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A Quantitative Resolution of the Spectra of a Membrane Potential Indicator, diS-C_3-(5), Bound to Cell Components and to Red Blood Cells

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Summary. Cationic cyanine dyes have been widely used to measure electrical potentials of red blood cells and other membrane preparations. A quantitative analysis of the binding of the most extensively studied of these dyes, diS-C_3-(5), to red blood cells and their constituents is presented here. Absorption spectra were recorded for the dye in suspensions of isolated red cell membranes and in solutions of cell lysate. The dependence of the spectra on the concentrations of dye and cell constituents shows that the dye binds to these membranes as monomers with an absorbance maximum at 670 nm instead of 650 nm as for free aqueous dye and that the dye binds to oxyhaemoglobin partly as monomer but primarily as dimer, with absorbance maxima ca. 670 and 595 nm, respectively. Quantitative estimates are derived for all binding constants and extinction coefficients. These estimates are applied to suspensions of whole cells to predict the dye binding, absorbance spectra, and calibration curves of binding and fluorescence *vs.* membrane voltage. Satisfactory agreement is found with binding and absorbance data for whole cells at zero membrane potential and with the binding and fluorescence data reported by Hladky and Rink (*J. Physiol. (London)* **263**:287, 1976) for cells driven to positive and negative potentials using valinomycin. The marked tendency of oxyhaemoglobin to bind dye as dimer is not shared by some other proteins tested, including deoxyhaemoglobin and oxymyoglobin.

Although the cyanine dyes have been used as photographic emulsion sensitizers and as anthelmintics (Swartzwelder *et al*, 1957), it was not until 1973 that Hoffman and Laris (1974) following the work of Davila, Salzberg, Cohen and Waggoner (1973) demonstrated that these dyes could be used to measure physiological membrane potentials. Hoffman and Laris, working on human and *Amphiuma* erythrocytes, found that the fluorescence of suspensions of red cells plus dye increased when the cell interior became more positive (depolarized) and decreased when the interior became more negative (hyperpolarized). Sims, Waggoner, Wang and Hoffman (1974) discovered that hyperpolarization increased the proportion of the dye

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cations which could be centrifuged out of a suspension of red cells and that this cell-associated dye fluoresced less strongly and at longer wavelengths than free aqueous dye. Sims *et al.* (1974) noted that diS-C_3-(5), the dye they studied most extensively, has a red-shifted fluorescence in organic solvents, but that such solutions invariably show higher, not lower, fluorescence efficiencies. They proposed formation of nonfluorescent dye aggregates as the explanation of the discrepancy, but were unable to say whether the aggregate was forming on the outside face, core, or interior face of the red cell membrane, or in the intracellular medium. Hladky and Rink (1976*a*, *b*, *c*) using diS-C_3-(5) found that most of the dye associated with red cells was actually bound inside the cell and that the amount bound was empirically proportional to the 1.6^{th} power of the internal dye activity. The dye in either a suspension of whole cells or in a solution of membrane-free cell lysate showed a prominent absorbance peak at 595 nm, some 55 nm blue-shifted from the normal absorption. The ratio of blue-shifted to normal absorbance increased with dye concentration. Dye bound to haemoglobin-free membrane fragments had its absorbance shifted to the red.

DiS-C_3-(5) and related dyes have been used in investigations of changes in membrane potential of red cells (Hoffman & Laris, 1974; Callahan & Hoffman, 1976; Hladky & Rink, 1976, *a*, *b*, *c*; Freedman & Hoffman, 1977), synaptosomes (Blaustein & Goldring, 1975), bacteria (Kashket & Wilson, 1974; Laris & Pershadsingh, 1974; Brewer, 1976), dinoflagellates (Adamich, Laris & Sweeney, 1976), mitochondria (Colonna, Massari & Azzone, 1973; Tedeschi, 1974; Laris, Bahr & Chaffee, 1975; Kinally & Tedeschi, 1976), Ehrlich ascites tumor cells (Laris, Pershadsingh & Johnstone, 1976), thin slices of bovine adrenal medulla (Baker & Rink, 1975), envelope vesicles from Halobacterium halobium (Renthal & Lanyi, 1976), squid axons (Davila *et al.*, 1973; Cohen *et al.*, 1974), and single muscle fibers (Oetliker, Baylor & Chandler, 1975). The use of polymethine dyes to monitor membrane potentials has recently been reviewed by Waggoner (1976).

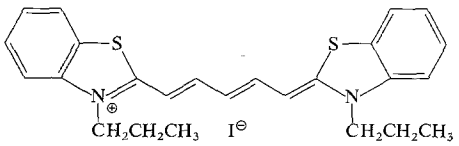
In the present study, oxyhaemoglobin is shown to be the major site of diS-C_3-(5) binding to red cells and quantitative estimates are obtained both for the binding constants of dye as monomer and dimer to membranes and oxyhaemoglobin and for the extinction coefficients for all the bound forms. With these numbers, it is then possible to build an explicit mathematical model for dye binding to the whole cell as the sum of its parts. This model, based entirely on absorption spectra, is tested by comparison of its predictions for dye binding with independent experiments in which dye binding is measured by centrifugation. The observed

fluorescence changes with potential are also compared with those expected from the changes in the concentrations of free dye, bound monomer, and bound dimer. Finally the binding of diS-C_3-(5) to a number of other proteins is considered and the use of permeant, fluorescent dyes as voltage-specific probes of other membrane systems is discussed.

Materials and Methods

Chemicals and Materials

3,3′-dipropylthiadicarbocyanine iodide, diS-C_3-(5),



was a gift from Dr. Alan Waggoner of Amherst College to Dr. T.J. Rink. The "diS-C_3-(5)" abbreviation was introduced by Sims *et al.* (1974). The Chemical Abstracts systematic name for this dye is benzothiazolium, 3-propyl-2-(5-(3-propyl-2(3 H)-benzothiazolylidene)-1,3-pentadienyl), iodide. Thin-layer chromatography of diS-C_3-(5) on silica gel eluted with chloroform-methanol (9:1 v/v) revealed only a single blue spot which faded to pink after a few hours exposure to light.

Solutions were made from glass-distilled water and reagent grade salts. K-gluconate was made up as a 1 molar stock solution, twice shaken with activated charcoal and filtered, and then used to prepare solutions as indicated. Tris-ATP and Tris-ascorbate were prepared by titrating Na_2H_2ATP or ascorbic acid with Tris base to pH 7.5. The standard medium for spectral measurements on red cell components contained 150 mmoles/liter KCl and 4 mmoles/liter Na-HEPES, pH 7.3. (4 mmoles/liter Na-HEPES, pH 7.3 means 4 mmoles/liter 4-hydroxyethyl-1-piperazine-ethanesulfonic acid adjusted to pH 7.3 with NaOH.) Tris base, Na_2H_2 ATP, K-gluconate, ascorbic acid, and the free acid of HEPES were obtained from Sigma. Dimethyl sulfoxide (DMSO) was Fisons Laboratory Reagent dried over Molecular Sieves 4 A. Dye solutions in DMSO were prepared at different concentrations by dilution from a master stock solution. These dilutions were monitored on a five-figure analytical balance to ensure accuracy to better than 1 %.

Blood drawn from a single donor (T.J.R.) was collected in a heparinized syringe, centrifuged and aspirated three times to remove plasma and buffy coat, and resuspended to provide a stock suspension of erythrocytes. Cell contents were prepared (Hladky & Rink, 1976*a*) by osmotic lysis and centrifugation to remove the membranes. Cell membranes, kindly supplied by Dr. T.J. Rink, were isolated by repeated washing in 30 mmoles/liter Tris Cl, 1 mmoles/liter EDTA, pH 7.3, as described by Hladky and Rink. Methaemoglobin was prepared by sodium nitrite treatment of whole blood. The excess nitrite was removed by the subsequent standard washing procedure. Deoxyhaemoglobin solutions or suspensions were prepared by nitrogen bubbling and were transferred by nitrogen pressure through double-ended syringe needles into serum-capped cuvettes (Shriver, 1969). Cell suspensions were freeze-thawed in glass vials by immersion in liquid nitrogen and gradual rewarming in room air. Metmyoglobin (type I, from horse skeletal muscle), cytochrome *c* (type VI, from horse

heart), and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co. Metmyoglobin was converted to oxymyoglobin by reduction with sodium dithionite and subsequent air oxidation. Myosin (from rabbit skeletal muscle) was kindly provided by Dr. Brian Pope of the Medical Research Council Laboratory of Molecular Biology, Hills Rd., Cambridge, and was supplied in 0.6 moles/liter NaCl, 10 mmoles/liter Na phosphate, pH 7.0 in 50% (v/v) glycerol/water. The glycerol was removed by dilution 10-fold into distilled water, centrifugation, and resuspension of the precipitated myosin in 475 mmoles/liter NaCl, 4 mmoles/liter Na-HEPES, pH 7.3. Rabbit F-actin was kindly donated by Dr. Paul Wagner, of the same laboratory, as a solution in 1.5 mmoles/liter $MgCl_2$, 6 mmoles/liter KCl, 3 mmoles/liter imidazole, and 3 mmoles/liter azide. F-actin was depolymerized to G-actin by standing for several days at 4 °C in a solution containing 0.2 mmole/liter Tris-ATP, 0.2 mmole/liter Tris-ascorbate, and 1 mmole/liter Na-HEPES, pH 7.45, then centrifuging 3 hr at $100{,}000 \times g$ to bring down any residual F-actin (Carsten & Mommaerts, 1963). Liposomes in standard media were prepared from lecithin-cholesterol in 3:1 mole ratio by the method of Deamer and Bangham (1976) but with further sonication to reduce the light scattering. All experiments were carried out at room temperature (ca. 20 °C).

