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Impermeant Potential-Sensitive Oxonol Dyes: III. The Dependence of the Absorption Signal on Membrane Potential

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Summary. We have measured potential-dependent changes in the absorption of light by oxidized cholesterol bilayer lipid membranes in the presence of impermeant oxonol dyes. The magnitude of the absorption signal increased linearly with the size of potential steps over a range of 500 mV. The signal also increased when the offset voltage of the pulse train was increased from −150 to +150 mV. The data are consistent with the "on-off" mechanism proposed by E. B. George et al. (*J. Membrane Biol.* **103**:245–253, 1988) in which the probe undergoes potential-dependent movement between a binding site in the membrane and an aqueous region just off the surface of the membrane. An equilibrium thermodynamic analysis of the experimental data indicates that the negatively charged oxonol chromophore senses only 5–10% of the total membrane potential difference across the membrane when it is driven into a nonpolar binding site on the membrane.

Key Words potential-dependent dye · oxonol dye · mechanism · lipid bilayer · oxidized cholesterol · membrane potential

Introduction

This paper describes the third of a series of studies on the mechanism of membrane potential sensitivity of the impermeant oxonol dyes. Potential-dependent light absorption changes have been determined with oxidized cholesterol bilayer lipid membranes bathed in an aqueous solution containing the dye. The previous papers were concerned with the dependence of the absorption signal on the wavelength and polarization of the illumination and on the length of alkyl groups attached to the chromophore structure (George et al., 1988; Nyirjesy et al., 1988). The present paper reports experimental measurements of the dependence of the absorption signal on the size of the voltage steps and on the offset voltage of the pulse train used to generate the signal. An equilibrium thermodynamic analysis is used to examine the experimental data in the context of an "on-off" model, in which dye molecules undergo a potential-dependent movement between sites on the membrane and an aqueous region just off the membrane. Analysis of the data with the theory leads to the conclusion that the probe molecules sense at the most about 5–10% of the total electrical potential drop across the membrane.

Materials and Methods

Experiments were performed according to the methods described by George et al. (1988). The hemispherical bilayer lipid membrane system was originally developed by Dragsten and Webb (1978) and the optical detection system is similar to that described by Waggoner, Wang and Tolles (1977). The structures of the three membrane potential probes used in this work are given in Nyirjesy et al. (1988). In order to identify systematic variations in the signal as a function of time (Materials and Methods section, George et al., 1988), membrane voltage settings were regularly returned to a reference setting. The data presented in this paper were obtained with stable membranes with which the signal drifted less than 5% during the course of the measurements. Data could usually be acquired within 1 to 2 min. Blocks of voltage measurements over time were normalized to the reference settings in order to further account for drift.

Results and Analysis

Pyrazolone barbituric acid dyes were added to the exterior compartment of spherical oxidized cholesterol bilayer lipid membranes (George et al., 1988). Trains of square-wave voltage steps of size ΔV were applied across the membrane and the absorbance

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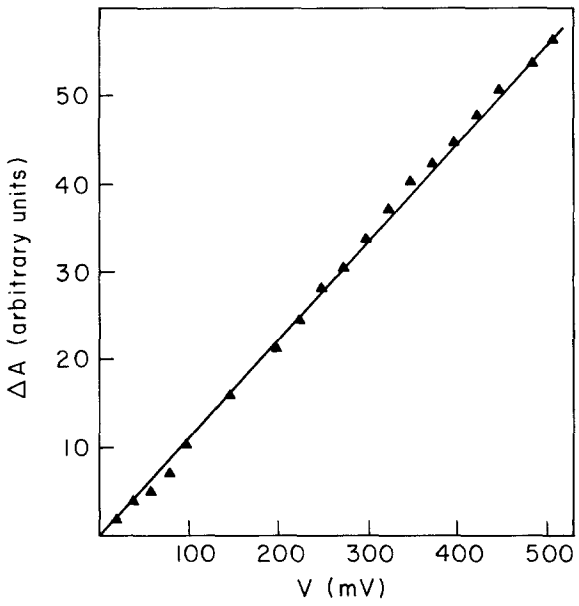

Fig. 1. Magnitude of light absorption ΔA *vs.* ΔV for 6 μM RGA461 measured at 620 nm. The vertical scale is in arbitrary units. The offset voltage was held at 0 mV during this experiment

signal ΔV was monitored at 620 nm. The offset voltage V_b of the train of potential steps could also be varied during the experiments (Fig. 5 of Nyirjesy et al., 1988). Voltages are relative to a reference potential of 0 mV in the bathing medium surrounding the spherical bilayer.

Experimental ΔA *vs.* ΔV with no Offset

The strength of the optical signal for dye RGA461 varies linearly with the size of the voltage steps applied to the membrane up to a ΔV of 500 mV (Fig. 1). Since the steps were symmetrical about 0 mV, the maximum magnitude of the voltage applied to the membrane during the positive and negative steps was 250 mV before the membrane ruptured. Several of the other pyrazolone barbituric acid dyes with closely related structures (Nyirjesy et al., 1988) gave similar results. Gupta et al. (1981) found that WW781, which belongs to this class of dyes, gives a linear response over a voltage range of +100 to −100 mV when used with squid giant axons.

