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# DiO-$C_3$-(5) and DiS-$C_3$-(5): Interactions with RBC, Ghosts and Phospholipid Vesicles

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**Summary.** The experiments presented below compare the interaction of diO-$C_3$-(5) and diS-$C_3$-(5) with erythrocytes, erythrocyte ghosts and phospholipid vesicles derived from erythrocyte membranes. The results confirm earlier reports of diS-$C_3$-(5) dimerization in the presence of hemoglobin and of dye aggregate formation in erythrocyte suspensions. DiO-$C_3$-(5), on the other hand, binds to vesicles and ghosts freed of hemoglobin in a potential-dependent manner but without forming dye aggregates. The two dyes bind to the different preparations in similar proportions, but diS-$C_3$-(5) is bound in amounts 3–40 times greater depending on the degree of polarization.

The results show that a mechanism other than binding to hemoglobin must occur in order to explain the potential-dependent binding of both dyes to ghosts and vesicles. A primary interaction must exist between the dye molecule and the lipid bilayer in a biological membrane, and this would be expected to occur in the presence of hemoglobin or other cytosolic components. DiO-$C_3$-(5) is a better dye to use than diS-$C_3$-(5) for mechanistic studies, in order to avoid problems associated with formation of complex aggregates of the latter dye, especially in hyperpolarized membrane suspensions.

Interest in exploring biological potentials has led to the development of several techniques for measurement of transmembrane potentials. The older and more established methods include the use of microelectrodes, $Cl^-$ distribution ratios and organic ion distribution ratios. More recently, fluorescent probes have come into vogue as convenient noninvasive indicators of membrane potential [1, 14, 19]. Davila et al. [3] reported on the use of several merocyanine and carbocyanine dyes in giant squid axons. Fluorescence from stained axons was found to be linearly dependent upon membrane potential as measured by microelectrodes. This work led Hoffman and Laris [7] to the use of carbocyanine dyes as a means of monitoring membrane potentials in erythrocyte suspensions.

Since then, carbocyanine dyes have been widely employed for estimating membrane potentials in cell or organelle suspensions [2, 9, 13, 15]. The underlying nature of the dye-cell interaction and consequent "slow" fluorescent signals [2, 19,20] is not understood although it is generally presumed that structurally similar dyes interact in a fundamentally similar fashion. For this reason, mechanistic descriptions of the interaction of these probes with biological suspensions have often focused on thiocarbocyanines as representative of the carbocyanines in general. DiS-$C_3$-(5) is often employed because it exhibits a larger amplitude change in fluorescence than related dyes [6, 17, 18]. On the other hand, a careful comparison of dye-binding characteristics for two or more types of carbocyanines under identical conditions has not been reported, although preliminary information from our laboratory indicates that differences exist [5].

The work presented here was undertaken as a means of defining comparative properties of carbocyanine-membrane interactions for a thiacarbocyanine and an oxacarbocyanine dye. The potential-dependent binding of both dyes to intact erythrocytes, erythrocyte ghosts and phospholipid vesicle preparations was studied.

## Materials and Methods

### *Membrane Preparation*

Intact human erythrocytes were prepared by collecting whole blood on the morning of use in heparinized centrifuge tubes. The tubes were centrifuged immediately for 4 min at $1000 \times g$, and the supernatant plasma and buffy coat (of white cells) were removed by aspirations. The packed cells were washed 3 times in 5 volumes

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of an isotonic buffer containing 60 mM $K_2HPO_4$, 50 mM $NaH_2PO_4$, 1 mM $MgSO_4$, pH 7.4 at 0 °C. The final cell pellet was suspended at 50% hematocrit in the same buffer and kept on ice until used in fluorescence measurements.

Ghosts were prepared by adding an intact cell pellet (1–2 ml) to 100 ml of a chilled lysing solution (6 mM $K_2HPO_4$, 4 mM $KH_2PO_4$, 1 mM $Na_2EDTA$, pH 7.4 at 0 °C) for 5 min. The lysate was centrifuged for 15 min at $30,000 \times g$ at 4 °C. The supernatant was aspirated and the ghosted membranes were washed two times in the lysing solution. The final membrane pellet was suspended in 100 ml of a resealing solution (60 mM $K_2HPO_4$, 50 mM $NaH_2PO_4$, 1 mM ATP, 1 mM $MgSO_4$, 5 mM glucose at pH 7.4) and incubated at 37 °C for 120 min in an oscillating water bath. The resealed ghosts were then centrifuged at $30,000 \times g$ for 15 min and washed in the resealing solution twice before making the final 50% hematocrit ghost suspension. The ghosts were kept refrigerated (4–9 °C) for 48 hr before being used for fluorescence measurements. Potential-dependent dye uptake by the ghosts gradually increased with time during the early refrigeration immediately following the resealing incubation and plateaued after 36–48 hr of refrigeration.

Phospholipid vesicles were prepared by extracting one ml of the final ghost suspension in a volume of a 1:1 mixture of chloroform and methanol such that the final solution was a single phase with a flocculent protein precipitate. The precipitate was centrifuged at $25,000 \times g$ for 15 min at 25 °C. The supernatant was mixed with enough chloroform and water to bring the final component ratios to 8:4:3 (chloroform/methanol/water). The solution separated into two phases: methanol and water over chloroform. Phase separation was accelerated by a $25,000 \times g$ centrifugation for 15 min at 25 °C. The lipids were extracted in the chloroform phase which was collected and evaporated to dryness under a steady stream of $N_2$. The lipids were redissolved in 1 ml of chloroform and redried under $N_2$. One ml of an isotonic buffer containing 60 mM $K_2HPO_4$, 50 mM $NaH_2PO_4$, 1 mM $MgSO_4$, pH 7.4 was mixed with dried lipids and sonicated at constant power output for up to 90 min at 37 °C in an L&R Maxomatic sonicator. The vesicle suspension was kept at 0 °C until used in fluorescence measurements.

### *Absorption Spectroscopy*

Spectra were recorded on a Cary-118C split beam recording spectrophotometer. Two and one-half ml of a buffer containing 140 mM NaCl, 5 mM $Na_2HPO_4$, 1 mM $MgSO_4$ at pH 7.4 were placed in each of two quartz cuvets positioned in the spectrophotometer. The instrument was zeroed at the monomer absorption maximum for the dye being tested. The dye was then added to the same cuvet and scanned after an initial equilibration period. The final dye concentration was 1 μM and the scanning ranges were from 500 to 750 nm for diO-$C_3$-(5) and from 500 to 800 nm for diS-$C_3$-(5). An aliquot of intact erythrocytes, ghosts or vesicles was added to both cuvets making the final suspension 0.1% (or its equivalent) and rescanned after an equilibration period of 2 min. The membranes were then hyperpolarized with 12.5 μg of valinomycin (in 5 λ of 100% ethanol). The suspensions were mixed and scanned after another 2 min equilibration. The amount of valinomycin used for all preparations was determined by titrating a 2.5 ml aliquot of 0.1% cells to maximum hyperpolarization.

