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Mechanism of Potential-Dependent Light Absorption Changes of Lipid Bilayer Membranes in the Presence of Cyanine and Oxonol Dyes

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Summary. The mechanism by which the light absorption of cyanine and oxonol dyes changes in response to changes in transmembrane electrical potential has been studied. Trains of membrane potential steps produce changes in the intensity of light passing through glycerylmonooleate (GMO) bilayer lipid membranes (BLM) in the presence of these dyes. The size of the signal-averaged absorbance change for one of the cyanine dyes diS-C_2-(5) is $\sim 10^{-5}$. The response time for the absorbance change of all of the dyes was ≤ 10 μsec. In order for an absorption signal to be observed, the concentration of dye on both sides of the membrane must be different. Since GMO bilayer membranes are permeable to the charged dyes that were studied, the dye concentration asymmetry necessary for the optical signal had to be maintained with a constant dc membrane potential, onto which the trains of potential steps were superimposed. The more hydrophobic dyes were the most permeant. Inclusion of cholesterol in the GMO bilayers decreased the permeance of the positively charged cyanine dyes, but increased the permeance of the negatively charged oxonol dyes. The magnitude and the size of the BLM absorbance change depended on the wavelength of illumination. Comparisons of the wavelength dependence of the BLM spectra with absorption difference spectra obtained with model membrane systems allow us to postulate a mechanism for a BLM absorbance change. For the cyanine and oxonol dyes, the data are consistent with an ON-OFF mechanism where a quantity of dye undergoes a rapid potential-dependent movement between a hydrocarbon-like binding site on the membrane and the aqueous salt solution near the membrane. For some dyes, which readily aggregate on the membrane, part of the absorbance change may possibly be explained by a potential dependent change in the state of aggregation of dye molecules localized on the membrane. Mechanisms involving a potential dependent change in the polarizability of the environment of membrane-localized dye molecules cannot be excluded, but seem unlikely.

Optical membrane-potential probes provide a novel approach for measuring potentials in cells and organelles too small for microelectrode measurements. They may also offer a way to simultaneously monitor spike activity in a large number of cells where it is too cumbersome

or impossible to insert a microelectrode into every cell (Cohen *et al.*, 1974).

The classes of potential-sensitive dyes that have been most extensively studied to date are the cyanine, merocyanine, and styryl dyes (Cohen *et al.*, 1974; Waggoner, 1976) and the naphthalene sulfonates (Conti, 1975). Of these dyes, the cyanines have so far proved most useful for studying potentials of cells, organelles, and vesicles in suspension. For example, cyanine dyes have been used to study transmembrane electrical potentials of red blood cells (Hoffman & Laris, 1974; Sims *et al.*, 1974), bacterial cells (Kashket & Wilson, 1974; Laris & Pershadsingh, 1974; Brewer, 1976), synaptosomes (Blaustein & Goldring, 1975), purple membrane vesicles (Renthal & Lanyi, 1976), ascites cells (Laris, Pershadsingh & Johnstone, 1976), and mitochondria (Laris, Bahr & Chaffee, 1975). In these systems the fluorescence changes take place over a period of a few seconds. On the other hand, merocyanine and oxonol dyes, as well as cyanine dyes, have been used to detect rapid potential changes in single nerve axons (Cohen *et al.*, 1974), invertebrate central neurons (Salzberg, Davila & Cohen, 1973; Salzberg *et al.*, 1976) and muscle cells (Oetliker, Baylor & Chandler, 1975; Vergara & Bezanilla, 1976; Salama & Morad, 1976). In fact, some dyes show absorption and fluorescence changes large enough so that a single spike in a nerve fiber can be observed optically with better than a 100:1 signal to noise ratio (Ross *et al.*, 1976).

Although it is possible to use the probes in certain cases without knowledge of how they work, an understanding of the mechanism of the potential sensitivity of each dye would aid interpretation of observed optical changes, as well as synthesis of better dyes. Sims *et al.* (1974) have provided insight into the mechanism by which cyanine dyes respond to potentials of red blood cells in suspension. The fluorescence response of the dyes was shown to result from potential-dependent partition of dye molecules between the cells and the extracellular medium. Cell hyperpolarization results in uptake of the positively charged dye by the cells, while depolarization results in release of dye. The emission from cell-associated dye becomes significantly quenched as the amount of cell-associated dye increases. The authors present evidence that suggests that the quenching of cell-associated dye is due to the formation of dye aggregates that are not fluorescent. They propose a model to explain the potential-dependent partition of dye between the inside and outside of vesicles and red blood cells that assumes that the cyanine behaves as a permeant cation.

There has been some support for the proposal of Sims *et al.* (1974) that cyanine (and oxonol) dyes readily pass through bilayer membranes. Waggoner *et al.* (1975) reported that these dyes penetrate within seconds into the interior of multiconcentric phosphatidylcholine liposomes to quench the fluorescence of liposome-bound energy donor molecules. They found that as the N-alkyl chain attached to the chromophore was lengthened from 2 to 6 carbons, the rate of liposome penetration increased to a maximum, but as the chain length became longer, the rate of penetration decreased. Szabo (1974, 1976) studied the conductance of glycerylmonooleate bilayers containing cholesterol in the presence of the cyanine dye diO-C_5-(3). He found that the dye carries current across the bilayer and that addition of cholesterol decreases the current carried by the dye.

Although changes in dye emission have most often been used to detect potential changes, the absorption properties of many dyes are also extremely sensitive to membrane potential (Chance & Baltscheffsky, 1975; Ross *et al.*, 1976). This paper concentrates on the mechanism of the potential-dependent light absorption changes that take place with lipid bilayer membranes in the presence of cyanine and oxonol dyes. We will show that the dye concentration in the aqueous region adjacent to the two membrane interfaces must be different in order to observe a light absorption change. We propose that the absorption change may result from a potential-dependent partition of dye between a hydrocarbon binding site on the membrane and the aqueous regions adjacent to the membrane on the side of the membrane where the dye is most concentrated.

Materials and Methods

Dyes

The preparation and properties of most of the dyes used in this paper are described in either Cohen *et al.* (1974) or Sims *et al.* (1974).

Membranes

Bilayer lipid membranes were formed across a 2 mm diameter hole in the bottom of a teflon cup in the optical apparatus. The aqueous solution was unbuffered but contained a salt. The membrane forming solution contained 2% glycerylmonooleate from Sigma Chemical Co. dissolved in glass distilled decane. In most experiments the membrane forming solution also contained the dye to be studied. This was accomplished by adding a quantity of the ethanol stock solution containing the dye directly to the membrane forming solution

to give a dye concentration of 50–100 μM. The purpose of this procedure was to speed up the rate at which the optical signals and membrane currents reach equilibrium after the membrane is formed. Egg phosphatidyl choline vesicles were formed in 100 mM KCl or distilled water by light sonication as described in Sims *et al.* (1974). The egg phosphatidyl choline was prepared by the method of Singleton *et al.* (1965). Glycerylmonooleate sonicates were prepared by essentially the same procedure.

Optical Apparatus

The equipment for the measurement of potential dependent absorption changes in black lipid membranes is shown in Fig. 1. A 12-V quartz halogen lamp (Sylvania FCR) in an Oriel Corporation Model 6325 lamp housing was powered by a Kepco JQE 15-12M low noise dc power supply. Monochromatic light was obtained with 3-cavity, 10 nm bandwidth interference filters purchased from Ditric Optics, Inc., or Oriel Corporation. The teflon cup supporting the membrane was immersed in a plastic petri dish containing 100 mM KCl. The petri dish was mounted on the stage of Unitron MIC-2313 inverted microscope, which was equipped for vertical illumination. A United Detector Technology, Inc., ultra low noise Pin-040A photodiode mounted in "camera optics" section of the microscope collected the light that passed through the membrane. The apparatus above was isolated from floor vibrations with a bicycle inner tube. Current from the photodiode was converted to a voltage with an Analog Devices 40J operational amplifier with a 100-k Ω feedback resistor. The signal was amplified with a Tektronix 3A9 oscilloscope amplifier and passed to a Kiethley model 822 phase sensitive detector and to a Princeton Applied Research TDH-9 Waveform Eductor. The phase sensitive detector provides an analog output of the average magnitude of the optical changes that result from membrane potential changes. This optical signal was recorded with one pen of a Brinkmann 2572 dual pen recorder. The Waveform Eductor provides the averaged waveform of the signal in 100 channels. The time constant for the optical detection system was 3 μsec.

