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Kinetics of the Association of Potential-Sensitive Dyes with Model and Energy-Transducing Membranes: Implications for Fast Probe Response Times

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Summary. The second-order rate constants characterizing the association of potential-sensing dyes of the cyanine, merocyanine, and oxonol classes with glycerylmonooleate suspensions, azolectin vesicles, or submitochondrial particles have been measured and the implications for redistribution type mechanisms proposed to explain the potential-dependent optical signals of these probes considered. The second-order rate constants obtained for the cyanines and oxonols are compatible with microsecond probe response times only on the assumption that a high local dye concentration exists in the aqueous phase immediately adjacent to the membrane surface. Calculations based on a surface charge density induced by a bias potential suggest that the necessary local concentration cannot be attained by a diffusion polarization mechanism. A model based on the rapid recombination of ejected dye with the membrane bilayer seems capable of explaining microsecond probe response times in systems where the potential is rapidly changing polarity; calculations suggest that an ejected dye molecule would not diffuse out of an unstirred layer of 100 microns thickness on a millisecond time scale. Microsecond probe responses are also compatible with a first-order potential-dependent dye ejection from the membrane with no rapid recombination when the potential is not changing polarity. The apparent first-order rate constants describing the interaction of merocanine M-540 with a glycerylmonooleate suspension are independent of dye concentration; the reaction may be diffusion limited. The high local dye concentration need not be met in this case for a mechanism based on the transfer of dye onto the membrane from the aqueous phase to describe the microsecond signals of this dye, but other mechanisms have been proposed to explain such signals. The mechanism leading to potential-dependent signals from optical probes appear to differ substantially between suspensions of energy-transducing biological membranes and those involving excitable membranes such as the squid giant axon or model black lipid membranes.

A number of charged dyes of the polyene class are useful optical indicators of electrical activity in a variety of biological preparations ranging from the brains of small animals (Chance, Mayevsky & Smith, 1976; Bashford, et al., 1979), leach ganglia (Grinvald, Salzberg & Cohen, 1977; Salzberg, Davila & Cohen, 1973) to model membrane systems (*see* below). Cohen and Salzberg (1978), Waggoner (1976) and Bashford and Smith (1979) have reviewed the use of polyene probes in biological preparations. Dyes of the cyanine, merocyanine, and oxonol classes are capable of responding spectrally on a microsecond time scale to potential gradients applied across the membrane of the giant axon from the squid *Loligo peali* by means of microelectrodes (Cohen, et al., 1974; Ross, et al., 1977; Chance, 1975). Waggoner, Wang, and Tolles (1977) have also demonstrated that a number of cyanines and oxonols used in axon and ganglia work exhibit microsecond spectral changes when a single 75 mV potential step, following a train of such steps, is applied across a black lipid membrane formed from a glycerylmonooleate solution containing the appropriate dye. Dragston and Webb (1978) have also found that the merocyanine M-540 responds spectrally in microseconds to a potential applied across a hemispherical bilayer. Salama and Morad (1976, 1979) have also shown that the M-540 fluores-

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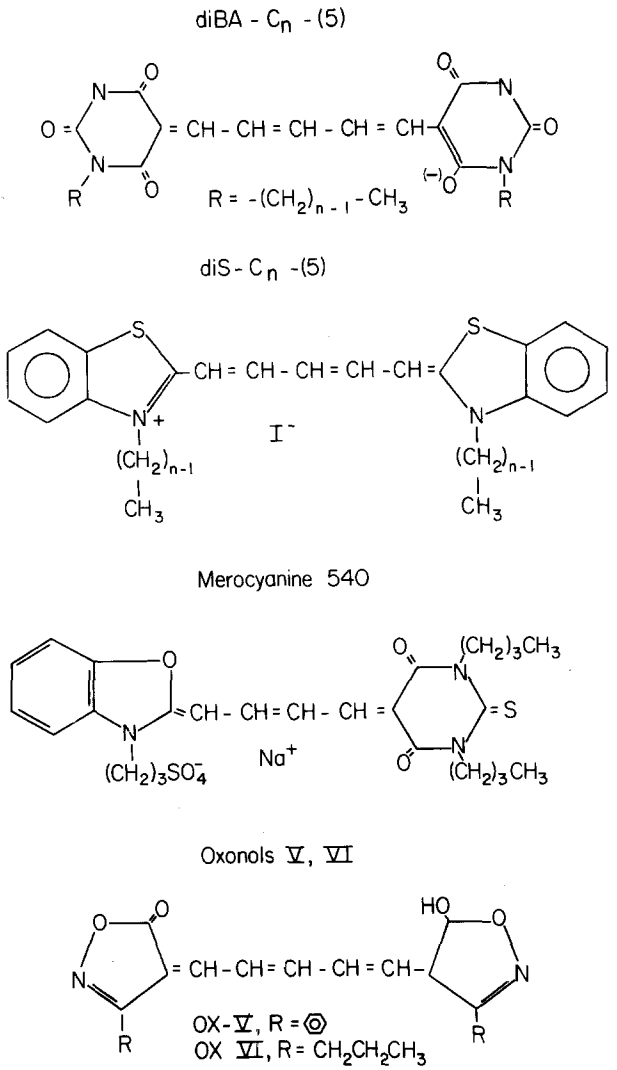

Fig. 1. The structures of the cyanine, merocyanine, and oxonol dyes studied in this report. The value of n in the structures shown is 3 and 5 for diS-C_3-(5) and diS-C_5-(5), respectively. At the pH values used in the binding measurements, oxonols V and VI exist as symmetrical anions since the pK of the hydroxyl proton is approximately 4 (Smith et al., 1976). The oxonol diBA-C_4-(5) also bears a negative charge.

cence response to the action potential in the frog heart closely follows the signal from electrodes placed in the heart.

The purpose of the present report is to investigate the kinetic consequences of redistribution type mechanisms that have been suggested to account for the probe response in excitable tissues, mitochondrial, chromatophore, and model membranes as described above. In particular, Waggoner et al. (1977) have suggested that the absorption spectrum changes exhibited by certain potential-sensitive probes can be explained by the rapid association with or dissociation of the dye from hydrophobic binding sites near the aqueous medium immediately adjacent to the membrane, that is, the unstirred layer. This mechanism is illustrated in Fig. 5. for the case of positively charged probes. In this report, the time course of the interaction of representative dyes of the cyanine, merocyanine, and oxonol classes with suspensions formed from either glycerylmonooleate, soybean phospholipid, or sonicated beef heart mitochondria have been investigated, using rapid mixing techniques. The implications of this investigation for the mechanism proposed above are considered. The structures of the dyes employed in these investigations are shown in Fig. 1. All of these probes are capable of responding to a potential applied across a model or axon membrane on a microsecond time scale.

