Studies on the Mechanism by Which Cyanine Dyes Measure Membrane Potential in Red Blood Cells and Phosphatidylcholine Vesicles†

Peter Jay Sims,‡ Alan S. Waggoner,* Chao-Huei Wang, and Joseph F. Hoffman

ABSTRACT: Presented are the results of studies with 29 positively charged cyanine dyes that fluorimetrically respond to valinomycin-induced changes in the membrane potentials of red blood cell and phospholipid vesicle suspensions. Several of the dyes exhibit fluorescence changes in excess of 80% upon membrane hyperpolarization (inside becomes negative). The fluorescence response of the dyes is shown to result from potential-dependent partition of dye molecules between the cells and the extracellular medium. Cell hyperpolarization results in uptake of the dye molecules by the cells, while depolarization results in release of dye. The emission from cell-associated dye becomes significantly quenched as the amount of cell-associated dye increases. We present evidence that suggests that the quenching of cell-associated dye is due to formation of dye aggregates, which have reduced fluorescence.

The successful use of fluorescent probes that noncovalently interact with membranes to study membrane potentials in intact cell preparations has been reported for the squid giant axon (Cohen *et al.*, 1974; Davila *et al.*, 1973; Cohen, 1973; Tasaki *et al.*, 1972; Conti *et al.*, 1971) and the leech segmental ganglion (Salzberg *et al.*, 1974). Membrane potentials in human and *Amphiuma* red blood cells (Hoffman and Laris, 1974) and in synaptosome preparations (Goldring and Blaustein, 1974) have also been investigated using oxacarbocyanine dyes. These results suggest that nondestructive fluorescent probes may become useful tools for monitoring membrane potentials in small cells, organelles, and vesicle preparations that cannot be penetrated or are damaged by penetration with microelectrodes.

In experiments with human and *Amphiuma* red blood cells, Hoffman and Laris (1974) demonstrated that the fluorescence of 3,3′-dihexyloxacarbocyanine (henceforth, diO-C_6-(3))[1] added to stirred suspension of cells changes its fluorescence by as much as 50% when a large hyperpolarization of the cell is induced by valinomycin. In these fluorescence experiments, hyperpolarization of the cell was always accompanied by a decrease in fluorescence, and depolarization produced an increase in fluorescence. With both human and *Amphiuma* cells the fluorescence changed in proportion to the membrane potential calculated from the constant field equation, and with *Amphiuma* cells the fluorescence change was also in proportion to the potentials measured with microelectrodes. Furthermore, estimates of the membrane potential from the fluorescence measurements were in agreement with the values calculated for comparable conditions from the distribution of chloride at Donnan equilibrium.

An understanding of the molecular mechanisms by which different fluorescent probes sense membrane potential is important, for several reasons. Such knowledge will be useful for designing probes that are more sensitive to potential changes and less harmful to the membranes. Information concerning the mechanism is likely to be useful for assessing the meaning of fluorescence changes observed with different kinds of biological membranes and for relating the amount of fluorescence or the emission wavelength to the true membrane potential. It is also possible that knowledge of the potential sensing mechanism of the probes will make the probes useful for investigating the molecular structure and dynamics of excitable membranes.

With these thoughts in mind, we have focused our attention on the cyanine dyes and have examined the relative potential sensing capabilities of structurally modified members of this class of dyes. We have done this by adding the dyes to red blood cell preparations and measuring fluorescence changes that result when the red cell potentials are altered with the use of valinomycin (Hoffman and Laris, 1974). We have studied changes in the spectral properties with changing membrane potential of several cyanine dyes when they are associated with the red cells and with phospholipid vesicles. The partitioning of dye between the red cells and the medium external to the cells was also investigated as a function of dye structure, dye concentration, and membrane potential.

We found that the fluorescence changes result primarily from a potential-dependent partition of dye between the red cells and the suspension medium, and we suggest several possible mechanisms to explain the potential-dependent partition. Finally we demonstrate that the fluorescence quenching of cell-associated dye is dependent upon the quantity of dye associated with the cell and is probably due to the formation of dye aggregates with reduced fluorescence.

Methods

Preparation of Normal Human Red Blood Cells. Blood was drawn into heparin (0.15 mg/ml) from normal young male adults and was centrifuged at 10,000*g* for 2 min at 4°. The serum and buffy coat were removed by aspiration and the red

† From the Department of Chemistry, Amherst College, Amherst, Massachusetts 01002 (P. J. S., A. S. W., and C-H. W.), and the Department of Physiology, Yale University School of Medicine, New Haven, Connecticut 06510. *Received March 14, 1974.* This work was sponsored by National Institutes of Health Grants EY-00930 and NS-10439 to A. S. W. and Public Health Service Grant HL-09906 to J. F. H. and National Science Foundation Grant GB-18924 to J. F. H.

‡ Present address: Duke University School of Medicine, Durham, N. C. 27710.

[1] Abbreviations used are: diO-C_6-(3), 3,3′-dihexyloxacarbocyanine; KCl–Tris medium, 153 mM KCl and 17 mM Tris-Cl; PCMBS, *p*-chloromercuribenzenesulfonic acid; ANS^-, 8-anilino-1-naphthalenesulfonate$^-$.

TABLE I: Spectral Properties of Dyes.

Dye	Mol Wt	Solvent	λ_{abs}^{max} (nm)	ϵ_{abs}^{max} [a] ($\times 10^3$)	λ_{Fl}^{max} [b] (nm)	F_{rel}^{max} [c]	Photostability [d]
diO-C_2-(3)	460.33	H_2O	478	142	496	0.24	S
		EtOH	483	149	499	0.35	
		OctOH	488	140	504	1.1	
diO-C_2-(5)	486.36	H_2O	574	212	597	2.0	MS
		EtOH	579.5	237	604	2.8	
		OctOH	586	229	610	3.0	
diO-C_2-(7)	512.40	H_2O	676	185	701	2.0	U
		EtOH	684	220	714	3.6	
		OctOH	690.5	162	720	2.4	
diI-C_2-(3)	512.49	H_2O	540	133	556	0.13	S
		EtOH	546	133	563	0.29	
		OctOH	551	127	567.5	0.90	
diI-C_2-(5)	538.53	H_2O	636	190	657	0.89	S
		EtOH	639	200	664	1.7	
		OctOH	645.5	192	668	2.3	
diI-C_2-(7)	564.57	H_2O	735	198	756	0.68	S
		EtOH	741	240	768	1.5	
		OctOH	747	216	774.5	1.2	
diS-C_2-(3)	492.45	H_2O	551	138	568	0.21	S
		EtOH	557	161	575	0.32	
		OctOH	563	158	579	0.81	
diS-C_2-(5)	518.49	H_2O	643	194	666	1.8	MS
		EtOH	652	249	675	2.6	
		OctOH	658	233	682	2.6	
		Lutidine			688		
diS-C_2-(7)	544.53	H_2O	747				U
		EtOH	758	212	786	1.3	
		OctOH	767	203	792	1.0	

[a] The extinction values for dyes in water are subject to some error because of the tendency of the dyes to form dimers in water even at low concentrations. [b] Uncorrected for instrument sensitivity variation with wavelength, but within 3 nm of corrected value. [c] F_{rel}^{max} is the number of photons emitted in a narrow bandwidth at the emission maximum per photon absorbed, relative to a standard merocyanine dye, 5-{[3-ethyl-2*H*-benzoxazolylidene]-2-butenylidene}-1,3-diethyl-2-thiobarbituric acid. These values are corrected for excitation lamp intensity, monochrometer characteristics, and photomultiplier sensitivity (see Methods). [d] S = stable, decomposition <2%; MS = moderately stable, 2% < decomposition < 5%; U = unstable, decomposition >25%. The per cent decomposition represents the fraction of absorbance at λ_{abs}^{max} that disappears after 1 hr irradiation under standard conditions (see Methods).

cells were washed three times, each time with a five-volume solution containing 153 mM KCl and 17 mM trishydroxymethylaminomethane chloride (hereafter called KCl–Tris medium) at pH 7.4. The cells were suspended in KCl–Tris (pH 7.4) at a hematocrit of 50% and kept on ice until use that same day.

Preparation of Potassium-Depleted Red Blood Cells. The cationic composition of human red blood cells was altered to form low potassium containing cells by using the PCMBS (*p*-chloromercuribenzenesulfonic acid) method of Garrahan and Rega (1967) as modified by Sachs (1972). Cellular K and Na concentrations were determined on appropriately diluted packed cells by flame photometry with lithium as an internal standard. The total cellular concentrations were held constant by replacing K with either Na or choline chloride (see Hoffman and Laris, 1974).

Preparation of Phosphatidylcholine–Cholesterol Vesicles. Egg phosphatidylcholine was prepared by the method of Singleton *et al.* (1965), and stored in chloroform at −20°. The cholesterol was obtained from Eastman Chemicals (Primary Standard). In brief, the appropriate mixture of phosphatidylcholine and cholesterol was coated on the walls of a conical centrifuge tube and the chloroform solvent was removed under vacuum. One milliliter of 153 mM KCl–Tris was added per 4 mg of lipid and the mixture was sonicated for 3 min at 0–10° using the microtip of a Branson sonifier Model W140 at power setting 3. A 15-μl aliquot of the stock vesicle suspension formed above was added to 3 ml of NaCl–Tris solution containing 0.75 μg of dye for the absorption and fluorescence experiments.

Spectroscopic Measurements. Absorption spectra were obtained with either a Cary 14 or a Cary 15 spectrophotometer. Fluorescence measurements were performed with a Hitachi Perkin-Elmer MPF-3 spectrofluorimeter equipped with a Hamamatsu R-446 photomultiplier tube. A correction curve for the relative photon intensity exciting the sample as a function of wavelength was obtained with a YSI-Kettering Model 65 radiometer. The correction curve for emission monochrometer and photomultiplier tube sensitivity as a function of wavelength was obtained using an EG&G, Inc., standard lamp. The determinations of emission maxima and relative fluorescence intensities quoted in Table I were carried out with dilute dye samples exciting on a short-wavelength shoulder of the main absorption band. The absorbance of the sample at the wavelength of excitation was always less than 0.05. Although the emission maximum wavelengths quoted are uncorrected for in-

strument sensitivity, they are within 3 nm of the corrected value. The relative fluorescence intensities are corrected for the intensity of exciting light and sensitivity of the detection system. Although all of the dyes tested were soluble in ethanol and octanol, a number had a tendency to stick to the cuvet faces when dissolved in aqueous solutions. To account for this loss when determining extinction coefficients and relative fluorescence intensities, the aqueous contents of the cuvet were removed and ethanol was added to extract and measure the dye that had adsorbed to the cuvet.

Fluorescence Measurements on Intact Cells. The fluorescence experiments were performed with intact cell suspensions stirred magnetically with a glass fly inside a standard 1-cm path-length quartz fluorescence cuvet. All recordings were made with the excitation and emission slits set at 6 nm. The temperature was maintained constant at 23° by water circulating through the cuvet holder of the spectrofluorimeter. A 3-ml volume of suspension medium (NaCl–Tris, KCl–Tris, or combinations of the two) was added to the cuvet and after the temperature equilibrated, 10 μl of cells at a hematocrit of 50% in KCl–Tris was added to the stirred suspension medium to make a final hematocrit of 0.17%. The dye was added directly to the cell suspension from a stock ethanol solution while emission intensity (with time) was recorded. The excitation and emission wavelengths used for each dye are shown in Table II. Valinomycin (Sigma or Calbiochem) dissolved in ethanol (0.33 mg/ml of EtOH) was added directly to the cell suspension while stirring. The final concentration of valinomycin was 1.0×10^{-6} M unless noted otherwise. The ethanol concentrations in the cell suspensions were generally 0.5% and never exceeded 1.0%.

