

# The Relation between Dicarbocyanine Dye Fluorescence and the Membrane Potential of Human Red Blood Cells Set at Varying Donnan Equilibria

JEFFREY C. FREEDMAN and JOSEPH F. HOFFMAN

From the Department of Physiology, Yale University School of Medicine, New Haven, Connecticut 06510

**ABSTRACT** The fluorescence,  $F$ , of two dicarbocyanine dyes, diS-C<sub>3</sub>(5) and diI-C<sub>3</sub>(5), depends both on the membrane potential,  $E$ , and on the intracellular pH,  $\text{pH}_c$ , of human red blood cells. Compositions of isotonic media have been devised in which the equilibrium Donnan potential,  $E$ , varies at constant  $\text{pH}_c$  and in which  $\text{pH}_c$  varies at constant  $E$ . Dye fluorescence measurements in these suspensions yield calibrations of  $+1.7\% \Delta F/\text{mV}$  for diS-C<sub>3</sub>(5) and  $+0.6\% \Delta F/\text{mV}$  for diI-C<sub>3</sub>(5). While  $\text{pH}_c$  does not affect  $F$  of either dye, changes in  $\text{pH}_c$  of 0.1 unit at constant  $E$  cause changes of  $F$  equivalent to those induced by 2–3 mV. Based on these results, a method is given for estimating changes in  $E$  from dye fluorescence in experiments in which  $E$  and  $\text{pH}_c$  co-vary. The relation of  $F$  to  $E$  also depends in a complex way on the type and concentration of cells and dye, and the wavelengths employed. The equilibrium calibration of dye fluorescence, when applied to diffusion potentials induced by  $1\ \mu\text{M}$  valinomycin, yields a value for the permeability ratio,  $P_{\text{K.VAL}}/P_{\text{Cl}}$ , of  $20 \pm 5$ , in agreement with previous estimates by other methods. The calibration of  $F$  is identical both for diffusion potentials and for equilibrium potentials, implying that diS-C<sub>3</sub>(5) responds to changes in voltage independently of ionic fluxes across the red cell membrane. Changes in the absorption spectra of dye in the presence of red cells in response to changes in  $E$  show that formation of nonfluorescent dimers contributes to fluorescence quenching of diS-C<sub>3</sub>(5). In contrast, only a hydrophobic interaction of dye monomers need be considered for diI-C<sub>3</sub>(5), indicating the occurrence of a simpler mechanism of fluorescence quenching.

## INTRODUCTION

This paper describes the quantitative relation between fluorescence and membrane potential for two dicarbocyanine dyes, diS-C<sub>3</sub>(5) and diI-C<sub>3</sub>(5) (see structures in Fig. 7), both of which previously were shown to give large changes of fluorescence dependent on the membrane potential of human red blood cells (Hoffman and Laris, 1974; Sims et al., 1974). In previous work on dye calibration (Hoffman and Laris, 1974) with *Amphiuma* red cells, at an

external K concentration,  $[K]_o$ , denoted the "null point," at which no fluorescence change occurs after addition of valinomycin, the internal-to-external K ratio,  $[K]_i/[K]_o$ , was within 7% of  $[Cl]_o/[Cl]_i$ . Moreover, the computed Nernst potential agreed closely with both the equilibrium Cl ratio as determined prior to addition of valinomycin, and with a direct microelectrode measurement of  $-15$  mV (Hoffman and Lassen, 1971; Lassen, 1972). With human red cells, the small size of the cells and an apparent lack of resealing after being punctured (Lassen, 1972) have precluded the use of microelectrodes as a primary standard for the new optical technique. From the null point of diS-C<sub>3</sub>(5) fluorescence, the human red cell resting potential was estimated to be  $-9$  mV, again in agreement with the Cl ratio (Hoffman and Laris, 1974). The agreement of the potential derived from the fluorescence null point with that computed from the Cl ratio and with microelectrode measurements, where possible, indicates that at the null point neither the dyes employed nor valinomycin create an offset from the normal resting potential.

To extend the range of calibration from a single point, the percentage change of fluorescence,  $\% \Delta F$ , after valinomycin addition at different K gradients was fit (Hoffman and Laris, 1974) with the constant field equation (Goldman, 1943; Hodgkin and Katz, 1949). In addition to depending on the theoretical assumptions, this method for dye calibration requires independent knowledge of all significant ionic permeabilities, including  $P_K/P_{Cl}$ , or the assumption of a linear relation between fluorescence and potential. However, due to the finite amount of dye available for partitioning, the fluorescence changes are nonlinear for large potential changes, so that the method is most accurate at high  $K_o$  near the null point. Hladky and Rink (1976 *a,c*) proposed a different calibration method using dye-binding measurements, assuming that unbound internal dye is in ideal Donnan equilibrium with external dye, and that voltage-dependent changes in intracellular dye distribution are negligible.

In order to avoid the assumptions required for the use of diffusion potentials or dye-binding measurements, and to check previous dye calibrations, our approach in this paper is to use equilibrium potentials for dye calibration. In the preceding paper (Freedman and Hoffman, 1979) it was shown that the Jacobs-Stewart (1947) equations for red cell Donnan equilibria, when appropriately modified with osmotic and activity coefficients, accurately describe cell volume and Donnan ratios. Consequently, the use of Gibbs-Donnan equilibrium potentials for dye calibration has a rigorous theoretical foundation, the only fundamental limitation being the small uncertainty due to single ion activity coefficients. In the present studies of calibration, dye fluorescence is measured in human red cell suspensions in which the composition of the extracellular solution is manipulated such that equilibrium membrane potentials are varied systematically either by titrating hemoglobin, Hb, and organic phosphates, or by substituting impermeant electrolytes for external NaCl. Simultaneous adjustments of  $pH_o$  and total tonicity allow  $pH_i$  and cell volume to be varied independently or held constant. Further information pertaining to the mechanism by which the dyes respond to membrane

potential has been obtained by examining changes in the absorption spectra of the two dyes in the presence of human red cells as the membrane potential is varied.

Preliminary reports of this study have been presented previously (Freedman and Hoffman, 1977, 1978*a*).

#### MATERIALS AND METHODS

##### *Relation of Dye Fluorescence, $F$ , to Equilibrium Membrane Potential, $E$ and to Intracellular $pH$ , $pH_c$*

DiS-C<sub>3</sub>(5) (3,3'-dipropylthiadicarbocyanine iodide) was first studied by procedure A (below) in which both  $E$  and  $pH_c$  vary simultaneously in red cells titrated with HCl and NaOH in media containing nystatin and sufficient impermeant sucrose to prevent hemolysis. It is important to note that nystatin renders the red cell membrane highly permeable to Na, K, and Cl (Cass and Dalmark, 1973), thus allowing the cells to come to a complete Donnan equilibrium, the characteristics of which have been described in the accompanying paper (Freedman and Hoffman, 1979). Thus the use of nystatin enables the calculation of equilibrium membrane potentials directly from Donnan ratios without possible complications due to ionic gradients. Also, the Donnan potentials vary with  $pH_o$  with much smaller cell volume changes in the presence of nystatin than in its absence. DiS-C<sub>3</sub>(5) and diI-C<sub>3</sub>(5) (3,3'-dipropylindodicarbocyanine iodide) were subsequently calibrated according to procedure B (below) in which  $pH_o$  and impermeant tartrate or Mg are simultaneously adjusted so as to vary  $E$  at constant  $pH_c$ , or to vary  $pH_c$  at constant  $E$ . The compositions of the solutions needed were calculated using a nonideal thermodynamic model of red cell ionic and osmotic equilibria (Freedman and Hoffman, 1979).

##### *Procedure A: Changes of $F$ When $E$ and $pH_c$ Co-Vary in Nystatin-Treated Cells*

From the Nernst equation,  $E = (RT/\mathcal{F})\ln r$ , where  $r$  is the Donnan ratio, and  $R$ ,  $T$ , and  $\mathcal{F}$  have their usual meanings, both  $E$  and  $pH_c$  will change whenever the Donnan ratio is altered by titrating the charge on impermeant cellular solutes, mainly Hb and organic phosphates. For this purpose, human blood from healthy donors was drawn by venipuncture, transferred to a heparinized flask, and immediately centrifuged at 12,000  $g$  for 3–5 min at 0–4°C, followed by aspiration of the plasma and buffy coat. The red cells were then washed three times in about 5 vol of a chilled solution containing 145 mM NaCl, 5 mM KCl, 5 mM glucose, and 5 mM HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid, pH 7.4 at 22°C). The washed cells were resuspended to 5% hematocrit in the same ice-cold medium, but including an additional 37 mM sucrose. In order to allow cation equilibrium, nystatin (E. R. Squibb & Sons, Inc., New York) was added from a stock solution (5 mg/ml methanol) to a final concentration of 50  $\mu$ g/ml suspension (Cass and Dalmark, 1973), and Na, K,  $pH$ , and cell volume were allowed to equilibrate at 0°C for at least 15 min. The suspension was then divided into a number of 10-ml tubes, and the cells in each were washed twice by centrifugation at 7,500  $g$  for 3 min at 0–4°C and resuspension to 5% hematocrit in media containing 145 mM NaCl, 5 mM KCl, 5 mM glucose, 37 mM sucrose, 35 mg/ml nystatin, and 5 mM HEPES. The charge on impermeant cell solutes was altered, since these media were previously titrated with HCl or NaOH to obtain suspension  $pH$ s between 6.5 and 8.5. These last washings were done to ensure that no change in solution composition or  $E$  would occur upon dilution at the same

pH to 0.4% hematocrit for dye fluorescence measurements. The suspensions were then equilibrated for 15 min at 25°C and the pH determined at 25°C (Radiometer model 26 pH meter with GK 2321C combination electrode, The London Company, Cleveland, Ohio). For some experiments simultaneous monitoring of pH and fluorescence, using a miniature combination pH electrode with a 1.2-mm tip diameter (Microelectrodes, Inc., Londonderry, N.H., model MI-410), was obtained on a 2-channel recorder (Leeds & Northrup Co., North Wales, Pa., model XL-624).

The membrane potentials of cells in these suspensions were estimated from equilibrium Na and K, or Cl Donnan ratios. Na and K were determined flame-photometrically in supernates of the cell suspensions and in lysates of weighed packed cell pellets (Freedman and Hoffman, 1979).  $^{36}\text{Cl}$  ratios were determined as previously described, and the microcentrifuge tube sampling technique was also used as previously described (Freedman and Hoffman, 1979).

One of the above suspensions, set aside on ice for fluorescence measurements, was then used to determine the dependence of fluorescence on pH as  $E$  was varied at 25°C by titration with HCl or NaOH (see Figs. 4 and 5).

#### *Procedure B: Changes of $F$ When $E$ and $\text{pH}_c$ Vary Independently*

In these experiments,  $E$  was depolarized (inside made more positive) by substituting external impermeant tartrate for permeant Cl. In order to prevent  $\text{pH}_c$  from rising,  $\text{pH}_o$  was simultaneously reduced.  $E$  was hyperpolarized (inside made more negative) by substituting external impermeant Mg for Na, and raising  $\text{pH}_o$  to maintain constant  $\text{pH}_c$ . Isotonicity was preserved where necessary with sucrose.