Measurement of Absorption Spectra and of Dye Binding to Cells

Absorption spectra were measured using 1 cm glass or quartz cuvettes placed in the auxiliary sample position of a Pye Unicam SP800B recording spectrophotometer. The SP800B accepts pairs of cuvettes either in the standard position or in an auxiliary position immediately in front of the photomultiplier. The auxiliary position was always used because it minimizes any apparent absorption due to scattering. To get the best possible cancellation of haemoglobin absorbances and the residual scattering signals, a single batch of cells, haemoglobin, or membrane suspension was divided into two equal parts to fill the sample and reference cuvettes. The optical matching between the two paths was checked frequently. Dye was added during brief magnetic stirring to the sample cuvette and the difference spectrum recorded after sufficient time for equilibration (practically instantaneous for haemoglobin or membranes, up to three or four minutes for whole cells), but before significant dye losses could intervene. Dye absorbances in standard media were checked by iteration as described in Hladky and Rink (1976*a*). The SP800B gave the same absorbance readings for a 0.5 μmole/liter dye solution in the standard position with or without cuvettes containing a suspension of 10 μl packed cells/ml in the auxiliary position.

While the normal SP800B output is on a fixed absorbance scale from 0 to 2.0, there is an internal potentiometer which allows a 10× absorbance scale expansion. The wavelength scale was calibrated with holmium and didymium glass filters and checked by spot comparisons with a Zeiss M4QII single beam spectrophotometer with MM12 double monochromator. The Zeiss spectrophotometer was used to measure dye absorbances in solutions of haemoglobin for which the haemoglobin absorbance at 580 nm was greater than 2.0. The SP800B could not cope with such interference. The Zeiss gave the same absorbance readings for a dilute dye solution with or without cuvettes containing lysate from 125 μl of packed cells/ml in series with the dye sample and buffer blank.

Dye binding and absorption spectra were measured for cells suspended in 105 mmoles/liter KCl, 45 mmoles/liter K-gluconate, 4 mmoles/liter K-HEPES, pH 7.3. This medium was chosen to reduce the membrane potential to zero (*see*, for instance, Hladky & Rink, 1977) and thus also to make pH_i equal to pH_0. After the absorption spectrum was recorded, the cells were spun out of the light path, and the absorption and fluorescence of the supernatant were noted. For 1% haematocrit suspensions the apparent absorbance due to scattering was ca. 1 for wavelengths greater than 600 nm. Thus, most of the incident light was scattered and an

appreciable portion of the light reaching the photomultiplier had been scattered more than once. It follows that the distance the light reaching the photomultiplier had travelled through the cuvette was, on average, greater than 1 cm and thus that the extinctions of absorbing substances appeared larger than their correct values. The size of this error was estimated in two ways. Firstly, the absorbance of Blue Dextran 2000 (Pharmacia Ltd., London), which does not bind to the cells, is 1.15 times larger in a 1% haematocrit cell suspension than in standard saline. Secondly, the ratio for the 650 or 670 nm absorbances of diS-C_3-(5) in 1% haematocrit suspensions before and after freeze-thawing was 1.27. At lower haematocrits the ratio was 1.0.

Measurement of Fluorescent Efficiency of Dye Bound to Whole Cells

Fluorescence was measured using a Perkin-Elmer MPF44A spectrofluorimeter with emission and excitation slits set to 5 nm. A standard curve of fluorescence versus aqueous dye concentration, $F(c)$, was determined for the conditions of the experiment using the procedure described by Hladky and Rink (1976*a*). The inverse function to $F(c)$ is denoted $c(F)$. The scattering S from cells without dye was also measured at the wavelengths of the fluorescence measurement. To determine the fluorescence of bound dye relative to dye in the aqueous medium, a known amount of dye c_0 in 5 µl of DMSO was added to 2.5 ml of a 0.2% haematocrit suspension of cells in standard KCl medium. The fluorescence F_i was noted after 3 min, the suspension centrifuged and the fluorescence F_f of the supernatant noted. The amount of bound dye was then calculated as $c_0 - c(F_f)$, the fluorescence of this bound dye as $F_i - S - F_f$, and the relative fluorescent efficiency as $(F_i - S - F_f)/(F(c_0) - F_f)$. For the lowest dye concentration (0.063 µmolar) and the smallest difference in fluorescences, $F_i - F_f$, the scattering correction to the numerator was 15%. With excitation at 622 nm and emission at 670 nm, the total fluorescence, F_i, in the presence of cells never exceeded 65% of that from the same amount of dye in aqueous solution, $F(c_0)$. For 630 nm/665 nm the maximum percentage was 60%.

Analysis of Dye-on-Haemoglobin and Dye-on-Membrane Absorbances; Calculation of the Binding and Dimerization Constants and the Extinctions

Computer curve-fitting of the absorbance data was done by a FORTRAN IV program on the University IBM 370/165 computer, using the nonlinear iterative least-squares subprograms E04GAF or E04BAF from the Nottingham Algorithms Group library. Consider first the procedure for analyzing dye + membrane-free cell lysate experiments. During each iteration the program used its current estimates of the monomer and dimer binding constants to calculate for each cuvette sample the distribution of the known total quantity of dye between free aqueous monomer, bound monomer, and bound dimer, according to the equation

$$\begin{aligned}&[\text{aq. m.}] + (\text{Hb-m. const.}) \times \langle \text{Hb} \rangle \times [\text{aq. m.}] \\ &\quad + (\text{Hb-d. const.}) \times \langle \text{Hb} \rangle \times [\text{aq. m.}]^2 = [\text{total dye}]\end{aligned}$$

where [aq. m.] is the concentration of free aqueous dye monomer, and the two constants in parentheses ( ) are to be determined by the curve-fitting routine. ⟨Hb⟩ denotes concentration of haemoglobin present. The absorbance at each of three wavelengths (595, 650 and 670 nm) was then calculated using the current estimate of the extinction coefficients as

$$\begin{aligned}&[\text{aq. m.}] \times (\text{aq. extnctn. c.}) \\ &+ (\text{Hb-m. const.}) \times \langle \text{Hb} \rangle \times [\text{aq. m.}] \times (\text{Hb-m. extnctn. c.}) \\ &+ (\text{Hb-d. const.}) \times \langle \text{Hb} \rangle \times [\text{aq. m.}]^2 \times (\text{Hb-d. extnctn. c.}).\end{aligned}$$

The aqueous extinction coefficients (aq. extnctn. c.) had been determined separately from spectra of dye in buffer alone. The extinction coefficients for bound dye were left to the curve-fitting routine to adjust. The difference between the absorbance predicted above and the actual measured absorbance gave the absorbance error. Each absorbance error was divided by the estimated accuracy of the experimental reading, and then squared. Derivatives of the final sum-of-squares with respect to each of the adjustable parameters were then calculated in order to choose new values for the eight parameters (Hb-m. const.), (Hb-d. const.), and (Hb-m. extnctn. c.) and (Hb-d. extnctn. c.) for $\lambda = 595$, 650 and 670 nm to minimize the sum of squares. When the successive values for the sum-of-squares became sufficiently constant, iteration was stopped. The statistical uncertainty in each parameter value was derived by the standard formula (Wolberg, 1967; Nottingham Algorithms Group Manual, 1974) based on diagonal elements of the inverse of the Hessian matrix, which measure the curvature of the hypersurface at its minimum.

For dye-on-white-membrane experiments, the above equations remain valid if "memb." replaces "Hb". The same program is used.

In the above equations, ⟨Hb⟩ or ⟨memb.⟩ should actually be the concentrations of unbound haemoglobin or membrane, not the total added. For all the membrane spectra and almost all the haemoglobin spectra, the binding sites were so far from saturation that the free concentration of binding sites could in fact be approximated by the total added. Saturability has been explicitly included in the haemoglobin curve-fits though it gave no significant changes in the fitted parameters.

Predicting the Behavior of Dye on Whole Red Cells

The mathematical model for describing the dye on normal oxygenated red cells is cumbersome but obvious once the following assumptions are made:

a) The membrane potential controls the equilibrium ratio between internal and external dye activities, but not the binding curve of dye to haemoglobin. Similarly, the relation between the amount bound to either face of the membrane and the dye activity in the adjacent solution is assumed to be independent of membrane potential. While experiments on black lipid membranes (Waggoner, 1976) indicate that a direct but weak voltage-dependence of binding may cause fast, small changes in dye absorbance, such a weak voltage-dependence would be negligible here.

b) Since the membrane may be asymmetrical, the binding to the two faces may be different. In the model, the asymmetry is described by b, the proportion of total membrane bound dye which resides on the inside face of the membrane at zero potential. This proportion is assumed to be independent of dye concentration.

c) As described explicitly in the text, the constants used are based on those in Table 2. Extinction coefficients which came out slightly negative (in all cases not significantly different from zero) are left as such, not forced to zero.

