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Erythrosin and pH Gradient Induced Photo-Voltages in Bilayer Membranes

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Summary. Erythrosin and light flashes induce voltage transients across bilayer membranes in the presence of transmembrane pH gradients. Fast voltage transients, which rise in <50 nsec and fall in ~ 500 nsec, are attributed to photo-deprotonation of dye sorbed in the glycerol region of phospholipid membranes. Six other halogenated xanthene dyes induce similar effects, which apparently resulted from triplet states of monoanionic dye. No photo-effects were observed with fluorescein.

Key words bilayer membranes · dye photo-deprotonation · erythrosin · fluorescein · membrane photo-voltages · xanthene dyes

Introduction

The impetus for this work came from two areas of study: (i) Synthetic dyes used in food, drugs, and cosmetics, such as erythrosin B or F, D & C red number 3, have been implicated in behavioral abnormalities in susceptible children and inflict damage on various organisms and tissue [1, 6, 18, 20, 22, 25, 26]. This work provides information on the sorption sites of erythrosin in lipid bilayer membranes. (ii) The effects of light on dyes and pigments in bilayer membranes have provided useful models for studying photosynthesis and vision [17, 21, 23, 24, 31, 32, 35, 37]. The dyes described in this study produce interesting photo-physical actions which may give useful comparatives for bacteriorhodopsin and rhodopsin [17, 21, 23, 35, 37] and for studying transmembrane pH gradients [24, 30].

The lowest excited singlet state of phenols is more acidic than the ground state, owing to electron shifts, which causes phenol deprotonation under some conditions following light absorption [3, 39]. Changes in acidity may also be expected for triplet states. Photo-voltages resulting from transverse displacement of electrons and ions in membranes, including displacements which cross only part of the membrane, have been considered by a number of investigators [2, 5, 7, 13, 14, 34, 40]. Making assumption that are not restrictive here [2], the voltage variations expected from such charge displacements in a membrane are

$$V(t) = n\, e\, x(t)/C_m \tag{1}$$

where n is the number of charges e that are displaced transversely in the membrane by $x(t)$, where $x(t)$ is measured as the distance the charge moves divided by the membrane thickness. C_m is the membrane capacitance.

The photo-voltages resulting from bilayer membranes with cyanine dye sorbed to both sides have been studied by Huebner [14]. No photo-voltages resulted unless some asymmetrical condition was imposed on the membrane. When asymmetrical conditions were imposed, the resulting photo-voltage waveforms were equal to the photo-voltage resulting with dye only on the positive (or inside, *see* below) side of the membrane, minus the photo-voltage resulting with dye only on the negative (or outside) side. Experimental evidence is presented below which indicates that erythrosin permeates the bilayer membranes used here. Thus the photo-voltages expected are

$$V(t) = (n_i - n_o)\, e\, x(t)/C_m \tag{2}$$

where n_i and n_o are the number of charges e displaced by illumination in the inside and outside surfaces of the membrane, respectively.

Theory

The theory presented here assumes: (i) Dye permeates the membrane and sorbs to both membrane surfaces where it is exposed to the aqueous solutions so it is influenced by the pH of the adjacent solution. (ii) The dye has an excited state which has a life time T and pK_a of pK_e. (iii) $pK_e < pK_g$, where pK_g is the pK_a of the ground state. (iv) Photo-deprotonated dye

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remains fixed on the membrane. (v) The values of n may be expressed as

$$n_i = N_i\ F_i\ E_i\ I\ Q_i \tag{3}$$

and

$$n_o = N_o\ F_o\ E_o\ I\ Q_o \tag{4}$$

where N is the number of dye molecules sorbed to that side of the membrane, F is the fraction of sorbed dye in the ground state and protonated, E is the dyes' molecular absorption cross section (in cm^2), I is the light intensity (in photons per cm^2 per flash), and Q is the quantum efficiency for photo-deprotonation.