**NYSTATIN-TREATED CELLS** Human blood from healthy donors was drawn by venepuncture, transferred to a heparinized flask, and immediately washed three times as before in medium containing 145 mM NaCl, 5 mM KCl, and 5 mM HEPES (pH 7.4 at 25°C). The cells were then incubated at 5% hematocrit in medium containing 145 mM NaCl, 5 mM KCl, 37 mM sucrose, 5 mM HEPES (pH 7.4 at 25°C), and 50  $\mu\text{g}/\text{ml}$  nystatin for 10 min at 2°C. The suspension was then divided into 10 portions, 9 of which were washed twice by centrifugation in the solutions described in Table I, and  $^{36}\text{Cl}$  (0.1  $\mu\text{Ci}/\text{ml}$ ) was added to each. After 15 min at 25°C, the pH of each tube was measured and adjusted to  $\pm 0.02$  with 0.3 M HCl or NaOH, if necessary, and then assayed in triplicate for Cl ratios as previously described (Freedman and Hoffman, 1979). Membrane potentials,  $E_{\text{Cl}}$ , were estimated from  $E_{\text{Cl}} = (RT/\mathcal{F})\ln r_{\text{Cl}}$ , and  $\text{pH}_c$  from  $\text{pH}_c = \text{pH}_o + \log r_{\text{Cl}} - 0.034$  (loc. cit., eq. A12). A suspension set aside was used for fluorescence measurements with 250  $\mu\text{l}$  of 5% hematocrit suspensions added per 2.75 ml of calibration solution (Table I). The pH was adjusted as before at 25°C if necessary, and the fluorescence was measured in triplicate 3–5 min after addition of 10  $\mu\text{l}$  diS-C<sub>3</sub>(5) (1.2  $\mu\text{M}$  final concentration).

**CELLS OF NORMAL CATION PERMEABILITY** After washing fresh cells as described above, a 50% hematocrit suspension was prepared in the same washing medium and was kept on ice. To 24 ml of each media described in Table II was added 200  $\mu\text{l}$  of cell suspension, and the temperatures brought to 25°C. The pH was checked at 25°C and adjusted to  $\pm 0.02$  if necessary with dropwise addition of 0.3 M HCl or NaOH with frequent swirling. Dye fluorescence was measured (see Fig. 6) in duplicate 3 min after addition of 10 or 20  $\mu\text{l}$  of diS-C<sub>3</sub>(5) or diI-C<sub>3</sub>(5). For all experiments, the stock solutions of both dyes contained 0.2 mg/ml ethanol.

#### *Fluorescence Measurements*

The technique and apparatus used for fluorescence measurements was basically the same as described by Hoffman and Laris (1974), but with two modifications. A

TABLE I  
CALIBRATION SOLUTIONS FOR NYSTATIN-TREATED RED CELLS

pH <sub>o</sub>	Na <sub>2</sub> -tartrate <i>mM</i>	MgCl <sub>2</sub> <i>mM</i>	Sucrose <i>mM</i>	pH <sub>c</sub>		E <sub>ci</sub>	
				Calculated	From <i>r</i> <sub>ci</sub>	Calculated	From <i>r</i> <sub>ci</sub>
				<i>mV</i>			
A. Variation of <i>E</i> at constant pH <sub>c</sub>							
7.10	0	23	0	6.99	6.98	-4.4	-5.3
7.07	0	11.5	14	6.99	7.00	-2.9	-2.4
7.04	0	0	26	6.99	6.96	-1.1	-2.9
7.01	11.5	0	14	6.99	6.96	-0.8	-1.2
6.98	23	0	0	6.99	6.97	+2.5	+0.9
B. Variation of pH <sub>c</sub> at constant <i>E</i>							
7.00	0	22	1	6.90	6.88	-3.8	-5.1
7.50	0	2	27	7.40	7.40	-3.8	-5.1
8.00	13	0	20	7.90	7.90	-3.8	-5.0
8.47	27	0	11	8.37	8.34	-3.8	-6.5

Solutions for calibrating dye fluorescence with independent variation of equilibrium membrane potentials, *E*, and pH<sub>c</sub> in suspensions of human red cells with high cation permeability induced by nystatin. In addition to the concentrations (millimolar) indicated, all solutions contained 75 mM NaCl, 75 mM KCl, 5 mM HEPES, and 50 μg/ml nystatin. All solutions were calculated to give *E* (millivolts) and pH<sub>c</sub> as indicated and to give normal cell volumes at 25°C. The values for E<sub>ci</sub> and pH<sub>c</sub> determined from *r*<sub>ci</sub> are from an experiment described in Materials and Methods, procedure B, for nystatin-treated cells. In this experiment, the water contents were 0.696 ± 0.008 g H<sub>2</sub>O/g cells (SD, *n* = 9) and hemolysis was 0.7 ± 0.7% (SD, *n* = 9).

TABLE II  
CALIBRATION SOLUTIONS FOR RED CELLS OF NORMAL  
CATION PERMEABILITY

pH <sub>o</sub>	NaCl <i>mM</i>	Na <sub>2</sub> -tartrate <i>mM</i>	MgCl <sub>2</sub> <i>mM</i>	pH <sub>c</sub>	<i>E</i> <i>mV</i>
7.40	142	0	0	7.28	-5.2
7.15	79	42	0	7.28	+9.6
6.90	43	66	0	7.28	+24.6
6.64	23	79	0	7.28	+39.6
6.39	12	87	0	7.28	+54.6
B. Variation of pH <sub>c</sub> at constant <i>E</i>					
6.90	81	0	52	6.78	-5.2
7.40	142	0	0	7.28	-5.2
7.90	99	17	0	7.78	-5.2
8.40	56	34	0	8.28	-5.2

Solutions for calibrating dye fluorescence with independent variation of equilibrium membrane potentials, *E*, and pH<sub>c</sub> in suspensions of human red cells with normal cation permeability. In addition to the concentrations (millimolar) indicated, all solutions contained 2 mM NaHCO<sub>3</sub> and 5 mM HEPES. All solutions were calculated to give *E* (millivolts) and pH<sub>c</sub> indicated and to give cell volumes constant at 0.67 g H<sub>2</sub>O/g cells at 25°C. The mean fluorescence, measured in duplicate 3 min after addition of dye (1.2 μM) to each solution in the absence of cells and expressed relative to the value at pH 7.40, was 1.07 ± 0.07 (SD, *n* = 7) for diS-C<sub>3</sub>(5) and 1.00 ± 0.02 (SD, *n* = 7) for diI-C<sub>3</sub>(5).

problem of the signal jumping to a new level at erratic times due to arc wander of the xenon lamp was eliminated with a magnetic stabilizer (American Instrument Co., Silver Springs, Md.). A second modification was the use of filters to reduce scattering interference. The overlap of the fluorescence emission peak of either dye in the presence of cells with the very large peak of scattered excitation light (see Fig. 1 A and

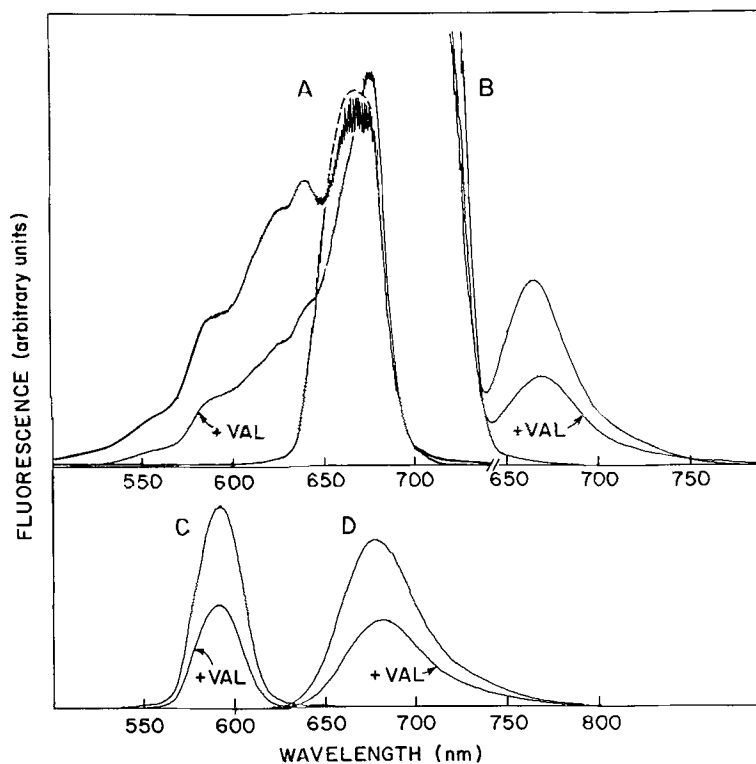


FIGURE 1. Excitation and emission spectra of diI-C<sub>3</sub>(5). (A, B). After adding 10  $\mu$ l of an ice-cold 50% hematocrit human red cell suspension, prepared as described in Materials and Methods, procedure B, to a cuvette containing 3 ml of 149 mM NaCl, 1 mM KCl, and 5 mM HEPES (pH 7.4 at 25°C), excitation (A,  $\lambda_{em} = 680$  nm) and emission (B,  $\lambda_{ex} = 600$  nm) spectra were recorded (lower curves) at 2-4 nm/s. Then 20  $\mu$ l diI-C<sub>3</sub>(5) (0.2 mg/ml EtOH, 2.4  $\mu$ M final concentration) was added and the spectra recorded again (upper curves). After addition of 10  $\mu$ l valinomycin (VAL) ( $3 \times 10^{-4}$  M in EtOH, 1  $\mu$ M final concentration), the spectra were again recorded (middle curves). Upper, middle, and lower refer to 600 nm in A and 650 nm in B. The slit arrangement was as follows: 5 mm entrance to the photomultiplier tube, 3 mm at both the excitation monochromator exit and emission monochromator entrance positions, and in A, 1 mm preceding the cuvette while in B, 1 mm immediately following the cuvette. Curves are uncorrected for spectral dependence of source intensity and detector sensitivity. (C, D) Same protocol as in A and B except that instead of the 1-mm slits near the cuvette, a narrow bandpass interference filter on the excitation side and a sharp cut-on filter on the emission side were employed to reduce interference due to scattered excitation light (see Materials and Methods). With filters, the cells in the absence of dye gave a signal (not shown) at all wavelengths less than 0.2% of the peak emission shown in D.

