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DETERMINATION OF MEMBRANE POTENTIALS IN HUMAN AND *AMPHIUMA* RED BLOOD CELLS BY MEANS OF A FLUORESCENT PROBE

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SUMMARY

1. Changes in the fluorescent intensity of the dye, 3,3'-dihexyl-2,2'-oxacarbocyanine, added to suspensions of human and *Amphiuma* red blood cells were measured in parallel with changes in the membrane potentials of these cells. In these studies the membrane potential was altered in three different ways: by the addition of valinomycin to alter the ratio, P_K/P_{Cl}, by a change in the pH of the medium to alter the ratio, Cl_c/Cl_0, and by the substitution of impermeant anions for Cl_0 again to alter the ratio, Cl_c/Cl_0. In each case hyperpolarization led to a decrease and depolarization to an increase in fluorescent intensity.

2. The change in fluorescence with the addition of valinomycin was dependent on the concentration of K in both the cells and the medium. Changes in fluorescence were not observed when valinomycin was added to suspensions of frozen-thawed, haemoglobin-free ghosts with dye in KCl or NaCl solutions. Such changes were observed with reconstituted ghosts provided that there was a K concentration gradient across the membrane.

3. From values of cellular K and the corresponding external K concentrations for which there were no changes in fluorescence with valinomycin, estimations of membrane potentials were made. The potential was -5 to -8 mV for the human red cell and -19 mV for *Amphiuma*. These values are in good agreement with the potentials estimated from the Cl ratios (-9 mV for human and -17 to -20 mV for *Amphiuma*) and from those obtained by direct electrical measurements (-15 mV for *Amphiuma*).

4. Fluorescent intensity of the dye in suspensions of human red cells was shown to be a linear function of the log Cl_c/Cl_0.

5. The dye ($2{\cdot}9 \times 10^{-6}$ M) increased the inward rate constants for ^{24}Na

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(3–4-fold) and ^{42}K (0·5–2-fold) for human red cells. In addition, the dye was found to be haemolytic (5–6 % in 1 hr) at 22° C.

6. In contrast, the dye did not alter the rate of $^{35}SO_4$ efflux at 37° C from human red cells previously equilibrated with a Cl-free SO_4 medium.

7. The dye was also seen to interact with certain impermeant anions and other compounds, e.g. inhibitors of anion permeability, of interest. These interactions and other limitations of the use of this dye are discussed.

INTRODUCTION

The use of electrophysiological techniques to measure membrane potentials in small cells such as red blood cells and Ehrlich ascites tumour cells has been hampered by a number of problems (Lassen, Nielson, Pape & Simonsen, 1971; Hoffman & Lassen, 1971). Impalement of these cells with electrodes may lead to a relatively severe ionic leak which would cause a rapid discharge of the normal membrane potential to the junction potential between cytoplasm and the external medium. This junction potential is probably similar in value to the membrane potential of the normal cell (Lassen *et al.* 1971). Hence although the potentials measured in these cells by Lassen & Sten-Knudsen (1968) and by Jay & Burton (1969) approximate expected values, the origin of the potentials is uncertain. We sought to avoid these difficulties by using non-destructive fluorometric methods to estimate the membrane potential of human and *Amphiuma* red cells. The method is based on observations that the fluorescence of a number of fluorochromes is directly proportional to the membrane potential in the squid axon (Davila, Salzberg, Cohen & Waggoner, 1973; Cohen, 1973).

The membrane potential of red blood cells can be expected to follow the constant field eqn. (1) (Goldman, 1943; Hodgkin & Katz, 1949):

$$E = \frac{RT}{F} \ln \frac{P_{Na}[Na]_0 + P_K[K]_0 + P_{Cl}[Cl]_c}{P_{Na}[Na]_c + P_K[K]_c + P_{Cl}[Cl]_0}, \quad (1)$$

where R = gas constant; T, absolute temperature; F, Faraday's constant and P_{Na}, P_K, P_{Cl} the permeability constants for Na, K and Cl; $[Na]_0$, $[K]_0$, $[Cl]_0$ = Na, K and Cl concentrations (more strictly speaking activities) of the medium and $[Na]_c$, $[K]_c$, $[Cl]_c$ = those of the cell water. In the course of our experiments P_{Na} always remains very much lower than P_K or P_{Cl} and hence eqn. (1) can be simplified to eqn. (2) where $\alpha = P_K/P_{Cl}$:

$$E = \frac{RT}{F} \ln \frac{\alpha[K]_0 + [Cl]_c}{\alpha[K]_c + [Cl]_0}. \quad (2)$$

In the normal cell P_{Cl} for ionic diffusion is estimated to be about 100 times greater than P_K (Hunter, 1971) and, provided that chloride is distributed

passively, the membrane potential should be equivalent to the chloride equilibrium potential. Hence the membrane potential in an erythrocyte is generally estimated by measuring the chloride ratios. In keeping with this assumption the results of many studies indicate that diffusible anions such as chloride are distributed in accordance with a Donnan equilibrium (Warburg, 1922; Van Slyke, Wu & McLean, 1923; Funder & Wieth, 1966). Direct measurement of the membrane potential in *Amphiuma* red blood cells (Hoffman & Lassen, 1971) has also provided evidence supporting the idea that the membrane potential is equivalent to the chloride equilibrium potential in these cells.

We have attempted to determine whether or not changes in membrane potentials in human and *Amphiuma* red blood cells lead to parallel changes in the fluorescent intensity of suspensions of these cells containing the dye, 3,3′-dihexyl-2,2′ oxacarbocyanine. If the membrane potential of these cells is described by eqn. (2), then it should be possible to change the membrane potential by altering α or by altering the chloride ratio (Cl_c/Cl_0). In our experiments fluorescent intensity was measured while the membrane potential was altered in three different ways. First, α, the P_K/P_{Cl} ratio, was changed by the addition of valinomycin. The addition of this compound greatly increases P_K (Harris & Pressman, 1967; Scarpa, Cecchetto & Azzone, 1968, 1970; Hunter, 1971) and hence the potential should move in the direction of the K equilibrium potential. Secondly, if the potential is a Donnan potential, then a change in the ratio, Cl_c/Cl_0, by the substitution of an impermeant anion for chloride in the medium, should shift the membrane potential towards a more positive internal potential. And thirdly, the chloride ratio can also be altered by changing the pH of the medium.

A preliminary account of this work has already been presented (Laris & Hoffman, 1973).

METHODS

Fresh human blood was drawn into heparin (0·15 mg/ml.) from normal young adults and was centrifuged at approximately 10,000 *g* for 2 min at 4° C. After removal of the buffy coat by aspiration, the red cells were washed three times with a medium containing 153 mM-NaCl + 17 mM-Tris (hydroxymethylaminomethane) chloride (hereafter called NaCl-Tris) at pH 7·4 (note: KCl-Tris is used to designate a solution containing 153 mM-KCl + 17 mM-TrisCl). The cells were suspended in NaCl-Tris (pH 7·4) and kept on ice until use that same day. *Amphiuma* blood was drawn by heart puncture and immediately mixed with a modified amphibian Ringer solution containing 115 mM-NaCl, 2·5 mM-KCl, 1·5 mM-$CaCl_2$ and 10 mM morpholinopropane sulphonic acid (MOPS) at pH 7·2 (hereafter called Na-Ringer) (note: K-Ringer is used to designate a solution in which NaCl has been replaced by KCl). The blood was centrifuged at approximately 200 *g* for 5 min at room temperature. After the buffy coat was removed by aspiration, the red cells were washed three times with Na-Ringer. The cells were then suspended in Na-Ringer at pH 7·2

at a haematocrit of approximately 50 % and kept on ice until use that day. The *Amphiuma means* used in this study were obtained mainly from Mogul-Ed Company, Oshkosh, Wisconsin.

The cationic composition of human red blood cells was altered by the PCMBS (*p*-chloromercuribenzene-sulphonic acid) method of Garrahan & Rega (1967) as modified by Sachs (1972). Cellular K and Na were determined on appropriately diluted packed cells by flame photometry with Li as an internal standard. The percent cell water was measured on a weight/weight (w/w) basis by determination of the weight of approximately 0·2–0·4 ml. of packed cells (centrifuged 10 min at 27,000 *g*) before and after drying for 24 hr at 80° C. The percent cell water was then converted to a volume/volume (v/v) basis by multiplying the percent H_2O (w/w) by 1·097, the density of packed cells. Reconstituted human red cell ghosts were prepared by the methods of Bodemann & Passow (1972) as modified by Lepke & Passow (1972). Haemoglobin-free, frozen-thawed ghosts were prepared from human red cells, by the method given in Heinz & Hoffman (1965). Protein concentrations of suspensions of isolated ghosts were determined by the method of Lowry, Rosenbrough, Farr & Randall (1951).

The dyes employed in these studies were obtained from Dr Alan Waggoner of Amherst College. The dye used in most of the experiments reported here is 3,3′-dihexyl-2,2′ oxacarbocyanine (hereafter referred to as 'the dye' or CC_6). Unless noted the final concentration of this dye was $2{\cdot}9 \times 10^{-6}$ M. In a few experiments another carbocyanine dye, 3,3′-dipropyl-2,2′ thiadicarbocyanine iodide (referred to as Di-S-C_3 (5)), was used.

In experiments with intact cells and with the haemoglobin-free ghosts the dye was added directly to cell or ghost suspensions as a solution in ethanol. In work with resealed ghosts an ethanolic solution of dye was placed in a beaker and dried by evaporation. A 3 % suspension of resealed ghosts was then added to the beaker and stirred for 5 min. These ghost suspensions which now contained dye were then transferred to a cuvette and placed in the fluorimeter. Valinomycin dissolved in ethanol was also added directly to the cell or ghost suspensions while they were stirred. The final concentration of valinomycin was 1×10^{-6} M unless noted otherwise. The ethanol concentrations in the cell suspensions were generally 0·5 % and never higher than 1·0 %.

Fluorescence was measured with an Aminco Bowman Spectrophotofluorimeter with a 150 W Hanovia Xenon Arc lamp. An RCA 1P21 photomultiplier tube was used in the studies with CC_6 and a Hamamatsu 446 tube with Di-S-C_3 (5). The fluorescent intensity was recorded with a Hewlett Packard *X-Y* recorder and tracings of actual records obtained are presented as in Fig. 1 or in composite as in Fig. 2. The compositions of the incubation mixtures are given in the Figure legends and text. Initial volume in the cuvette was 3 ml. Except where indicated, excitation was at 460 nm and emission at 505 nm for CC_6 and 622 nm and 670 nm respectively for Di-S-C_5 (3). The slit arrangement used gave band widths at the half-height of 10–15 nm. The experiments were carried out at room temperature (22–23° C). The cell or membrane suspensions were stirred magnetically with a circular stirrer inside a standard 1 cm path length quartz cuvette. Three ml volumes of suspension medium were added to the cuvette and for intact cells, 10 μl. packed cells (at an haematocrit of 40 %) were added to make a final haematocrit of 0·33 %.

^{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 the method of Sachs & Welt (1967). The cells were at 0·33 % suspension in a medium containing 148 mM-NaCl, 5 mM-KCl, 17 mM-TrisCl (pH 7·5) and 1×10^{-4} M ouabain in the presence and

absence of $2{\cdot}9 \times 10^{-6}$ M dye. Measurements of the outward rate constant for K in human and *Amphiuma* 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-TrisCl (at pH = 7·5) at 37° C for 2–3 hr and of *Amphiuma* cells in a Na-Ringer at 22° C for 2 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° C 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 haematocrit during equilibration was approximately 10 %. After equilibration, the cells were washed five times at 0° C with tracer-free Na_2SO_4-$Tris_2SO_4$ medium. The packed cells were then suspended at 0·3 % haematocrit in tracer-free SO_4 media at either 22 or 37° C. 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. The outward rate constant was calculated by the method described by Gardos, Hoffman & Passow (1969).