Measurement of Dye Partition between Cells and Medium. Determination of the relative molar distribution of the dye, diS-C_n-(5) (see Results for structure), between the red cells and the extracellular suspension medium necessitated the development of a procedure to measure the quantity of cell-associated dye coincident with equilibrium fluorescence measurements on the cell suspension. Because this dye and several of the other cyanines are notorious for their affinity for glass surfaces—in particular the quartz faces of the fluorescence cuvet and laboratory glassware—an indirect approach to the experimental determination of cell-associated dye was found most satisfactory. In these experiments, fluorescence measurements with the dye on intact cells in suspension were performed as described above. The corresponding quantity of dye associated with the cells during the fluorescence measurements on the suspension was obtained by subtracting from the total quantity of dye initially added to the suspension the experimentally determined values for the quantity of dye in the aqueous medium (extracellular) and the quantity of dye adsorbed to the surfaces of the cuvet. The concentration of dye in the extracellular medium was determined by centrifuging the suspension in the fluorescence cuvet to remove the cells from the optical path of the fluorimeter. The cuvet was centrifuged for 2 min at 2400 rpm in an International Clinical Centrifuge (Model CL) with the bottom of the cuvet held 10 cm from the rotor axis by a Plexiglas adapter fitted into the swinging bucket. The fluorescence intensity at 670 nm of the cell-free supernatant was compared to a standard curve, plotting emission intensity (at 670 nm) *vs.* dye concentration for diS-C_3-(5) in aqueous medium to obtain the quantity of dye in the medium. To determine the quantity of dye adsorbed to the cuvet surface the cells were resuspended in the medium by magnetic stirring, the suspension was completely removed from the cuvet by aspiration, and the dry cuvet was extracted for 15 min with stirred 3 ml of butanol. The fluorescence intensity at 670 nm of the butanol extract was compared with a standard curve, plotting emission intensity (670 nm) *vs.* dye concentration in butanol, to obtain the quantity of dye adhered to the surfaces of the cuvet. A second 3-ml butanol extraction of the cuvet established that the first 15-min extraction was 97–98% complete.

Determination of Hemolytic Activity of the Dyes. Each dye tested for hemolytic activity was added to a separate stirred suspension of 0.17% fresh human red cells in 3 ml of NaCl–Tris (pH 7.4). The final dye concentration was always 6.1×10^{-7} M and the final ethanol concentration was always less than 0.5%. Control samples were prepared by adding 0.5% ethanol to the cell suspension with no dye. Samples containing dye and controls were incubated at 23° with gentle stirring for 15 min, and then centrifuged at 10,000*g* at 4° for 2 min. The absorbance of the supernatant was measured at the 410-nm Soret band of hemoglobin. A reference sample with 100% hemolysis was obtained by lightly sonicating a 3-ml suspension of 0.17% red cells until the suspension was clear. This sample was then centrifuged at 15,000*g* for 20 min and the absorbance of the supernatant at 410 nm was obtained from a sevenfold dilution.

^{24}Na and ^{42}K Fluxes. Simultaneous measurements of the inward rate constants for Na and K in human red cells were made with ^{24}NaCl and ^{42}KCl (both isotopes obtained from International Chemical and Nuclear Corp.) by use of the method of Sachs and Welt (1967) as described by Hoffman and Laris (1974). The cells were at 0.33% suspension in a medium containing 148 mM NaCl, 5 mM KCl, 17 mM Tris-Cl (pH 7.5), and 1×10^{-4} M ouabain in the presence and absence of 2.9×10^{-6} M dye. Measurements of the outward rate constant for K in human cells were made with ^{42}K by the method of Hoffman (1962) adapted for use with intact cells. The cells were first loaded with ^{42}K during incubation of human cells in a medium which contained 148 mM NaCl + 5 mM KCl + 17 mM Tris-Cl (pH 7.5) at 37° for 2–3 hr. After incubation the cells were washed five times in ice-cold tracer-free media. After the final centrifugation aliquots of the packed cells were added to flasks under various conditions as indicated.

$^{35}SO_4$ Efflux. Human red blood cells were washed four to five times with a solution containing 93 mM Na_2SO_4 and 20 mM Tris adjusted to pH 7.5 with H_2SO_4 (Na_2SO_4–$Tris_2SO_4$ medium). The cells were then equilibrated during a 4-hr incubation at 37° in Na_2SO_4–$Tris_2SO_4$ medium containing a tracer quantity of carrier-free $^{35}SO_4$ in aqueous solution (obtained from Amersham Searle Corp.) and a trace of chloramphenicol. The hematocrit during equilibration was approximately 10%. After equilibration, the cells were washed five times at 0° with tracer-free Na_2SO_4–$Tris_2SO_4$ medium. The packed cells were then suspended at 0.3% hematocrit in tracer-free SO_4 media at either 22 or 37°. Immediately after mixing (zero time) and at 20-min intervals (up to 180 min), aliquots of the cell suspensions were removed and centrifuged. A portion of the supernatant fluid was then mixed with Triton X-100–toluene fluor and the radioactivity determined by liquid scintillation counting. From the rate of $^{35}SO_4$ appearance in the supernatant the outward rate constant was calculated by the method described by Gardos *et al.* (1969).

Dye Photosensitivity Measurements. Ethanol solutions of the dyes were irradiated in a 1-cm absorption cuvet placed 9 cm from the filament of a 75-W clear glass tungsten light bulb. A heat filter was placed between the sample and the light and the dye solution temperatures remained near 30°. The optical density of the solutions tested was generally 0.8. The relative decomposition of dye was determined by measuring the absorbance of the sample after 1-hr irradiation.

TABLE II: Cyanine Dyes: Per Cent Change with Valinomycin.[a]

Dye	Dye Concn ($\times 10^{-7}$ M)	C_2	C_3	C_4	C_5	C_6	C_7	C_8
diO-C_n-(3)								
λ_{Exc} = 470 nm	3.05	Slow increase	Slow increase	Fast increase	Fast increase	0	−6	−4
λ_{Em} = 500 nm	6.1	Slow increase	Slow increase	0	−8	−30	−20	−17
	12.2	Slow increase	Slow increase	−16	−39	−50	−31	−24
	18.3	Slow increase	0	−29	−46	−56	−34	−14
	24.4	Slow increase	−5	−30	−37	−52	−23	*b*
	30.5	0	−17	−31	−36	−51	*b*	*b*
	36.6	−8	−25	−29			*b*	
	48.8	−12				*b*		
diS-C_n-(3)								
λ_{Exc} = 520 nm	3.05	Slow increase	Slow increase	Fast increase	0	0	−6	−6
λ_{Em} = 595 nm	6.1	Slow increase	−25	0	−40	−32	−16	−24
	12.2	Slow increase	−46	−47	−54	−47	−17	−19
	18.3	0	−49	−58				
	24.4	−10	−52	−67	−60	−33	−4.5	−12
	30.5	−12	−58	−72				
	36.6		−58	−73		−25	−2	−5
	48.8	−19		−76	−60		*b*	*b*
diO-C_n-(5)	3.05	−45	−53.5	−60.5	−45	*b*		
λ_{Exc} = 560 nm	6.1	−57.5	−63	−61	−67	*b*		
λ_{Em} = 600 nm	9.15	−56	−67	−63	−67			
	12.2	−53	−68	−57	*b*			
	18.3	−48	−63	−52	*b*			
	24.4		−63		*b*			
	30.5	−42.5	−64	−47				
diS-C_n-(5)	3.05	−67	−66	−64	−55	−26		
λ_{Exc} = 622 nm	6.1	−82	−75	−75	−74	−50		
λ_{Em} = 670 nm	9.15	−82	−78	−80	−77	−50		
	12.2	−82	−83	−85	*b*	−50		
	18.3	−79	−86	−86	*b*	*b*		

Final Concn ($\times 10^{-7}$ M)	diI-C_5-(3) λ_{Exc} = 515, λ_{Em} = 560	diI-C_3-(5) λ_{Exc} = 600, λ_{Em} = 660	diI-C_2-(7) λ_{Exc} = 690, λ_{Em} = 760	diO-C_2-(7) λ_{Exc} = 640, λ_{Em} = 700	diS-C_2-(7) λ_{Exc} = 700, λ_{Em} = 770
3.05	Small	−44	−59	−79	−61
6.1	−24	−56	−74	−80	−78
9.15	−33	−61			
12.2	−37	−68	−77	−81	−85
18.3	−38	−70	−76	−79	−88
24.4	−38	−71	−74	−76	−88
30.5	−37	−72	−73		
46.6		−72			

[a] Changes in fluorescence (in per cent) recorded after the addition of valinomycin to cell suspensions equilibrated with cyanine dyes in K-free medium. Each dye was added in ethanol (final ethanol concentration <0.5%) to 0.17% suspensions of normal red cells in 153 mM NaCl plus 17 mM Tris (pH 7.4) at 23°. Final dye concentrations are given in the tables. The fluorescence was recorded at the wavelengths of excitation and emission given in the tables and after a steady level of fluorescence was observed (approximately 3–5 min) valinomycin was added at a final concentration of 1.0×10^{-6} M. The resulting change in fluorescence was recorded and expressed at a per cent of the level of fluorescence observed prior to the addition of valinomycin. [b] Indicates an unstable response, characterized by a fluorescence decrease after the addition of valinomycin, followed by a gradual increase in fluorescence to the level observed prior to the addition of valinomycin to the suspension.

Dye Syntheses. The cyanine dyes were synthesized from either 2-methylbenzothiazole, 2-methylbenzoxazole, or 2,3,3-trimethylindolenine; triethyl orthoformate, 1,3,3-trimethoxypropene, or 1-anilino-5-anilopenta-1,3-diene hydrochloride; and the appropriate alkyl iodide or alkyl sulfonate according to general procedures described in a review by Hamer (1964) and references therein. With the exception of the ethyl derivatives, the synthesis and properties of most of the dyes used in this work are not in the literature. The details of the synthetic procedures for a trimethine dye, a pentamethine dye, and a heptamethine dye are described below as examples.

3,3'-Dihexylthiocarbocyanine Iodide, diS-C_6-(3). The 2-methylbenzothiazole (7.5 g, 50 mmol) and 1-iodohexane (12.7 g, 60 mmol) were heated together at 130° for 2 days. The solid was filtered, washed well with ether, and recrystallized from methanol–ether solution to give 12.5 g of 3-hexyl-2-methylbenzothiazolium iodide, mp 115–117°.

The 3-hexyl-2-methylbenzothiazolium iodide (3.6 g, 10 mmol) and triethyl orthoformate (5 g, 30 mmol) in 20 ml of pyridine were refluxed for 2 hr and poured into 100 ml of cold water. The tar which separated was recrystallized from ethanol to give 2.1 g of pure dye in large greenish prisms, mp 198–200°.

3,3'-Dipropyloxadicarbocyanine Iodide, diO-C_3-(5). The 2-methylbenzoxazole (13.3 g, 0.1 mol) and 1-iodopropane (20 g, 0.12 mol) were refluxed for 10 hr. The solid was collected and washed with ether to give 19 g of 3-propyl-2-ethylbenzoxazolium iodide, mp 194–197°.

The propyl-2-methylbenzoxazolium iodide (3 g, 10 mmol) and 1,3,3-trimethoxypropene (3 g, 22 mmol) in 40 ml of pyridine were refluxed for 15 min. At the end a hot solution containing 10 g of potassium iodide in 30 ml of water was added and the whole solution was stirred for another 10 min. The crystals which formed the next day were collected and recrystallized from acetone to give 1.5 g of pure dye in crystals with blue luster, mp 233–234°.

3,3'-Dipropylindotricarbocyanine Iodide, diI-C_3-(7). The 2,3,3-trimethylindolenine (4.8 g, 30 mmol) and 1-iodopropane (8.5 g, 50 mmol) were refluxed for 4 hr. The solid was collected, washed with ether, and recrystallized from methanol–ether solution to give 6.6 g of 1-propyl-2,3,3-trimethylpseudoindolium iodide, mp 150–151°.