Materials and Methods

Suspensions of glycerylmonooleate, the same material as used by Waggoner et al. (1977) to form black lipid membranes as previously discussed, or azolectin vesicles were formed by sonication of the appropriate material in aqueous media using either a probe type model W 185 Heat Systems Ultrasonics cell disrupter at medium power or a bath type model G 112 SP1 Laboratory Supply Co. sonicator. Sonication was generally about one-half h to 1 h when the bath type device was used and 5 min when the probe type cell disrupter was employed; sonication was continuted until no further clarification of the suspensions could be detected. The glycerylmonooleate suspensions were subjected to electron microscopy (EM). The results indicated that the preparations were vesicular; no evidence for micelle formation could be detected upon inspection of the EM negatives. The details of the experiments are given in the appropriate figure legend or table.

Electrolytes were obtained from the J.T. Baker Chemical Co. and were of reagent grade quality. HEPES[1] buffer was purchased from the Sigma Chemical Co. Merocyanine 540 was purchased from the Eastman Kodak Co.; the cyanines diS-C_3-(5) and diS-C_5-(5) and the oxonol diBa-C_4-(5) were the kind gifts of Dr. Alan Waggoner. Oxonols V and VI were synthesized according to the procedures described by Smith et al. (1976). Dye solutions in aqueous media were prepared by diluting aliquots of stock solutions in ethanol with the appropriate aqueous solvent. Well-coupled submitochondrial particles were prepared according to the method of Hansen and Smith (1964) by sonicating heavy beef heart mitochondria; this preparation retains an intact F_1 and exhibits respiratory control (Thayer, Tu & Hinkle, 1977).

The observation of the time course of dye interaction with the membrane system studied in the present work is based on the red shift (and, to some extent, the change in shape of the dye absorption spectrum when the probe associates with the membrane). Such spectral changes caused by the latter interaction are illustrated in reports by Smith et al. (1976, 1978), Bashford, Chance, and Prince (1979*a*), and Sims et al. (1974). The time course of the spectral shift was followed by monitoring the differential transmission of the dye

[1] *Abbreviations used:* ANS: 1-anilino-8-naphthalene sulfonate; ATP: adenosine 5′-triphosphate, disodium salt, from equine muscle; CCCP: carbonyl cyanide *m*-chlorophenyl hydrazone; diS-C_3-(5): 3,3′dipropylthiadicarbocyanine iodide; diS-C_5-(5): 3,3′dipentylthiadicarbocyanine iodide; HEPES: N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; oxonol V: bis [3-phenyl-5-oxoisoxazol-4-yl]pentamethine oxonol; oxonol VI: bis[3-propyl-5-oxoisoxazol-4-yl]pentamethine oxonol.

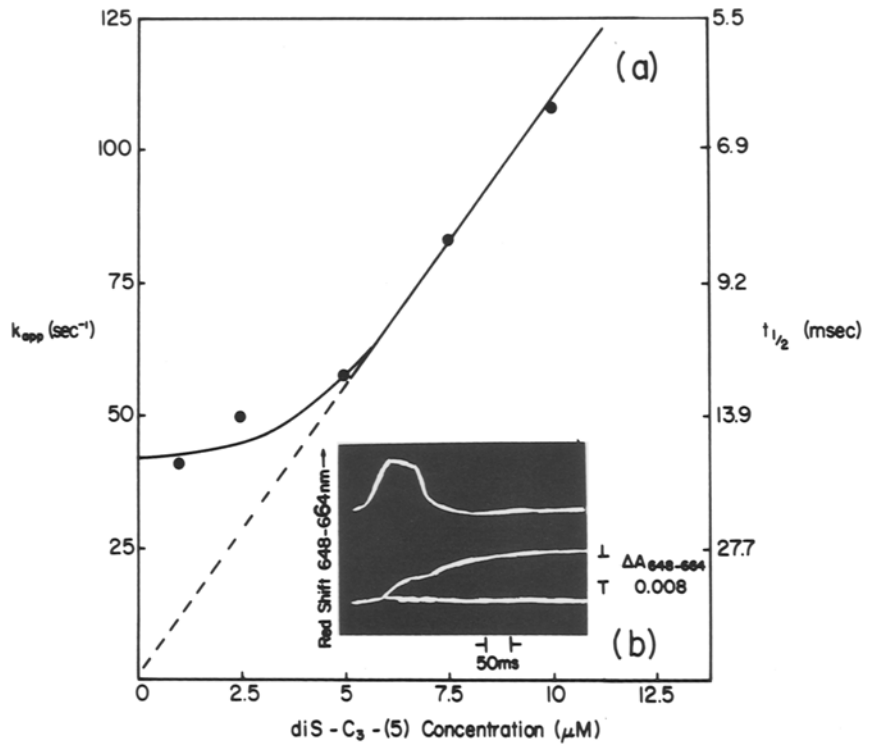

Fig. 2. (*a*): A plot of the apparent first order rate constant k_{app} *vs.* dye concentration for the binding of diS-C_3-(5) to a glycerylmonooleate dispersion, 0.03 mg/ml (final concentration after mixing), in water, pH ~6.5. The second order rate constant is obtained from the slope of the line as described in Eq. (3) in the text. The time course of the dye interaction with the dispersion was followed by monitoring the differential transmission at the 648–664 nm wavelength pair. (*b*): A typical oscilloscope record of the interaction of diS-C_3-(5) with a glycerylmonooleate (GMO) suspension. Dye concentration was 1 μM; the final suspension concentration was 0.03 mg/ml. Experimental conditions were as described in (*a*). The mixing ratio of the model E rapid mixing device is 80:1

at wavelength pairs using time sharing spectrometers. Wavelength choices at which the spectral changes were maximal were obtained from equilibrium titrations of dye solutions of fixed concentration with membrane suspensions. Scanned dye spectra were recorded after addition of each aliquot of the stock suspension, using either a Cary Model 15 or a Beckman Model UV 5270 spectrometer.

Mixing experiments were carried out using a Johnson Foundation Model E rapid-mixing device, adapted to either a Johnson Foundation 200-Hz time-sharing double-beam spectrometer equipped with 500 mm Bausch and Lomb monochromators or to a Johnson Foundation spinning disk (filter) spectrometer that was operated at approximately 200 Hz. The detector output was displayed on a type 564 Tektronix storage oscilloscope. The optical changes were monitored in the transmission mode but have been expressed as absorbance changes indicated by the calibration marks in Figs. 2 and 4.