The membrane potential pulses were generated with a Hewlett Packard model 3310A or 8011A pulse generator. The voltage steps were applied directly to Ag-AgCl electrodes on each side of the membrane. The time constant for charging the membrane was usually

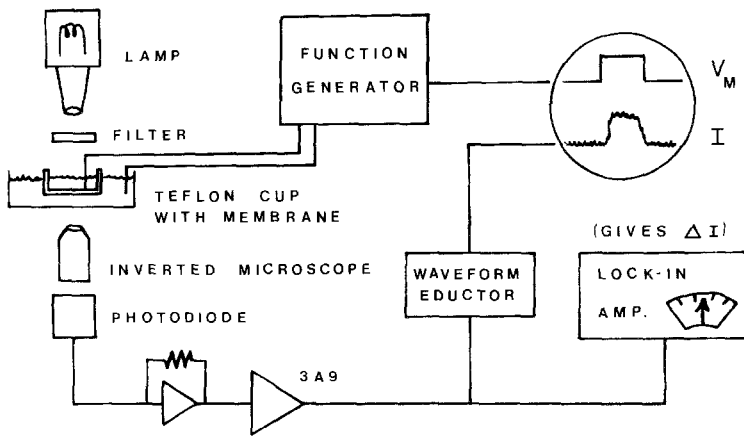


Fig. 1. Schematic diagram of optical BLM apparatus used to detect absorption signals from lipid bilayer membranes in the presence of potential-sensitive dyes

10 μsec. Membrane currents were detected with an Analog Devices 41K operational amplifier. Current transients were obtained using a 10^4 Ω feedback resistor which yielded response time for the current measuring system of 20 μsec. Transient signals were amplified with a Tektronix 7A22 oscilloscope amplifier, digitally recorded with a Biomation 802 transient recorder, and plotted with a Hewlett-Packard 7004B XY recorder. In addition, membrane current signals from the 41K were passed through a Datel lowpass filter to the second pen of the Brinkmann recorder, and thus were recorded concurrently with the optical signals. The current measuring system was checked with standard resistors substituted for the membrane.

Absorption Spectra

All dye absorption spectra were recorded with either a Cary 14 or a Cary 15 absorption spectrophotometer. After each spectrum of an aqueous dye solution was obtained, the solution was removed from the cuvet by aspiration and the amount of dye sticking to the walls was measured by extracting the cuvet with 3 ml ethanol and measuring the absorption of the ethanol extract. The dimer spectrum was calculated by two methods. In the first method the dimer dissociation constant (1.5×10^{-5} M for diS-C_2-(5) in distilled water) of West and Pearce (1965) was assumed and the dimer absorption spectrum was calculated according to their procedure. In the second method a computer was used to generate a dimer spectrum and a monomer spectrum from a series of spectra obtained experimentally at different dye concentrations. The program assumed that a simple dimer-monomer equilibrium was obeyed and then searched for a dimer spectrum and a monomer spectrum which, when combined in the proper proportions, produced most correctly all the experimental spectra at different dye concentrations. By this method the dimer dissociation constant was 2×10^{-5} M for diS-C_2-(5) in distilled water.

The absorption spectrum of "membrane-bound dye" was determined by the following procedure. A baseline was obtained by adding a large quantity (1.0 mg/ml) of sonicated egg phosphatidyl choline vesicles to both the sample and reference cells of the Cary spectrophotometer. A small quantity (0.5 μM) of dye was then added to the sample cell and an absorption spectrum was obtained. A virtually identical spectrum was obtained when glyceryl-monooleate sonicate was used instead of phosphatidyl choline vesicles.

Calculated difference spectra were obtained by adding or subtracting the appropriate monomer or dimer spectra with an IBM 1130 computer and graphing the results with Calcomp plotter.

Results

The intensity of light, I, passing through glycerylmonooleate (GMO) bilayer membrane formed in salt solutions containing 1–5 μM concentrations of certain cyanine and oxonol dyes is modulated by changes in the transmembrane potential difference. We find it convenient to discuss the optical signal observed during step changes in membrane potential in terms of an absorbance[1] change, ΔA, rather than a transmittance change, ΔI. Since $\Delta I/I$ is small,[2] typically $\leqq 3\times 10^{-5}$ for dyes in this

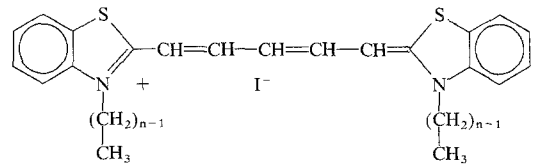
1 The absorbance change, ΔA, is not the same as the absorption change of Cohen *et al.* (1974).

2 Signal averaging was necessary to obtain good absorption signals for the cyanine and oxonol dyes. No signal was observed in the absence of dye.

work, $\Delta A = -\Delta I/2.3I$. For potential steps that move from 0 mV (ground) to either positive or negative voltages, we define ΔA for a given wavelength as the absorbance at the highest absolute value of membrane potential minus the absorbance at 0 mV.

Direction and Size of the Optical Change

In most of the following discussion we will refer to the results obtained with the cyanine dyes series, diS-C_n-(5).



For these dyes, trains of square potential steps that go positive *or* negative from 0 mV result in a decrease in absorbance during the step at 670 nm (Fig. 2). However, trains of alternating symmetrical potential steps as large as 200 mV that are centered about 0 mV produce no absorption change.

The magnitude of ΔA for diS-C_2-(5) at 670 nm varies nonlinearly with the size of the potential steps when the bottom of the steps is held at 0 mV (Fig. 3*a*). In this experiment both the step size and the average voltage [$V_{avg} = (V_{max} + V_{min})/2$] were varied and the absorbance

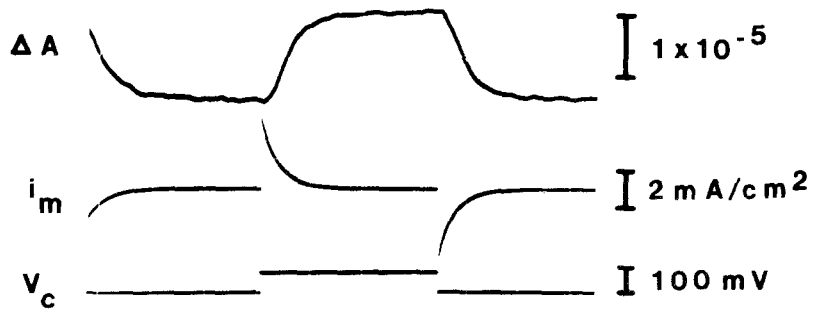


Fig. 2. Top trace: signal averaged light absorption change ΔA, of diS-C_2-(5) during a 100 μsec, 75 V membrane potential pulse. Dye concentration was 3 μM on both sides of the membrane. The solution electrolyte was 100 mM KCl. Middle trace: membrane current determined from voltage drop across a 100 Ω resistor in series with the membrane. Bottom trace: the 75 mV potential pulse applied to the membrane

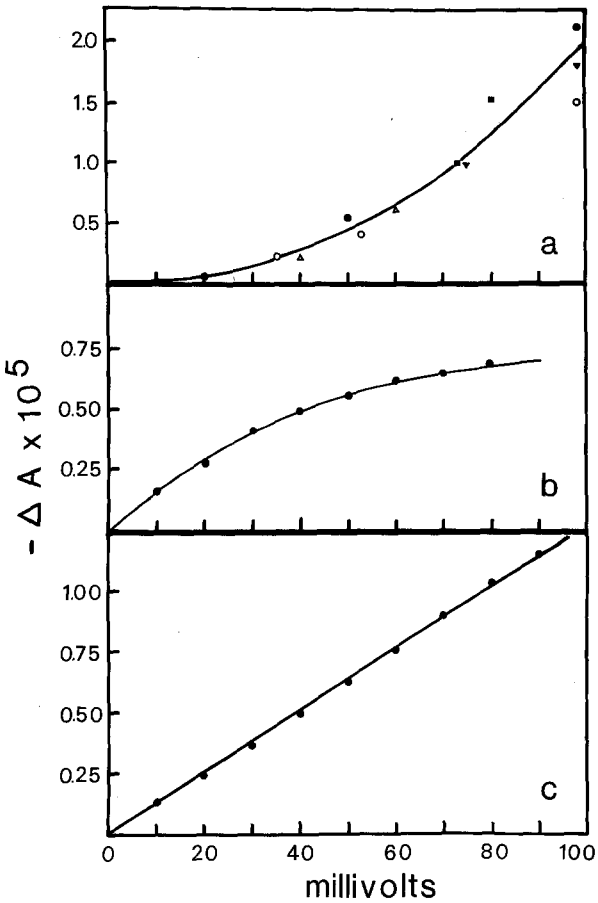


Fig. 3. (*a*) Magnitude of the absorbance change, ΔA, *vs.* the size of the potential steps in the pulse train. The 1 msec potential steps went 0 mV to the voltage given; thus, the average potential is half the value given on the horizontal axis. For each point on the graph the absorbance value was recorded 30 sec after the membrane potential was set, so that the signal had reached nearly full strength. Different symbols represent measurements on different membranes. The diS-C_2-(5) concentration was 1.5 μM on both sides of the membrane and the electrolyte was 100 mM KCl. (*b*) Same as in Fig. 3*a* except that ΔA values (given by the points) were recorded using constant 40 mV steps and the *average* membrane potential, $V_{avg}=(V_{max}+V_{min})/2$, was offset to the value given on the horizontal axis. (*c*) Same as in Fig. 3*a* except ΔA was obtained with a constant V_{avg} (37 mV) and the size of the potential step was set at the value given on the horizontal axis

change per mV potential change is larger at larger membrane potentials. If the size of the potential steps is held constant (40 mV) and the "offset" voltage of the pulse train (same as V_{avg}) is varied, ΔA increases with V_{avg} but seems to saturate at high V_{avg} (Fig. 3*b*). On the other hand, if V_{avg} is held constant (37 mV), ΔA was found to vary linearly with

the voltage of the steps in the pulse train (Fig. 3*c*). The latter result has also been observed by Cohen *et al.* (1974) with the squid giant axon. In the experiments in Fig. 3*a* and *b*, negative potential steps gave the same results (ΔA had the same sign) as positive potential steps. The absorbance changes for members of the series diS-C_n-(5) were in the ratio 1:0.2:0.4:0.3 for n=2, 3, 4, 5, respectively.

