rose Bengal, dyes applied externally sensitized the same changes as dyes applied internally. Concentrations ranged from 0.001 to 0.01%. A concentration dependence was not evident. The rate of modification, however, was very much lower with external application.

Table 1. Mean rate constants ±SEM for light-induced decrease in peak sodium current comparing internal *vs.* external application of Rose Bengal and Eosin Y

	Rose Bengal $K(sec^{-1})$	Eosin Y $K(sec^{-1})$
Internal	0.617 ± 0.01 n=9	0.262 ± 0.14 n=6
External	0.008 ± 0.003 n=5	0.019 ± 0.008 n=6
Inter/ext	78	13.8

This was assayed by comparing rate constants for the decline in values of peak sodium current during illumination, assuming an exponential time course during illumination as described for lobster axons (Pooler, 1972). Experiments confirmed this relationship for squid axons. As shown in Table 1, internal application of Rose Bengal and Eosin Y was 78 and 14 times more effective, respectively, than external application.

Rose Bengal Modifies Inactivation Only from the Inside

Rose Bengal was the most potent sensitizer of sodium channel block, but differed from the other dyes used in that it sensitized changes in sodium channel inactivation only from the inside. In contrast, Eosin Y, Napthol green B, and Methylene blue led to inactivation changes from either side. Figs. 6 and 7 compare Rose Bengal and Eosin Y in this regard. Fig. 6*a* shows semilogarithmic plots of sodium currents from axons exposed to Eosin Y externally. Illumination induced a decline in current magnitude and a slowing of inactivation kinetics, evidenced by a decrease in slope and shift of time-to-peak sodium current. With Rose Bengal as sensitizer applied externally (Fig. 6*b*), only the reduction in current magnitude is seen. A foot in the steady-state sodium inactivation curve developed after external Eosin Y photosensitization (Fig. 7*a*), but did not develop in axons illuminated after external exposure to Rose Bengal (Fig. 7*b*), in contrast to its effect when applied internally (Fig. 5). The small shift of the inactivation curve for Rose Bengal after light may have resulted at least in part from an uncompensated series resistance (Goldman & Schauf, 1972; Binstock *et al.*, 1975) estimated to be 3–4 Ω cm^2.

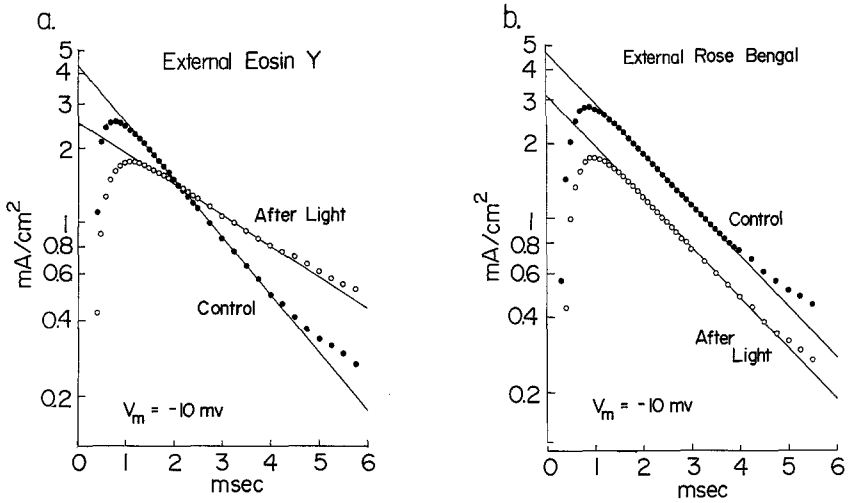

Fig. 6. Time course of sodium current (log scale) during a test pulse to −10 mV before and after 8 sec illumination for axons exposed externally to Eosin Y (*a*) and Rose Bengal (*b*), demonstrating absence of Rose Bengal sensitization of inactivation kinetics

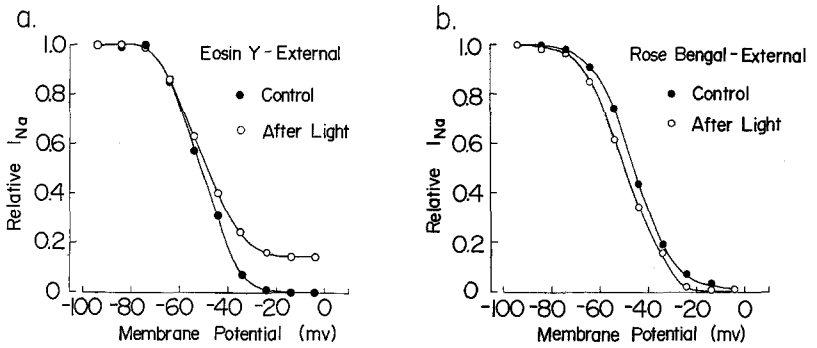

Fig. 7. Steady-state sodium inactivation *vs.* voltage relation for axons treated externally with Eosin Y (*a*) and Rose Bengal (*b*), demonstrating absence of external Rose Bengal sensitization of inactivation