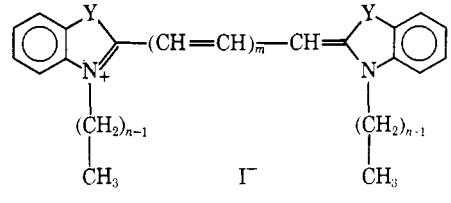
The above quaternary salt (3.3 g, 10 mmol), 1-anilino-5-anilopenta-1,3-diene hydrochloride (1.45 g, 5 mmol), and 1 g of anhydrous sodium acetate in 30 ml of acetic anhydride were refluxed for 1 hr and poured into a hot solution containing 10 g of potassium iodide in 30 ml of water. The crude dye which had precipitated after chilling was collected, yield 1.8 g. The crude dye was recrystallized from an ethanol–petroleum ether solution to give 1 g of pure dye as red-brown crystals, mp 170–173°.

When chromatographed on Eastman Chromatogram silica gel thin-layer sheets with several different solvent systems, each dye showed a single visible spot. Iodine staining revealed no additional spots.

The melting points of the cyanine dyes used in this study are listed in Table III.

TABLE III: Melting Points of the Cyanine Dyes.

X	*n*	Recrystallization Solvent	Mp (°C)
	a. Carbocyanine Dyes, diX-C_n-(3)		
O	2	MeOH	272–273
O	3	MeOH	283–284
O	4	MeOH	233–234
O	5	MeOH	214–215
O	6	EtOH	222–224
O	7	EtOH	194–197
O	8	EtOH	201–203
S	2	EtOH	252–253
S	3	Dimethylformamide–CCl_4	283–284
S	4	$CHCl_3$–CCl_4	278–280
S	5	EtOH	213–214
S	6	EtOH	198–200
S	7	EtOH	216–217
S	8	EtOH	222–223
I	5	EtOH–ether	163–165
	b. Dicarbocyanine Dyes, diX-C_n-(5)		
O	2	MeOH	231–232
O	3	Acetone	233–234
O	4	Acetone	217–218
O	5	Acetone–ether	172–175
O	6	Acetone–petroleum ether	165–168
S	2	MeOH	241–243
S	3	MeOH	248–249
S	4	MeOH	225–228
S	5	EtOH	208–210
S	6	EtOH	144–145
I	3	MeOH–ether	226–229
	c. Tricarbocyanine Dyes, diX-C_n-(7)		
O	2	EtOH–ether	160–161
O	3	EtOH–ether	144–145
S	2	MeOH	221–222
S	3	MeOH	172–174
I	2	EtOH–petroleum ether	175–177
I	3	EtOH–petroleum ether	170–173



where Y = O, S, or $(CH_3)C(CH_3)$; *n* varies from 2 to 18; *m* is 1, 2, or 3; and the counterion is iodide. The shorthand notation we have adopted for these dyes is diY—C_n—$(2m + 1)$, where diY indicates the particular heterocyclic nucleus of the symmetric dye, C_n gives the number of carbon atoms in the alkyl chains attached to the nitrogen atom of each nucleus, and the value in parentheses is the number of methine (—CH=) groups bridging the two nuclei. When the nucleus is an indolenine, Y = $(CH_3)C(CH_3)$≡I.

The absorption and emission properties of the dyes with ethyl groups on the nuclei (C_2- derivatives) together with the photostability of the dyes are shown in Table I. The dyes with *n*

Results

Dye Structure and Spectral Properties. For this paper 29 members of the cyanine class of dyes (Hamer, 1964) were examined for their possible usefulness as probes of membrane potential. The general structure of these dyes is

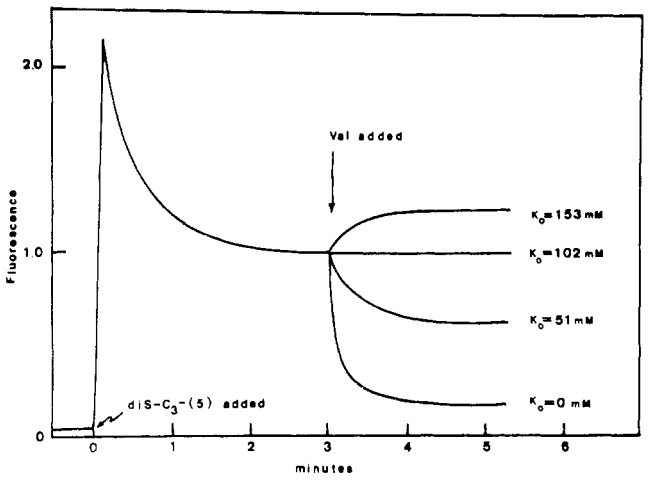


FIGURE 1: Characteristic changes in fluorescence intensity with time of diS-C_3-(5) (6.1×10^{-7} M) in a 0.17% suspension of normal human red cells in NaCl-Tris medium (pH 7.4) and in mixtures where KCl-Tris was substituted for NaCl-Tris to give the $[K_0^+]$ values listed in the figure. $[K_c^+]$ = 152 mmol/l. of cell H_2O. Dye and valinomycin (final concentration, 1.0×10^{-6} M) were added where indicated. This figure presents the tracings of actual recordings obtained in a single experiment that have been normalized to the same steady level of fluorescence prior to the addition of valinomycin. Fluorescence was recorded at 670 nm with excitation at 622 nm.

greater than 2 are not listed in this table because their spectral properties are essentially the same as the ethyl derivatives. A number of observations can be made from the data in Table I. First, the wavelengths of maximum absorption (λ_{abs}^{max}) and emission (λ_{Fl}^{max}) of the different dyes range over more than 300 nm and depend on both Y and the number of methine groups in the bridge between the nuclei. λ_{abs}^{max} and λ_{Fl}^{max} increase as Y is changed from O to I and from I to S, and when the methine bridge is lengthened from 3 to 5 and from 5 to 7 carbon atoms. Second, the extinction coefficients for all the dyes are large, greater than 10^5, with the pentamethines and heptamethines having values about 50% larger than the trimethine dyes. The relative fluorescence intensity at the emission maximum, F_{rel}^{max}, is higher by a factor of 10 for the penta- and heptamethine dyes as compared with the trimethine dyes. The higher fluorescence efficiencies of the dyes that emit at longer wavelengths is desirable for membrane experiments because many vacuum photocathodes (but not photodiodes) become much less sensitive at longer wavelengths. Although the F_{rel}^{max} values in Table I are not the quantum yields for the dyes, the values are expected to be roughly proportional to the quantum yields because the emission peak shapes and widths at half-maximum of the dyes are similar on a wavenumber scale. Since the quantum yields in methanol of diO-C_2-(3) and diO-C_2-(5) are 0.04 and 0.49, respectively (courtesy of D. Hesseltine, Eastman Kodak), the trimethine dyes in Table I probably have quantum yields in ethanol between 0.02 and 0.06, and the penta- and heptamethine dyes probably have quantum yields between 0.3 and 0.8. Thus the dyes listed, and particularly the penta- and heptamethine dyes, are very efficient absorbers and emitters of photons.

A third observation concerns the solvent sensitivity of the spectral properties of the dyes in Table I. Both the absorption and emission maxima of each of the dyes shifts to the red between 10 and 20 nm in going from water to a relatively nonpolar solvent, octanol. The emission intensity of the cyanine dyes is not as sensitive to changes in solvent as other fluorescent molecules, such as ANS^-, has been found to be (Weber and Laurence, 1954; Stryer, 1965). F_{max} for the cyanine dyes studied increases by approximately 50–100% in going from water to ethanol. In going from ethanol to octanol, F_{max} increases by a factor of 3 for the trimethine dyes, remains nearly constant for the pentamethine dyes, and decreases slightly for the heptamethine dyes. Generally speaking, the cyanine dyes are 1.5–4 times more fluorescent in organic alcohols than in water.

Finally, the tri- and pentamethine cyanine dyes in ethanol are moderately stable in the presence of high intensity irradiation in the region of their absorption maximums. The heptamethine dyes are much less stable, but bubbling the ethanol solution with N_2 reduced the rate of photodecomposition by more than half. The dyes with the indolenine nucleus are the most stable of the dyes for any constant methine bridge length.

Dependence of diS-C_3-(5) Fluorescence on Red Cell Membrane Potential. The membrane potential of red blood cells at 23° can be expected to obey the constant field eq 1 (Goldman, 1943; Hodgkin and Katz, 1949), adapted for use with red cells at 23° (see Hoffman and Laris, 1974)

$$V_M = 59mV \log \frac{\alpha[K]_0 + [Cl]_c}{\alpha[K]_c + [Cl]_0} \qquad (1)$$

where $[K]_0$ and $[Cl]_0$ are the K^+ and Cl^- concentrations in the medium; $[K]_c$ and $[Cl]_c$ are the K^+ and Cl^- concentrations in the cell water; and $\alpha = P_K/P_{Cl}$ is the ratio of the permeability constants of K^+ and Cl^-. In the normal cell P_{Cl} is estimated to be about 100 times greater than P_K (Hunter, 1971) and, provided that Cl^- is distributed passively, the resting membrane potential should be equivalent to the chloride equilibrium potential. Since chloride is distributed in accordance with a Donnan equilibrium, the membrane potential of a red cell is calculated to be, under normal conditions, about −8 mV, inside negative. Hoffman and Laris (1974) using diO-C_6-(3) and diS-C_3-(5) have fluorimetrically estimated the resting potential to be between −5 and −9 mV.

Addition of valinomycin, a cyclic antibiotic known to selectively transport potassium across membranes (Harris and Pressman, 1967; Andreoli *et al.*, 1967; Tosteson *et al.*, 1967), to a suspension of red cells will greatly increase P_K. Thus V_M will move from −8 mV toward the K^+ equilibrium potential when valinomycin is added. By altering the K^+ concentration of the medium, while maintaining Na plus K, and therefore the total osmolarity constant, V_M, can be altered over a wide range when valinomycin is added. For example, the largest hyperpolarization (the inside of the cell becomes more negative) is obtained by adding valinomycin to normal cells suspended in a K^+-free medium (153 mM NaCl). Using an α value of 3 (Hoffman and Laris, 1974) and the experimentally measured concentrations $[K]_c$ = 153 mmol/l. of cell H_2O, $[Cl]_0$ = 153 mmol/l. of cell H_2O, and $[Cl]_c$ = 117 mmol/l. of cell H_2O, V_M after addition of valinomycin to cells in a potassium free medium is expected to be around −40 mV. The maximum depolarization of normal cells is obtained when the cells are suspended in 153 mM KCl and is expected to be +3 to +5 mV. It should be noted that these calculated potentials are only approximate since considerable uncertainty is associated with α (Hoffman and Laris, 1974) especially since α may not be an independent variable. Thus, the extremes of V_M accessible with valinomycin could be twice the values just calculated.

Most of the dyes listed in Table I and their longer chained derivatives show changes in fluorescence if they are added to a stirred suspension of red blood cells and the potential across the cell membrane is modified by addition of valinomycin. An example of the changes that can be observed is shown in Figure 1. In this figure the fluorescence of 6.1×10^{-7} M diS-C_3-(5), added to normal red cells in four different suspension media, is

monitored with time. Within three minutes of the time that the dye is added to the suspension the fluorescence at 670 nm decreases to a steady intensity, which is assigned a relative value of 1.0. After this time a new membrane potential is established across the cell membrane, by addition of valinomycin. The fluorescence rapidly attains a new value. It is clear from Figure 1 that when the cells are *depolarized* in 153 mM KCl–Tris the fluorescence *increases*. As the cells are *hyperpolarized* in low K^+ media the fluorescence *decreases*. This is the same result observed for diO-C_6-(3) by Hoffman and Laris (1974).

