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Optical Probes of Membrane Potential

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Summary. There are two basically different mechanisms for the fluorescence and absorption changes of merocyanine, cyanine and oxonol dyes. The permeant dyes (cyanine and oxonol dyes, with delocalized charges) work by a potential-dependent accumulation mechanism. These dyes show large (up to 80%) fluorescence and absorption changes with suspensions of cells, and the changes are complete in seconds. The impermeant dyes (merocyanine dyes, with localized charges) and the permeant dyes also show optical changes that take place in fractions of milliseconds. The rapid optical changes are relatively small ($\leqq 5 \times 10^{-3}$) but can often be easily detected in experiments with single cells. The rapid, nonaccumulative, optical changes result from membrane-localized dye movements. Cyanine dye-absorption changes occur because of a potential-dependent partition of dye between the membrane and the adjacent aqueous region at the high dye-concentration side of the membrane. Dimers and larger aggregates are formed in the aqueous region during the change. Merocyanine dyes may also work by the same mechanism.

DiS-C_3-(5) is presently the best dye for measuring membrane potentials of cells, organelles, and vesicles in suspension, but several other cyanines work nearly as well (P.J. Sims, A.S. Waggoner, C.-H. Wang, J.F. Hoffman, *Biochemistry* **13**:3315, 1974). For each system, the ratio of dye to membrane must be varied until the optimum fluorescence change is found. A separate calibration curve must be obtained for each system. For measuring fluorescence and/or absorption changes in single cells, merocyanine 540 and diBA-C_4-(5) work well but produce some photodynamic damage with high intensity illumination. A rhodanine merocyanine (WW-375) gives very large absorption changes and does not damage tissue during strong illumination. As the mechanisms of the optical changes are worked out, it should be possible to design and synthesize more sensitive, less toxic dyes that are easier to calibrate. And, as the mechanisms of the optical changes are worked out, these dyes may be useful for studying the structure and dynamics of excitable membranes.

In the past few years it has become possible to measure changes in the electrical difference across membranes of cells, organelles, and vesicles that are too small to permit the use of microelectrodes. One new technique involves the use of certain dye molecules whose absorption and/or fluorescence is sensitive to membrane potential[1]. These "optical probes" have been used to detect membrane-potential changes in single nerve and muscle fibers and in suspensions of cells, organelles, and

1 A more comprehensive review of this new area of research will be published by B.M. Salzberg and L.B. Cohen in *Rev. Physiol. Biochem. Pharm.*

vesicles. This review will center mainly on three classes of dyes: merocyanines, cyanines, and oxonols (shown in Fig. 1). These dyes are of most practical value at this time simply because their optical responses to potential changes are much larger than other dyes that have been studied, such as the naphthylamine sulfonates. Experiments with the naphthylamine dyes have been reviewed by Conti (1975).

Detection of Single Cell Potential Changes

In experiments with single nerve or muscle fibers, an intense beam of monochromatic light is focused on a cell previously stained by bathing in a medium containing the dye (Fig. 2). Either a change (ΔF) in cell fluorescence (F), or a change (ΔI) in light transmitted (I) through the cell can be measured in this apparatus, depending on placement of photodetectors. An example

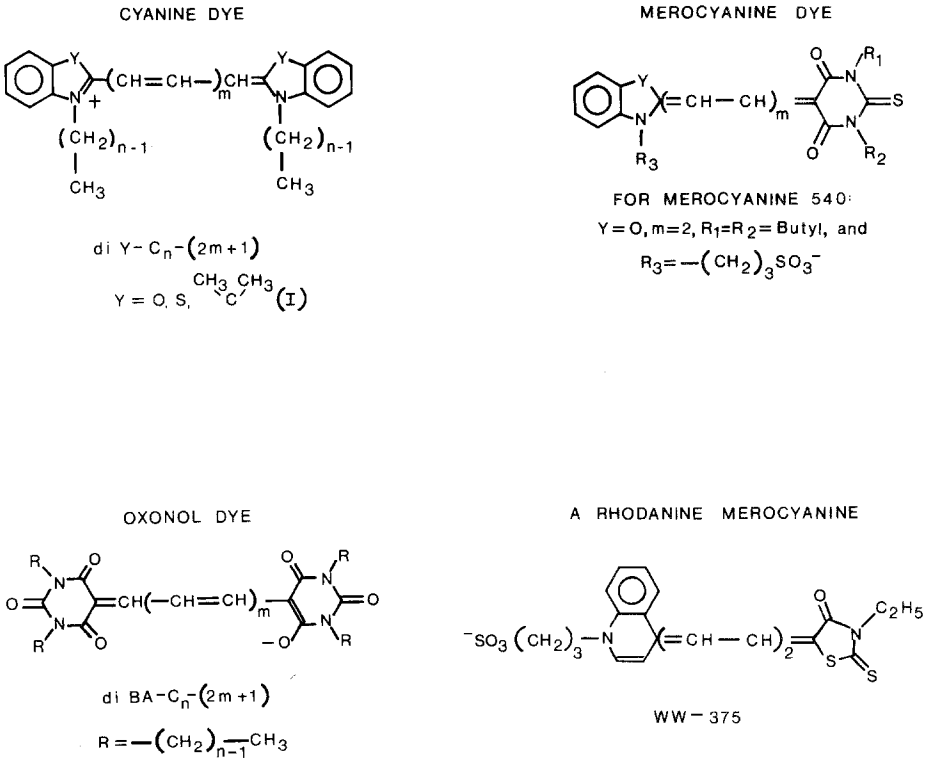

Fig. 1. The molecular structures of selected merocyanine, cyanine, and oxonol dyes that are discussed

