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Impermeant Potential-Sensitive Oxonol Dyes: I. Evidence for an "On-Off" Mechanism

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Summary. This series of papers addresses the mechanism by which certain impermeant oxonol dyes respond to membrane-potential changes, denoted ΔE_m. Hemispherical oxidized cholesterol bilayer membranes provided a controlled model membrane system for determining the dependence of the light absorption signal from the dye on parameters such as the wavelength and polarization of the light illuminating the membrane, the structure of the dye, and ΔE_m. This paper is concerned with the determination and analysis of absorption spectral changes of the dye RGA461 during trains of step changes of E_m. The wavelength dependence of the absorption signal is consistent with an "on-off" mechanism in which dye molecules are driven by potential changes between an aqueous region just off the membrane and a relatively nonpolar binding site on the membrane. Polarization data indicate that dye molecules in the membrane site tend to orient with the long axis of the chromophore perpendicular to the surface of the membrane. Experiments with hyperpolarized human red blood cells confirmed that the impermeant oxonols undergo a potential-dependent partition between the membrane and the bathing medium.

Key Words oxonol · potential-sensitive dyes · mechanism · bilayer lipid membrane · red blood cells · absorption spectrum

Introduction

Voltage-sensitive dyes have been used as optical probes to study excitable and nonexcitable membranes for more than 10 years (Cohen & Salzberg, 1978; Waggoner, 1979, 1985; Freedman & Laris, 1981, 1987; Salzberg, 1983; Grinvald, 1985). In this method individual cells, whole tissues, or cells in suspension may be stained with a potential-sensitive probe and, depending on the particular probe selected, changes in the intensity of transmitted light (ΔI) or fluorescence (ΔF) from the preparation are used to indicate changes in transmembrane electrical potential, or E_m. The transmission signal can be expressed as a fractional change of the light reaching the photodetector ($\Delta I/I$) or as an absorbance change (for small signals, $\Delta A = -\Delta I/2.3I$). Useful optical signals have fractional changes ranging from 0.001 to 0.05 for a ΔE_m of 100 mV. The times required for most of these excitable membrane probes to respond to sudden ΔE_m are from <2 to 100 μsec (Ross et al., 1977; Loew et al., 1985); thus these probes are sometimes called "fast dyes" in order to distinguish them from other potential-sensitive probes that respond with longer time constants. The "slow dyes" are more useful for measuring ΔE_m that take place in times of seconds in cells, organelles, and vesicles (Freedman & Laris, 1981; Waggoner, 1985) and will not be discussed here.

A number of studies have been undertaken to determine the mechanisms of the fast dyes. The best understood mechanism is that of Merocyanine 540. This probe has a large permanent dipole moment and is known to reorient when the electric field in the membrane changes (Ross et al., 1974; Tasaki & Warashina, 1976; Waggoner & Grinvald, 1977; Dragsten & Webb, 1978; Wolf & Waggoner, 1986). Reorientation shifts dye molecules between a population where the chromophore is oriented more perpendicular to the plane of the membrane and another population that is oriented more parallel to the membrane. At higher concentrations, nonfluorescent dimers are present in the membrane. Transmembrane potential changes alter the dimer concentration as well as the populations of mono-

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mers oriented parallel and perpendicular to the surface of the membrane. The absorption and fluorescence signals from Merocyanine 540 on excitable cells result from the potential-dependent shifts between these populations. There is no indication that E_m significantly affects the partitioning of Merocyanine 540 between the membrane and the aqueous region adjacent to the membrane. The negatively charged sulfonate group, which is attached to the chromophore by a short alkyl chain, apparently does not sense potential changes and seems to function only as an anchor to keep the probe from crossing to the opposite side of the membrane (Ross et al., 1977; Smith, Graves & Williamson, 1984). Substitution of a positively charged ammonium group for the sulfonic acid has only a minimal effect on the absorption signal (Wolf & Waggoner, 1986). For simplicity, this mechanism can be called the "dimer-rotation" mechanism.

Membrane permeant cyanine and oxonol dyes, which have neither large permanent dipole moments nor sulfonate anchoring groups, respond rapidly to ΔE_m by a different mechanism. Comparisons of the wavelength dependence of ΔA obtained from glycerol monoolein membranes with simple absorption spectra of the dyes in solvents that model the membrane environment suggest that these probes, unlike Merocyanine 540, may undergo a potential-dependent change in partitioning between the membrane and the aqueous medium near the membrane (Waggoner, Wang & Tolles, 1977). This mechanism has been called the "on-off" mechanism.

