The Effects of Ionophores on the Fluorescence of the Cation 3,3'-Dipropylloxadycarbocyanine in the Presence of Pigeon Erythrocytes, Erythrocyte 'Ghosts' or Liposomes

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1. Pigeon erythrocytes, resealed lysed erythrocytes or liposomes derived from erythrocyte lipids were suspended in solutions containing up to $2\text{ }\mu\text{M}$ -3,3'-dipropylloxadycarbocyanine iodide. Gramicidin, valinomycin, nigericin or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, or combinations of these, were used to induce electrical diffusion potentials dependent on Na^+ , K^+ or protons. In each instance hyperpolarization of the cell membrane lowered the fluorescence of the cell suspension, a process that was completed in about 1 min. Subsequent depolarization caused an increase in fluorescence. 2. Quenching of the fluorescence of the cell suspension appeared to be due to the reversible binding of the dye to the cells. Much larger amounts of dye were bound, both to the intact and to the resealed erythrocytes, than would be expected if partitioning of the dye cation followed the Nernst equation. The dependence of the binding on the extracellular dye concentration was studied in the presence and absence of valinomycin. The results were consistent with the suggestion of Sims, Waggoner, Wang & Hoffman [(1974) *Biochemistry* 13, 3315–3330] that the dye was bound at both membrane surfaces and that, at low dye concentrations, hyperpolarizing the cells promoted dye binding at the inner membrane surface. 3. The applications of the technique are limited by the circumstance that the direct effect of the electric field on the uptake of the dye into the cells is amplified by a binding process that may be affected by other physiological variables.

There has been considerable interest in the possibility of using fluorescent carbocyanine dyes as a means of monitoring changes in cell-membrane potential, especially in situations where micro-electrode techniques are inapplicable (Waggoner, 1976; Cohen *et al.*, 1974; Sims *et al.*, 1974). The basis of the method appears to be that a change in membrane potential causes a change in the distribution of the cationic dye molecules between the cells and the extracellular fluid, which in turn causes a change in the fluorescence of the suspension, because the fluorescence of the cellular dye is quenched in comparison with a free solution of the dye (Sims *et al.*, 1974; Pick & Avron, 1976).

Changes in carbocyanine-dye fluorescence have been correlated with the expected changes in membrane potential caused by the addition of valinomycin to suspensions of human and *Amphiuma* erythrocytes (Hoffman & Laris, 1974; Sims *et al.*, 1974), hamster liver mitochondria (Laris *et al.*, 1975), mouse ascites-tumour cells (Philo & Eddy,

1975; Laris *et al.*, 1976), certain bacteria (Kashket & Wilson, 1974; Laris & Pershadsingh, 1974; Brewer, 1976), phosphatidylcholine/cholesterol vesicles (Sims *et al.*, 1974) and *Rhodospirillum* chromatophores (Pick & Avron, 1976).

We have now studied the effects of various ionophores, acting singly or in combination, on the fluorescence of 3,3'-dipropylloxadycarbocyanine iodide [1-(3-propylbenzoxazolidin-2-ylidene)-5-(3-propylbenzoxazol-2-ylonium)-penta-1,3-dien-5-ylidene iodide] (Sims *et al.*, 1974) in the presence of pigeon erythrocytes, resealed erythrocyte 'ghosts' or liposomes prepared from these. The ionophores selected were gramicidin, nigericin, valinomycin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Reid *et al.* (1974) used these to demonstrate the role of the membrane potential in the Na^+ -dependent absorption of amino acids by mouse ascites-tumour cells. The latter also exhibited fluorescence signals with 3,3'-dipropylloxadycarbocyanine iodide (Philo & Eddy, 1975), and we wished to obtain further information about the underlying mechanisms. Accordingly the binding of the fluorescent dye to the erythrocytes was studied in relation to a model that

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Sims *et al.* (1974) have proposed of the way in which the membrane potential affects the fluorescence of the system.

Materials and Methods

Carbocyanine dye

The dye 3,3'-dipropylloxadicarbocyanine iodide was prepared as described by Sims *et al.* (1974) and recrystallized twice from ethanol. A 200 μ M stock solution of the dye in ethanol was kept for up to 4 months at room temperature (approx. 295 K) in a light-tight cabinet to prevent photodecomposition, and 60 μ M working solutions in ethanol were made shortly before each day's experiments. The dye was relatively soluble in standard Ringer solution (Reid *et al.*, 1974) at 37°C, forming at least a 30 μ M solution.