The Rapid Response

The response of the optical signal of diS-C_2-(5) during each individual potential step is rapid (Fig. 2). The time constant (time to reach 0.63 of maximum), τ_R, of this rapid response was less than or equal to 10 μsec for all of the dyes described in this work. Since the capacitative charging time for the membranes was also 10 μsec in these experiments, τ_R is certainly less than 10 μsec for these dyes. In a few experiments we reduced the membrane charging time to 3 μsec. This reduced τ_R for diS-C_2-(5) to ~4 μsec. After the initial rise, the signal for most of the dyes remained level for up to 10 msec, the maximum pulse length used.

The Slow Appearance of the Optical Signal

When dye is on both sides of the membrane, the optical signal does not appear at the beginning of a train of potential pulses applied to the membrane. Rather the signal develops slowly over a period ranging from less than 0.8 sec to 150 sec, depending on the particular dye used and whether cholesterol is present in the membrane. This phenomenon is shown schematically in Fig. 4. When the alternating pulses *centered about 0 mV* are switched to pulses going *positive or negative from 0 mV,* the envelope enclosing the individual optical absorbance changes appears with a time course that is slow compared with the rapid response of the optical signal during a single potential step. The time required for the envelope of the individual optical responses to reach 63% of the maximum value after the pulse train is switched on is τ_S, the time constant for the *slow* appearance of the signal. The relatively slow appearance of ΔA can be seen in experiments where the phase-sensitive detector output, which is proportional to ΔA, is recorded during a train of 1 msec square pulses (Fig. 5). The τ_S values obtained from recordings in this figure together with τ_S values for other members of the diS-C_n-(5) series are tabulated in Table 1. The trend is clear. As the length of the alkyl

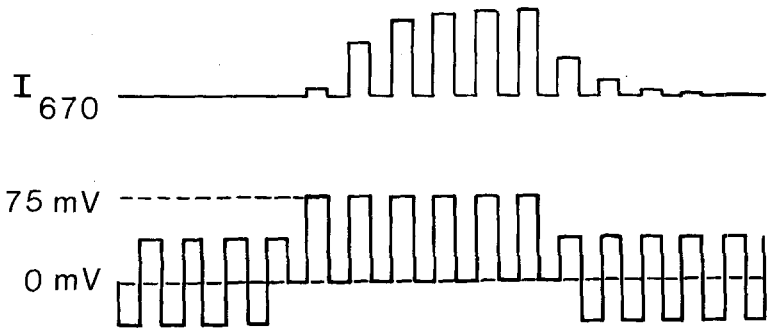


Fig. 4. Diagram illustrating the relatively slow development of the optical absorption responses when the potential steps, initially centered about 0 mV, are suddenly made to go between 0 and +75 mV. The time for ΔA, the magnitude of the absorption changes, to reach 63% of the plateau value, is τ_S. If the pulses went from 0 to −75 mV, the optical signals were in the same direction as shown in this diagram

chain (C_n) on the dye increases the optical signal develops faster. And, for any individual dye, addition of cholesterol to the membrane decreases the speed at which the optical signal appears.

Membrane Currents that Are Related to τ_S of the Absorbance Signal

Szabo (1974) has demonstrated that the presence of cholesterol decreases the permeance of the positively charged cyanine dye, diO-C_5.(3), in GMO bilayer membranes. Waggoner *et al.* (1975) and D. Sirkin (*unpublished*) have observed that the rate at which cyanine dyes of the series diO-C_n-(3) and diS-C_n-(5) penetrate into inner layers of multiconcentric PC liposomes and quench the fluorescence of lipid soluble energy donor molecules depends on C_n. The penetration rate increases by a factor of 5 as n goes from 2 to 6. These results when compared with the τ_S data for the absorbance signal suggest that τ_S may be proportional to the rate at which the dye moves across the membrane. In order to more fully understand the relationship between the time course for absorbance changes and dye movement across the membrane, we have measured membrane currents either during the optical experiments or under the same conditions (trains of 1 msec steps) in which the optical experiments were carried out. In addition we have investigated the kinetics of dye movement by observing the transient membrane currents that occur during single square potential steps.

In Fig. 5 it can be seen that following the onset of a train of 1 msec

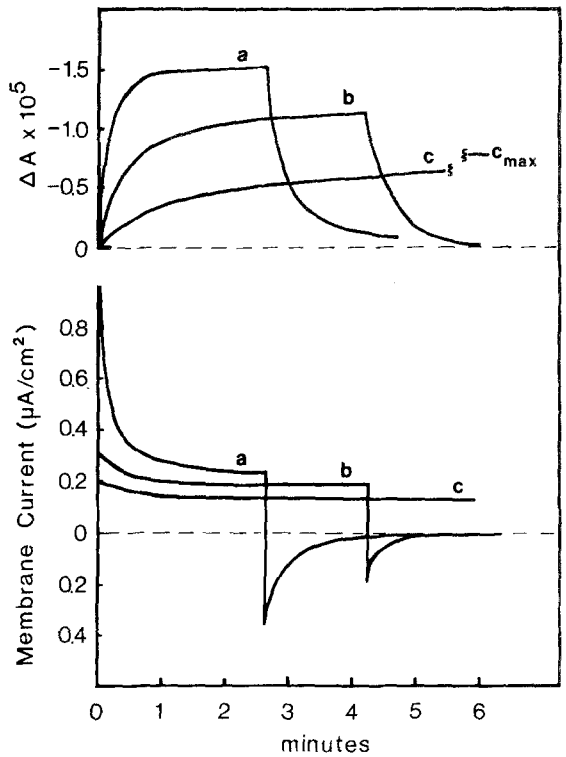


Fig. 5. Simultaneous measurement of the absorbance change, ΔA, and membrane current following the onset of a train of 75 mV, 1 msec potential pulses. The concentration of diS-C_2-(5) on both sides of the membrane was 3 μM and the electrolyte was 100 mM KCl. The mole percentages of cholesterol in the GMO-decane membrane forming solutions were (*a*) 0%, (*b*) 17%, and (*c*) 28%. The absorbance signal presented is the output of the phase-sensitive detector. A high frequency cutoff filter has removed the rapid current changes that occur during the 1 msec voltage steps in the pulse train

Table 1. τ_s for diS-C_n-(5), in seconds[a]

	Mole percent cholesterol in membrane-forming solution				
	0	17	23	28	38
C_n 2	9	38	45	70	160
3	≦0.8	2	9	20	25
4	≦0.8	2	5	8	8
5	≦0.8	1	1.5	1.5	2

[a] Trains of 1 msec square pulses stepping from 0 to 75 mV were used to obtain τ_S. Thus the average membrane potential during the pulse train was 37 mV. The dye concentration used to obtain the τ_S values was 1.5 μM. The solution electrolyte was 100 mM KCl. τ_S did not vary significantly for twofold changes in dye concentration. Values were reproducible to within 10%.

long potential pulses the membrane current[3] decays to a steady state value. This figure shows that the current transients at the onset of the pulse train, and after completion of the pulse train, decay at nearly the same rate as the absorbance signal appears and disappears, respectively. It is therefore worthwhile to try to understand the origin of these membrane current transients that are closely related to the time course of the appearance of the absorbance change.

Assuming the dye to be the predominant current carrier in the membrane, it is reasonable to expect that the current will be limited by movement of dye across three barriers: (1) a barrier at the center of the membrane (between the dye binding sites on the membrane), (2) barriers at the aqueous interfaces (between the dye binding sites and the aqueous solution immediately adjacent to the membrane), and (3) the barrier that occurs because of diffusion limited movement of dye through the unstirred aqueous regions near the membrane. The magnitude and time dependence of the membrane current carried by a permeant dye will depend on the relative heights of these barriers and upon the relative concentrations of the ion in the regions between the barriers and in bulk solutions. The kinetics of membrane currents carried by other permeant ions have been studied by Ketterer, Neumcke and Läuger (1971), Anderson and Fuchs (1975), Krasne and Eisenman (1977) and others. For example, it has been shown (Ketterer *et al.*, 1971; Andersen & Fuchs, 1975; Szabo, 1976) that sudden potential changes across membranes formed in solutions containing dilute, highly permeant lipid soluble ions, such as tetraphenylborate (TPB), will lead to current transients that are due primarily to movement of the permeant ion from an electrochemical potential energy well on one side of the membrane to a similar well on the other side of the membrane. With TPB it is observed that within a few msec of the beginning of a potential change the membrane current decays exponentially to a small, more-or-less steady value as the permeant ions shift across the membrane. During the early part of the TPB current transient, the rate limiting step is movement of the ion across the center of the membrane. After several msec, the rate limiting step is movement of TPB from the aqueous solution into the TPB-depleted well and movement of the ion from the TPB-loaded well into the aqueous solution on the other side of the membrane.