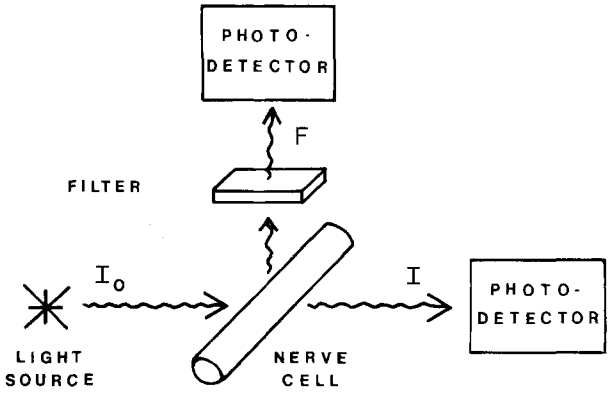

Fig. 2. Schematic diagram of basic apparatus for measuring absorption and for measuring absorption and fluorescence changes during stimulation of a nerve cell. I_o is the incident light intensity, I is the transmitted light and F is the intensity of dye fluorescence from the cell

of the fluorescence changes that can be observed with a squid giant axon is shown in Fig. 3. Notice that the fluorescence signal from the electrically stimulated axon stained with merocyanine 540 (Fig. 1) is superimposable on the voltage trace obtained with microelectrodes. Thus, the fluorescence of merocyanine 540 responds rapidly and accurately to changes in nerve membrane potential (the time constant τ for the optical signal of merocyanine 540 to reach 63% of the maximum change during a square potential step is 33 μsec in the squid axon at 13 °C, and the magnitude of the fluorescence change is linear with the size of the potential step over a range of 200 mV (Cohen, Salzberg, Davila, Ross, Landowne, Waggoner & Wang, 1974).

Although certain dyes show large fluorescence changes in nerve, other dyes respond with large changes in absorption. The quantity $-\Delta I/I$ has been termed the absor*ption* change by Ross, Salzberg, Cohen and Davila (1975) and is related to the absorbance change, ΔA, by $\Delta A = -\Delta I/2.3\,I$, when $\Delta I/I$ is small.[2] Although the fractional changes, $\Delta I/I$ and $\Delta F/F$, have always been small ($< 10^{-3}$) for single fibers, the best indicator of the ease with which an optical signal from a single cell can be detected is the signal-to-noise ratio, S/N. With a high-intensity, low-noise light source, a low-noise photodetector, and vibration control, optical signals resulting from a *single spike* in a squid axon can be detected easily. For example, S/N for the fluorescence change of merocyanine 540 is

2 Waggoner, A.S., Wang, C.-H., Tolles, R.L. 1976. The mechanism of rapid light absorption changes of cyanine dyes in black lipid membranes. (*in preparation*)

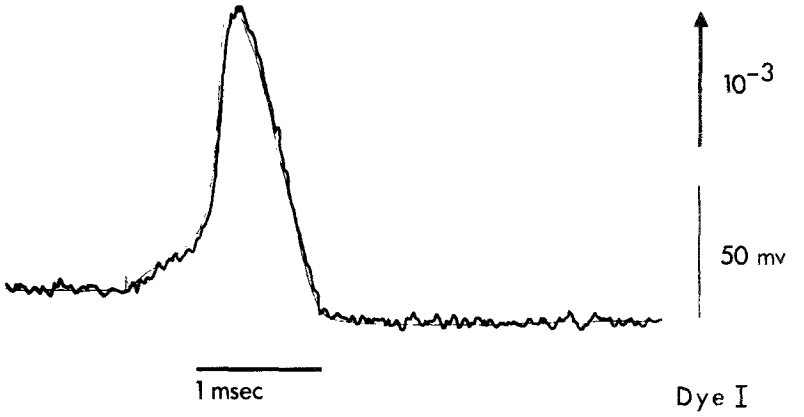

Fig. 3. Fluorescence change of merocyanine 540 during squid giant axon action potentials. The heavy trace of the record of fluorescence intensity and the light trace is the membrane potential, recorded simultaneously. The vertical arrow indicates the direction of increasing fluorescence and the size of the arrow indicates the magnitude of the fluorescence change divided by the resting fluorescence intensity. $\Delta F/F$ (from Cohen *et al.*, 1974)

10:1. The absorption change of this dye is about 10× smaller than the fluorescence change. However, the signal-to-noise ratio for the absorption change was 20× larger than that found for the fluorescence measurements on the same apparatus with the same incident light intensity (Ross *et al.*, 1975). This surprising result occurs because the intensity of light reaching the photodetector in the absorption experiment is several orders of magnitude larger than that in the fluorescence experiments. A recently discovered rhodanine merocyanine dye, WW-375 (*see* Fig. 1), yields a S/N of better than 50:1 for the absorption change during a single nerve impulse in the squid axon (Salzberg, Cohen, Ross, Waggoner & Wang, 1976).