Preparation of erythrocytes

Erythrocytes were prepared from Barbary doves (*Geotrygon mystacea*) by collecting whole blood from decapitated birds in 5.0 ml of chilled saline containing 1000 units (1 unit defined as having activity equal to 8.446 μ g of First British Standard Preparation) of heparin (Evans Medical Ltd., Liverpool, U.K.). The saline contained 150 mM-NaCl, 20 mM-Tris/HCl, pH 7.4, 1 mM-MgSO₄ and 1 mM-CaCl₂. Care was taken to avoid drainage of fluid from the crop into the collection vessel. The whole blood was centrifuged at 1000g for 5 min in an MSE clinical centrifuge to sediment the cells. Serum and the buff layer at the top surface of the pellet were removed by aspiration, and the cells resuspended in 2 vol. of fresh saline. After recentrifugation, and a similar wash cycle, the cells were suspended at 50% haematocrit in the saline at 0°C. The suspension was stored at 0°C.

Preparation of erythrocyte 'ghosts'

Erythrocyte 'ghosts' were prepared by haemolysing the intact cells by introducing 1 ml of the cell suspension into 9.0 ml of a solution containing 5 mM-Na₂HPO₄, 1 mM-MgSO₄ and 1 mM-CaCl₂, adjusted to pH 7.4 with HCl. The haemolysate was kept at 0°C for 15 min, and the osmoticity was then restored by the addition of either (a) 1.8 ml of 1 M-NaCl or 1 M-KCl or (b) 3.0 ml of 0.5 M-H₃PO₄ adjusted to pH 7.4 or pH 4.5 by the addition of KOH, NaOH or Tris base as required. The restored cells were incubated at 37°C for 30 min to seal the membranes, sedimented by centrifugation at 1000g for 5 min and washed in a solution of the composition used in the resealing process. The washed preparation was suspended at 50% haematocrit in the appropriate chilled medium, and kept at 0°C. 'Ghosts' prepared by this procedure retained less than 5% of the haemoglobin present in the intact cells. Haemoglobin was estimated in the original erythrocyte lysate and in each of the washings

by measuring the A_{540} of appropriately diluted samples.

Preparation of liposomes

Erythrocyte lipids were prepared from haemolysed cells washed free of released haemoglobin. The membrane pellet (about 1 ml) was extracted with 10 ml of chloroform/methanol (2:1, v/v) at 37°C for 30 min, with intermittent vigorous stirring. The extract was clarified by filtration through a small pad of glass-wool held in a capillary pipette, and the clarified extract evaporated to dryness under a stream of N₂. The residue was dissolved in 5 ml of chloroform/methanol (2:1, v/v) and portions (1 ml) were placed in small glass test tubes. The solvent was evaporated under N₂, and the dry lipids were stored in the tightly capped tubes under a N₂ atmosphere.

Liposomes were made by adding 2 ml of the appropriate salt solution containing 1 mM-MgSO₄ to a tube containing the dry lipids, and sonicating the suspension in a reservoir-type ultrasonic cleaning bath (Jencons, Hemel Hempstead, Herts., U.K.) at a frequency of 80 kHz for 2 min.

Assay of K⁺

The erythrocytes were suspended in 5% (w/v) trichloroacetic acid, and the K⁺ content of the extract was determined by flame photometry (Reid *et al.*, 1974). Intracellular volume was estimated as 80% of the volume of tightly packed cell pellets, by the use of [³H]inulin (The Radiochemical Centre, Amersham, Bucks., U.K.) as an extracellular-space marker.

Fluorimetry

Fluorescence measurements were made with an EEL fluorimeter, model 244 (Corning-Medical; Corning Ltd., Halstead, Essex CO9 2DX, U.K.), by using the 578 nm emission line from the mercury lamp for excitation of the sample. An Ilford 606 gelatin filter film (560–610 nm) selected the appropriate incident wavelength, and an Ilford 607 orange filter in conjunction with a setting of 630 nm (13 nm half-bandwidth) on the interference filter of the instrument selected the emission wavelength. Unless otherwise noted, 20 μ l of cell, 'ghost' or liposome suspension was used in 3.0 ml of incubation medium contained in a cuvette. This was housed in a block maintained at 37°C by the circulation of water at that temperature. A continuous recording of fluorescence was obtained on a Servoscribe potentiometric recorder, model RE 541.20 (Smiths Industries, Cricklewood, London NW2 7UR, U.K.). The contents of the cuvette were mixed by a Teflon-coated magnetic 'flea', driven by a small motor under the fluorimeter. In certain experiments the erythrocytes were removed from the light-path by spinning the cuvette in a clinical centrifuge for 1 min at 500g. The cuvette was warmed in a water

bath at 37°C for 30s before replacing it in the fluorimeter and measuring the fluorescence with the stirrer switched off. Starting the stirrer resuspended the erythrocytes.