Results and Data Analyses

The time course of dye interaction with the model or energy-transducing membrane suspensions was investigated under pseudo first-order conditions. The association of the dyes with the suspensions was sufficiently rapid that the data could be analyzed in the continuous flow case (Chance, 1973). A typical oscilloscope record for the interaction of diS-C_3-(5) and M-540 with the glycerylmonooleate suspension is illustrated in Figs. 2*b*) and 4*a*, respectively. For the continuous flow case, the apparent first order rate constant k_{app} is given by

$$k_{app} = \frac{2.3}{t_m} \log (D_2/D_1). \tag{1}$$

This displacements D_1 and D_2 are defined in Fig. 4*b* and the time t_m is related to t_f, which is obtained from the flow velocity trace (Fig. 4*b*) by

$$t_m = t_f (V/V_d) \tag{2}$$

where V is the volume from the point of observation to that of mixing and V_d is the volume discharged in time t_f. These characteristic volumes must be determined for the particular flow device in use. Since the second order rate constant describing the dye-membrane association was to be obtained, the suspension concentration was maintained constant and the concentration of dye kept sufficiently high that it would be the reactant in excess. Under these conditions, the apparent first order rate constant is related to the second order rate constant by

$$\ln 2/t_{1/2} = k_{app} = k_{2nd} X_D \tag{3}$$

where X_D is the reactant in excess, the dye in this investigation. A plot of k_{app} *vs.* X_D will be a straight line, the slope of which gives the second order rate constant, k_{2nd}. Using the procedures described above, the second order binding rate constants for a number of dye-membrane systems have been obtained. A typical plot based on Eq. (3) is illustrated in Fig. 2 for

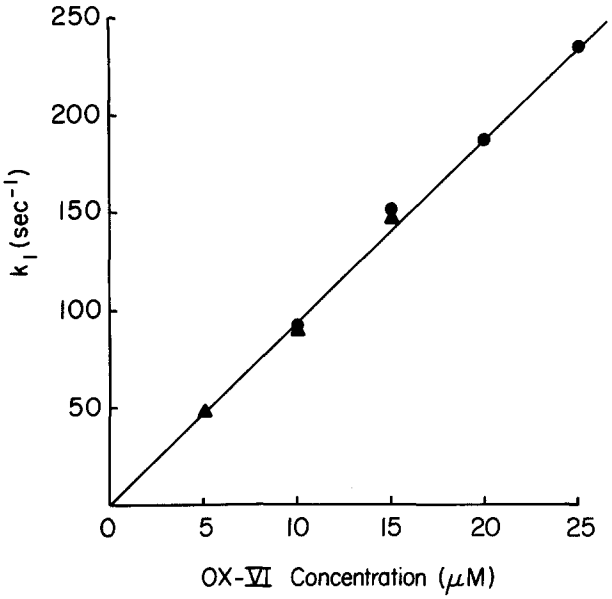

Fig. 3. A plot of the apparent first order rate constant k_{app} *vs.* dye concentration for the passive association of oxonol VI with beef heart submitochondrial particles with the dye in excess. Medium: 25 mM K_2SO_4, 10 mM K-HEPES, pH 7.5, 0.25 M sucrose. The medium contained 0.09 mg/ml submitochondrial particle protein and, when present, 3 μM CCCP. The instrument time constant was 3 msec. Differential transmission changes were followed at the 603–630 nm wavelength pair. Data taken in the absence (●) and presence (▲) of the uncoupler CCCP fall on the same line, indicating the lack of endogenous substrates in the submitochondrial particle preparation. Temperature: 23 °C. The figure is reproduced from Smith and Chance, 1979, by permission

the interaction of diS-C_3-(5) with a glycerylmonooleate suspension. The experimental points fall on a straight line until the dye concentration is reduced to below 5 μM. The apparent first order rate constant then becomes, within experimental error, independent of dye concentration. The latter deviation from the straight line relationship is a consequence of the dye concentration no longer exceeding that of the suspension, or, more precisely, the number of binding sites represented by the suspension. Since the latter becomes the reactant in excess at dye concentrations below approximately 5 μM and the suspension concentration is maintained constant, the observed apparent first order rate constant becomes independent of dye concentration. At diS-C_3-(5) concentrations above approximately 10 μM, the reaction becomes too fast to permit a precise determination of the apparent first order rate constant because the magnitude of D_1 displacement became comparable to that of the noise and could not be measured with sufficient precision. For comparison, data for the passive binding of oxonol VI to beef heart submitochondrial particles is illustrated in Fig. 3, where the pseudo first order condition is maintained over the entire range of concentrations used.

The results of the rapid mixing experiments and experimental conditions are summarized in Table 1. The on-rate constant values for all the dyes employed in these investigations are quite similar, with the

Table 1.

Dye	Wavelength pair (nm)	k_{2nd} (M^{-1} sec^{-1})	Effective concentration (mM)	Membrane system	Final membrane concentration	Medium
diBA-C_4-(5)	595–615	2.3 ($\pm$0.3) $\times 10^6$	30	Azolectin vesicles	1.25 mg/ml	Water, pH ~ 6.5
diS-C_3-(5)	648–664	1.08 ($\pm$0.07) $\times 10^7$	6.4	Glycerylmonooleate suspension	0.03 mg/ml	Water, pH ~ 6.5
diS-C_5-(5)	660–697	2.25 ($\pm$0.22) $\times 10^6$	30.8	Azolectin vesicles	0.13 mg/ml	1 mM KCl, pH ~ 6.5
Oxonol V	591–605	9.04 ($\pm$0.36) $\times 10^6$	7.7	Azolectin vesicles	0.31 mg/ml	10 mM Na-HEPES, pH 7.5
Oxonol VI	603–630	9.53 ($\pm$0.35) $\times 10^6$	7.3	Beef heart submitochondrial particles	0.09 mg/ml protein	0.25 M sucrose, 25 mM K_2SO_4, 10 mM K-HEPES, pH 7.6
Merocyanine M-540	566–542.5	k_{app} independent of dye concentration	Diffusion limited (?)	Glycerylmonooleate suspension	0.03 mg/ml	Water, pH ~ 6.5

Temperature for all measurements was 23 ($\pm$2) °C. The values for k_{2nd} were obtained from a fit of the data to Eq. (3) using a linear regression program. The numbers in parentheses are the standard deviations. The second order rate constants for oxonols V and VI have been taken from Bashford et al. (1979*b*) and Smith and Chance (1979), respectively.