B for diI-C<sub>3</sub>[5]; cf. Hoffman and Laris, 1974, for diO-C<sub>6</sub>[3]), results in the scattered light contributing as much as 10% of the total optical signal, depending on hematocrit, even with optimal slit and wavelength settings. With diS-C<sub>3</sub>(5) (Figs. 2,4-6,9 A) a 10-nm bandpass interference filter centered at 647 nm (Baird Corp., Bedford, Ma.) was

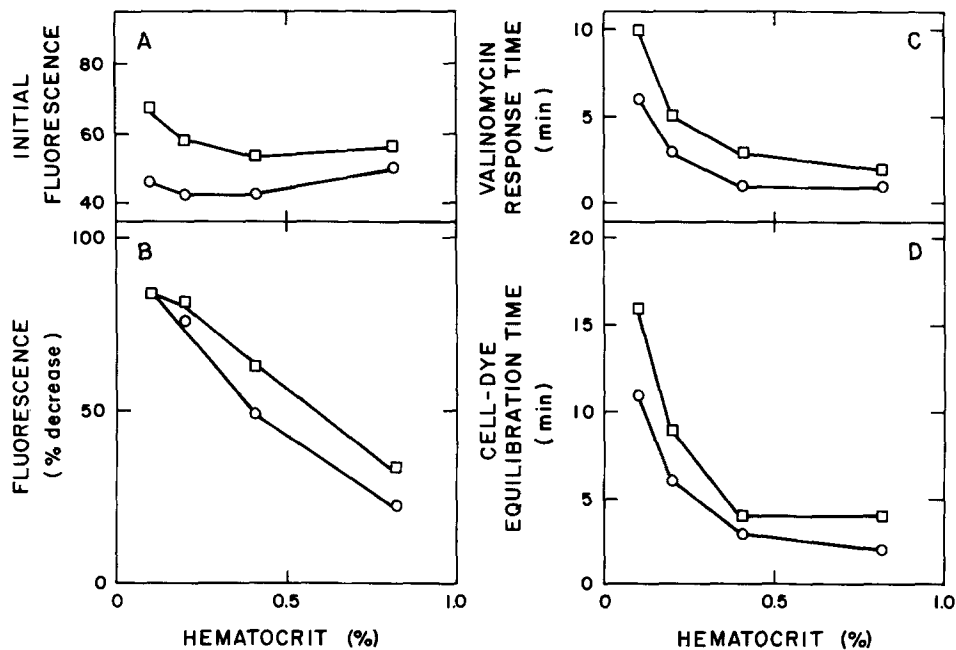


FIGURE 2. Optimization of cell and dye concentrations. To cuvettes containing 3 ml of media consisting of 149 mM NaCl, 1 mM KCl, and 5 mM HEPES (pH 7.4 at 25°C) was added 5, 10, 25, or 50  $\mu$ l of an ice-cold 50% hematocrit red cell suspension. After equilibration to 25°C, 10 or 20  $\mu$ l of diS-C<sub>3</sub>(5) (0.2 mg/ml EtOH) was injected, giving final dye concentrations of 1.2  $\mu$ M (O) or 2.4  $\mu$ M (□). Each of the eight traces of fluorescence vs. time was characterized by four measured parameters, shown in A-D. After injection of dye, the fluorescence (measured in arbitrary units and using filters, see Materials and Methods) rose through a peak and then fell to a steady level,  $F_i$ , denoted "initial fluorescence," shown in A. The time from addition of dye to stabilization of the signal is denoted "cell-dye equilibration time," and is shown in D. After injection of 10  $\mu$ l valinomycin ( $3 \times 10^{-4}$ M in EtOH), the fluorescence fell to a new steady level,  $F_f$ , (as in Fig. 11, *inset*), giving a percent change shown in B. The time ( $\pm 0.2$  min) for this change, denoted "valinomycin response time," is shown in C. The percentage change in fluorescence,  $\% \Delta F$ , is defined to be  $100(F_f - F_i)/F_i$ .

placed between the cuvette and the 3-mm exit slit of the excitation monochromator, and a 3-mm thick cut-on filter (RG 665, Schott Optical Glass Inc., Duryea, Pa.) was placed between the emission monochromator and the 5-mm entrance slit to the photomultiplier tube. With diI-C<sub>3</sub>(5) (Figs. 6 and 11), the interference filter was centered at 620 nm and the cut-on filter was RG 645, 4 mm thick. With these optics, the filtered excitation and emission peaks are well separated (e.g., Fig. 1 C and D for

diI-C<sub>3</sub>[5]), with scattering interference reduced to less than 0.5% of the fluorescent emission signal. Also, the signals are a little less noisy both because of reduced scattering noise and because the excitation wavelength now corresponds more closely with the absorption peak, enabling use of lower amplifier gains. When filters were used, the excitation and emission monochromators were set to correspond with the peaks at 647 and 690, respectively, for diS-C<sub>3</sub>(5), and at 620 and 680 for diI-C<sub>3</sub>(5), except where otherwise noted in the figure legends. While the magnitude of the step decrease in fluorescence after hyperpolarization with valinomycin at low K<sub>o</sub> was the same with and without filters, this improvement (Freedman and Hoffman, 1978 *b*)

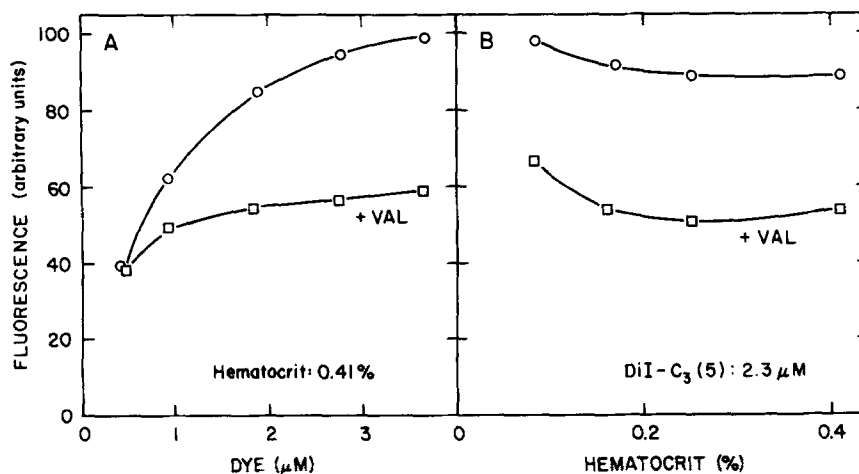


FIGURE 3. Fluorescence of diI-C<sub>3</sub>(5) with varying concentrations of dye (A) and cells (B). Differential fluorescence was used with excitation at 620 nm and with emission detected through a 4-mm-thick RG 645 cut-on filter (see Materials and Methods). The reference cuvette contained 2.4 μM diI-C<sub>3</sub>(5) in 3 ml EtOH and the sample cuvette initially contained 3 ml of 145 mM NaCl, 5 mM KCl, 5 mM HEPES (pH 7.4 at 25°C), with (□) or without (○) 1 μM valinomycin (VAL). In A, 4- or 8-μl aliquots of dye (0.2 mg/ml EtOH) were injected successively to give the indicated final concentrations with the hematocrit constant at 0.41%. In B, 5- or 10-μl aliquots of ice-cold 50% red cell suspension were injected successively to give the indicated final hematocrits with the dye concentration constant at 2.3 μM. The fluorescence (arbitrary units) was noted after the signal stabilized (1-2 min) following each successive addition to the same cuvette.

effectively eliminated a subsequent, small, slowly rising component of the signal due to increased scattering concomitant with net KCl loss and cell shrinkage. The rate of shrinkage is reported to be about 1.5%/min at 37°C and about a factor of 3 less at ambient temperature (Hunter, 1977).

The magnitude of the percentage decrease of fluorescence after valinomycin addition to cells in a given medium for both dyes is strongly dependent on the hematocrit and dye concentration employed (Figs. 2 B and 3), implying that a calibration relating fluorescence and membrane potential is valid only for a defined set of conditions. The sensitivity of response of diS-C<sub>3</sub>(5), but not of diI-C<sub>3</sub>(5), increases by about 50% when the hematocrit is reduced from 0.41 to 0.17% (Figs. 2 B and 3 B). Systematic variation of hematocrit between 0.1 and 0.8% at two different concentra-



tions of diS-C<sub>3</sub>(5) showed that the hematocrit markedly affects the time required for the fluorescence to stabilize after addition of dye to cells, denoted the "cell-dye equilibration time" (Figs. 2 D), as well as the response time to valinomycin (Fig. 2 C), but has a smaller effect on the magnitude of the initial fluorescence (Fig. 2 A). With diS-C<sub>3</sub>(5), greater sensitivity is obtained with more dilute cell suspensions and with higher dye concentrations, but at the expense of slower cell-dye equilibration times. The pattern of results shown in Figs. 2 and 3 enabled a choice of optimal conditions for subsequent calibration experiments. With 1.2  $\mu$ M diS-C<sub>3</sub>(5), optimum kinetic responses to valinomycin with a cell-dye equilibration time of 3–5 min are achieved when 25  $\mu$ l of a 50% hematocrit suspension is added to 3 ml of buffered medium. As seen in Fig. 3 A, a dye concentration of 2.4  $\mu$ M gives a slightly better sensitivity with diI-C<sub>3</sub>(5). Optimum wavelengths may be selected from excitation and emission spectra determined in the presence and absence of valinomycin (e.g., Fig. 1 A and B).

It should be mentioned that downward drifts of fluorescence ranging from 1–3 % $\Delta F$ /min after addition of diS-C<sub>3</sub>(5) to 0.4% hematocrit suspensions could be reduced to 0–0.2 % $\Delta F$ /min by thorough cleaning of the quartz cuvettes. In procedure A (see above), nystatin in the cuvettes caused a small concentration-dependent downward drift of fluorescence reaching 0.6 % $\Delta F$ /min at 50  $\mu$ g/ml suspension, but the fluorescence intensity measured 3 min after dye addition or 5 min after a pH adjustment was comparable with nystatin-treated cells whether or not nystatin was included in the cuvette. Other controls showed that the fluorescence of either dye without cells was constant in media buffered between pH 5.0 and 8.6. Also, the fluorescence of both dyes was not affected by any of the calibration solutions utilized.

After injection into continuously stirred medium containing 145 mM NaCl, 5 mM KCl, and 5 mM HEPES (pH 7.4 at 25°C) in the absence of cells, the fluorescence of diS-C<sub>3</sub>(5) falls to 70% of the initial value in about 10 min and by an additional 10% over the next half hour, as does diO-C<sub>6</sub>(3) (Hoffman and Laris, 1974). This decline, which is identical in the light and in the dark, is due to dye binding to the pyrex cuvette and Teflon stir bar. Under identical conditions, diI-C<sub>3</sub>(5) fluorescence falls only 10% in 5 min and an additional 2% over the next half hour. The much weaker binding of diI-C<sub>3</sub>(5) to the cuvette and stir bar facilitates the study of spectral properties in the absence of cells. In the presence of cells, drifts were negligible with both dyes.

All fluorescence measurements were at 25°C.

#### *Absorbance Measurements*

Absorption spectra (see Figs. 7 and 10) at 25°C were determined with an Aminco DW-2 spectrophotometer (American Instrument Co.), using a 3.0 nm bandpass. This instrument was equipped with an accessory for 90° differential fluorescence measurements. For this purpose deflecting mirrors could be mounted on the magnetic stirrer and a cut-on filter was placed between the cuvettes and the photomultiplier tube. For kinetic traces of small absorbance changes, scattering interference was minimized with improved signal-to-noise ratios using dual wavelength spectroscopy (see Fig. 8). For dual wavelength measurements, cells and dye were in a single cuvette. One monochromator was set at an isosbestic point to minimize contributions from scattering, while the second monochromator was set at the wavelength of a maximum spectral shift, and the difference in absorbance was recorded with time.

#### RESULTS

The experimental observations are described in two parts: (a) changes in the dye fluorescence,  $F$ , when human red cell equilibrium membrane potentials,

$E$ , and intracellular pH,  $pH_c$ , are varied either simultaneously or independently (procedures A and B, respectively, in Methods) and (b) a description and comparison of changes in the absorption spectra of two dicarbocyanine dyes in the presence of the cells at varied membrane potentials. After measuring the extent to which changes in  $pH_c$  can affect the apparent relation between  $F$  and  $E$  in red cells titrated with acid and base in the presence of nystatin (Fig. 5 and Table III), and showing that  $F$  varies with  $pH_c$  at constant  $E$  in nystatin-treated cells, experiments are presented which calibrate the response of  $F$  to  $E$  at constant  $pH_c$ , and of  $F$  to  $pH_c$  at constant  $E$ , for two dyes in cells of normal cation permeability (Fig. 6). It is then shown that the separation of variables accounts quantitatively for the observed  $F$  in the controlled circumstance in which  $E$  and  $pH_c$  covary. Comparison of the absorption spectra of the two dyes in cells at varied membrane potentials extends previous work in attempting to delineate the mechanism by which the dyes respond to membrane potential.