^{36}Cl ratios and $^{35}SO_4$ ratios. For determination of ^{36}Cl ratios, washed human or *Amphiuma* red cells (less than 1 % haematocrit) were first equilibrated at 22° C with the different media as specified in the Results section. After equilibration the haematocrit of the suspension was increased to approximately 10 % by centrifugation at 22° C and a tracer quantity of $H^{36}Cl$ (obtained from Cambridge Nuclear Corp., Cambridge, Mass.) added and the suspension mixed. The cells were then packed by centrifugation at 27,000 *g* at 22° C for 10 min. Samples of 0·094 ml. packed cells or supernatant fluid were pipetted (using an 'Aliquanter' obtained from the Hamilton Co., Whittier, Calif.) and mixed with 1·0 ml. H_2O. After the cells had haemolysed 1·0 ml. 10 % trichloroacetic acid was added with mixing. The samples were centrifuged and a 1·0 ml. aliquot of the clear supernatant fluid was added to 10 ml. Triton-X-100-toluene fluor and counted by liquid scintillation.

The $^{35}SO_4$ ratios were determined on cells which had been equilibrated with Cl-free SO_4, as described before for the measurement of $^{35}SO_4$ efflux. After incubation at 37° C the cells were further incubated for 4 hr at 22° C. The medium and cells were then separated as described above for ^{36}Cl ratio determinations. Similarly aliquots of the cells and supernatant were analysed by the same method as described for ^{36}Cl.

RESULTS

Human red blood cells

Fluorescent intensity with time. The fluorescent intensity of CC_6 following its addition to NaCl-Tris at pH 7·4 is given in Fig. 1. There is an initial rapid appearance of fluorescence with the mixing of the dye followed by a period of slow decline in light emission until a steady level is reached. When the same amount of dye is added to a 0·33 % suspension of red blood cells in the same medium, the changes in fluorescent intensity are similar. However, as seen in Fig. 1, the initial rise is lower; the decline in light

emission is faster and the steady level is reached more rapidly in the presence of cells than in their absence. The substitution of KCl for NaCl in the medium did not appreciably change the fluorescent intensity of the dye in a medium alone or in cell suspensions.

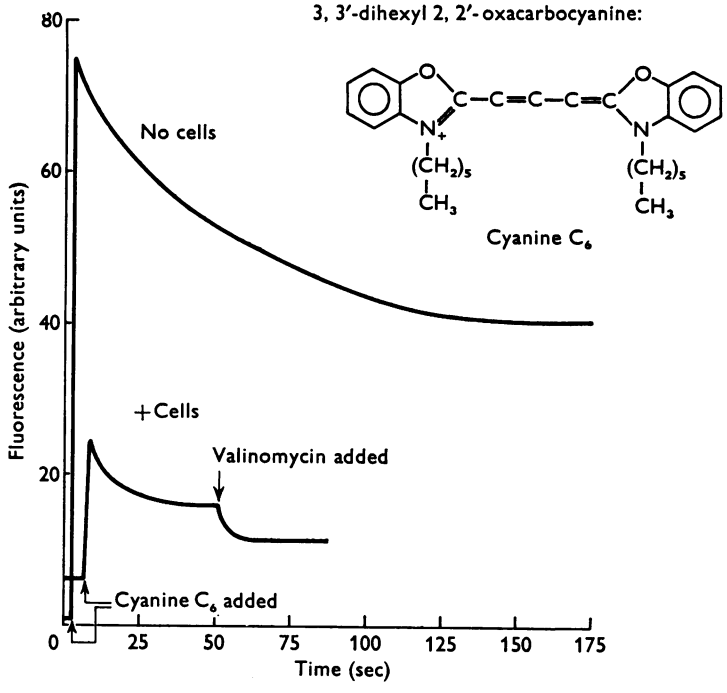

Fig. 1. Characteristic changes in fluorescent intensity of the dye CC_6 as a function of time in a 0·33 % suspension of human red blood cells in NaCl-Tris medium at pH 7·4 or in the NaCl-Tris medium alone. Excitation at 460 nm and emission recorded at 505 nm. Dye (final concentration of $2{\cdot}9 \times 10^{-6}$ M) and valinomycin (final concentration 1×10^{-6} M) were added as indicated.

Addition of valinomycin. The fluorescent intensity of normal red cells in NaCl-Tris at pH 7·4 containing CC_6 was markedly influenced by the addition of valinomycin. A rapid drop (approximately 30 %) in fluorescent intensity was observed (Fig. 1) when valinomycin (added in 10 μl. ethanol) was added to cell suspensions which had reached the steady level of fluorescence. This drop was not observed when 10 μl. ethanol alone was added to cell suspensions nor were changes seen upon the addition of valinomycin to the dye in the absence of cells. The magnitude of the fluorescence change upon the addition of valinomycin was found to be dependent on the external K concentration. As shown in Fig. 2, with increasing K (sub-

stituting KCl for NaCl in the Tris medium), the decrease in fluorescence was smaller. At 115 mM-K there was no change in fluorescence and above this concentration of K, the fluorescence increased. The addition of valinomycin produces a highly selective increase in K permeability in red cell membranes (Harris & Pressman, 1967), the magnitude of which can even exceed the control value for P_{Cl}. This increase in P_K should result in a

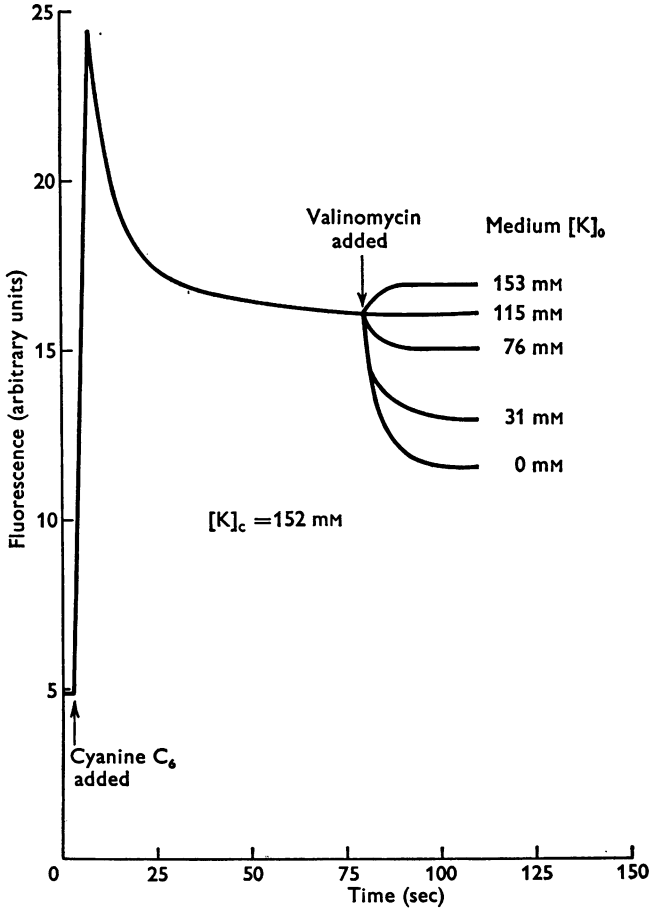

Fig. 2. Fluorescent intensity of the dye with time in a 0·33 % suspension of normal human red blood cells in NaCl-Tris medium at pH 7·4 and in mixtures where KCl was substituted for NaCl to give the K_o values listed on the figure. K_c equals 152 m-mole/l. cell water. Dye (final concentration of $2{\cdot}9 \times 10^{-6}$ M) and valinomycin (final concentration 1×10^{-6} M) were added where indicated. This Figure (as in others of similar design) presents tracings of actual recordings obtained in a single experiment which have been normalized to the same steady level of fluorescence prior to the addition of valinomycin.

change in the membrane potential when the KCl products are unequal since in the absence of valinomycin P_{Cl} is 100 times greater than P_K (Hunter, 1971). If the changes in fluorescence which occur upon the addition of valinomycin are related to changes in the membrane potential, expected from the action of valinomycin, then a decrease in fluorescence should be associated with hyperpolarization of the membrane and an increase in fluorescence with depolarization.

The changes in fluorescence observed after the addition of valinomycin

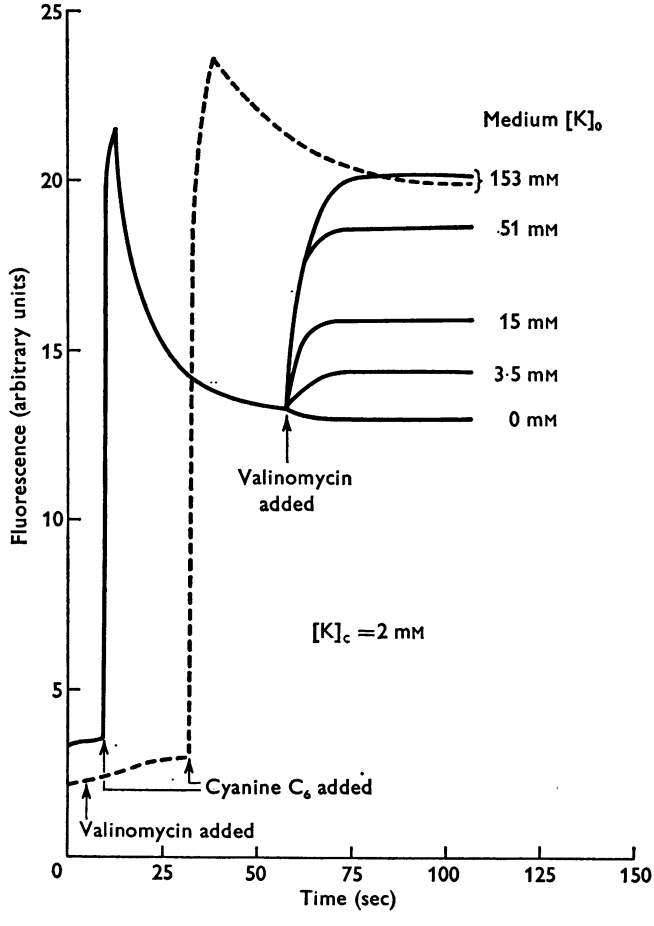

Fig. 3. Fluorescent intensity of the dye with time in a 0·33 % suspension of human red blood cells in NaCl-Tris medium at pH 7·4 and in mixtures where KCl was substituted for NaCl to give the K_0 values listed on the Figure. K_c altered to equal 2 m-mole/l. cell water. Dye (final concentration of $2{\cdot}9 \times 10^{-6}$ M) and valinomycin (final concentration of 1×10^{-6} M) were added where indicated. Note that in the curve with the dashed line, valinomycin was added prior to the dye and K_0 = 153 mM.

also vary with the cellular K concentration as shown in Fig. 3. In this series of measurements the cellular concentration was changed to 2 mM by treatment with PCMBS and dithiothreitol (see Methods). When the external medium was K-free there was either no change or just a small decrease (2 %) in the fluorescent intensity. With increasing external K the fluorescence increased when valinomycin was added and was 50–60 % greater when the external K equalled 153 mM. Since the results obtained from cells also treated with PCMBS and dithiothreitol but maintained at normal (high) K concentration were similar to untreated cells (i.e. no change in fluorescence with valinomycin at 115 mM-K), it was concluded that the differences seen with high and low K cells can be attributed to the difference in K content and not to the PCMBS-dithiothreitol treatment *per se*. Again an increase in fluorescence appears to be correlated with depolarization (or reversal) of the membrane potential.