Oxonol dyes can be made membrane impermeant by addition to the chromophore of a localized charge, such as a sulfonate group, attached to a spacer arm. The asymmetrical pyrazolone-barbituric acid and pyrazolone-thiobarbituric acid oxonols bearing sulfonate groups are impermeant oxonol dyes first suggested by A. Grinvald and synthesized by C.H. Wang (Gupta et al., 1981). One of these, WW781, has been found to given particularly large signals with the squid giant axon (Gupta et al., 1981), heart muscle (Dillon & Morad, 1981), and red blood cells (Freedman & Novak, 1983). Another, RGA461, gives big absorption signals with the oxidized cholesterol lipid bilayer system. In order to better understand the mechanism of RGA461, WW781, and their analogs, we have studied their spectral behavior on oxidized cholesterol bilayers, in which ΔE_m can be closely controlled.

In this paper the dependence of ΔA on the concentration of RGA461 and on the direction of polarization of illumination passing through the membrane has been determined. The spectral studies reveal that there is no evidence for involvement of dimers of RGA461 in the mechanism. The probe appears to undergo potential-dependent movement between a nonpolar region of the membrane and an aqueous region just off the surface of the membrane. Red blood cell experiments indicate that about 17% of the dye on the membrane is redistributed into the medium when the cells are hyperpolarized.

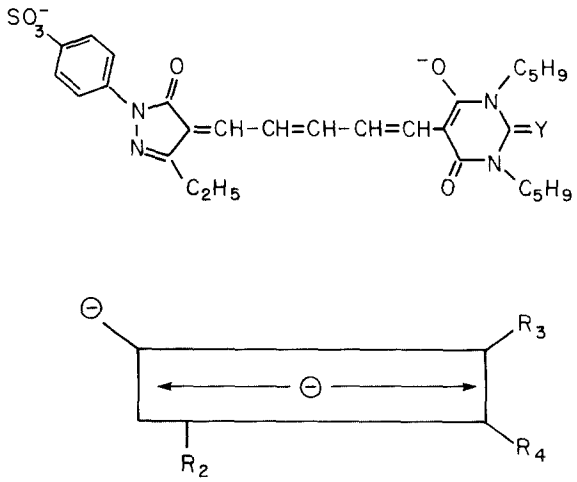

Fig. 1. Structure of the pyrazolone-barbituric acid dye RGA461. A representation of the dye structure is included beneath the actual structure. The box represents the chromophore, the circle is the sulfonate group, and the arrow is the assumed direction of the absorption transition moment. For WW781, R_3 and R_4 are butyl groups and R_2 is a methyl group

The dependence of the optical signals of the impermeant oxonol dyes on the size of the voltage change, the average membrane potential, and the structure of substituents attached to the chromophore is addressed in two subsequent papers.

Materials and Methods

The potential-sensitive dyes, RGA461 and WW781 (*see* structures in Fig. 1), used in this study were synthesized by the procedure of Gupta et al. (1981). Concentrated dye in an ethanol or distilled water stock solution was added with a microsyringe to the aqueous bathing medium surrounding the hemispherical bilayer membrane. A small stir bar in the bottom of the cuvette mixed the dye in the bathing medium but the stirrer was turned off before optical measurements were made.

The experimental apparatus for carrying out the optical experiments is shown in Fig. 2. Details on its construction and use are given by Wolf and Waggoner (1986) and by Waggoner et al. (1977). Dragsten and Webb (1978) were the first to use the hemispherical oxidized cholesterol bilayer membrane to study potential-sensitive dyes. The time constant for the optical detection system was 0.3 msec.

The membrane potential is defined as the voltage of the internal compartment of the hemispherical bilayer relative to an external reference voltage of 0 mV. Thus the transmittance change ΔI is equal to the transmittance during a positive potential minus the transmittance during a negative potential.