Detection of Potential Changes of Cells in Suspension

In a collaborative effort by the laboratories of Cohen, Hoffman and Waggoner, a series of cyanine dyes were developed which turned out to be remarkably sensitive to membrane potentials of cells in suspension. The optical changes from these dyes are large enough so that the experiments can be carried out with unsophisticated fluorometers and spectrometers. For example, when erythrocytes are hyperpolarized by addition of valinomycin to the cells suspended in a potassium-free medium, the fluorescence of the cyanine dye diS-C_3-(5) (*see* Fig. 1 for structure)

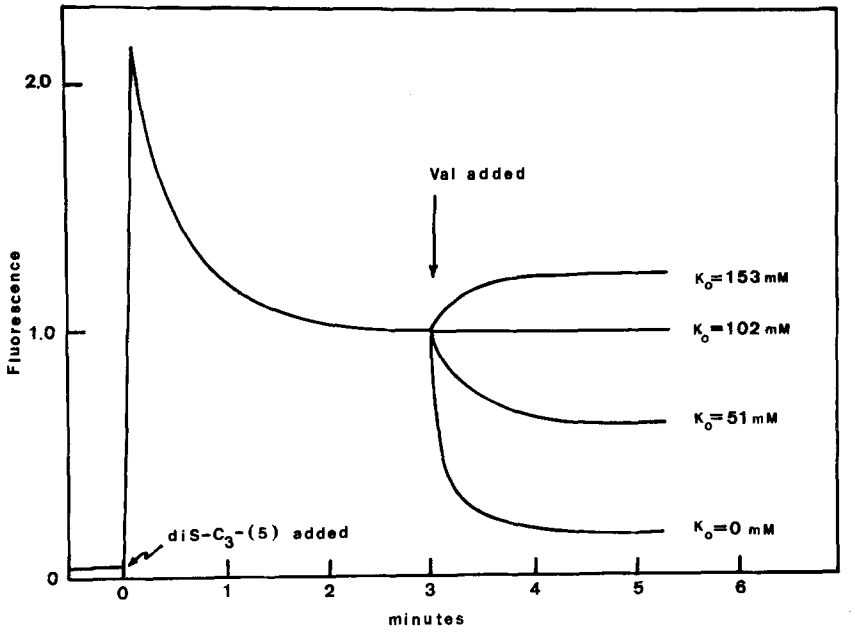

Fig. 4. Characteristic changes in fluorescence intensity with time of diS-C_3-(5) in a 0.17% suspension of normal human red cells in NaCl-Tris medium (pH 7.4) and in mixtures where KCl-Tris was substituted for NaCl-Tris to give the [K_o] values listed in the Figure. The cellular potassium concentration, [K_c]=152 mmoles/liter of cell H_2O. Dye and valinomycin were added where indicated. Fluorescence was recorded at 670 nm with excitation at 622 nm (from Sims *et al.*, 1974)

decreases by more than 80% (Fig. 4). The size of the fluorescence change after valinomycin addition varies nearly linearly with the log of the external potassium concentration over a 10-fold concentration range. Significant cyanine dye absorption changes can also be observed under the same conditions in which the fluorescence experiments were carried out (Sims & Chance, *unpublished*).

It is worth noticing that these large, cyanine-dye optical responses from cells in suspension occur in seconds, whereas some components of the optical responses in nerve are complete in tens of microseconds. Thus, there appear to be two components to optical changes with cyanine dyes. This matter will be discussed further later.

DiS-C_3(5) and related cyanine dyes have been used to estimate membrane potentials in suspensions of red blood cells, mitochondria, synaptosomes, bacterial cells, purple membrane-containing vesicles, and ascites cells. Each of these studies will be reviewed later. First I will introduce the dyes that may prove to be most practical for use by physiologists

and biochemists and then discuss their properties and the propable mechanisms by which they respond optically to changes in membrane potential.

Dye Properties

Cyanine, merocyanine and oxonol dyes were developed mainly for use as sensitizers by the photographic industry (Hamer, 1964). They have large extinction coefficients, typically $1–2 \times 10^5$, and relatively narrow absorption and emission peaks. Some have quantum efficiencies as high as 0.8. When cyanine and oxonol dyes are transferred from water to nonpolar solvents like octanol, their absorption and emission maxima do not usually shift by more than 20 nm, and the extinction coefficients and quantum yields seldom change by more than 50%, provided that the dye remains in a monomeric form (West & Geddes, 1964; Sims *et al.*, 1974). On the other hand, the spectral properties of merocyanines are much more sensitive to solvent polarity. For example, the fluorescence of merocyanine 540 decreases by a factor of 20 when the dye is transferred from ethanol to water (Cohen *et al.*, 1974). The optical properties of all three classes of dyes depend strongly on the state of dye aggregation. Dye aggregates are frequently not fluorescent and absorb at different wavelengths (West & Pearce, 1965; Sims *et al.*, 1974).