The dye, valinomycin and the other ionophores used were each added in solution in ethanol. The final ethanol concentration in the cuvette did not exceed 3% (v/v), and up to 5% (v/v) did not affect the fluorescence of the system. Valinomycin and gramicidin were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was obtained from Boehringer Corp. (London) Ltd., London W5 2T2, U.K. Dr. Harned of the Research Department, Commercial Solvents Corp., Terre Haute, IN, U.S.A., kindly gave us a sample of compound X464, which is identical with nigericin (Henderson, 1971).

Mathematical methods

Numerical solutions to the system of equations used were obtained by expressing dye concentrations D_1 , D_3 and D_4 in terms of D_2 and solving for D_2 by using the subroutine CO5 AAF from the Nottingham Algorithm Group Subroutine Library (NAG Central Office, 13 Banbury Road, Oxford OX2 6NN, U.K.).

Results

Intact pigeon erythrocytes and valinomycin

When 20 μ l of the suspension of intact pigeon erythrocytes was added to the cuvette containing 2 μ M-dye, dissolved in the buffered 0.15M-NaCl solution used for the cell preparation, a rapid decrease in fluorescence occurred to a steady value reached by about 4 min (Fig. 1). At this point separation of the cells caused a rise in fluorescence (Fig. 1), which indicates that part of the initial decrease was due to the scattering and absorption of light by the erythrocytes. The addition of the K^+ -selective ionophore valinomycin (Henderson, 1971) caused a further rapid fall in fluorescence. Separation of the cells now (8 min; Fig. 1) caused a fall in fluorescence. This was presumably because the fluorescence of the cell-associated dye more than compensated for the optical interference of the cells. The addition of successive portions of 1 M-KCl (Fig. 1), but not of 1 M-NaCl (results not shown), caused an increase in fluorescence. Further additions of KCl had little effect when extracellular KCl was about 0.1 M. The fluorescence of the extracellular phase was then lower than that obtaining before the addition of the cells. Thus a fraction of the dye remained associated with the cells.

The effect of varying the membrane potential, by replacing extracellular Na^+ by K^+ in the presence of valinomycin, is illustrated in Fig. 2. Similar observations were made by Sims *et al.* (1974) using 3,3'-dipropylthiadicarbocyanine iodide and human erythrocytes, by Hoffman & Laris (1974) using 3,3'-

Fig. 1. Effect of erythrocyte-membrane hyperpolarization or depolarization on the fluorescence of 3,3'-dipropylloxadicarbocyanine iodide before and after separation of the cells

The washed pigeon erythrocytes were introduced into 3 ml of medium containing 150 mM-NaCl, 20 mM-Tris/HCl (pH 7.4), 1 mM-MgSO₄, 1 mM-CaCl₂ and 1.4 μ M-dye at the time indicated. Key to additions: C, erythrocytes (20 μ l of a 50%-haematocrit suspension); S, cells removed from the light-path by centrifuging the cuvette at 500g for 30s; M, cells resuspended by re-starting the magnetic stirrer; V, 9 nmol of valinomycin; A, 10 μ mol of KCl; B, 30 μ mol of KCl; D, 100 μ mol of KCl.

dihexyloxacarbocyanine iodide and *Amphiuma* erythrocytes, and by Philo & Eddy (1975) using 3,3'-dipropylloxadicarbocyanine iodide and mouse ascites-tumour cells. From experiments of this type the external K^+ concentration at which the addition of valinomycin causes no change in fluorescence may be found. The membrane potential at this 'null point' can be calculated from the K^+ gradient acting across the cell membrane (Hoffman & Laris, 1974). For the pigeon erythrocyte we found the external K^+ concentration ($\pm$ S.E.M.) at the 'null point' to be 48 ± 2.8 mM (four experiments) and the internal K^+ concentration to be 97.8 ± 3.3 mM (eight experiments). Hence the membrane potential was about -18 mV under these conditions.