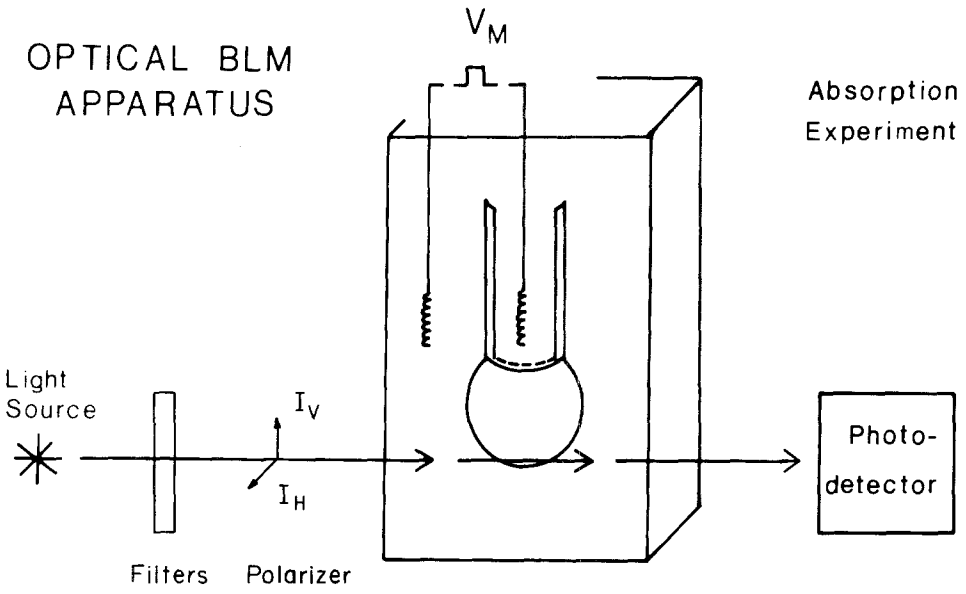

Fig. 2. Schematic diagram of the optical bilayer lipid membrane (BLM) apparatus (described more fully by Wolf and Waggoner, 1986). Hemispherical membranes are formed at the tip of a Teflon® tube in a 1 × 1 × 3 cm fluorescence cuvette containing 100 mM KCl by exerting pressure from a mechanical pipette. The solution within the Teflon tube was in contact with one Ag/AgCl electrode and a second Ag/AgCl electrode was placed in the bathing medium. Voltage pulses were applied to these electrodes. A highly regulated 100 W quartz halogen light source provided collimated illumination. After passing through 10 nm bandwidth interference filters and a polarizer, the quasimonochromatic light was focussed on the bilayer with a 10× long working length microscope objective. Light passing through the bilayer was collected with an identical objective and directed to a silicon photodiode. The electronic signal was amplified and averaged as described by Wolf and Waggoner (1986)

The magnitude of the optical signal varies from membrane to membrane and over time after the membrane is formed. The time-dependent changes seem to be related to the thinning process which occurs as solvent moves out of the bilayer and into the torus. Invariably the absorption signal increased during thinning and leveled out after a period of a minute to several minutes. Presumably, the optical signal becomes greater because thinning increases the electric field strength within the membrane, even with the transmembrane potential difference held constant. The thinning process was followed by monitoring the sharpening of membrane capacitance spikes with an oscilloscope. The optical signals reported in this paper were recorded after the capacitance spikes became visibly sharp. In order to account for further drift of the signal, data for different wavelengths were obtained in a somewhat random sequence. After five or six filters were used the signal was determined at a reference wavelength (usually the wavelength with the largest signal) and another sequence of filters was used, and so on. At the end of an experiment data were normalized for the change of the reference signal over time, which was usually not more than 15%.

Membrane-to-membrane variations were more difficult to control. The variations can be due to the extent of membrane thinning and to differences in the region of the membrane illuminated. For example, if a focussed beam of illumination, intended to pass tangentially through the bottom edge of the spherical bilayer, extended too far into the aqueous region below the membrane, $\Delta I/I$ was reduced. If the beam was too high the signal shape was distorted and the long wavelength negative $\Delta I/I$ peak was reduced. A binocular microscope was used to adjust membrane diameter and the location of illumination. Signals for RGA461 over a period of several months varied at most by a factor of 2, but usually by not more than 20–30% from membrane to membrane.

Another potential source of error is the tendency of some analogs, particularly the more nonpolar dyes, to form dimers and higher order aggregates in the bathing medium. Signals from aggregated dyes are reduced (Nyirjesy et al., 1988). The rate and extent of aggregation varies from dye to dye and is difficult to monitor during bilayer experiments. Signals from membranes that thinned quickly may be larger than signals from membranes that thinned after some of the dye aggregated.

Absorption spectra for dyes in solvents and micelles were obtained with a Varian/Cary 2200 spectrophotometer. Difference spectra were generated with a computer. Lubrol PX was purchased from Sigma Chemical Co.

Dye binding to red blood cells was determined as follows. Fresh human blood was washed 3–4 times by centrifugation and resuspension in chilled media containing 149 mM NaCl, 1 mM KCl, and 5 mM HEPES buffer, pH 7.4 at 23°C. The red cells were adjusted to 50% hematocrit (HCT) and kept on ice for use on the same day.

WW781 (1 mg/ml in EtOH) was added to polyacrylic cuvettes containing 2.3 ml of the washing solution to produce final concentrations ($[dye]_{initial}$) of 0.5, 1.5, 3.0, 4.5 and 6.0 μM. The initial absorbance was measured at 600 nm (Cary 219 spectrophotometer), and the resultant curve was used to calibrate dye concentrations.

A 0.2-ml portion of the 50% HCT cell suspension was then added to each dye solution to produce 4% HCT suspensions. In order to equilibrate cells with dye, the cuvettes were capped with Teflon® lids and inverted three times. For each $[dye]_{initial}$, three suspensions received no valinomycin (VAL), and three other

suspensions received 3.2 μl of 2.5 mM VAL in EtOH, and each cuvette was capped and inverted twice more. The cuvettes were then centrifuged (Beckman Model TJ-6) at 2500 rpm for 6 min in a swinging bucket rotor (Beckman TH-4). The concentration of dye in the supernatant ($[dye]_{free}$) was determined from its absorbance and the calibration curve.