Theoretical Analysis of the Linear ΔA *vs.* ΔV with no Offset

The ΔA *vs.* ΔV data contain information about how the probes interact with the membrane. In Part I (George et al., 1988) of this series, the "on-off" model for this interaction was presented. The model, which is depicted in Fig. 4 of Nyirjesy et al. (1988), describes the voltage-dependent movement of dye between population I, which is in the membrane hydrocarbon, and population II, which is in the unstirred aqueous region of thickness ∂ just off the surface of the membrane. The dye in population II is in equilibrium with the dye in the bathing solution and has the same average concentration. In Part II of this series, Nyirjesy et al. (1988) developed an equilibrium thermodynamic equation based on the "on-off" model in order to analyze the dependence of the signal on voltage and on the membrane-binding properties of the dye. According to the theory, the absorbance signal is proportional to the amount of probe Δn moving between populations I and II and is given by their Eq. (7). Below we have rewritten that equation specifically for the analysis of the voltage dependence of the absorption signal. The conversion was accomplished by substituting $\exp(-\gamma\beta V_b)$ for $K(V_b)$ (Nyirjesy et al., 1988):

$$\Delta A \propto \Delta n \sim \frac{K(N)e^{-\gamma\beta V_b}[1 + K(N)e^{-\gamma\beta V_b}][e^{-\gamma\beta\Delta V/2} - e^{+\gamma\beta\Delta V/2}]}{[1 + K(N)e^{-\gamma\beta(V_b-\Delta V/2)}][1 + K(N)e^{-\gamma\beta(V_b+\Delta V/2)}]}. \quad (1)$$

In this Eq. (1), $\gamma = ZF/RT$, where Z is the charge on the chromophore part of the dye, F is Faraday's constant, R is the gas constant, and T is the Kelvin temperature. We assume that the sulfonate charge on the dye cannot penetrate into the membrane to sense the electric field, but the oxonol chromophore can. For the oxonol chromophore, $Z = -1$, so γ has the value -0.04 mV^{-1}. β is the fraction of the total membrane potential drop that the negative charge on the oxonol chromophore part of the dye senses as it moves between populations I and II. If the potential drops linearly across the membrane, β would be equal to the "depth" of the chromophore in population I divided by the membrane thickness (*see* Fig. 4 in Nyirjesy et al., 1988). The larger the value of β the greater the driving force of the membrane potential to displace the chromophore to a new location. $K(N)$ is the equilibrium constant for dye binding to the membrane that is independent of membrane potential. $K(N)$ depends on the basic chromophore structure, which remains relatively unchanged for analogs in this three-part study, and upon the number of hydrophobic groups attached to the chromophore. In particular, the $K(N)$ depends on the number of CH_2 groups (N) attached to the chromophore (Nyirjesy et al., 1988).

Nyirjesy et al. (1988) showed that the dependence of signal on N has a "sigmoidal" shape. They

Table 1. Experimental and theoretical values for the fractional change of the absorbance signal per millivolt change in membrane potential

Dye name	N	Calc.[a] $K(N)$	Exp. $f_{\Delta A}$	Calc.[b] $f_{\Delta A}$	Calc.[c] f_M
RGA461	12	2.2	0.0012	0.0010	0.0038
WW802	11	1.0	0.0016	0.0016[d]	0.0032
RGA459	10	0.42	0.0017	0.0022	0.0024

[a] $K(N) = K(N = 0)\exp(-N * \Delta G_{CH_2}/RT)$, where $K(N = 0) = 10^{-4}$ and $\Delta G_{CH_2} = -0.5$ kcal/mol.
[b] Assumes same β for each dye. Then $f_{\Delta A} = 0.0016 * [2/(1 + K(N))]$.
[c] $f_M^{calc} = [1 + K(N)] * f_{\Delta A}^{exp}$.
[d] Assumed (*see* text).

were able to fit the ΔA *vs.* N data for 43 dyes by substituting the following expression for $K(N)$ into their Eq. (7), which is equivalent to Eq. (1) of this paper:

$$K(N) = K(N = 0)E^{-N \cdot \Delta G_{CH_2}/RT}. \quad (2)$$

In this equation, $K(N = 0)$ is the association constant for a dye molecule without any alkyl substituents and ΔG_{CH_2} is the free energy change for transfer of a CH_2 group of an alkyl substituent from water to the hydrocarbon binding site of the membrane. Nyirjesy et al. (1988) found that the data could be approximately fit by using $K(N = 0) = 10^{-4}$ and $\Delta G_{CH_2} = -0.5$ kcal/mol. By inserting these two values into Eq. (2) it is possible to estimate the association constants for the three dyes studied in this paper. The calculated $K(N)$ values for RGA459 ($N = 10$), WW802 ($N = 11$), and RGA461 ($N = 12$), are 0.42, 1.0 and 2.2, respectively (Table 1). These data will be used later.

We have calculated the theoretical variation of ΔA *vs.* ΔV for RGA461 using Eq. (1) with $V_b = 0$ mV. The results are shown graphically in Fig. 2 and should be compared to the experimental results in Fig. 1. It can be seen that the value of β and also the value of $K(N)$ each affects the shape of these curves. However, if β is less than 0.10, the lines are nearly linear for values of $K(N)$ ranging from 0.05 to 20. If $K(N)$ for RGA461 is close to our estimate of 2.2, then panels *C* and *D* of Fig. 2 would best represent the data. In this case, linearity is seen only if β is 0.1 or smaller. In summary, comparison of the linear experimental data in Fig. 1 with the theoretical curves of Fig. 2 suggests that the probe RGA461 senses less than 10% ($\beta < 0.1$) of the transmembrane potential change. This conclusion is relatively insensitive to the value of $K(N)$ used.