The total fluorescence of the cell suspension was

Fig. 2. Effect of valinomycin on dye fluorescence in erythrocytes incubated at various extracellular K^+ concentrations

Conditions were as described for Fig. 1, except that part of the NaCl in the incubation medium was replaced by KCl to produce the indicated K^+ concentrations (mM). The arrow indicates the point at which valinomycin was added. The initial fluorescence readings varied by less than 5% for the five cases. The fluorescence changes after valinomycin addition were complete in 1 min and stable for at least 10 min.

related in a complex fashion to the dye distribution. For example, in the experiment illustrated in Fig. 1, the addition of valinomycin caused a 75% change in the fluorescence of the cell suspension, whereas the extracellular dye concentration changed by 90%. The effect was more marked at low dye concentrations (Fig. 3). The fluorescence of a cell suspension containing $0.2 \mu\text{M}$ -dye changed by only 19% in the presence of valinomycin, whereas the fluorescence of the extracellular solution changed by 100%.

The marked effect of valinomycin on the distribution of the dye was evident when the amount of cell-associated dye was assayed as a function of the extracellular dye concentration (Fig. 4). Valinomycin caused a 16–17-fold increase in the amount of dye bound at a given dye concentration.

Gramicidin

In contrast with the effect of valinomycin, a K^+ -selective ionophore, the addition of gramicidin,

Fig. 3. Variation of fluorescence with added dye concentration: erythrocyte suspensions in the presence and absence of valinomycin, before and after separation of the cells. Conditions and procedures were as described for Fig. 1. $\blacksquare$, $\square$, Suspension fluorescence; $\bullet$, $\circ$, fluorescence after separation of the erythrocytes. Open symbols, no valinomycin; filled symbols, $+3 \mu\text{M}$ -valinomycin. The solid lines were drawn by eye.

Fig. 4. Effect of valinomycin on the amount of dye associated with the pigeon erythrocytes at different extracellular dye concentrations

(a) $\square$, No valinomycin; (b) $\blacksquare$, $+3 \mu\text{M}$ -valinomycin. The buffered saline contained no added K^+ . Other conditions were as described in the legend of Fig. 1. Cellular dye was determined as the difference between the initial amount of dye present and that remaining in the supernatant solution after centrifugal removal of the cells, allowance being made for dye binding to the cuvette. The solid lines were calculated from the model described in the text.

which distinguishes poorly between Na^+ and K^+ (Henderson, 1971), resulted in a small initial decrease in fluorescence that partially reversed after about 0.5 min (Figs. 5a and 5b). Hyperpolarization of the erythrocyte was not expected to occur with gramicidin in these circumstances. However, gramicidin produced a large fluorescence decrease when the

erythrocytes were suspended in Tris/HCl (Fig. 5c), when they would be hyperpolarized. The fact that the subsequent addition of either Na^+ or K^+ increased the fluorescence is consistent with the relative in-

ability of gramicidin, as opposed to valinomycin, to distinguish between these two ions. The behaviour in the NaCl medium when gramicidin was added after valinomycin (Fig. 5b) can be explained in similar

Fig. 5. Effect of gramicidin on dye fluorescence in pigeon erythrocyte suspensions

In (a) and (b) the incubation medium was the NaCl medium described for Fig. 1. In (c) 150 mM-Tris/HCl replaced all of the NaCl. Key to additions: G, gramicidin (3 nmol); V, valinomycin (9 nmol); E, KCl (50 μmol); X, NaCl (10 μmol); Y, NaCl (30 μmol); Z, NaCl (100 μmol); A, KCl (10 μmol).

Fig. 6. Effects of nigericin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone on dye fluorescence in pigeon erythrocyte suspensions

(a) Suspension in the buffered NaCl as for Fig. 1. (b) Suspension in the buffered solution containing Tris/HCl instead of NaCl. Additions: N, nigericin (0.3 nmol); P, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (3 nmol); A, KCl (10 μmol); X, NaCl (10 μmol).

terms. It further supports the notion that the fluorescence monitored changes in membrane potential.

Nigericin

Nigericin, which catalyses an electroneutral exchange of cations (Henderson, 1971), caused no change in the fluorescence of cells suspended in NaCl medium (Fig. 6*a*) and a slow change when Tris/HCl was used (Fig. 6*b*). The fluorescence fell rapidly after the addition of the proton conductor carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The combination of nigericin and the proton conductor is electrogenic in the sense that it catalyses a net efflux of alkali cations that would hyperpolarize the system (Henderson, 1971; Reid *et al.*, 1974). The response to Na⁺ or K⁺ is consistent with that interpretation (Fig. 6*b*).