*Relation of Dye Fluorescence,  $F$ , to Equilibrium Membrane Potentials,  $E$ , and to Intracellular pH,  $pH_c$ .*

**SIMULTANEOUS CHANGES OF  $E$  AND  $pH_c$**  In order to minimize cell volume changes dependent on pH and the influence of any diffusion potentials,  $E$  and  $pH_c$  were varied simultaneously by titrating red cells with HCl and NaOH in the presence of nystatin with sufficient impermeant sucrose to prevent hemolysis, allowing cations, anions, and water to come to equilibrium (Freedman and Hoffman, 1979). Tracings of diS-C<sub>3</sub>(5) fluorescence (Fig. 4), recorded simultaneously with  $pH_o$  (not shown) before and after injections of small aliquots of HCl or NaOH, show the expected increase of dye fluorescence with depolarization and decrease with hyperpolarization. In the presence of cells, the fluorescence of diS-C<sub>3</sub>(5) was stable between pH 6.5 and 9.3 (Fig. 4), and reversible for at least seven alternate additions of acid and base (not shown). Moreover, the fluorescence changes were additive in the sense that the same final value was reached with a single addition of 20  $\mu$ l HCl as with two sequential additions of 10  $\mu$ l HCl. Typical fluorescence values and membrane potentials estimated from the Donnan ratios of Na and K, as obtained in the same experiment at varied  $pH_o$ , are shown in Fig. 5 and in Table III, columns 1-3. Over a range of  $pH_o$  from 6.8 to 8.8, the ratio  $([Na]_c + [K]_c)/([Na]_o + [K]_o)$  increased such that  $E_{Na+K}$  decreased by 8.4 mV. In this range the fluorescence decrease may be described by the linear regression  $F = 60 + 3.76 E$ . The apparent relation between  $F$  and  $E$ , as given by the percentage change in fluorescence per mV, or  $\% \Delta F/mV$ , is then  $100 \Delta F/F^0 \Delta E = 376/(60 + 3.76 E^0)$  or 7.6%  $\Delta F/mV$ , referred to an initial  $E^0$  of -3 mV at  $pH_o = 7.30$ . Note that the numerical value of  $\% \Delta F/mV$  depends on the choice of the initial value, denoted by superscript zero. Thus, the apparent relation between  $F$  and  $E$  obtained from titrating nystatin-treated cells (procedure A in Materials and Methods) is substantially greater than the 0.7-1.0  $\% \Delta F/mV$  estimated for diO-C<sub>6</sub>(3) with valinomycin or tartrate (Hoffman and Laris, 1974; Freedman and Hoffman, 1978), and values around 1%  $\Delta F/$

mV from diS-C<sub>3</sub>(5) binding studies (Hladky and Rink, 1976; Tsien and Hladky, 1978). This large difference indicates the extent to which the quantitative dye response can appear to vary with experimental conditions, and, since  $F$  does not depend on  $pH_o$  in the absence of cells, suggests  $pH_c$  as an interfering variable. Hladky and Rink (1976 *b, c*) reported that pH affects the equilibrium of diS-C<sub>3</sub>(5) monomers and dimers in dilute hemolysates, but it is not possible to estimate from their data whether or not our results with intact cells can be quantitatively explained.

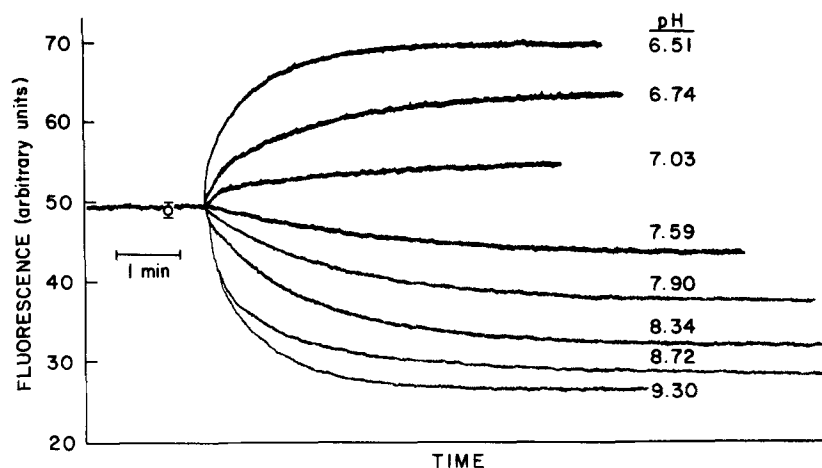


FIGURE 4. Effect of pH on diS-C<sub>3</sub>(5) fluorescence in red cell suspensions containing nystatin. The tracings above are for the experiment described in Table III and in Materials and Methods, procedure A. For each of the above tracings, 250  $\mu$ l of an ice-cold 5% hematocrit suspension in medium with nystatin and sucrose at pH 7.3 was added to a cuvette containing 2.75 ml of the same medium. After equilibration at 25°C, 10  $\mu$ l diS-C<sub>3</sub>(5) (0.2 mg/ml EtOH) was injected. The initial fluorescence (see Materials and Methods) is shown after 3–5 min for equilibration and was  $49 \pm 1$  (SD,  $n = 9$ ) at pH 7.30. The fluorescence rose to a final stable level after injections of 20–60  $\mu$ l of 0.1 N HCl or fell after additions of 30–120  $\mu$ l 0.1 N NaOH. The pH was recorded simultaneously (final value shown). The reproducibility of a fluorescence change in response to a given pH change was  $\pm 2\%$  for a triplicate determination.

**INDEPENDENT VARIATION OF  $E$  AND  $pH_c$**  In order to measure directly the extent to which changes in  $pH_c$  influence dye fluorescence in red cells, a series of solutions was designed (Tables I and II) in which  $pH_c$  varies at constant  $E$ , an experiment complimentary to one of Hoffman and Laris (1974) in which  $E$  was varied at constant  $pH_c$ . The mean difference between membrane potentials computed from the measured ratios  $[Cl]_c/[Cl]_o$  with those calculated from the compositions of the solutions employed was  $-1.3 \pm 0.7$  mV (SD,  $n = 9$ ), and the mean difference between estimated and calculated  $pH_c$  was  $0.02 \pm 0.01$  pH units (SD,  $n = 9$ ). This agreement (Table I) provides

further confidence in the ability of the nonideal thermodynamic model (Freedman and Hoffman, 1979) to predict red cell equilibrium properties from the composition of the suspending media. With nystatin-treated cells an increase of  $\text{pH}_c$  from 6.9 to 8.3 at constant  $E$  was associated with a decrease in fluorescence amounting to  $-31\% \Delta F/\text{unit } \Delta \text{pH}_c$  at a reference  $\text{pH}_o = 7.0$ . The variation of  $E$  at constant  $\text{pH}_c$  in this experiment was over a range of only 7 mV, and the equilibrium fluorescence data was of insufficient accuracy to calibrate the response to  $E$ . A greater range of potentials could be obtained

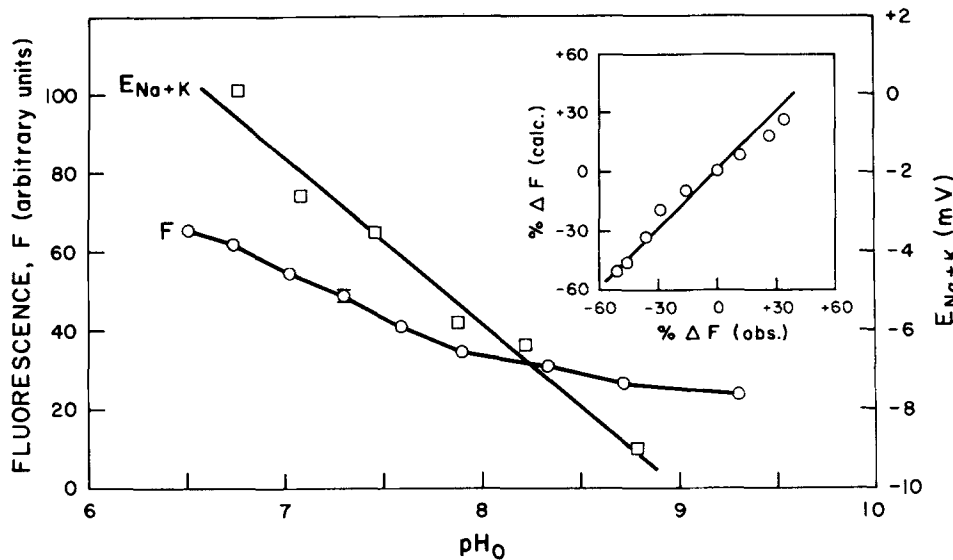


FIGURE 5. Effect of  $\text{pH}_o$  on diS-C<sub>3</sub>(5) fluorescence,  $F$ , and on membrane potential,  $E_{\text{Na+K}}$ , of nystatin-treated red cells. The cells were prepared and incubated as described in Materials and Methods, procedure A. The data refer to the same experiment as do Table III and Fig. 4. The inset shows that the  $\% \Delta F$  (obs.), as observed upon titrating cells in the presence of nystatin (Fig. 4), is completely accounted for by the values of  $(\partial F/\partial \text{pH}_c)_E$  and  $(\partial F/\partial E)\text{pH}_c$  obtained in the experiment described in Fig. 6, taken together with the values of  $\Delta \text{pH}_c$  and  $\Delta E$  shown in Table III (see Discussion).  $\% \Delta F$  (calc.) refers to  $100 \Delta F/F^0 = 100(\partial F/F^0 \partial E)_{\text{pH}} \Delta E + 100(\partial F/F^0 \partial \text{pH}_c)_E \Delta \text{pH}_c$ , where  $F^0$  is  $49 \pm 1$ ,  $\text{pH}_o = 7.30$  in Table III and Fig. 4, and where  $F^0 = 24$  at the corresponding values of  $\text{pH}_c = 7.23$  and  $E = -3$  mV in Fig. 6 (see Text and Discussion).

in nystatin-treated red cells by using hypertonic solutions of impermeant electrolytes, but since it was considered desirable to maintain constant cell volume, dye calibration was determined in cells of normal cation permeability without exposure to nystatin.