Another property distinguishes the merocyanine dyes from the cyanine and oxonol dyes. The chromophore structure of the merocyanine dyes is uncharged. To synthesize charged merocyanine dyes, charged functional groups, such as sulfonate or quaternary ammonium groups, must be covalently attached to the chromophore. The charge on these dyes remains localized on the attached functional group. On the other hand, the positive charge of the cyanine dyes and the negative charge of the oxonol dyes is delocalized over the entire chromophore structure. The property of charge localization *vs.* delocalization has important implications regarding the membrane permeance of the dyes and the mechanisms of their potential-dependent optical changes. For example, the relative conductances of glycerol monoolein bilayer membranes in the presence of merocyanine, cyanine and oxonol dyes is shown in Table 1. 8-anilino naphthalene-sulfonate (ANS) is included for comparison. Where the conductance values are large, the dye is the major membrane current carrier (Waggoner *et al.*, 1976). The permeance of the fastest cyanine and oxonol dyes (with delocalized charges) is 10^2 times the permeance

Table 1. Relative conductance values for glyceryl monoolein bilayer membranes in the presence of selected dyes (1–1.5 μM)[a]

Dye	Salt (0.08 M)	Relative conductance
ANS	$CaCl_2$	0.05
WW 375	$CaCl_2$	0.03
Merocyanine 540	$CaCl_2$	0.08
diBA-C_2-(5)	$CaCl_2$	0.13
diBA-C_4-(5)	$CaCl_2$	1.2
diS-C_2-(5)	Na_2SO_4	1.0
diS-C_3-(5)	Na_2SO_4	1.6
diS-C_4-(5)	Na_2SO_4	8.1
diS-C_5-(5)	Na_2SO_4	10.5
diS-C_8-(5)	Na_2SO_4	0.2

[a] Measurements were made with 75-mV potential steps. Membrane currents of the more permeant dyes may be limited by diffusion through unstirred aqueous layers. More complete data will appear in Waggoner *et al.* (*see* footnote 2)

of the merocyanines and ANS (with localized charges) and addition of a localized charge ($C_n{=}CH_2CH_2CH_2SO_3^-$ instead of $C_n{=}CH_2CH_3CH_3$) to a highly permeant cyanine reduces its permeance by 10^2.

There are also changes within homologous series of cyanine and oxonol dyes. As the length of the alkyl chains bound to the dye increases, the permeance increases until a maximum is reached. This trend can be explained by the increased solubility of the more hydrophobic dyes in the hydrocarbon region of the bilayer. It is more difficult to explain the decreased permeance observed when the chain length is greater than six carbons. Flip-flop of the longer chained dyes across the membrane may be slow because these dyes may be firmly "anchored" in the membrane by the long alkyl chains *or*, solutions of these dyes may contain predominantly dye aggregates or micelles which cannot cross the membrane.

The Mechanism of the "Slow" Optical Changes From Cells in Suspension

The mechanism of the large potential-dependent fluorescence changes exhibited by cyanine dyes in suspensions of red blood cells has been investigated by Sims *et al.* (1974). 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 dye molecules 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 the quenching of cell-associated dye is due to 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 cells. The cyanine dye behaves like a permeant cation, they suggest, and therefore is distributed between the inside and outside of the cell in accordance with the transmembrane potential.

The conductance values in Table 1 lend support to the notion that the cyanine dyes can rapidly pass through membranes and therefore behave as permeant cations. The conductance data also explain the dependence of the time constants for the red blood cell fluorescence changes on the hydrocarbon chain length of the different cyanine dyes. The time constants observed by Sims *et al.* (1974) for members $n=2$ to 6 of the diS-C_n-(5) series were 30, 8, 6, 4, and <2 sec, respectively. The conductance values of Table 1 suggest that the rate-limiting step for the red cell fluorescence change is the movement of dye across the membrane. Furthermore, the rate at which these same dyes penetrate into the interior of multiconcentric egg-lecithin liposomes and quench the fluorescence of a dye incorporated within the liposome parallels the trend observed in Table 1 and in the red blood cell experiments (Waggoner, Sirkin, Tolles & Wang, 1975).

One would predict that the oxonol dyes, which are also permeant, should also be distributed between the inside and outside of cells according to the membrane potential. Unfortunately it has been difficult to evaluate the usefulness of oxonols in cell suspensions because the dyes, which are negatively charged, form complexes with many ionophores.