### *Fluorescence and Dye Binding*

The cyanine dyes used [diS-$C_3$-(5) and diO-$C_3$-(5)] were prepared by Dr. Roger D. Philo after the procedure described in Sims et al. [17]. Stock solutions of 100 μM in 100% ethanol were used. All fluorescence measurements were taken on a J4-8960 Aminco-Bowman spectrophotofluorometer with a xenon lamp source. The excitation and emission band passes were 5.5 and 11.0 nm, respectively. The excitation and emission wavelengths were 622 and 670 nm for diS-$C_3$-(5) and 576 and 596 nm for diO-$C_3$-(5). Aliquots of stock dye were placed in a cuvet containing a $K^+$-free isotonic NaCl buffer in a 22 °C water-jacketed compartment in the fluorometer. A calibration curve of fluoreacence *vs.* concentration was constructed for each dye. For binding studies the concentration range investigated is limited by the form of the fluorescence calibration curve. Under the experimental conditions described here, this curve is linear from 0–300 nM, peaks at 1300–1400 nM and diminishes at higher concentrations due to the inner filter effect [4]. Cyanine dye concentrations higher than 1–2 μM have recently been shown to alter certain bioactivities [2, 12]. For these reasons the dye concentrations used here were less than 1 μM.

For experiments involving intact cells, the cells were added to the cuvet making the final hematocrit 0.1%. After the fluorescence stabilized the cuvet was centrifuged at $1000 \times g$ for 2 min and the supernatant fluorescence level was recorded. The cells were then hyperpolarized by adding 12.5 μg of valinomycin (in ethanol) and recentrifuged for 2 min. The fluorescence level of the supernatant over the hyperpolarized cells was then recorded. The amount of dye bound to the normal and hyperpolarized cells could be determined from the supernatant fluorescence changes recorded before and after the addition of cells and valinomycin by reference to the dye concentration-fluorescence calibration curve.

Dye binding measurements to ghosts and vesicles were somewhat easier since centrifugation steps were unnecessary. The hemoglobin in whole cells substantially absorbed (and/or scattered) the excitation energy in whole cell suspensions. In contrast, the addition of ghosts or vesicles to cuvets placed in the excitation or emission beam paths resulted in no fluorescence change for dye solutions in the central compartment. Since fluorescence of bound dye is almost completely quenched [17] or shifted from the aqueous recording wavelengths [6, 18] the suspension fluorescence of ghosts and vesicles was equated to supernatant fluorescence. Determinations of bound dye were made using fluorescence differences before and after valinomycin and the concentration-fluorescence calibration curves.

## Results

When intact human erythrocytes are added to a 1 μM solution of dye in a $K^+$-free NaCl buffer and then hyperpolarized by the addition of valinomycin, the spectra shown in Fig. 1*A* and 1*B* are obtained. The free-dye spectra in each case are dominated by single peaks centered about the aqueous monomer absorption maximum with shorter wavelength absorption shoulders. With diO-$C_3$-(5) there is little change in the spectrum when cells are added, but for diS-$C_3$-(5) a new peak appears which is 55 nm blue-shifted compared to the diminished monomer peak. After hyperpolarization the new peak in the diS-$C_3$-(5) spectra is further enhanced while the nonomer peak diminishes and broadens to a position 12–15 nm red-shifted compared to the aqueous monomer peak. In the diO-$C_3$-(5) spectra, hyperpolarization induced a new 50 nm blue-shifted peak and an 8–10 nm red shift in the remaining monomer peak.

Similar spectra were recorded for suspensions of ghosts and vesicles made from lipids extracted from