All members of diS-C_n-(5) show transient currents immediately fol-

3 A low-pass active filter in the current measuring circuit removes the rapid transients that occur during the 1 msec pulses but passes current fluctuations with time constants > 0.3 sec.

lowing the onset and following completion of trains of potential pulses or single potential steps. For the moment we will assume that during the membrane current transient the dyes described in this paper are undergoing interface-to-interface transfer (much like TPB) *together with* depletion in a narrow aqueous region adjacent to the membrane interface[4]. The relative contributions of these two factors for each different dye will depend on the relative membrane permeance of the dye. We will term this entire process "concentration polarization". It is schematically shown in Fig. 6. The fact that membrane current, both during the pulse train and after completion of the pulse train, follows the same time course that the absorbance signal follows (*refer* again to Fig. 5) suggests that concentration polarization of the dye is essential for the absorbance change. That is, *a transmembrane asymmetry in dye concentration is essential for the absorbance signal.* Two additional experiments lend support to this hypothesis. First, if diS-C_2-(5) is added to only one side of the membrane, *symmetrical* potential steps, which produce no optical signal if dye is on both sides of the membrane, produce an *immediate* and substantial absorbance signal at the onset of the pulse train. In order to see this effect the pulse train must be applied soon after addition of the dye. The absorbance signal disappears after several minutes. Second, when dye is initially at the same concentration on both sides of the membrane, a train of positive/negative potential steps produces an *immediate* absorption signal *provided that* the membrane was held for a period of time at a positive/negative dc potential prior to the pulse train. In both of these experiments, a dye concentration asymmetry was created before the pulse train was applied. The latter

4 Furthermore, we are asserting that most of the membrane current we measure is carried by dye. Membrane currents in the absence of dye are 100 times less than in the presence of the least permeant dye. It is unlikely that the presence of dye on the membrane is greatly facilitating the movement of electrolyte across the membrane since experiments in which KCl is substituted with Na_2SO_4, $CaCl_2$, K_2SO_4, NaH_2PO_4, Na_2HPO_4, and sodium 1,3,5-benzenetrisulfonate provide membrane currents that differ at most by a factor of 2. And with each of these salts the same trends in membrane current with changes in C_n and membrane cholesterol content are observed. It is possible that some electrically neutral anion transport is taking place as dye-anion complexes. This would serve to replenish dye that has been removed from the unstirred layers but would not otherwise alter the membrane conductance. Transient current measurements to be presented below suggest this effect may be negligible. It is also possible that the torus contributes up to 20% of the steady state current since we find that thick membranes saturated with dye pass considerable current. With membranes not constructed from GMO, particularly those that have more positive dipolar potentials, ionic species other than dye might be the major current carriers. This appears to be the case in recent unpublished experiments of S. Krasne with membranes formed from phosphatidylethanolamine in the presence of the diS-C_n-(5) dyes.

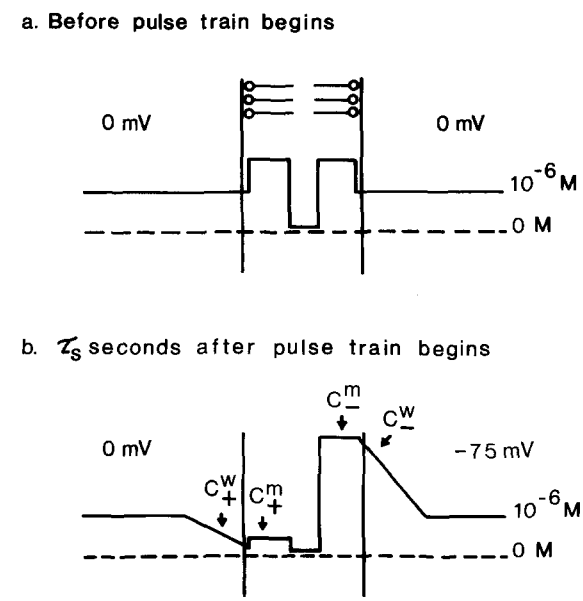


Fig. 6. Illustration of the change in permeant dye concentration near the membrane after a potential difference is imposed on the membrane. The vertical axis represents the steady state dye concentration as a function of distance from the membrane bilayer. We believe the dye concentration in the center of the membrane is low because the cyanine dyes are insoluble in hydrocarbon solvents such as decane. τ_S is the time needed to approach steady state after the membrane potential has been applied. $C_+{}^w$ and $C_-{}^w$ represent the dye concentrations in a narrow aqueous region adjacent to the positive and negative sides of the membrane and $C_+{}^m$ and $C_-{}^m$ represent the corresponding concentrations for the membrane associated dye

experiment is analogous to experiments conducted with these dyes with the squid axon (Cohen *et al.*, 1974). The resting potential of the axon produces the constant dye concentration asymmetry so that the optical signal appears with the full size during a single nerve impulse.

The following data are intended (1) to support the notion that concentration polarization is occurring during trains of potential pulses, (2) to further demonstrate the dependence of τ_S on membrane current, and (3) to show how dye permeance depends on the hydrophobicity of the dye and the cholesterol content of the membrane.

In Table 2 are given the membrane currents observed at the time at which the optical signal has reached its maximum value during a train of 75 mV pulses. From this table it can be seen that the steady state current increases as the hydrocarbon chain on the dye is lengthened and decreases as cholesterol is included in the membrane. Thus the steady state current data suggest that (1) the dyes with longer hydrocar-

Table 2. Steady state membrane currents (nA/cm^2) with diS-C_n-(5)[a]

	Mole percent cholesterol in membrane-forming solution			
	0	17	28	38
C_n 2	30	20	10	2
3	60	40	20	10
4	290	140	60	30
5	760	380	280	110

[a] The steady state current densities were recorded when the absorbance signal had reached at least 90% of its maximum value. Trains of 75 mV, 1 msec potential steps were used to collect this data; therefore, the average membrane potential was 37 mV. The currents for most of the dyes in this table were linear with voltage up to 60 mV. Therefore the conductances can be estimated by dividing the current densities in this table by 0.037 V. In all experiments the dye concentration was 1.5 μM and the electrolyte was 100 mM KCl.

bon chains are more permeant and (2) cholesterol decreases the permeability of GMO membranes to the diS-C_n-(5) dyes. A comparison of the τ_S data in Table 1 and the steady state current data in Table 2 indicates that large steady state currents are found under conditions where the absorbance signals appear the fastest. Thus, the rate of appearance of the absorbance signal at the onset of a pulse train is proportional to the dye permeance. This observation is not surprising since dye permeance determines the rate of concentration polarization.

Transient Current Measurements

A criticism of the conclusions drawn above from steady state current measurements is that the rate of concentration polarization resulting from a change in membrane potential ought to be predicted more accurately from the *initial part* of the current transient rather than from the steady state current, which exists after diffusion polarization is completed. This consideration is particularly relevant for the more permeant dyes since for these dyes the initial current in the transient is much greater than the steady state current. For this reason we have measured membrane current transients during single potential steps for several members of the diS-C_n-(5) series. A typical current transient together with the parameters recorded is shown in Fig. 7. The values of these parameters are given in Table 3 and are discussed below. The experiments were carried out in Na_2SO_4, with the hope of minimizing

Table 3. Current transient parameters for diS-C_n-(5)[a]

C_n	Pulse length	i_0	i_{ss}	i_{rec}	τ_{rec}	Q_{rec}
2	8	0.15	0.06	0.06	3.0	0.14
3	8	0.40	0.11	0.17	2.5	0.43
4	2	1.7	0.17	1.2	0.4	0.52
5	2	4.5	0.20	3.7	0.2	0.80
	sec	$\mu A/cm^2$	$\mu A/cm^2$	$\mu A/cm^2$	sec	$\mu C/cm^2$

[a] The membrane current parameters presented in this table are indicated in Fig. 7. Single 60-mV pulses were used to obtain the data. The pulse length was chosen so as to bring the current to near steady state toward the end of the pulse. In experiments with longer pulses the membrane current continued to drop slightly over a period of a min. The value of i_0 given does not represent the capacitative current that flows during the first 20–30 μsec of a potential pulse. Rather, i_0 is the value of the current density about a msec after the beginning of the step. Q_{rec}, in μcoulombs/cm^2, was obtained by measuring the area under the recovery current transient. The dye concentration in each experiment was 1.5 μM and the electrolyte was 80 mM Na_2SO_4.

the contribution of anion movement to the total membrane current. Similar trends were found using KCl, NaCl, $CaCl_2$, and sodium 1,3,5-benzenetrisulfonate.

The magnitude of the initial current, i_o, depends on "true" membrane permeance of the dye and also on the quantity of dye bound at the membrane interface (Ketterer *et al.*, 1971; Andersen & Fuchs, 1975; Szabo, 1976). The initial current should be free of contributions from electrically neutral transport of dye-anion complexes, since at the beginning of the potential pulse there is no dye concentration gradient to drive movement of the complexes across the membrane. It was found that the initial current increases by a factor of 30 as the alkyl chain is lengthened from C_2 to C_5.