Bound dye was determined from $[dye]_{initial}$ and $[dye]_{free}$ as follows: $dye_{bound} = (2.3 \times [dye]_{initial} - 2.4 \times [dye]_{free})/(0.1 \times 0.34)$, where 2.3 is the volume of initial dye solution, 2.4 is the volume of supernatant after adding cells, 0.1 is the volume of the cells in the cuvette, and 0.34 is g Hb/ml cells. The units of dye_{bound} are nanomoles per gram of hemoglobin (nmol/g Hb). A plot of dye_{bound} against $[dye]_{free}$ is the binding curve of dye on the red blood cells.

Results and Discussion

Structure of the Probe

RGA461 and WW781 are two of 43 pyrazolone-barbituric acid and pyrazolone-thiobarbituric acid analogs that have been screened for sensitivity to E_m (Gupta et al., 1981). These probes belong to the oxonol class of dyes (Hamer, 1964) and have the basic chromophore structure shown in Fig. 1. A negative charge is delocalized over the three oxygen atoms. Many cyanine and oxonol dyes with delocalized charges behave like tetraphenyl boron and triphenylmethyl phosphonium ions and can partition into hydrocarbon regions of membranes (Sims et al., 1974; Szabo, 1974; Waggoner et al., 1977). Such molecules are usually membrane permeant unless restricted. However, the arylsulfonate group on RGA461 and WW781 act as an anchor to prevent the chromophores from crossing the membrane. Previous studies of Merocyanine 540 suggest that the sulfonate groups on the impermeant oxonols remain outside the membrane hydrocarbon region and do not sense E_m (Wolf & Waggoner, 1986).

For simplicity the chromophore structure is symbolized by a box in Fig. 1. The arrow in the box represents the region of the delocalized negative charge. The absorption transition moment of the chromophore is probably in the same direction as the extended polymethine chain of the dye (Bucher et al., 1967; Yguerabide & Stryer, 1971) and is therefore parallel to the arrow. Light polarized with the electric vector in this direction has the maximum probability of being absorbed by the chromophore. The sulfonate group is indicated on the upper left corner of the chromophore box.

Concentration Dependence of the BLM Difference Spectra

Studies with Merocyanine 540, cyanine, and oxonol dyes have shown that absorption difference spectra are useful for identifying the microenvironments

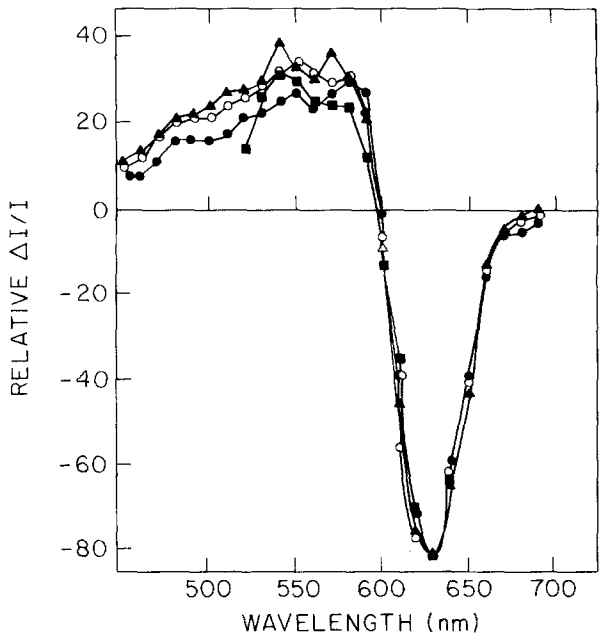

Fig. 3. BLM transmission difference spectra for different concentrations of RGA461. The spectra were normalized to give the same $\Delta I/I$ at 630 nm as was obtained at 12 μM dye. The transmission difference spectrum is the inverse of the absorption difference spectrum, and represents the relative change in transmission when dye moves from the medium onto the membrane (*see* text). The bottom half of the membrane was illuminated with unpolarized light. The probe concentrations were 0.5 μM, ■; 1 μM, ●; 6 μM, ▲; 12 μM, ○

that probes experience during changes in E_m. Difference spectra for RGA461 were obtained by adding the probe to the 100 mM KCl solution surrounding hemispherical oxidized cholesterol BLM's (bilayer lipid membranes) in the apparatus diagrammed in Fig. 2. Trains of 5 msec voltage steps varying between +50 and −50 mV were applied to the membranes and fractional changes in transmitted light $\Delta I/I$ were recorded at different wavelengths to produce transmission difference spectra. The transmission difference spectra for RGA461 at different concentrations (*see* Fig. 3) were obtained by illuminating the bottom half of the spherical BLM with unpolarized light. The shapes of the difference spectra in Fig. 3 are similar over a wide concentration range, even up to 15 μM where the signal for RGA461 saturates (Fig. 4). This result suggests that, unlike the situation for Merocyanine 540, dimers are not involved in the mechanism for RGA461.