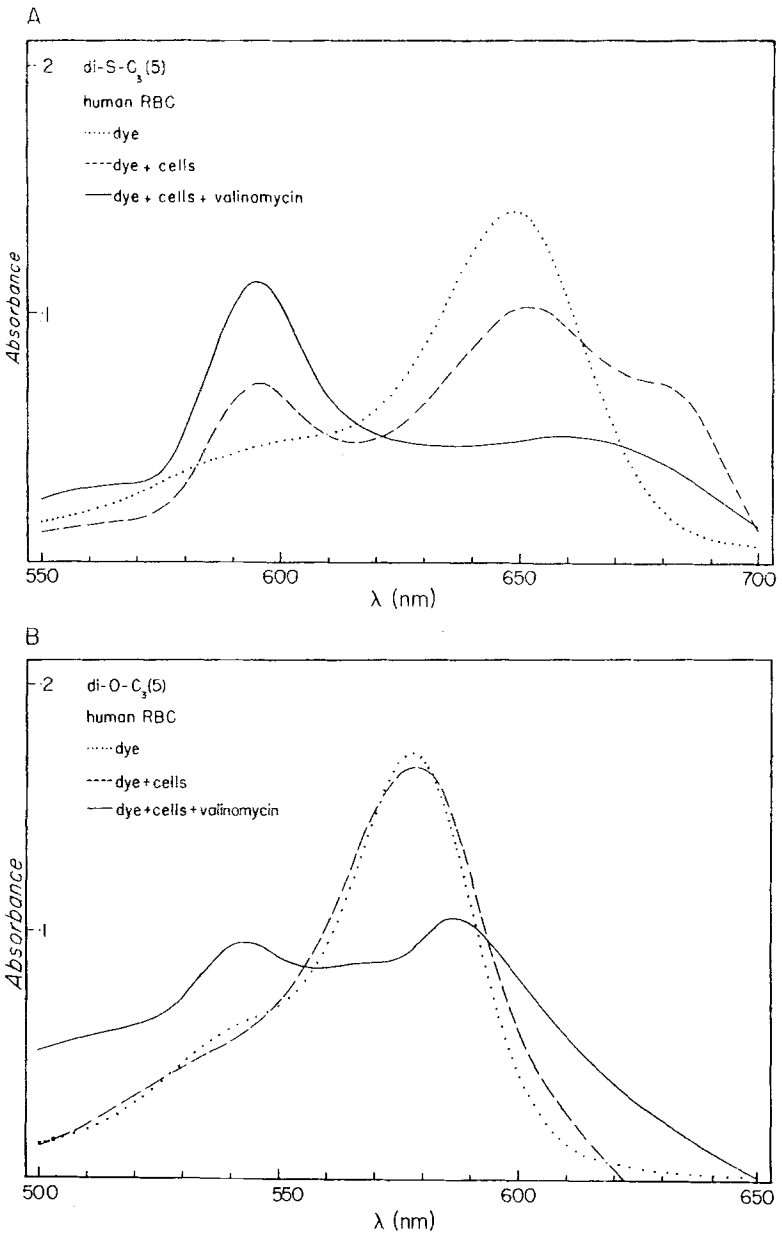


**Fig. 1.** Absorption spectra of diS-$C_3$-(5) and diO-$C_3$-(5) in the presence of normally polarized and hyperpolarized human erythrocytes. In *A* the dotted curve represents the spectra of 1 μM diS-$C_3$-(5) in 2.5 ml of 140 mM NaCl, 20 mM TrisCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, pH 7.4 buffer. The dashed curve represents the spectra of the same dye solution after human erythrocytes were added to make the final suspension 0.1%. The solid curve is the suspension spectra after 5 λ of 2.5 mg/ml valinomycin had hyperpolarized the cells. In *B* diO-$C_3$-(5) was substituted for diS-$C_3$-(5) and spectra were run under conditions identical to those described for *A*. All spectra were run at room temperature (20 °C)

ghost membranes. Fig. 2*A* and 2*B* are the spectra obtained in the presence of 0.1% ghost suspensions and $K^+$ transmembrane gradients. The significant features of these spectra are the absence of blue-shifted monomer peaks in all polarization states and the progressive red-shifting of the monomer absorption maximum with the degree of hyperpolarization. In the diS-$C_3$-(5) spectra for the hyperpolarized ghost suspension there is a long red-shifted (compared to the monomer absorption maximum), low intensity, broad absorption band centered about 760 nm. This absorption is not detected in the normally polarized ghost suspension, nor in any of the diO-$C_3$-(5) spectra.

The spectra (not shown) obtained in the presence of vesicle suspensions with $K^+$ transmembrane gradients are qualitatively identical to the ghost spectra for both dyes. There are no instances of new blue-shifted absorption peaks. Monomer absorption peaks progressively red-shift with hyperpolarization (although to lesser degrees compared to ghost suspensions) and long red-shifted absorption bands are only found in the diS-$C_3$-(5) hyperpolarized vesicle suspension.

### *Fluorescence Pattern Responses of Membrane Suspensions*

The fluorescence responses of whole cells, ghosts and vesicles to the addition of valinomycin are qualitative-

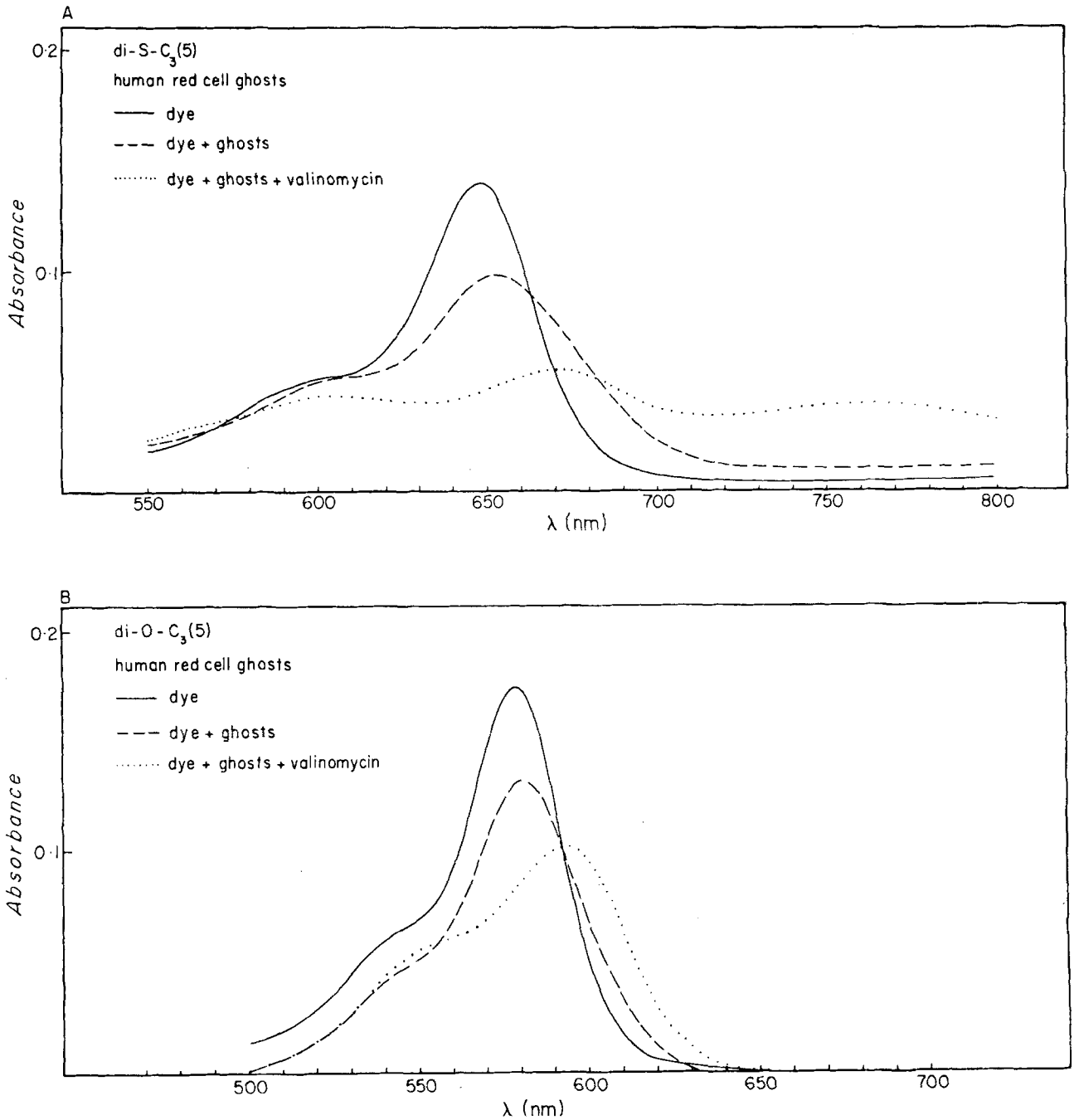


**Fig. 2.** Absorption spectra of diS-$C_3$-(5) and diO-$C_3$-(5) in the presence of normally polarized and hyperpolarized resealed ghosts. In *A* the solid curve represents the spectra of 1 μM diS-$C_3$-(5) in 2.5 ml of 140 mM NaCl, 5 mM $Na_2HPO_4$, 1 mM $MgSO_4$, pH 7.4 buffer. The dashed curve represents the spectra of the same dye solution after 10 λ of a 20% ghost suspension (determined by PEG exclusion as described in Materials and Methods) was added. The final ghost suspension was 0.08%. The dotted curve is the suspension spectra after 5 λ of 2.5 mg/ml valinomycin had hyperpolarized the ghosts. In *B*, 1 μM diO-$C_3$-(5) substituted for diS-$C_3$-(5). The rest of the conditions remained identical. The ghosts were resealed in media containing 60 mM $K_2HPO_4$ and 50 mM $NaH_2PO_4$. The whole cell/ghost hemoglobin ratio was 127. All spectra were run at room temperature (20 °C)