With cells of normal cation permeability,  $\text{pH}_c$  and  $E$  were independently varied at constant cell volume over a range of 2 pH units and 55 mV (Fig. 6), again utilizing impermeant external tartrate and Mg with simultaneous adjustment of  $\text{pH}_o$  and total tonicity to achieve the desired conditions

(Materials and Methods, procedure B). Both diS-C<sub>3</sub>(5) and diI-C<sub>3</sub>(5) showed a marked response to pH<sub>c</sub> of around  $-20\% \Delta F/\text{unit } \Delta \text{pH}_c$  (cf. Hladky and Rink, 1967 *b, c*). Under the conditions employed, diI-C<sub>3</sub>(5) showed a smaller response than diS-C<sub>3</sub>(5) to membrane potential, the calibrations being  $+0.6\% \Delta F/\text{mV}$  for diI-C<sub>3</sub>(5) (Fig. 6 A and B) and  $+1.7\% \Delta F/\text{mV}$  for diS-C<sub>3</sub>(5) (Fig. 6 C). Around pH 7 a change of pH<sub>c</sub> of 0.1 unit would therefore mimic a membrane potential change of 2–3 mV. The independently determined relations of *F* to *E* and to pH<sub>c</sub> (Fig. 6) totally account for the fluorescence in the experiment in which *F* and pH<sub>c</sub> co-varied (Fig. 5, inset) and thus provide the basis for a method of determining *E* from dye fluorescence in the normal

TABLE III  
EFFECT OF pH<sub>o</sub> ON diS-C<sub>3</sub>(5) FLUORESCENCE AND ON MEMBRANE POTENTIAL OF NYSTATIN-TREATED RED CELLS

pH <sub>o</sub>	<i>F</i>	<i>E</i> <sub>Na+K</sub>	pH <sub>c</sub>	Δ <i>E</i> <sub>Na+K</sub>	ΔpH <sub>c</sub>	Δ <i>F</i> / <i>F</i> <sup>0</sup>
		<i>mV</i>		<i>mV</i>		
6.51	65	+0.5	6.49	+3.3	-0.74	+0.34
6.74	62	-0.5	6.71	+2.4	-0.52	+0.27
7.03	55	-1.7	6.98	+1.1	-0.25	+0.12
7.30	49 ± 1	-2.9	7.23	0	0	0
7.59	41	-4.1	7.50	-1.2	+0.27	-0.16
7.90	35	-5.4	7.78	-2.5	+0.55	-0.29
8.34	31	-7.3	8.19	-4.4	+0.96	-0.36
8.72	26	-8.9	8.54	-6.0	+1.31	-0.46
9.30	24	-11.3	9.08	-8.5	+1.85	-0.51

Human red cells were incubated at 5% hematocrit in media containing 145 mM NaCl, 5 mM KCl, 37 mM sucrose, 5 mM HEPES, and 50 μg/ml nystatin at the indicated pH<sub>o</sub> and at 25°C. The cells were washed twice in the same medium and the fluorescence was determined as described in Materials and Methods, Procedure A. The membrane potential, *E*<sub>Na+K</sub>, was calculated from  $E_{\text{Na+K}} = -(RT/\mathcal{F}) \ln r_{\text{Na+K}}$ , where  $r_{\text{Na+K}} = ([\text{Na}]_c + [\text{K}]_c)/([\text{Na}]_o + [\text{K}]_o)$ , and is taken from a linear regression of the points shown in Fig. 5. The pH<sub>c</sub> was estimated from  $\text{pH}_c = \text{pH}_o - \log r_{\text{Na+K}} - 0.023$  (Freedman and Hoffman, 1979, Eq. A12 *b*). Δ*E*<sub>Na+K</sub> and ΔpH<sub>c</sub> represent the changes in *E* and in pH<sub>c</sub>, respectively, between the indicated pH<sub>o</sub> and a reference pH<sub>o</sub> of 7.30. Δ*F*/*F*<sup>0</sup> is the relative change in fluorescence between the indicated final pH<sub>o</sub> and the initial value, *F*<sup>0</sup>, at pH 7.30, and is taken from the traces shown in Fig. 4. The values for *r*<sub>Na</sub> and cell water contents ( $0.668 \pm 0.015$ , SD, *n* = 6) for this experiment compare well with the predictions of a nonideal thermodynamic model (Freedman and Hoffman, 1979, Fig. 3, expt. B). Comparable fluorescence changes for nystatin-treated cells titrated between pH 6.1 and 8.8 were seen in three similar experiments, and comparable cation concentration ratios in three other similar experiments.

circumstance of simultaneous changes of *E* and pH<sub>c</sub> in red cells (see Discussion).

#### *Absorption Changes in Response to Membrane Potential*

From the study of Sims et al. (1974) it is known that red cells hyperpolarized with valinomycin have an increased amount of diS-C<sub>3</sub>(5) cations associated with the cells, and that the quantum yield of fluorescence decreases with increasing amounts of cell-associated dye. In extension of this work, we found (Freedman and Hoffman, 1977) that in the presence of red cells, the monomer absorption peak of diS-C<sub>3</sub>(5) is red-shifted by 10 nm and the dimer peak at

595 nm is greatly enhanced (Fig. 7 C) as compared with the spectrum of dye in the absence of cells. These peak assignments are based on comparisons with dyes of closely related structure (West and Pearce, 1965; Waggoner et al., 1977). Hladky and Rink (1976) reported that the enhanced absorption at 595 nm is due to binding of diS-C<sub>3</sub>(5) dimers to oxyhemoglobin, and Tsien and Hladky (1978) have further modelled the binding to Hb and to membranes so as to account, at least in part, for the absorbance and fluorescence at varied cell and dye concentrations, and at varied membrane potentials. A direct correlation between the change in the membrane potential and the shift in

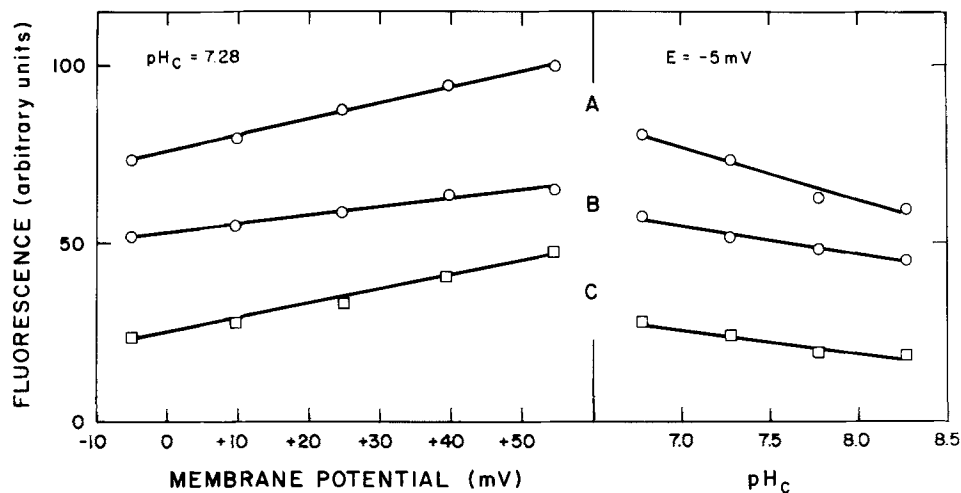


FIGURE 6. Calibration of fluorescence,  $F$ , of diS-C<sub>3</sub>(5) (□) and diI-C<sub>3</sub>(5) (○) with independent variation of membrane potential,  $E$ , and pH<sub>c</sub>. Cells of normal cation permeability were prepared as described in Materials and Methods, procedure B, and suspended in the media described in Table II.  $E$  was calculated according to the Jacobs-Stewart model, as modified for nonidealities (Freedman and Hoffman, 1979), and  $F$  was determined as described in Materials and Methods. Left panel: relation of  $F$  to  $E$  with pH<sub>c</sub> constant at 7.28. Right panel: relation of  $F$  to pH<sub>c</sub> with  $E$  constant at -5 mV. (A) 2.4 μM diI-C<sub>3</sub>(5); (B) 1.2 μM diI-C<sub>3</sub>(5); (C) 1.2 μM diS-C<sub>3</sub>(5). All points are the average of duplicate determinations.

the monomer-dimer equilibrium is shown by the absorption shifts in Figs. 7 C, 8, and 9. After hyperpolarization by addition of valinomycin at 5 mM K<sub>o</sub>, the monomer peak at 648 nm decreases and is red-shifted by an additional 8 nm, while the peak at 595 nm increases (Fig. 7 C). Smaller shifts in the opposite direction occur with depolarization at 150 mM K<sub>o</sub> (Fig. 8). Dual wavelength absorption traces (Fig. 8) show that the percentage decrease of the dual wavelength monomer signal (Fig. 9 B, lower curves) parallels the percentage decrease in fluorescence (Fig. 9 A) after valinomycin addition as K<sub>o</sub> is lowered. The reciprocal increases in the 595 nm peak (Fig. 9 B, upper curves) shows

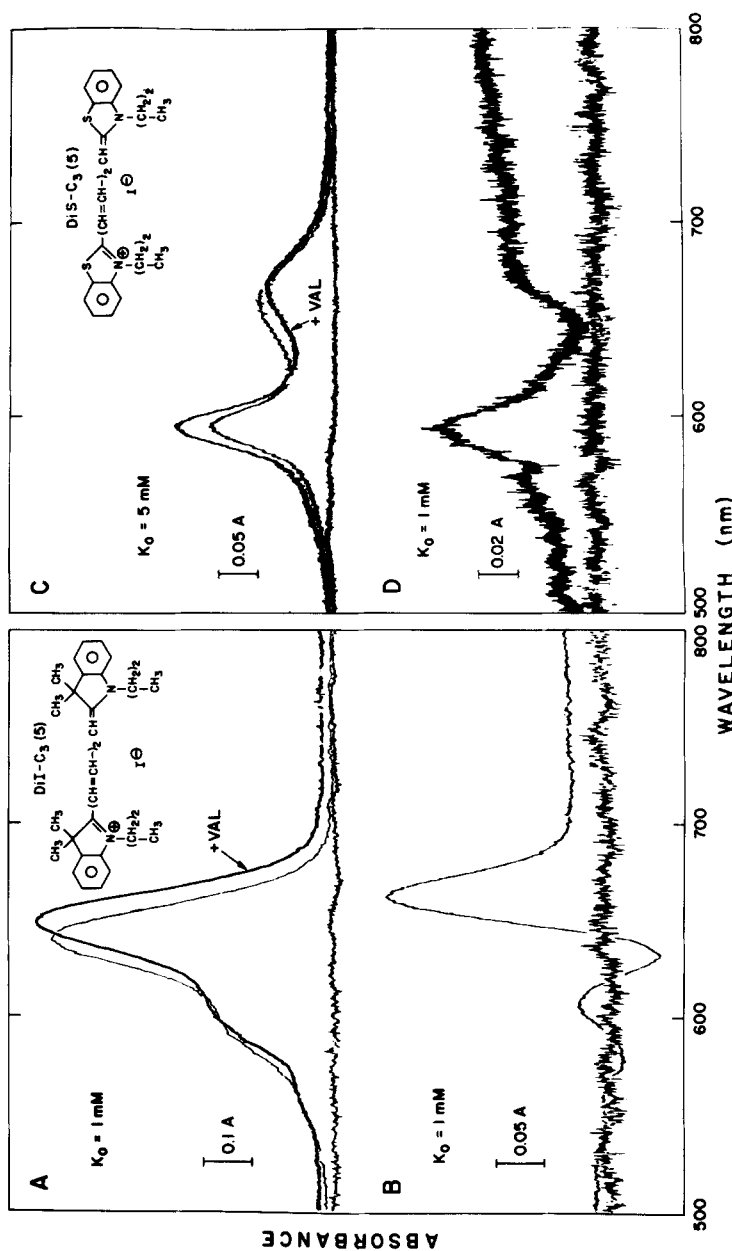


FIGURE 7 Potential-dependent absorption shifts of (A, B) diI-C<sub>3</sub>(5) and (C, D) diS-C<sub>3</sub>(5) (A) Split beam absorption spectra before and after hyperpolarization. To two cuvettes initially containing 3 ml of 145 mM NaCl, 5 mM KCl, and 5 mM HEPES (pH 7.4 at 25°C) was added 25  $\mu$ l of an ice-cold 50% red cell suspension, followed by addition of 10  $\mu$ l of dye (0.2 mg/ml EtOH, 1.2  $\mu$ M final concentration) to the sample cuvette alone. Spectra were recorded (1 nm/s) before and 1 min after addition of 10  $\mu$ l of valinomycin (VAL) (300  $\mu$ M in EtOH) to both cuvettes, a technique chosen to minimize scattering artifacts due to cell shrinkage. (B) Difference spectrum following addition of valinomycin to sample cuvette only. Both cuvettes initially contained cells plus dye in 149 mM NaCl, 1 mM KCl, and 5 mM HEPES (pH 7.4 at 25°C). The potential-dependent spectral shift is superimposed on the time-dependent rise in scattering during the scan. (C) Split beam absorption spectra of 1.2  $\mu$ M diS-C<sub>3</sub>(5) recorded (0.5 nm/s) before and 1 min after hyperpolarization by addition of valinomycin, with same conditions as in A. (D) Difference spectrum recorded following addition of valinomycin to sample cuvette only, as in B.

directly that dimers are at least one product of the fluorescence-quenching reaction(s).