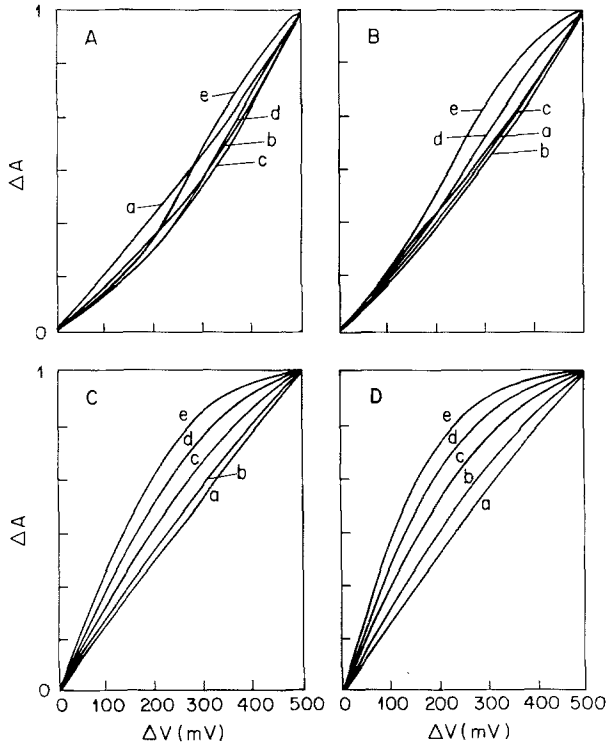

Fig. 2. Computer-generated graphs for the predicted by Eq. (1) for ΔA *vs.* ΔV. The $K(N)$-values for the respective panels are (*A*) 0.05 and 20; (*B*) 0.10 and 10; (*C*) .33 and 3.0; (*D*) 1.0. In each panel β increases from 0.1 to 0.5 in 0.1 unit steps beginning with curve (*a*)

Experimental Dependence of ΔA on V_b with Step Size Constant

In the experiments shown in Fig. 3, the voltage steps (ΔV) are held constant at 100 mV but the DC offset V_b is varied. For the three dyes tested, ΔA at 620 nm increases as V_b increases to make the inside of the bilayer more positive.

It is interesting that the probes that are more sensitive to V_b also are the most sensitive to increases in ΔV. For example, RGA461 has the greatest slope of the three dyes tested in Fig. 3 and it also has the largest ΔA for the 100 mV pulse train when $V_b = 0$ mV. It is useful to define $f_{\Delta A}$ as the fractional increase in ΔA per mV increase in V_b (Eq. 3). The value of $f_{\Delta A}$ can be determined by dividing the slope of the curves by the values of ΔA for the curves measured at the point where V_b is equal to 0 mV. While it is possible that the value of $f_{\Delta A}$ for any particular experiment may also depend to some extent on ΔV, Eq. (1) indicates that this dependence will be small if ΔV is kept small. Thus, ΔV of the pulse train can be thought of as producing a carrier signal that can be used to study modulation of ΔV

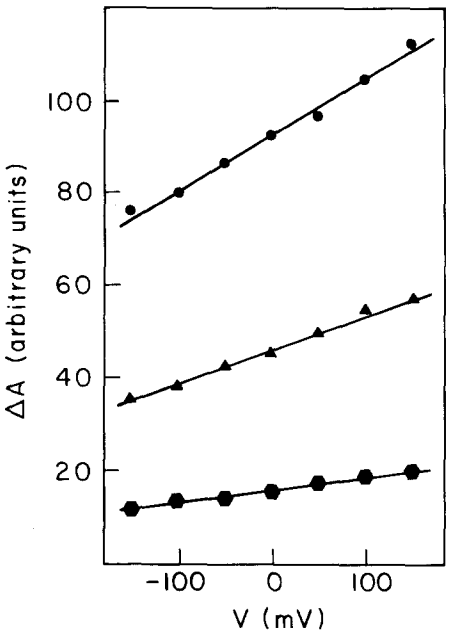

Fig. 3. Magnitude of ΔA *vs.* V_b, for 6 μM dye measured at 620 nm. The dyes studied are RGA459; ●; WW802, ▲; RGA461, ●. The size of the voltage steps of the pulse train was maintained at 50 mV as V_b was changed