ly similar to each other and are dependent on the presence of a $K^+$-gradient across the membrane. Fig. 3 shows the typical fluorescence patterns of diS-$C_3$-(5) and diO-$C_3$-(5) with cells, ghosts and vesicles in the presence of transmembrane $K^+$-gradients. Fluorescence is recorded as a function of time during an interval in which dye, the membrane preparation, valinomycin and KCl are added sequentially. Cell suspension (panel *A*) requires 2-min centrifugation steps in order to remove cells so that dye fluorescence

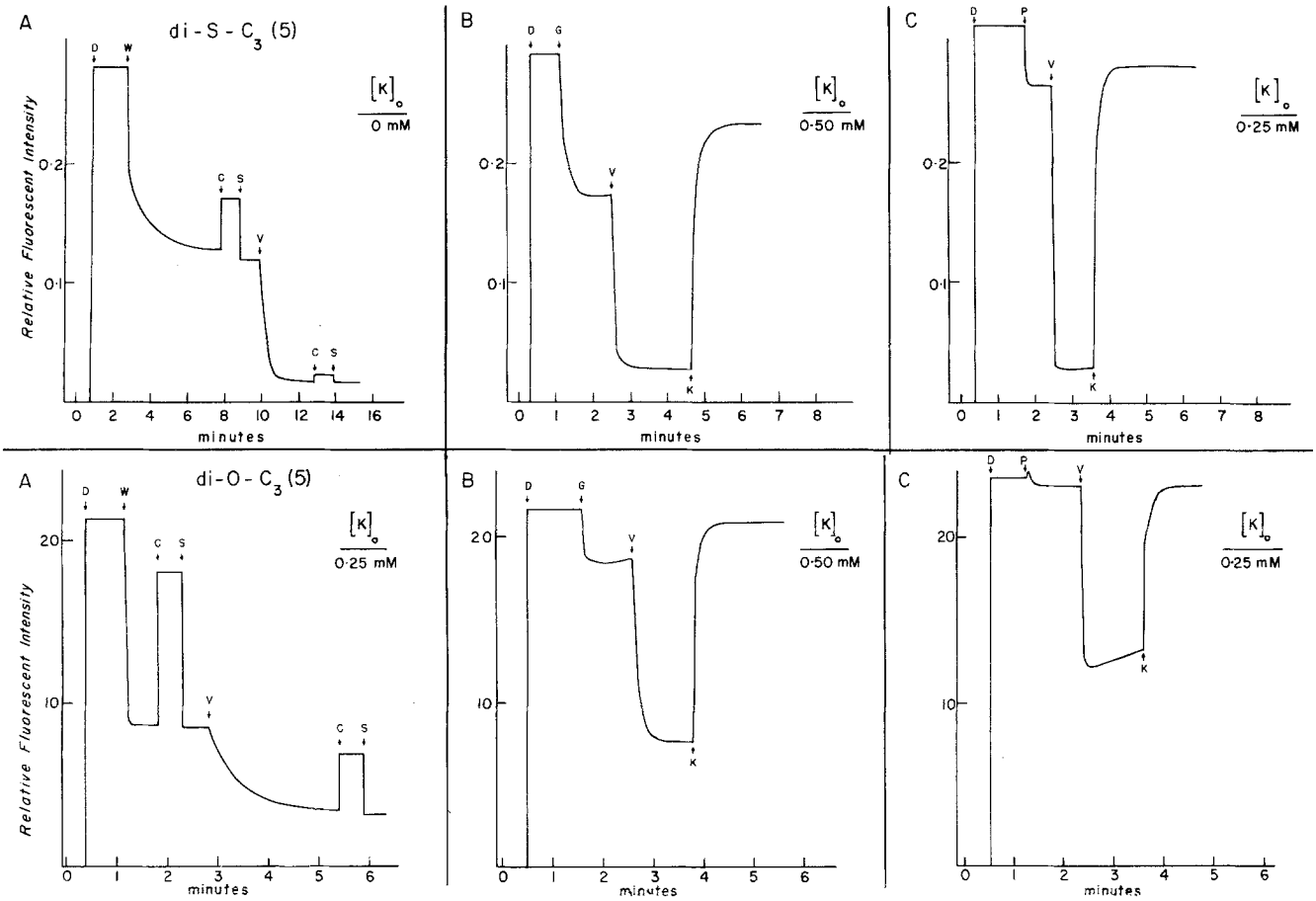


**Fig. 3.** DiS-$C_3$-(5) and diO-$C_3$-(5) fluorescence in erythrocyte, ghost and phospholipid vesicle suspensions in response to valinomycin addition in the presence of transmembrane $K^+$ gradient. The buffer volume in each case was 2.5 ml. The buffer in each case was 140 mM NaCl, 5mM $Na_2HPO_4$, 1 mM $MgSO_4$, pH 7.4. $[K]_0$ in each figure is the external potassium concentration after the addition of cells, ghosts or vesicles. For diS-$C_3$-(5), 25 $\lambda$ of 100 μM dye was added at *D* to produce an initial dye concentration of 1 μM. For diO-$C_3$-(5), 10 $\lambda$ of 100 μM dye was added at *D* to produce a final dye concentration of 0.4 μM. In panel *A*, 5 $\lambda$ of a 50% suspension of whole cells was added to the dye solution at *W*. In panel *B* 10 $\lambda$ of a 50% ghost suspension (measured by PEG exclusion) was added to the dye solution at *G*. In *C*, 5 $\lambda$ of a 5 mg/ml phospholipid vesicles suspension was added to the dye solution at *P*. In each case 5 $\lambda$ of 2.5 mg/ml valinomycin was added at *V* for *B* and *C* and 50 $\lambda$ of 2 M KCl was added at *K*. In *A* a 2-min centrifugation at $100 \times g$ was performed at *C* and the suspensions were restirred at *S* by a glass-coated magnet and a magnetic mixing device. The ghosts and phospholipid vesicles were prepared in 60 mM $K_2HPO_4$, 50 mM $NaH_2PO_4$, 1 mM $MgSO_4$, pH 7.4 buffer. The whole cell/ghost hemoglobin ratio was 171. The fluorescence excitation wavelength was 622 nm or 576 nm and the emission wavelength was 670 nm or 596 nm for diS-$C_3$-(5) and diO-$C_3$-(5), respectively. The temperature was held constant at 22 °C

can be studied without interference due to light-scattering. Each membrane preparation causes an initial decrease in fluorescence as a portion of the total dye equilibrates into the membranes. Valinomycin added after the fluorescence reaches a new steady state causes an immediate and rapid drop in extracellular fluorescence. A stable fluorescence level is reached within one minute and the further addition of KCl causes an immediate increase in the extracellular fluorescence for all hyperpolarized preparations (shown only for ghosts and vesicles). In the absence of an asymmetric $K^+$-gradient valinomycin addition and subsequent KCl cause little or no change in suspension or supernatant fluorescence with either dye (data not shown).