Other Dyes and Dark Effects

With a few exceptions dyes in the absence of light produced no changes in excitability properties. Bromothymol blue exhibited some local anesthetic-like activity, decreasing maximum sodium and potassium conductances by approximately 50 and 30%, respectively, during 3-min external application. Further decreases in conductances upon illumination

were small and indistinguishable from continued exposure to Bromothymol blue in the dark. Crystal violet and Methyl orange were relatively ineffective in sensitizing the squid axon membrane. Eosin Y in high concentration (140 μM) reversibly hyperpolarized the axon membrane by as much as 15 mV when applied externally in the dark.

β-Carotene as a Protective Agent

The role of singlet oxygen ($^1\Delta g$) generated from the physical interaction of ground state oxygen with excited dye triplet molecules is well established in photodynamic processes involving certain simple amino acid and peptide systems (Nilsson *et al.*, 1972). In aqueous solution these molecules serve as substrates for chemical oxidation by the relatively long lived (~2 μsec) singlet oxygen species. Other substances which can physically or chemically react with singlet oxygen at rates greater than these biochemical substrates effectively compete for singlet oxygen, thus protecting the substrate from photooxidation. One widely recognized agent, β-carotene, has a high quenching rate constant in many solvents (Farenholtz *et al.*, 1974) and was examined as a possible inhibitor of photodynamic effects on squid axons. An internal solution saturated with β-carotene was perfused through a squid axon for 30 min followed by a 3 min perfusion with β-carotene and 100 μM Methylene blue. Some reduction in both sodium and potassium currents (Fig. 8*a*) occurred

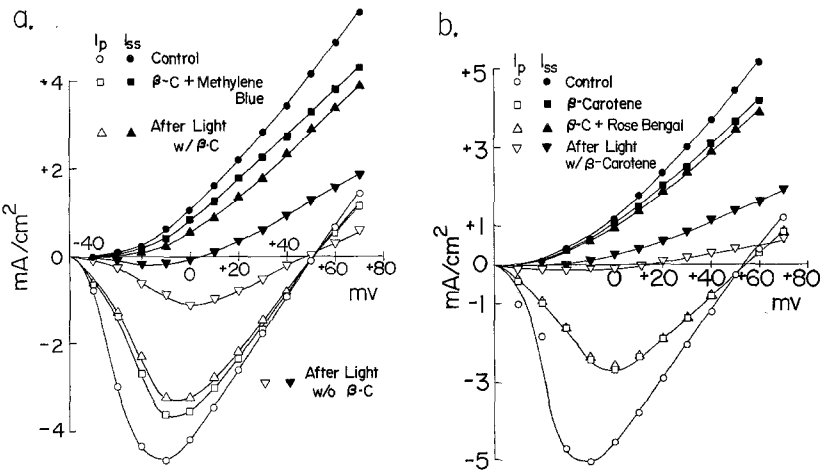

Fig. 8. Current-voltage relations for peak transient current (I_p) and maximum steady-state current (I_{ss}) associated with various stages of sensitization by Methylene blue (*a*) and Rose Bengal (*b*) in the presence and absence of β-carotene. *See text* for detailed explanation

in the dark during the perfusion of β-carotene, indicating a nonphotochemical effect. Illumination of the Methylene blue stained axon in the presence of β-carotene for 2 min produced only minimal further changes in sodium and potassium currents. Following a 5-min rinse with a solution containing Methylene blue but no β-carotene, additional exposure to light (30 sec) decreased both sodium and potassium conductances dramatically (▽ and ▼, Fig. 8*a*). Thus β-carotene effectively protects the axon from photochemical modification with internally perfused Methylene blue in a reversible manner.

The same experiment was performed with Rose Bengal as the sensitizer (Fig. 8*b*). In this case, however, β-carotene did not protect the axon. The same result was obtained using Merocyanine 540, a voltage-sensitive optical probe (Salzberg *et al.*, 1973). Thus in contrast to the protection afforded axons perfused with Methylene blue, β-carotene fails to prevent photodynamic effects of Rose Bengal or Merocyanine 540.

Discussion

The present work establishes that photosensitization of squid axons with many dyes proceeds in a manner similar to that described in more extensive experiments on lobster axons. Since *Sepia* axons also appear to behave similarly (*see* Pooler & Oxford, 1973) one may hypothesize that excitable membranes in general become photochemically modified in the same characteristic manner. This includes an irreversible reduction in sodium and potassium conductances, destruction of inactivation in some sodium channels, and a slowing of remaining sodium inactivation kinetics. The degree to which these various modifications occur, or whether they occur at all, depends on the structure of the sensitizer and the side of membrane to which it is applied. The voltage clamp experiments on squid and lobster axons have not revealed reversibility of the characteristic changes and the nature of the reported reversibility on some other nonvoltage clamped preparations (e.g., Arvanitaki, Romey & Chalazonitis, 1968) remains unclear.