The equations are as follows:

$$[\text{int. aq. m.}] = [\text{ext. aq. m.}] \times \exp(-\text{membrane voltage}/25.6\ \text{mV})$$
$$[\text{ext. memb. m.}] = (\text{memb.-m. const.}) \times (1-b) \times \langle\text{memb}\rangle \times [\text{ext. aq. m.}]$$
$$[\text{ext. memb. d.}] = (\text{memb.-d. const.}) \times (1-b) \times \langle\text{memb}\rangle \times [\text{ext. aq. m.}]^2$$
$$[\text{int. memb. m.}] = (\text{memb.-m. const.}) \times b \times \langle\text{memb}\rangle \times [\text{int. aq. m.}]$$
$$[\text{int. memb. d.}] = (\text{memb.-d. const}) \times b \times \langle\text{memb}\rangle \times [\text{int. aq. m.}]^2$$
$$[\text{Hb.-m.}] = (\text{Hb-m. const.}) \times \langle\text{Hb}\rangle \times [\text{int. aq. m.}]$$
$$[\text{Hb.-d.}] = (\text{Hb-d. const.}) \times \langle\text{Hb}\rangle \times [\text{int. aq. m.}]^2$$

[Total dye introduced] = [ext. aq. m.] × (1.0-haematocrit)
+ [int. aq. m.] × (hcrit) × 0.7
+ [ext. memb. m.] + 2 × [ext. memb. d.]
+ [int. memb. m.] + 2 × [int. memb. d.]
+ [Hb-m.] + 2 × [Hb-d.]
[total aq. m.] = [ext. aq. m.] × (1.0-hcrit) + [int. aq. m.] × hcrit × 0.7
[total memb. m.] = [ext. memb. m.] + [int. memb. m.]
[total memb. d.] = [ext. memb. d.] + [int. memb. d.]
fluorescence at standard wavelengths
= [total aq. m.] + (rel. efficiency of red-shifted memb-m.)
× [total memb.-m.] + (rel. efficiency of red-shifted Hb-m.)
× [Hb-m.]
absorbance at any of the three set wavelengths
= [total aq. m.] × (aq. extnctn. c. at that wavelength)
+ [total memb. m.] × (memb.-m. extnctn. c.)
+ [total memb. d.] × (memb.-d. extnctn. c.)
+ [Hb-m.] × (Hb.-m. extnctn. c.)
+ [Hb-d.] × (Hb.-d. extnctn. c.)
[bound dye] = [total dye introduced] − [ext. aq. m.] × (1.0-hcrit).

Quantities in square brackets again mean concentrations of dye species in moles per liter of cuvette suspension, except for [ext. aq. m.] and [int. aq. m.] which are in moles per liter of extracellular solution and intracellular water, respectively.

Properties of the Dye

Lability of diS-C_3-(5) and its Handling

DiS-C_3-(5) as solid crystals was not observed to undergo any decomposition. However, its solutions lose optical density remarkably easily. This lability is displayed in at least four ways:

1) The dye is slowly and irreversibly destroyed if illuminated in the presence of oxygen. This photo-oxidation limits the long-term stability of dye solutions in ethanol (Sims *et al.*, 1974), but seems not to be a significant problem in aqueous solutions for experiments lasting less than an hour.

2) The dye in aqueous saline tends to adsorb strongly to polyethylene or Teflon surfaces, or to any hydrophobic film on glass or quartz cuvettes. This dye can hardly be dislodged by any amount of rinsing with aqueous solutions, but is easily extracted by polar organic solvents such as ethanol, butanol, or dimethyl sulfoxide (DMSO). The easiest way to reduce adsorption losses to acceptably low levels is to avoid contact with plastics and to rinse all glassware and cuvettes with dichromate-sulfuric acid mixture just before use. The chromic acid must in turn be exhaustively rinsed off since any residual trace destroys dye. With such precautions correction for dye bound to the cuvette walls was not necessary when cells or their components were present during a single dye addition. Fortunately, plastic surfaces do not absorb dye from organic solvents, so that disposable pipette tips may be used for dispensing concentrated stock solutions in DMSO.

3) Any bubbling of an aqueous solution produces marked, rapid losses of optical density, sometimes as much as 50 % in a minute or two. This disappearance of dye cannot be photo-oxidation, as shown by four criteria: a) it is too fast; b) pure oxygen, air, or natural gas (methane) are equally effective at removing optical density; c) the decay is much less in distilled

water than in buffered saline; d) if the cuvette is covered with a glass cover slip during the bubbling, all the lost dye can be extracted from the cover slip, the upper walls of the cuvette, and the outside of the pipette used for the bubbling. This concentration of a surface-active material into the spray from breaking bubbles which is carried into the air is a well-known phenomenon in oceanography (MacIntyre, 1974). Although only a few microliters of solution are sprayed upwards by the bubbles breaking at the surface, they seem to contain a large share of the total dye. The practical consequence is that aqueous dye solutions must not be bubbled or needlessly agitated. Stirring is best done by small glass-enclosed magnetic bars at the bottom of the cuvette.

4) Stock solutions of dye in DMSO gradually become more dilute, losing strength at up to perhaps 10 % per day. This loss seems irreversible and proceeds at a significant rate even during storage in the dark in the freezer, though it is greatest at room temperature when the solution is being used frequently. Deteriorated solutions often have lowered freezing points, implying that moisture picked up from the atmosphere has diluted the sample. Quantitative work therefore requires freshly prepared stock solutions of dye and frequent checks of absorbance during any extended series of spectra.

Spectra of diS-C_3-(5) in Various Solvents

Absorption spectra of diS-C_3-(5) in several organic solvents are shown in Fig. 1. All show a peak extinction ε of 200,000–230,000 at 660–680 nm. The short-wavelength shoulder is present in all spectra at all concentrations and presumably reflects the vibrational fine structure of the individual dye molecules (West & Geddes, 1964). The dye behaves in a more

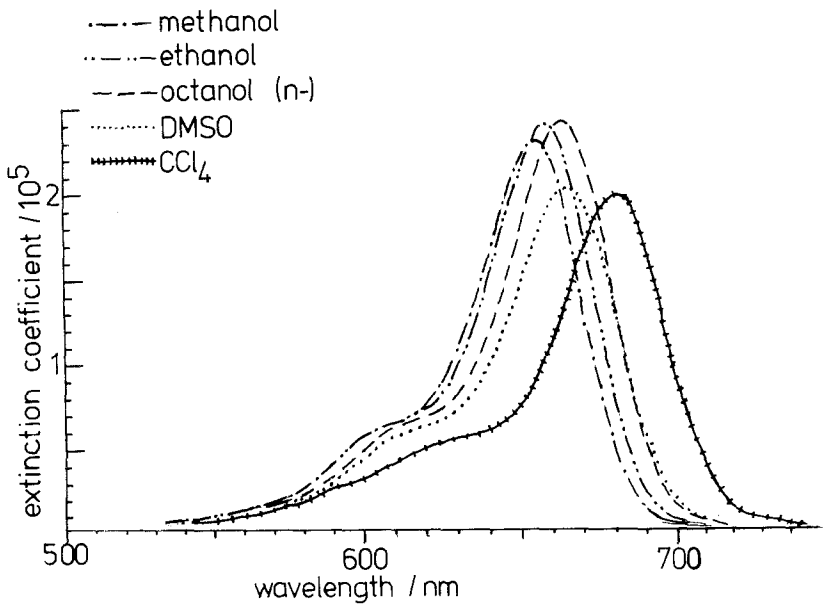


Fig. 1. Absorption spectra of diS-C_3-(5) in various solvents. 3.0 µl of a 5.0 mmoles/liter solution of dye in DMSO was stirred into 3.0 ml of solvent in a 1.0 cm cuvette, so that the final concentrations of dye and DMSO were 5.0 µmoles/liter and 0.1 % v/v. (– · – · –) methanol; (– · · – · · –) ethanol; (– – –) n-octanol; (· · · · · · · · ·) DMSO; (|||||||||) CCl_4