Estimate of membrane potential. The percentage change in fluorescence upon the addition of valinomycin to cells suspended in various external K concentrations was reasonably reproducible, and data similar to those presented in Fig. 4 were obtained in a number of experiments. The results presented in Fig. 4 demonstrate that the relationship between percent change and log K_0 is non-linear. The relationship between the membrane potential calculated from eqn. (2) and log K_0 shows a similar non-linearity unless $P_K \gg P_{Cl}$. The percent change in fluorescence and changes in membrane potential following the addition of valinomycin were compared by fitting curves calculated from various values of α through the points given in Fig. 4.

The first step in fitting the curves was the calculation of the original membrane potential (the potential before the addition of valinomycin) from eqn. (2) with various values for α and the concentration of K_0, K_c, Cl_0 and Cl_c at which the percent change in fluorescence was zero. The values of K_c and Cl_c used were those determined on cells which were not exposed to valinomycin, for it was assumed that no changes in the concentrations of these ions would occur in the time period of the experiments with valinomycin (less than 1 min to a new level of fluorescence). Then the changes in membrane potential expected upon the addition of valinomycin were computed from eqn. (2) for various values of α and K_0 (K_c, Cl_c and Cl_0 are constant). These calculated changes in potential were compared to the changes in fluorescence observed upon the addition of valinomycin. The relationship between change in potential (mV) for various values of α and percent change in fluorescence was estimated in the region of high K_0 where the points appear to fall on a straight line. The relationship was determined by equating the difference in the calculated membrane potential for two high K values (within the linear portion of the curve) and the difference in percent change in fluorescence for the same K_0 values. With the initial membrane potential equal to zero percent change in fluorescence and the calculated changes in potential with valinomycin calibrated in terms of percent change in fluorescence, curves could be constructed through the observed points for different values of α.

This method assumes of course that the relationship between change in potential and change in fluorescence is constant. The lines drawn in Fig. 4 are the results of these calculations where $\alpha = 3$ for the high K cells (lower curve) and 25 for the low K cells (upper curve). A 1·0% change in fluorescence is equivalent to a change of 1·0 mV for the high K cells and to a change of 1·4 mV for the low K cells. (It is not clear why there are

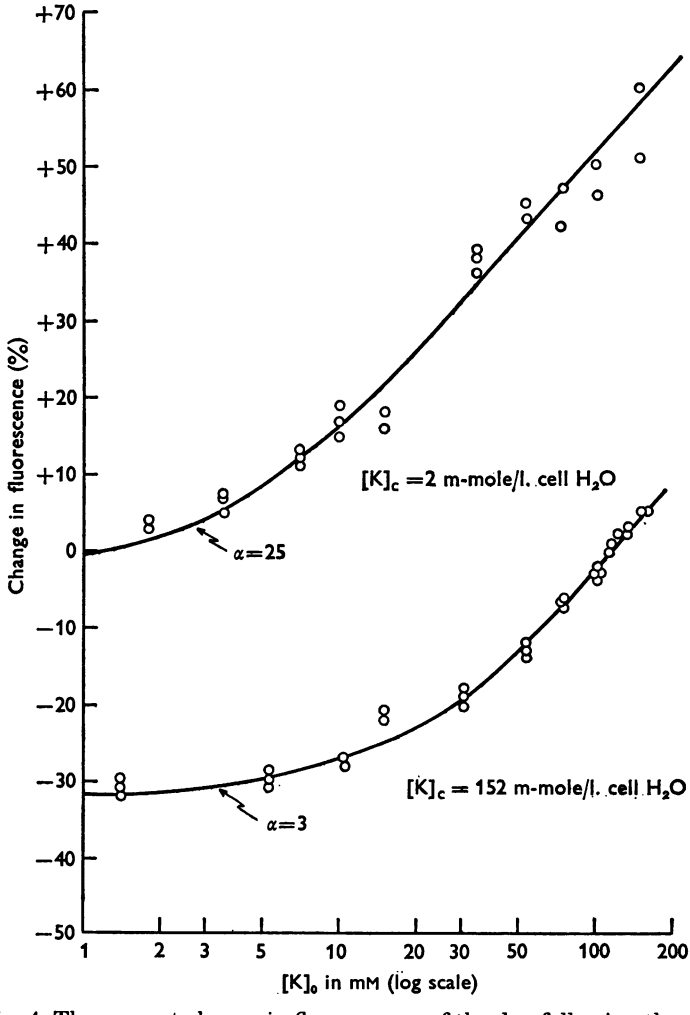

Fig. 4. The percent change in fluorescence of the dye following the addition of valinomycin (same conditions as given in Figs. 2 and 3) plotted as a function of log K_0. K_c upper curve = 2 m-mole/l. cell H_2O. K_c lower curve = 152 m-mole/l. cell H_2O. Lines represent an attempt to equate the membrane potential and change in fluorescence for different values of α. See text for details.

differences between these values for the two types of red cells but it could be related to the choice of using the percent change in fluorescent intensity rather than some other relationship.) The calculated membrane potentials (eqn. (2)) based on the K_0 in which there were no changes in fluorescence after the addition of valinomycin and the values of α mentioned previously are -8 mV for the high and -10 mV for the low K cells. These values compare very favourably with the value -9 mV calculated from the chloride ratio measured at pH 7·4 and room temperature (22° C). Obviously the calculation of the membrane potential from eqn. (2) is independent of α when $K_cCl_c = K_0Cl_0$ (eqn. (2)).

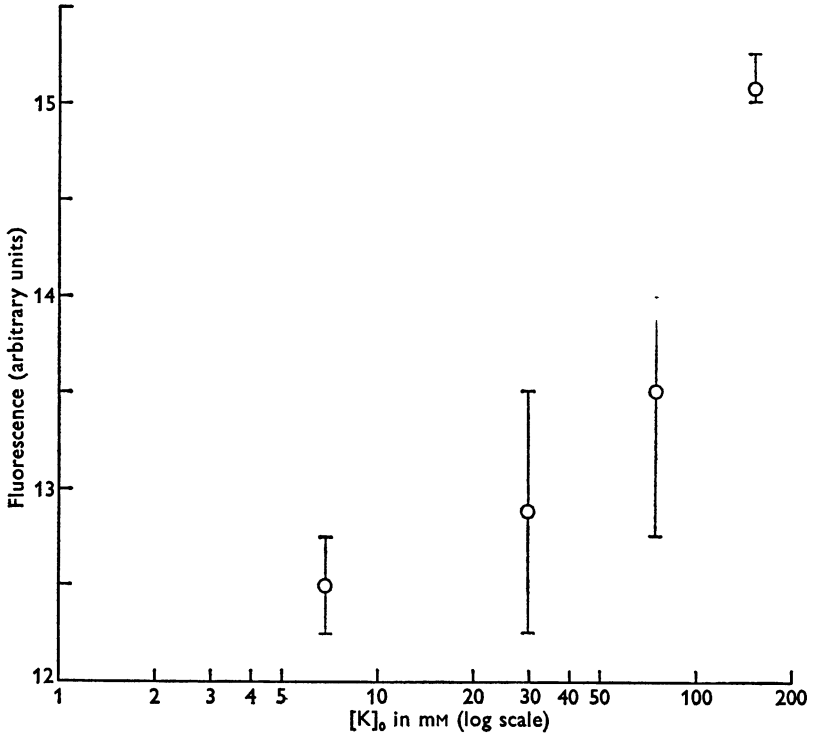

Fig. 5. The steady level of fluorescence of dye ($2{\cdot}9 \times 10^{-6}$ M) in 0·33% suspensions of human red cells in mixtures of NaCl-Tris and KCl-Tris pH 7·4 containing K concentrations given on the abscissa. In this series valinomycin (final concentration 1×10^{-6} M) was added prior to the dye. The circles represent the average of four determinations. The lines (Ⴗ) indicate the extreme values found.

Valinomycin added before dye. In some experiments the cells were treated with valinomycin (10^{-6} M) before the addition of dye. Under these conditions the change in fluorescent intensity with time (see Fig. 3) exhibited the same pattern of response as recorded in the absence of valinomycin. The steady level of fluorescence, however,

now varied with the external K concentration. The tracings in Fig. 3 demonstrate that the steady level of fluorescence in the presence of valinomycin was approximately the same regardless of whether valinomycin was added before the dye or after the fluorescence had attained a steady level. In Fig. 5 the steady level of fluorescence attained with normal cells when valinomycin was added before the dye is plotted as a function of log K_o. Although there is considerable spread in the data (the reproducibility of measurements of absolute levels of fluorescence in these experiments is generally poorer than measurements of percent change in fluorescence) the relationship is again non-linear and is comparable to that seen in Fig. 4.

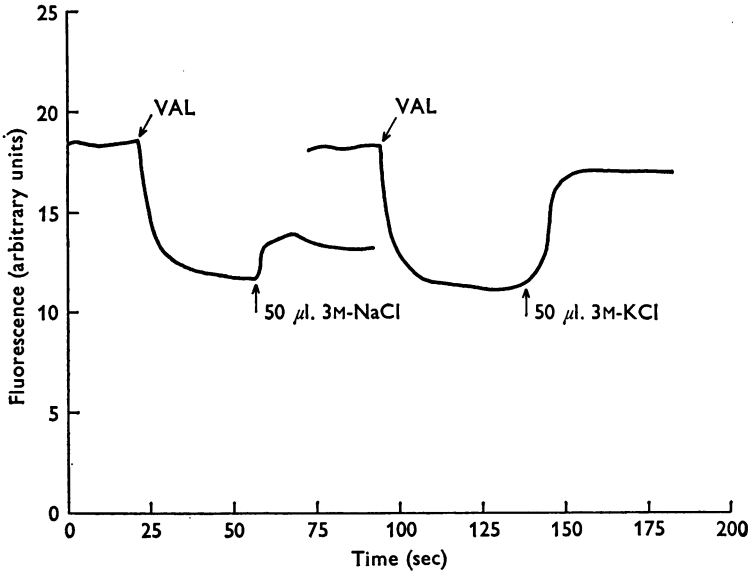

Fig. 6. Fluorescent intensity of the dye ($2{\cdot}9 \times 10^{-6}$ M) in 0·33 % suspensions of human red cells in NaCl-Tris medium. Valinomycin (VAL) added (final concentration 1×10^{-6} M) where indicated. After the change in fluorescence following the addition of valinomycin was recorded, NaCl (left) or KCl (right) was added as indicated. See text for details.

Fluorescence and relative cell volume. Upon the addition of valinomycin to normal cells suspended in NaCl-Tris medium containing dye, a new level of fluorescence was reached which was steady at this level for at least 5 min (longest time period over which measurements were made). If KCl is added during this time, the membrane should be depolarized and an increase in fluorescence would be expected from our previous studies. To test this prediction the K concentration of the medium was made 50 mM by the addition of 50 µl. 3 M-KCl. As shown in Fig. 6 the expected increase in fluorescence was recorded. In other experiments it was demonstrated that the magnitude of the change with the addition of KCl increased with increasing concentrations of added KCl. An increase in fluorescence was also seen (Fig. 6) upon the addition of 50 µl. 3 M-NaCl to cells under identical conditions. But the change was smaller, being only about one third of that seen with the same amount of KCl. Changes in fluorescence were also observed when either KCl or NaCl was added to a cell suspension containing dye in the absence of valinomycin. In these experiments

the changes with the two salts were identical. Since similar changes were observed when the medium was made hypertonic with the addition of non-electrolyte, it was concluded that cell shrinkage resulted in a change in light scattering which, in turn, caused an increase in light emission. However, it should be noted that in the presence of valinomycin the change was greater with KCl than with an osmotically equivalent amount of NaCl and therefore this enhanced fluorescence agreed with the prediction that an increase in fluorescence occurs with depolarization.