The photo-voltages are expected to be dependent upon the pH values of both solutions, since pH will affect F, E, Q and possibly also N. With the above assumptions in force, and with solution pH values in both solutions equal and between pK_e and pK_g, illumination will cause deprotonation of sorbed dye. The free protons released by illumination, being relatively polar, will move toward the adjacent aqueous solutions. Since an equal number of charges move through both surfaces of the membrane, no variation in the membrane voltage is expected. Photo-voltages may be obtained by either raising or lowering the pH of one solution. If the pH of the outside solution is $\gg pK_g$, then dye on that side of the membrane will not be protonated prior to the light flash so it will not be able to photo-deprotonate (i.e., F_o will be zero). Thus the membrane voltage will vary as protons from dye on the inside (the $pH \sim pK_e$ side) of the membrane are displaced. The voltage will reach a peak value

$$V = n_i\ e\ X/C_m \tag{5}$$

where X is the maximum value of $x(t)$. The voltage will return to the baseline as dye reabsorbs protons from the adjacent aqueous solution. The rise time will be the time required for protons to move away from the dye; the fall time will be the time required for protons to move back, which is approximately the dyes' excited state life time.

If the pH of the outside solution is $\ll pK_e$, then excited dye on that side of the membrane will not deprotonate (i.e., Q_o will be zero). E_o will also be reduced (*see* below). Q is thus seen to be the quantum efficiency for excited state formation times the probability for deprotonation. Reversing these pH gradients is expected to reverse the polarity of the photo-voltages. No photo-voltages are expected when one solution pH is $\ll pK_e$ and the other is $\gg pK_g$.

Materials and Methods

Bilayer membranes were prepared across a 1.3-mm diameter hole in a Teflon cup held inside a glass cell by the syringe method using 10 mg lipid/ml decane in apparatus described in detail elsewhere [15]. Briefly, experiments were performed on a 10-nsec apparatus and a 5-μsec apparatus. The 10-nsec apparatus incorporates a tunable pulsed dye laser (model SP-10, Molectron Corp., Sunnyvale, Calif.), model 3553 buffer amplifier (Burr-Brown, Tucson, Ariz.) and an electrometer output filter. The filter reduced the flash induced electrical noise to <1 mV, but limited the rise time to $\gtrsim 50$ nsec. The laser delivered 7 $\mu J/mm^2$ at 526 nm in 10 nsec, as determined with a Molectron J3–O2 joulemeter. The 5-μsec apparatus incorporates a stroboslave (Gen Rad, West Concord, Mass.) with P-4 capacitors. The energy delivered by the stroboslave which can be absorbed by erythrosin can be estimated by using a quartz filter filled with an aqueous erythrosin solution. The filter had a peak absorption of 50% and reduced the light energy reaching the membrane by 13 $\mu J/mm^2$.

The experiments were performed at 22 ± 2 °C in air, which gave solution pH values of ~ 5.5 (from dissolved CO_2) unless modified by dye, HCl or NaOH additions. Such additions were made after membrane formation using stock solutions of dye in ethanol and diluted HCl and NaOH. The final ethanol content was $\lesssim 2\%$. Unless otherwise noted, erythrosin was added (to 10 μM final concentration) only to the solution inside the Teflon cup, which is subsequently identified as the inside solution. This solution was in contact with the positive electrometer electrode, so positive charges moving through the membrane toward that solution gives a positive photo-effect. The light path passed through the other or outside solution to the membrane. The dye could normally not be detected ($<0.2\%$ absorption in 1-cm path) in the outside solution after the membrane experiments were completed. The solution pH values were determined with a pH meter (Model 7, Corning Glass Works, Corning, N.Y.) and miniature electrode. The lipids were obtained from Sigma Chemical Co. (St. Louis, Mo.). Unless otherwise specified, the membrane lipid was phosphatidylethanolamine. All dyes were obtained from Eastman Kodak Co. (Rochester, N.Y.). The structures of erythrosin and ethyl eosin are illustrated in Fig. 1.

The experimental procedure routinely included observing the noise traces from i) light flashes and bilayer membranes with no dye present, ii) bilayer membrane and dye, with light to the membrane blocked by an opaque card, and iii) light flashes with dye present, after the membrane had been ruptured. The noise traces in all three cases were similar and are illustrated below.