In order to determine whether a monomer-dimer shift is essential for the large fluorescence changes obtained with dicarbocyanine dyes, A. S. Waggoner of Amherst College suggested using diI-C<sub>3</sub>(5), since this dye might not dimerize or polymerize as readily as diS-C<sub>3</sub>(5) due to steric interference of the parallel or nearly parallel stacking by the two methyl groups which are attached to a carbon atom in place of the sulphur of diS-C<sub>3</sub>(5), and which project perpendicular to the plane of the conjugated chain (see structures in Fig. 7). As with

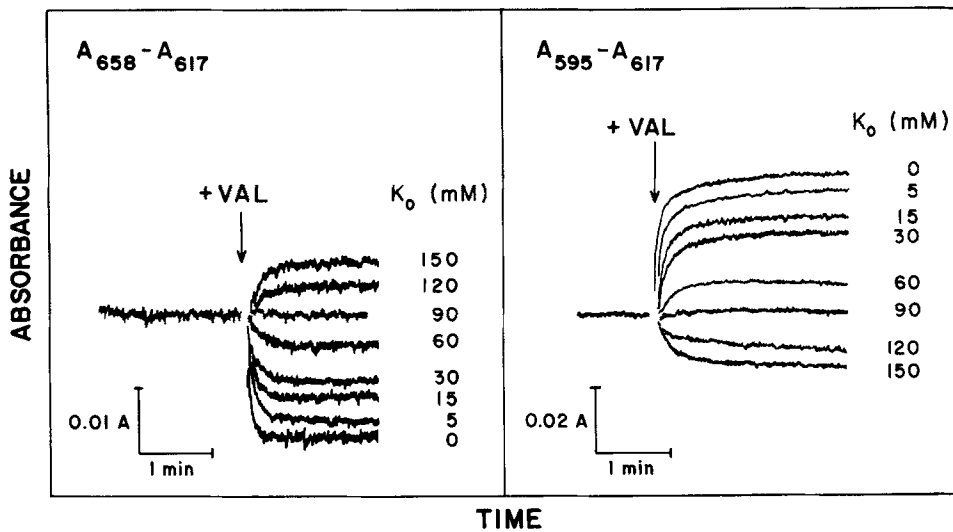


FIGURE 8. Potential-dependent dual wavelength absorbance shifts of diS-C<sub>3</sub>(5). To cuvettes containing 3 ml of (150 - *x*) mM NaCl, *x* mM KCl as indicated, and 5 mM HEPES (pH 7.4 at 25°C) was added 10  $\mu$ l (not shown) or 25  $\mu$ l of ice-cold 50% hematocrit suspensions. After equilibration to 25°C, 10  $\mu$ l of dye (0.2 mg/ml EtOH) was added and the initial dual wavelength absorbance recorded. Then 10  $\mu$ l of valinomycin (VAL) (300  $\mu$ M in EtOH) was added and the absorbance recorded until a new stable level was reached. Each tracing has been slightly shifted to the same initial absorbance. Left panel: monomer peak ( $A_{658} - A_{617}$ ); right panel: dimer peak ( $A_{595} - A_{617}$ ).

diS-C<sub>3</sub>(5), the absorption spectrum of diI-C<sub>3</sub>(5) in buffered isotonic solution (Fig. 10) shows a prominent monomer peak (639 nm) with a blue-shifted shoulder (600 nm); however, unlike diS-C<sub>3</sub>(5), (Sims et al., 1974), as dye concentration is increased, the absorption of the shoulder does not increase relative to the monomer peak and no higher order aggregates absorbing at high wavelengths (700–800 nm) were detected. Although it is not proved that diI-C<sub>3</sub>(5) does not form dimers, there is no indication of dimerization which leads to coupling of the dipole transition moments with a shifted absorption frequency. Furthermore, the linearity of the Beer's law plot (Fig. 10, lower inset) would imply the improbable properties that presumptive dimers must



have the same absorption frequency and exactly twice the extinction coefficient of monomers. More likely, the equilibrium constant for dimerization is so small that the number of dimers present can be neglected in the concentration range employed. That  $A_{639}/A_{600}$  is independent of dye concentration (Fig. 10, upper inset) indicates that the shoulder is most probably part of the monomer absorption of diI-C<sub>3</sub>(5) and is not due to dimers.

With diI-C<sub>3</sub>(5) the changes of fluorescence induced by valinomycin at

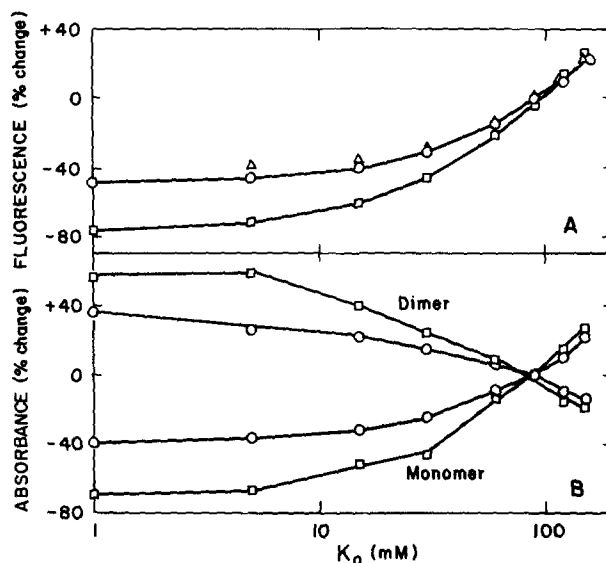


FIGURE 9. Correlation of potential-dependent changes in fluorescence and dual wavelength absorbance of diS-C<sub>3</sub>(5). (A) Percentage change in single beam fluorescence (○, □) after addition of valinomycin (1  $\mu$ M final concentration), determined as described in Fig. 2 with additions of 10  $\mu$ l (□) or 25  $\mu$ l (○, △) of cell suspension, giving 0.17% and 0.41% hematocrits, respectively, and using 1.2  $\mu$ M dye. The media contained (150 -  $x$ ) mM NaCl,  $x$  mM KCl, and 5 mM HEPES (pH 7.4 at 25°C) to give the indicated log  $K_o$ . The same results (△) are obtained with the differential fluorescence accessory to the split beam absorbance spectrophotometer, using the 3-mm-thick RG 665 cut-on filter. (B) Percentage changes in dual wavelength absorbance signals of monomer ( $A_{658} - A_{617}$ ) and dimer ( $A_{595} - A_{617}$ ) peaks, obtained from experiment described in Fig. 8.

varied  $K_o$  (Fig. 11) are comparable to those obtained with diS-C<sub>3</sub>(5) (Fig. 9 A), but the absorption spectrum (Fig. 7 A) shows no enhanced dimer peak in the presence of cells and no detectable monomer-dimer shift with hyperpolarization. The peak at 639 nm was red-shifted by 4 nm when diI-C<sub>3</sub>(5) was added to cells and by an additional 8 nm upon hyperpolarization, indicating a hydrophobic interaction. This red-shifting of the monomer peak was the only absorption change with diI-C<sub>3</sub>(5), demonstrating that stable dimers and

dye aggregates need not be implicated as a general feature of fluorescence quenching of dicarbocyanine dyes in red cell suspensions.

In order to clarify further the interactions responsible for fluorescence quenching of diI-C<sub>3</sub>(5), its absorption and fluorescence were measured in membrane-free hemolysates in comparison with diS-C<sub>3</sub>(5) (Table IV). The absorption spectrum of diI-C<sub>3</sub>(5) was unaltered in hemolysates, and the ratio of absorptions at the peak and shoulder ( $A_{639}/A_{600}$ ) was unaffected by pH, in contrast to 50% changes shown by diS-C<sub>3</sub>(5). The fluorescence of diI-C<sub>3</sub>(5) was not quenched in hemolysates even when the mole ratio of Hb to dye was

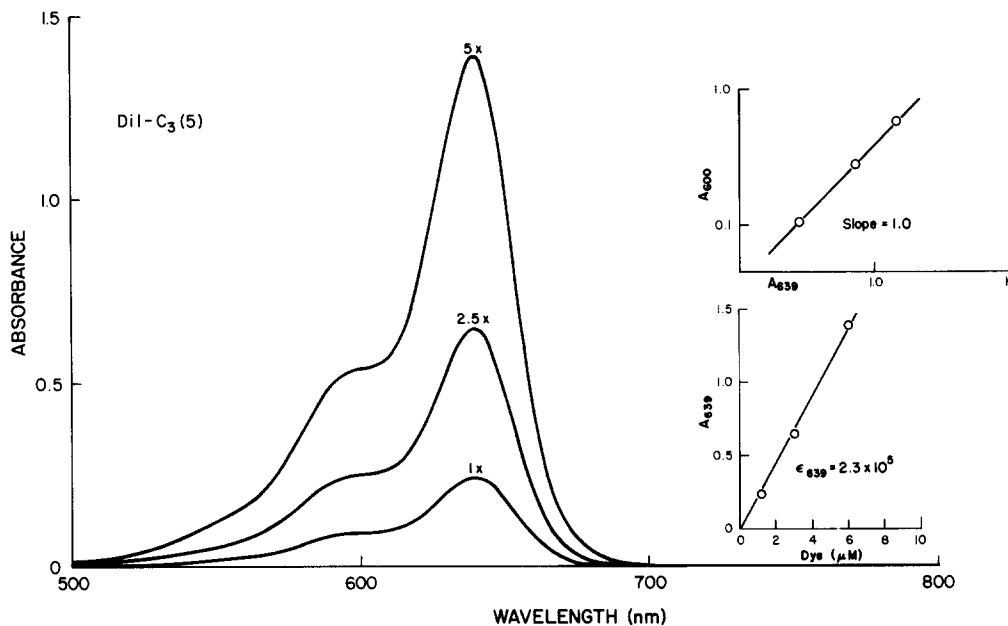


FIGURE 10. Split beam absorption spectra of diI-C<sub>3</sub>(5) in 145 mM NaCl 5 mM KCl, and 5 mM HEPES (pH 7.4 at 25°C) without cells at varying dye concentrations ( $1 \times = 1.2 \mu\text{M}$ ). The upper inset is a log-log plot of  $A_{600}$  vs.  $A_{639}$ , with the slope of unity indicating that  $A_{600}$  is part of the monomer absorption. The lower inset is a Beer's law plot giving the extinction coefficient of the main peak.

as high as 8. In contrast, at varying Hb and dye concentrations, diS-C<sub>3</sub>(5) fluorescence was quenched by 20–40%. Thus, unlike diS-C<sub>3</sub>(5), diI-C<sub>3</sub>(5) does not appear to interact with Hb in dilute hemolysates. The extent to which diI-C<sub>3</sub>(5) fluorescence is quenched by Hb at higher concentrations approaching that present in red cells (5 mM) and by interaction with the membrane remains to be tested

#### DISCUSSION

The most significant result in this paper is the ability to calibrate dye fluorescence vs. membrane potential in red cell suspensions (Fig. 6) with an

empirical method free from assumptions about relative ionic permeabilities, about the mechanism of dye response, or about the range of linearity between fluorescence and potential, and without requiring dye binding measurements.