TABLE 1. Influence of the concentration of dye CC_6 on percent change in fluorescent intensity upon the addition of valinomycin (final concentration 1×10^{-6} M) to 0·33 % suspensions of human red blood cells. In low K cells, K_c equals 2 m-mole/l. cell H_2O. High K cells are normal red cells. Dye added in ethanol (final concentration 0·5 %)

	% change	
Dye concentration (10^{-6} M)	Low K cells in KCl-Tris	High K cells in NaCl-Tris
8·7	—	−9
7·0	—	−14
5·8	+34	−19
2·9	+31	−25, −28, −24, −24
1·5	+11, +11	−15
0·6	−18, −18	0

Fluorescence and dye concentration. The magnitude of the change in fluorescent intensity resulting from the addition of valinomycin was also influenced by the dye concentration (Table 1). With high K cells in NaCl-Tris medium, the percent change was maximal at $2{\cdot}9 \times 10^{-6}$ M and hence this concentration was employed throughout our experiments. (The fluorescent intensity increases as a function of dye concentration and therefore the percent change in fluorescence upon the addition of valinomycin, and hence the sensitivity of the method, is maximal at approximately $2{\cdot}9 \times 10^{-6}$ M.) No change was seen with $0{\cdot}6 \times 10^{-6}$ M dye. With low K cells suspended in KCl-Tris medium, the change diminished with decreasing dye concentration and, in addition, the direction of the change was altered (from an increase at $1{\cdot}5 \times 10^{-6}$ M to a decrease at $0{\cdot}6 \times 10^{-6}$ M dye). At this concentration of dye the pattern of change in fluorescence with time was also different from the changes recorded at higher concentrations. In $0{\cdot}6 \times 10^{-6}$ M dye the fluorescence of the cell suspension rose rapidly to a level which was then maintained. At high concentrations of dye, the initial rise was greater and was followed by a period of slow decline until a steady level was reached. The reasons for this type of difference found with different dye concentrations are unknown.

Fluorescence change and valinomycin concentration. The magnitude of the change in fluorescence following the addition of valinomycin was also dependent upon the concentration of valinomycin used. In preliminary studies with normal high K cells the change in fluorescence increased with increasing concentrations of valinomycin up to 1×10^{-6} M. Since higher concentrations did not yield significantly higher changes in fluorescence, 1×10^{-6} M valinomycin was employed throughout the experiments. In further studies of the relationship between percent change in fluorescence and valinomycin concentration, low K cells were used because large changes in fluorescence could be measured with these cells. As seen in Fig. 7 a change was observed with these cells with 1×10^{-7} M valinomycin and apparent saturation was reached with 2×10^{-6} M.

Interaction between dye, valinomycin, K and membrane. Although we have related the changes in fluorescence upon the addition of valinomycin to changes in membrane potential, there was the possibility that the changes in fluorescence resulted from interactions of the membrane, valinomycin, K and dye. To test this possibility the fluorescence of the dye was measured in the presence of haemoglobin-free, frozen-thawed ghosts. With this ghost preparation there will be no potential difference across the membrane because it is freely permeable to all ions. Thus if changes in fluorescence were observed with the addition of valinomycin to ghosts suspended in KCl-Tris with dye, they would have to be attributed directly to an interaction of these components with the membrane and not to a change in membrane potential. However, it was found that no changes in fluorescence could be observed when valinomycin (final concentration 1×10^{-6} M) was added to mixtures of dye ($2{\cdot}9 \times 10^{-6}$ M) and varying amounts of ghosts (6–725 μg protein) suspended in solutions of

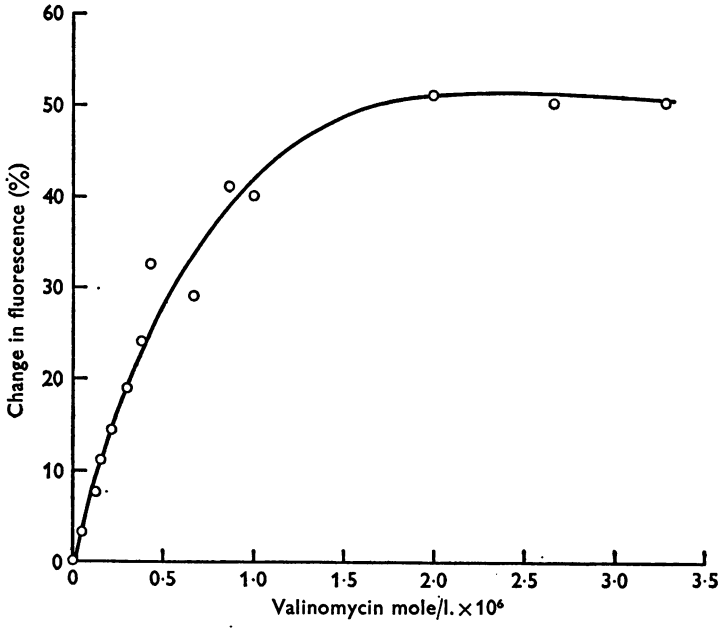

Fig. 7. The percent change in fluorescent intensity of the dye ($2{\cdot}9 \times 10^{-6}$ M) following the addition of various amounts of valinomycin (final concentration given on abscissa) in 10 μl. ethanol to 0·33% suspensions of human red cells. External medium equals 76·5 mM-KCl, 76·5 mM NaCl and 17 mM-TrisCl, pH 7·4. K_c equals 1 m-mole/l. cell H_2O.

KCl-Tris and/or NaCl-Tris at various pHs (pH 6·5–7·9). The amount of membrane protein employed in the experiments described above using intact cells (estimated to be 75 μg from values given in Dodge, Mitchell & Hanahan, 1963) lies within the range tested. Hence the effects of valinomycin observed with intact cells do not appear to be the result of an interaction of K, valinomycin and dye in the membrane.

Changes in fluorescent intensity of the dye in suspensions of reconstituted ghosts were observed upon the addition of valinomycin provided that there was a significant K gradient across the membrane. Resealed ghosts were prepared containing either high K or low K by using either KCl or NaCl respectively to restore the lysed cells

to isotonicity. With high K resealed ghosts the changes were −27% in NaCl-Tris and 0% in KCl-Tris. With low K resealed ghosts the changes were −16% in KCl-Tris and 0% in NaCl-Tris. Why the changes in the two cases should be in the same direction is not clear but was not explored further.

Changes in the pH of the medium. As described earlier, since the charge on haemoglobin, and therefore the magnitude of the Donnan equilibrium, changes with pH the chloride concentration of red cells varies with the pH of the medium, increasing with decreasing pH. These changes in Cl_c should lead to changes in the membrane potential with depolarization (or reversal) expected in increasing acidity and hyperpolarization in decreasing acidity. We would predict from the results presented above that the fluorescent intensity of cell suspensions containing dye should increase upon the addition of acid and decrease with base. To test these predictions normal red cells were first suspended in a medium consisting of 19 parts of 170 mM-NaCl and 1 part of 170 mM-MOPS buffered to pH 7·2 with NaOH. After the fluorescence had reached the steady level, HCl or NaOH was added to change the pH to 6·5 or 7·9 respectively. In keeping with prediction the fluorescence increased (6·0%) with acid and decreased (5·5%) with base. When the pH of solutions containing dye but no cells was altered in the same way, a change in fluorescence of approximately 1% was observed with the addition of acid. If the pH was shifted from 7·2 to 7·9, there was a slow decline in fluorescence. The change in light emission over 10 sec (time during which the fluorescence change in the presence of cells was seen) was of the order of a 1% decrease.

A second prediction could be made about the fluorescence of the dye in suspensions of cells equilibrated with media of different pH values. Since the chloride ratio (Cl_c/Cl_0) increases (membrane potential becomes more positive) with decreasing pH, it was predicted that a decrease in pH should shift the curves relating percent changes in fluorescence with valinomycin to log K_0 towards higher values of K_0. In other words the K_0 for which there is no change in fluorescence with valinomycin should increase with decreasing pH. To test this prediction cells were washed 4 or 5 times with solutions of 170 mM-NaCl MOPS (19:1) at pH 7·9, 7·2 or 6·5 to equilibrate them at these pH values. Dye ($2{\cdot}9 \times 10^{-6}$ M) was added to the cells in the usual manner and followed by valinomycin (1×10^{-6} M) after the steady level of fluorescence was attained. Measurements of the pH in cell suspensions identical to those on which the fluorescence measurements were made showed the pH of the solutions to be 7·8, 7·17 and 6·6 respectively. The data presented in Fig. 8 confirmed the predictions made for the shift of the relationship between percent change in fluorescence and log K_0. The K_0 for no change in fluorescence was 95 mM at pH 7·8, 138 mM at pH 7·17 and 165 mM at pH 6·6.

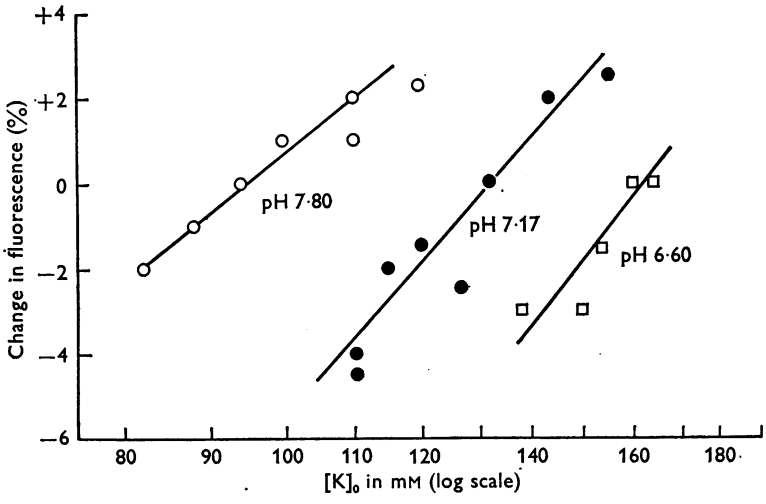

Fig. 8. The percent change in fluorescent intensity of the dye ($2{\cdot}9 \times 10^{-6}$ M) following the addition of valinomycin (1×10^{-6} M) to 0·33% suspensions of human red cells in mixtures of (165 mM-NaCl + 5 mM-MOPS) and (165 mM-KCl + 5 mM-MOPS). Media buffered with TrisOH to pH values indicated: ○ = pH 7·8, ● = pH 7·18 and □ = pH 6·6.

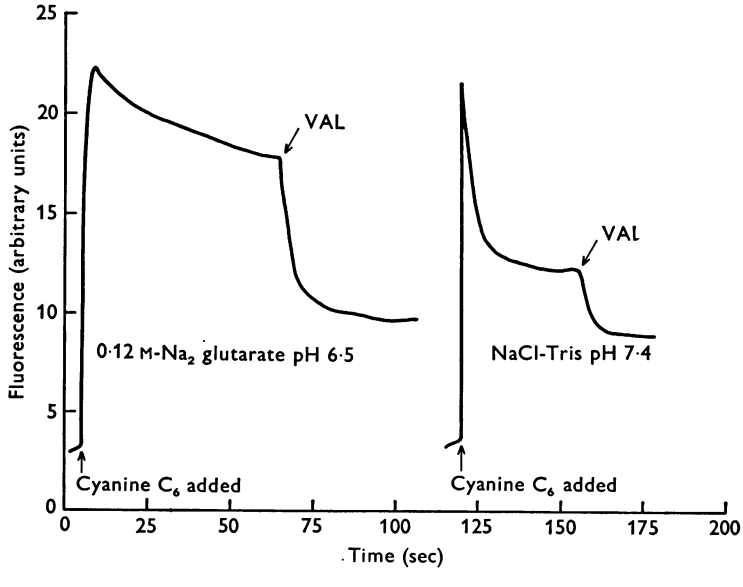

Fig. 9. Fluorescent intensity of the dye with time in 0·33% suspensions of human red cells in 120 mM-Na_2 glutarate pH 6·5 (left) or NaCl-Tris medium pH 7·4 (right). Where indicated, dye added to a concentration of $2{\cdot}9 \times 10^{-6}$ M. Valinomycin (final concentration of 1×10^{-6} M) added as shown.