Fluorescence of 3,3'-dipropylxadicarbocyanine in resealed erythrocyte 'ghosts'

To obtain reproducible results, we found it advantageous to replace cellular chloride by the relatively impermeant phosphate anion by resealing the system in 0.11M-H₃PO₄ adjusted to pH7.4 with KOH.

Comparison of Fig. 7 with Figs. 1 and 2 shows that the effect of valinomycin on the fluorescence of these preparations and on the dye distribution resembled that shown by the intact erythrocytes. Similar amounts of dye were associated with the intact cells

and with the resealed preparations in the presence of valinomycin at the two concentrations of K⁺ used (Table 1).

The effects of imposed pH gradients were also studied (Figs. 8*a* and 8*b*) in circumstances where the diffusion of protons would either hyperpolarize (Fig. 8*a*) or depolarize (Fig. 8*b*) the system. When there was an outward H⁺ gradient and an inward K⁺ gradient the proton conductor carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone caused a decrease in fluorescence that was reversed by valinomycin (Fig. 8*a*). When the ionic gradients were reversed, so were the responses to the ionophores. The absence of both gradients abolished the responses to both ionophores (results not shown). These observations with the resealed cells provide further evidence of the inverse relation between the magnitude of the membrane potential and the fluorescence of the system.

Fluorescence of 3,3'-dipropylxadicarbocyanine iodide in liposome preparations

Mixtures of the dye with liposomes prepared from the erythrocyte lipids also responded to the ionophores in a manner consistent with the behaviour of both the intact cells and the resealed cells. For instance, valinomycin lowered the fluorescence when an outward gradient of K⁺ was imposed (Figs. 9*a* and 9*b*), and gramicidin reversed the fluorescence decrease caused by valinomycin, provided NaCl was present (Figs. 9*c* and 9*d*).

Fig. 7. Effect of valinomycin and K⁺ on dye fluorescence in suspensions of the pigeon erythrocyte 'ghosts' resealed in the potassium phosphate buffer, pH 7.4

(a) Suspension in iso-osmotic Tris/HCl. (b) Suspension in 100mM-H₃PO₄ adjusted to pH7.4 with KOH. Both systems contained 1 mM-CaCl₂ and 1 mM-MgCl₂ as well. Additions: H, resealed erythrocyte 'ghosts' (amount equivalent to the amount of intact cells used in Fig. 1); V, valinomycin (0.3 nmol); S, cells spun down; M, cells resuspended; A, KCl (10 μmol); E, KCl (50 μmol); D, KCl (100 μmol).

Table 1. Comparison of 3,3'-dipropylxadicarboyanine iodide binding and fluorescence changes observed for intact pigeon erythrocytes and the equivalent number of erythrocyte 'ghosts' in the presence of 3 μ M-valinomycin: effect of separating the cells by centrifugation

The erythrocytes and the resealed 'ghost' preparations were prepared as described in the Materials and Methods section and were suspended in the standard saline modified as indicated by the replacement of NaCl by KCl. The haemolysed 'ghost' preparations were resealed in the presence of the potassium phosphate buffer at pH7.4.

System	Dye concentration (μ M)	Extracellular [K ⁺] (mM)	Bound dye (nmol)	Relative decrease in fluorescence caused by valinomycin (as % of original fluorescence)	
				Cells+solution	Solution
Intact cells	1	1	5.11	71	73
	1	140	0.80		
	0.2	1	0.45	25	96
Resealed 'ghosts'	1	1	4.51	67	71
	1	140	0.80		
	0.2	1	0.43	54	76

Fig. 8. Effect of uncoupler and valinomycin on dye fluorescence in suspensions of pigeon erythrocyte 'ghosts' resealed in phosphate solutions containing Tris or K⁺ ions

Suspension media were: (a) 3.3 mM-KCl/110 mM-H₃PO₄ adjusted to pH7.4 with Tris; (b) 3.3 mM-KCl/110 mM-H₃PO₄ adjusted to pH5.0 with Tris. In (a) the 'ghosts' contained the phosphate buffer adjusted to pH5.0 with Tris, and in (b) they contained the phosphate buffer adjusted to pH7.4 with KOH. Additions: H, the resealed erythrocyte 'ghosts'; P, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (3 nmol); V, valinomycin (0.3 nmol); D, KCl (100 μ mol).