Note that 1) the relative fluorescent intensity scale is 100 times larger for diO-$C_3$-(5) than for diS-$C_3$-(5) (due to different $\lambda_{ex}$ and $\lambda_{em}$ in relation to monomer absorption and emission maxima) and 2) the hyperpolarized fluorescent levels are relatively higher for diO-$C_3$-(5), possibly reflecting a lower membrane affinity for diO-$C_3$-(5) compared to diS-$C_3$-(5).

### *Dye Binding as a Function of Extracellular Concentration and Membrane Polarization*

The supernatant dye levels were recorded before and after the addition of whole cells for a variety of initial dye concentrations. By using the fluorescence concentration calibration curve and the volume of solution

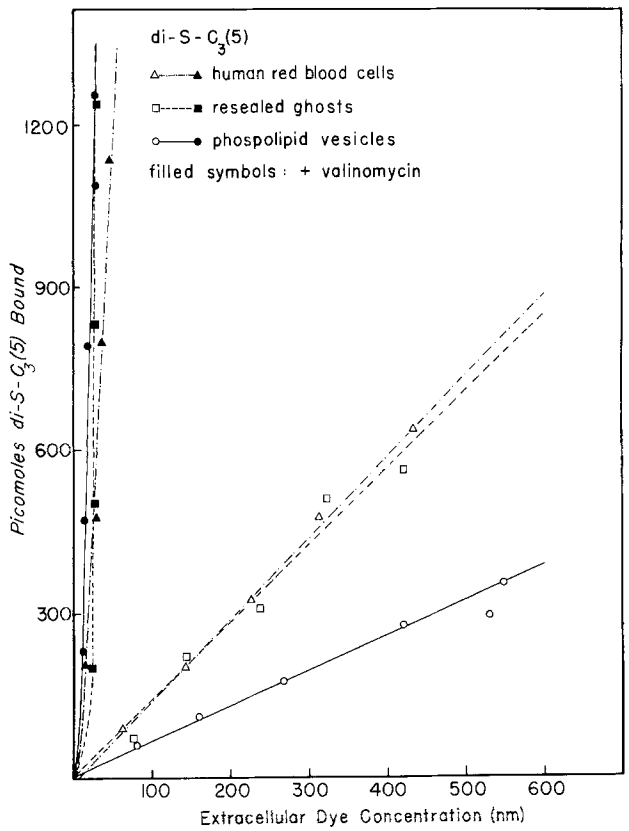


**Fig. 4.** Comparison of the binding amounts of diS-$C_3$-(5) by normally polarized and hyperpolarized human erythrocytes, ghosts and phospholipid vesicles. The total binding amounts and extracellular dye concentrations were determined using the fluorometric assay techniques described in Materials and Methods. The symbols represent binding to normally polarized erythrocytes (△---△), ghosts (□---□), phospholipid vesicles (○—○), and hyperpolarized erythrocytes (▲---▲), ghosts (■---■) and phospholipid vesicles (●—●). The lines were drawn by eye. The extracellular buffer and conditions for ghost and phospholipid vesicle preparations were the same as those described for Fig. 3. Total volume in the cuvet was 2.5 ml and hyperpolarization was induced by adding 5 λ of a 2.5 ml valinomycin solution

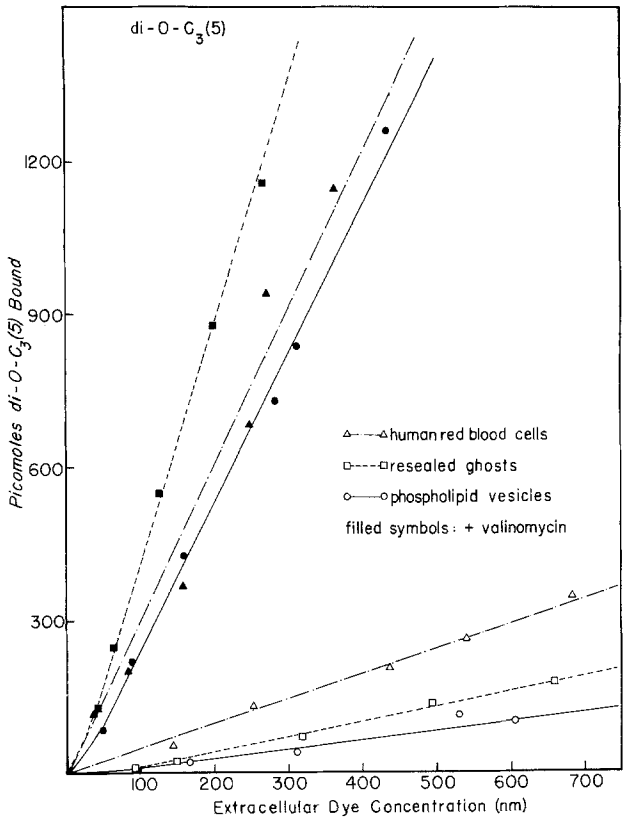


**Fig. 5.** Comparison of the binding amounts of diO-$C_3$-(5) by normally polarized and hyperpolarized human erythrocytes, ghosts and phospholipid vesicles. The conditions under which the binding of diO-$C_3$-(5) was measured to each membrane preparation and the symbol legends are identical to those described in the legend for Fig. 4. Again, the lines were drawn by eye

in the cuvet, a binding curve relating picomoles of dye bound (to cells in 0.1% suspension) to extracellular dye concentration was constructed. This procedure was repeated before and after the addition of 12.5 μg of valinomycin. In Fig. 4 the diS-$C_3$-(5) binding curves are shown for whole cells, ghosts and vesicles. The whole cell curves were calculated based on supernatant fluorescence measurements while the ghost and vesicle curves were from suspension measurements. The lines were drawn by eye.