It is evident from Fig. 6 that the fluorescence,  $F$ , of diS-C<sub>3</sub>(5) and of diI-C<sub>3</sub>(5) depends both on  $E$  and on  $\text{pH}_c$ , or

$$F = F(E, \text{pH}_c). \quad (1)$$

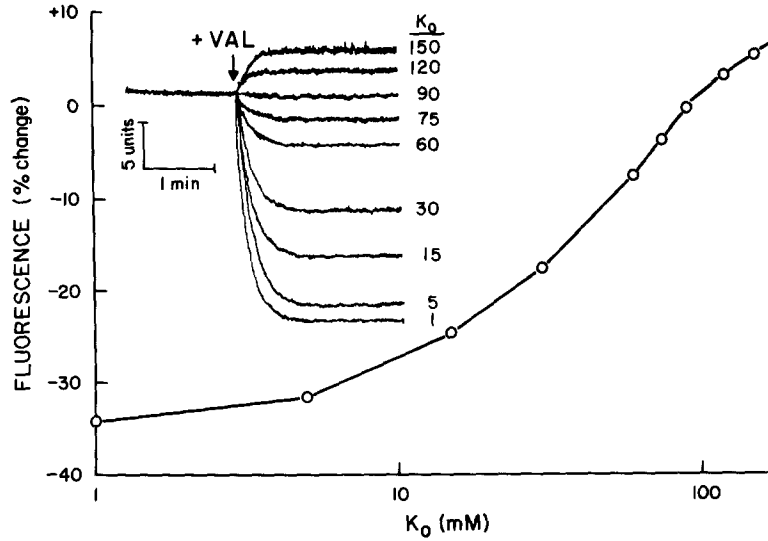


FIGURE 11. Percentage change of fluorescence of diI-C<sub>3</sub>(5) (1.2  $\mu\text{M}$  final concentration) following the addition of valinomycin (VAL) (1  $\mu\text{M}$  final concentration) to red cell suspensions (0.41% hematocrit) in  $x$  mM KCl (as indicated),  $150 - x$  mM NaCl, and 5 mM HEPES, (pH 7.4 at 25°C). The inset shows the initial level of fluorescence (after 3–5 min for equilibration) and the time-course to new steady levels after addition of valinomycin at various  $K_0$  (same protocol as in Fig. 2). Downward deflections indicate hyperpolarization and upward deflections indicate depolarization.

We now show how  $E$  can be determined from  $F$  in experiments in which  $\text{pH}_c$  also changes. The total differential of  $F$  is given by,

$$dF = \left( \frac{\partial F}{\partial E} \right)_{\text{pH}_c} dE + \left( \frac{\partial F}{\partial \text{pH}_c} \right)_E d\text{pH}_c. \quad (2)$$

From linear regressions of the data in Fig. 6 C, the partial derivatives for diS-C<sub>3</sub>(5) evaluated at  $\text{pH}_c = 7.28$  and  $E = -5$  mV are as follows:

$$\left( \frac{\partial F}{\partial E} \right)_{\text{pH}_c=7.28} = 0.40; \quad \left( \frac{\partial F}{\partial \text{pH}_c} \right)_{E=-5} = 6.5. \quad (3)$$

Thus, in the restricted regions where  $F$  is linear with both  $E$  and  $\text{pH}_c$ , we have,

$$\Delta F = 0.40 \Delta E + 6.5 \Delta \text{pH}_c. \quad (4)$$

In order to ascertain how well the partial derivatives determined in Fig. 6 account for the fluorescence of diS-C<sub>3</sub>(5) when  $E$  and  $\text{pH}_c$  change simultaneously (Fig. 5 and Table III), it is necessary to normalize Eq. 4 so as to allow comparison between the two experiments. In Table III, column 7, the fluores-

TABLE IV  
ABSORBANCE AND FLUORESCENCE OF diS-C<sub>3</sub>(5) AND diI-C<sub>3</sub>(5)  
IN MEMBRANE-FREE HEMOLYSATES OF RED BLOOD CELLS

		Absorbance	
pH		diS-C <sub>3</sub> (5) $A_{648}/A_{595}$	diI-C <sub>3</sub> (5) $A_{638}/A_{600}$
7.0		1.49	2.47
7.4		0.93	2.49
8.6		0.45	2.32
Hemoglobin $\mu\text{M}$	Dye $\mu\text{M}$	Fluorescence diS-C <sub>3</sub> (5)	$F/F_0$ diI-C <sub>3</sub> (5)
5	2.4	0.86, 0.65	0.96
5	1.2	0.66, 0.71	1.03
5	0.6	0.75, 0.61	1.03
16	2.4	0.53, 0.56	0.90, 0.85

Absorbance: the split-beam absorption spectrum of membrane-free hemolysate (3 ml) containing 5  $\mu\text{M}$  Hb and 1.2  $\mu\text{M}$  dye was recorded vs. hemolysate without dye. The pH of both cuvettes was then adjusted with less than 0.1 ml of 0.1 M NaOH or HCl and the spectra were recorded again. Hemolysates were prepared by adding 0.1 ml of a suspension of washed red cells at 50% hematocrit to 25 ml of cold 40 mM KCl, buffered with 5 mM HEPES (pH 7.4 at 25°C). After 5 min, sufficient 1 M KCl was added to restore the KCl concentrations to 50 mM. The membranes were sedimented at 20,000 rpm and the clear supernate was decanted.

Fluorescence: the fluorescence of dye in 3 ml of lysate,  $F$ , or in buffer alone,  $F_0$ , was measured 3 min after addition of dye. A membrane-free hemolysate, more concentrated than described above, was prepared by adding 1 vol of cells at 50% hematocrit to 9 vol of hemolyzing solution.

cence changes,  $\Delta F/F^0 = (\text{final-initial})/\text{initial}$ , are given normalized to an initial  $\text{pH}_o = 7.30$ , which was the initial  $\text{pH}_o$  for the fluorescence traces shown in Fig. 4. Since  $\text{pH}_c = 7.23$  and  $E = -2.9$  mV at  $\text{pH}_o = 7.30$ , the fluorescence data from Fig. 6 C may be normalized by dividing Eq. 4 by  $F^0 = 24$ , yielding

$$\frac{\Delta F}{F^0} = 0.017 \Delta E - 0.27 \Delta \text{pH}_c. \quad (5)$$

Eq. 5 states that the calibration of diS-C<sub>3</sub>(5) is +1.7 % $\Delta F/\text{mV}$  and -27 % $\Delta F/\text{unit } \Delta \text{pH}_c$ , as referred to an initial state where  $\text{pH}_o = 7.30$ . The inset

on Fig. 5 shows that the observed fluorescence changes (abscissa) agree well with the fluorescence changes (ordinate), as calculated by Eq. 5, when  $\Delta E$  and  $\Delta \text{pH}_c$  were estimated from Donnan ratios (Table III, columns 3-6).

Eq. 5 may be solved for  $\Delta E$  as follows:

$$\Delta E = 59 \frac{\Delta F}{F^0} + 16 \Delta \text{pH}_c. \quad (6)$$

While Eqs. 3 and 5 define the selectivity of dye fluorescence to  $E$  and to  $\text{pH}_c$ , Eq. 6 may be used to derive values for  $\Delta E$  from the fluorescence when  $\Delta \text{pH}_c$  is also known.

If  $\text{pH}_c$  is at equilibrium with  $\text{pH}_o$ , then the  $\Delta \text{pH}$  across the red cell membrane is covariant with  $E$  as follows:

$$E = (2.303RT/\mathcal{F})(\text{pH}_c - \text{pH}_o). \quad (7)$$

While a net movement of H or OH will occur until Eq. 7 is satisfied, the resultant changes in  $\text{pH}_c$  and  $\text{pH}_o$  depend on the relative buffering capacity and the volumes of the intracellular and extracellular solutions. The stronger the extracellular buffering, the more  $\text{pH}_c$  will change and the less  $\text{pH}_o$  will change. Under certain conditions, such as with valinomycin, the red cell membrane is able to maintain pH gradients and thus prevent rapid pH equilibration (Callahan and Hoffman, 1976), in which case  $\text{pH}_c$  changes less than it would if equilibrium were reached.

Differentiating Eq. 7 and substituting into Eq. 2, we obtain upon rearrangement an expression relating  $F$  to  $E$  and  $\text{pH}_o$ :

$$dF = \left[ \left( \frac{\partial F}{\partial E} \right)_{\text{pH}_c} + \frac{\mathcal{F}}{RT} \left( \frac{\partial F}{\partial \text{pH}_c} \right)_E \right] dE + \left( \frac{\partial F}{\partial \text{pH}_c} \right)_E d\text{pH}_o. \quad (8)$$

Eq. 8 states that at equilibrium, it is only necessary to know how  $\text{pH}_o$  changes in order to derive values of  $\Delta E$  from measurements of  $\Delta F$ , once the partial derivatives have been estimated. When Eqs. 6 or 8 are used to extract values of  $\Delta E$  from the measured values of  $\Delta F$  for the experiment in Fig. 5, the deviation from the values of  $\Delta E$  as estimated from the Donnan ratios averages  $4 \pm 3$  mV (SD,  $n = 8$ ). This agreement is satisfactory considering that the values of  $\Delta E$  are small and are calculated as small differences between large numbers. The conclusions to be drawn from this analysis are that Eq. 2 appears to be valid for the experiments reported and may prove useful for testing other dyes for selective responses to  $E$ . Also, a method has been developed which enables estimation of  $E$  from diS-C<sub>3</sub>(5) fluorescence in experiments in which there are simultaneous changes in  $\text{pH}_c$ . The resultant values of  $E$  will of course be more accurate when  $\Delta E$  is large and  $\Delta \text{pH}_c$  is small.

The mechanism of the interference by  $\text{pH}_c$  does not necessarily depend on dimerization of dye, since in intact red cells both dyes showed a fluorescence dependent on  $\text{pH}_c$ . In dilute hemolysates only diS-C<sub>3</sub>(5), and not diI-C<sub>3</sub>(5), showed pH sensitive absorption changes and fluorescence quenching indica-

tive of binding to cell solutes. The pH sensitive fluorescence suggests that there is a titratable component in the fluorescence quenching reaction for both dyes.