Results

The photo-voltage waveforms consisted of two parts, which are identified here as fast and slow. This paper is concerned with the fast part, which appears in each of the waveforms illustrated in Figs. 2*D–H* and 3*A–E*. The fast and slow parts both appeared in the waveforms obtained with phospholipid membranes and low salt concentrations in the aqueous solutions, as illustrated in Fig. 3*F*. The rise and fall times of the fast part depended upon the speed of the apparatus used.

The rise time is defined here as the time required after the flash onset for the photo-voltage to reach

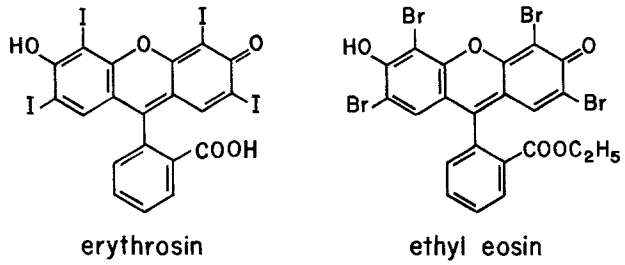

Fig. 1. The structure of two dyes used in this work

80% of its maximum amplitude. The fall time is the time required after the flash for the voltage to fall back to one-half the maximum amplitude. Figure 2*A–C* illustrates that the 10-nsec apparatus, as used here, can resolve ~1 mV transients with ~50 nsec resolution, providing the aqueous solution ionic strength is ≧1 M NaCl. Below 1 M salt concentration, the solution and electrode resistance limit the rise time [15, 33]. Experiments were conducted in 1 M NaBr, CsCl, and LiCl, 0.1 mM, 1 mM, 10 mM, 0.1 M, 1 M, 2 M and 5 M NaCl, all in H_2O; and in 1 and 3 M NaCl in D_2O. When the salt concentration was ≧1 M, the rise time was ~50 nsec, the apparatus limit, independent of the mass of salt or hydrogen ions present. The results presented here suggest that faster apparatus would be able to observe faster rise times which, if the apparatus were fast enough, would vary with the mass of the hydrogen ions present. Figure 3 illustrates that the 5-μsec apparatus could resolve ~0.1 mV transients with ~5-μsec resolution. The rise and fall times of the fast part observed on the 5-μsec apparatus were essentially the times required for the xenon lamp to turn on and off, respectively [36].

The fast part was observed with ≲3 mV amplitudes in phosphatidylcholine, phosphatidylethanolamine, and glycerol monoolein membranes and with reduced amplitude (≲0.2 mV) in oxidized cholesterol membranes, using ≳10 mM NaCl aqueous solutions, from approximately pH 3 to 9. The amplitude and polarity of the fast part depended upon the pH values, gradient magnitude, and polarity. The largest amplitudes occurred with the outside solution at pH 3 and with dye in the inside solution at pH ~5.5, in which case the polarity was such as to make the pH ~5.5 solution positive relative to the pH 3 solution. The amplitude was not increased by lowering the outside solution pH below ~3. The membranes often became unstable below pH 2. With one solution at pH ~5.5 and the other at pH 9, smaller (~1.5 mV) photoeffects resulted which also made the pH ~5.5 solution positive. The amplitude was not increased by raising the pH of the high pH solution above ~9. No photoeffects were observed with one solution at pH 3 while