Binding to normally polarized whole cells is approximately equal to the binding to normally polarized ghosts. These two preparations bind approximately twice as much diS-$C_3$-(5) as the normally polarized vesicles. Both the hyperpolarized ghosts and vesicles bind slightly more dye than the whole cells at each extracellular concentration, except the lowest, where whole cells bind slightly more than ghosts but less than vesicles. All the hyperpolarized preparations bind substantially more dye than the respective normally polarized suspensions.

Figure 5 demonstrates the diO-$C_3$-(5) binding under conditions identical to those in Fig. 4. Dye binding to normally polarized whole cells is at least twice the binding to ghosts or vesicles. After hyperpolarization vesicle and whole cell binding is approximately equal and both are less than binding to ghosts.

It is probable that a part of the binding observed for normally polarized preparations may be nonspecific and unrelated to the membrane potentials. On the other hand, that binding which is induced by valinomycin represents binding which has been induced by a change in potential. This potential-dependent binding is referred to here as valinomycin-in-

**Table 1.** Binding parameters of both diO-$C_3$-(5) and diS-$C_3$-(5) to whole cells, ghosts and phospholipid vesicles

| | 80 mM $K^+$ (150 mM $Cl^-$) | | | 120 mM $K^+$ (110 mM $PO_4^{2-}$) | | |
|---|---|---|---|---|---|---|
| | $\frac{\text{pmoles}}{\text{nmolar}} \pm$ SD | ($r^2$) | hp/np | $\frac{\text{pmoles}}{\text{nmolar}} \pm$ SD | ($r^2$) | hp/np |
| diO-$C_3$-(5) | | | | | | |
| *Normally polarized* | | | | | | |
| whole cells | 0.537 ± 0.054 | (0.935) | | 0.528 ± 0.027 | (0.992) | |
| ghosts[1] | 0.189 ± 0.032 | (0.895) | | 0.303 ± 0.011 | (0.996) | |
| PLV[1] | | | | 0.213 ± 0.040 | (0.935) | |
| *Hyperpolarized* | | | | | | |
| whole cells | 4.06 ± 0.26 | (0.973) | 7.57 | 3.57 ± 0.42 | (0.961) | 6.76 |
| ghosts | 2.02 ± 0.59 | (0.749) | 10.69 | 4.64 ± 0.12 | (0.998) | 15.30 |
| PLV | | | | 2.95 ± 0.11 | (0.995) | 13.84 |
| diS-$C_3$-(5) | | | | | | |
| *Normally polarized* | | | | | | |
| whole cells | 1.46 ± 0.05 | (0.995) | | 1.50 ± 0.03 | (0.998) | |
| ghosts | 0.794 ± 0.110 | (0.929) | | 1.46 ± 0.16 | (0.964) | |
| PLV | | | | 0.584 ± 0.044 | (0.978) | |
| *Hyperpolarized* | | | | | | |
| whole cells | 33.8 ± 1.3 | (0.994) | 23.1 | 31.1 ± 3.0 | (0.981) | 20.7 |
| ghosts | 48.1 ± 11.8 | (0.806) | 60.5 | 190.1 ± 27.8 | (0.959) | 130.6 |
| PLV | | | | 56.6 ± 8.8 | (0.954) | 96.9 |

[1] Ghosts were resealed and vesicles were sonicated in the $K^+$ anion solutions indicated at the top of the table. All slopes were derived from data taken from experiments such as those depicted in Figs. 5 and 6. Each slope was determined from a linear regression of binding levels for at least 5 different extracellular dye concentrations. Each slope measures the change in the amount of bound dye (in picomoles) per standard change in extracellular dye concentration (nanomolar). The standard deviations (SD) and correlation coefficients ($r^2$) were calculated according to standard statistical formulas. The hp/np ratios were calculated by dividing the binding slope after hyperpolarization by the binding slope before hyperpolarization (normal polarization) for each membrane preparation in the different $K^+$ resealing and sonicating solutions. Phospholipid vesicles were only sonicated in phosphate solutions.

duced dye uptake and is determined by subtracting the dye bound prior to valinomycin addition from the total dye bound after valinomycin addition. Measurements of valinomycin-induced dye uptake were made for several different initial dye concentrations and the induced dye uptake was plotted against initial dye concentration. The resulting linear plots (not shown) were similar to the dye-binding plots shown in Figs. 4 and 5. For both dyes, the induced binding to ghosts or vesicles is as high or higher than that observed for intact cells.

Other experiments were performed to compare dye binding to ghosts and vesicles resealed at different $K^+$ concentrations. The three basic resealing solutions were: (a) 80 mM KCl, 70 mM NaCl, (b) 60 mM $K_2HPO_4$, 50 mM $NaH_2PO_4$ and (c) 60 mM $K_2HPO_4$, 50 mM $KH_2PO_4$. The technique used in standardizing ghost suspensions was to use $^{14}$C-polyethylene glycol to determine a 50% exclusion volume and define it as a 50% ghost suspension. The dye-binding plots were qualitatively similar to Fig. 4 and 5 and exhibited linearity. Linear regressions were performed on each set of binding points (no fewer than 5 points). This yielded a slope ± standard deviation and correlation coefficient for each membrane preparation in each resealing solution. The slopes for normally polarized (np) and hyperpolarized (hp) cells were calculated in each case and a ratio of the two (hp/np) was computed for purposes of comparison. Binding to ghosts and vesicles was compared to binding to intact cells for each different resealing solution.

Table 1 summarizes all the binding data and includes the slopes derived from data in Fig. 4 and 5. Table 2 summarized the induced dye uptake (after hyperpolarization with valinomycin) for all membrane preparations and resealing solutions. The amount of valinomycin-induced dye uptake was a linear function of external dye concentration (not shown). All induced dye binding data were treated in a manner similar to the binding data summarized in Table 1. A linear regression of each set of data points yielded a slope ± standard deviation and a correlation coefficient of binding for both dyes and all membrane preparations. These data are summarized in Table 2.