The strikingly greater sensitivity of sodium channels in squid axons to internal dye application compared to external application is probably even greater than indicated in Table 1, since the light-induced response with internal dye was too fast to permit measurement of rate constants in some cases. Part of the lower sensitivity with external dye could

be attributed to shielding of the membrane by dye taken up by the Schwann cell layer. It seems unlikely, however, that shielding could account for the almost two orders of magnitude difference observed with Rose Bengal, particularly in view of the ability of light to penetrate through dye in the external bath in experiments where no pre-illumination rinse was used. Therefore, it may be that the main site of sensitization for block of sodium channels is located close to the internal membrane surface, where one might expect a higher sensitizer concentration to be reached with internal application.

In these experiments sodium conductance could be blocked while sodium inactivation could be slowed and/or prevented, depending on the dye used and the membrane surface to which it was applied. For example, Rose Bengal sensitized only block when applied externally and block plus removal of inactivation when applied internally. This implies the existence of physically separate sites of alteration corresponding to these behaviors. Physically separate membrane regions for the permeability and gating properties of sodium channels has also been suggested as a result of differential sensitivity to changes in divalent cation concentration (Begenisich, 1975).

The data on steady-state inactivation in Fig. 5 suggests that some channels lose the ability to inactivate while the rest inactivate normally. Furthermore, the parallel between the voltage dependencies for the development of noninactivated channels and peak sodium conductance implies that only opened channels fail to inactivate. That is, opened channels are held in the open conformation while unopened channels inactivate normally. This is consistent with several recent models of sodium channel gating which, in contrast to the Hodgkin-Huxley formulation, incorporate two routes of inactivation: one coupled via channel opening and one independent of the opening of channels (More & Cox, 1976; Jakobsson, 1976; Oxford & Pooler, 1975). If coupled inactivation is blocked, but noncoupled inactivation remains unaltered, the parallel between blocked inactivation and peak conductance must occur. In the Hodgkin-Huxley model no such parallel is required. However, an approximate parallel occurs due to the coincidental fall of h and rise of m over the same general voltage range. The data from the present experiments are not sufficiently precise to allow a definitive choice between models but suggest an approach to the question. Future experiments, perhaps involving a combination of photochemical modification and differential shifting of activation and inactivation curves by pharmacological agents, may provide evidence offering a clear choice.

In experiments on lobster axons Pooler (1968) reported a shift of the inactivation curve in the depolarizing direction and a decrease in steepness of the curve, with no development of a foot. This is an apparent conflict with the present squid axon data, but may be an artifact of Pooler's leakage correction method for separating sodium current from total membrane current, which could have concealed the existence of a foot. In the present work sodium currents were separated by repeating a voltage step schedule before and after block of sodium current with TTX and then subtracting currents at corresponding potentials. This latter method gives the true sodium current independent of its time course.

The effective protection by β-carotene of the Methylene blue sensitization is strong circumstantial evidence that singlet oxygen is the reactive intermediate responsible for the membrane alteration (Foote, Chang & Denny, 1970). However, carotenoids can directly interact with excited triplet dye molecules in addition to physically quenching singlet oxygen (Anderson & Krinsky, 1974) and the protective action could conceivably proceed independent of singlet oxygen. The inability of β-carotene to protect with Rose Bengal and Merocyanine 540 is surprising, especially in view of the known effectiveness of Rose Bengal in sensitizing the creation of singlet oxygen (Blossey *et al.*, 1973).

It has been demonstrated that the photodynamic inactivation of enzymes sensitized by dye molecules bound to the substrate can differ signficantly from that involving free dye molecules (Kepka & Grossweiner, 1973). Singlet oxygen appears to preferentially react with molecules to which its parent triplet dye molecules are bound. Perhaps in the present studies the site of sensitization by Rose Bengal or Merocyanine is near a binding site for the dye within the membrane interior, shielded from the protective action of β-carotene, whereas Methylene blue sensitizes at a region near the inner membrane surface in either bound or free form. The observation that the axons appear heavily stained following experiments with Rose Bengal or Merocyanine 540 but do not retain a color after Methylene blue experiments indirectly supports such a mechanism.

The failure of β-carotene to affect the sensitization by Merocyanine 540 indicates that its use as a protective agent during optical measurements of nerve cell membrane potentials is not feasible. Some other substances such as bilirubin have been found to have higher rates of quenching singlet oxygen than β-carotene (Matheson, Curry & Lee, 1974). Their protective action on axon membranes could prove to be very useful.

The authors wish to express their appreciation to Drs. J.Z. Yeh and C.H. Wu for helpful discussion and to Gillian C. Cockerill, Arlene McClenny, Lila Munday and Claire Guest for secretarial assistance. This study was supported by NIH grants (NS09040) to Dr. Pooler and (NS10823) to Dr. Narahashi.

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