It should also be mentioned that K^+-depleted red cells (see Methods) which contain only 2 mM K show increases in diS-C_3-(5) fluorescence as large as 200% when these cells are suspended in 153 mM KCl–Tris and valinomycin is added. When the K^+ concentration of the medium in this circumstance is lowered, the cell should depolarize as anticipated on the basis of the constant field equation (eq 1). As expected, the size of the fluorescence change decreased as the magnitude of the depolarization decreased.

Dye Survey. Table II presents the fluorescence responses of structurally related cyanine dyes to the same degree of hyperpolarization of the red cell membrane as induced by valinomycin. The maximal degree of hyperpolarization is established by the addition of valinomycin (1.0×10^{-6} M) to normal cells suspended in K-free medium. This survey serves two purposes. It provides data indicating which dye structures are most responsive (*i.e.*, show largest changes in fluorescence intensity) to alterations in membrane potential, and are, therefore, most likely to be of use in monitoring membrane potentials. The survey also provides clues to the mechanism(s) by which such dyes respond to membrane potential. In particular, by comparing the response of analogous structures with modifications in either the "Y-group" or the length of the methine bridge of the chromophore, the importance of the chromophore structure in the response of the dye to membrane potential can be evaluated. The table also compares dyes with the same chromophore but with different alkyl chain lengths. Since this alteration does not affect the electronic properties of the chromophore, the importance of the membrane affinity of the dye, which is expected to be strongly affected by the hydrocarbon chain length, can also be evaluated.

From the data of Table II, it is apparent that the ability to respond to membrane potential is common to at least 9 different chromophore structures (Y = O, I, and S; m = 1, 2, and 3) of the cyanine class of dyes. It is also apparent that for each of the basic chromophore structures the fluorescence response to cell hyperpolarization strongly depends upon both the length of the alkyl chain attached to the chromophore as well as upon the concentration at which the dye is added to the cell suspension. In general, it can be seen that dyes with longer alkyl chains exhibit maximal fluorescence changes at lower dye concentrations than their short-chain analogs. It is interesting to note that at low concentrations, the trimethine dyes (diY-C_n-(3)) show an *increase* in fluorescence with cell hyperpolarization when the alkyl chain is 2 or 3 carbons long. With these structures, however, as n becomes larger the increase in fluorescence is no longer observed and the usual decrease with cell hyperpolarization is exhibited. As n becomes even larger (long alkyl chains) the magnitude of the fluorescence change begins to diminish. This trend is most apparent with diO-C_n-(3) at 18.3×10^{-7} M. The observations suggest that the mechanism responsible for the fluorescence change may depend partly on the partition of dye between the membrane and the extracellular medium, because increasing the alkyl chain length, as we shall show later, increases the ratio of cell-associated dye to dye in the medium. It is also likely that the total amount of dye associated with the cells is an important feature of the mechanism of the fluorescence change since the magnitude of the change is concentration dependent. In support of this conclusion is the observation that in experiments with the dyes using suspensions containing 0.33% red cells instead of the usual 0.17% suspension the concentrations of dye necessary for optimum fluorescence responses was found to approximately double.

Solely on the basis of the magnitude of fluorescence change that can be elicited with hyperpolarization, many of the dyes surveyed in Table II can be considered as possibly useful probes of membrane potential. The dyes diS-C_2-(5), diS-C_3-(5), and diS-C_4-(5), however, are exceptional, showing fluorescence changes in excess of 80% under the appropriate conditions. Although fluorescence responses of this magnitude were observed with the heptamethine diS-C_2-(7) (see Table II), its instability in aqueous solution severely limits its potential usefulness.

The time required for the fluorescence change to be completed after hyperpolarization was found to depend strongly upon the length of the alkyl chain attached to the dye. In general, the longer the alkyl chain the faster the change in fluorescence. For the dyes diS-C_n-(5), the times in seconds for half the total fluorescence change to take place were C_2 = 30, C_3 = 8, C_4 = 6, $C_5 \sim 4$, and $C_6 < 2$. The estimated time for valinomycin mixing was 1–2 sec. The time courses of fluorescence changes of other cyanine dyes depended, in a similar manner, upon alkyl chain length.

Hemolytic Activity. If a probe is to be useful for measuring membrane potentials it is desirable that it not damage the membrane or alter normal ion fluxes. We have assayed the hemolytic effects of the dyes incubated for 15 min with normal red cells in suspension at 23°. Table IV shows the percent of total cell hemoglobin that is released from cells incubated with the dyes (dye concentration, 6.1×10^{-7} M with a 0.17% red cell suspension) as determined by the hemoglobin content of the supernatant after centrifugation of the cell suspension (see Methods). The hemolysis of cells incubated with all of the C_2 and C_3 dye structures did not exceed the extent of hemolysis detected in the control (dye free) samples, suggesting that the lytic effects of these dyes upon the membrane is negligible. The diS-C_n-(3) and diO-C_n-(3) dyes showed little membrane damage (hemolysis less than 1%) up through the C_5 structure. Some dyes, for example diS-C_5-(5) and other dyes which are underlined, were very hemolytic. The greater hemolytic activity often observed to occur at intermediate alkyl chain lengths for dyes with the same chromophore is not understood. Nevertheless, the data were reproducible within ±5% in assays performed in three separate experiments. In order to reduce the likelihood of contaminants in the dye samples, attempts were made to assure the purity of these dyes by multiple solvent recrystallizations and sometimes column chromatography. Since we do not know the mechanism by which these dyes disrupt the membrane, we cannot speculate about the apparently specific interaction that changes so abruptly with alkyl chain length to produce hemolysis.

Investigation of the Potential-Sensitive Fluorochrome diS-C_3-(5). The dye diS-C_3-(5) has been shown to exhibit a substantially large and reasonably fast fluorescence change in response to changes in membrane potential when the dye is added at relatively low concentrations to cell suspensions (Table II). Its absorption and emission maxima are far into the red region of the spectrum, away from the major hemoglobin absorption bands, and the dye has been shown to be negligibly

TABLE IV: Per Cent of Total Cell Hemolysis[a]

Dye	C_2	C_3	C_4	C_5	C_6	C_7	C_8
diO-C_n-(3)	0.67	0.27	0.33	0.39	0.73	*3.9*	*3.15*
diO-C_n-(5)	0.82	0.60	1.0	*1.45*	*5.5*		
diO-C_n-(7)		*1.2*					
diI-C_n-(3)				0.95			
diI-C_n-(5)		0.90					
diI-C_n-(7)		*1.4*					
diS-C_n-(3)	0.55	0.24	0.41	0.77	*4.8*	0.79	0.53
diS-C_n-(5)	0.40	0.29	*1.9*	*12.7*	*2.5*	0.93	0.95
diS-C_n-(7)	0.79						

Controls (no dye): 0.5 ± 0.2%

[a] Per cent of total cell hemolysis after 15-min incubation at 23° with cyanine dyes at a concentration of 6.1×10^{-7} M. Data obtained from normal human red cells suspended at 0.17% in 153 mM NaCl–17 mM Tris (pH 7.4) with 0.5% ethanol. For each basic chromophore structure, per cent hemolysis after 15 min is presented as a function of the length of the alkyl chain substituent (C_n). Control suspensions of cells incubated as above but with no dye show 0.5 (±0.2)% hemolysis. See Methods for experimental procedure.

TABLE V: diS-C_3-(5): Effect on Ion Flux.

	$^{i}k_{Na}$[a] (hr^{-1})		$^{i}k_{K}$[a] (hr^{-1})		$^{o}k_{Na}$[b] (hr^{-1})	$^{o}k_{K}$[b] (hr^{-1})	$^{o}k_{SO_4}$[c] (min^{-1})	
Expt	22°	37°	22°	37°	22°	22°	22°	37°
No dye	0.0073 ± 0.0019	0.0032 ± 0.0003	0.0409 ± 0.0101	0.0447 ± 0.0019	0.0416 ± 0.0011	0.0166 ± 0.0005	0.0029	0.047
With dye	0.0054 ± 0.0008	0.0049 ± 0.0018	0.0087 ± 0.0049	0.0360 ± 0.0039	0.0458 ± 0.0020	0.0224 ± 0.0008	0.0032	0.054
							(single expt)	

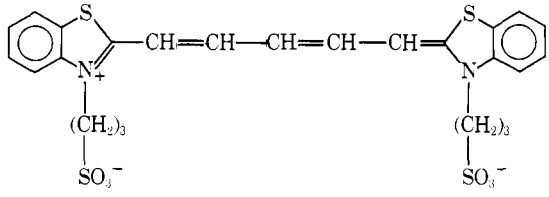
[a] Simultaneous measurements of inward rate constants for Na and K, using ^{22}Na and ^{42}K, into human red blood cells in medium containing 148 mM NaCl, 5 mM KCl, 17 mM Tris (pH 7.5), and 1×10^{-4} M ouabain in the presence and absence of 6.1×10^{-7} M diS-C_3-(5). Measurements made over a 30-min period (5 and 35 min) at 22°, and in separate experiments, at 37° (see Methods). [b] Simultaneous measurements of outward rate constants for Na and K, obtained under conditions described in footnote *a* (see Methods). [c] Measurements of the outward rate constant for SO_4, using $^{35}SO_4$ for human red cells suspended in 93 mM Na_2SO_4–20 mM Tris (pH 7.5) in the presence and absence of 6.1×10^{-7} M diS-C_3-(5). Cells were previously loaded with Na_2SO_4 and a tracer quantity of carrier-free $^{35}SO_4$ (see Methods). Measurements were made at five points over an 180-min period in separate experiments at 22 and 37° (see Methods).

lytic when incubated with cells under the conditions employed during fluorescence measurements. Further investigation of the effects of this dye upon the normal ion fluxes across the cell membrane confirmed that the dye alters the normal physiological parameters of the membrane to a negligible extent at the concentrations employed in these experiments (Table V). The remainder of this paper will be concerned with investigations of the nature of the interaction of this potential-sensitive probe with the red cells.

Potential Dependence of the Cell-Medium Partitioning of diS-C_3-(5). As part of an effort to determine the mechanism underlying the fluorescence change observed when these cells are hyperpolarized we have investigated in detail the emission spectra of the diS-C_3-(5) suspended with normal cells in a K-free medium. Figure 2a shows the emission spectra (with excitation at 622 nm) of the total cell suspension before and after the addition of valinomycin. The dashed line is an emission monochrometer scan of the red cell suspension prior to the addition of dye, and gives an indication of the contribution of scattered light to the results. Addition of valinomycin to this dye-free cell suspension was found not to alter the cell-scattering spectrum. It can be seen from the figure that dye in equilibrium with the cell suspension before the addition of valinomycin exhibits a moderately broad emission peak with maximum at 670 nm. When valinomycin is added to the suspension, the emission spectrum changes markedly, with the maximum shifting 16 nm to the red, and decreasing in intensity by a factor of 4. Because of the significant red shifting of the emission peak, it is apparent that the magnitude of fluorescence change observed when monitoring membrane potential with this probe will depend upon the selection of emission wavelength. For example, fluorescence monitored at 700 nm decreases only 40% with the addition of valinomycin to the cell suspension, but an 80% change is recorded at 670 nm. The red shifting of the emission peak, concomitant with a fourfold decrease in emission intensity, indicates that the molecular environment of at least some of the chromophores has been altered as the cells are hyperpolarized. To further explore this possibility, we designed a procedure to separate the emission spectra of Figure 2a into emission spectra due to fluorescence of cell-associated dye and emission spectra due to nonbound dye in the extracellular medium.

Figure 2b shows the fluorescence contributions from the unbound dye in the medium, in the absence and presence of valinomycin, and from the dye associated with the cells, in the absence and presence of valinomycin. The fluorescence contribution of unbound dye was obtained by centrifuging the cells to the bottom of the fluorescence cuvet (see Methods) and obtaining the emission spectrum of the cell-free supernatant. The emission spectrum that is due only to cell-associated dye (dotted spectra, Figure 2b) was obtained by correcting the corresponding fluorescence spectrum of the total suspension (Figure 2a) for light scattering, and then subtracting from this corrected spectrum the component of fluorescence due to the unbound dye in the medium.