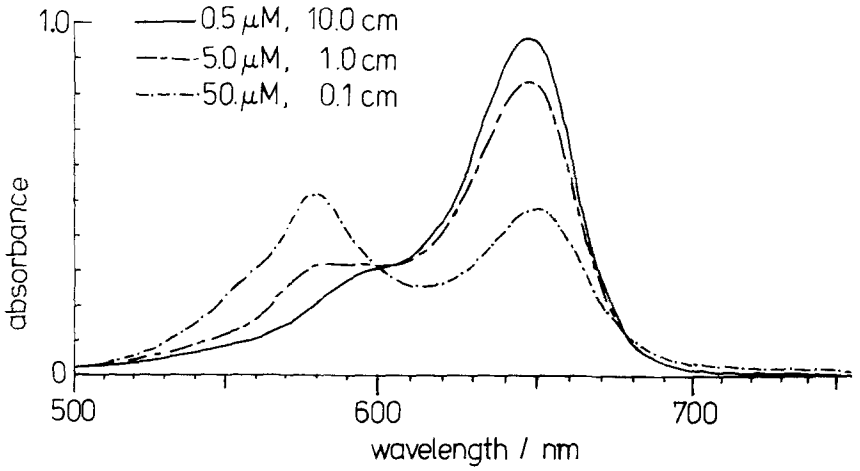


Fig. 2. Absorption spectra of diS-C_3-(5) at different concentrations in distilled water. The solid curve was obtained with a 0.5 μmole/liter solution in a 10 cm cuvette, the dashed curve corresponds to 5 μmoles/liter solution and 1 cm path length, the dash-dot curve to 50 μmoles/liter solution and 0.1 cm path length

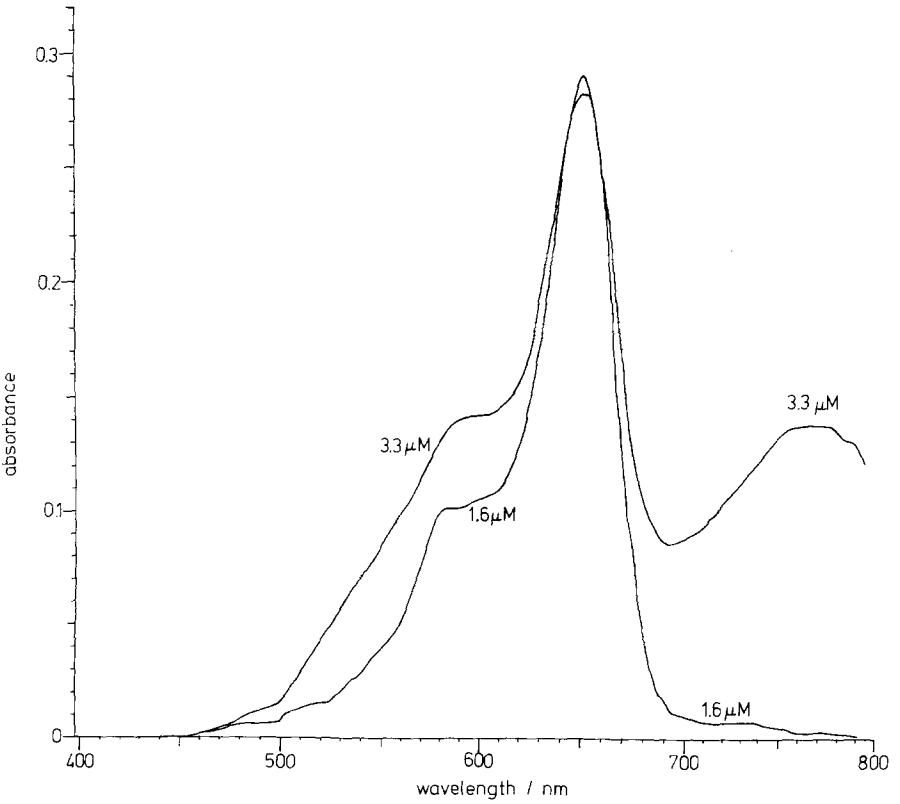


Fig. 3. Absorption spectra of diS-C_3-(5) in 150 mmoles/liter KCl, 4 mmoles/liter K-HEPES, pH 7.4, demonstrating the aggregation threshold. The total dye concentrations are 3.3 and 1.6 μmoles/liter for the upper and lower curves, respectively. Path length 1 cm. Doubling the total dye concentration produced a tiny rise in 650 nm absorbance, a larger increase at 590–600 nm, and a very large increase at 760–780 nm. The DMSO concentration was 0.17 % (v/v) in each solution

complicated manner in aqueous solutions. In distilled water (Fig. 2) or dilute (< 0.5 M) solutions of salts such as sodium sulfate or fluoride, low dye concentrations give a peak at 647 nm, $\varepsilon = 175{,}000$. Higher dye concentrations cause the shoulder at 590–600 nm to grow into a separate peak. Though it has not been studied quantitatively, this new peak looks qualitatively very much like the known examples of dimerization of this and other cyanine dyes (West & Pearce, 1965; Padday, 1968). Injection of dye into solutions containing bromide, iodide, or hydrophobic anions such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), gives broad ill-defined peaks extending to short wavelengths. Solid dye soon precipitates, suggesting that the spectra corresponded to colloidal aggregates in the process of precipitation. Dye in 150 mmoles/liter Na or KCl gives the usual aqueous monomer spectrum up to about 2 μmoles/liter but as the concentration is raised above 2 μmoles/liter, a broad peak appears at about 760 nm (Fig. 3). The suddenness of its onset as a function of concentration suggests that it represents some form of polymeric aggregate, though the broadness of the peak is not at all like the very sharp J-band of other cyanine aggregates (West & Carroll, 1966). The threshold concentration for aggregation is strongly dependent on the anions in the solutions, chloride being one of the more effective promoters. Once formed, the 760 nm peak cannot be quickly removed by redilution to less than 2 μmoles/liter or addition of dye-binding materials. Whatever the precise molecular structure of the aggregate, its indefinite stoichiometry and slowness of equilibration mean that its presence upsets any careful quantitative studies of the distribution and behavior of the rest of the dye. Therefore the concentration of the free aqueous monomer was always kept below the polymerization threshold. At such low concentrations, the dye obeys Beer's Law to within experimental accuracy. No significant formation of dimer is observed, because the presence of 150 mmoles/liter chloride has lowered the aggregation threshold so much that avoidance of 760 nm polymer means avoidance of 595 nm dimer as well.

Results

The Interaction of diS-C_3-(5) with Cells and Cell Components

If whole red cells are added both to the reference cuvette containing standard saline (150 mmoles/liter NaCl, 4 mmoles/liter K-HEPES, pH 7.3) and to the sample cuvette containing 1 μmole/liter diS-C_3-(5) in standard saline, the small concentration-independent shoulder at 595 nm which occurs in the absorbance spectra of the dye alone (sample minus reference) grows dramatically to become a major peak. If the dye concentration in a cell suspension is varied from zero upwards, the ratio of the absorbance at 595 nm to that at 650 nm grows and the 595 peak soon dominates the spectrum, suggesting that it represents dye dimer. Addition of red cells also shifts the 650 nm peak towards 670 nm. The analysis of dye absorption spectra on separated components of the cell demonstrates that dimerization occurs in the cell contents (Table 1) but not significantly on washed membranes. It will be shown below that the dimers are bound to oxyhaemoglobin. Table 2 lists the computed estimates of the monomer and dimer binding constants, and the extinction coefficients for bound monomer and dimer at 595, 650, and 670 nm. Examples of spectra are displayed in Fig.4

Table 1. Absorbances of diS-C_3-(5) in various dilutions of red cell lysates

Dye conc.	Lysate conc.	Absorbances × 1000 595 nm	650 nm	670 nm
0.42	899	29. 25	52, 49	61, 56
0.42	300	44, 41	39, 40	42, 42
0.20	300	12, 14	18, 23	20, 24
3.29	146	−, 605	152, 143	110, 121
3.29	146	520, 605	160, 143	140, 121
1.63	146	250, 273	60, 90	40, 80
1.63	146	−, 273	103, 90	98, 80
0.83	146	−, 121	65, 57	47, 53
0.43	146	−, 52	40, 36	28, 34
0.21	146	−, 20	14, 21	14, 21
0.20	88.9	17, 20	16, 19	14, 17
1.63	48.7	320, 294	80, 81	47, 58
0.83	48.7	134, 135	42, 52	34, 38
0.43	48.7	63, 60	26, 33	21, 25
0.21	48.7	29, 25	21, 20	14, 16
3.29	14.6	680, 632	150, 148	92, 76
1.63	14.6	300, 292	85, 92	49, 50
0.83	14.6	128, 134	56, 58	34, 33
0.43	14.6	58, 60	30, 37	16, 21
0.21	14.6	25, 25	21, 22	12, 13
1.63	4.87	280, 265	118, 121	57, 56
0.83	4.87	116, 119	78, 74	36, 35
0.43	4.87	54, 53	39, 45	19, 22
0.21	4.87	18, 21	24, 26	9, 13
1.63	1.62	230, 216	152, 169	75, 73
0.83	1.62	80, 96	105, 98	45, 43
0.43	1.62	41, 42	63, 57	29, 25
0.21	1.62	19, 17	38, 31	18, 14
1.63	0.49	120, 155	250, 227	115, 96
0.83	0.49	63, 71	138, 121	65, 52
0.43	0.49	29, 32	74, 66	31, 28
0.21	0.49	12, 14	35, 34	15, 15