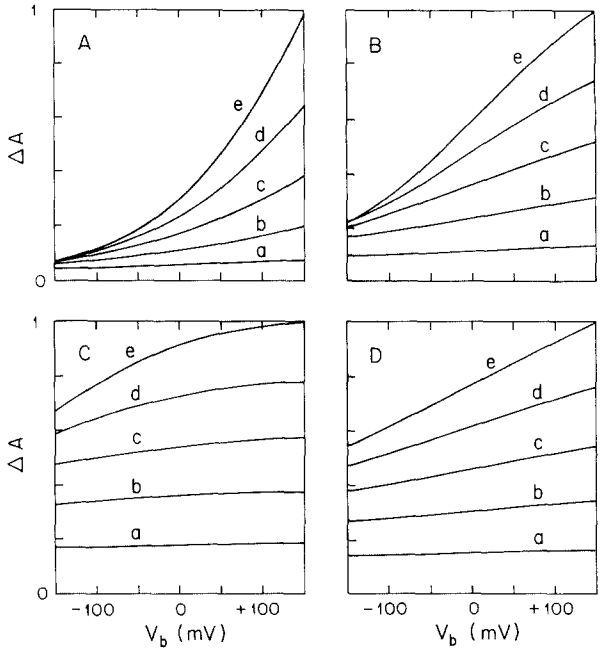

Fig. 4. Computer-generated graphs for ΔA *vs.* V_b as predicted by Eq. (1). The $K(N)$ values for the 4 panels are (*A*) 0.1; (*B*) 1.0; (*C*) 10; (*D*) 1.0. In panels (*A–C*), *a–e* indicate curves with β ranging from 0.05 to 0.25, respectively, in 0.05 unit steps. In panel (*D*) *a–e* indicate values of β ranging from 0.02 to 0.10 in 0.2 unit steps. The respective $f_{\Delta A}$ values for curves (*a*) to (*e*) are as follows: Panel (*A*): 0.18, 0.36, 0.54, 0.72, 0.89; Panel (*B*): 0.10, 0.20, 0.30, 0.40, 0.50; Panel (*C*): 0.02, 0.04, 0.06, 0.08, 0.11; Panel (*D*): 0.04, 0.08, 0.12, 0.16, 0.20

by V_b. In any case, ΔV was kept at 50 mV for the experiments of Fig. 3:

$$f_{\Delta A} = \left[\frac{d(\Delta A)/d\ V_b}{\Delta A}\right]_{V_b=0}. \tag{3}$$

For RGA459, WW802, and RGA461 the values of $f_{\Delta A}$ are 0.0017, 0.0016, and 0.0012, respectively. Thus we conclude that for the three dyes the sensitivity of ΔA to V_b is about 15% per 100 mV and the sensitivity remains near this value even though the signal size changes by nearly a factor of 6 for these dyes.

Analysis of the Dependence of ΔA on V_b

A qualitative explanation for the results in Fig. 3 can be suggested again using the "on-off" model. A more positive bias voltage (inside of the bilayer more positive) would drive more of the negatively charged chromophore from the bathing solution into the membrane, thereby increasing the average amount of dye in site I, $n_{\mathrm{I}}^{\mathrm{avg}}$. Although $n_{\mathrm{II}}^{\mathrm{avg}}$ remains constant and in equilibrium with the bathing solution, the total amount of dye that can participate in the potential-dependent shift of probe between sites I and II is increased and ΔA increases as a result.

By using Eq. (1), which is based on the "on-off" model, we can generate families of curves for ΔA as a function of V_b. The two parameters that determine the shapes of the curves are $K(N)$ and β. The curves are shown in Fig. 4 and should be compared to the experimental data in Fig. 3. For the extreme values of $K(N)$ examined, 0.1 to 10, it is clear that the curves are only linear when β is 0.1 or smaller (Fig. 4*C*). When $K(N)$ is 1.0, the curve is nearly linear, even when β is 0.2 (Fig. 4*B*).

In addition to linearity it is worthwhile to compare the $f_{\Delta A}$ values of the experimental curves (Fig. 3) and the computer-generated curves (Fig. 4). The experimental values range from 0.0017 to 0.0012 (Table 1). The values of $f_{\Delta A}$ obtained from the slopes of the computer-generated curves are shown in the caption of Fig. 4. The only computer-generated curves that are both linear and have values in a range that corresponds to the experimental data are found in Fig. 4*D*, where $K(N) = 1.0$ and β is between 0.06 and 0.10.

Thus, analysis of the offset data using the "on-off" model suggests that the probe molecules occupying the hydrocarbon binding site sense between 5% and 10% of the transmembrane potential drop. This is in the same range as the β values that give a good fit for the ΔA *vs.* ΔV data in Fig. 1.

Further Analysis of the Data

The data in Fig. 3 can be approached from a different angle. Since ΔV (50 mV) and β are small, Eq. (1) can be simplified by allowing $\exp(-\gamma\beta\Delta V/2)$ to equal $1 - \gamma\beta\Delta V/2$. The dependence of signal size on ΔV, N, and other factors held constant during the experiments can be lumped in the constant Q. The signal size is then:

$$\Delta A = Q \cdot [K(N)e^{-\gamma\beta V_b}/(1 + K(N)e^{-\gamma\beta V_b})]. \quad (4)$$

By taking the derivative of ΔA with respect to V_b, then dividing by ΔA, and finally evaluating the result at $V_b = 0$ mV, we obtain a theoretical expression for $f_{\Delta A}$:

$$f_{\Delta A} = \left[\frac{d(\Delta A)/d\ V_b}{\Delta A}\right]_{V_b=0} = \frac{-\gamma\beta}{1 + K(N)}. \quad (5)$$

By assuming that $K(N) = 2.2$ for RGA461 and by substituting the experimental value of $f_{\Delta A}$ for this dye, 0.0012, into Eq. (5), it is found that β is approximately 0.10. This value is within the range of β (0.06–0.10) obtained by curve-fitting in Fig. 4.

Interestingly, Eq. (5) suggests that the different membrane association constants of RGA461, WW802, and RGA459 may account for the decrease in the experimental $f_{\Delta A}$ values for this series. The estimates of $K(N)$ and the resulting values of $f_{\Delta A}$ calculated with Eq. (4) (β kept constant) are listed in Table 1. While these estimates are crude, the calculated trends for $f_{\Delta A}$ parallel the experimental values, which are also shown in Table 1.