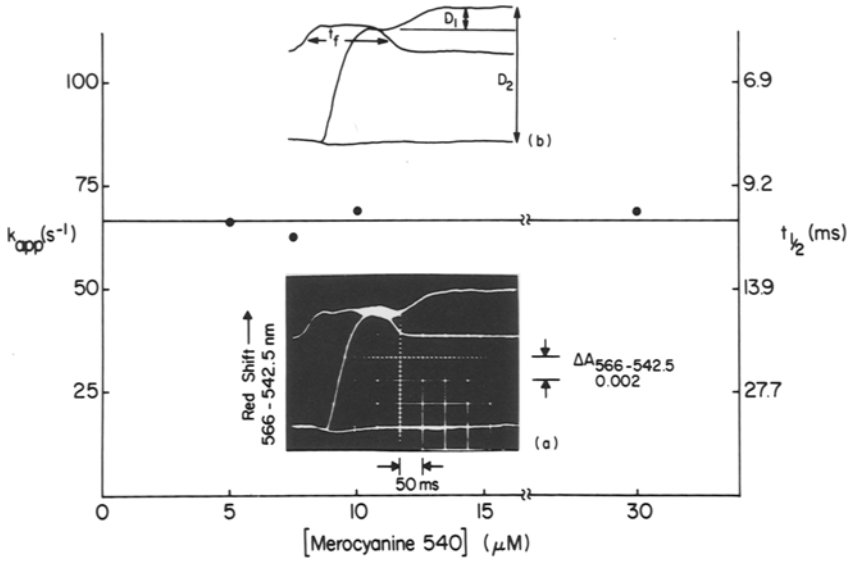

Fig. 4. The interaction of merocyanine M-540 with a glycerylmonooleate suspension. (*a*): The time course of the interaction of M-540 with a glycerylmonooleate suspension in water, pH ~6.5. The dye concentration is 10 μM and the final suspension concentration 0.03 mg/ml. Temperature: 23 °C. (*b*): A diagram illustrating the determination of the apparent first order rate constant from the oscilloscope record using Eqs. (1) and (2). The flow velocity trace defines the quantity t_f. The trace beginning at the base line is the optical signal. The horizontal line represents the average value of the experimental points obtained at the indicated dye concentrations. The average value of k_{app} from a total of 20 experiments at the dye concentrations indicated in the figure is 66.7 ($\pm$11.7) sec^{-1}

notable exception of merocyanine M-540, even though the nature of the membrane system and experimental conditions varied widely.

In the case of M-540, the apparent first order rate constant observed at the lowest glycerylmonooleate concentration at which the signal due to dye binding could be detected was invariant with dye concentration in the 5 to 30 μM range (Fig. 4). Although the possibility that the pseudo first order condition, the dye being the reactant in excess, was not reached cannot be completely eliminated, these observations suggest that the on-rate for the M-540 association with glycerylmonooleate may be independent of dye concentration and, perhaps, diffusion limited. It should be noted that in work with other dyes, the limit of time resolution was determined by the sampling period of the time-sharing spectrometer, approximately 5 msec. The apparent first order rate constant for the M-540 concentrations is well below that corresponding to the 5 msec limit, so the invariance of the apparent first order-rate constant with dye concentration does not appear to reflect inadequate time resolution of the instrument. Haynes and Simokowitz (1977) have also found that the association of ANS, which also bears a sulfonate group, with phospholipid vesicles occurs at a rate too fast to measure with rapid mixing techniques.

For mechanistic considerations, the quantity of interest is the effective dye concentration that has been calculated for an assumed 10 μsec halftime for the dye spectral response to an applied potential gradient. Response times near this value are typical of probes shown in Fig. 1 when they are used in both excitable tissue (Cohen, et al., 1974; Ross, et al., 1977) and in black lipid membrane work (Waggoner et al., 1977; Dragston & Webb, 1978). The concentration values listed in Table 1 were obtained by using Eq. (3), into which the apparent first order rate constants corresponding to a halftime of 10 μsec have been substituted. The values so obtained for the effective dye concentration fall into the millimolar range; such large values greatly exceed not only the bulk concentrations used in virtually all probe work where the dye concentration is typically 5 μM or less, but also, in some cases, the actual bulk solubility limit of these probes in aqueous media. The implications of these results will be considered in the Discussion section.

Discussion

The second order rate constants for representative dyes belonging to the cyanine, merocyanine, and oxonol classes of potential-sensing probes have been measured under pseudo first order conditions for a number of membrane systems and experimental conditions. The values of the rate constants are similar, however, for all dyes employed in this investigation, with the exception of the merocyanine M-540, which appears to have a diffusion limited rate of membrane association. An effort was made in the case of diS-C_5-(5) to measure the rate constant under conditions as close as possible to those employed by Waggoner et al. (1977) in work with dyes in black lipid membranes. The KCl concentration for rapid mixing work unfortunately, had to be kept significantly below that used by Waggoner et al. (1977) (1 mM *vs.* 100 mM). Because of severe aggregation problems

with glycerylmonooleate dispersions prepared by sonication of the ester in 100 mM KCl, the resulting suspensions were unsuitable for experiments based on transmission spectroscopy. With the exceptions of oxonols V and VI, rate measurements were made in water for the reasons cited above.

The time course of the passive interaction of potential-sensitive dyes with biological and model membranes is often observed to be biphasic, consisting of a rapid phase that cannot be resolved in the time of hand mixing techniques, or, in some cases, even with rapid mixing techniques, and a slower phase that usually develops over a period of several seconds or, in some instances, even minutes. Potential-dependent signals that are biphasic have been observed in certain energy transducing systems. Smith and Chance (1979) and Ross et al. (1977) have reported such signals in work with submitochondrial particles and squid giant axons, respectively. The development of the slower phase signal is favored by increasing the dye to membrane binding site concentration ratio and has been interpreted in a number of cases as being associated with movement of the probes through the membrane bilayer. Cyanine dyes such as diS-C_3-(5) appear to accumulate in the interior of red blood cells and phospholipid vesicles over a period of minutes (Sims et al., 1974). When oxonol V associates with phospholipid vesicles, the slower phase can be observed only when the temperature is above that necessary for the gel transition to occur. Under these conditions, oxonol V can be trapped in the interior of the vesicles (Bashford et al., 1979*b*). The conditions that favor the accumulation of the dye are also those that lead to the formation of a slower phase optical signal.

In substrate pulse work using oxonol VI with submitochondrial particles, Smith and Chance (1979) have found that the slower phase potential-dependent signal follows first order kinetics and is described by a rate constant, 0.3 sec^{-1}, similar to that characterizing the valinomycin facilitated transport of ANS across phospholipid vesicles as reported by Haynes and Simkowitz (1977). This rate constant is much smaller than any of those reported in this work for the association of dye with various suspensions. The onset of the slower phase signal could not be observed over the time intervals of Figs. 2 and 4. The fast and slower components are thus well separated in time. There seems little likelihood that the faster forming signals have any significant contribution from dye transport processes and can be treated with considerable confidence as reflecting the initial transfer of dye from the aqueous phase to the initial membrane binding sites.

It is assumed in the following interpretation of the data that the rate constants obtained from rapid mixing techniques adequately describe the rate at which the probe can be transferred from the aqueous phase to the membrane bilayer under conditions in which microsecond optical signals are observed (Waggoner et al., 1977; Cohen et al., 1974; Ross et al., 1977). Supporting evidence for this assumption has been cited previously in this section. In one sense, however, the rate constants summarized in Table 1 likely represent the maximal rate at which dye can be transferred to the black lipid or excitable membrane in the presence of an applied potential gradient because of the effect of the surface potential caused either by the presence of charged dye bound to the bilayer during its formation or to the dye added to the supporting medium. The effect of the surface potential Ψ_0 due to passively bound dye will be shown later in this section to reduce the value of dye concentration immediately adjacent to the bilayer by approximately 35%; the value of the second order rate constant deduced from Eq. (3) will be underestimated by this factor when bulk dye concentrations are used in this equation. An uncertainty of this magnitude will not affect the interpretation of the binding data to be offered subsequently. The second order rate constant $\sim 3 \times 10^{-5}$ M^{-1} sec^{-1} characterizing the transfer of oxonol VI to the ATP-energized submitochondrial particle membrane, for example, is substantially smaller than that corresponding to passive binding of the probe to the dye-free particle, $\sim 9 \times 10^{6}$ M^{-1} sec^{-1} (Smith & Chance, 1979), presumably due, at least in part, to the effect discussed above.