If excitation and emission wavelengths are red-shifted by an appropriate amount it is possible to monitor the fluorescence properties of membrane-as-

**Table 2.** A comparison of the valinomycin-induced dye uptake for both dyes at different internal $K^+$ concentrations

$$\frac{\Delta \text{ valinomycin-induced dye uptake}}{\Delta \text{ initial extracellular dye concentration}}$$ [a]

| | 80 mM $K^+$ (150 mM $Cl^-$) | | 120 mM $K^+$ (110 mM $PO_4^{2-}$) | |
|---|---|---|---|---|
| | $\frac{\text{pmoles}}{\text{nmolar}} \pm$ SD | ($r^2$) | $\frac{\text{pmoles}}{\text{nmolar}} \pm$ SD | ($r^2$) |
| diO-$C_3$-(5) | | | | |
| Whole cells | 1.112 ± 0.046 | (0.988) | 1.049 ± 0.080 | (0.983) |
| Ghosts | 1.028 ± 0.142 | (0.929) | 1.354 ± 0.018 | (0.999) |
| PLV | | | 1.095 ± 0.034 | (0.997) |
| diS-$C_3$-(5) | | | | |
| Whole cells | 1.406 ± 0.020 | (0.999) | 1.366 ± 0.009 | (0.999) |
| Ghosts | 1.787 ± 0.060 | (0.995) | 1.548 ± 0.069 | (0.994) |
| PLV | | | 1.875 ± 0.022 | (0.999) |

[a] The calculations were made as described under the legend for Table 1.

sociated dye monomers. Figure 6 shows the results obtained when this is done for $Na^+$-loaded asolectin vesicles suspended with diO-$C_3$-(5) and hyperpolarized with gramicidin D. The wavelengths employed for this experiment were 20 nm and 12 nm longer than those used for dye in free solution for excitation and emission, respectively. Note that hyperpolarization still results in a pronounced fluorescence decrease indicating that a true fluorescence quenching has occurred rather than simple shifting of the fluorescence spectrum. With wavelengths set for dimer or higher order aggregates we have not been able to detect the presence of these molecular forms in the hyperpolarized suspensions. When suspensions of ghosts or erythrocyte phospholipid vesicles were employed in place of asolectin vesicles qualitatively similar but lower magnitude changes were observed.

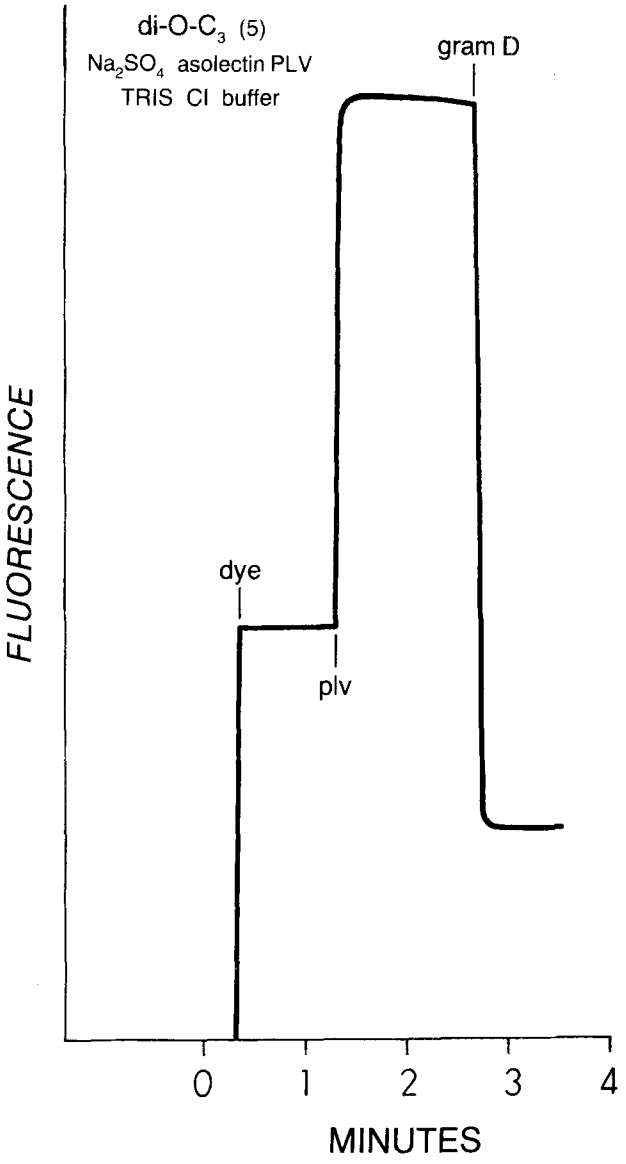


**Fig. 6.** Fluorescence response of diO-$C_3$-(5) to gramicidin D addition to a suspension of asolectin vesicles at wavelengths appropriate for monitoring dye monomers in the membrane. The excitation wavelength was set at 597 nm and the emission wavelength was set at 612 nm. At the indicated time, 25 $\lambda$ of stock 100 μM diO-$C_3$-(5) was added to 2.5 ml of a 150 mM Tris Cl, 1 mM $MgSO_4$, pH 7.4 buffer. At "*plv*" 5 $\lambda$ of a 16 mg/ml asolectin vesicle suspension was added to the dye solution making the final vesicle suspension 32 μg/ml. At "gram D" 2.1 $\lambda$ of a 50 μg/ml gramicidin D-ethanol solution was added to hyperpolarize the vesicle suspension. The asolectin vesicles were sonicated in 98 mM $Na_2SO_4$, 15 mM $TrisSO_4$, 1 mM $MgSO_4$, pH 7.4 media. The tracing was run at 22 °C

## Discussion

### *Absorption Spectra*

Sims et al. [17] were the first to suggest that the potential-sensing carbocyanines became membrane-associated or form large dye aggregates when in suspension with hyperpolarized erythrocytes. Later, Hladky and Rink [6] and Tsien and Hladky [18] demonstrated that diS-$C_3$-(5) forms dimers in the presence of human hemoglobin and they suggested that dimerization on hemoglobin may account for most of the dye binding to intact erythrocytes. On the other hand, Kimmich et al. [8] used diO-$C_3$-(5) with pigeon erythrocytes and found that approximately equal amounts of dyes could be bound to intact erythrocytes or erythrocyte ghosts suggesting that binding to hemoglobin for this dye is not a factor.

The results described above represent the first systematic comparison of the two carbocyanines under identical experimental conditions. The absorption spectra presented in Figs. 1 and 2 show that both dyes exhibit a 10–15 nm shift of monomer absorption

peaks following membrane hyperpolarization indicating that a change has occurred in the polarity of the molecular environment of the chromophore. Similar shifts were reported for diS-$C_3$-(5) by Sims et al. [17] and Hladky and Rink [6]. Because the monomer shifts are qualitatively similar for intact erythrocytes, ghosts, and phospholipid vesicles, one can assume they represent interaction between dye monomers and membrane components rather than interaction with hemoglobin.

Both dyes also show some evidence of dimer formation following hyperpolarization of intact cells as evidenced by the appearance of a new absorption peak at 592 nm for diS-$C_3$-(5) and at 542 nm for diO-$C_3$-5. The latter fact was surprising because we have previously shown that diO-$C_3$-(5) does not form dimer when lysed erythrocytes are used [5]. Neither dye shows evidence of dimer formation in the ghost or vesicle preparations which are free of hemoglobin. Only diS-$C_3$-(5) exhibited higher order aggregate formation with the appearance of a broad absorption peak at 760 nm after hyperpolarization. These aggregates were only observed in ghost and vesicle preparations although they probably form in intact cells but remain undetected due to cell scattering and consequent masking of the long wavelength absorption signal.