Dependence of the Average Concentration of Membrane-Bound Dye on V_b

Let us now consider how n_I^{avg} changes with V_b. Define f_M as the fractional change in n_I^{avg} per unit change in V_b measured at $V_b = 0$ mV:

$$f_M = \left[\frac{d(n_I^{avg})/d\ V_b}{n_I^{avg}}\right]_{V_b=0}. \quad (6)$$

As was done for $f_{\Delta A}$ we can use the "on-off" model to obtain an expression for f_M. The relationship $n_I^{avg} = n_w\partial K(N)K(V_b)$ describes the equilibrium distribution of dye between the bathing solution and the membrane, where again, $K(V_b) = \exp(-\gamma\beta V_b)$ (Nyirjesy et al., 1988). By taking the derivative of this expression with respect to V_b, then dividing by n_I^{avg}, and finally evaluating the expression at $V_b = 0$ mV, we get Eq. (7):

$$f_M = -\gamma\beta. \quad (7)$$

Thus the fractional change in the average amount of dye bound to the membrane per change in membrane potential is proportional only to the fraction of the total membrane potential sensed by the dye.

By equating Eq. (7) and Eq. (5), the relationship between $f_{\Delta A}$ and f_M [Eq. (8)] is obtained:

$$f_M = f_{\Delta A}\ (1 + K(N)). \quad (8)$$

George et al. (1988) found that the fractional change in binding of the analog WW781 to human red blood cells per millivolt change in membrane potential change was in the range of 0.0039 to 0.0068. In those experiments, the potential-dependent change in dye binding was divided by the amount of dye bound at −10 rather than at 0 mV. This small difference can be neglected for the comparisons below. While WW781 was among the better probes for red cell experiments it was not among the most sensitive probes in the lipid bilayer experiments, where it gave a signal 30% smaller, for example, than RGA459. However, WW781 ($N = 9$) and RGA459 ($n = 10$) are very similar in structure. Therefore, we have incorporated the experimental value of $f_{\Delta A}$ from oxidized cholesterol bilayers and the estimated value for $K(N)$, also obtained from the bilayer experiments (Table 1), into Eq. (8) in order to predict an f_M value for RGA459. The calculated values for RGA459 and the other two dyes with larger N values are listed in Table 1. While the f_M value calculated for RGA459 from bilayer experiment data is roughly half the fractional change in binding of WW781 to red blood cells, the values estimated for RGA461 (0.0038) and WW802 (0.0032) are closer.

It is also interesting in this regard to note that when the red blood cell values of f_M are inserted into Eq. (7), β is determined to be in the range of 0.10 to 0.17. Thus the fraction of the transmembrane potential sensed by WW781 in the red cell membrane may be somewhat larger than the fraction sensed by impermeant oxonol analogs in oxidized cholesterol bilayers. Alternatively, the red cell membrane potential generated with valinomycin is at the large end of the range that has been estimated (Freedman & Novak, 1983; George et al., 1988) so that f_M is closer to 0.0038 and $\beta \sim 0.10$ for WW781 in red cells.

Changes in n_I During Step Potential Changes can be Compared with Changes in n_I^{avg} During Bias Potential Changes

The number f_M obtained with impermeant oxonols using bilayers is useful for estimating the number of binding sites for the three dyes, as we shall see

Table 2. Estimates of the number of membrane binding sites for various hydrophobic ions (data are from various literature sources)

Membrane	Hydrophobic ion	Binding sites (pmol/cm^2)	References
diO-PC blm	dipicrylamine$^+$	6.7	a
diO-PC blm	TPB$^-$	8.3	a
Egg PC ves	TPP$^+$	2	b
Egg PC ves	TPB$^-$	5	b
PC ves	TNS$^-$	230	c
PC ves	TNS$^-$	30	d
GMO blm	diSC$_3$(5)$^+$	3*	e
GMO blm	diSC$_4$(5)$^+$	9*	e
GMO blm	diSC$_5$(5)$^+$	16*	e
Egg PC ves	dilC$_2$(5)$^+$	4	f
Egg PC ves	dilC$_3$(5)$^+$	7	f
Egg PC ves	dilC$_4$(5)$^+$	20	f
Bacterial PE blm	TPB$^-$	25	g
Soybean PC ves	OX-V$^-$	28	h
Ox. Chol. blm	RGA 461^{-2}	0.7	this work

* Membrane binding sites may not be completely saturated at this density.

Abbreviations: blm (bilayer lipid membrane), ves (vesicle), PC (phosphatidylcholine), PE (phosphatidylethanolamine), PL (phospholipid), diO- (diolyoyl-), GMO (glycerylmonoolein), Ox. Chol (oxidized cholesterol), TPB (tetraphenylborate), TPP (tetraphenylphosphonium), TNS (2,6-toluidinylnaphthalenesulfonate), diSC$_n$(5) and dilC$_n$(5) (membrane permeant cyanine dyes defined by Sims et al. 1974), OX-V membrane permeant oxonol dye described in reference h).

References: (a) Ketterer et al. 1971; (b) Flewelling and Hubbell, 1986; (c) McLaughlin and Harary, 1976; Huang and Charlton, 1972; (e) Waggoner et al., 1977; (f) P. Howard and A. Waggoner, *unpublished;* (g) Andersen et al., 1978; (h) Bashford et al., 1979.

below. The f_M values range from 0.0022 to 0.0038. Thus, for a 100 mV change in V_b, the ratio $[\Delta n_I^{avg}/n_I^{avg}]$ would be between 0.22 and 0.38, indicating that the amount of dye on the membrane increases by about 20–40% as dye from the aqueous region moves onto site I during an increase in V_b of 100 mV.