Waggoner et al. (1977) have proposed a mechanism for the microsecond response time of cyanines and oxonols to a single potential step across a black lipid membrane that involves in part the transfer of dye from an aqueous region near the surface of the membrane to a hydrophobic binding site in the bilayer (*see* Fig. 5). The effective dye concentrations in this region that are required for a typical 10 μsec halftime for the optical response of the probe are those collected in Table 1. (Although the effective dye concentrations corresponding to a 10 μsec halftime for probe spectral response were calculated on the assumption that the dye was the reactant in excess, the use of the second order rate constants obtained from rapid mixing experiments is also applicable to the case in which the membrane – binding site – is in excess or when the dye and membrane binding site concentrations are comparable. *See* Appendix.) Since these values are greatly in excess of bulk phase dye concentration, the portion of the mechanism involving transfer of dye from the aqueous phase to the membrane is compatible with the second order rate

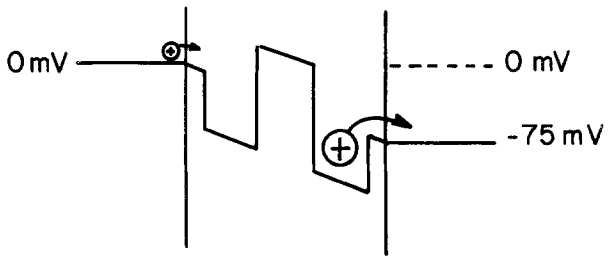

Fig. 5. A diagram illustrating the movement of charged dye from the aqueous phase into binding sites on the left side of the membrane and the ejection of dye from binding sites on the right side of the bilayer when a single −75 mV step potential is applied across the membrane. The binding sites on the right-hand side of the bilayer are said to be occupied by the slow electrophoretic movement of the dye through the bilayer under the influence of a train of potential pulses not illustrated in this figure. The illustration is for a positively charged dye but is applicable to a negatively charged one if the sign of the step potential is reversed. The illustration has been reproduced from Waggoner et al., 1977 by permission

constants obtained from rapid mixing work only on the assumption that a high local concentration of dye exists near the membrane bilayer. Since the bulk dye concentrations in experiments where microsecond optical signals are observed (Waggoner et al., 1977; Cohen et al., 1974; Ross et al., 1977) were a few micromolar and those in Table 1 are in the millimolar range, the ratio of the local to bulk concentrations is of the order of 10^3; this ratio is subject to the possible underestimate of the second order rate constants caused by the repulsive surface potential due to dye bound passively to the bilayer. The uncertainty in k_{2nd}, however, is small compared to the large value of the dye ratio required.

At least two possible kinetic schemes can be envisioned for the processes proposed by Waggoner, et al. (1977) and illustrated in Fig. 5. The first case is the possibility that the rate of transfer of dye from the aqueous phase to a hydrophobic binding site and the ejection of probe from binding sites on the opposite side of the bilayer that have been occupied by the dye that has moved electrophoretically across the membrane are sequentially coupled. The rate at which the optical signal develops is given by the slower transfer process, or rate limiting step. A second possibility is that the on and off transfer processes represent mechanisms operating independently and in parallel. The case of sequential coupling will be considered first. Since the rate of transfer of dye from the aqueous phase to the bilayer binding site is concentration dependent, a dye concentration will exist at which this part of the total transfer process would be the rate limiting step. It thus remains to investigate the possibility that the local dye concentration that is necessary for a nominally 10-μsec probe response halftime can be achieved.

One possibility for accumulating the probe at the necessary concentration is a diffusion polarization process dependent on the surface potential induced by an electrostatic potential applied across the membrane. In the case of the experiments of Waggoner et al. (1977), a value of 37.5 mV can be used for this potential, since it is the mean value of the series of potential steps applied across the bilayer prior to the rapid development of the single potential step leading to the 10-μsec probe response. The value σ_i of the surface charge density induced by an electric field E within a dielectric slab in an external electric field E_0 is given by (Corson & Lorrain, 1962)

$$\sigma_i = \varepsilon_0 E(K_e - 1) = \varepsilon_0 E_0 (K_e - 1)/K_e \tag{4}$$

where K_e is the dielectric constant and ε_0 the permittivity constant. For the 37.5 mV average potential difference across the bilayer as used by Waggoner et al., $E_0 = 3.75 \times 10^4$ V/cm for an assumed bilayer thickness of 100 Å; it is further assumed that the voltage drop is virtually entirely across the bilayer, although electrolyte is present between the electrodes and the bilayer surface. From Eq. (4)

$$\sigma_i = 3.3 \times 10^{-9}(K_e - 1)/K_e \text{ C/cm}^2 \tag{5}$$

At this point, two limiting cases can be considered. One is that the total membrane surface charge density is determined by the induced charges, the passively bound charged dye being cleared from the membrane surface by the electrophoretic movement of the probe to the interior and the opposite side of the bilayer by the action of the average bias potential. The second case is that the total charge density is determined by the sum of the induced charge and that due to passively bound dye, i.e., the passively bound dye is not significantly depleted by the bias potential.

The effect of the induced charge density alone will be considered first. Using a value of 2.5, the value for oleic acid at 20 °C (Gabler, 1978), for K_e, σ_i is $\sim 2 \times 10^{-9}$ C/cm^2. (The value obtained for σ_i is not sensitive to the value chosen for K_e; for example, for water, $K_e = 78$ and $\sigma_i = 3.3 \times 10^{-9}$ C/cm^2.) The surface charge density σ can be related by the Gouy equation (McLaughlin & Harary, 1976) to the surface potential Ψ_0 in the aqueous medium immediately adjacent to the membrane surface:

$$\sinh[F\Psi_0/(2RT)] = A\sigma/\sqrt{C} \tag{6}$$

where $RT/F = 25$ mV at 25 °C. A is a constant, dependent on temperature and dielectric constant; at 25 °C, $A = 8.54 \times 10^4$ M$^{1/2}$ cm^2/C. C is the molar concentration of monovalent electrolyte. At equilibrium, the ratio of the dye in the aqueous medium im-

mediately adjacent to the membrane surface $[D]_{x=0}$ to the bulk concentration $[D]$ is then given by the Boltzmann relationship assuming a uniform distribution of charge on the membrane surface:

$$[D]_{x=0}/[D] = \exp[F\Psi_0/(RT)]. \quad (7)$$

Using $\sigma_i = 2 \times 10^{-9}$ C/cm^2 and $C = 0.1$ M (KCl), the value of Ψ_0 obtained from Eq. (6) is 2.8×10^{-2} mV, or from Eq. (7), $[D]_{x=0}/[D] = 1.001$, which is negligible in comparison to the required value of 10^3 for this ratio. The preceding calculation has assumed that the polarity of the electrostatic potential is such that the sign of the surface charge density σ_1 is opposite to that of the charged dye. Furthermore, the dipole nature of the induced charge density has been ignored in using Eq. (6), which considers only the half of the charge nearer the membrane surface, so the value of Ψ_0 obtained above is an overestimate of this quantity.