Polarization Dependence of the Difference Spectra

To extract more information from the difference spectrum, polarized light can be focussed on a small

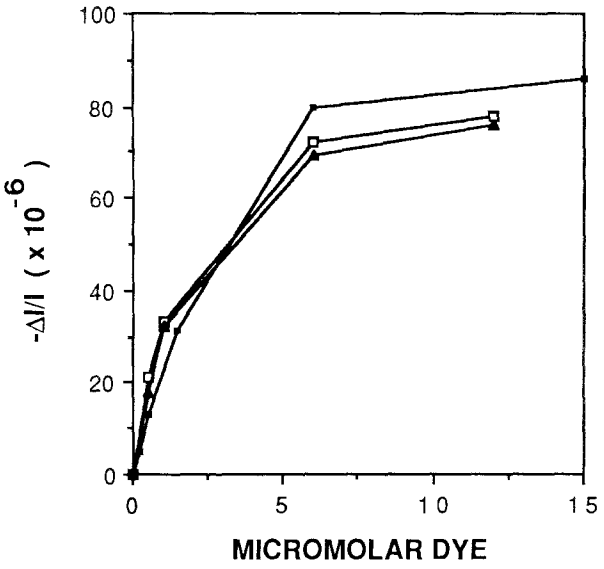

Fig. 4. Saturation of the absorption signal as the concentration of RGA461 increases. Signal was measured at 630 nm. Experiments by Nyirjesy, ■. Experiments by Bassom, □ (average of all values of signal obtained for all membranes, 3 to 10 values per membrane, two to eight membranes at each concentration); ▲ (averaged maximum values of signal obtained for each membrane, two to eight membranes at each concentration)

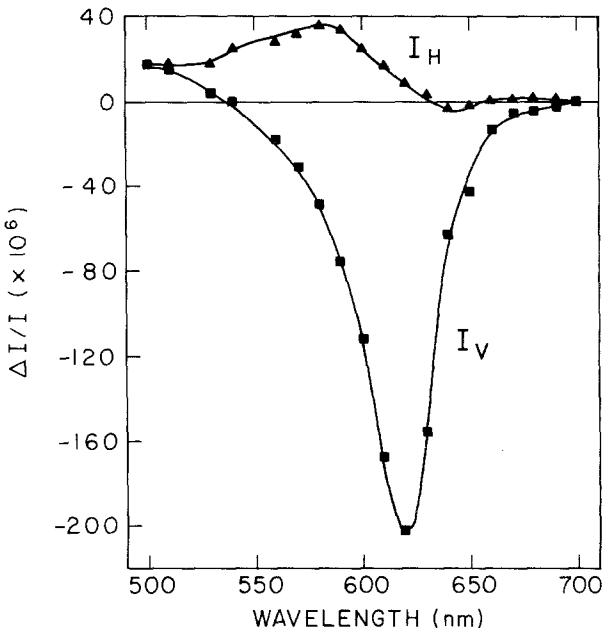

Fig. 5. Dependence of the BLM transmission difference spectrum on the polarization of the illumination. The bottom 10% of the hemispherical bilayer was illuminated with light polarized either perpendicular (I_V) or parallel (I_H) to the plane of the membrane at the bottom of the bilayer

region at the bottom of the bilayer or at the front face of the hemispherical BLM. Polarization difference spectra can be used to identify potential-dependent changes in the orientation of dye molecules relative to the plane of the membrane (Tasaki & Warashina, 1976; Ross et al., 1977; Dragsten & Webb, 1978; Loew et al., 1985; Wolf & Waggoner, 1986].

Transmission difference spectra for RGA461 obtained with polarized light are shown in Fig. 5. A large signal is obtained at 620 nm when vertically polarized light (I_V) is used to illuminate only the bottom 10% of the spherical BLM. When the polarization of the light is rotated 90°, so that the electric vector is parallel to the plane of the membrane (I_H), a broad peak appears in the opposite direction with a maximum at about 580 nm.

The polarization results may be explained as follows. The large negative signal corresponding to increased absorption at 620 nm, which occurs during positive potential steps takes place because of an increase in the number of dye molecules with their transition moments oriented more perpendicular to the membrane. The positive peak at 580 nm that is obtained with horizontally polarized light indicates a decrease in a population of dye molecules oriented in a direction more parallel to the plane of the membrane. Dye molecules in the "parallel population" must absorb at shorter wavelengths (below 580 nm) and those in the "perpendicular population" must absorb at longer wavelengths (above 620 nm). From these data alone the exact distribution of orientations of the "perpendicular" and the "parallel" populations cannot be determined. However, since wavelength shifts characteristically occur when oxonols change solvent environments, we conclude that there are changes in both the orientation and the polarity of the microenvironment of the probe molecules when the potential changes.