Quantitative aspects

The accumulated dye might form non-fluorescent complexes by self-aggregation (West & Pearce, 1965), possibly in association with polyanions (Bucher & Kuhn, 1970). However, a model based on dye aggregation in solution predicts that the fractional change in extracellular dye concentration, for a given change in membrane potential, would increase with the total

dye concentration, which is the reverse of what we observed. Sims *et al.* (1974) suggested that the cellular dye was mainly associated with the cell membrane rather than the cellular contents and that hyperpolarization caused the accumulation of the dye on the inner membrane surface. The fact that the erythrocytes and the resealed cells bound similar amounts of dye (Table 1), as well as the observed

Fig. 9. Dye-fluorescence changes in suspensions of liposomes generated from pigeon erythrocyte lipids and containing 0.11 M H_3PO_4 adjusted to pH 7.4 with KOH

Suspension media (a) and (d) 110 mM- H_3PO_4 adjusted to pH 7.4 with Tris; (b) 110 mM- H_3PO_4 adjusted to pH 7.4 with KOH; (c) 150 mM-NaCl. The system in each case also contained 1 mM-MgCl₂. Additions: L, liposomes; V, valinomycin (90 nmol); G, gramicidin (0.3 nmol); A, KCl (10 μ mol); B, KCl (30 μ mol); E, KCl (50 μ mol); D, KCl (100 μ mol); W, NaCl (160 μ mol).

magnitude of the fluorescence changes exhibited by the liposomes (Fig. 9), are consistent with that interpretation, which we developed as follows.

Consider four pools of dye concentration D_1 , D_2 , D_3 , D_4 , where subscript 1 refers to the extracellular solution, 2 to the outer membrane surface, 3 to the inner membrane surface and 4 to the intracellular fluid. Let both the inside and the outside of the membrane each have the same fixed concentration, S , of dye-binding sites of intrinsic dissociation constant K_d for the dye-membrane-site complex. The distribution of dye between pools 1 and 4 exhibits an equilibrium constant of 1 in the absence of a membrane potential, and a value Q , where $Q = \exp(-VF/RT)$, in the presence of a potential V ; F is the Faraday constant, R the gas constant and T the temperature. These assumptions lead to the equations:

$$D_1 = \frac{D_2 K_d}{S - D_2} \quad (1)$$

$$D_3 = \frac{Q D_2 S}{S + D_2(Q - 1)} \quad (2)$$

$$D_4 = \alpha Q D_1 \quad (3)$$

where the factor α is the ratio of the amounts of cellular water to total water and D_4 is expressed as a concentration in the total water.

The observations shown in Fig. 4 were fitted to eqns. (1), (2) and (3), the value of Q relevant to line (a) in Fig. 4 having been shown to be 2.04 by means of the null-point assay (see above). The curve-fitting procedures showed that Q rose to 150, which represents 0.13 V, in the presence of valinomycin (Fig. 4, line b). The solid lines shown in Fig. 4 correspond to

$K_d = 18 \mu M$, $S = 3.16 \mu M$ (10 nmol/mg dry wt. of cells). Thus the model satisfactorily fits these few observations.

Discussion

If it is assumed that the membrane potential of avian erythrocytes obeys the 'constant field' equation (Goldman, 1943) then, with an outwardly directed K^+ gradient, the addition of valinomycin would lead to hyperpolarization of the erythrocyte membrane. Gramicidin would cause this effect with either an outwardly directed Na^+ gradient or a K^+ gradient. According to the model of Sims *et al.* (1974) hyperpolarization leads to more of the positively charged dye molecules becoming associated with the cells, as indeed the observations illustrated in Figs. 1 and 7 show. There is, moreover, a decrease in the total fluorescence because the fluorescence of cell-associated dye is quenched compared with that of the dye in free solution (Sims *et al.*, 1974; Hoffman & Laris, 1974; Pick & Avron, 1976). In no case did we observe a decrease in the fluorescence of the cellular suspension that was not correlated with an increase in the amount of dye associated with the cells.

Depolarization can be effected by either the addition of a second ionophore (Fig. 5b) or increasing the extracellular concentration of any cation for which the ionophore used to induce hyperpolarization has specificity. Thus K^+ but not Na^+ depolarized the system when valinomycin was used, and both cations depolarized it when gramicidin was used (Fig. 5c). Depolarization causes a release of dye and increases the fluorescence of the suspension (Fig. 1).