The second case in which the contribution of bound charged dye to the surface potential is included in σ is now considered. For the cyanines diS-C_3-(5) and diS-C_5-(5) at 1.5 μM concentration, Waggoner et al. (1977) have estimated that the surface charge density due to bound dye is 9×10^{-12} and 16×10^{-12} mole/cm^2 or $\sigma = 8.7 \times 10^{-7}$ and 1.5×10^{-6} C/cm,^{2} respectively. Since at higher bulk dye concentrations, the value of σ can only increase, it is concluded that the induced surface charge density due to the electrostatic potential σ_i is negligible compared to that of the bound dye at concentrations above 1.5 μM. Using the values of σ given above and $C = 0.1$ M (KCl) the values of Ψ_0 obtained from Eq. (6) are -12 and -13.3 mV for diS-C_3-(5) and diS-C_5-(5), respectively. The negative sign indicates a repulsive surface potential since the surface charge density and the dyes in solution have the same sign. From Eq. (7), $[D]_{x=0}/[D] = 0.63$ and 0.45 for diS-C_3-(5) and diS-C_5-(5), respectively. It is seen that the concentration of dye near the membrane surface has been somewhat diminished relative to the bulk concentrations due to the repulsive surface potential of the bound dye. Assuming that the oxonols listed in Table 1 give rise to surface charge densities of the same order of magnitude as those for diS-C_3-(5) and diS-C_5-(5), it is clear that the ratio $[D]_{x=0}/[D] \sim 10^3$ required from the second order binding rate constants cannot be achieved for either of the two surface charge density cases considered. As will be shown later in this section, 2.5×10^{-8} mole/cm^2 of oxonol V is associated with azolectin vesicles. This value corresponds to a surface charge density of 2.4×10^{-3} C/cm^2. The latter value is much larger than the surface charge density induced by the bias potential. A value of ~ 170 mV for Ψ_0 would be required for this ratio for $t_{1/2} \sim 10$ μsec; this value is large in comparison to the resting potentials maintained by a number of biological systems, -9 mV in the red cell (Hoffman & Laris, 1974; Sims et al., 1974), -90 mV in cardiac cells (Draper & Weidemann, 1951), and -70 mV in the giant axon (Hodgkin, 1958), so it seems unlikely that a surface charge density large enough to give this value for Ψ_0 could be induced by resting potentials of this magnitude.

The preceding calculations have assumed that the membrane surface is initially neutral; this assumption seem to be a reasonable one for the glycerylmonooleate ester since the remaining glycerol hydroxyl groups are not easily ionized. The conclusions drawn from these calculations are nevertheless subject to modification if the surface charge density has significant contributions from either ionized groups at the membrane surface or from passively associated charged species.

It should be noted that the case in which the ejection of the dye from the bilayer under the influence of a potential gradient is the rate limiting step need not be considered in a sequential transfer scheme since the on transfer rate of dye would be required to be even larger than that considered in the preceding discussion. On the basis of the preceding calculations, it is seen that the second possibility, in which the on and off transfer processes are independent ones and the rate of transfer of dye from the membrane to the unstirred layer determines the probe response halftime, would at least avoid the difficulties of achieving high local dye concentrations. Since this transfer process should follow a first order rate law, the halftime for the probe response should be independent of dye concentration in the well (Fig. 5), but this concentration should increase as the magnitude of the bias potential increases. Thus, one would expect the size of the step potential-dependent signal to increase as the bias potential increases but the halftime of the probe response to be independent of the magnitude of this potential. These predictions in principle offer a means of testing the mechanism described in Fig. 5.

Within the context of the discussion of potential dependent ejection of charged dye from a membrane bilayer, the possibility of the ejected dye itself giving rise to the high local concentration within the unstirred layer should be considered, especially in those cases where sequential potentials of alternating polarity are being developed, as in the train of pulses employed by Waggoner et al. (1977), since a rapid reassociation of the dye with the membrane could occur when the potential changes sign. A bias potential in model systems or the resting potentials in

biologically active preparations will insure an unequal exchange of dye on the two sides of the membrane, if both are exposed to the bathing dye solution, as illustrated by Waggoner et al., using black lipid membranes, so that a net optical signal is observed. At least two questions must be considered in evaluating such a mechanism: (1) Does the ejected dye diffuse out of the unstirred layer during the time span of the repulsive pulse? (2) Is the amount of dye ejected sufficient to produce local concentrations in the millimolar range? In order to explore the first question, the following calculations of the distance x a dye molecule would diffuse in water after ejection during a repulsive pulse are presented. The dye molecules immediately after ejection will form a sharp boundary; the time and distance dependence of the dye concentration C for the movement of the probe into a volume of uniform cross section is given by (Tanford, 1967):

$$C=\frac{C_0}{2}\left[1-\frac{2}{\sqrt{\pi}}\int_0^{x/2\sqrt{Dt}}\exp(-y^2)\,dy\right] \tag{8}$$

where C_0 is the initial dye concentration and the integral is the familiar error function. Equation (8) assumes that the diffusion coefficient is not concentration dependent. The value of x for which the dye concentration C has decreased to 1% of the initial value C_0 is taken as a generous estimate of the distance the probe has moved during a time t. Since $C/C_0=0.01$ in Eq. (8), the value of the error function under these conditions is 0.98. The value of $y=x/[2\sqrt{Dt}]$ corresponding to this error function value is 1.65. The diffusion coefficient D must then be determined in order to estimate the distance x. This coefficient is given by

$$D=kT/f \tag{9}$$

where f is the frictional coefficient of the dye molecule in water in this case. With the exception of M-540, which was treated as a sphere, the dye molecules were treated as prolate ellipsoids of major semiaxis a and minor semiaxis b. The ratio of the frictional coefficient f to that for a sphere of the same volume as the ellipsoid f_0 is given by (Tanford, 1967)

$$f/f_0=\frac{[1-b^2/a^2]^{1/2}}{(b/a)^{2/3}\ln\left[\dfrac{1+(1-b^2/a^2)^{1/2}}{b/a}\right]} \tag{10}$$

where $f_0=6\pi\eta r$. The radius $r=(ab^2)^{1/3}$ for a prolate ellipsoid, and η is the viscosity of water, 1 centipoise at 23 °C. The diffusion coefficients are obtained by solving Eq. (10) for f and substituting the resulting expression into Eq. (9). The results of the calculations of x are summarized in Table 2 for selected values of the repulsive pulse width t.