In comparison with cyanine dye optical changes, only small changes in fluorescence and absorption have been observed with merocyanine dyes in *Neurospora* cell suspensions (Naparstek & Slayman, 1976) and submitochondrial particle suspensions (Chance, Baltscheffsky, Vanderkooi & Cheng, 1974). And, with red blood cells and phospholipid vesicles, under the same conditions where we see 80% changes in cyanine dye fluorescence, we have been unable to detect significant fluorescence changes for merocyanine 540. We think that the accumulation mechanism which gives large cyanine dye optical changes in cell suspensions does not work for the impermeant merocyanine dyes. Merocyanine optical

changes may be localized to the membrane (to be discussed more later) and therefore involve a smaller quantity of dye.

The Mechanism of "Rapid" Optical Responses from Single Membranes

In a search for sensitive optical probes of membrane potential, more than 600 dyes have been screened with the squid axon by Cohen and his colleagues. Dyes from all three classes (Merocyanine, cyanine and oxonol) have been found that show large, rapid ($\tau < 1$ msec) fluorescence and absorption changes with the squid axon. Three notable examples, one from each class, are merocyanine 540, diI-C_1-(5), and diBA-C_4-(5). Although we are uncertain of some details in the mechanism of the merocyanine dye optical change, we know how the cyanine, and probably oxonol, dyes respond to rapid potential changes.

Waggoner *et al.* (*see* footnote 2) have used glycerol monoolein black lipid membranes (BLM) to study the dependence of cyanine and oxonol dye absorption on membrane potential. When diS-C_2-(5) was at μM concentrations on both sides of the membrane a train of 1 msec square potential steps moving from ground to either positive *or* negative voltages produced a decrease in absorbance at 670 nm. The absorbance change was typically 10^{-5} for 75 mV steps and had a response time, $\tau < 10$ μsec. Symmetrical steps (centered at 0 mV), however, produced no absorbance change under these experimental conditions. The complicated dependence of the sign and magnitude of the absorption signal can be explained with the assumption that the dye concentration at the two membrane interfaces must be *unequal* in order for an optical signal to be observed, even if the bulk concentration on both sides is identical. Since the cyanine and oxonol dyes are relatively permeant, it would not be surprising to find a diffusion polarization[3] occurring in the unstirred layers at the two membrane interfaces when there is a net membrane potential. With symmetrical potential steps there is no net membrane potential across the membrane, and the average dye concentration at the two membrane interfaces remains the same.

If diffusion polarization does account for the dye concentration asymmetry at the membrane surfaces, and if the asymmetry is a requirement

3 Diffusion polarization results from diffusion-limited movement of dye through the unstirred (typically 10^{-3}–10^{-1} cm) layer adjacent to the membrane interfaces. When membrane current is limited by diffusion through unstirred layers, the current carrier is depleted at one interface and concentrated at the other.

for an absorption change, a prediction can be made. When a train of pulses is turned on, the absorption signal should increase towards a steady state with a rate parallel to the rate at which the diffusion polarization approaches a steady state. The time for the diffusion polarization to reach steady state is proportional to the membrane current density squared (Vetter, 1967). Therefore, the data of Table 1 predict that the rate of appearance of the optical signal should increase as the hydrocarbon chain length of diS-C_n-(5) increases. This trend is observed: the absorbance signal of diS-C_2-(5) nears its maximum value in 10 sec while the signal of diS-C_5-(5) appears in less than 0.8 sec.

Once a local dye concentration asymmetry is present, one need only look to the "high dye concentration" side of the membrane to understand the mechanism of the rapid absorption change that occurs during each individual potential step. Absorption difference spectra [$\Delta A(\lambda)_{75}$ mV − $\Delta A(\lambda)_0$ mV] in conjunction with solvent model studies indicate that the rapid absorbance change results from a potential-dependent movement of dye between the membrane and the aqueous region on the high dye concentration side of the membrane. At low bulk-dye concentrations, monomer dye in the membrane moves to form dimers in the aqueous region as the potential steps go positive or negative from 0 mV. The absorption of light passing through the membrane is altered during this process because the absorption spectrum of membrane-associated dye is markedly different from the absorption spectrum of dimers in water. The mechanism of the oxonol dye-absorption change in bilayer membranes appears to be similar although oxonol dyes were not studied as extensively as the cyanines.

The mechanism of the rapid cyanine dye absorption change (and perhaps fluorescence change) observed in the squid axon (Cohen *et al.*, 1974) is probably the same as that of the bilayer membrane. However, the −60 mV resting potential of an axon assures that the dye concentration asymmetry exists even before a potential change occurs across the nerve membrane. Thus, it is not surprising that a single action potential produces an immediate optical change with the squid axon.

An important implication of this mechanism is that the membrane-associated cyanine dye molecules must reside deep enough in the membrane to sense a substantial fraction of the membrane potential change.