Valinomycin and gramicidin catalyse electrogenic ion translocations, and the fluorescence changes induced by these agents are rapid. Nigericin, on the other hand, catalyses an electroneutral exchange of cations, but can cause a depolarization, albeit more slowly, by dissipating an existing gradient of permeant ion. Further, the presence of both nigericin and the proton conductor lowered the fluorescence in circumstances where hyperpolarization of the system was expected to occur (Fig. 6). All these observations strongly support the view that in avian erythrocytes, the derived liposomes and the resealed cell preparations the ionophores produced changes in dye fluorescence that reflected changes in the membrane potential. The latter were probably much faster than the changes in fluorescence, which were completed in about 1 min (Figs. 1 and 2).

Our observations are also consistent with the suggestion by Sims *et al.* (1974) that a large fraction of the bound dye is associated with the cell membrane (Table 1). The behaviour of the liposomes (Fig. 9), also studied by Sims *et al.* (1974), shows that phospholipids may be involved. When the present work with 3,3'-dipropylthiadicarbocyanine iodide was completed we became aware of a study by Hladky & Rink (1976) of the mechanism by which another cation, the carbocyanine dye 3,3'-dipropylthiadicarbocyanine iodide, monitors the membrane potential in human erythrocytes. In contrast with the observations illustrated in Table 1, these workers observed that considerably smaller amounts of 3,3'-dipropylthiadicarbocyanine iodide were bound by the resealed human erythrocyte 'ghosts' than by the intact erythrocytes, apparently because the dye binds to the cellular contents. We also noted the change in the spectrum of this dye in the presence of haemoglobin that Hladky & Rink (1976) reported, but we found no similar change with 3,3'-dipropylthiadicarbocyanine iodide, suggesting that this dye does not bind to haemoglobin.

Our preliminary analysis was consistent with the model based on two pools of bound dye, separated by an electric field corresponding to the membrane potential. It appears that the inner and outer surfaces of the membrane may carry similar numbers of binding sites. Much further work would be needed to prove this. Indeed, inspection of line (a) in Fig. 4 suggests that saturation of the proposed outer sites was not achieved in these assays and that the tendency to saturation exhibited in line (b) of Fig. 4 involves the hypothetical inner sites.

An important property of the model concerns the way the distribution of the dye between the extracellular solution and the cells would vary with the membrane potential when different concentrations of dye are used with a fixed concentration of erythrocytes (Fig. 10). The problem of relating dye distribution to the observed fluorescence of the suspension is

Fig. 10. Mathematical model of the relationship between the fluorescence of the extracellular dye and membrane potential

The fluorescence values shown were calculated for values of the membrane potential from 0 to -120 mV by using the model based on eqns. (1)–(3), with the parameters $K_d = 18 \mu\text{M}$, $S = 3.16 \mu\text{M}$. The total dye concentration was $0.4 \mu\text{M}$ for the line on the extreme left, increasing in steps of $0.4 \mu\text{M}$ to $4.0 \mu\text{M}$ for the line on the extreme right.

complex, and we do not have a rigorous solution to it. The largest fractional change in extracellular dye concentration for a given change in membrane potential is predicted to occur at low dye concentrations (Fig. 10). However, the fluorescence of bound dye is then relatively large (Fig. 3). The combination of these two effects would result in the largest fractional change in suspension fluorescence occurring at intermediate dye concentrations, as Hoffman & Laris (1974) observed for the human erythrocyte and Pick & Avron (1976) for chromatophores.

A similar family of curves to those shown in Fig. 10 was obtained when both the dye concentration and the cell concentration were fixed and the number of hypothetical binding sites on the cell membrane was varied. The conclusion of physiological relevance is that, in a constant environment, a change in the fluorescence of the system does not necessarily mean that the membrane potential has changed, unless there are reasons for thinking that both the number of dye-binding sites and their affinity for the dye have remained constant. The fluorescence is likely to be governed by other cellular parameters besides the membrane potential. This general conclusion is relevant to the problem of interpreting the changes in fluorescence that Philo & Eddy (1975) observed when glycolysis was initiated in suspensions of mouse ascites-tumour cells containing 3,3'-dipropylthiadicarbocyanine iodide, and where there was the further complication that the mitochondria and the plasma-

lemma both appeared to contribute to the fluorescence signal (Eddy *et al.*, 1976).

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