Suppose we assume that the fractional increase in population I $[\Delta n_I^{step}/n_I^{avg}]$ during individual steps (ΔV^{step}) of a 100 mV pulse train is identical to the fractional increase in n_I^{avg} during a 100 mV increase in V_b. This assumption is weak because the aqueous pool of dye n_{II}, would become depleted, then supersaturated, during alternate steps of ΔV, whereas, this pool is always constant and in equilibrium with dye in the bulk bathing solution during the slow changes in V_b used in our experiments. In other words, there are important kinetic differences between the bias voltage change effects and the step voltage change effects. Nevertheless, by using this assumption, the three quantities in Eq. (9) are equal to each other and to a value in the range of 0.2 to 0.4 (for a 100 mV potential change).

$$\left[\frac{\Delta n_I^{step}}{n_I^{step}}\right]_{\Delta V=100} = \left[\frac{\Delta n_I^{avg}}{n_I^{avg}}\right]_{\Delta V_b=100} = f_M \cdot 100. \quad (9)$$

An independent estimate of Δn_I^{step} can be obtained from experiments in which the illumination is passed through a region of the bilayer that faces the incoming beam. The light passes perpendicularly through two layers of membrane on opposite sides of the spherical bilayer to produce an absorbance change of 2 times ΔA. Experiments with 6 μM RGA461 yielded a value for ΔA of 2×10^{-5} for a single bilayer at 590 nm when 100 mV steps were used. Since $\Delta A = 100\, \Delta E \Delta n$ (Nyirjesy et al., 1988), Δn can be obtained from the experimental ΔA if an appropriate value of ΔE is used. ΔE can be estimated as follows. Since dye molecules bound to the bilayer are oriented with their absorption transition moments more or less perpendicular to the bilayer surface (George et al., 1988), they do not absorb light from the beam because it is coming from a direction perpendicular to the membrane. Thus the absorption change that is observed results mainly because of changes in the number of dye molecules in the aqueous phase during the potential steps. The absorption spectrum in Fig. 6 of George et al. (1988) indicates that the extinction coefficient of RGA461 at 590 nm in 100 mM KCl is about 10^{+5} liter/mol cm. The final calculation using $\Delta E = 10^{+5}$ and $\Delta A = 2 \times 10^{-5}$ gives $\Delta n_I^{step} = 0.2 \times 10^{-12}$ moles/cm^2 per 100 mV potential step. Using $[\Delta n_I^{step}/n_I^{avg}] \sim 0.3$ per 100 mV (Eq. 9), we can now calculate that $n_I^{avg} = 0.7 \times 10^{-12}$ moles/cm^2. The signal for RGA461 is nearly saturated at 6 μM (Fig. 4, George et al., 1988) which means that the membrane binding sites are almost saturated at this concentration. Thus, 0.7 pmol/cm^2 represents an approximation to the maximum number of RGA461 binding sites on an oxidized cholesterol membrane in 100 mM KCl. This value is on the low end of the range of surface densities for other hydrophobic ions on membranes (Table 2). It is possible that repulsions between doubly charged RGA461 molecules on the membrane may account for the lower maximum surface density at saturation.

Can Enough Dye Move Between Sites I and II to Account for the Signal?

We can now answer an important question in regard to the "on-off" mechanism. Can a sufficient quan-

tity of dye diffuse during a potential step from region II to the membrane in order to account for the experimental value of Δn? This question has been addressed by several authors who have been concerned with the mechanisms of potential-sensitive probes (Smith *et al.*, 1980; Cabrini & Verkman, 1986). Region II has been defined as the volume to and from which the probe moves during pulses of length t. The thickness of this region ∂ is determined by how far probe molecules can diffuse during time t. Nyirjesy et al. (1988) estimated ∂ to be approximately 2×10^{-4} cm for impermeant oxonols under these conditions. Thus the total amount of probe in region II is equal to the average dye concentration (6 μM) times ∂, or about 1.2×10^{-12} moles/cm^2. This, then, is the maximum amount of dye that could be involved in a potential-driven population shift lasting 5 msec. Since the experimental value of Δn was calculated above to be 0.2×10^{-12} moles/cm^2 for 100 mV pulses, we can see that the amount of dye available in region II is approximately 6× the amount that is actually transferred to the membrane during the 100 mV pulses. Thus it appears that there is barely sufficient probe in population II to justify consideration of the "on-off" mechanism. Interestingly, if the magnitude of ΔV is increased well above 100 mV, we expect a point will be reached where Δn approaches the size of the pool of dye in region II. The signal must saturate when this occurs. However, according to Fig. 1 this point is not reached when ΔV is 500 mV. Analysis of the kinetics of the absorption signal would prove useful for detecting depletion of population II in response to large, long potential steps.

Addition of Dye to the Inner Compartment of the Bilayer

The mechanism presented for modulation of ΔA by V_b, explains another interesting observation we have made. When bilayers are formed so that the dye concentrations inside and outside of the membrane are equal, no absorption signal is obtained with a symmetrical pulse train centered at $V_b = 0$. This is expected because population shifts on opposite sides of the membrane are reversed and the signals for each cancel. However, increasing or decreasing V_b leads to a signal, presumably because a population asymmetry is generated at the inner and outer membrane binding sites so that one signal is larger than the other and there is no cancellation. From this result, we would expect that addition of an impermeant oxonol at equal concentrations to both the inside and outside of a squid axon would give a substantial signal because the axon resting potential gives a V_b of nearly −70 mV. Other dyes that do not behave by the "on-off" mechanism and are not driven into or out of the membrane by V_b would not be expected to give significant signals when added to both sides of the squid membrane. Such dyes would include M540 analogs (Cohen et al., 1974).