Solvent Dependence of the Absorption Spectrum of RGA461

Because there is a shift in the absorption spectrum of dye molecules that move between the two potential-sensitive sites, we wanted to obtain the absorption spectra of RGA461 in each of the two sites. The difference between these two spectra should closely resemble the BLM absorption difference spectrum shown in Fig. 3. In order to test the "on-off" mechanism, one absorption spectrum was obtained for RGA461 in the 100 mM aqueous KCl medium bathing the membranes (Fig. 6*a*, lower curve). The absorption spectrum for the other site should ideally be obtained for the probe bound to the oxidized cholesterol BLM. However, we were unable to disperse small fragments of this membrane in 100 mM KCl solutions for absorption spectrometry. Micelles of the nonionic detergent Lubrol PX may pro-

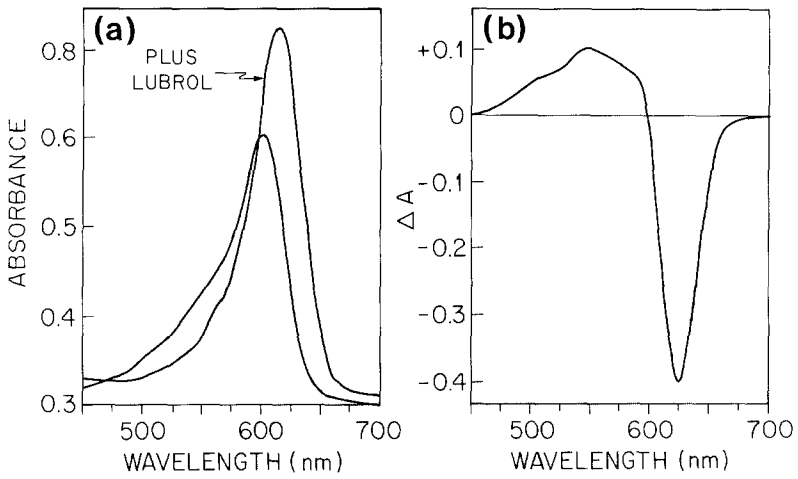

Fig. 6 (*a*) Absorption spectra of 6 μM RGA461 in 100 mM KCl (shorter peak) and in 100 mM KCl containing 1% Lubrol. (*b*) The absorption difference spectrum obtained by subtracting the spectrum of the solution containing Lubrol from the spectrum of the solution without Lubrol, and represents the change in absorption when dye moves out of the micelle (*see* text)

vide a suitable alternative model for studying the interaction of the impermeant oxonol dyes with oxidized cholesterol. Lubrol PX is a condensate of polyethyleneoxide and fatty alcohols. The common features of Lubrol micelles and oxidized cholesterol membranes are (1) an aqueous bathing medium, (2) a fluid hydrocarbon membrane interior, and (3) a surface that consists of polar hydroxyl groups protruding from the hydrocarbon phase into the aqueous phase.

The absorption spectrum of RGA461 in 100 mM KCl containing 1% Lubrol is shown in Fig. 6(*a*), upper trace. Notice the 15 nm red shift of the absorption peak for the probe when it associates with the Lubrol micelles. Sodium dodecyl sulfate and cetyltrimethylammonium bromide micelles, as well as nonpolar solvents like octanol and lutidine, produce a red shift in the absorption spectrum of nearly the same magnitude. Subtraction of the Lubrol spectrum from the aqueous spectrum generates the model absorption difference spectrum (Fig. 6*b*) for transfer of this probe from the Lubrol micelle into 100 mM KCl. The absorption difference spectrum of the model system (Fig. 6*b*) and the BLM transmission difference spectrum (Fig. 3) are very similar. [Notice that the direction of subtraction for Fig. 6(*b*) was selected to obtain an absorption difference spectrum with the same polarity as the BLM transmission difference spectrum shown in Fig. 3. The direct comparison can be made because the absorption difference spectrum $\Delta A(\lambda)$ is proportional to the negative of the transmission difference spectrum $-\Delta I(\lambda)/I(\lambda)$, when the transmission change is small (Waggoner & Grinvald, 1977).] The similarity of the difference spectra suggests that positive voltage changes across the BLM are driving the negatively charged probe from an aqueous region just off the membrane surface into a nonpolar region in the oxidized cholesterol membrane. Negative potential steps drive dye back off the membrane. Thus the solvent model studies support an "on-off" mechanism for the impermeant oxonol dyes.

Potential-Dependent Binding of the Impermeant Oxonol WW781 to Human Red Blood Cells

In preliminary experiments with human red blood cells, RGA461 exhibited greater interactions with valinomycin than did the structural analog WW781. Moreover, WW781 was useful in monitoring ΔE_m associated with Ca-induced increases in K conductance (Freedman & Novak, 1983). This dye is thought to be impermeant to red cells because of its two separated negative charges, and because it responds within the mixing time in cuvettes when the K-ionophore valinomycin (VAL) is used to hyperpolarize the cells. Figure 7 compares the mixing time of WW781 (upper trace) with the response time to VAL-induced hyperpolarization (lower trace). The traces confirm that the half-time of mixing and the half-time for the voltage-dependent fluorescence responses are within 1 sec (*cf.* Freedman & Novak, 1983, Fig. 3, inset *A*). Note that for the traces in Fig. 7 the recorder speed was slowed before and after the change in fluorescence to ensure that the complete fluorescence change was recorded. The rapidity of the response seems inconsistent with a permeation mechanism (Sims et al., 1974) for a divalent anionic dye with red cells, but is more indicative of a mechanism involving only the membrane and the medium.