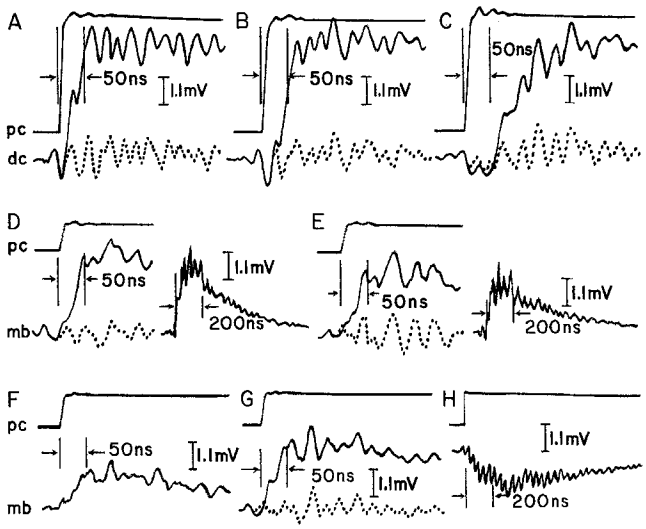

Fig. 2. Photo-voltage transients recorded on the 10-nsec apparatus. Traces *A* through *C* illustrate the apparatus performance with a dummy membrane cell and; (*A*) 3 M NaCl solutions and electrodes, (*B*) 0.1 M NaCl solutions and 3 M NaCl electrodes, and (*C*) 0.1 M NaCl solutions and electrodes. The *"pc"* traces were produced by a photo-cell driving one oscilloscope vertical amplifier. Beam splitters provided the same illumination to an identical photo-cell on a dummy membrane cell, which drove the other oscilloscope vertical amplifier through a 200/1 voltage divider, the cell solutions, electrodes, electrometer, and filter, to produce the *"dc"* traces. Dotted traces were recorded with light to the dummy cell blocked. Traces *D* through *H* were made with phosphatidylethanolamine membranes. (*D*) Photo-cell and membrane *"mb"* traces induced by erythrosin and pH ~5.5 inside, 3 outside, in 3 M NaCl solutions. The dotted trace was recorded with light to the membrane blocked. (*E*): Traces recorded under the same conditions as *D*, except in D_2O. (*F*): Traces recorded with erythrosin and pH ~5.5 inside, 3 outside, in 5 M NaCl solutions. (*G*): Traces recorded with ethyl eosin and pH ~5.5 inside, 3 outside, in 3 M NaCl D_2O. (*H*): Traces recorded with erythrosin and pH 3 inside, ~5.5 outside, with 0.1 M NaCl solutions

the other solution was at pH 9. Photo-effects could be restored by adjusting either solution toward neutral pH values, in which case the polarity of the photoeffect was such as to make the neutral pH solution positive.

The fast part was also observed with erythrosin in both solutions in equal concentrations, provided a pH gradient was created. In this case the amplitude was larger with acid added to the outside solution, where it reduced erythrosin's optical absorption, allowing more light to reach the membrane. The photovoltage action spectrum and erythrosin's optical absorption are shown in Fig. 4. Erythrosin is useful as an indicator dye [8], because of the color change associated with pK_2. The value of pK_2 in pure water, determined spectrophotometrically [27] is 3.7. In dioxane/water (2:3) pK_2 is 4.5 [29], so the pK_a values may be expected to vary with different sorption sites

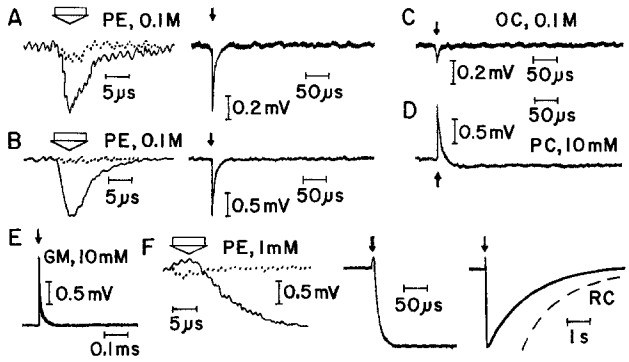

Fig. 3. Photo-voltage transients recorded on the 5-μsec apparatus. Vertical arrows mark the flash illuminations. (*A*): Traces recorded with phosphatidylethanolamine (PE) membranes, pH ~4.5 inside, ~5.5 outside in 0.1 M LiCl. The dotted trace was recorded with light to the membrane blocked. (*B*): Traces recorded with the same membrane used in *A* after lowering the inside pH to 3. (*C*): Traces recorded with oxidized cholesterol (*OC*) membrane, pH 3 inside, ~5.5 outside in 0.1 M NaCl. (*D*): Traces recorded with phosphatidylcholine (PC) membrane, pH ~5.5 inside, 3 outside in 10 mM NaCl. (*E*): Trace recorded with glycerol monoolein (*GM*) membrane, pH ~5.5 inside, 3 outside in 10 mM NaCl. (*F*): Traces recorded with PE membrane, pH ~5.5 inside, 3 outside, in 1 mM NaCl. The dashed "*RC*" trace shows the resistance-capacitance discharge of the membrane through a 10^9 Ω shunt resistor [15]. The fast part of the waveforms is apparent in each trace. In trace *B*, the photo-voltage amplitude *vs.* time trace may be seen to closely follow the well-known stroboscope light flash intensity *vs.* time curve [36]. The slow part is seen in part *F*, and with reduced amplitude in *D*