The recovery current, i_{rec}, results from relaxation of the dye concentration polarization. This current must be carried by dye alone since (1) there is no membrane potential and (2) no electrolyte concentration gradients should exist after completion of the potential step because of the high concentration (100 mM) and high mobility of the electrolyte. The values of i_{rec} parallel the values of i_o as C_n is varied. And as C_n is varied the steady state currents, i_{ss}, vary in parallel with i_o. The latter observation indicates that i_{ss} provides at least a rough estimate of the relative permeance of the dyes, even with the more permeant dyes. This lends credence to the comparisons of the steady state currents

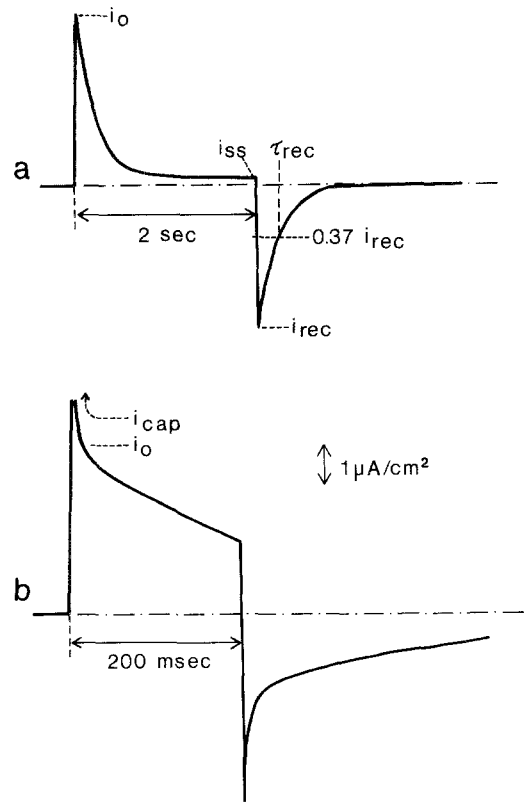


Fig. 7. Membrane transient currents in the presence of 3 μM diS-C_5-(5). In (*a*) a 2 sec 60 mV step potential change was used while in (*b*) the pulse length was 0.2 sec. The time constant of the current measuring system was 0.1 msec in (*a*) and 0.01 msec in (*b*). The electrolyte was 80 mM Na_2SO_4. Most of the capacitative current, i_{cap}, extends off the scale in Fig. 7*a* and *b*

with the time courses of absorbance changes that were made in the previous section.

The time constant for the recovery current transient, τ_{rec}, is a measure of the time for dye concentration polarization established during the potential step to relax toward equilibrium values by the process of dye diffusion back across the membrane and to some extent through unstirred layers. Following the trend predicted by i_{rec}, i_0, and i_{ss} data, the relaxation time is shorter for the more permeant dyes.

Finally, the quantity of charge, Q_{rec}, that moves across the membrane during the recovery is largest for the most permeant dye. This may be due to the fact that a greater quantity of the more hydrophobic diS-C_5-(5) binds to the membrane.

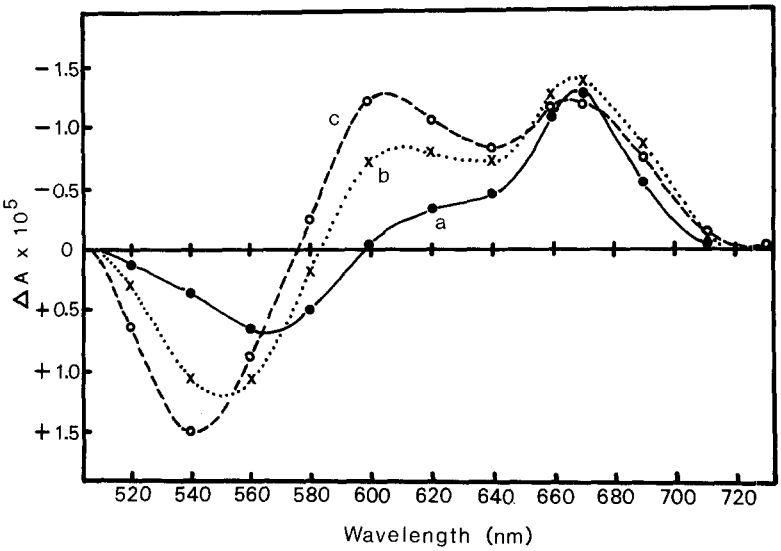


Fig. 8. BLM absorption difference spectrum for diS-C_2-(5). Vertical axis indicates the absorbance at 75 mV minus the absorbance at 0 mV. Bulk solution dye concentrations were (*a*) 3 μM; (*b*) 6 μM: (*c*) 9 μM

In experiments (data not shown) in which cholesterol was included in the membrane i_0, i_{rec}, and i_{ss} diminished while τ_{rec} became larger. These results support other evidence presented earlier in this paper and by Szabo (1974, 1976) that cholesterol in the membrane decreases cyanine dye permeance.

Difference Spectra

Both the sign and the magnitude of ΔA are wavelength dependent. A plot of ΔA *vs.* λ for three different concentrations of diS-C_2-(5) is shown in Fig. 8. In the bilayer lipid membrane (BLM) difference spectra, the absorbance always decreases at long wavelengths and increases at shorter wavelengths. However, the exact shape of the BLM difference spectrum depends on the particular cyanine or oxonol dye used and upon the concentration of the dye. For example, three regions in the BLM difference spectrum are markedly altered when the concentration of diS-C_2-(5) is changed. As the dye concentration increases a shoulder develops at 600 nm, the point at which the absorbance change crosses zero shifts to shorter wavelengths, and the 570 nm peak shifts to shorter wavelenghts.

The shape and concentration dependence of the BLM difference spectra contain information about the mechanism of the absorbance change. Undoubtedly, changes in membrane potential produces changes in the environment of a fraction of the dye molecules associated with the membrane, and these changes result in the alteration of the absorption properties of the dye molecules. In principle, it should be possible to determine what kinds of environment changes a dye undergoes in the BLM during a potential step by comparing the shape of the BLM difference spectrum with other difference spectra obtained with the dye in "known" environments (e.g., pure solvents, phospholipid vesicles, dye in a pure monomer state, or pure dimer state). For difference spectra that simulate the BLM difference spectrum, the "known" environment used in the simulation can serve as a model for the actual environments between which the dye shifts during potential changes across the BLM. For example, if we suppose that a particular dye moves from a hydrocarbon environment in the membrane to water during the membrane potential change, we should be able to simulate the BLM difference spectrum by subtracting the absorption spectrum of the dye when it is dissolved in a hydrocarbon solvent (or some other appropriate solvent that models the membrane binding site) from the spectrum of the same quantity of dye in water. We have used the approach just described to search for mechanisms capable of explaining the wavelength dependence of BLM difference spectra such as those in Fig. 8. First, we shall consider two possible mechanisms for these absorbance changes: (1) potential-dependent movement of dye between a membrane binding site and an aqueous region off the membrane (an ON-OFF mechanism) and (2) potential-dependent changes in dye aggregation localized on the membrane (originally proposed by Ross *et al.*, 1974, to explain potential changes in merocyanine dye absorption and fluorescence). Then we will consider whether there might be other mechanisms that can explain the BLM absorption difference spectra.

Let us first hypothesize that the BLM difference spectra in Fig. 8 arise from the movement of diS-C_2-(5) from a membrane hydrocarbon environment to 100 mM KCl when the membrane potential moves away from 0 mV. Thus we are considering an ON-OFF mechanism. At dilute concentrations diS-C_2-(5) absorbs maximally at 643 nm in both distilled water and 100 mM KCl. The absorption spectrum is shifted to the red in hydrocarbon solvents (West & Geddes, 1964; Sims *et al.*, 1974). Dye associated with phosphatidyl choline (PC) or GMO sonicates or in decane-GMO absorbs maximally at 660 nm, 17 nm to the red

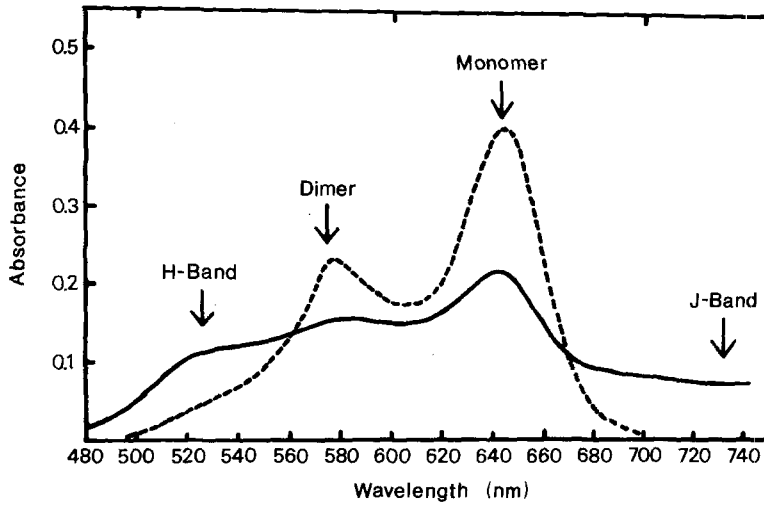


Fig. 9. Absorption spectra of 1.5×10^{-5} M diS-C_2-(5) in distilled water (dotted spectrum) and 100 mM KCl (solid spectrum)

of the water absorption peak. We shall assume that when this dye is in the BLM membrane it has the same absorption spectrum as when it is associated with the PC vesicles, GMO sonicates, or GMO-decane. This spectrum, absorbing maximally at 660 nm, we call the *membrane monomer absorption* spectrum. In effect, we are assuming that the polarity (or more properly, polarizability; West & Geddes, 1964) of the dye binding sites in the BLM and in the solvent environments provided by the model membrane systems are the same and that dye molecules on the BLM are not preferentially aligned in any particular direction.