*Fluorescence Patterns and Dye Binding*

The fluorescence patterns presented in Fig. 3 are qualitatively similar for both dyes and cell membrane preparations. As originally reported by Hoffman and Laris [7] for diS-$C_3$-(5) hyperpolarization of the membrane leads to sharp decreases in suspension and supernatant fluorescence and extracellular dye concentration. Depolarization reverses these changes. Sims et al. [17] interpreted the fluorescence decrease as a true fluorescence quenching on the basis of experiments in which only the aqueous monomer was monitored. Hladky and Rink [6] showed that the peak monomer fluorescence is red-shifted by approximately 20 nm so that when wavelengths are set to monitor aqueous monomer the fluorescence will decrease as chromophores shift to an environment in which the emission peak is also red-shifted.

The same phenomenon of peak shifting as dye associates with membranes is also observed with diO-$C_3$-(5) (*see* Figs. 1 and 2). However, in this case if the fluorescence characteristics of the membrane-bound monomer are monitored, the fluorescence decrease after hyperpolarization appears to be due to true fluorescence quenching and not a result of spectral shifting (Fig. 6). Quenching studies with whole cells suffer from interference by hemoglobin absorption and scattering. The simplified experimental design reported here for diO-$C_3$-(5) and asolectin vesicles avoids these problems.

Ghost or vesicle membrane dye quenching becomes important in measuring binding to ghosts and vesicles since the values are determined from suspension fluorescence levels. In the absence of scattering the fluorescence of membrane-associated dye becomes the main difference between extracellular dye fluorescence and total membrane suspension fluorescence. As membrane-dye fluorescence quenching approaches 100%, suspension fluorescence approaches extracellular dye fluorescence. The reported values for bound dye to these preparations, as calculated from suspension fluorescence, will tend to be underestimated since the extracellular dye concentrations will be slightly overestimated, more so before hyperpolarization than after.

Figures 4 and 5 and Table 1 clearly show that ghosts and vesicles take up as much or more dye than whole cells under similar conditions. Table 1 illustrates that whole cells tend to accumulate more dye than ghosts or vesicles in the normally polarized state, but with hyperpolarization the reverse occurs. In the normally polarized state, several factors contribute to the greater dye uptake by whole cells. Intracellular hemoglobin, no doubt, accounts for part of the excess uptake. One might expect that in the ghost and vesicle preparations there will be a certain percentage of nonfunctional or "leaky" membrane units which won't maintain either a normally polarized or a hyperpolarized transmembrane potential. In addition, the resting cell membrane potential measured with microelectrodes [10] and other dye and ion distribution methods [7] has been shown to be in the range of $-8$ to $-15$ mV. The resting potential of ghost and vesicle preparations is problematic but could easily be less negative than that for whole cells.

The above reasons may explain why whole cells accumulate more dye than ghosts or vesicles in the normally polarized state. These same reasons point to the usefulness of interpreting the binding data via considerations other than absolute binding amounts. This leads to the self-standardizing hp/np ratios and the valinomycin-induced dye uptake values shown in Tables 1 and 2. The hp/np ratios tend to minimize the effect of variation in cell, ghost or vesicle density while the induced dye uptake measurement more directly reflects dye uptake sensitive to potential. This value eliminates that portion of dye bound to the native preparation in a potential-independent manner. The excess dye bound after the addition of valinomycin is primarily due to a transmembrane hyperpolarization of functional membrane units.

When one compares the binding parameters of

whole cells to those of ghosts and vesicles it is apparent that the latter demonstrate a capacity to bind in a potential-sensitive manner which is at the least equal to that observed for whole cells (Table 2). Bearing in mind that the binding estimates for ghosts and vesicles are lower limits, these findings are most surprising in view of Hladky and Rink's [6] and Tsien and Hladky's [18] contention that dimer formation on hemoglobin is responsible for the major portion of dye uptake. There must exist one or more other mechanisms which are responsible for dye association to whole cells and functional analogs. For diS-$C_3$-(5) binding this may include the formation of higher order aggregates as suggested by spectral evidence given above and by Sims et al. [17]. The formation of dye aggregates does not seem to explain all diS-$C_3$-(5) binding to ghosts or vesicles because of the relatively small aggregate absorption signals found for these membrane suspensions. Neither dimer nor higher order aggregate formation would seem to explain any of the binding of diO-$C_3$-(5) to ghosts or vesicles because of the lack of appropriate absorption signals in any of the diO-$C_3$-(5) spectra. DiO-$C_3$-(5) dimer formation in whole cells seems to play only a minor role in the overall dye binding in the intact cell suspensions. Furthermore, it is easy to show that dye aggregate or dimer formation as a primary reason for cellular dye accumulation would lead to an exponential relationship between cellular dye and dye concentration rather than the linear relationship observed.

Since dye binding to vesicles is approximately equivalent to that of whole cells and ghosts it is likely the bilayer membrane phospholipid is the major site of dye-membrane interaction. A model originally described by Läuger and Neumcke [11] for lipophilic ions in a bilayer can be adapted for the binding of diO-$C_3$-(5) to intact rat erythrocytes. This model considers the potential energy of lipophilic ions across phospholipid bilayers and can accommodate the experimental binding data quite well. According to the model most of the membrane-associated dye is located at the phospholipid-aqueous interfaces, where potential energy mnima exist for lipophilic ions. Hyperpolarization alters these energy minima making the inside interface more negative, thereby inducing more dye accumulation. This model is attractive in that it can accommodate binding to such simple structures as hemoglobin-depleted ghosts and protein-depleted phospholipid vesicles.

In order to evaluate the parameters of the model and its applicability and usefulness it is necessary to use a dye which compartmentalizes in the simplest molecular forms. Di-O-$C_3$-(5) is most suitable because it does not form high-order aggregates and, in rat erythrocytes, does not dimerize to hemoglobin [5]. Such a model could be generalized to both diO-$C_3$-(5) and diS-$C_3$-(5) if appropriate corrections for non-membrane dye forms can be derived. One would predict this because the monomer absorption studies for both dyes indicate similar mechanisms with regard to membrane interaction (Fig. 1 and 2) and the fluorescence patterns (Fig. 3) for both dyes are identical with regard to potential manipulations.

In light of the evidence reported here, work directed at elucidating this interaction can be best pursued with those dyes which form the fewest molecular species following hyperpolarization of the membrane and would point toward the use of oxacarbocyanines rather than thiacarbocyanines.

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