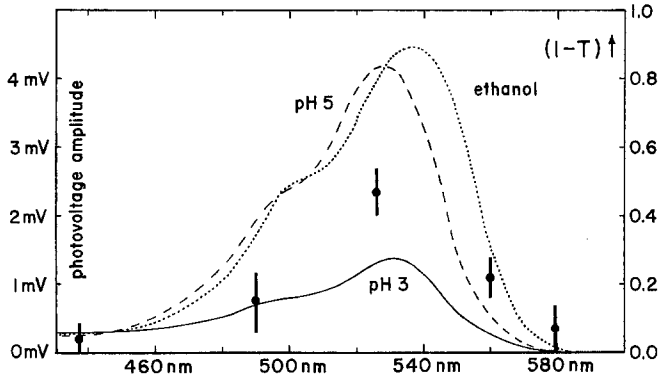

Fig. 4. The photo-voltage action spectrum and one minus transmission of 10 μM erythrosin (1 cm path length) in ethanol, and water (2% ethanol) at pH 5 and 3. The fast part of the photo-voltage amplitudes were measured with approximately constant (±10%) laser flash energy, produced by Molectron laser dyes # 70355-2 (437 nm), # 70368-2 (490 nm), # 70370-2 (526 nm), # 70369-2 (560 nm) and # 70360-2 (579 nm), using phosphatidylethanolamine membranes, pH ~5.5 inside, 3 outside in 1 M NaCl. Four membranes were evaluated at each wavelength. The error bars give ±SD

in membranes. The results described above indicate that the fast photo-voltage transients reported here are associated with pK_3, which in dioxane/water is 5.9 [29]. At pH values above pK_3, erythrosin is a divalent anion, while below pK_3 it is monovalent [12, 29].

Photo-voltages observed on the 5-μsec apparatus with 10 μM dye, pH ~5.5 inside, 3 outside, in 10 mM NaCl, has fast-part amplitudes (in mV) of: erythrosin (1.7), ethyl eosin (1.3), rose bengal (1.2), eosin Y (0.6), phloxine B (0.5), 4′,5′-dibromofluorescein (0.3) and 2′,7′-dichlorofluorescein (0.1). No photo-voltages (<0.1 mV) were detected using bromophenol blue, fluorescein, resorufin, resazurin and tetrabromophenolphthalein under the same conditions. Figure 5 shows variations in the fast-part amplitude *vs.* NaCl concentration for both apparatus used here.

Experiments with neutral density filters indicated the photo-voltage amplitudes from erythrosin decreased linearly with light intensity reductions. The amplitude of the fast part of the photo-effects were ~15% larger with light polarized (electric vector) in the membrane plane than they were with the light polarized 45° from that plane. This indicates the optical absorption moment of the dye inducing these effects favors the membrane plane. The photo-electric effect quantum efficiency is estimated to be 0.4±0.1 by procedures described elsewhere (J.S. Huebner & W.E. Varnadore, *submitted*).

The resistance of the phosphatidylethamolamine membranes were 3 to 10×10^9 Ω. These values were not changed by erythrosin and the pH gradients used here in the absence of illumination. No transmembrane potentials were generated by erythrosin and pH gradients in the absence of illumination. No photo-voltages were observed with membranes that had been reformed but had not thinned to the black (bilayer) state using the 10 nsec apparatus, 50 pF (10 ml volume, 1 mm thick, Chemware) cup, pH ~5.5 inside, pH 3 outside, and 1 M NaCl. Smaller photo-voltages (~1 to 1.5 mV) reappeared as the membranes thinned to the black state, which typically required 5 to 20 min. These photo-voltages increased to ~3 mV in ~5 min after the membrane was black. Reduced photo-effect amplitudes are expected from thicker membranes, since, as the membrane becomes very thick, X from the above equations approaches zero, while C_m approaches the cup capacitance. The capacitance of the black membranes used here were typically 5 nF. These results demonstrate that illuminating the torus of bilayer membranes did not contribute significantly to the photo-effects reported here.