Discussion

In the first paper of this series, George et al. (1988) propose that the impermeant oxonol dyes respond to potential changes by an "on-off" mechanism. Membrane potential changes drive the oxonol chromophore between a membrane binding site and an aqueous region off the surface of the membrane as illustrated in Fig. 7 of George et al. (1988). The absorption change occurs because the chromophore in the membrane binding site absorbs 15 nm to the red of the dye molecules in the aqueous phase. In the second paper, Nyirjesy et al. (1988) show that the absorbance signal depends strongly on the amount of hydrocarbon attached to the dye structure. They propose that the hydrocarbon groups control the relative affinity of the dye for the membrane and, up to a point, the absorbance signal increases for the more nonpolar dyes that have high affinity for the bilayer. The results of the second study are consistent with the "on-off" mechanism.

In this study, we have experimentally determined the dependence of the signal on the potential difference across the bilayer membrane. The size of the absorbance signal was found to vary linearly with the size of the potential steps over a range of 500 mV. The magnitude of the absorbance signal is also sensitive to the DC offset voltage of the pulse train. According to the "on-off" model, this sensitivity occurs because the offset voltage affects the average (steady state) concentration of dye at the potential-sensitive site on the membrane. Thus a positive offset voltage which drives more dye onto the membrane increases the total amount of dye that participates in the rapid on-off movement of dye that occurs during the train of potential pulses. The average amount of dye in the narrow aqueous region just off the membrane is independent of the size of the potential pulses and the offset voltage and instead remains constant because this population is in equilibrium with the large pool of dye in the bathing solution.

The theoretical analysis of the ΔA *vs.* ΔV and ΔA *vs.* V_b data indicate that only 5–10% of the transmembrane potential drop is sensed by the impermeant oxonol dyes. This percentage is similar to that estimated for permeant ions, such as tetraphenyl borate (TPB^-) and tetraphenyl phosphonium (TPP^+) (Ketterer, Neumcke & Läuger, 1971;

Anderson et al., 1978; Hladky, 1979; Flewelling & Hubbell, 1986; Honig, Hubbell & Flewelling, 1986). This would not be surprising since the chromophore of the impermeant oxonols is also a hydrophobic ion. One might be tempted to say that oxonol and cyanine chromophores, TPB^-, TPP^+, and probably many other hydrophobic ions that are drugs, toxins, or metabolites, have essentially the same membrane binding region. However, it is difficult to define a binding region for molecules that range in size from 10 to 20 Å, nearly half the thickness of the bilayer.

The chromophore of the pyrazalone-barbituric acid oxonols together with the phenyl ring on the pyrazolone nucleus and the alkane substituents on the barbituric acid nucleus is about 20 Å long. The negative charge of the chromophore is delocalized over a region that includes the carbonyl group on the pyrazolone nucleus and the two oxygens near the methine chain of the barbituric acid nucleus. The center of this charge distribution is probably midway between the nuclei and therefore is about 10 Å from the point where the sulfonate group, which has a localized negative charge, is attached to the probe. If the hydrated sulfonate group lies in the aqueous phase and the rest of the probe, which is nonpolar except for the delocalized charge on the chromophore, is buried in the membrane and is oriented with its long axis parallel to the oxidized cholesterol molecules and perpendicular to the surface of the membrane, the center of negative charge density on the chromophore would be about 10 Å deep in the 40–50 Å thick membrane. Thus we would expect the probe to sample 20–25% of the membrane potential change rather than the 5–10% we estimate from the voltage dependence of the optical signal. A number of explanations can be made for the difference. (1) The transmembrane potential drop may not be linear between the two aqueous interfaces. This would not be surprising if water penetrates significantly into the bilayer (Griffith, Dehlinger & Van, 1974). (2) The probe may not sink completely in the bilayer. This, however, would be surprising because the phenyl group and other nonpolar regions of the probe would be exposed to the water. (3) The probe may sit at an angle in the membrane. Yet it seems more probable that the long axis of the probe would be colinear with the oxidized cholesterol molecules (George et al., 1988). (4) The negative charge may not be evenly distributed on the buried chromophore and may be shifted towards the pyrazolone nucleus near the membrane surface. We believe that a major charge shift has not taken place because it would produce a significant blue shift in the absorption maximum of the membrane associated dye (Platt, 1956). There is no evidence for such a blue shift (George et al., 1988).

While it is likely that the alkyl groups on the barbituric acid nucleus penetrate into the decane interior of the membrane, it is improbable that the whole probe sinks to the center of the membrane. First of all, the oxonol chromophore part of the dye is essentially insoluble in very nonpolar solvents like hexane and decane and, secondly, the sulfonate group is unlikely to move very far into the membrane. Although the oxonol chromophore part of the dye is expected to be insoluble in the hydrophobic interior of the membrane, it apparently has a high affinity for the particular region of the membrane it occupies. In fact, the first 10 Å of the membrane may offer an ideal environment of intermediate polarity. As described previously (George et al., 1988) the oxonol chromophore partitions strongly from water or from hexane into hydrogen-bonding solvents like ethanol, butanol, dimethyl sulfoxide, and dimethylformamide. It is likely that the first 10 Å of the oxidized cholesterol membrane provides such an environment. The hydroxyl groups and ketone groups of the oxidized cholesterol molecules together with penetrating water molecules must account for intermediate polarity of this region. Biological and other synthetic membranes probably also contain such a region (Honig et al., 1986).