The dye concentrations are in μmoles/liter of final suspension, the amount of lysate is specified as the final concentration (μmoles/liter) of haemoglobin in the cuvette (assuming $\varepsilon_{578} = 61{,}600$ per tetramer). For each solution composition and wavelength, the observed absorbance is listed before the value predicted by the "best fit" values of the constants in Table 2.

for lysate and Fig. 5 for isolated cell membranes. The extinction coefficients for haemoglobin bound monomer could only be determined from spectra at low dye and high haemoglobin concentrations (haemoglobin

Table 2. Computer-fitted parameters at pH 7.3, 20 °C, assuming that one haemoglobin tetramer binds a maximum of one dye monomer or dimer:

haemoglobin-dye monomer constant (Hb-m. const.) 0.023 ± 0.005 $(\mu M)^{-1}$
haemoglobin-dye dimer constant (Hb-d. const.) 0.338 ± 0.047 $(\mu M)^{-2}$
membrane-dye monomer constant (memb.-m. const.) 0.13 ± 0.05 per membrane unit; 1 membrane unit is the concentration of membrane present in a 0.1 % haematocrit suspension of cells.
membrane-dye dimer constant (memb.-d. const.) 0.015 ± 0.04 $(\mu M)^{-1}$ $(m.u.)^{-1}$

Extinction coefficients/(10^3 liters × $mole^{-1}$ cm^{-1}):

	595 nm	650 nm	670 nm
aqueous buffer	58.4 ± 2.0	172.2 ± 4.2	72.4 ± 2.4
Hb-bound monomer	-10.0 ± 52.0	158.9 ± 30.0	189.2 ± 39.0
Hb-bound dimer	465.8 ± 33.0	22.1 ± 16.0	4.6 ± 16.0
memb.-bound monomer	35.4 ± 15.8	104.3 ± 12.1	173.3 ± 26.1
memb.-bound dimer	327.0 ± 818.0	70.5 ± 700.0	-82.4 ± 1290.0

Monomer bound to any component of cell lysate is referred to, without proof, as bound to haemoglobin. The extinction coefficients for dimers refer to moles of dimers *not* moles of dye molecules.

580 nm absorbance greater than 1). Both the membrane and haemoglobin monomer spectra resemble those for the dye in ethanol or octanol though the peak extinction is substantially less. The extinctions for monomer bound to isolated membranes are the same as for monomer bound to liposomes.

The fluorescence of dye bound as monomer to membranes was determined by adding dye to suspensions containing an excess of either liposomes or isolated cell membranes. In both instances, membrane-bound dye excited at 630 nm fluoresced one-third as much at 665 nm as the same amount of dye in standard aqueous medium. No fluorescence has been detected for dye bound to haemoglobin as dimer. Dye bound as monomer to oxyhaemoglobin has little or no fluorescence either. Thus a solution with very little dye, 0.125 μmole/liter, but a very high haemoglobin content, 500 μmole/liter, had only 15 % as much fluorescence (excitation 630, emission 665 nm) as dye in standard medium. However, the absorbance at 670 nm indicated that at least 75 % of the dye was bound as monomer. Since 0–25 % could still be free aqueous monomer, 15 % is an upper limit on the relative fluorescence efficiency of haemoglobin-bound monomer.

For whole cells with zero membrane potential, the composite absorption spectrum, the binding to the different components, and the

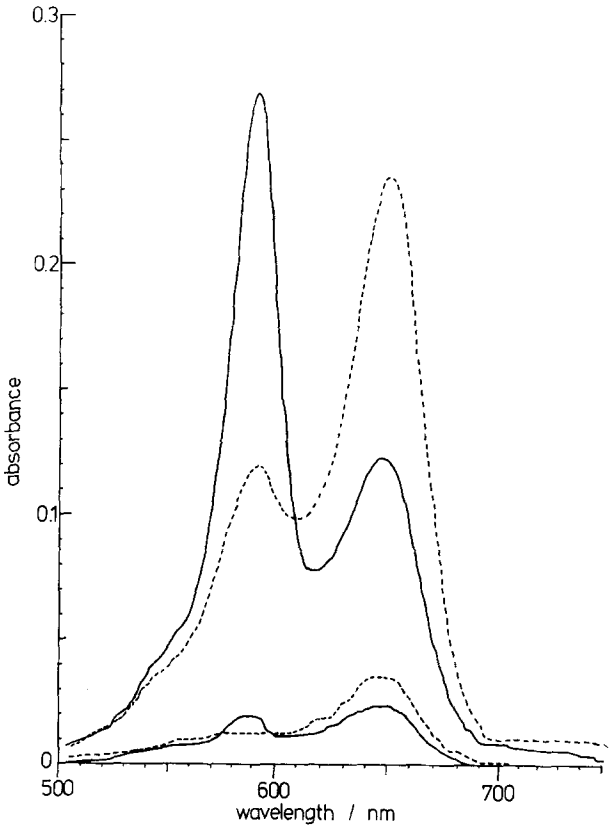


Fig. 4. Absorption spectra of diS-C_3-(5) plus cell lysate in 150 mmoles/liter KCl, 4 mmoles/liter K-HEPES. The solid lines correspond to 4.87 μmoles/liter haemoglobin tetramers, the dashed lines to 0.487 μmoles/liter. The upper curves are for 1.64 μmoles/liter dye, the lower for 0.209 μmoles/liter. The height of the 590 nm peak (assigned to haemoglobin-bound dimer) increases relative to the 650 nm peak (mostly aqueous monomer) as either the dye or haemoglobin concentration increases. The absorption peaks from the haemoglobin itself have been cancelled by putting matching dye-free solutions of cell lysate in the reference beam of the double-beam spectrophotometer

relative fluorescence efficiency of cell-associated dye may be calculated from the binding constants, the fluorescence efficiencies, and the extinction coefficients derived for separated cell components. The predicted binding, spectrum, and fluorescence may then be compared with independent experimental measurements (Table 3). With the values of the constants listed in Table 2 the model predicts binding curves indistinguishable from the curve for zero potential in Fig. 6 but it also predicts consistently larger absorbances at 595 nm than were observed. The concentrations and absorbances in Table 3 and Fig. 6 were, therefore, calculated using the

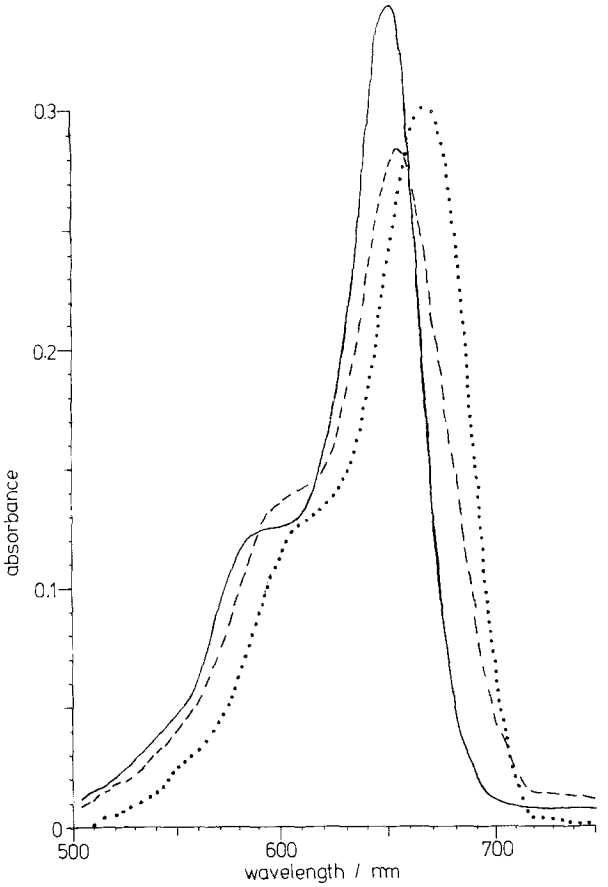


Fig. 5. Absorption spectra of diS-C_3-(5) plus haemoglobin-free fragments of red cell membranes ("white membranes"). The dye concentration in the membrane suspensions was 2.09 μmoles/liter. The dotted curve corresponds to the membranes from 33 μl packed cells per ml of suspension (33 "membrane units"), the dashed curve the membranes from 8 μl cells per ml, and the solid line to standard medium without membranes. Note the red shift of the absorption as the membrane content increases, and the absence of any distinct dimer peak at 590–600 nm

maximum values within the statistical uncertainties for the binding constants of monomer species (0.0277 l/μmole to haemoglobin, 0.18 $(m.u.)^{-1}$ to membranes) and the minimum values within the uncertainties for the binding constants of the dimer species (0.291 $l^2/\mu moles^2$ to haemoglobin, 0 to membranes). The estimates of the relative fluorescence efficiency of dye bound to separated cell components predict that the maximum relative fluorescence of dye bound to cells should be between 0.2 and 0.25 (ex. 630 nm, em. 665 nm). The relative fluorescence efficiency of