The photo-effects observed with membranes formed in dye solutions not containing ethanol were indistinguishable from those which contained 2% ethanol. The use of 10 mM acetate of phosphate buffers reduced the amplitude and modified the photo-voltage waveforms. The fast part photo-voltage amplitude

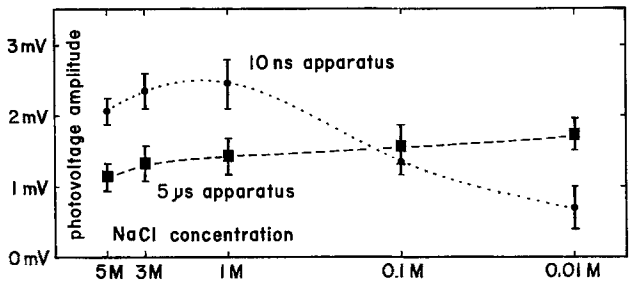

Fig. 5. The mean photo-voltage amplitude for the fast part induced by erythrosin in phosphatidylethanolamine membranes with pH ~5.5 inside, 3 outside, *vs.* NaCl concentration. Traces on the 10-nsec apparatus were recorded using 526 nm light. Three to six membranes were evaluated for each data point shown. The error bars give ± SD

remained unaltered from 50 to 100 laser flashes. Several hundred flashes reduced both the photo-voltage amplitude and the membrane resistance. The fast part photo-voltages were not modified by bubbling N_2 or O_2 in the membrane cell for 30 min prior to membrane formation.

The slow part of the photo-voltage waveforms was observed using $\lesssim 10$ mM NaCl solutions with phosphatidylcholine membranes. It was not observed with glycerol monoolein or oxidized cholesterol membranes. The slow part varied with a large number of parameters, including the salt concentration, membrane lipid, dye location, pH, buffer concentration, time, and membrane illumination history. It is not well understood. It is described here briefly to distinguish it from the fast part and to alert others who may reproduce these experiments to its presence. The slow part may mask the presence of the fast part on low speed apparatus. The limited speed of apparatus which records current transients under voltage clamped conditions, imposed by the aqueous solution resistance and membrane capacitance [16], will prevent such apparatus from detecting the time constants of the phenomena reported here.

Discussion

Bilayer membranes, as used here, serve as transducers which convert electric charge displacements in membranes into externally observable voltage transients. The fast part of the observed voltage transients reported here may be explained by the above theory and known properties of erythrosin. Erythrosin presumably crossed the membrane as a neutral species. The rise times observed on the 10-nsec apparatus were limited to $\gtrsim 50$ nsec by the apparatus output filter for ≥ 1 M NaCl solutions, and by the solution and electrode resistance for < 1 M NaCl. The limited rise time with reduced solution and electrode conductance also accounts for the decreased amplitude observed at low salt concentrations on the 10-nsec apparatus. No fast part would be expected on this apparatus, for example, when the rise time was sufficiently longer than the excited state life time T. On the other hand, the longer flash duration on the 5-μsec apparatus allowed time for the membrane-generated signal to appear on the cup and electrometer input capacitors. The action of the aqueous solution resistance-cup capacitance, and the electrode resistance-electrometer input capacitance as low pass filters limiting the rise time of observed membrane voltage transients has been discussed elsewhere [15]. The decreased amplitude observed with > 1 M NaCl solutions may result from increased membrane capacitance [9, 19, 38] and decreased X values (*see* above, and ref. 2). Reduced erythrosin sorption to the membrane may also occur with high salt concentrations, as has been observed for a carbocyanine dye (2).

The displacement of divalent azo and monovalent carbocyanine dye following photo-isomerization result in voltage transients with rise times in the 2 to 20 μsec [7] and 0.3 to 3 msec [2] ranges, respectively. Erythrosin is of comparable size. It is also believed to be sorbed further from the aqueous solution than the azo dye (*see* ref. 7 and below), which would cause it to move slower toward that solution [14]. Thus photo-deprotonated erythrosin molecules in the membrane are not expected to move significantly in ~500 nsec. The ~500-nsec fall time of the photo-voltages are attributed to dye returning to its ground state, becoming a weaker acid, and reabsorbing protons. Proton equilibrium for dyes in aqueous solutions is usually assumed into the nsec range [3].