Changes in fluorescence using non-penetrating anions and non-electrolytes. When red cells are suspended in media in which non-penetrating anions are substituted for chloride, the reduction in external chloride results in the membrane potential becoming more positive than it is in 170 mM-NaCl-Tris. Hence, we should expect from the studies reported above that the steady levels of fluorescence attained in suspensions of cells in such media would be higher than the levels seen in NaCl-Tris medium. This expectation was confirmed by the records presented in Fig. 9. Here the fluorescence of CC_6 with cells suspended in 120 mM-Na glutarate (pH = 6·5) or in 170 mM-NaCl-Tris (pH 7·4) is compared. (The pH of the glutarate solution was brought to 6·5 so as to maintain the internal pH of the cells in both media at approximately the same level.) The difference observed between the two media is considerably larger than differences seen when cells in NaCl-MOPS are compared at 7·4 and 6·5.

If chloride is added to cells suspended in impermeant anion, the membrane potential should become more negative and should result in a decrease in the fluorescent intensity. To test this prediction cells were first suspended in 170 mM-Na gluconate, pH 6·5; dye was then added and, after a time interval of 1–2 min (during which the steady level is approached), 50 μl. 1·7 M-NaCl were added. The addition of NaCl (final concentration 29 mM) resulted in a 6–7 % decrease in fluorescence. With 100 μl. 1·7 M-NaCl (58 mM) the change was -8 to -9 %. As already discussed, studies with cells in NaCl-Tris media have demonstrated that cell shrinkage caused by the addition of hypertonic NaCl led to an increase in light emission. The opposite effect on light emission noted with cells suspended in Na gluconate can be attributed to the change in potential which occurred when chloride was added. Since the two effects (on membrane potential and cell volume) of the addition of hypertonic NaCl have an opposite influence on light emission, some of the change expected from a change in membrane potential is undoubtedly 'masked' by the change in cell volume. It would be difficult to correct the changes in fluorescence resulting from changes in membrane potential for those resulting from cell shrinkage and hence no attempt was made to follow this line of experiment. The basic observation of a decrease in fluorescence with the addition of chloride to cells suspended in non-penetrating anion was, however, in agreement with prediction.

According to eqn. (2), the membrane potential, in normal conditions when α is taken as zero, is a linear function of $\log Cl_c/Cl_0$. If the dye monitors potential, then fluorescence levels should also vary linearly with $\log Cl_c/Cl_0$. To test this hypothesis the fluorescence of the dye was measured in cell suspensions of media composed of an impermeant anion, tartrate (Na salt) and NaCl. In these experiments both Cl_0 and external pH were altered so that Cl_c and internal pH remained constant (120 mM at pH 7·24). Under these circumstances the membrane potential should vary linearly with $\log Cl_0$. In most cases where Cl_0 is lowered the level of fluorescence continued to show some slow decline rather than reach some sharply defined steady level. Hence the fluorescent intensity 50 sec after the addition of dye (the time when cell suspensions in NaCl-Tris reached a

steady level) was chosen as a point for comparison. The results of a series with mixtures of Na tartrate and NaCl are given in Fig. 10, where the fluorescence 50 sec after addition of dye is plotted as a function of log Cl_0. Although this kind of experiment is hampered by the problem of poor reproducibility encountered before in other experiments where the absolute level of fluorescence was measured, the fluorescence level at 50 sec does nevertheless decrease with increasing extracellular chloride and the

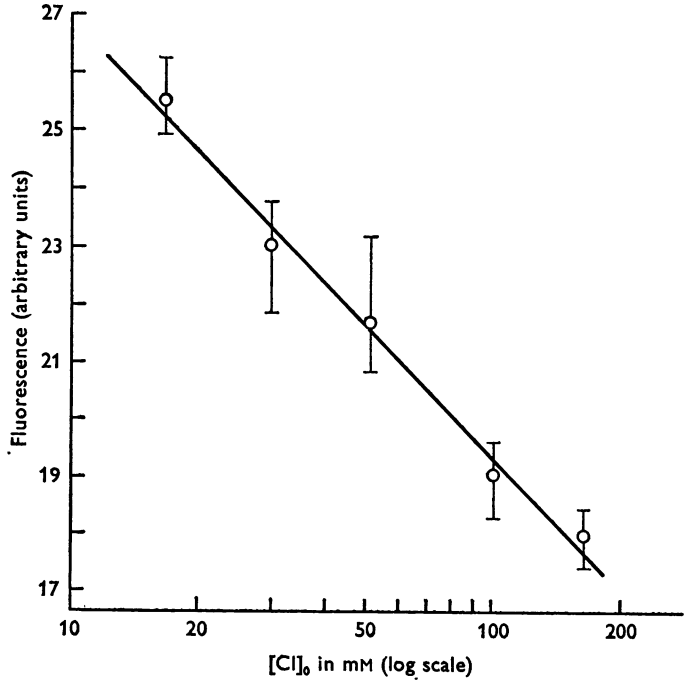

Fig. 10. Fluorescent intensity 50 sec after the addition of dye (final concentration $2{\cdot}9 \times 10^{-6}$ M) to 0·33% suspensions of human red cells in isotonic mixtures of 128 mM-Na tartrate, 170 mM-NaCl, and 170 mM-MOPS. Final MOPS concentration was always 5 mM. Cl_c and pH varied as follows: 17 mM at pH 6·40, 30 mM at pH 6·65, 54 mM at pH 6·74, 100 mM at pH 7·18 and 165 mM at pH 7·40. The circles represent the average of five determinations. The lines (⚲) indicate the extreme values found. See text for details.

relationship is logarithmic. Similar results were obtained in experiments in which gluconate, glutarate, citrate and sucrose:MOPS (19 parts 300 mM sucrose:1 part 170 mM-MOPS at pH 6·5) were used to replace chloride.

The addition of valinomycin to cells suspended in impermeant anion solution should lead to the membrane potential becoming more negative. In our system this addition should result in a decrease in fluorescence.

The results of an experiment to test this prediction are given in Fig. 9. Here valinomycin was added to cells suspended in Na glutarate after the fluorescence had begun to level off. The new level of fluorescence reached is comparable to that seen with cells treated with valinomycin in NaCl-Tris at pH 7·4. Results similar to those obtained using glutarate media were also obtained with Na tartrate, Na citrate and sucrose-MOPS media.

The relationship between percent change in fluorescence with valinomycin addition and log K_0 was determined for cells suspended in various mixtures of chloride and tartrate. From the results above we would expect that a decrease in Cl_0 would result in a shift in the relationship so that

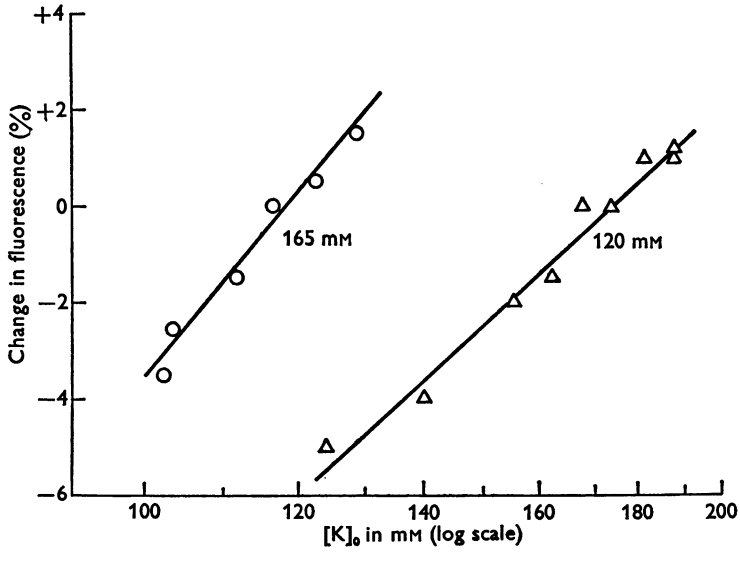

Fig. 11. The percent change in fluorescent intensity of the dye ($2·9 \times 10^{-6}$ M) following the addition of valinomycin (1×10^{-6} M) to 0·33% suspensions of normal human red cells plotted as a function of log K_0. External media for curve at right (120 mM-Cl_0) are mixtures of all Na or K solutions of 34 mM tartrate, 120 mM-Cl + 5 mM-MOPS buffered to pH 7·24 with TrisOH, to give values indicated on abscissa. External media for curve at left (165 mM-Cl_0) are mixtures of 165 mM-NaCl + 5 mM-MOPS at pH 7·4 and 165 mM-KCl + 5 mM-MOPS at pH 7·4 to give K values presented on abscissa.

higher K would be required to produce an equal percent change with valinomycin, that is, the K_0 for which there is no change in fluorescence should increase with decreasing Cl_0. Most of these studies were carried out with low K cells so that a wider range of Cl_0 could be employed. The media consisted of mixtures of isotonic NaCl, Na tartrate and MOPS (final concentration 5 mM) buffered to a pH which varied with the chloride

concentration of the medium. Again the adjustments in pH were made to keep internal pH and chloride constant. The results presented in Fig. 11 for normal cells and in Fig. 12 for low K cells are in agreement with the prediction.

Studies were also conducted in which the fluorescence of the dye in isosmotic solutions of various anions (without cells) was compared to that in 170 mM-NaCl-Tris. The fluorescent intensity in tartrate, gluconate,

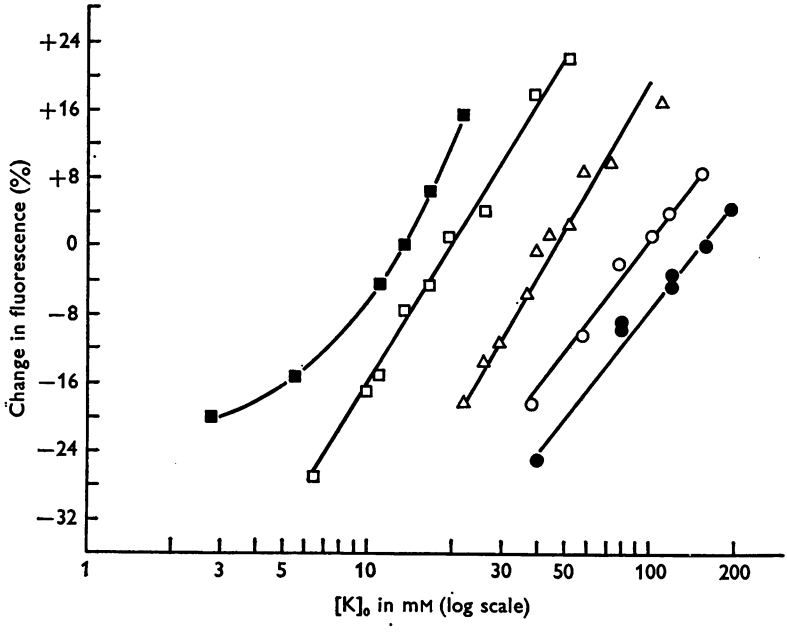

Fig. 12. The percent change in fluorescent intensity of the dye ($2{\cdot}9 \times 10^{-6}$ M) following the addition of valinomycin (1×10^{-6} M) to 0·33% suspensions of human red cells (K_c equals 23·6 m-mole/l. cell H_2O) plotted as a function of log K_0. External media are mixtures of a Na–tartrate–chloride–MOPS buffered with TrisOH solutions (isotonic mixtures of 128 mM-Na tartrate, 170 mM-NaCl and 170 mM-MOPS) and a K–tartrate–chloride–MOPS solution buffered with TrisOH (isotonic mixtures of 128 mM-K tartrate, 170 mM-KCl and 170 mM-MOPS) to give the K_0 values indicated on the abscissa and Cl_0 and pH as indicated: ● = 17 mM, pH 6·40; ○ = 30 mM, pH 6·65; △ = 54 mM, pH 6·91; □ = 108 mM, pH 7·18; ■ = 165 mM, pH 7·40. MOPS always equals 5 mM.