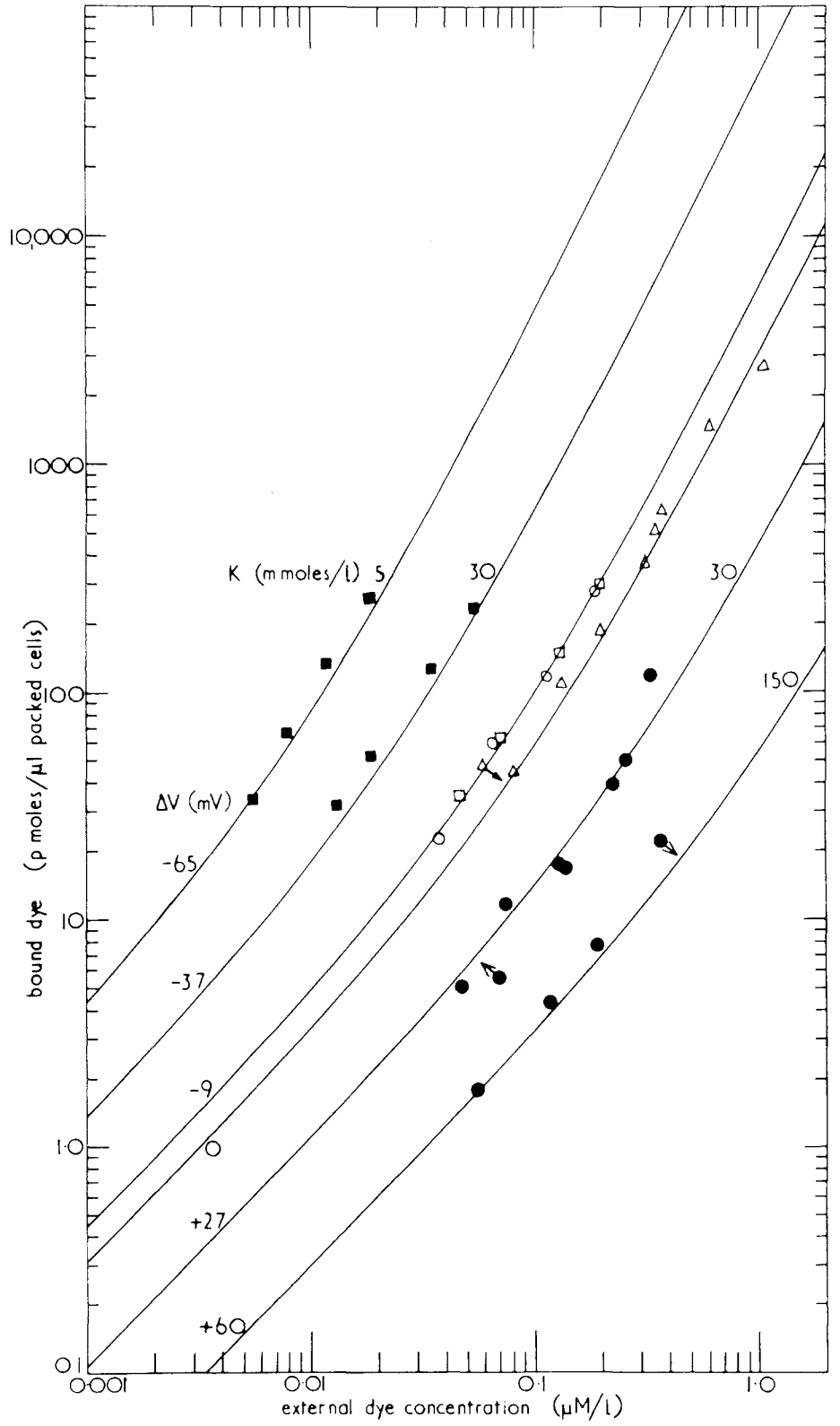
Table 3. Dye bindings and absorbances on whole cells in 105 mmoles/liter K-gluconate, 45 mmoles/liter KCl, 4 mmoles/liter K-HEPES, pH 7.29, predicted by the mathematical model and compared with independent experimental values

μl packed cells/ml:	0.32	0.32	0.96	0.96	0.96	3.2	3.2	9.6	9.6	9.6
total dye conc. (μM):	0.506	1.99	0.251	0.998	1.99	0.506	1.99	0.506	1.99	3.98
Predicted:										
monomer on Hb	0.014	0.033	0.018	0.049	0.074	0.062	0.150	0.101	0.255	0.386
dimer on Hb	0.054	0.384	0.028	0.222	0.545	0.100	0.613	0.089	0.576	1.35
monomer on membr.	0.021	0.065	0.026	0.074	0.122	0.089	0.225	0.144	0.371	0.573
free aqueous dye	0.364	1.13	0.150	0.431	0.706	0.155	0.390	0.084	0.215	0.332
Experimental values for aqueous dye after centrifugation, measured										
by absorbance:	0.343	1.13	0.134	0.395	0.714	0.136	0.383	0.081	0.226	0.369
by fluorescence:	0.35	1.15	0.14	0.38	0.63	0.13	0.36	0.06	0.19	0.33
Predicted absorbance at 595 nm:	0.047	0.247	0.023	0.131	0.299	0.058	0.315	0.050	0.29	0.66
Exptl. abs. at 595 nm:	0.050	0.192	0.019	0.113	0.235	0.052	0.285	0.044*	0.26*	0.63*
Predicted absorbance at 650 nm:	0.068	0.214	0.032	0.095	0.158	0.048	0.128	0.047	0.13	0.21
Exptl. abs. at 650 nm:	0.080	0.274	0.033	0.107	0.172	0.057	0.139	0.055*	0.14*	0.22*
Predicted absorbance at 670 nm:	0.033	0.101	0.019	0.054	0.089	0.039	0.098	0.051	0.131	0.20
Exptl. abs. at 670 nm:	0.032	0.105	0.016	0.047	0.084	0.044	0.099	0.046*	0.12*	0.21*

All predicted concentrations of dye species are in μmoles per liter of cuvette suspension. Values marked (*) have been reduced by 15 % as an approximate correction for the increase in optical path length due to scattering (*see* Materials and Methods).

dye bound to whole cells has been determined as described in Materials and Methods. In agreement with the predictions and with Sims *et al.* (1974) the efficiency decreases rapidly as the concentration of cell-associated dye increases. Measured at 630/665 nm, the value observed for the lowest amount of bound dye, 17 pmoles per μl of packed cells (45 ng dye per 5 μl of packed cells) was 0.2–0.3. The relative fluorescence efficiency at 622/670 nm was 0.3–0.45. Our methods do not produce values as high as the 0.9+ shown in Fig. 4 of Sims *et al.* (1974).

In order to consider the effect of potential on the binding and spectra it is necessary to specify separately the constants for the binding of the dye to the inside and outside faces of the membrane. This is conveniently done by specifying *b*, the proportion of membrane bound dye which is inside the cell at zero potential. Hladky and Rink (1976*a*) discuss in detail the evidence that comparatively little dye binds to the outside face of the membrane, i.e., in the present model that *b* is nearly 1. Experimental binding points from Table 3 and the data used by Hladky and Rink (1976*a*) in


10,000
1000
100
10
1·0
0·1
bound dye (p moles/μl packed cells)
K (m moles/l) 5
30
30
150
ΔV (mV)
-65
-37
-9
0
+27
+60
0·001
0·01
0·1
1·0
external dye concentration (μM/l)

their Fig. 10 are compared in Fig. 6 with the computer model predictions using $b=1$ and the values of membrane potential determined by Hladky and Rink. There is good agreement. Evidently the empirical result that for negative potentials the amount of dye bound is proportional to the 1.6^{th} power of the free external concentration can be explained as the sum of linear and quadratic terms in the binding. The model predictions assuming that the membrane is symmetrical ($b=0.5$) are indistinguishable from those in Fig. 6 ($b=1.0$) except for the curves for $+27$ and $+60$ mV. At these potentials, the model with $b < 0.8$ predicts substantially more binding than is observed. The data presently available do not allow an accurate estimate of the amount of dye bound to the outside face of the membrane.