In resolving the emission spectrum of dye in the cell suspension into the components representing dye in the medium (supernatant) and the calculated cell-associated fraction, it was essential for us to consider how the presence of red cells might alter the shapes of the dye spectra. Absorption and scattering of excitation and emitted light by particulate species in suspension can, in many cases, change the shape and intensity of an emission spectrum. We have measured the absorbance of a lightly sonicated 0.17% red cell suspension to be approximately 0.02 at 622 nm, the wavelength at which we excite diS-C_3-(5) in these experiments. Therefore, the presence of hemoglobin alone does not significantly interfere with emission measurements employing red-absorbing dyes such as diS-C_3-(5) that can be excited above 620 nm. The effects of cell scattering are more difficult to assess. We attempted to investigate the extent of scattering interference with our measurements by determining the scattering component introduced by the presence of cells with a dye exhibiting the identical absorption and emission properties as diS-C_3-(5), but which does not bind to the cells. The dye, diS-$C_3SO_3^-$-(5), which has high water solubility (see structure below), was added at different concentrations



to K-free medium. The emission spectrum of this dye solution was recorded with excitation at 622 nm for each of several different dye concentrations, prior to the addition of red cells to the solutions to give 0.17% cell suspensions. After three minutes of mixing, the emission spectra of the cell suspensions was recorded. The cells were then centrifuged to the bottom of the cuvet to permit measurement of the fluorescence from the cell-free supernatant. For each concentration of dye employed (3×10^{-7} to 3×10^{-6} M) the emission spectrum of the dye in the medium before the addition of cells was found to be identical with the emission spectrum of the cell-free supernatant obtained after incubation with the cells, confirming that the dye does not bind to the cells. Furthermore, subtraction of the cell-scattering spectrum (dashed line in Figure 2a) from the emission spectra obtained from the red cell suspensions at each of the dye concentrations employed produced spectra that were identical to the spectra obtained from this dye (emission from 640 to 720 nm) in cell-free medium. Hence we are confident that we have accurately determined the spectra for the unbound and cell-associated components of diS-C_3-(5) shown in Figure 2b.

It is clear from Figure 2b that most of the fluorescence detected from the cell suspension before the addition of valinomycin is due to dye in the aqueous medium. However, we have

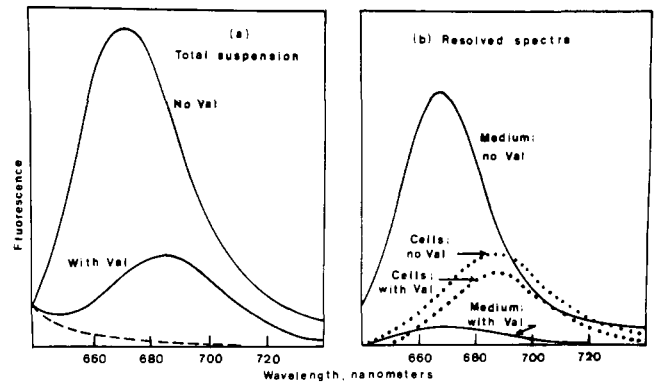


FIGURE 2: (a) Fluorescence emission spectra of suspensions of diS-C_3-(5) with red cells in NaCl-Tris (pH 7.4). Upper solid-line trace was obtained 3 min after mixing of the dye (6.1×10^{-7} M) with the 0.17% suspension of red cells. Also shown in this figure is the trace of the emission spectrum obtained 2 min after the addition of valinomycin (final concentration, 1×10^{-6} M) to the suspension of cells with dye. The dashed line is a trace of cell scattering obtained from the cell suspension before the addition of dye. Excitation is at 622 nm. (b) The aqueous (solid-line traces) and cell-associated (dotted-line traces) components of the dye emission spectra presented in part a. The solid-line traces were obtained from the cell-free supernatants obtained from centrifugation of the cell suspensions with dye (or dye + valinomycin) as described in Methods. The dotted-line traces present the calculated emission from cell-associated dye in the presence and absence of valinomycin. See text for explanation.

found that quantity of dye in the aqueous medium (not bound to cells) represents less than half of the total quantity of dye in the suspension. In twelve experiments in which 1 μg of dye was added to the cell suspension, we determined by extraction (see Methods) that 53 (±4)% of the total dye in suspension was associated with the cells, 47 (±4)% in the extracellular medium. Although as much as 10% of the dye was found to adhere to the surfaces of the cuvet, the ratio of cell-associated dye to dye in the medium remained remarkably constant throughout the twelve experiments. Thus we have determined that prior to hyperpolarization the 53% of the dye in suspension that is associated with the cells contributes only 18% of the fluorescence at 670 nm. And, although less than half (47%) of the dye is in the medium, it accounts for 82% of the total fluorescence recorded at that wavelength.

After the addition of valinomycin to red cells equilibrated with the diS-C_3-(5) in K-free medium, the emission spectrum of the suspension exhibited a 16-nm red shift with a fourfold decrease in fluorescence at the emission maximum (Figure 2a). From the resolved spectra shown in Figure 2b it is clear that the fluorescence changes of the suspension are mostly due to a tenfold decrease in the quantity of free dye in the medium. Under these conditions, the fluorescence from dye associated with the cells (shown by the dotted spectra in Figure 2b) is found to decrease by a factor of $\frac{1}{3}$, even though the percentage of dye associated with the cells determined by extraction increases from 53 (±4)% before hyperpolarization to 97(±1)% after the addition of valinomycin. It is important to note that the cell-associated components of fluorescence determined both prior to and after the addition of valinomycin exhibit the *same* emission maximum at 688 nm, a red shift of over 20 nm from the dye's emission peak in aqueous medium.

If the potential-dependent fluorescence changes shown in Figure 1 are due to a potential-dependent partition of dye between the cells and medium, as is suggested by experiments involving maximal hyperpolarization of the cells in K-free medium, then the relative percentages of dye distributed between the cells and extracellular medium ought to depend in a pre-

TABLE VI: Partition of diS-C_3-(5) as a Function of $[K_0^+]$.[a]

$[K_0^+]$ (mM)	Cell-Associated Dye (%)		Dye in Medium (%)	
	No Val	With Val	No Val	With Val
0	53 ± 5	97 ± 1	47 ± 5	3 ± 1
51	55 ± 3	70 ± 2	45 ± 3	30 ± 2
102	52 ± 2	53 ± 4	48 ± 2	47 ± 4
153	42 ± 2	28 ± 3	58 ± 2	72 ± 3

[a] Influence of the extracellular potassium concentration $[K_0^+]$ on the binding of diS-C_3-(5) to normal cells in suspension prior to and after the addition of valinomycin. The amounts of cell-associated dye and nonbound dye (in the medium) are expressed as a per cent of the total dye in the suspension. The data were obtained with normal cells suspended to 0.17% at 23° in medium containing potassium at concentrations of 0–153 mM. $[K_0^+]$ = 0 refers to medium containing 153 mM NaCl–17 mM Tris (pH 7.4). KCl was substituted for NaCl to obtain the potassium concentrations given in the table. In each experiment, 1 μg (1.83 μmol) of dye was added in ethanol (final ethanol concentration, 0.5%) to 3 ml of stirred cell suspension in a standard fluorescence cuvet and mixed for 4 min. Determinations of the cell-associated and nonbound fractions of dye were made according to the procedures given under Methods. The experiments were then repeated with cell suspensions prepared as described above but with the addition of valinomycin (final concentration, 1.0×10^{-6} M).

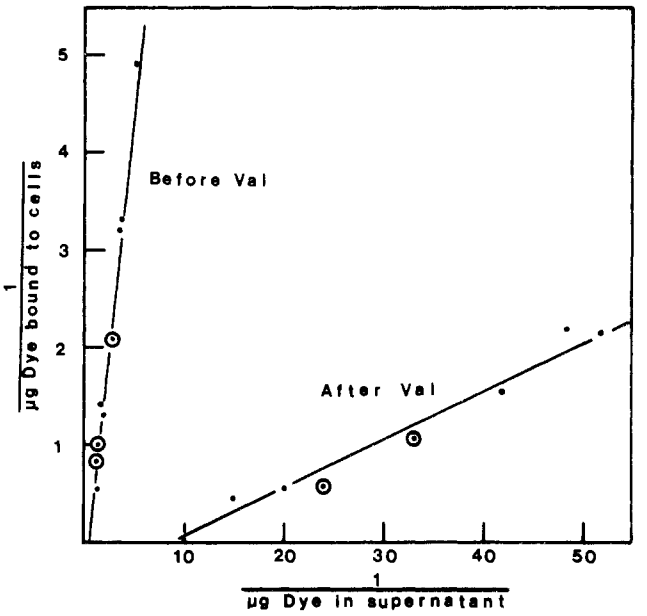


FIGURE 3: Double-reciprocal plot of the quantity of dye associated with the cells against the quantity of (unbound) dye in the extracellular medium. Data were obtained from 0.17% suspensions of normal cell suspended with diS-C_3-(5) (final concentrations from 3.05 to 30.5 × 10^{-7} M) in NaCl–Tris medium and from cells suspended with dye plus valinomycin (1.0×10^{-6} M) in NaCl–Tris medium. Solid lines represent least-squares fits of the two sets of data. Double data points are represented by the symbol ⊙. See text for details.

dictable way on the size of the potential change elicited by the addition of valinomycin. Table VI shows the partitioning of diS-C_3-(5), with and without valinomycin, with suspensions of cells in medium containing the same series of K_0^+ concentrations used to generate Figure 1. It can be seen that before the addition of valinomycin, the partition of the dye shows only a small dependence on K_0^+. This is reasonable, since for normal cells, where P_K is about 100 times smaller than P_{Cl}, the constant field equation predicts that the membrane potential is relatively insensitive to K_0^+. Upon the addition of valinomycin the amount of dye taken up by the cells changes proportionally with the size of the potential (and fluorescence) change generated in the cells, which are suspended in media of different K^+ concentration. The uptake of dye by cells is maximal under conditions eliciting the largest fluorescence decrease (maximal hyperpolarization in K-free medium), and diminishes proportionally as K_0 increases. When the cells are suspended in 153 mM KCl, dye is actually *released* from the cells upon the addition of valinomycin. *Thus we conclude that there is a potential-dependent partition of diS-C_3-(5) between cells and medium. The dye is released from cells upon depolarization and taken up by cells upon hyperpolarization.*

We have futher examined the uptake of diS-C_3-(5) by hyperpolarized red cells in K-free medium and have found that the fraction of dye associated with the cells remains approximately constant over a tenfold range (3.05×10^{-7} to 30.5×10^{-7} M) of total dye concentration in the suspension. Figure 3 is a double-reciprocal plot of the quantities of cell-associated dye *vs.* unbound dye in the medium. The apparent dye/cell dissociation constant, K_a, can be obtained from the slope assuming that the data can be represented by the Klotz equation (Weber and Young, 1964; Klotz, 1947): $1/\nu = 1/K_aD + 1/n$, where ν = dye associated with the cells (in μg), D = μg of dye in the medium (μg/3 ml of medium), and n is a constant. The apparent K_a value determined from this plot in the absence of valinomycin is 14 times the K_a value obtained when the cells are hyperpolarized. This change in the dissociation constant demonstrates the striking increase in the affinity of the red cell for the dye upon hyperpolarization. Since the data fall approximately on straight lines, we infer that the affinity of dye for cell binding sites remains essentially constant over at least a tenfold dye concentration range.

Since other members of the diS-C_n-(5) homologous series show large fluorescence decreases with maximal hyperpolarization (Table II), it is not surprising to find that they also show changes in cell-medium partitioning with hyperpolarization. Table VII shows the percentages of each dye associated with the cells and with the medium, before and after addition of valinomycin. In each case the dye is taken up by the cells upon hyperpolarization with the dye concentration of the water phase decreasing by a factor of ten. As we would predict, simply on the basis of expected water/hydrocarbon solvent solubility, and assuming the dyes associate with membrane hydrocarbon, the dyes with longer alkyl chains partition more toward the cells, before hyperpolarization as well as after hyperpolarization.