For a 1-msec repulsive pulse width, the value used by Waggoner et al. (1977), and also the approximate duration of the upstroke and plateau retions of the action potential in the squid giant axon (Cohen et al.,

Table 2. Summary of calculated diffusion coefficients and distances for polyene dyes

Dye	a (A)	b (A)	D (cm^2/sec)	x (cm) $y=x/2\sqrt{Dt}$	Time (msec)	σ (cm) $D=\sigma^2/(2t)$
diBA-C_4-(5)	7.3	4.3	4.1×10^{-6}	2.1×10^{-4}	1	9.1×10^{-5}
	7.3	4.3	4.1×10^{-6}	3.0×10^{-3}	200	1.3×10^{-3}
diS-C_3-(5)	6.5	3.7	4.7×10^{-6}	2.3×10^{-4}	1	9.7×10^{-5}
	6.5	3.7	4.7×10^{-6}	3.2×10^{-3}	200	1.4×10^{-3}
diS-C_5-(5)	6.5	5.2	3.9×10^{-6}	2.1×10^{-4}	1	8.8×10^{-5}
	6.5	5.2	3.9×10^{-6}	2.9×10^{-3}	200	1.2×10^{-3}
M-540[a]	5.5	5.3	4.0×10^{-6}	2.1×10^{-4}	1	8.9×10^{-5}
	5.5	5.3	4.0×10^{-6}	2.9×10^{-3}	200	1.3×10^{-3}
Oxonol V	5.0	2.0	6.4×10^{-6}	2.6×10^{-4}	1	1.1×10^{-4}
	5.0	2.0	6.4×10^{-6}	3.7×10^{-3}	200	1.6×10^{-3}
Oxonol VI	5.1	2.4	6.7×10^{-6}	2.7×10^{-4}	1	1.2×10^{-4}
	5.1	2.4	6.7×10^{-6}	3.8×10^{-3}	200	1.6×10^{-3}

The dyes were treated as prolate ellipsoids with major semiaxis a and minor semiaxis b.

[a] M-540 was treated as a sphere with $R=5.4$ A since $a\simeq b$. Bond angles and lengths taken from the literature, e.g., Cram and Hammond (1959) were used to estimate a and b. In calculating b, the average of the distance across the ring structures and R groups relative to the conjugated carbon chain axis was used. The calculations are for a temperature of 23 °C. Water was assumed to be the medium in which the diffusion was occurring.

1974), the value of x is approximately 10^{-4} cm. Since the width of the unstirred layer near a bilayer in a stirred supporting medium is nominally 1×10^{-2} cm (S. Krasne, *personal communication*), the calculations indicate that the diffusion of the ejected dye out of the unstirred layer and the resulting equilibration with the bulk dye concentration in the medium will not occur. Similar conclusions apply in the case of a 3-msec pulse duration, the pulse width used by Cohen et al. (1974) in a number of experiments on the giant axon in which potentials were applied using microelectrodes.

The diffusion distance x has also been estimated for a repulsive pulse width of 200 msec, which is the approximate duration of the upstroke and plateau regions and the slow diastolic depolarization phase of the action potential observed in the sinoatrial node or Purkinje fiber (West, 1972). Since relative to the threshold potential at which the action potential fires, the time course of the potential leads to a change in polarity, the model based on dye ejection from a membrane followed by rapid reassociation may have some applicability in the cases described above. Salama and Morad (1976, 1979), for example, have shown that M-540 can be used to monitor the time course of the action potential in the frog heart. The value of x corresponding to a 200 msec pulse width is tabulated in Table 2 for the series of dyes employed in the rapid mixing work. For the dyes shown in Fig. 1, the results indicate that the diffusion distance is well short of the 1×10^{-2} cm width of an unstirred layer, so by the criterion of question (1), a mechanism based on dye ejection followed by rapid reassociation with the membrane seems feasible for both the 1 msec and the 200 msec action potential cases and for the train of 1 msec symmetrical pulses used by Waggoner et al. (1977).

The second question, dealing with the amount of dye ejected into the unstirred layer, is much more difficult to address than the first one. The quantity ejected depends on the amount of dye bound to the membrane, which is a function of the bulk free dye concentration, the bias or resting potential, the affinity of the probe of interest for the membrane, and the magnitude of the repulsive potential. Data describing the interaction of the probes listed in Fig. 1 with membranes is available for diS-C_3-(5), diS-C_5-(5), and oxonol V. Waggoner et al. (1977) have found that 9×10^{-12} mole diS-C_3-(5)/cm^2 and 16×10^{-12} mole diS-C_5-(5)/cm^2 is bound to a glycermonooleate bilayer when the free dye concentration is 1.5 μM. For ~1 μM free dye, ~30 nmol oxonol V/mg lipid is associated with azolectin vesicles (Bashford & Smith, 1979) in a medium containing 100 mM Na_2SO_4 and 10 mM Na-HEPES, pH 7.5. Assuming an average molecular weight of 500 for the lipid and that the membrane area associated with one phospholipid molecule is 1/60 Å^2 (McLaughlin & Harary, 1976), the oxonol V binding affinity becomes 1.5×10^{-8} mole/cm^2. The amount of bound dye/cm^2 is designated P_0 in the following analysis.

It is assumed that the bias potential gradient falls primarily between the two wells illustrated in Fig. 5 and that the distribution of dye between these wells can be described by a Nernst relationship. In the presence of a bias potential Ψ, P_0 will be altered by an amount P as the dye distributes between the two wells:

$$\Psi = -0.0592 \log \frac{P_0 - P}{P_0 + P}. \tag{11}$$

This analysis assumes that both sides of the bilayer are exposed to the dye containing medium. For $\Psi = 37.5$ mV, the value used by Waggoner et al. (1977), the solution to Eq. (11) yields $P = 0.63\, P_o$. The amount of dye/cm^2 associated with the deeper well in Fig. 5 is then $P_o + P$. The resulting values for the latter sums are 2.5×10^{-8} mole oxonol V/cm^2, 1.5×10^{-11} mole diS-C_3-(5)/cm^2, and 2.6×10^{-11} mole diS-C_5-(5)/cm^2.

The molar concentration of dye ejected into the unstirred layer from a membrane area of Acm^2 is then given by

$$1{,}000 \cdot (P + P_o) \cdot A/A \cdot \sigma) \tag{12}$$

where σ, the dye diffusion distance in cm, is the standard deviation of the Gaussian curve describing the dye gradient distribution as a function of distance. The latter value for the diffusion distance is used as a representative one, since the value of x corresponding to $C/C_o = 0.01$ is near the maximal distance a dye molecule would diffuse during a time t. The values of σ are collected in Table 2 for 1 and 200 msec pulse widths. The resulting dye concentrations in the unstirred layer resulting from the preceding calculations are ~0.2 M for oxonol V and ~0.2 and ~0.3 mM for diS-C_3-(5) and diS-C_5-(5), respectively, when the repulsive pulse width is 1 msec; when the width is 200 msec, the concentrations given above are reduced by approximately an order of magnitude. These values further assume that all dye in the deeper well, Fig. 5, is expelled by the repulsive potential, which is, of course, unlikely. If only 1% of the oxonol V in this well is ejected by a 1 msec pulse, however, the local concentration will be near the ~7 mM value estimated to be required from the data derived from rapid mixing work (Table 1). Even if all of the diS-C_3-(5) or diS-C_5-(5) were expelled from the deeper well by a 1 msec pulse, the local concentration of these dyes would still be about an order of magnitude below the values listed in Table 1.