There are two major implicit assumptions in this mathematical reconstruction of the red cell from its parts: 1) Some of the parts of the real red cell are not properly included in either the lysate or the white membranes, in particular the extrinsic proteins of the membrane, which were stripped off the white membranes during the extensive washing needed to remove the haemoglobin. Total binding to these is assumed to be negligible. 2) The model has tacitly assumed that the haemoglobin packed inside a red cell has the same intrinsic affinity for dye as dilute haemoglobin in lysate. To test whether dilution alone could change the molecular affinity of haemoglobin for dye, cells were suspended in a 105 mmoles/liter chloride, 45 mmoles/liter gluconate, 4 mmoles/liter Na HEPES, pH 7.3 medium. The cells in an aliquot of this suspension were lysed by freeze-thawing. Equal quantities of dye were added to the lysed and control suspensions and the two spectra compared. For 0.3 % haematocrit suspensions the

Fig. 6. Dye binding to whole cells as predicted by the model, compared with experimental determinations using the centrifugation assay. Note log axes. Open triangles, cells suspended in 105 mmoles/liter KCl, 45 mmoles/liter K-gluconate, pH 7.29 with 4 mmoles/liter K-HEPES, which should have zero membrane potential. The same data are used in Table 3. The circles and squares are the data points from Fig. 10 of Hladky and Rink (1976*a*). The squares are for cells filled with KCl by equilibration with 150 mmoles/liter KCl, 4 mmoles/liter K-HEPES, 27 mmoles/liter sucrose, pH 7.3, plus nystatin; the circles are for cells filled in a similar manner with NaCl. The nystatin was washed out of the cells before the experiment. Open circles and squares are for cells without valinomycin which should be at a normal Donnan equilibrium with membrane potential ca. -9 mV. Filled symbols are for cells suspended in mixtures of standard Na and K media containing 1 μmol/liter valinomycin and the K concentrations indicated. The curves have been drawn using constants determined as described in the text and the potentials indicated. For the curves through the filled symbols, the potentials were predicted by the Goldman-Hodgkin-Katz equation using $P_{K\text{-}Val}/P_{Cl}=16$ as determined by Hladky and Rink from the data represented by the squares

Table 4. Survey of proteins tested with diS-C_3-(5)

Protein	Gives red shift of 650 nm peak	Gives dimer peak near 595 nm
human oxyhaemoglobin	weak	yes
human methaemoglobin	weak	yes
human deoxyhaemoglobin	none detected	no
horse oxymyoglobin	very slight	no
horse metmyoglobin	none detected	no
horse cytochrome *c*	none detected	no
bovine serum albumin	yes	no
rabbit myosin	yes	no
rabbit actin	yes	yes

A slight red shift of the 650 nm peak is hard to detect because of jitter in the wavelength axis of the scale expansion recording.

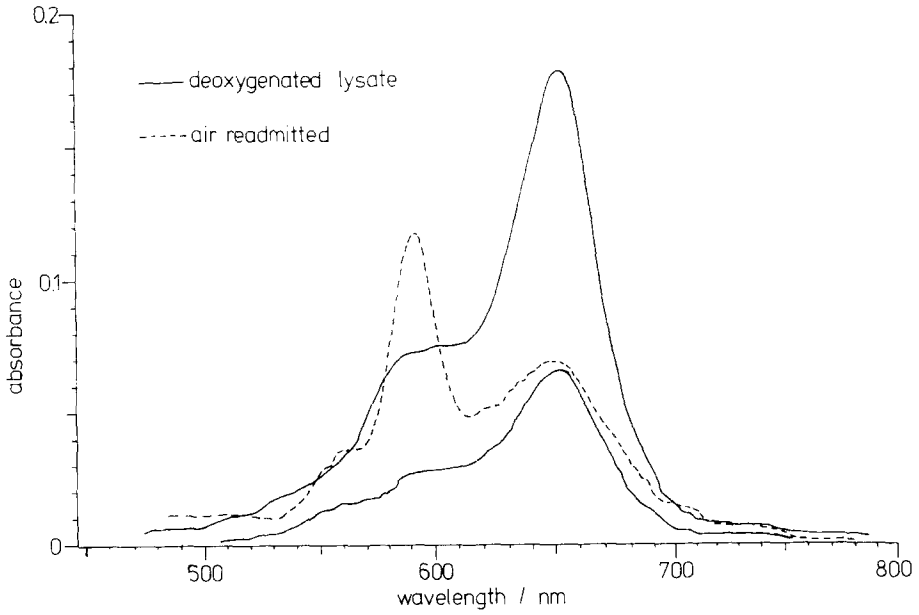


Fig. 7. Absorption spectra of diS-C_3-(5) in deoxygenated cell lysate (solid lines), 14 μmoles/liter in haemoglobin tetramers. (Similar spectra may be obtained using whole cell suspensions.) The lower solid line is for 0.5 μmoles/liter dye. Two further equal aliquots of dye were added successively to raise the nominal dye concentration to 1.5 μmoles/liter (upper solid line), though the actual concentration was less than 1.5 μmoles/liter due to the losses from stirring and adsorption to glass. Note the absence of a 595 nm peak and the approximate conformity to Beer's Law. The serum cap on the cuvette was then removed and the solution left for 3 hr to re-aerate, after which the dashed spectrum was recorded. The dimer peak at 595 nm gained at the expense of 650 nm absorbance

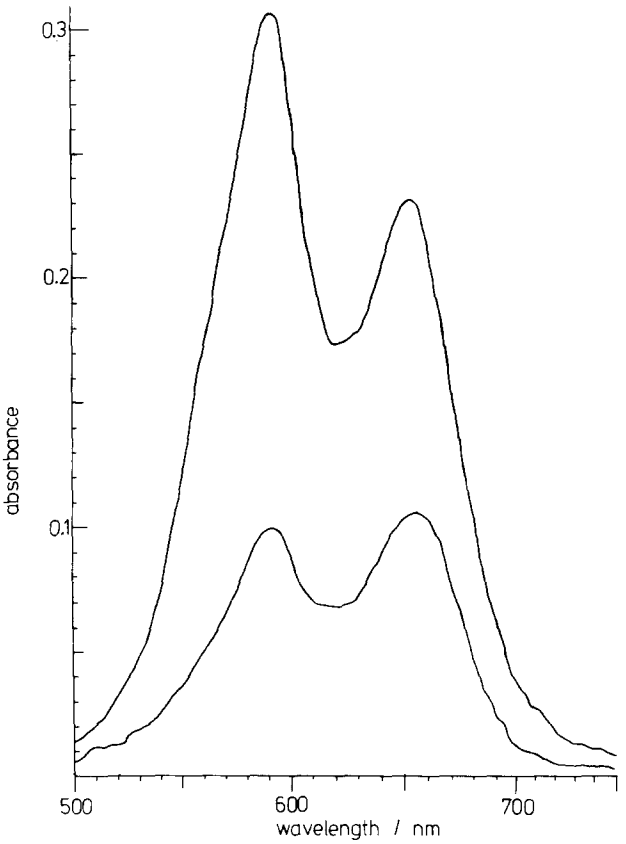


Fig. 8. Absorption spectra of diS-C_3-(5) at 1 μmole/liter and 3 μmoles/liter and rabbit F-actin, 125 μg/ml (2.6 μmoles/liter in actin monomers), freshly diluted into 0.2 mmoles/liter Tris-ATP, 0.2 mmoles/liter Tris-ascorbate, 1 mmole/liter Na-HEPES, pH 7.45. The spectra were run within 3–5 min of the dilutions into the ATP solution, so that depolymerization, which takes hours, should have been negligible. No significant difference was found when dye was added to an equivalent solution of actin which had been centrifuged at 100,000 × *g* for 3 hr after standing for 6 days in ATP solution. Note the peak at 595 nm and the increase in the Abs (595):Abs (650) ratio with increasing dye concentration

dye showed either the same or slightly more dimerization with the intact cells than with the lysed, demonstrating that dilution of the haemoglobin hardly affected the dye binding.

The finding that the most important interaction of diS-C_3-(5) with red cells was with cell contents (Hladky & Rink, 1976*a*, *b*), not with the membrane as had generally been assumed, prompted a study of the dye interaction with haemoglobin and other proteins. The substances tested are listed in Table 4. A striking finding is that simple deoxygenation either

of red cells or lysate prevents the formation of the 595 nm dimer peak (Fig. 7). This is a useful confirmation that the binding partner for the dye dimer in the red cell is really oxyhaemoglobin, not some other protein or cell constituent. It also shows that dimer formation is surprisingly sensitive to protein conformation. Dye binding to whole cells is correspondingly much weakened by deoxygenation, the ratio of bound-to-free dye decreasing about threefold at 1 μM total concentration of dye. Methaemoglobin has the same dimerizing ability as haemoglobin, which is not surprising considering the great similarity of their tertiary structure. The only other protein found to show dimer promotion is F-actin (Fig.8). No significant difference was found when the F-actin filaments were depolymerized to globular G-actin monomers. In myosin solutions, only red-shifted monomer appears.

Discussion

The red cell membrane potential controls the ratio between external and internal diS-C_3-(5) activities according to the Nernst relation. Internal dye activity in turn controls the binding to internal proteins or membranes. Bound molecules of diS-C_3-(5) absorb and fluoresce differently from free molecules and thus the binding (and in favorable circumstances the potential) can be determined from the spectra.

The present study has been concerned with the characterization of the binding and prediction of the potential dependence of the binding and fluorescence. The absorption spectra of diS-C_3-(5) bound to membrane fragments show no significant dimerization or deviations from Beer's law, just a simple red shift relative to the spectrum for dye in the aqueous phase. The fluorescence excitation and emission spectra are also red-shifted, without decreasing quantum yield. Though the dye adsorbed to the membrane is at a much higher volume concentration than in free solution, that crowding in itself is not sufficient in a nonaqueous environment to bring about aggregation. By contrast, dye bound to oxyhaemoglobin is predominantly present as nonfluorescent (Sims *et al.*, 1974; Hladky & Rink, 1976*a*) dimers which absorb strongly at 595 nm, some 55 nm blue-shifted from the maximum absorbance of dye in aqueous solution. Overall fluorescence amplitude of dye bound to red cells decreases because most of the dye is bound as nonfluorescent dimers to haemoglobin. The little remaining fluorescence arises from dye bound as monomer to the membrane and hence is as expected red-shifted from its

aqueous position. Proteins as well as membranes are able to cause red shifts of bound dye; even haemoglobin causes observable red shifting when the haemoglobin-to-dye ratio is sufficiently high to suppress dimerization. Cytochrome *c* may reject dye simply because it already carries a net positive charge. Negative charge alone is, however, not sufficient to cause strong binding, since the dye spectrum is not affected by the anionic polysaccharide heparin, which is reported to bind some other cationic dyes (Jeanloz, 1970).