In order to determine whether the "on-off" mechanism described above for hemispherical bilayers pertains to red cell suspensions, experiments were conducted to measure the amount of dye associated with red blood cells before and after hyperpo-

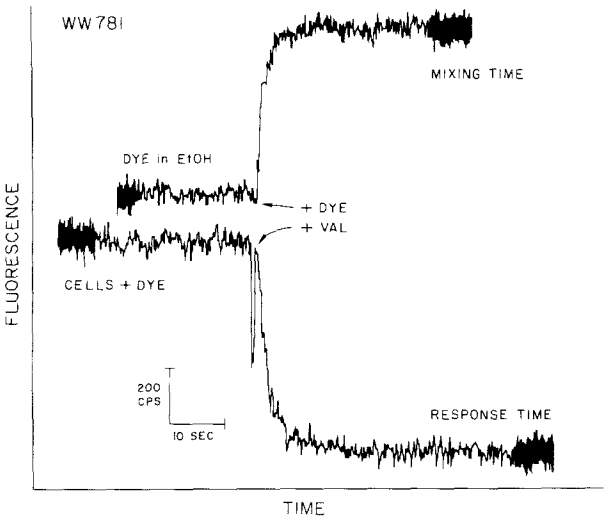

Fig. 7. Comparison of mixing time of WW781 in EtOH (upper trace) to the response time when human red blood cells with WW781 are hyperpolarized with VAL (lower trace). For the mixing time, sufficient WW781 (1 mg/ml in EtOH) was added to 2.5 ml EtOH to give a fluorescence approximating that of the dye with the cells. Then, about 1 μl of dye was added to give a change in fluorescence approximating that obtained when the cells are hyperpolarized with VAL. For the response time, 1 μl WW781 was added to 2.5 ml of 1.2% hematocrit red cells in 149 mM NaCl, 1 mM KCl, 5 mM HEPES buffer, pH 7.4 at 23°C. After 3–5 min, the cells were hyperpolarized by adding 1 μl of 2.5 mM VAL in EtOH. The transient drop in fluorescence during addition of VAL is due to the syringe needle blocking the light path. The voltage-dependent fluorescence decreased by 9%. For both traces the recorder was slowed by 10-fold before and after the change in order to verify that new equilibria were attained. The bottom trace confirms that the half-time for the voltage-dependent fluorescence response of WW781 in human red blood cells is approximately 1 sec or less

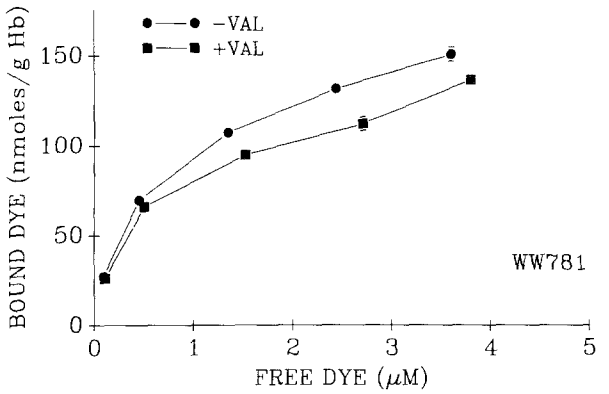

Fig. 8. Representative binding curve of WW781 before and after addition of VAL to human red blood cells. The binding of dye to red cells in the absence (circles) and prescence (squares) of VAL was determined as described in Materials and Methods. Error bars represent standard errors of the mean for triplicate determinations. Points without error bars have errors smaller than the symbol size

Table. Binding of WW781 by red blood cells[a]

Experiment	Bound dye (nmol/g Hb)[b]	
	-VAL	+VAL
1	152 ± 15	114 ± 6
2	165 ± 4	136 ± 5
3	151 ± 4	137 ± 1
mean	156 ± 6	129 ± 11

[a] A 0.2-ml aliquot of 50% HTC red blood cell suspension was added to a cuvette containing 2.3 ml of media consisting of 149 mM NaCl, 5 mM HEPES buffer (pH 7.4 at 23°C), and 6 μM WW781. The complete binding curve for experiment 3 is given in Fig. 8.

[b] Bound dye is the difference between the total initial dye and the dye remaining in the supernatant after centrifugation of the cells. Errors represent standard errors of the mean for triplicate determinations.

larization by VAL. In three experiments, the absorbance of dye was measured before adding cells and also in the supernatants after centrifuging the cells both with and without adding VAL. The results in Fig. 8 are a typical binding curve for WW781 by red blood cells before and after addition of VAL. For the three experiments, the Table gives the bound dye before and after VAL at an initial dye concentration of 6 μM, the highest used in Fig. 8. Figure 8 gives the complete binding curves for the third experiment. The change in binding upon addition of VAL at 6 μM initial dye was significant with $P < 0.1$, 0.025, and 0.05 in the three experiments, respectively. The average decrease in cell-associated dye after VAL was 17 ± 7%.