We must now consider the spectra that the dye might exhibit when ejected into an aqueous region near the membrane. Many of the cyanine dyes are well known for their tendency to aggregate in aqueous solutions. Below 10^{-4} M diS-C_2-(5) exists mainly as dimers and monomers in distilled water. The dimer dissociation constant, K_D, for this dye is 1.5×10^{-5} M in distilled water (West & Pearce, 1965). As the solution ionic strength increases, screening effects facilitate further dye aggregation. In 100 mM KCl, dimers form more readily and *H*-aggregates (small aggregates but not dimers), and *J*-aggregates (extended aggregates) appear when the dye concentration is above 10^{-5} M (Fig. 9, solid spectrum). The absorption spectra of pure monomer and pure dimer have been obtained by the procedure of West and Pearce (1965) and are shown in Fig. 10.

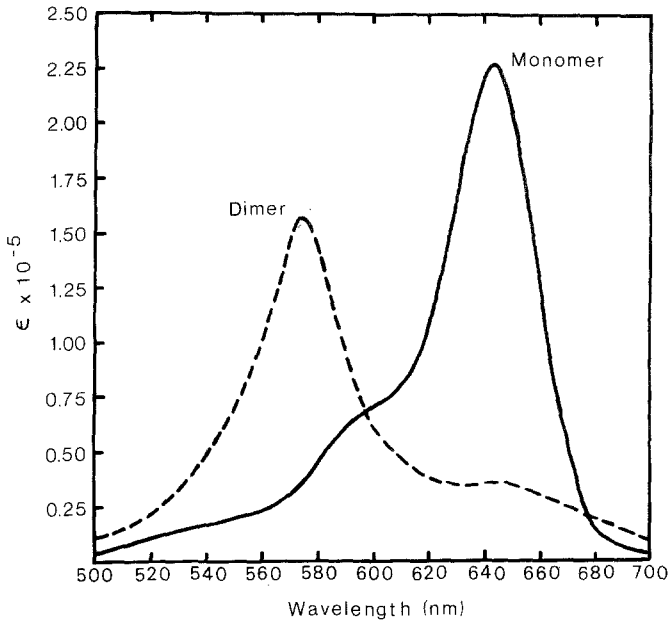


Fig. 10. Monomer and dimer absorption spectra for diS-C_2-(5) in distilled water

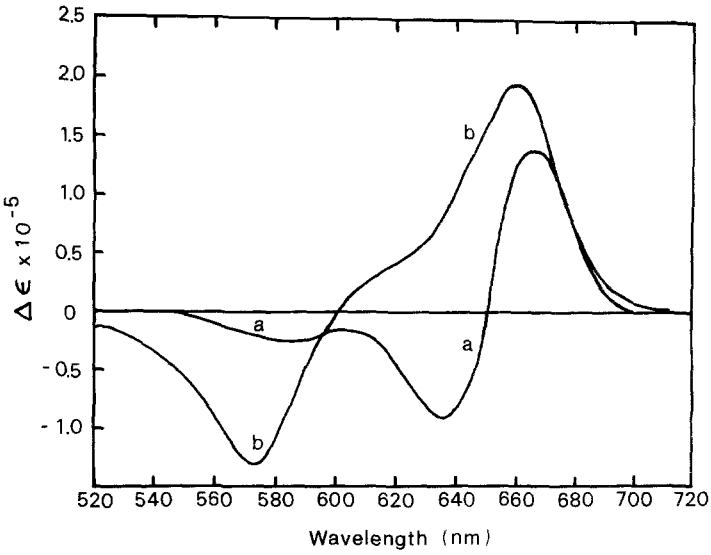


Fig. 11. Computed absorption difference spectra for movement of diS-C_2-(5) from a monomer state in membrane hydrocarbon to different states of aggregation in water. Difference spectrum (*a*) monomer in water; (*b*) all dimer in water. *See* text for details

In Fig. 11*a* we have generated a difference spectrum by subtracting an aqueous monomer absorption spectrum of diS-C_2-(5) from the membrane monomer spectrum that we described above. The difference spectrum generated by this procedure clearly does not match any of the BLM difference spectra shown in Fig. 8. However, the difference spectrum generated by subtracting an aqueous dimer spectrum from the membrane monomer spectrum (Fig. 11*b*) is similar to the BLM difference spectrum obtained with a low concentration of diS-C_2-(5). Thus, *at low dye concentrations, the BLM spectrum is consistent with a mechanism where dye monomers on the GMO-bilayer membrane move to form dimers in 100 mM KCl when the membrane potential increases or decreases from 0 mV.*

We were not able to compute a difference spectrum to match the high dye concentration bilayer difference spectra because it was not possible to obtain an absorption spectrum of pure *H*-aggregates, which are formed at high dye concentrations in 100 mM KCl. However, from the absorption spectrum of diS-C_2-(5) at a relatively high concentration in 100 mM KCl (Fig. 9) it can be seen that one would indeed expect the appearance of a negative peak near 520–540 nm in the bilayer difference spectrum when the dye concentration is raised if, in fact, the dye undergoes a potential dependent partition between the membrane and 100 mM KCl. The negative shoulder in the BLM absorption spectrum at 600 nm can be explained by assuming that at high dye concentrations a fraction of the dye that moves from the membrane to the 100 mM KCl was originally in the form of dimers within the membrane. It is reasonable to expect that at higher dye concentrations, dimers begin to appear on the membrane. And, if membrane-associated dimers absorb 17 nm to the red of the dimer absorption peak in water, exhibiting the same size shift as the dye monomers, a peak should appear in the region 590–600 nm. Although we have not been able to isolate the absorption spectrum of membrane-associated diS-C_2-(5) dimers, unpublished experiments with merocyanine 540, where a membrane associated dimer has been observed, suggest that the analysis above may be valid.

As a result of the analysis given above we can say that the shape and concentration dependence of the BLM spectra for diS-C_2-(5) are consistent with an ON-OFF mechanism. However, as far as we know, the BLM spectra in Fig. 8 may be also consistent with a membrane localized shift in dye aggregation. The latter mechanism is difficult to evaluate because we do not know the exact shapes of the spectra of diS-C_2-(5) when the dye is in different states of aggregation in a hydrocar-

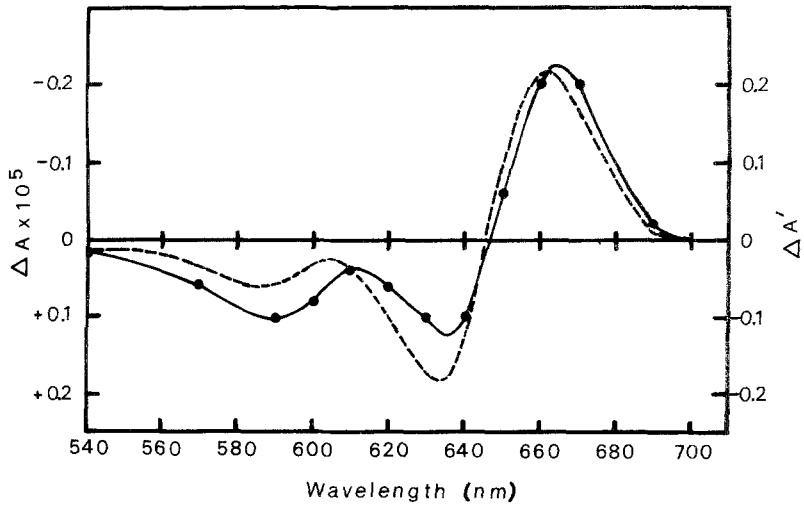


Fig. 12. Solid spectrum: BLM absorption difference spectrum (ΔA) for 1.5 μM diI-C_2-(5) with 100 mM KCl as the electrolyte. Dotted spectrum: absorption difference spectrum ($\Delta A'$) for 25 μM diI-C_2-(5) in both the sample and reference cells of a Cary spectrophotometer and with phosphatidyl choline vesicles (110 μM PC) in the sample cell

bon environment. It is not unreasonable, however, to expect that the spectra of diS-C_2-(5) aggregates in hydrocarbon solvents are similar to the spectra of dye in the same state of aggregation in water, except shifted to the red about 17 nm. If we made this assumption, the BLM spectra of diS-C_2-(5) at low concentrations might result from a shift of membrane bound dye in a monomer state to a state of aggregation greater than dimer. At higher diS-C_2-(5) concentrations the BLM spectra might result from a shift of membrane bound monomers and dimers to higher order aggregates on the membrane.

In order to try to distinguish between the ON-OFF mechanism and the membrane localized aggregation-disaggregation mechanism for the cyanine dyes we have obtained BLM difference spectra for an indodicarbocyanine dye, diI-C_2-(5). This dye is not as sensitive to membrane potential changes as diS-C_2-(5) but it has remarkably little tendency to form dimers and higher order aggregates. This feature may result from the presence of the two methyl groups at the 3 position of diI-C_2-(5) that sterically inhibit stacking interactions between the dye molecules. Even at concentrations as high as 40 μM (30× the concentration used to obtain the BLM difference spectrum in Fig. 12) in 100 mM KCl the dye does not show the blue shifted dimer band that is characteristic of other cyanine dyes (West & Pearce, 1965). Nor do we see any evidence

of aggregation of this dye on PC vesicles with 40 μM dye in 100 mM KCl. Since diI-C_2-(5) has the same spectral line shape and solvent senstivity as diS-C_2-(5), if there is indeed a potential-dependent ON-OFF shift of diI-C_2-(5) involving only dye monomers, a BLM difference spectrum with the characteristic shape shown in Fig.11*a* should be seen.