*Determination of  $P_{K,VAL}/P_{Cl}$*

After addition of valinomycin at varying  $K_o$  (Figs. 9 and 11) the membrane potential of human red cells may be described by the constant field equation (Goldman, 1943; Hodgkin and Katz, 1949). Considering K and Cl fluxes to be the only significant membrane currents, then the ratio of permeability constants,  $\alpha_{VAL} = P_{K,VAL}/P_{Cl}$ , is given as follows:

$$\alpha_{VAL} = (Cl_o - Cl_c D) / (K_o D - K_c), \quad (9)$$

where  $D = \exp(-\mathcal{F}E/RT)$ . Thus with  $K_c$ ,  $Cl_c$ , and  $Cl_o$  constant, each  $K_o$  for which  $E$  has been determined from the calibrated fluorescence gives an independent estimate of  $\alpha_{VAL}$ .

The equilibrium calibration (Fig. 6 and Table II) covers a range of primarily positive potentials (-5 to +55 mV), a range which overlaps the negative potentials in the valinomycin experiment (-79 to +2 mV; see Fig. 12) only at high  $K_o$ . But at high  $K_o$ ,  $\alpha_{VAL}$  is a very insensitive function of  $E$ , as evident in plots (not shown) of  $E$  vs.  $\alpha_{VAL}$  at various values of  $K_o$  (Eq. 9). However, the equilibrium calibration does overlap with the range of diffusion potentials induced by valinomycin for low-K cells in which the bulk of intracellular K has been replaced with Na by using *p*-chloromercuribenzenesulfonic acid. From the equilibrium data of Hoffman and Laris (1974, their Fig. 10), we estimated (Freedman and Hoffman, 1978 *b*) that the fluorescence of diO-C<sub>6</sub>(3) is linearly related to  $E$  between -5 and +55 mV according to the expression  $F = 18 + 0.13E$ , with constant  $pH_c = 7.26 \pm 0.06$  (SD,  $n = 5$ ). With a reference potential of -9 mV, the equilibrium calibration relating  $F$  and  $E$  is  $+0.77 \% \Delta F/mV$ , quite close to the value of  $0.71 \% \Delta F/mV$  originally obtained from the diffusion potentials at high  $K_o$ . Using the equilibrium calibration to obtain  $E$  for the eight values of  $K_o$  ranging from 3.5 to 100 mM (data of Hoffman and Laris, 1974, Fig. 4) with the low-K cells, for which case  $E$  is a sensitive function of  $\alpha_{VAL}$ , we obtain (Eq. 9)  $\alpha_{VAL} = 20 \pm 5$  (SD,  $n = 8$ ).

In this analysis of  $P_{K,VAL}/P_{Cl}$ , no corrections of the fluorescence were included for possible changes in  $pH_c$ , as might result from proton fluxes driven by the altered membrane potential. From a measured  $\Delta pH_o$  after addition of valinomycin to an unbuffered suspension,  $\Delta pH_c$  may be estimated from proton conservation and the known buffering capacity,  $m$ , of the cells as follows:

$$\Delta pH_c = [(1 - h)/h][Hb]wm \Delta(H^+)_o. \quad (10)$$

In the above expression,  $h$  is the hematocrit,  $w$  is the cell water content (0.719 liter/liter cells), and  $m = -10$  eq/mol Hb/pH<sub>c</sub> (Dalmark, 1975). Thus, from the reported  $\Delta pH_o = +0.1$  for a valinomycin-treated 1% hematocrit suspension in 160 mM NaCl (Kaplan and Passow, 1974),  $\Delta pH_c$  would be only  $-5(10^{-5})$ , and hence no corrections of the fluorescence data are needed (Eq. 5).

By using Donnan potentials to calibrate dicarbocyanine dye fluorescence, the analysis given above validates the assumption made previously (Hoffman and Laris, 1974) that  $E$  and  $\% \Delta F$  are linearly related in a range of positive potentials, thus allowing the determination of  $\alpha_{VAL}$  in low-K cells. The value we obtain from the calibrated dye fluorescence agrees with previous estimates obtained by different techniques (Hoffman and Laris, 1974; Hladky and

Rink, 1976 *c*; Hunter, 1977; Knauf et al., 1977), a result which supports the view that dicarbocyanine dyes may be used for quantitative measurements of membrane potential.

Hoffman and Laris (1974) estimated that  $\alpha_{\text{VAL}} = 3$  for valinomycin-treated red cells with normal  $K_c$ . This value, which is evidently an underestimate, was obtained by assuming that the calibration factor estimated at high  $K_o$  is constant over the entire range of  $K_o$ , and then by fitting curves similar to Fig. 9 A with the best value

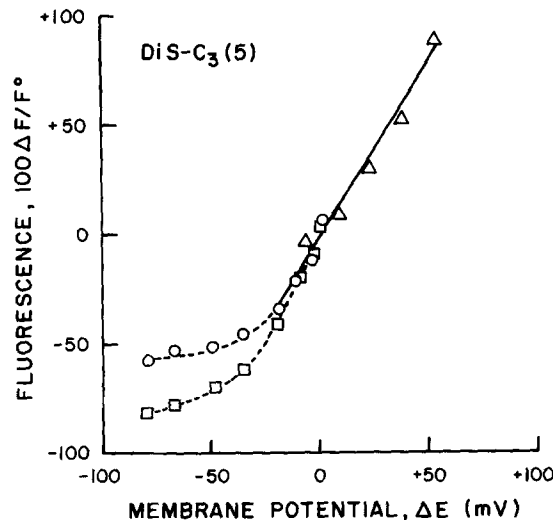


FIGURE 12. Relation of percentage changes of fluorescence of diS-C<sub>3</sub>(5) to changes in equilibrium and diffusion potentials,  $E$ . The circles and squares represent percentage changes of the single-beam fluorescence,  $100 \Delta F/F^0$ , induced by valinomycin at hematocrits of 0.41% and 0.17%, respectively, from the experiment described in Fig. 9 A. The diffusion potentials (-79 to +2 mV) were estimated from the constant field equation,  $E = 58 \log (\alpha K_o + Cl_o) / (\alpha K_c + Cl_o)$ , using  $\alpha = P_{K.VAL} / P_{Cl} = 20$  (see Discussion). From an assumed initial potential of -9 mV,  $Cl_o$  was estimated to be 105 mM at 150 mM  $Cl_o$ . Since the "null point," or  $K_o$  at which addition of valinomycin induced no change of  $F$ , was 95 mM,  $K_c$  was estimated to be 136 mM. The triangles represent changes in  $F$  obtained at 0.41% hematocrit when the equilibrium potential was altered (-6 to +54 mV) at constant  $pH_c$  by substituting impermeant external tartrate for  $Cl_o$  in the experiment of Fig. 6 C. The data are shown normalized to  $E = 0$  mV (see Discussion). A linear regression (solid curve) through the five tartrate points and the upper four valinomycin points at 0.41% hematocrit gives  $(\Delta F / F^0 \Delta E) = 0.016$ .

of  $\alpha_{\text{VAL}}$ . But this procedure results in values of  $\alpha_{\text{VAL}}$  which differ by a factor of 7 to account for the altered curvature when the hematocrit is reduced by a factor of 2 (Fig. 9 A). The reason that  $\alpha_{\text{VAL}}$  was underestimated for the high-K cells is that the calibration factor becomes nonlinear at large negative potentials, as may be seen in Fig. 12 (circles and squares), in which the measured  $\% \Delta F$  at two hematocrits is plotted against  $E$ , as calculated from the constant field equation with  $\alpha_{\text{VAL}} = 20$ . Similar plots show that for  $\alpha_{\text{VAL}}$  between 10 and 50, the relation between fluorescence and

potential is nonlinear for fluorescence changes greater than about 30% at 0.41% hematocrit and greater than about 50% at 0.17% hematocrit. Nonlinearity is predictable from the presence of a finite amount of dye available for partitioning with large fluorescence changes.

The results with diS-C<sub>3</sub>(5) (Fig. 12, solid line) also show that for a given hematocrit (0.41%) the same dye calibration,  $(\Delta F/F^0\Delta E) = 0.016$ , pertains both to the equilibrium potentials obtained by substituting external impermeant tartrate for Cl<sub>o</sub> at constant pH<sub>c</sub> (Fig. 12, triangles), and to the valinomycin-induced diffusion potentials (Fig. 12, circles) on the linear part of the curve at high K<sub>o</sub>. Thus, it appears that diS-C<sub>3</sub>(5) responds identically to changes in voltage, independently of whether or not ions are passing across the red cell membrane.

#### *Mechanism of Dye Response*

Although Hladky and Rink (1977) claim to have established the mechanism by which diS-C<sub>3</sub>(5) reports the membrane potential of human red blood cells (cf. Sims et al., 1974), the computer model of dye binding to membranes and to cell solutes (Tsien and Hladky, 1978) leaves a few questions unresolved. As discussed by Brand and Witholt (1967), fluorescence quenching may be either static or dynamic. In static quenching, stable complexes are formed between dye molecules in the ground state and some other cell component; the quantum yield of fluorescence of bound dye is less than of free dye. Alternatively, in a system as close-packed as the red cell interior, quenching may be dynamic, with energy dissipation due to formation of short-lived transient complexes between excited dye molecules and cell components. The absorption shifts (Fig. 7) are consistent with static quenching, but it is not known whether or not dynamic quenching is also operative in red cells. While stable complexes of diS-C<sub>3</sub>(5) with cell solutes undoubtedly account for a large component of quenching of cell-associated dye (Hladky and Rink, 1976 *a, c*; Tsien and Hladky, 1978), evidence also exists for a second reaction. Sims et al. (1974) reported that at a constant amount of cell-associated dye, valinomycin alters the quantum yield of fluorescence of cell-associated dye particularly at low dye concentrations. That effect cannot depend on dye in Donnan equilibrium changing its distribution across the membrane in accordance with an altered membrane potential, but suggests that a voltage-dependent association or redistribution of dye within the membrane also contributes some component to the signal. The finding that diI-C<sub>3</sub>(5) gives large fluorescence changes (Fig. 11) without detectable dimerization or aggregation (Fig. 7 A) may provide an important simplification in further studies of mechanism by enabling consideration only of reactions of dye monomers. Even diS-C<sub>3</sub>(5) does not require intracellular protein for fluorescence quenching since large fluorescence changes associated with a shift in the monomer-polymer equilibrium were obtained with liposomes using valinomycin and K-gradients (Sims et al., 1974). Guillet and Kimmich (1978) found that diS-C<sub>3</sub>(5) does not show dimers in avian red cell hemolysates and that diO-C<sub>3</sub>(5), which responds to diffusion potentials induced by ionophores in avian red cells (Kimmich et al., 1977),



also does not dimerize in human, chicken, or rat cell hemolysates. These observations and the findings that diS-C<sub>3</sub>(5) shows a shift in the monomer-dimer equilibrium correlating with the membrane potential of human red cells (Figs. 7–9), but a different spectral shift in mouse Ehrlich ascites tumor cells,<sup>1</sup> while diI-C<sub>3</sub>(5) responds by a different mechanism, all indicate that the same mechanism and calibration will not be applicable for different vesicles, organelles, cells, and dyes. In each system the mechanism and calibration will have to be independently evaluated.

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