Where is the Membrane-Binding Site for the Chromophore?

It is likely that the sulfonate group on the probe prevents the chromophore from sinking too deeply in the membrane. If the sulfonate sits at the surface, the molecular dimensions of RGA461 would place the center of the chromophore about 10 Å into the membrane when the probe is oriented with its long axis perpendicular to the surface. This depth is about 20% of the total thickness of the membrane. If the center of the distributed charge on the chromophore is near the center of the chromophore, the charged part of the probe that can be influenced by the membrane electric field may sense at most 20% of the transmembrane potential drop, assuming that the potential changes linearly across the entire thickness of the membrane. Unfortunately, it is difficult to say exactly where the center of the charge distribution is located. The charge may have a tendency to move away from the low dielectric interior of the bilayer toward the more polar surface region.

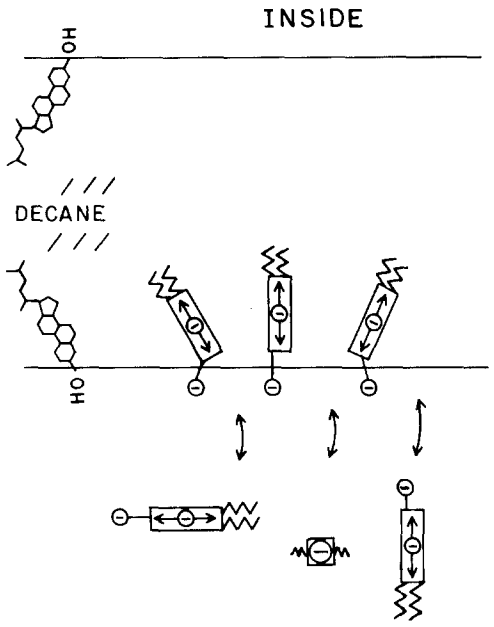

Fig. 9. Proposed "on-off" mechanism for RGA461 and other impermeant oxonol analogs. This illustration shows three probe molecules oriented more or less perpendicular to the plane of the membrane. When the inside of the bilayer (top of figure) moves to a negative potential, probe molecules are driven off the membrane and assume random orientations. The center of the bilayer contains an undetermined amount of decane solvent (Robinson & Strickholm, 1978)

The boundary and dipolar potentials of the membrane may also affect the charge distribution. The fraction of the transmembrane potential change sensed by the dye will be discussed further in a subsequent paper (George et al., 1988).

Solubility considerations also weigh against the possibility that the chromophore sinks deeply in the membrane. Cyanine and oxonol chromophores, with their delocalized charges, are very insoluble in pure hydrocarbon solvents like hexane and benzene but are very soluble in nonpolar alcohol solvents like ethanol, butanol and octanol (Sims et al., 1974; Waggoner et al., 1977). The pyrazolone oxonol chromophores behave similarly. For example, the analog in which R_2 = ethyl, R_3 = R_4 = butyl, and in which the sulfonate group on the phenyl ring is absent, partitions in a two-phase solvent system more than 95% into 100 mM KCl leaving the hexane phase colorless but in a separate experiment partitions more than 95% into butanol leaving the 100 mM KCl phase colorless. Therefore it seems likely that the chromophore part of RGA461 probably resides in an adsorption plane only part way between the surface and the hydrocarbon center of the membrane. The adsorption plane is not as nonpolar as the decane interior but contains occasional water molecules (Griffith, Dehlinger & Van, 1974), and the hydroxyl and keto groups on the 3 and 7 positions of oxidized cholesterol.

It is not surprising that the membrane-associated chromophore is oriented more or less perpendicular to the membrane surface. This orientation places the charged chromophore the deepest in the membrane in response to the driving force of potential changes in the positive direction. Furthermore, the chromophore part of the probe together with its alkyl substituents constitutes an elongated, relatively nonpolar structure which has approximately the same shape as an oxidized cholesterol molecule. Therefore, packing considerations, which would maximize lateral interactions between the lipid molecules and the dye molecules, would also favor the perpendicular orientation.

Figure 9 illustrates the "on-off" mechanism we propose for RGA461. We think it is probable that the other 42 impermeant analogs of RGA461, including WW781, also operate by the "on-off" mechanism (Nyirjesy et al., 1988). All the analogs have roughly the same shape and charge distribution. They differ mainly in the length of the alkyl groups attached to the chromophore. Thus each involves a sulfonate group attached by a linker to an oxonol chromophore possessing a distributed negative charge.

The following two papers (Nyirjesy et al., 1988; George et al., 1988) are concerned with the dependence of the signal size on the length of the alkyl groups bound to the chromophore and with the dependence of the signal on the membrane voltage.

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