The mechanism for rapid absorption and fluorescence changes of the merocyanines has not been clearly determined. The size of fluorescence signals of a series of merocyanine analogs depends on the hydrophobic nature of the dye, its charge, and the structure of the chromo-

Table 2. Effect of alkyl group substitution on the signal-to-noise ratio (S/N) of the merocyanine 540 fluorescence change in the squid axon

R_1[a]	R_2[a]	S/N
methyl	methyl	0.02
ethyl	ethyl	1.0
butyl	butyl	5.0
pentyl	pentyl	4.9
hexyl	hexyl	4.5
ethyl	octyl	4.7
octyl	octyl	0.7
ethyl	octadecyl	0.00

[a] R_1 and R_2 are the N-substituted alkyl groups on the merocyanine 540 structure shown in Fig. 1.

phore itself (Table 2; Cohen *et al.*, 1974). The variation of $\Delta F/F$ with hydrocarbon chain length of merocyanine 540 may indicate that there is an optimum partition coefficient for a maximum signal change. As has been the case for most of the 600 dyes screened, there is no optical change if the dye is not charged. In contrast to the permeant cyanine dyes, merocyanine 540 does not yield an optical signal when dye is at equal concentrations on both sides of the membrane. Absorption and fluorescence difference spectra in squid axon (Ross *et al.*, 1974) and BLM absorption-difference spectra (Waggoner, *unpublished results*) indicate that a potential-dependent monomer-dimer shift is involved in the optical change. Although the dye monomers appear to be associated with a hydrophobic region of the membrane, it is yet unclear whether the dimers formed during hyperpolarization are also membrane-associated or are instead formed in the aqueous region adjacent to the membrane. If the dimers are formed off the membrane, then the mechanism of the merocyanine optical change is essentially the same as that of the *ON-OFF* mechanism of the rapid, cyanine dye optical change discussed in the last section. Although dichroism and birefringence studies (Ross, Cohen, Salzberg, Kohn & Grinvald, 1976) on merocyanine 540 with nerve are not completely consistant with a simple *ON-OFF* mechanism, model solvent studies presently being conducted (Ross & Waggoner, *unpublished*) may help distinguish between these two possible mechanisms. Potential-dependent reorientation of the charged merocyanine monomers is unlikely to account for the entire optical changes of merocyanine 540. This mechanism has been suggested by Conti (1975) and his colleagues to explain fluorescence changes of naphthalene sulfonate analogs in nerve and bilayer membranes.

Uses of Cyanine Dyes to Determine Membrane Potentials of Cells, Organelles, and Vesicles in Suspension

The cyanine dyes, diS-C_3-(5), diO-C_5-(3) and diO-C_6-(3) have been used to estimate potentials in suspensions of red blood cells, synaptosomes, and purple membrane-containing vesicles. These dyes have also been useful for studying metabolism-related membrane potentials in bacterial cells, mitochondria, and ascites cells. One of the most critical problems in these studies lies in the generation of a calibration curve that relates fluorescence intensity to known membrane potentials. In the simplest systems, where the membrane is impermeable to all the ionic species present, the "known" membrane potentials for the calibration are calculated with the Nernst equation from the magnitude of potassium concentration gradients that are experimentally generated across the membrane in the presence of valinomycin. This approach has been used by a number of authors in experiments to be described below. The accuracy of the calibration curve depends on a careful assessment of both the internal potassium concentration during the experiment, and the permeabilities of ionic species present relative to the permeability of the potassium-valinomycin complex. If the ion permeabilities are significant, the constant field equation should provide a better estimate of the membrane potential (Hoffman & Laris, 1974). Other ionophore-ion combinations can be helpful in establishing calibration curves.

Hoffman and Laris (1974) measured changes in the fluorescence intensity of diO-C_6-(3), added to suspensions of human and *Amphiuma* red blood cells while they varied the membrane potential of cells. Cell potentials were altered in three different ways: by addition of valinomycin to alter the permeability ratio P_K/P_{Cl}, by a change in pH of the medium to alter the ratio of internal to external chloride (Cl_i/Cl_o), and by substitution of impermeant anions for Cl_o, again to alter the ratio, Cl_i/Cl_o. In each case hyperpolarization led to a decrease and depolarization to an increase in fluorescence intensity. The authors plotted the magnitude of the fluorescence change upon valinomycin addition against the log of the external potassium concentration. This graph is shown in Fig. 5. From values of cellular potassium and the corresponding external potassium concentrations for which there were no changes in fluorescence with valinomycin, estimations of membrane potentials were made. The potential was -5 to -8 mV for the human red cells and -19 mV for *Amphiuma*. These calculated values are in good agreement with the potential estimated from the Cl ratios (-9 mV for human and -17 to -20 mV for *Amphiuma*) and from those obtained by direct electrical