The solid spectrum in Fig. 12 is the BLM difference spectrum of 1.5 μM diI-C_2-(5). This difference spectrum is remarkably constant for diI-C_2-(5) concentrations ranging between 0.5 and 3 μM. The shape of the BLM difference spectrum for the transfer of diI-C_2-(5) from 100 mM KCl to PC vesicles (this spectrum is also identical to the difference spectrum for transfer of this dye from 100 mM KCl to GMO-decane). Although the BLM difference spectrum and the model system difference spectrum are not superimposable at shorter wavelengths, the overall shapes of the spectra and the positions of the maximum, the minima, and the crossover point are similar[5]. The fact that the amplitudes of the peaks in the blue region of the difference spectra are not superimposable may be due to preferencial orientation of a fraction of the dye bound to the BLM. We should be able to test the latter hypothesis by using spherical bilayer membranes rather than planar bilayers. Thus the results with diI-C_2-(5) are consistent with an ON-OFF mechanism. It is possible that the absorbance changes of diS-C_2-(5), and perhaps other cyanine dyes, have the same mechanism. However, since diS-C_2-(5) has a greater tendency to aggregate compared with diI-C_2-(5) it is also possible that a membrane localized change in dye aggregation may contribute to the absorbance signal of the thiadicarbocyanine.

The BLM difference spectra do not exclude the possibility that the cyanine dyes may shift between two environments of different polarizability yet remain within the membrane. For example, dye might move during potential changes between a hydrocarbon environment deep in the membrane and polar head group region. However, it must be remembered that the cyanine dye molecules are large (10–15 Å along the length of the chromophore) compared with the 40 Å thick membrane. Thus *small* shifts in position may not appreciably alter the environment of the dye.

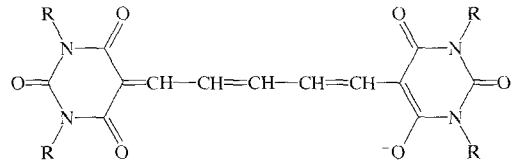
The fact that the magnitude of the absorbance signal strongly depends

5 The slit width of Cary 14 spectrophotometer cannot be opened wider than 3 nm whereas 10 nm band interference filters were used to obtain the BLM difference spectrum. PC model membrane difference absorption spectra as per Fig. 12 have also been obtained using the interference filters. The shape of the latter spectrum even more closely resembles the BLM difference spectrum.

on dye concentration polarization makes a mechanism based only on an electrochromic shift (Chance & Baltcheffsky, 1975) unlikely. Furthermore, since the dipole moments of the cyanine dyes are small and the dyes described in the following section have no dipole moments, a mechanism explaining the absorbance change in terms of a potential-dependent dipole rotation (Conti, 1975) is also unlikely.

Results with Oxonol Dyes

The absorption changes of the negatively charged oxonol dyes of the series diBA-C_n-(5), n=2–6, are similar to the changes observed with the cyanines.



R = $-(CH_2)_{n-1}-CH_3$

Changes are only observed when the average membrane potential is greater than zero during the pulse train. The signal requires time to develop after the pulse train has been applied, although $\tau_R \leqq 10$ μsec for an individual step. For the oxonol dyes τ_S also varies inversely with the membrane current density during the experiment. However, in accordance with the prediction of Szabo (1976) the permeance of the negatively charged oxonol dye increases as the membrane cholesterol content increases. The difference absorption spectra of the oxonol dyes in the GMO bilayers are similar to those for diI-C_2-(5) at low concentrations. Although the latter dyes were not studied in as much detail as the cyanine series diS-C_n-(5), it is probable that they respond optically by essentially the same mechanism.

Discussion

We have studied potential-dependent changes in the intensity of light passing through GMO bilayer membranes in the presence of cyanine dyes with the structure diS-C_n-(5). In all the experiments the bulk dye concentration, typically 1–5 μM, was the same on both sides of the membrane. Trains of square potential steps (0.1, 1.0 or 10 msec in length)

produced an optical signal that depended on the average potential $[(V_{max}+V_{min})/2]$ of the pulses as well as upon the length of time the pulse train had been on. Trains of potential changes that move between 0 mV and positive potentials or between 0 mV and negative potentials in both cases produced an increase in light absorption at shorter wavelengths and a decrease in light absorption at longer wavelengths. Trains of symmetrical potential steps (average potential = 0 mV) produced no absorbance change. The dependence of the magnitude of the absorbance signal on average membrane potential, as illustrated in Fig. 3, is of practical importance to physiologists considering using permeant cyanine dyes to study single rapid potential changes across cell membranes. The ΔA per mV potential change will be largest for cells with the largest resting potentials (Fig. 3*a*). However, with a constant resting potential (analogous to Fig. 3*c*) the absorbance change is linear with the size of the voltage change. This observation has been noted numerous times for cyanine, merocyanine, and oxonol dyes in studies with the squid axon (Ross *et al.*, 1976).

The time constant, τ_R, of the optical signal during any *single* potential step in the train was less than 10 μsec for all of the dyes studied in membranes with and without cholesterol (similar to the time constant of the fast phase of optical changes observed by Cohen *et al.*, 1974, using similar dyes with the squid axon). However, the envelope covering the maxima of the individual optical responses rose relatively slowly after the pulse train was initiated. The time constant, τ_S, for the development of the optical signal envelope depended on which dye was used and on the mole fraction of cholesterol in the membrane.

Membrane currents (positively charged dye is carrying the bulk of the membrane current) were measured during the optical experiments. The time constants for relaxation of membrane currents at the onset and immediately following the pulse train were similar to the τ_S values for the appearance and decay, respectively, of the absorbance signal. These data, together with observations of transient membrane currents that occur during single potential steps, are consistent with the notion that dye concentration polarization is occurring in the region of the two membrane interfaces during a train of potential steps. *Thus, the appearance and disappearance of the absorbance signal depends on the buildup and decay of a dye concentration asymmetry across the membrane.* The two phenomena have the same time courses and their rate depends on the permeance of the dye. More permeant dyes give higher membrane currents, faster current transients, and optical signals that appear most

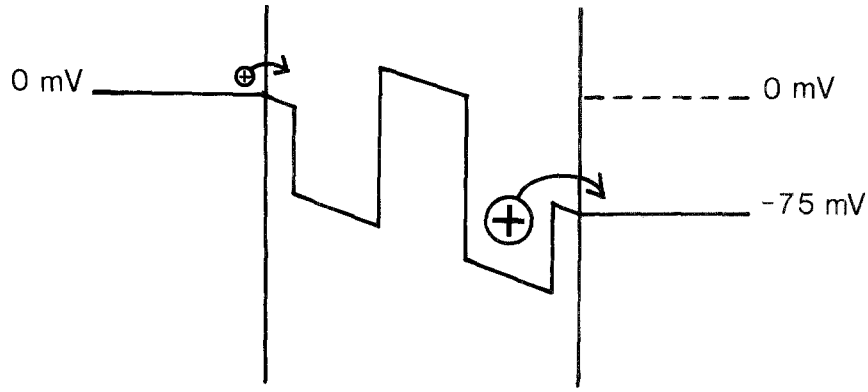


Fig. 13. Diagram illustrating the movement of a small amount of dye into binding sites on the left side of the membrane and movement of a larger amount of dye from binding sites on the right side of the membrane into the aqueous interface near the membrane when the membrane potential moves from 0 to −75 mV (and after the steady state dye concentration asymmetry illustrated in Fig. 6 has been reached). Note that the positively charged dye senses, and moves down the electrical potential gradient within the membrane. The relative depths of the electrochemical potential wells and the height of the energy barrier in the center of the membrane are hypothetical

rapidly. Again it should be emphasized that we are describing the sequence of events that result during a pulse train when dye is initially at equal concentrations on both sides of the membrane. The development of a dye concentration asymmetry is schematically illustrated in Fig. 6.

We now turn our attention toward the mechanism by which the absorption signal changes in response to a rapid change in membrane potential. Although the mechanism we will discuss is based primarily on experiments with the diS-C_n-(5) and diI-C_2-(5) dyes, results with other cyanine dyes and oxonol dyes indicate that the same mechanism may explain their optical changes as well.