Similar photo-effects resulting with ethyl eosin indicate that these effects are caused by phenol and not carboxyl protons. Triplet states are implicated as the deprotonating species by the inferred ~500-nsec life time of the excited states and by the reduced amplitudes which resulted from dyes which lacked the heavier halide atoms. Such dyes exhibit decreased triplet yields [4, 10, 28]. Photo-deprotonation of membrane-sorbed dye in singlet excited states probably also occurs [3], but the contribution to the photo-voltage amplitude is expected to be reduced by the shorter excited state life times (*see* Appendix). The number of photo-protons required to produce the observed photo-voltages could be estimated if X were known. Assuming charge is displaced one-tenth of the membrane thickness (i.e., $X=0.1$), erythrosin and the light flashes used here produce bursts of $\sim 10^9$ protons/mm^2.

Dye locations in membranes may be inferred from photo-voltage measurements with different solution Debye lengths. The membrane thickness, as defined

by capacitance [9, 19] and photo-effect measurements [2, 7], is approximately that of the hydrocarbon membrane core plus a Debye length on each side. Charge movements occurring more than a Debye length from the core are effectively in the bulk aqueous solution and thus do not alter the membrane voltage [2, 7]. The fact that the fast part was observed with 5 M NaCl solutions (Debye length ~ 1 Å), yet sorbed dye was exposed to the pH of the adjacent aqueous solutions suggests dye was in the glycerol regions of the membranes. The fact that oxidized cholesterol membranes give only small amplitudes is consistent with this view. Spin-label experiments with erythrosin and brain liposomes also support this view [11].

The fact that the slow part was not observed in phospholipid membranes with ≥ 0.1 M NaCl indicates the sites for sorbed dye which induced the slow part were more than ~ 10 Å from the membrane core and thus in the lipid head group region of phospholipid membranes. The fact that the slow part was not observed with glycerol monoolein and oxidized cholesterol membranes is consistent with this interpretation.

Quantitative accounts of the effects of buffers on these photo-effects requires knowledge of the buffer distribution in membrane surfaces, since the buffer could prevent charge movement either by reacting with protons before they move transversely or by changing the pH of the microenvironment around the dye.

Conclusion

This work illustrates that: (i) flash-induced voltage transients of membranes prepared in solutions of different salt concentrations can provide information on xanthene dye locations in membranes, a result described previously for the azo dye Bis-Q [7]; (ii) erythrosin permeates and sorbes both in the glycerol and head-group regions of phosphatidylethanolamine bilayer membranes where it is in contact with the pH of the adjacent aqueous solutions; and (iii) weak acids which are also dyes can be used to induce voltage transients and transient proton gradients across membranes. This may provide useful model systems for comparison to rhodopsin and bacteriorhodopsin, which also induce photo-effects in membranes through proton movements [21].

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Appendix

This appendix shows that the contribution to photo-voltage amplitudes from short-lived states decreases with the lifetime of the state. It is assumed that (i) the excited state lifetime T is much less than the flash duration τ, (ii) the charge transport is instantaneous with photon absorption, and (iii) dye in the excited state does not absorb light. The symbols are defined above, and F is equated to unity. The rate of charge movement during the flash is

$$e\, dn/dt = (N - N_E)\, e\, E\, I\, Q/\tau - e\, N_E/T$$

where N_E is the number of dye molecules in the excited state. A steady state will be reached in a few times T, in which

$$N_E = (N\, E\, I\, Q\, T/\tau)/(1 + E\, I\, Q\, T/\tau) \sim N\, E\, I\, Q\, T/\tau.$$

The approximation follows since $E\ I\ Q$ is usually less than one. Thus, the photo-voltage amplitude from short-lived states, such as singlet states where $T \lesssim 10^{-9}$ sec, is expected to be much smaller than for longer lived states, such as triplet or isomeric states.

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