glutarate or sucrose was the same as that in NaCl-Tris while the fluorescence with citrate was somewhat lower. With other anions of interest, *p*-aminohippurate and MOPS (170 mM at pH 6·5), the fluorescent intensity tended to remain elevated, showing only a very slow decline after the initial peak was reached. It would also have been of interest to determine whether or not changes in fluorescence would occur when inhibitors of

anion permeability were employed to change membrane potential. However, difficulties arose when the three inhibitors, salicylate (Wieth, 1970), dipyridamole or phloretin (Deuticke, 1970) were tested. With salicylate there was no initial sharp rise in fluorescence in the presence or absence of cells and it was not possible to interpret the fluorescent measurements recorded in the presence of this compound. The intrinsic fluorescence of persantin precluded its use and the addition of phloretin to dye in the presence or absence of cells led to a very sharp and pronounced quenching of fluorescence.

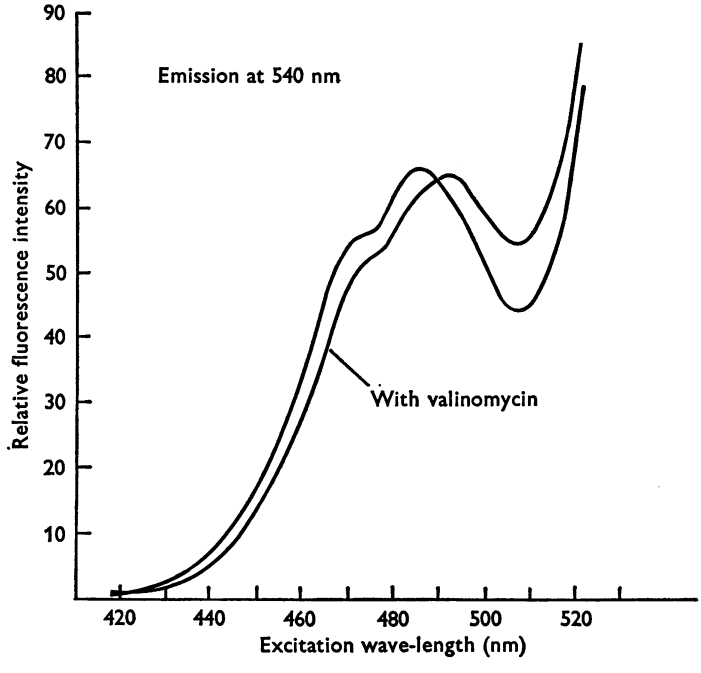

Fig. 13. Excitation spectrum of the dye ($2{\cdot}9 \times 10^{-6}$ M) in 0·33% suspension of normal human red blood cells in NaCl-Tris pH 7·4 in the presence and absence of 1×10^{-6} M valinomycin. Fluorescent intensity measured at 540 nm.

Excitation and emission spectra. The excitation spectrum for CC_6 with a 0·33% cell suspension in NaCl-Tris (pH 7·4) is presented in Fig. 13. A peak is reached at 485 nm. When valinomycin is added to a concentration of 1×10^{-6} M this peak is shifted toward the red to 495 nm. The emission spectrum for the dye under the same conditions shows a maximum at 498 nm (Fig. 14) which does not appear to change with the addition of valinomycin.

Simultaneous measurements of K_0/K_c and Cl_c/Cl_0. At the K_0 for which

there is no change in fluorescence of dye upon the addition of valinomycin (called the null point hereafter) it is expected that

$$E = \frac{RT}{F} \ln \frac{\alpha K_0 + Cl_c}{\alpha K_c + Cl_0} = \frac{RT}{F} \ln \frac{Cl_c}{Cl_0},$$

therefore

$$\frac{\alpha K_0 + Cl_c}{\alpha K_c + Cl_0} = \frac{Cl_c}{Cl_0}$$

and

$$K_0 Cl_0 = K_c Cl_c \quad \text{(the Donnan Law)}$$

and

$$K_0/K_c = Cl_c/Cl_0 \text{ or } r_K = r_{Cl},$$

where

$$r_K = K_0/K_c \text{ and } r_{Cl} = Cl_c/Cl_0.$$

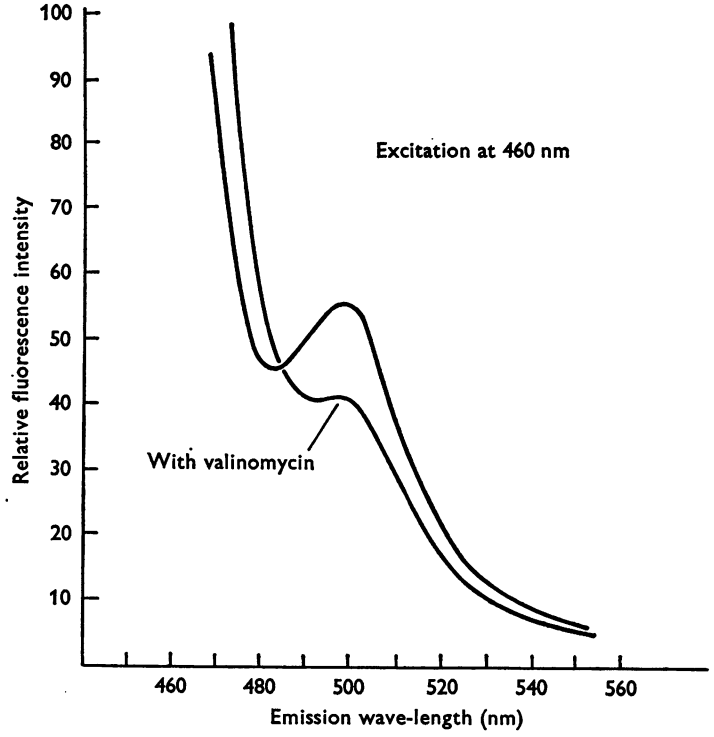

Fig. 14. Emission spectrum of the dye ($2{\cdot}9 \times 10^{-6}$ M) in 0·33% suspensions of normal human red blood cells in NaCl-Tris pH 7·4 in the presence and absence of 1×10^{-6} M valinomycin. Fluorescent intensity was measured with excitation at 460 nm.

To test for the equality of these ratios, measurements were made at 22° C of K_c and $^{36}Cl_c/^{36}Cl_0$ in a medium containing 115 mM-K (K_0 at the null point), 38 mM-Na, 17 mM-TrisCl at pH 7·4, $2{\cdot}9 \times 10^{-6}$ M dye and

TABLE 2. Comparison of the ratios, K_0/K_c and Cl_c/Cl_0, in normal human and *Amphiuma* red blood cells. Cells exposed to solutions given below where listed. VAL equals 1×10^{-6} M valinomycin and DYE equals $2{\cdot}9 \times 10^{-6}$ M-CC_6. The results of two different experiments are given for human cells. See text for details

Cell type	Suspension medium	% H_2O (v/v)	K_c (m-mole/l. cell H_2O)	K_0/K_c	$^{36}Cl_c/^{36}Cl_0$
Human, expt. *A*	NaCl-TrisCl (pH 7·4)	71·5	129·4	—	0·765
	115 mM-KCl, 38 mM-NaCl, 17 mM-TrisCl (pH 7·4) + VAL + DYE	72·3	131·5	0·874	0·761
Human, expt. *B*	NaCl-TrisCl (pH 7·4)	69·2	138·2	—	0·695
	115 mM-KCl, 38 mM-NaCl, 17 mM-TrisCl (pH 7·4) + VAL + DYE	70·3	138·7	0·829	0·672
Amphiuma	Na-Ringer	73·3	118·4	—	0·446
	60 mM-KCl, 56 mM-NaCl, 1·5 $CaCl_2$, 10 mM-MOPS (pH 7·2) + VAL + DYE	73·5	115·8	0·519	0·486

TABLE 3. Comparison of r_K, K_0/K_c, where K_0 is the value at the null point in fluorescence studies and r_{Cl}, Cl_c/Cl_0, is determined in NaCl-MOPS at various pH values. Cells were washed 5 times in NaCl-MOPS solutions at the pH tested. Null points determined as in Fig. 4. Final CC_6 concentration, $2{\cdot}9 \times 10^{-6}$ M. Final Di-S-C_3 (5) concentration, $1{\cdot}52 \times 10^{-6}$ M

Dye	pH	% H_2O (v/v)	K_c (m-mole/l. cell H_2O)	K_0/K_c	$^{36}Cl_c/^{36}Cl_0$
CC_6	6·60	73·3	118	1·37	1·02
CC_6	7·17	70·9	134	0·99	0·80
CC_6	7·80	68·2	151	0·62	0·55
Di-S-C_3 (5)	6·48	73·8	115	1·09	1·07
Di-S-C_3 (5)	7·18	70·8	134	0·83	0·79
Di-S-C_3 (5)	7·83	68·1	152	0·59	0·54

1×10^{-6} M valinomycin. The measurements given in Table 2 were made after an exposure of the cells to the medium for approximately 30 min. There was a difference between the measured r_K and r_{Cl} under these condi-

tions and this discrepancy was observed on a number of occasions. The products predicted by the Donnan Law:

$$K_0Cl_0 = K_cCl_c$$
$$(115)\,(170) = (132)\,(129)$$
$$19{,}550 = 17{,}028$$

differ by approximately 13%.

When r_K using K_0 at the null point was compared to r_{Cl} (determined in NaCl-MOPS) at various pH values, differences were also observed (Table 3). Comparable differences were recorded in 4 experiments. Although these differences were typical of measurements obtained with the dye, CC_6, measurements of r_K using the dye, Di-S-C_3 (5), in contrast, gave results which were in excellent agreement with r_{Cl}. It is not clear why the K_0 values used at the null point with the two dyes should differ (it could be, for instance, that the relationship between membrane potential and the fluorescence of the dye CC_6 is non-linear or offset by a constant factor) but because the difference is small we consider the two sets of results to be in general agreement.

Measurements of K_0/K_c and SO_{4_c}/SO_{4_0}. In SO_4 loaded cells suspended in SO_4 media at the K_0 for which there is no change in fluorescence upon the addition of valinomycin

$$K_0/K_c = (SO_{4_c}/SO_{4_0})^{\frac{1}{2}} \text{ or } r_K = r_{SO_4} \text{ where } (SO_{4_c}/SO_{4_0})^{\frac{1}{2}} = r_{SO_4}.$$

To test for the equality of these ratios, the K_c values of cells at equilibrium and loaded with SO_4 in the Na_2SO_4-$Tris_2SO_4$ medium or K_2SO_4-$Tris_2SO_4$ medium were measured and the corresponding K_0 values for the null point were determined by the usual methods. The r_{SO_4} was measured as the $^{35}SO_{4_c}/^{35}SO_{4_0}$ on cells equilibrated in Na_2SO_4-$Tris_2SO_4$. The data presented in Table 4 demonstrate that r_K and r_{SO_4} were very different when CC_6 was used in the studies of fluorescence. However, once again values of r_K and r_{SO_4} are in good agreement when the dye Di-S-C_3 (5) was used.