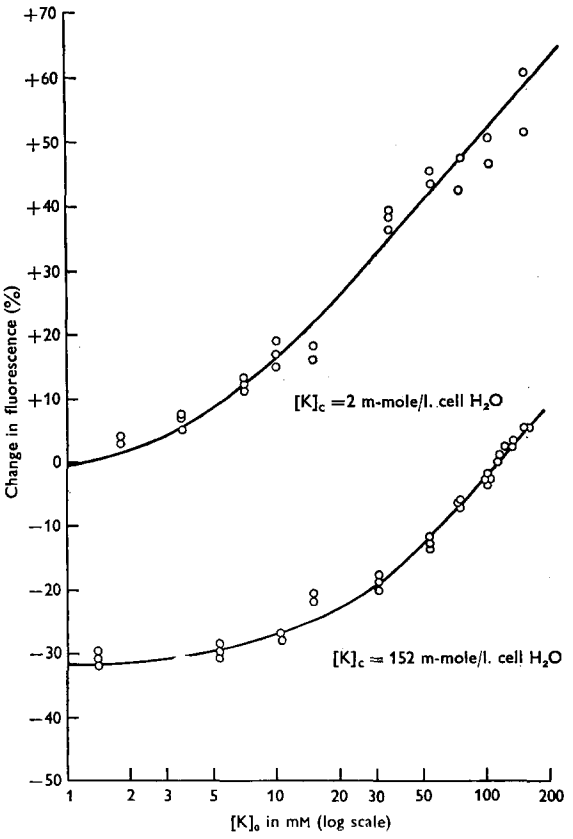

Fig. 5. The percent change in fluorescence of diO-C_6-(3), following addition of valinomycin to a suspension of red blood cells. The data are plotted as a function of the log of the potassium concentration in the medium, $[K_o]$. The cellular potassium concentration, $[K_c]$ is indicated for the two experiments (from Hoffman & Laris, 1974)

measurements (−15 mV for *Amphiuma*). While diO-C_6-(3) was found to be slightly hemolytic and to increase the inward rate constants for sodium and potassium for human red cells, diS-C_3-(5) was found to be considerably less toxic (Hoffman & Laris, 1974; Sims *et al.*, 1974).

Large decreases in the fluorescence intensity of diS-C_3-(5) were found in suspensions of hamster liver mitochondria upon the development of the energized state by the addition of succinate or ATP (Laris, Bahr & Chaffee, 1975)[4, 5]. The changes seen with the addition of succinate or ATP were comparable to those recorded upon the addition of valino-

4 We have observed (*unpublished*) that diS-C_3-(5) inhibits oxidation at Complex I of rat liver mitochondria.

5 However, Tedeschi (1974) observed only small changes with *Drosophila* mitochondria. But *see* Kinnally, K.W., Tedeschi, H. (1976) *Biophys. J.* **16**:18*a*.

mycin to mitochondria suspended in media containing low concentration of K. NaCN inhibited the succinate fluorescence change and Oligomycin inhibited the ATP-generated change. The change observed with succinate was partially reversed by the addition of either 2,4-dinitrophenol or ADP. Oligomycin prevented the reversal seen with ADP. With rat liver submitochondrial particles, however, increases in fluorescence intensity were seen upon the addition of succinate or ATP. These observations are consistent with the idea that a large negative (internal) potential develops across the inner membrane of the mitochondrion during energization, and with other aspects of the chemiosmotic hypothesis.

Laris and Pershadsingh (1974) estimated membrane potentials in *Streptococcus faecalis* using diO-C_6-(3). The fluorescence experiments gave a membrane potential of -60 to -70 mV for normal cells in the absence of glucose. Addition of glucose hyperpolarized the cells to a new potential of -130 to -140 mV. N,N′-dicyclohexylcarbodiimide, a membrane ATPase inhibitor, prevented the hyperpolarization seen upon addition of glucose. These results suggest that glucose provides energy for an ATPase which is capable of generating a large membrane potential.

Kashket and Wilson (1974) used diO-C_6-(3) to measure membrane potentials in *Streptococcus lactis* fermenting glucose or arginine. The membrane potentials obtained from a fluorescence calibration curve, together with measurements of the pH gradient across the cell membrane, were used to calculate the proton-motive force driving accumulation of thiomethyl-β-galactoside. Their results could be explained with the chemiosmotic view of coupling of metabolic energy to the active transport of nutrients *via* electrochemical proton gradients.

Before returning to uses of cyanine dyes, it should be mentioned that a number of other dyes have been used to probe energization phenomena in mitochondria and chloroplasts. Chance *et al.* (1974) have used merocyanine 540 and an oxonol (OX-V) to study absorbance changes upon energization of chloroplasts and mitochondria. ANS has frequently been used to study mitochondria but the mechanism of observed fluorescence changes is mostly obscure [*see* reviews by Radda & Vanderkooi (1972), Azzi (1975) and Waggoner (1976)]. Cyanine and other cationic dyes have been used by Colonna, Masari and Azzone (1973), Schuldiner and Kaback (1975), and others (*see* Azzi, 1975) to study mitochondrial energetics. Some of these dyes may be responding to transmembrane electrical potentials by the same mechanism as diS-C_3-(5). In most of these studies no attempt has been made to determine calibration curves

for the optical changes, so no estimates of membrane potential were made.

Brewer (1976) has shown that the bacterial protein colicin K, when added to suspensions of *Escherichia coli* containing diO-C_6-(3), causes a doubling in fluorescence of the probe. Glucose and oxygen cause a decreased fluorescence while anoxia and cyanide cause a rise in fluorescence. These results, together with the work of other laboratories, suggest that colicin K causes a partial depolarization of the transmembrane electrical potential. Other results presented by the author suggest that colicin K does not interfere with the component of membrane energization by electron transport. The partial depolarization of the membrane may account for the inhibition of active solute transport caused by colicin K.