Quenching of Cell-Associated Dye Fluorescence. Figure 2b demonstrates that in absence of valinomycin the diS-C_3-(5) molecules associated with the cells are less fluorescent at 670 nm than the dye molecules in the medium. It is also apparent that when the cells have been maximally hyperpolarized, the fluorescence of cell-associated dye is even further diminished.

A quantitative measure of the apparent quenching of cell-associated dye fluorescence is given by the factor Q_λ. We define Q_λ as the ratio of the fluorescence intensity, at wavelength λ, of a unit quantity of dye associated with the cells (of a 0.17% red cell suspension) to the fluorescence intensity of the same amount of dye in 3 ml of cell-free medium. Q_λ becomes less than 1.0 as cell associated dye is quenched relative to its fluorescence in water, regardless of the quenching mechanism. For the experimental results presented in Figure 2b, Q_{670} is calculated to be 0.20 for the cells in suspension before the addition

TABLE VII: Cell Partition of Cyanines diS-C_n-(5).[a]

Dye diS-C_n-(5) n =	% Dye Bound to Cells		% Dye in Medium	
	No Val	With Val	No Val	With Val
2	32 ± 3	91 ± 1	68 ± 3	9 ± 1
3	53 ± 4	97 ± 1	47 ± 4	3 ± 1
4	71 ± 1	97.5 ± 0.5	29 ± 1	2.5 ± 0.5
5	82 ± 0.5	98.5 ± 0.5	18 ± 0.5	1.5 ± 0.5
6	95.5 ± 0.3	99.8 ± 0.1	4.5 ± 0.3	0.2 ± 0.1

[a] The binding of structural analogs of diS-C_3-(5) to normal cells in K^+-free suspension with and without valinomycin. The amounts of cell-associated dye and dye in the medium are expressed as a per cent of the total dye in suspension. The data were obtained with normal cells suspended to 0.17% at 23° in medium containing 153 mM NaCl–17 mM Tris (pH 7.4). In each experiment, 1.83 μmol of dye was added in ethanol (final ethanol concentration, 0.5%) to stirred 3-ml cell suspensions in standard fluorescence cuvet and mixed for 4 min. Determinations of the cell-associated and nonbound fractions of dye were made according to the procedures given under Methods. Experiments were also conducted for cell suspensions prepared as above but with the addition of valinomycin (final concentration, 1.0×10^{-6} M).

of valinomycin. A significant part of the apparent quenching of cell-associated dye fluorescence at 670 nm before valinomycin addition is due to a red shift of dye fluorescence to a new maximum of 688 nm. But the red shift cannot totally account for the quenching at 670 mm. Even at the red shifted emission maximum, the fluorescence intensity of cell-associated dye is less than the maximum intensity of the dye in water. Hence, there is a mechanism for fluorescence quenching before valinomycin addition that is not associated with the red shift.

After the addition of valinomycin to the red cell suspension (Figure 2b, maximum hyperpolarization), Q_{670}^{val} is calculated to be only 0.08. The change in Q_{670} with cell hyperpolarization reflects the fact that the emission from cells is less intense after the addition of valinomycin (*cf.* the dotted line spectra of Figure 2b), even though the quantity of dye associated with the cells has *increased* by a factor of nearly 2 (Table VI). Since the emission maximum of cell-associated dye remains at 688 nm after valinomycin addition, it is apparent that the dye molecules associated with the cells become even less fluorescent after hyperpolarization as a result of a quenching mechanism that does not depend on a red shift.

The question arises whether the increased quenching observed is directly related to the change in membrane potential, or is rather a consequence of the increased dye content of the cells after hyperpolarization. In order to resolve this question, we have investigated the change in Q_{670} as a function of the number of micrograms of dye associated with the cells. In three sets of experiments, dye was added in different amounts to 0.17% cell suspensions to give dye concentrations in the total suspension ranging from 3.05×10^{-7} to 15.3×10^{-7} M. After 4 min of mixing, the Q_{670} and the quantity of cell-associated dye were determined for each trial by the methods discussed earlier. The experiments were conducted with normal cells suspended under four conditions: cells in K-free medium without and with valinomycin, and cells in 51 mM K without and with valinomycin. Figure 4 presents plots of Q_{670} *vs.* micrograms of

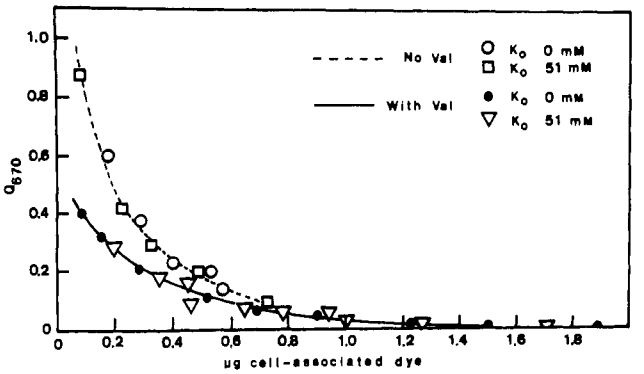


FIGURE 4: The change in Q_{670} as a function of the number of micrograms of dye associated with the cells. Q_{670} is a factor that quantitatively describes the apparent quenching (relative to an emission of 1.0 for dye in aqueous medium) of cell-associated dye. Data obtained from experiments with normal red cells (0.17% suspensions) suspended with diS-C_3-(5) (dye concentrations from 3.05 to 30.5×10^{-7} M) under four different conditions: in NaCl–Tris medium without valinomycin (○); in medium of 51 mM K^+, 102 mM Na^+ without valinomycin (□); in NaCl–Tris medium with valinomycin (●); and in medium of 51 mM K^+–102 mM Na^+ with valinomycin (▽). See text for an explanation.

cell-associated dye for each of the four experimental conditions.

If one assumes that the presence of dye does not significantly alter the membrane potential, it is clear from the data of Figure 4 that *the quenching of the fluorescence of cell-associated dye is directly related to the quantity of dye associated with the cell.* When the concentration of dye in the cell is low, Q_{670} approaches or even exceeds the reference (nonassociated) value of 1.0. As the concentration of dye in the cell increases, the fluorescence per molecule of dye drops sharply, Q_{670} approaching zero. Furthermore, the quenching of fluorescence as a function of the quantity of cell-associated dye is almost the same for each of the four experimental conditions. However, the data obtained from cells under conditions of hyperpolarization (see solid curve, Figure 4) show slightly greater quenching at each dye content as compared with the data from cells in the absence of valinomycin (see dotted curve, Figure 4). In other words, independent of changes in the dye content of the cells, hyperpolarization of the membrane seems to diminish the fluorescence of cell-associated dye. This is especially apparent when the quantity of dye associated with the cells is less than 0.4 μg.

Formation of Dye Aggregates That Do Not Fluoresce at 670 nm. From the results we have presented to this point, we have been able to relate the fluorescence changes observed with the dye in cell suspensions to a potential-dependent distribution of the dye between the cells and extracellular medium. But beyond an indication that cell-associated dye is observed to show red-shifted and highly diminished fluorescence (with quenching dependent on the quantity of cell-associated dye), we have yet to offer a molecular mechanism to account for the differences in fluorescence observed for cell-associated and nonbound dye. In fact, it is worthwhile to note an apparent discrepancy between our observations on the fluorescence of these dyes in association with cells and our earlier data on the spectral behavior of these dyes in organic solvents (Table I). Note that in octanol, diS-C_2-(5) shows a highly (22 nm) red-shifted fluorescence relative to its aqueous emission. Qualitatively, this is quite similar to the 20-nm red shift we have observed for the association of diS-C_3-(5) with the cells (Figure 2). But from the data of Table I, the dye shows nearly a 50% *increase* in the intensity of its emission on going from an aqueous environment to that of octanol.

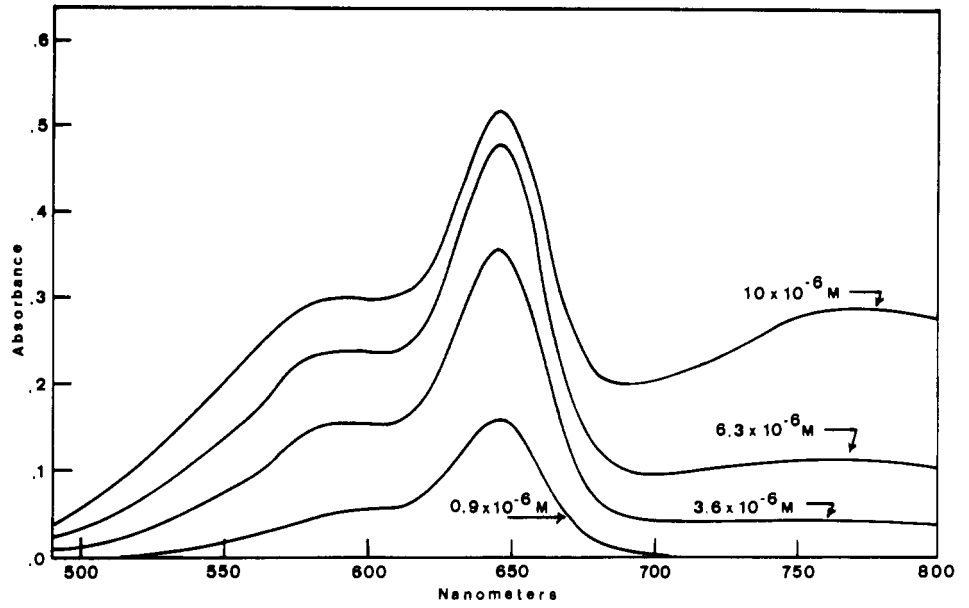


FIGURE 5: Absorption spectra for diS-C_3-(5) obtained at various dye concentrations in NaCl-Tris medium at 23°. Dye was added in ethanol (final EtOH concentration, 0.5%) to a 1-cm path-length quartz cuvet containing 1 ml of NaCl-Tris medium (pH 7.4) to give the dye concentrations indicated in the figure. Spectra were scanned 1 min after mixing of dye.

The fact that diS-C_3-(5) in association with the cells shows significantly *diminished* fluorescence relative to its emission in aqueous medium is a somewhat surprising result if one assumes that diS-C_3-(5) is associating with the hydrocarbon regions of the red cell membrane in a manner similar to the amphiphilic fluorochrome ANS^-. With the addition of cells to ANS^- in aqueous medium, the emission from this molecule is observed to greatly increase in intensity along with a distinct shift in the emission maximum upon binding to the membrane (Rubalcava *et al.*, 1969; Fortes and Hoffman, 1971). This change in ANS^- fluorescence on binding to the membrane is quite similar to the emission changes observed when ANS^- is removed from water and placed in a hydrocarbon solvent such as octanol (Stryer, 1968). diS-C_3-(5), by its 22-nm red shift on association with the cells, also appears to be in or near the hydrocarbon regions of the membrane. But instead of showing increased fluorescence as one would expect from its enhanced emission in octanol, the dye appears to be significantly quenched.