Within the uncertainty of the preceding rough calculations of the amount of dye that could be ejected from a membrane by a repulsive potential pulse, the results suggest that an on-off mechanism may be feasible for a train of pulses of alternating polarity, especially if the dye concentration and bias potentials are somewhat larger than those used in the preceding estimates.

It should be noted that in the case where only one side of a membrane is accessible to the dye, a bias potential is not necessarily needed, since no signal of the opposite sign to that due to reassociation of the dye could come from the dye free side of the bilayer.

For those cases in which an exciting pulse is used, such as in substrate pulse work (Smith & Chance, 1979) or single flash excitation experiments with chromatophores from photosynthetic bacteria (Bashford et al., 1979*a*), the preceding model is unlikely to be applicable, because any ejected dye would rapidly reassociate with the membrane because of the lack of a subsequent attractive potential. For these and similar cases, the ability of the probe to permeate the bilayer seems vital to the formation of microsecond probe response times in the scheme illustrated in Fig. 5. Biological systems such as the red blood cell are known to maintain resting potentials that may function in a manner similar to the bias potential in the black membrane work, thereby populating the deeper well.

If the binding sites on the opposite side of the bilayer from which dye transfer to the membrane occurs cannot be significantly populated, either due to the absence of a resting potential or the inability of the dye to permeate the membrane, the movement of the probe onto the membrane from the aqueous region near the surface is the only viable portion of the proposed mechanism for generating potential-dependent optical signals. The merocyanine M-540 moves across black lipid membranes much less rapidly than do cyanines and oxonols that possess delocalized charges (Waggoner et al., 1977). In terms of the mechanism illustrated in Fig. 5, the on transfer process from the aqueous phase is likely the only viable mechanism. The rapid mixing results, however, suggest that the binding rate of M-540 to membranes may be diffusion limited. Thus, no dye concentration requirements need be met since the transfer rate would be independent of these concentrations. Alternative mechanisms for M-540 optical signals have been suggested. Dragston and Webb (1978) have proposed that the potential-dependent loss of M-540 fluorescence yield observed in work with black lipid membranes is due to the rotation of the bound dye fraction into the plane of the bilayer followed by the formation of nonfluorescing aggregates. Conti (1975) has also suggested a rotation-dependent mechanism for probes. Since M-540 has a large dipole moment due to the localized charge of the sulfonate group (Fig. 1), the dye would experience significant torque when in the presence of an electric field. Ross et al. (1974) have also suggested that M-540 fluorescence changes that accompany a potential-dependent absorption spectrum shift that is observed in giant axons from the squid *Loligo peali* are due to a shift in the monomer-dimer distribution of the membrane bound fraction of dye. The rotation-dependent dimer formation described above is unlikely to be applicable to probes such as the oxonols and cyanines that bear delocalized charge distributions and hence have low permanent dipole moments. The potential-dependent formation of nonfluorescing or weakly fluorescing dye aggregates seems to be of importance in the case of a number of cyanines similar to diS-C_3-(5) (Sims, et al., 1974; Waggoner et al., 1977).

In systems such as beef heart submitochondrial particles and chromatophores from photosynthetic bacteria, oxonols V and VI exhibit potential-dependent absorption spectrum shifts and loss of fluorescence yield that, according to thermodynamic studies, are due to redistribution of the dye from the aqueous phase to the biological membrane (Smith et al., 1976; Bashford et al., 1979*b*; Smith & Chance, 1979). These preparations, however, do not maintain resting potentials. It is significant in light of the preceding discussion that no evidence for microsecond probe responses has been found when these preparations are energized either by substrate consumption or flash activation. The second order rate constants describing the potential-dependent absorption spectrum shifts do not exceed those given in Table 1 for passive binding [$\sim 3 \times 10^5$ M^{-1} sec^{-1} for submitochondrial particle (Smith & Chance, 1979); $\sim 2 \times 10^6$ M^{-1} sec^{-1} for chromatophores (Bashford et al., 1979*a*)]. These negative observations would be expected on the basis of the mechanism illustrated in Fig. 5, since no means of accumulating the oxonols on the opposite side of the membrane is available, nor is the rapid reassociation of ejected dye possible because the potential does not undergo a reversal of polarity. In the case of the submitochondrial particle preparation, the enzyme turnover numbers may be too small to allow a membrane potential to develop in microseconds. In chromatophores from photosynthetic bacteria, however, the initial charge separation is complete within 10 psec (Dutton et al., 1976), and a substantial fraction of the membrane potential has been developed within a few μsec (Jackson & Dutton, 1973), as judged by carotenoid band shifts. Even when a resting potential was developed in a chromatophore suspension by subsaturating background illu-

mination, Bashford et al. (1979*a*) were unable to observe an oxonol VI energy-linked spectral shift on a microsecond time scale contrary to the predictions of the scheme in Fig. 5, since it was shown that the probe is able to permeate the chromatophore membrane when a potential is generated. These results suggest that perhaps the dye cannot be ejected from the chromatophore membrane fast enough to produce optical signals on a microsecond time scale. The mechanisms leading to potential-dependent signals from optical probes may differ substantially between suspensions of energy-transducing biological membranes, such as submitochondrial particles or chromatophores, and those involving excitable membrane preparations, such as the giant squid axon or model black lipid membranes.

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Appendix

The second-order rate constant obtained under pseudo first-order conditions in which the membrane binding site is the reactant in excess is related to that obtained with the dye in excess by the expression (Bashford, et al., 1979*b*)

$$n k_{2nd}^{(membrane)} = k_{2nd}^{(dye)} \tag{A1}$$

where n is the binding stoichiometry for the association relation, i.e., the number of moles of dye that can bind to a unit weight of membrane or membrane protein. For the case of the membrane system in excess

$$k_{app} = k_{2nd}^{(membrane)} X_{membrane} = k_{2nd}^{(dye)} X_{membrane}/n = k_{2nd} X_D \tag{A2}$$

since dividing the effective membrane binding site concentration by n converts this quantity to the corresponding effective dye concentration, X_D.

For the case in which neither the membrane binding sites nor dye are greatly in excess, an approximate effective dye concentration can be obtained from the solution to the general second order rate law under the condition that the dye and membrane binding site concentrations are equal. For the latter case,

$$t_{1/2} = 1/k_{2nd} X_D \tag{A3}$$

for $t_{1/2} \sim 10\,\mu$sec and $k_{2nd} \sim 10^7\ M^{-1}\ sec^{-1}$, a typical value, the effective dye concentration X_D is nominally 10 mM, a similar value to those given in Table 1.

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