The fluorescence intensity of the diS-C_3-(5) iodide was measured in suspensions of Ehrlich ascites tumor cells in order to monitor membrane potential in these cells (Laris, Pershadsingh & Johnstone, 1976). The authors found that the fluorescence was influenced by the way in which cells were handled. Estimations of membrane potential on cells equilibrated for 1 hr at room temperature or 37 °C ranged from -18 to -42 mV, whereas estimations with fresh nonequilibrated cells were of the order of -50 to -60 mV. Estimations of membrane potential on the basis of chloride distribution ($Cl^-_{cell}/Cl^-_{medium}$) in equilibrated cells ranged from -13 to -32 mV. When glucose was added to cells equilibrated at 37 °C for 30 min in the presence of rotenone, a decrease in fluorescence intensity was observed indicating hyperpolarization. Addition of ouabain, in turn, led to a 70 to 100% reversal of fluorescence intensity. It was suggested that hyperpolarization, therefore, is due probably to the electrogenic activity of the sodium pump. Further fluorescence experiments indicated that the addition of amino acids, known to require external Na for transport, results in cell depolarization. The fluorescence change (ΔF) observed with addition of amino acids saturates at higher concentrations of amino acids. Plots of $1/\Delta F$ *vs.* 1/[glycine] were linear with an apparent K_m of 2–3 mM. These apparent K_m values compare favorably to the K_m values derived from studies of amino acid transport in these cells. Laris *et al.* (1976) suggest that these data indicate that the Na-dependent transport of amino acids in these cells is electrogenic.

Illumination of bacteriorhodopsin-containing vesicles prepared from *H. halobium* cells causes translocation of protons from the vesicle interior into the medium. A pH gradient and an electrical potential result from this process. Renthal and Lanyi (1976) have used diO-C_5-(3) to

estimate the electrical potential of these "purple membrane" vesicles. Valinomycin-induced potassium diffusion potentials were used to calibrate the fluorescence changes observed upon illumination of the bacteriorhodopsin. By calculating the total proton motive force from the sum of the pH term and the potential term obtained from the fluorescence measurements, the authors found that the vesicles can produce proton motive forces near -200 mV, inside negative.

Blaustein and Goldring (1975) used diO-C_5-(3) to study membrane potentials of synaptosomes. They conclude that synaptosomes may retain resting membrane potentials and the ability to increase sodium permeability.

Uses of Dyes to Measure Potentials of Single Cells

Oetliker, Baylor and Chandler (1975) used merocyanine 540 and the indodicarbocyanine diI-C_1-(5) to follow different steps in muscle activation. They found that the fluorescence signal from the fiber stained with merocyanine 540 was early and transient. This information is consistent with the idea (proposed by Landowne, 1974) that merocyanine 540 is an indicator of surface and T-system membrane potential. Unlike the merocyanine fluorescence change, diI-C_1-(5) gave a fluorescence change that appeared with a 10-msec delay and continued to change for a period of 30 msec. This fluorescence change, which required no signal averaging, paralleled changes in optical retardation which are thought (Oetliker *et al.*, 1975, and references therein) to result from membrane or ion changes associated with the sarcoplasmic reticulum. Since diI-C_1-(5) may be capable of moving across membranes into the cell, it is certainly possible that this dye is monitoring membrane potential changes or ionic events within the muscle fiber. It is also reasonable that the impermeant merocyanine responds only to potential changes occurring in membranes that are accessible to the bathing solution.

Salama and Morad (1976) demonstrated that frog hearts stained with merocyanine 540 show a 1.5 to 2.0% increase in fluorescence during the cardiac action potential. Fluorometric action potentials, similar to those recorded with intracellular microelectrodes in pacemaker, atrial and ventricular tissues, were recorded by focusing the excitation beam on various regions of the heart. The signal-to-noise ratio obtained for a single action potential ranged between 10/1 to 40/1. The authors, who used a lower incident light intensity than Cohen *et al.* (1974), found

merocyanine 540 to be biologically inert in their heart preparation. However, both Cohen *et al.* (1974) and Oetliker *et al.* (1975) observed rapid damage to single nerve and muscle fibers stained with this dye under intense illumination. Only one-hundredth the damage was found with the rhodanine merocyanine (WW 375) in the squid axon (Salzberg *et al.*, 1976).

Salzberg *et al.* (1973) demonstrated that it is possible to detect single action potentials in individual sensory neurons of leech segmental ganglia. The relative intensity change from neurons 50 μm in diameter was approximately the same as that found with squid axon ($\Delta F/F \sim 10^{-3}$). The success of these initial experiments suggest that it will be possible to employ an array of photodetectors so that many neurons can be studied simultaneously. Such experiments would facilitate mapping of functional connections within a ganglion.

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