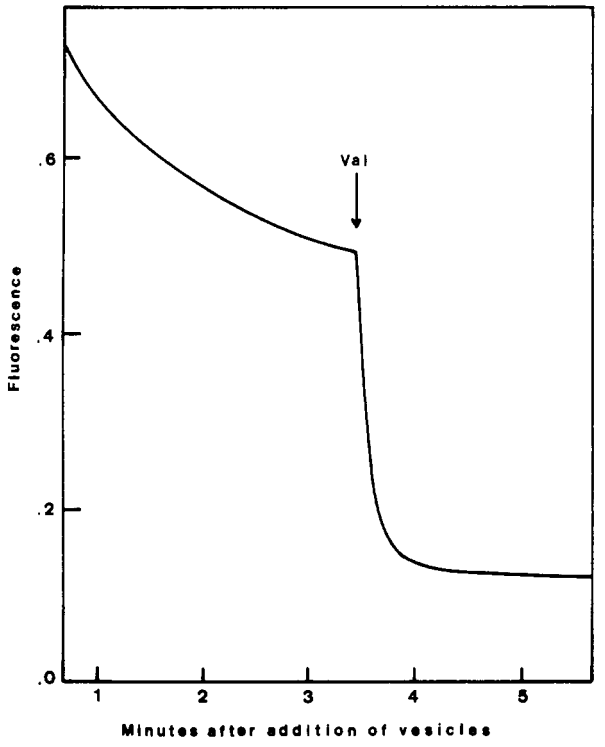


FIGURE 6: Fluorescence intensity as a function of time recorded for a suspension of KCl-filled phosphatidylcholine-cholesterol (3:1) vesicles (formed by light sonication in KCl-Tris medium, see Methods) suspended with diS-C_3-(5) in NaCl-Tris medium. Suspension contains 20 μg of lipid/ml. Dye was added to lipid suspension at time 0 (final dye concentration, 4.6×10^{-7} M). Valinomycin was added where indicated. Emission is at 670 nm with excitation of 622 nm. Fluorescence is in arbitrary units.

Accordingly, we have sought other explanations for the quenching of cell-bound dye fluorescence. It has been reported that a number of cyanine dyes with increased concentration in aqueous solutions, in association with silver bromide crystals and biological macromolecules, and in dye-octadecane monolayers, undergo aggregation to form dimers and multimers (West and Pearce, 1965; Kay *et al.*, 1964; Bücher and Kühn, 1970; West and Carroll, 1951). Quantum mechanical and extended dipole calculations assuming appropriate geometries of the aggregates have given absorption wavelengths which are in good agreement with experimental findings (Czikklely *et al.*, 1970). Figure 5 shows absorption spectra of diS-C_3-(5) obtained in aqueous solutions at increasing dye concentration. Note that at 0.9×10^{-6} M concentration, the monomer absorption peak at 643 nm is observed. With increasing concentration of dye, two new absorption peaks become evident: a blue-shifted peak at 583 nm, corresponding to the dimer absorption (West and Pearce, 1965), and a peak beyond 700 nm due to the existence of higher order aggregates and commonly referred to as the J band (Sheppard, 1942). Note also that with increasing concentration, the peak intensity at 643 nm is increasingly diminished relative to the absorption peaks for the dimer and multimer species. We also obtained the corrected excitation spectrum (emitting at 670 nm) of 10×10^{-6} M diS-C_3-(5) in an aqueous solution. Although Figure 5 shows that dye solution at this concentration contains dimers and higher order aggregates, the excitation spectrum is nearly superimposable on the monomer absorption spectrum. This gives indication that the dimers are essentially nonfluorescent at 670 nm. Since the formation of nonfluorescent (at 670 nm) multimeric

aggregates of dye is a concentration-dependent phenomenon, we were led to explore the possibility that the concentration-dependent quenching of dye associated with the red cells might be related to a process of dye aggregation.

Because light scattering from red cell suspensions makes small changes in the dye absorption spectrum difficult to measure, we turned our attention to mixed phosphatidylcholine–cholesterol (3:1) vesicle preparations which can be used as a model for the red blood cell system and which scatter far less light.

A stock suspension of vesicles containing K^+ was formed by coating a test tube with lipid, adding 153 mM KCl and 17 mM Tris, and lightly sonicating the mixture for 2 min. A 15-μl aliquot of the vesicle stock suspension was added to 3 ml of 153 mM NaCl–17 mM Tris along with 0.75 μg of diS-C_3-(5). Emission was monitored at 670 nm with excitation at 622 nm. Several minutes after the addition of the dye the fluorescence intensity became constant and valinomycin was added to hyperpolarize the vesicles. Figure 6 presents the recording obtained from a single such experiment. Note the rapid 80% decrease in fluorescence at 670 nm that is observed after the addition of valinomycin. Emission spectra obtained before and after the addition of valinomycin (not shown) revealed a red shift of emission to 686 nm after hyperpolarization. The fluorescence intensity of the hyperpolarized vesicles remained essentially constant for longer than 10 min.[2] In experiments where the external concentration of K^+ was increased by substituting KCl for NaCl in the medium, the size of the fluorescence change observed after the addition of valinomycin was correspondingly smaller. No change in fluorescence was observed when valinomycin was added to the vesicles suspended in 153 mM KCl–Tris. Therefore we assume that when the vesicles are hyperpolarized, the magnitude of the fluorescence change is proportional to the size of the valinomycin-induced potential change, as was seen in the red cell system.

The absorption spectra shown in Figure 7 were obtained from lipid–cholesterol vesicles under the same conditions employed during the fluorescence studies depicted in Figure 6. The absorption spectrum of diS-C_3-(5) in 153 mM NaCl–17 mM Tris was obtained 2 min after the addition of dye. The total absorbance continued to slowly decrease, probably because of adsorption of the dye to the cuvet surfaces. Despite this slow decline in total absorbance, the shape of the spectrum was observed to remain the same for more than 10 min. When vesicles were added to the dye solution, the absorption spectrum changed very rapidly for the first 30 sec and then decreased in absorbance very slowly over time. The spectrum of dye with the vesicles in Figure 7 was obtained 1 min after the addition of the vesicles to the dye solution and was observed to remain qualitatively the same for more than 10 min. The third absorption spectrum shown in Figure 7 was obtained 1 min

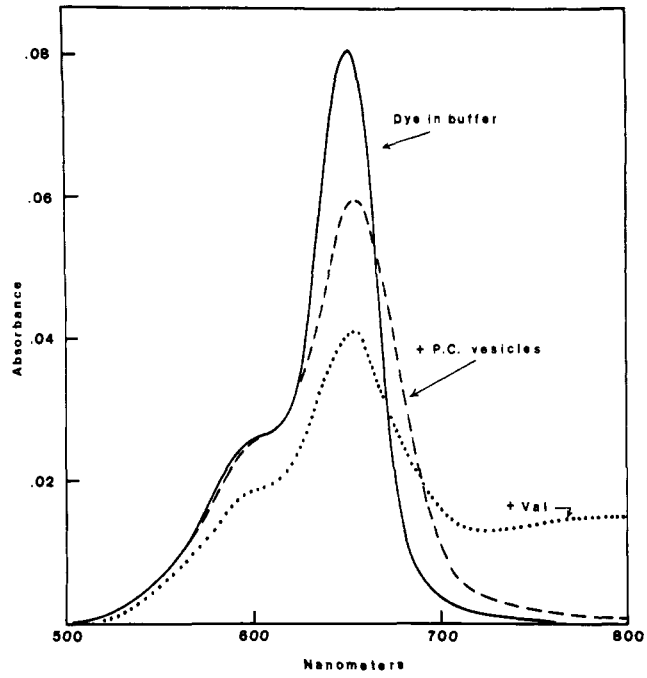


FIGURE 7: Absorption spectra obtained from diS-C_3-(5) with KCl-filled lipid–cholesterol vesicles under the same conditions described for the fluorescence measurements of Figure 6 (see legend, Figure 6). The solid line is a tracing of a spectrum obtained 90 sec after the addition of diS-C_3-(5) to 3 ml of NaCl–Tris medium in a 1-cm path-length quartz cuvet (final dye concentration, 4.6×10^{-7} M). A spectrum represented by a dashed-line tracing was obtained 3 min after the addition of the vesicles to the dye solution to give a suspension of 20 μg of lipid/ml. The dotted line is the spectrum obtained 2 min after the addition of valinomycin to the suspension of lipid with dye. The absorbance of each sample was measured against a reference cell containing a suspension of the identical composition of the sample cell, but with no dye present.

after the addition of valinomycin to the vesicle suspension with dye. Although no substantial change in the absorption spectrum had been recorded when the vesicles were added to the dye, the addition of valinomycin to the vesicle suspension caused a pronounced change in the shape of the dye absorption spectrum. As can be seen in the figure, the hyperpolarization of the vesicles with the addition of valinomycin results in the appearance of a new dye absorption peak to the red of 700 nm. This broad absorption peak appears similar to the J band that was observed (Figure 5) when diS-C_3-(5) was dissolved in aqueous medium at concentrations above 10^{-5} M. It appears, then, that with the vesicle suspension, aggregation of the dye takes place upon hyperpolarization. Presumably, the aggregation results from uptake of the dye by the vesicles in a manner similar to that observed for red blood cells.

Thus a plausible explanation for the quenching mechanism after hyperpolarization is the formation of dimers and higher order aggregates of dye that are nonfluorescent at 670 nm as the concentration of vesicle (or, cell) associated dye increases. We do not know where the aggregates of dye are formed; whether, for instance, on the surface of the membrane, in the interior regions of the membrane, in the aqueous interior of the vesicles, or in various combinations of these.

Discussion

Sensitivity of fluorescence to red cell membrane potential is common to a number of related cyanine dyes. Nine different chromophore structures we tested showed decreases in fluorescence greater than 50% with maximal cell hyperpolarization. But the sensitivity of each chromophore depended on the length of the hydrocarbon chains attached to the chromophore

[2] If cholesterol was omitted from the vesicle preparation, the fluorescence response to valinomycin-induced hyperpolarizations did not remain constant, but tended to rise to the fluorescence intensity observed before the addition of valinomycin to the suspension. This instability may be due to the fact that the vesicles formed without cholesterol may be more permeable to Na^+ or Cl^- (Papahadjopoulous and Watkins, 1967; Papahadjopoulos *et al.*, 1972), or that the dye can cross through the membrane and act as an anion carrier. On the other hand, when the cholesterol content of the vesicles is increased to 50% of the total lipid content, no fluorescence change was observed upon the addition of valinomycin to the vesicle suspension in 153 mM NaCl. This phenomenon could be explained by the fact that valinomycin is a less efficient carrier in high-cholesterol vesicles (DeGier *et al.*, 1970). It is also possible that the dye cannot permeate the membranes of vesicles that are high in cholesterol, and therefore cannot sense the potential across the membrane.

and the concentration at which the dye was used. One of the dyes, diS-C_3-(5), shows an exceptionally large fluorescence change (−80%) with hyperpolarization and was found to minimally alter membrane transport parameters such as ion fluxes and hemolytic activity. Therefore, we are interested in investigating the mechanism for the fluorescence change observed with the use of this dye.

We have found that the fluorescence changes of diS-C_3-(5) with membrane potential are related to two phenomena. First, the partitioning of dye between the cells and the external medium depends on the cell potential. Dye is taken up by the cells upon hyperpolarization and released upon depolarization. For example, with maximal hyperpolarization, a tenfold decrease of dye concentration in the medium is observed. Levin *et al.* (1968) and Nasonov (1959) have shown that the binding of Heliogen Blue and Neutral Red to nerves changed when the nerve was stimulated to give trains of action potentials. These binding changes may be similar to the potential-dependent changes described here. Second, cell-associated dye is significantly less fluorescent than the dye in the medium and the degree of quenching is dependent on the amount of dye associated with the cells. For example, before hyperpolarization cell-associated dye is $\frac{1}{5}$th as fluorescent as dye in the medium. But after maximal hyperpolarization the amount of dye associated with the cells is nearly doubled (because of dye uptake from the medium) and the fluorescence of cell-associated dye is found to be only $\frac{1}{12}$th of its relative intensity in the medium. We would expect, from our knowledge of the quenching of cell-associated dye, that upon *depolarization* the fluorescence increase observed at 670 nm results because the dye is ejected from the cells into the medium where it is more fluorescent, and also because the dye that remains associated with the cells is now relatively more fluorescent, since the cells are partially depleted of dye content.