The present study presents strong evidence that haemoglobin is the most important binding site for the dye in whole red cells, with the membrane playing a lesser role. In red cell ghosts, the binding curve and spectra obtained by Hladky and Rink (1976*a*, *b*) show that both the residual haemoglobin and the membrane surfaces are important. In vesicles prepared from frog rod outer segments (S.B. Hladky and W.T. Mason, *unpublished*), the absorption peak moves to 670 nm as if for monomer bound to the membranes of the vesicles. Whole muscle has not been tested, but our experience with membranes in general and with actin and myosin in particular suggests that dye will bind to the plasma membrane, the T system, sarcoplasmic reticulum, and myosin as monomer, to actin both as monomer and dimer, and possibly to other components. In lecithin-cholesterol liposomes, the absorption peak for dye at concentrations below 2 μmoles/liter is shifted towards 670 nm with no sign of dimerization. In experiments of Sims *et al.* (1974), the internal activity was raised above 2 μmoles/liter by strongly hyperpolarizing the vesicles. The far red-shifted absorbance in Fig. 7 of their paper clearly shows that aggregates had formed. In all cases investigated, binding of the dye has resulted either in a red shift of the absorption spectra for bound monomers or a blue shift of the absorbance and suppression of fluorescence for bound dimers. The far red-shifted aggregates are best regarded as a type of precipitate since in cell-free solutions they eventually coalesce to visible particles.

The direct method for measuring dye binding to cells in a suspension is to assay the supernatant for the dye left after centrifuging down the cells (Sims *et al.*, 1974). When this method is used, the requirements for a dye to be a potential indicator are much less stringent since the dye does not have to change its spectrum when it binds; it only has to be membrane-permeable, charged, and readily measurable in the supernatant (Hladky & Rink, 1976*a*). The disadvantages of centrifugation are that only a few cell or organelle types form properly dissociated suspensions which can also be spun down in a cuvette. Even when mechanically applicable, the centrifugation assay is a slow batch process unsuited to monitoring the

ongoing voltage response of a cell population. It also requires great care to be sure that the total amount of dye remains accurately conserved throughout the experiment.

Both centrifugation assay and whole-suspension spectroscopy can only measure membrane voltage if the relation between internal dye activity and internal binding either remains accurately constant or undergoes changes which are known exactly enough to allow compensation. Most previous workers have not taken care to characterize the binding. The red cell binding curve for diS-C_3-(5) is now known to be critically dependent on the quantity of haemoglobin and the internal pH (Hladky & Rink, 1976*a*, *c*; Freedman & Hoffman, 1977), and on the oxygenation of the haemoglobin. It should also be noted that the potentials calculated from either binding or spectral measurements are, strictly, potentials in the presence of the dye. It is easy to imagine that the presence of a lipid-soluble ion such as diS-C_3-(5) could change the permeability of the membrane. While no increases in membrane permeability have yet been reported for red blood cells, there is now evidence that a number of cyanine dyes can block the K channels of red cell ghosts opened by high internal Ca concentrations (Simons, 1976).

Fluorescence or absorbance measurements on whole tissues or cell suspensions are easier, faster, and more versatile in the variety of tissues acceptable than binding experiments, but more difficult to calibrate and vulnerable to artifact. The naive calibration procedure of plotting fluorescence *vs.* external potassium concentration in the presence of valinomycin leaves much to be desired, since no proof is usually presented that valinomycin has indeed done its job and overwhelmed all the other ionic conductances. A better procedure is available for cells in suspension since the centrifugation assay of binding may be used to calibrate the optical response as described by Hladky and Rink (1976*a*, *b*). One thus finds that in red cells the saturation of dye response at low external K is due both to the voltage not following log K_0 and the dye no longer responding linearly to potential. Even if the dependence of fluorescence on potential has been correctly determined, the resulting calibration curve remains valid only so long as the quantities of total dye and cells present, the excitation and emission wavelengths, and the internal binding curve remain constant. Sample calibration curves derived from the present model using the same values for the constants as in Fig. 6 and Table 3 are plotted in Fig. 9 and compared with experimental fluorescences reported by Hladky and Rink (1976*a*) at voltages measured by centrifugation assay. The curves have a nonlinear S shape because at extreme hyperpolarizations, when practically

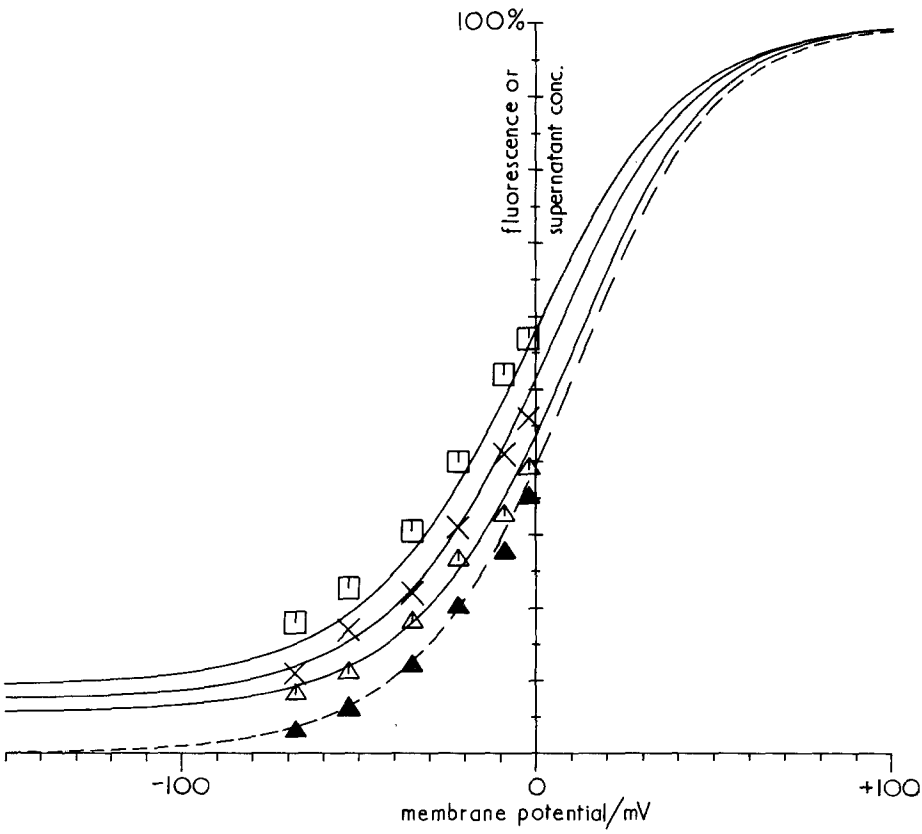


Fig. 9. Fluorescence and binding of dye to whole cells, predicted by the model as functions of voltage, compared with experimental determinations by Hladky and Rink (Fig. 12, 1976*a*). Filled triangles: percent dye left in supernatant after centrifugation of cells; total dye 0.463 μmoles/liter. Open triangles: fluorescence of suspension before centrifugation, measured as a percentage of fluorescence before addition of cells. 630 nm excitation, 665 nm emission. Crosses (X) and squares, fluorescence of suspensions with 0.231 and 0.116 μmoles/liter total dye, respectively. 2.0 μl packed cells per ml of suspension. The predicted curves have no free parameters, since all the constants have preassigned values as described in the text. The fluorescences are predicted on the basis that dye bound as monomer to membrane has an effective fluorescence efficiency (at the given wavelengths) 0.33 times that in water, while monomers bound to haemoglobin and dimers are nonfluorescent

all the dye has been sucked into the cell, further hyperpolarization can do no more; conversely at sufficiently positive internal potentials, essentially all the dye has been excluded from the cell interior, so that further voltage displacement is ineffective. Over a restricted range of intermediate potentials, the fluorescence is approximately linearly related to membrane potential, much as for the titration curve of an ordinary buffer where amount of titrant added is roughly linear with pH at the midpoint of the titration.

There is, however, no justification for a general claim that diS-C_3-(5) gives linear fluorescence responses to potential. The S curves in Fig. 9 shift to the left for decreasing total dye and would be shifted leftwards for decreasing cell count, decreasing internal pH, and decreasing degree of oxygenation. In suspensions which do not contain dimer-promoting protein, for example in guinea pig spermatozoa (D.P.L. Green & T.J. Rink, *unpublished*), even the qualitative sign of the response (fluorescence decrease implying hyperpolarization) can be turned upside down merely by exciting at 670 nm and detecting emission at 690 nm to select for red-shifted bound dye.

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