In summary, the "average region" occupied by the impermeant oxonol dyes in their dynamic interaction with the membrane is probably that depicted in the "on-off" model shown in Fig. 7 of George et al. (1988).

What is the evidence that the impermeant oxonols do not work by another mechanism?

(1) An electrostrictive mechanism where dye is squeezed out of the membrane when the potential increases, does not explain the impermeant oxonol signals. With this mechanism, the spectral changes would be similar to those described by George et al. (1988), but the bias and step voltage dependences of the signals shown in Figs. 1 and 3 of this paper are not expected. Partitioning of dye into the membrane would be inversely proportional to the square of the membrane potential and would lead to a nonlinear dependence of the signal on V_b. Furthermore, the electrostrictive model predicts that the absorption signal will have the same sign when dye is on either side of the membrane and V_b is zero. Neither of these is the case.

(2) The "dimer-rotation" mechanism (Ross et al., 1974; Dragsten & Webb, 1978; Wolf & Waggoner, 1986) is not in effect at the concentrations of dye used in these experiments. There is no spectral evidence of dimers (George et al., 1988).

(3) A "membrane-localized rotation" mechanism, in which the dye molecules that are inserted

into the hydrophobic region perpendicular to the bilayer simply rotate into the plane of the membrane at the membrane surface, can be ruled out for red cells because the release of dye from the membrane into the bathing medium is significant and nearly quantitatively accounts for the size of the optical signal. There is no obvious reason why the mechanism in oxidized cholesterol bilayers should be different from the "on-off" mechanism found with red cells. Additionally, the experimental voltage pulse-generated difference spectrum obtained with bilayers (George et al., 1988) can be explained better by dye molecules moving from membrane hydrocarbon to water than by moving to the membrane surface, which is probably less polar than water.

(4) An electrochromic response for oxonol dyes that are relatively fixed in the bilayer is ruled out for a number of reasons. First of all, these analogs are not structurally optimized to be electrochromic dyes (Loew & Simpson, 1981). Secondly, the polarization difference spectra of RGA461 is not consistent with an electrochromic response (*compare* George et al., 1988 with Loew & Simpson, 1981). Thirdly, the bias voltage dependence of the signal is more difficult to explain with an electrochromic mechanism, unless voltage-dependent changes in binding or dye location on the membrane are included. Fourthly, if the more hydrophobic oxonol analogs (large N) are indeed binding to the membranes, one would expect them to produce large signals in an electrochromic model, but they do not (Nyirjesy et al., 1988). Kinetic analysis of responses to fast voltage changes and alteration of the orientation in the oxonol dye on the membrane would provide additional ways to test this mechanism.

The discussion we have presented has implications for the design of membrane potential-sensitive probes. For the class of dyes that respond to potential changes by moving to a new environment, it is desirable that the dye sense as much of the transmembrane potential change as possible. When the permeant cyanines and oxonols are used in the "slow response" mode, usually with cells, organelles, or vesicles, the probe molecules are moving between compartments on the two sides of the membrane and therefore they sense nearly all of the potential drop across the membrane (Sims et al., 1974). On the other hand, the impermeant oxonols that have been developed so far cannot flip to the opposite side of the bilayer. Thus it seems that the fraction of impermeant oxonol molecules that can be affected by the potential change, and hence the size of the signal, is limited by the inability of this class of probes to sense more than 10% of the potential drop. It may be possible to increase sensitivity by lengthening to 50 Å the linker that attaches the sulfonate group to the oxonol chromophore so that the chromophore can sense nearly 90% of the potential drop by flipping to the opposite side of the membrane. It is not easy, however, to predict the effect of such a long alkyl chain on the speed at which such a probe would respond to potential changes or on how it might affect partitioning between the aqueous phase and the membrane.

There are other possible ways to improve the optical signal by redesigning the probe. For example, if the delocalized charge on the chromophore could be doubled there would be a significant increase in the driving force for shifting dye between environments. However, additional hydrocarbon groups might have to be placed on the chromophore to make sure the triply charged dye molecule partitions strongly enough into the membrane.

Another option for improvement is to try to increase the size of the optical signal by increasing the total number of dye molecules that sense the potential change. However, the results of this work and the previous paper (Nyirjesy et al., 1988) suggest that the membrane may already be saturated with probe under the conditions that have been used in most biological experiments.

Still another approach would be to increase the size of the extinction coefficient change ΔE of the dye when it moves between environments. However, the extinction coefficients of the cyanine and oxonol dyes are already larger than those of most other known chromophores and the wavelength shifts that occur upon membrane binding produce values of ΔE that are about as large as can be expected (George et al., 1988).

Efforts to increase the sensitivity of membrane potential probes are important because each improvement helps biologists obtain better information from more complex excitable systems. While the impermeant oxonols developed by Gupta et al. (1981) provide reasonably strong signals with minimal photodynamic damage to the biological preparation, we believe that radical changes will be required for significant improvements. Some of the features of the mechanism that have been illuminated by the experiments and analysis in this series of papers should help point the way for improvements. It should not be forgotten that there are other classes of potential-sensitive dyes, including the electrochromic dyes (Loew & Simpson, 1981; Grinvald et al., 1982; Grinvald, 1985; Loew, 1988), where it may be possible to enhance the size of the absorption signals. And fluorescence measurements still offer possible advantages for improving signal to noise (Waggoner & Grinvald, 1977). Certainly there is still need to increase the photostability of the probes and to reduce the pharmacologic and phototoxic effects of many existing dyes.

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