Other observations. Since the dye has a positive charge, it was of interest to determine whether or not the changes observed above depended on an interaction of the dye with the negatively charged sialic acid groups at the cell surface, responsible for its negative zeta potential. To test this possibility, changes in fluorescence upon the addition of valinomycin were measured on normal cells and cells which had been pre-treated with neuraminidase (see Knauf & Rothstein, 1971) to remove these negatively charged groups from the membrane. Since treated cells and untreated cells showed exactly the same changes in fluorescent intensity upon the addition of valinomycin, it was concluded that the sialic acid residues were not involved in the changes observed above.

It was also of interest to test whether the changes in fluorescence observed involved some photodynamic action (Blum, 1941) of the dye. To test this possibility changes in fluorescence with valinomycin were measured with cells suspended either in 60 mM-Na_2SO_3 (a reducing agent which will prevent photodynamic action (Blum, 1941)) + 63 mM-NaCl + 17 mM-TrisCl (pH 7·4) or 60 mM-Na_2SO_3 + 63 mM-KCl + 17 mM-TrisCl (pH 7·4) and compared to the same measurements in NaCl-Tris (pH 7·4) or 90 mM-NaCl + 63 mM-KCl + 17 mM-TrisCl (pH 7·4). Since no differences were seen between the solutions containing identical K, it was concluded that the changes observed did not involve a photodynamic effect of the dye.

TABLE 4. Comparison of r_K, K_0/K_c, where K_0 is the value at the null point in fluorescence studies and $\sqrt{r_{SO_4}}$, where r is $^{35}SO_{4c}/^{35}SO_{4o}$, determined in Na_2SO_4-$Tris_2SO_4$ pH 7·4. Human red cells equilibrated with Na_2SO_4 pH 7·4 medium or K_2SO_4-$Tris_2SO_4$ pH 7·4 medium. Final concentration CC_6 is $2·9 \times 10^{-6}$ M. Final concentration Di-S-C_3 (5) is $1·52 \times 10^{-6}$ M

Equilibration medium	K_c (m-mole/l. cell H_2O)	K_0/K_c		$(^{35}SO_{4c}/^{35}SO_{4o})^{\frac{1}{2}}$
		CC_6	Di-S-C_3 (5)	
Na_2SO_4	107·9	1·67	0·936	0·718
K_2SO_4	139·0	> 1·72	0·806	0·718

TABLE 5. 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-TrisCl (pH 7·5) and 1×10^{-4} ouabain in the presence and absence of $2·9 \times 10^{-6}$ M-CC_6. Measurements over 30 min period (5 and 35 min) at 22° C. $n = 4 \pm$ S.E. of mean. Results of two different experiments (*A* and *B*) are presented

Expt.	$^1k_{Na}$ (hr^{-1})		^1k_K (hr^{-1})	
	A	*B*	*A*	*B*
No dye	0·0023 ± 0·0005	0·0073 ± 0·0019	0·0128 ± 0·0014	0·0128 ± 0·0038
With dye	0·0037 ± 0·0004	0·0153 ± 0·0022	0·0170 ± 0·0009	0·0242 ± 0·0011

Influence of dye on ^{24}Na, ^{42}K and $^{35}SO_4$ fluxes. Since a correlation was made between the fluorescence of the dye and the membrane potential, it was of interest to determine whether or not the dye had any influence on the movements of ions across the red blood cell membranes. In order to examine this possibility the dye was tested for effects on ^{24}Na and ^{42}K influx, on ^{42}K efflux and on $^{35}SO_4$ efflux.

Simultaneous measurements of ^{42}K and ^{24}Na influx were made at 22° C on 0·33 % suspensions of cells in a medium which contained 148 mM-NaCl, 5 mM-KCl, 17 mM-TrisCl (pH 7·5) and 1×10^{-4} M ouabain in the presence

or absence of $2{\cdot}9 \times 10^{-6}$ M dye. The data given in Table 5 demonstrate that the inward rate constants for both ions increased in the presence of the dye, the increase being approximately twofold for ^{24}Na and 1·5 to twofold for ^{42}K.

Measurements of ^{42}K efflux were made at 15, 22 and 37° C on 0·33% suspensions of cells (preloaded with ^{42}K) into a medium which contained 148 mM-NaCl, 5 mM-KCl and 17 mM-TrisCl at pH 7·4. The influence of the dye on ^{42}K efflux from human red cells was dependent on temperature (Table 6). At 15° C the outward rate constant increased fivefold and at 37° C threefold. With human red cells the influence at 22° C was variable ranging from thirtyfold in this experiment to 2 in others. At 22° C 5–6% haemolysis was observed during the first hour of incubation whereas

TABLE 6. Measurements of the outward rate constants for K, using ^{42}K, from human and *Amphiuma* red blood cells at various temperatures. Human cells suspended in 148 mM-NaCl, 5 mM-KCl and 17 mM-TrisCl at pH 7·4. *Amphiuma* cells suspended in Na-Ringer. CC_6 concentration $2{\cdot}9 \times 10^{-6}$ M. For human, $n = 6 \pm$ S.E. of mean. For *Amphiuma*, $n = 7 \pm$ S.E. of mean

		${}^0k_K$ (hr^{-1})		
Cells	Dye	15° C	22° C	37° C
Human	None	0·0091 ± 0·0014	0·0125 ± 0·0008	0·035 ± 0·0007
	Present	0·0503 ± 0·0050	0·392 ± 0·073	0·085 ± 0·0190
Amphiuma	None	0·0687 ± 0·0105	0·1207 ± 0·0188	— —
	Present	0·1047 ± 0·0067	0·097 ± 0·0222	— —

negligible haemolysis was seen at 15 and at 37° C. The haemolysis at 22° C interfered with the determinations of ^{42}K efflux and makes the value presented questionable. Further work would be necessary to assess the effect of the dye on ^{42}K efflux from human red cells at this temperature.

Measurements of $^{35}SO_4$ efflux at 37° C were made on 0·3% cell suspensions in Na_2SO_4-$Tris_2SO_4$ medium. The cells had been equilibrated with $^{35}SO_4$ in a Na_2SO_4 medium as described in Methods. The rate constants, 0·0036 min^{-1} at 22° C and 0·047 min^{-1} at 37° C, for SO_4 efflux determined in a single experiment in the presence of dye were not very different from the rate constants, 0·0033 min^{-1} at 22° C and 0·048 min^{-1} at 37° C determined in the absence of the dye. More experiments would be needed to evaluate the significance of these differences.

Amphiuma red blood cells

Since Hoffman & Lassen (1971) have made direct measurements of the electrical potential across the membrane of the *Amphiuma* red blood cell using micro-electrode techniques, it was of interest to use these cells in our experiments in order to compare estimates of membrane potential obtained from measurements of fluorescence with those obtained by direct measurements.

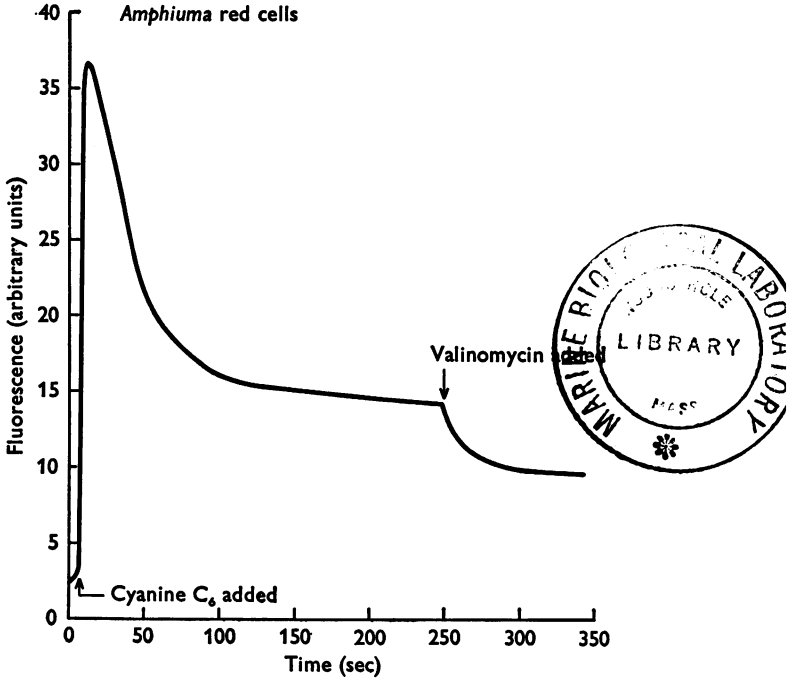

Fig. 15. Fluorescent intensity of the dye with time in a 0·33% suspension of *Amphiuma* red blood cells in Na-Ringer pH 7·2. Dye (final concentration of $2{\cdot}9 \times 10^{-6}$ M) and valinomycin (final concentration 1×10^{-6} M) were added where indicated.

Fluorescent intensity with time. The fluorescent intensity of the CC_6 following its addition to a 0·4% suspension of *Amphiuma* red cells in Na-Ringer is given in Fig. 15. The same pattern of quick rise and slow decline to a steady level observed with human cells is also seen here but the decline in *Amphiuma* cells is somewhat slower. In contrast to human cells the steady level of fluorescent intensity was about 5% higher in K-Ringer than in Na-Ringer. This difference between observations with human and *Amphiuma* red cells may be the result of a higher P_K/P_{Cl} ratio in the *Amphiuma* cell. The ratio is approximately 1 : 100 in human (Hunter,

1971) and 1:5 in *Amphiuma* (unpublished observations). Although P_K in *Amphiuma* is smaller than P_{Cl}, K cannot be eliminated in eqn. (2). The potential in K-Ringer would be somewhat more positive than in Na-Ringer. The differences in fluorescence observed in Na-Ringer again comply with the general observation that depolarization increases and hyperpolarization decreases fluorescent intensity. Addition of valinomycin after the steady level was reached in Na-Ringer resulted in a decrease in fluorescent intensity, with the decline occurring more slowly than the comparable change seen with human red blood cells. In a series of experiments the percent change in fluorescence of CC_6 was recorded when

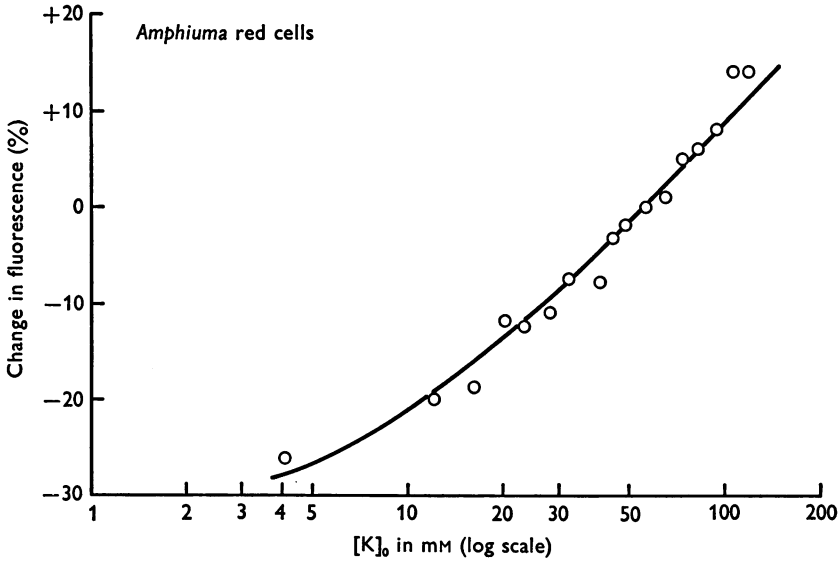

Fig. 16. The percent change in fluorescence of the dye following the addition of valinomycin (same conditions as given in Figs. 2 and 3) plotted as a function of log K_0. External media are mixtures of Na-Ringer and K-Ringer to give the K values indicated on the abscissa.

valinomycin was added (after the steady level was reached) to cells suspended in mixtures of Na-Ringer and K-Ringer. The results of these experiments are plotted in Fig. 16 as the percent change in fluorescence as a function of log external K. The line drawn in Fig. 16 is fitted through the points using eqn. (2) with $\alpha = 4$. The potential calculated using values for cellular compositions (K = 118 mM and Cl = 54 m-mole/l. cell water (Table 2)) and the K_0 and Cl_0 for no change in fluorescence is -19 mV. This potential is in good agreement with the value of -15 mV obtained by direct electrical methods (Hoffman & Lassen, 1971).