Mixed phospholipid–cholesterol vesicles containing high potassium concentrations and suspended in a low potassium medium containing diS-C_3-(5) show large decreases in fluorescence upon valinomycin addition. They also exhibit a red-shifted emission maximum to 686 nm, similar to the shift in emission maxima seen with the red cell preparations. It is not unreasonable, then, to expect that the very similar fluorescence changes, which take place under identical ionic conditions in both vesicle and red cell suspension, results from a similar interaction of the dye with the lipid bilayers of both systems. For this reason, we have used the vesicle preparation as a model for the red cell and have obtained information about dye interaction with the vesicles which may be useful for understanding the interaction of dye with the red cells. From absorption spectroscopic studies on the vesicle preparation we have found evidence of dimer and higher order aggregate formation upon hyperpolarization of the vesicles. The multimers, which absorb in the J band above 700 nm, do not contribute to fluorescence at 670 nm. The dimers, which absorb in the region of excitation (622 nm), are not fluorescent, as judged by the shape of the excitation spectrum of an aqueous solution of monomers, dimers and higher order aggregates. Most likely, then, the decrease in diS-C_3-(5) fluorescence at 670 nm upon hyperpolarization of the vesicles is due to uptake of dye by the vesicles with the following results. Some of the dye associated with hyperpolarized vesicles is probably in a hydrocarbon environment since the emission is red shifted. The fluorescence of the rest of the vesicle-associated dye is quenched because it has formed nonfluorescent aggregates due, most likely, to increased dye content.

If the mechanism for the fluorescence change of diS-C_3-(5) is the same for the vesicles and the red cells, then the dye is probably also forming nonfluorescent aggregates in the red cells when the cell-associated dye content becomes high. Certainly, the increase in fluorescence quenching of red cell-associated dye with increasing dye content is consistent with the usual concentration dependence of aggregate formation. A few rough calculations also support the contention that aggregation of dye may be taking place in the cells. If it is assumed that the dye is bound noncovalently to the outside surface of the membrane, 0.5 μg of dye per 4×10^7 cells (a typical experiment before hyperpolarization with a 0.17% cell suspension) assuming a surface area of 130 μ^2/cell results in a surface density of one dye molecule per 1000 Å^2. The dimensions of a diS-C_3-(5) molecule obtained from a space-filling molecular model are $20 \times 8 \times 4$ Å. Hence, there is enough dye associated with the cells to cover 10–20% of the external membrane surface of a red cell prior to hyperpolarization and 20–40% after maximal hyperpolarization results in a doubling of cell-associated dye. Of course, it is possible that only certain regions of the membrane surface are available as dye binding sites. This would increase the surface concentration of dye in these regions and further increase the likelihood of dye aggregation. It is conceivable that the negatively charged sialic acid residues could stabilize the adsorption of a large amount of positively charged dye to the outer membrane surface. We consider this situation unlikely, however, because no change in the total fluorescence of the dye with the cells is observed after neuraminidase treatment to remove the sialic residues.

On the other hand, if it is assumed that all the dye is accumulated in the cytoplasm of the cell, the concentration of dye in the intracellular fluids would be 2×10^{-4} M before valinomycin addition and 4×10^{-4} M after maximal hyperpolarization. These concentrations are 20–40 times larger than those used to generate the absorption spectrum of an aqueous solution of dimers and multimers (Figure 5). It is likely, therefore, that nearly all the accumulated dye in the cytoplasm would be in an aggregated form. At this time we have not experimentally determined the location of the dye binding sites within the red cell, and since we do not know whether the dye permeates the membrane we cannot estimate accurate surface or internal concentrations of dye. It is clear, however, that there is sufficient dye associated with the cells to produce aggregation and account for the quenching of cell-associated fluorescence.

A major question yet to be answered is how the dye "senses" the membrane potential and partitions between the cells and the medium according to the potential. Several possible mechanisms come to mind.

(1) Suppose the dye associates only with the external half of the membrane. Perhaps it is most reasonable to expect the dye, which bears a single positive charge, to interact with the negatively charged phospholipid head groups near the surface. The dye would then have to sense structural changes, such as reorientations of surface dipoles or changes in the surface potential that may take place with changing trans-membrane potential. The result would be the alteration of the number of binding sites on the membrane surface. Or, the dye might sense the part of the trans-membrane electrical potential change that occurs near the outer surface of the membrane. The altered potential energy of the charged dye at the surface of the membrane (relative to its potential energy in the external medium) would shift the equilibrium partition of dye between the cell and the medium. In either case, it is unlikely that the dye can experience the entire electrical field across the membrane because the largest part of the electrical potential drop will be across the hydrocarbon region of the membrane (Lauger and Neumcke, 1973).

(2) If the hydrocarbon regions of the membrane are accessible to the dye, it is possible that changes in membrane potential may change the solubility of the dye in these regions. Electrostrictive effects (White, 1970, and references therein) at high potentials might "squeeze" the dye from the membrane. It should be remarked that this mechanism seems unlikely because the dyes have been found to be insoluble in hydrocarbons such as decane, benzene, and ether, and are therefore unlikely to be soluble in the hydrocarbon interior of the membrane. Furthermore, according to this model, dye would be "squeezed" into the external medium when the potential goes *above* or *below* the zero potential. In other words, contrary to what we have observed, both depolarization *and* hyperpolarization of the membrane should result in increased dye content in the medium (dissociation of dye from the cell membrane) and more intense fluorescence.

(3) One might assume the dye is capable of passing through the membrane. According to this model, when the inside electrical potential is negative relative to the outside, the positively charged dye is accumulated in the intracellular space in an aggregated form, or complexes with negatively charged macromolecules such as hemoglobin. The relative concentrations of free monomeric dye inside and outside the cell should be in accordance with the equilibrium distribution of permeant ions. Upon depolarization of the cell, the aggregates and complexes of dye are broken down as the dye is expelled to the extracellular space.

It would not be surprising to find that the dyes can move *through* the membrane because the positive charge of the fluorochrome is distributed over the chromophore structure, which has two symmetric charge resonance forms. The fact that the major charge is delocalized should reduce the free-energy increase entailed in moving the dye from the polar medium into the hydrocarbon interior of the membrane.

(4) A model can also be devised in which the dye in the extracellular medium is in equilibrium with the interfaces at both sides of the membrane, but in which there is only a small accumulation of dye within the intracellular space. Figure 8a (similar to the potential energy profile for a positive hydrophobic ion presented by Lauger and Neumcke, 1973) depicts the electrochemical potential energy (W) of dye in a membrane with a −7 mV electrical potential. When the cell is hyperpolarized (to −40 mV), the potential profile of the dye in the membrane changes to the form represented in Figure 8b. The dye would be expected to move from the medium to the inner membrane surface, where, owing to increased surface concentration, aggregation would take place and the fluorescence quenched. If the membrane were depolarized, the dye on the inner membrane surface would be expelled across the membrane and into the medium; fluorescence would increase.

We feel that model 4 is the most probable mechanism for the potential-dependent partition of diS-C_3-(5) between cells and the medium. In view of the high concentration of dye associated with the cells it may be necessary to include some of the features of model 3, which allows for accumulation of dye intracellularly. It should be mentioned that the other dyes listed in Table II that show potential-dependent fluorescence changes similar to diS-C_3-(5), probably respond to the potential by the same mechanism. The amount of dye remaining in the supernatant after hyperpolarization and centrifugation is observed to decrease by more than 50% for each dye whose fluorescence in cell suspension is observed to decrease by more than 50%. Although these dyes show potential-dependent uptake, we have not examined their aggregation or fluorescence quenching behavior in the cell preparation.

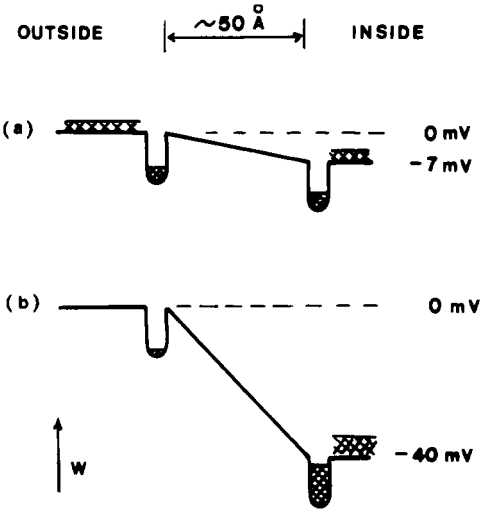


FIGURE 8: A hypothetical potential profile for the interaction of diS-C_3-(5) with a lipid bilayer membrane. The relative electrochemical potential energy, W, of the dye is the ordinate. Crosshatched marks represent the relative concentrations of dye in the membrane and outside of the membrane. The electrical potential difference between the two sides of the membrane changes linearly across the hydrocarbon region of the membrane in this model.

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Contribution of Carotenoids to the Optical Activity of Human Serum Low-Density Lipoprotein†

G. Chi Chen and John P. Kane*

ABSTRACT: Low-density lipoprotein (LDL) (1.024–1.045 g/cm^3) was prepared by ultracentrifugal flotation from serum of subjects on diets of varying carotenoid content. Absorption, circular dichroism (CD), and optical rotatory dispersion (ORD) spectra were measured at 2, 25, and 37°. LDL from subjects receiving a β-carotene-enriched diet has a marked increase in absorbance between 350 and 550 nm attributable to carotenoids. In the same region, LDL exhibits multiple CD bands, which appear below 37° and which increase in intensity with decreasing temperature. The corresponding ORD of carotenoid-poor LDL is monotonic at all three temperatures, whereas that of carotenoid-rich LDL shows multiple Cotton effects below 37°. However, the ORD becomes monotonic after subtraction of the contribution due to carotenoids using the Kronig–Kramers transform for CD to ORD. Since the magnitudes of the CD bands increase with enrichment of β-carotene, which itself lacks optical asymmetry, the observed optical activity of carotenoids appears to be induced by environmental constraint in the lipoprotein complex.

The optical activity of human serum low-density lipoprotein (LDL)[1] has been studied extensively. Most of the published studies of optical rotatory dispersion (ORD) and circular dichroism (CD) are confined to the ultraviolet region (Scanu and Hirz, 1968; Gotto *et al.*, 1968; Dearborn and Wetlaufer, 1969). However, Gotto *et al.* (1968) reported that the plot of their data of the ORD of LDL at room temperature according to the Drude equation was nonlinear and obeyed the Moffitt–Yang equation only over a narrow wavelength interval (300–425 nm). Kobozev and Troitskii (1967) observed that LDL showed temperature-dependent optical rotations at four wavelengths (405, 435, 546, 579 nm) in the visible region. Since LDL contains carotenoids (Oncley *et al.*, 1950; Krinsky *et al.*, 1958) which absorb light between 250 and 550 nm, these compounds could contribute to optical activity in the visible and near-ultraviolet regions. In this work, we have shown that the carotenoids in LDL exhibit optical activity which is temperature dependent. This optical activity is the basis of nonlinearity of data for LDL plotted according to the Drude equation for LDL between 300 and 600 nm. This optical activity also complicates the interpretation of data using the Moffitt–Yang equation for determination of the content of helix in the protein moiety of LDL (apoLDL). The appearance of optical activity attributable to the symmetrical molecule, β-carotene, at low temperature demonstrates temperature-dependent environmental constraint upon that compound within the lipoprotein complex.

Materials and Methods

Preparation of LDL. Serum was obtained from normal fasting male subjects on diets of varying carotenoid content and

† From the Specialized Center of Research in Arteriosclerosis of the Cardiovascular Research Institute, and Department of Medicine, University of California, San Francisco, California 94143. *Received March 22, 1974.* This work was supported by U. S. Public Health Service Grant HL-14237. Dr. Kane is an Established Investigator of the American Heart Association.

[1] Abbreviations used are: LDL, low-density lipoprotein; ORD, optical rotatory dispersion; CD, circular dichroism; apoLDL, the protein moiety of LDL.