Once a large dye concentration asymmetry is present it is necessary to consider only the "high dye concentration" side of the membrane in order to understand how the rapid optical response originates during each potential step. Small absorbance changes on the "low concentration" side of the membrane should simply subtract from the larger optical signals that result from events at the "high dye concentration" side of the membrane. The BLM absorption difference spectra obtained at low concentrations of diS-C_2-(5) are consistent with the "ON-OFF" model depicted in Fig. 13. When the membrane potential moves from 0 mV to a negative potential, say −75 mV, dye moves from binding sites on the membrane into the adjacent unstirred aqueous region on the "high concentration" side of the membrane. When the bulk dye

concentration is near 3 μM, diS-C_2-(5) that is ejected from the membrane predominantly forms dimers in the water. Since dye in water has an absorption maximum 17 nm to the blue (or even further to the blue if dimers, trimers, etc., are formed) of the absorption maximum of dye bound to membrane, the absorption increases at lower wavelengths when the membrane potential moves from 0 to −75 mV, while absorption at longer wavelengths decreases. A mechanism involving changes in aggregation of diS-C_2-(5) may also be consistent with the absorption data. In this mechanism, originally proposed by Ross *et al.*, 1974, the dye undergoes a potential dependent aggregation (at high membrane potentials)−diaggregation (when the potential moves toward 0 mV) *on* the membrane. The absorbance change, according to this mechanism, results from the difference in absorbance of dye monomer *vs.* dye aggregate. For another cyanine dye, diI-C_2-(5), the potential dependent absorbance change can be explained by an ON-OFF mechanism alone. Mechanisms involving potential dependent changes in the polarizability of the environment of membrane-localized dye cannot be excluded, but seem less likely. It is conceivable that some cyanine dyes may have complicated mechanisms involving both of the mechanisms described in this paper, or perhaps other mechanisms not discussed.

An important feature of the ON-OFF mechanism is that the membrane-associated dye must reside deep enough in the bilayer to sense a significant fraction of any transmembrane potential changes that occur. It is generally assumed (Läuger & Neumcke, 1973) that the electrical potential drop occurs predominantly across the hydrocarbon portion of the bilayer. Thus, the conclusion from the BLM experiments that membrane bound dye resides in a hydrocarbon environment is exactly what one might expect for a potential sensitive dye. The observation that the optical response is rapid ($\tau_R < 10$ μsec) suggests that the energy barrier between the hydrocarbon binding site and the aqueous solution near the interface may be small.

Assuming the ON-OFF mechanism to be correct for all the cyanine dyes studied in this paper, it is of interest to try to estimate the number of dye molecules that move between the membrane and the water during one pulse of the train of 75 mV, one msec pulses. A modification of Beers law, $A = \varepsilon Cl$, can be used for this purpose. We want to determine the quantity of dye, Δn (moles/cm^2), that moves in 10 μsec from an environment on the membrane, where the dye has an extinction coefficient, ε_m, to a new environment in the water near the membrane, where the extinction coefficient is ε_w. The pathlength, l, of the miniature absorp-

tion cell composed of the membrane and adjacent aqueous regions remains constant. If ε is expressed in liters/mole cm, then the absorbance change is given by $\Delta A = 1000\ (\varepsilon_m - \varepsilon_w)\ \Delta n$. The absorbance change observed (Fig. 8*a*) for diS-C_2-(5) at 670 nm is 10^{-5}. The value of $(\varepsilon_m - \varepsilon_w)$ obtained for from Fig. 11*b* at 670 nm is 2×10^5 (the difference in extinction at 670 nm between membrane-associated monomer and membrane-associated aggregate would be similar to this value). Substitution into the equation above gives a value for Δn of 5×10^{-14} moles/cm^2, or, one dye molecule per 3×10^5 Å^2. A similar calculation for diS-C_5-(5) gives $\Delta n = 2 \times 10^{-14}$ moles/cm^2.

If the dye moves to a new environment during the first 10 μsec of a potential pulse, one might expect to see the movement of the dye as an additional contribution to the capacitative transients that are observed with bilayer membranes in the absence of dye, which also have a time constant of 10 μsec. The capacitative charge movements we see for GMO membranes without dye are typically 4×10^{-8} coulombs/cm^2 for 75 mV pulses. Since the magnitude of the dye movement, Δn, predicted from the absorption change is 10 times smaller (5×10^{-14} moles/cm^2 $\times 9.6 \times 10^4$ coulombs/mole $= 5 \times 10^{-9}$ coulombs/cm^2) than this value, it is likely that the contribution of dye movement to the total capacitative transient will be hard to detect. In fact in our experiments we see no apparent difference between the shapes of the capacitative transients for membranes with and without dye present.

If the number of dye molecules bound to the membrane per unit surface area were known, it would be possible to estimate the fraction of the bound dye molecules that shift environments during the absorbance change. Highly permeant charged molecules, "permions" (Szabo, 1976), that partition largely into the membrane move for the most part only between the two membrane interfaces during potential changes. In such cases, integration of the current density during a single potential step can provide an estimate of the quantity of permion molecules adsorbed to the membrane interfaces. With these highly permeant molecules it is a common observation that the area under the recovery current transient is nearly identical to the area under the current transient observed at the onset of the potential step, as would be expected if the permion is localized at the membrane. Tetraphenylborate (TBP) is an example of such a molecule (Ketterer *et al.*, 1971; Andersen & Fuchs, 1975; Szabo, 1976).

Unfortunately the movement of the cyanine dyes during potential steps may not be limited to transfer between the membrane interfaces,

so it is not as easy to estimate the surface density of dye. However, if it is assumed that Q_{rec} provides a reasonable estimate of the quantity of dye moving from binding sites on the high-dye-concentration-side of the membrane back to the dye-depleted binding sites on the other side of the membrane after completion of a potential step, we can make an order of magnitude estimate of the surface density of dye on the membrane. Assuming that half the dye on one side of the membrane shifts to the other side during a 60 mV potential step, the density of dye on one side is approximately $2Q_{rec}$ in the absence of a potential difference. With the Q_{rec} values in Table 3 this calculation gives surface densities of 3×10^{-12}, 9×10^{-12}, 11×10^{-12} and 16×10^{-12} moles/cm^2 for diS-C_2-(5), diS-C_3-(5), diS-C_4-(5) and diS-C_5-(5), respectively[6]. Thus the surface density essentially doubles for each pair of -CH_2-groups added to diS-C_n-(5). This calculation places the diS-C_2-(5) molecules approximately 100 Å apart. At higher dye concentrations it would not be surprising to find spectroscopic evidence for the formation of dimers on the membrane.

During a train of 75 mV potential steps the surface concentration of dye, C_-^m, on the negative side of the membrane will be approximately twice the value at 0 mV, or, $\sim 4Q_{rec}$. By recalling our estimate for the number of dye molecules that change environments during a single potential step, Δn, we can calculate the fraction of dye molecules on the surface that are affected by the 75 mV potential change. For diS-C_2-(5) and diS-C_5-(5), respectively, $\Delta n/C_-^m$ are 0.02 and 0.001. Thus, it appears that the affinity of the dye for the membrane plays a major part in determining the size of the absorbance signal and dyes that bind to the membrane in higher surface concentrations may be two tightly bound to yield large optical signals. A similar dependence on signal size with dye hydrophobicity has been observed with the derivatives of merocyanine 540 (Cohen *et al.*, 1974). The dependence of the optical signal size on dye partition coefficient is being investigated (A. Grinvald, *unpublished*).

Finally we return our attention to the relative permeances of the dyes studied in this paper. It is not surprising to find that the more hydrophobic cyanine dyes are more permeant. Presumably these dyes with their greater hydrocarbon content are more soluble in the hydrocarbon region of the membrane. However, it is interesting that we have

6 Preliminary unpublished experiments of P. Howard and A. Waggoner yield approximate surface densities for similar dyes, diI-C_2-(5), diI-C_3-(5), and diI-C_4-(5), on phosphatidyl choline vesicles of 4×10^{-12}, 7×10^{-12}, and 20×10^{-12} moles/cm^2, respectively, when the dye concentration in the aqueous phase (100 mM KCl) is 1.5 μM.

found none of the dyes studied to be soluble in pure hydrocarbon solvents like hexane, decane or benzene. But, inclusion of GMO in the hydrocarbon solvent increases dye solubility to a startling extent. That these positively charged dyes are permeant at all probably depends on the fact that their charges are delocalized over much of the dye structure, a length of about 10–15 Å. Negatively charged oxonol dyes of the class diBA-C_n-(5) produce membrane currents similar in magnitude to those found with cyanine dyes. As with the cyanine dyes, the charge on the oxonol dyes is distributed over the chromophore structure. On the other hand, dyes with localized charge groups (negative sulfonate groups) such as merocyanine 540, 1-anilinonaphthalene sulfonate (ANS), 2-*p*-toluidinyl-6-naphthalene sulfonate (TNS), and merocyanine 375 (Ross *et al.*, 1976) show steady state and transient currents under similar conditions at least 20–100× smaller than the smallest current we have observed with the diS-C_2-(5), the least permeant cyanine dye studied in this paper. Nevertheless, the merocyanine dyes give large, rapid absorption signals (Ross *et al.*, 1976). Presumably the molecular events responsible for the optical signals of the latter dyes are restricted to one side of the membrane. In fact, signals from the merocyanine dyes added to the inside or outside of squid axons have opposite signs. Also, merocyanine 540 added to both sides of a BLM gives no absorbance signal, yet a significant signal is produced when this dye is added to only one side.

Our observation that cyanine dye permeance decreases while oxonol dye permeance increases when cholesterol is added to the membrane has also been observed by Szabo (*personal communication*). He explains this behavior on the basis of changes in the membrane dipolar potential with cholesterol addition. Cholesterol apparently increases the electrical potential within the bilayer.

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