Changes with pH. As with human red cells an alteration of the pH of

the medium of *Amphiuma* red cells should result in a change in the Cl_c/Cl_0 ratio and hence to a change in membrane potential. The addition of base should hyperpolarize and the addition of acid should depolarize the membranes of these cells. These changes should result in an increase in fluorescence with acid and a decrease in base. The pH of the cell suspensions in Na-Ringer was changed after fluorescence had reached a steady level. The addition of 8 μl. 0·1 N-NaOH to change the pH from 7·2 to 7·9 led to a decrease in fluorescence of 9·0 % while the addition of 10·5 μl. 0·1 N-HCl to change the pH from 7·2 to 6·5 resulted in an increase of 10·7 %. These predictions were in the direction predicted from the results of studies with valinomycin.

Changes with impermeant anions. Measurements of fluorescence were also made on cells in which the chloride ratio had been altered by the substitution of the impermeant anion, *p*-aminohippuric acid (PAH) at pH 6·5 for chloride of the Na-Ringer. Studies with PAH were of interest because direct measurements of the influence of the anion on the membrane potential of these cells had been made by Hoffman & Lassen (1971). The steady level attained with these cell suspensions was higher (80 %) than that obtained with cell suspensions in Na-Ringer. Although, as described earlier, there are difficulties analysing results with PAH, there are indications that the increase in fluorescent intensity is in some measure the result of a depolarization or reversal of the membrane potential. When valinomycin was added to cells in NaPAH, a decrease of 42 % in light emission was observed as compared to a 35 % decrease seen in Na-Ringer. A larger decrease would be expected if cells were at a more positive internal potential in PAH. In KPAH-Ringer the addition of valinomycin led to a 5 % decrease as compared to a 14 % increase with KCl-Ringer. Again, these observations are compatible with a depolarization (or reversal) in PAH so that the membrane potential is now more positive than it is in KCl-Ringer. Results were also obtained using either MOPS or tartrate as impermeant anions, which were qualitatively comparable to the results reported before for human red cells, but further studies are needed to evaluate their effects quantitatively.

Simultaneous measurements of K_0/K_c and Cl_c/Cl_0. As described earlier, at the K_0 at which there is no change in fluorescence upon the addition of valinomycin, it is predicted that $r_K = r_{Cl}$ and $K_0Cl_0 = K_cCl_c$. To test for the equality of these ratios and products, measurements were made at 22° C of K_c and r_{Cl} (measured as $^{36}Cl_c/^{36}Cl_0$ in a medium containing 60 mM-KCl (K_0 at the null point), 56 mM-NaCl, 1·5 mM-$CaCl_2$, and 10 mM-MOPS at pH 7·2, $2{\cdot}9 \times 10^{-6}$ M dye and 1×10^{-6} M valinomycin. The difference between the measured r_K (using CC_6 to determine the null point) and r_{Cl} seen earlier with human red cells was also observed with *Amphiuma* cells.

The products ($K_0Cl_0 = K_cCl_c$) predicted by the Donnan Law differed by approximately 7%.

Influence of dye on ^{42}K efflux. Measurements of the efflux of ^{42}K from *Amphiuma* red blood cells loaded with ^{42}K were made at 15 and 22° C in the presence and absence of $2{\cdot}9 \times 10^{-6}$ M dye. The results presented in Table 6 demonstrate that there was an increase of approximately 40% at 15° C with dye and a small or negligible decrease at 22° C.

DISCUSSION

The main findings of this work are, first that the fluorescence of the two cyanine dyes, CC_6 and Di-S-C_3 (5), as employed in our studies can be used to measure membrane potentials in red cells and, secondly, that estimates of the membrane potentials of human and *Amphiuma* red blood cells based on studies of fluorescence are in good agreement with those obtained with other methods (see Table 7). That the fluorescence of the dyes used changes

TABLE 7. Summary of membrane potential (mV) estimation based on three different methods: calculated from Cl_c/Cl_0, direct measurement using microelectrodes and calculated from null point fluorescence measurements, (1) below represent measurements reported in this paper and (2) taken from Hoffman & Lassen (1971)

Cell type	Cl_c/Cl_0	Direct electrical	Fluorescence	
			CC_6	Di-S-C_3 (5)
Human	$-9^{(1)}$	—	-5 to $-8^{(1)}$	$-9^{(1)}$
Amphiuma	$\left\{\begin{matrix}-20^{(1)}\\-17^{(2)}\end{matrix}\right\}$	$-15^{(2)}$	$-19^{(1)}$	—

systematically in accordance with expected changes in the membrane potential was evaluated from studies where the potential was changed either by the addition of valinomycin (which increased appreciably the partial conductance of the membrane to K, thereby increasing the ratio, P_K/P_{Cl}) or by changes in the chloride equilibrium ratio (Cl_c/Cl_0) either by variations in pH (which changes Cl_c relative to Cl_0) or by substitution of an impermeant anion for chloride (which changes Cl_0 relative to Cl_c). With all these different means of changing the potential an expected hyperpolarization led to a decrease, and depolarization to an increase in fluorescence. With *Amphiuma* estimates from fluorometric methods can be compared with those obtained directly by impalement with microelectrodes (Hoffman & Lassen, 1971; Lassen, 1972). According to these authors the average membrane potential recorded at pH 6·5 is -8 mV, at 7·2, -15 mV and at pH 7·9, -24 mV. With 1×10^{-6} M valinomycin the potential changed to -45 mV. Our estimates (based on the calibration with valinomycin where $\alpha = 4$, and 1% change in fluorescence,

equals a 1·3 mV changein potential) for these pH values are −4·5, −19, and −31 mV and for 10^{-6} valinomycin, −48 mV. Our estimates for pH 7·2 and 7·9 fall into the higher (more negative) range of potentials reported by Hoffman & Lassen (1971). Our value with 10^{-6} M valinomycin is also somewhat more negative. Technical problems related to the electrical recording of the true membrane potential, as discussed by Lassen *et al.* (1971), might account for the differences between our

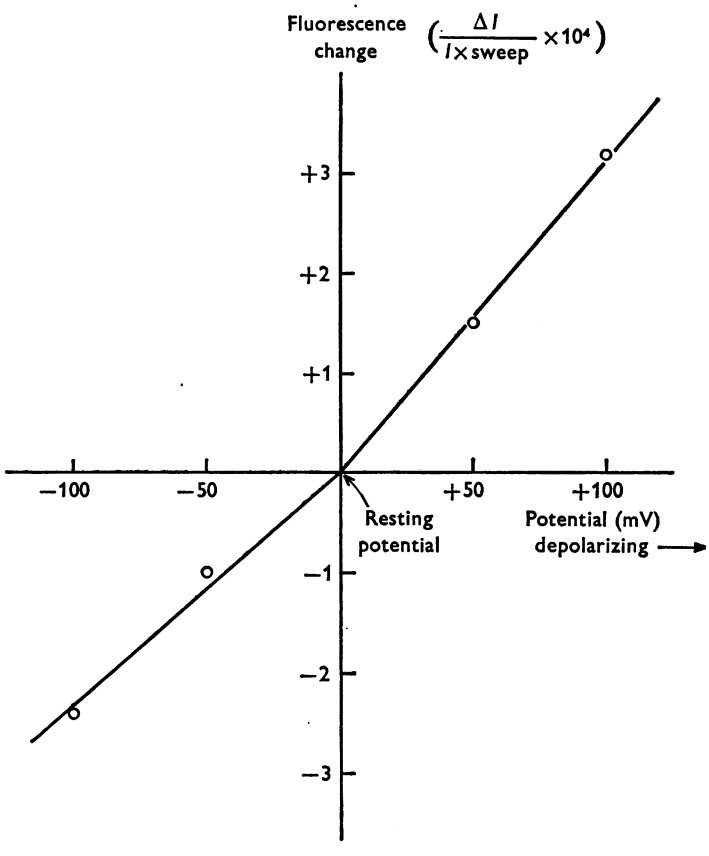

Fig. 17. Changes in CC_6 fluorescence *vs.* change in membrane potential in *Loligo pealii*. 5·8 mg of dye dissolved in 0·58 ml. hot ethanol and mixed with 350 mg melted (55° C) pluronic F127, then 58 ml. of 55° C sea water mixed in. Axon incubated for 10 min with this mixture and then washed with sea water. Experiment performed after 5 min wait. Incident wavelength is 480 ± 15 nm. Emission wave-lengths longer than 515 nm measured. For details see Davila, Cohen, Salzberg & Shrivastav (1974). The electrodes and amplifiers used for the voltage-clamp experiments were the same as described previously (Cohen, Keynes & Landowne, 1972). Fluorescence measured at the end of 2 msec steps. The resting potential is at zero mV on the potential scale. Temperature is 21° C. Axon diameter 550 μm.

estimates of the membrane potential and the direct measurements of Hoffman & Lassen (1971). Regardless of the reasons for the differences we consider the agreement between the two approaches rather good.

Studies with the voltage-clamp technique kindly performed for us by L. B. Cohen, H. V. Davila & B. M. Salzberg have demonstrated (see Fig. 17) that the fluorescence of the dye is also a function of the membrane potential of the squid axon. In this preparation, hyperpolarization also leads to a decrease and depolarization to an increase in fluorescence. Since the fluorescence of the dye exhibits the same relationship to potential in membranes from very different sources, it may be of use in monitoring changes of potentials across membranes in general.

It should be emphasized, however, that there are limitations to the use of these dyes. In the squid axon the relationship between fluorescence and membrane potential is not linear in the range studied. Although linearity was seen in a range of potentials (-9 to $+49$ mV) with the human red blood cell, it is not certain that the relationship is linear outside this range. The dye, CC_6, also has the drawbacks of increasing the fluxes of Na and K across the membrane and of being lytic at the concentration used in the fluorescence studies. In addition, the dye interacts in unknown ways with a number of compounds (e.g. anions and inhibitors of anion permeability) of interest in studies of potential. Because of these interactions it was not possible to use these compounds in our studies. On the other hand, some of the problems encountered during the studies with CC_6 are apparently avoided with the use of Di-S-C_3 (5). The fluorescent intensity of this dye shows greater sensitivity to changes in membrane potential than CC_6 and is considerably less noxious. Studies concerned with a systematic evaluation of various dyes in terms of molecular structure and other parameters as well as the mechanism by which these dyes monitor membrane potential will be taken up in a subsequent publication (P. Sims, J. Wang, A. Waggoner & J. F. Hoffman, in preparation).

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