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NON-LINEAR RELATIONSHIP BETWEEN FLUORESCENCE AND MEMBRANE POTENTIAL

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Summary

The fluorescence intensity of the cyanine dye DiO-C_6-(3) in a 0.32% suspension of Ehrlich ascites tumor cells was determined under various potassium concentrations. In addition, the fluorescence levels of cell-free buffer solutions and those of supernatants were measured. For every potassium concentration the partition of the dye between cells and medium was calculated and its relation to the potassium gradient was given. A model was developed which assumes the fluorescence of a cell suspension to be the sum of the fluorescence signal of dye in the extracellular medium and that of cell-associated dye. A calibration curve of fluorescence vs. membrane potential was constructed. Neither the fluorescence of the cell suspension nor that of the supernatants was a linear function of the membrane potential. The limitations of membrane potential determination by fluorimetric methods are discussed.

Introduction

Measurements of transmembrane potentials involve direct and indirect methods. Whereas direct microelectrode impalements become difficult with small cells, estimations of membrane potentials even of organelles are possible by determining the distribution ratio of labeled ions, provided they are distributed passively and equally throughout the cells or organelles. In the last few years several fluorescent dyes have been found which change their optical properties in response to changes in membrane potential. The cyanine dyes turned out to give considerably fast and large changes of fluorescence with cell suspensions [1]. These positively charged dye molecules show a potential-dependent partition between cells and the extracellular medium [2]. Uptake of dye molecules into the cells results in a quenching of fluorescence. Thus fluorescence measurements allow a continuous recording of a potential-depen-

dent signal. This paper is concerned with the changes of the fluorescence signal and their relation to membrane potential.

Material and Methods

Cells

Ehrlich ascites tumor cells were harvested 8—10 days after inoculation by peritoneal puncture and suspended in Krebs-Ringer phosphate buffer, pH 7.4, containing 11.4 mM K^+, 143.2 mM Na^+, 2.55 mM Ca^{2+}, 1.36 mM Mg^{2+}, 139.7 mM Cl^-, 1.36 mM SO_4^{2-}, and 12.5 μg/ml heparin. The cells were washed twice and then incubated for 30 minutes at 37° C in the same buffer but in the absence of heparin. After this incubation cells were spun down at 2000 $\times g$ for 5 min. The stock cell suspension contained 12.5 g of packed wet cells in 100 ml phosphate buffer, K^+ concentration being again 11.4 mM. All buffers contained 1% bovine albumin to prevent cell aggregation which occurred without albumin within 10 minutes of the incubation period.

Fluorimetric measurements

25—50 μl of a 0.5 mM ethanolic solution of 3,3′-dihexyl-2,2′-oxacarbocyanine iodide (DiO-C_6-(3)) were added to 12 ml of phosphate buffers with various K^+ concentrations, the sum of $[K^+]$ and $[Na^+]$ remaining constant at 155 mM. Aliquots of these solutions were taken to measure fluorescence of the buffer prior to the addition of cells. The fluorescence of the cell suspension was measured 10 and 30 min after the addition of the cells to give a final cytocrite of 0.32% (0.32 g packed wet cells/100 ml buffer). Parts of the probes contained 0.186 or 18.6 μM valinomycin. Immediately after the last fluorescence determination the cells were spun down to measure the fluorescence of the supernatants. All fluorescence measurements were performed in 1-cm path length plastic cuvettes at room temperature with a Beckman Fluorometer SF 1078. The dye was excited with a Xenon lamp at 470 nm, and emission was detected by the 1P28 tube at 502 nm at the cuvette face. The addition of freeze-dried cells shows that changes in absorbance do not alter the fluorescence signal in the applied cell concentration range.

Potassium was determined by flame photometry after extracting the freeze-dried cells with 3 ml distilled water.

DiO-C_6-(3) was a generous gift of Dr. A. Waggoner, Amherst College. Valinomycin and bovine albumin were obtained from Serva, Heidelberg.

Results

Time course of fluorescence

Fluorescence at 502 nm is proportional to the dye concentration in the tested concentration range (at least up to 2 μM). Albumin enhances the fluorescence with increasing concentration, showing a saturation at 1—1.5%. In the presence of 1% albumin a constant level of fluorescence is reached immediately after the dye is added to the buffer. In a 0.32% cell suspension, however, 1% albumin increases the half-time for reaching a constant fluorescence level from about 0.5 to about 1.5 min. After addition of the cells to the dye-containing

buffer solutions the fluorescence drops, passing through a minimum after about 5 min, and then slowly increases again to reach a new steady level after 20—30 min. If valinomycin is present, no minimum is seen and a constant level is attained after 10 min.

Influence of extracellular potassium concentration on the fluorescence level

The dependence of the constant fluorescence levels of cell suspensions and supernatants on the external K^+ concentration is shown in Fig. 1. In the presence of 18.6 μM valinomycin the fluorescence changes of the cell suspensions cover a range of 23 arbitrary units (the fluorescence of cell-free buffer with dye is set to be 100 arbitrary units). The fluorescence of the supernatants shows greater changes, when external potassium is varied. In the absence of valinomycin the fluorescence of cell suspensions increases only slightly with increasing external K^+.

Theory

Sims et al. [2] showed by a centrifugation technique that the dye is distributed between extracellular medium and red cells in response to the membrane potential: cells becoming more negative inside take up more of the positively charged dye molecules. In an Ehrlich cell suspension the dye is considered to show a comparable potential-dependent partition. In the following all the dye

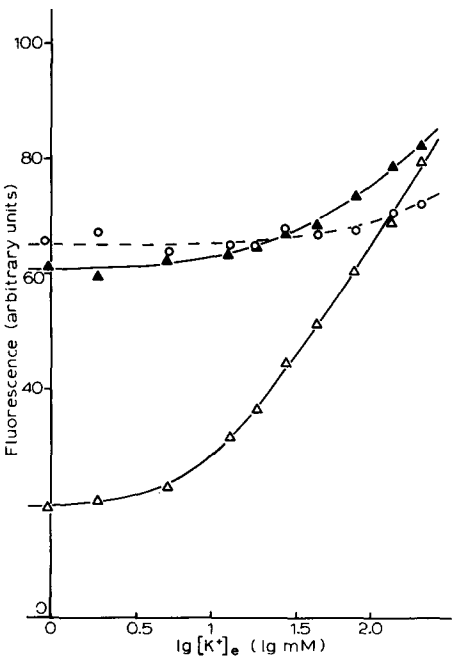


Fig. 1. The influence of the extracellular potassium concentration on the constant fluorescence level (F_S) of a 0.32% cell suspension. Fluorescence values were taken 30 min after the addition of cells to buffers with 1 μM DiO-C_6-(3) and are given in arbitrary units; cell-free buffer with dye = 100 arbitrary units. The sum of $[Na^+]_e$ and $[K^+]_e$ is always 154.6 mM. Cell suspensions without (open circles) and with 18.6 μM valinomycin (closed triangles). The open triangles represent supernatant fluorescence (F_{sn}) of the cell suspension with 18.6 μM valinomycin.

that is removed from the light path by centrifuging down the cells, is termed "cell-associated" without regard to its localisation within the cells.

The cell-associated dye can be expressed as a fraction Q of the total dye amount present in the cuvette:

$$\text{dye}_c = Q \cdot c_0 \cdot v_e \tag{1}$$

c_0 and v_e are dye concentration and buffer volume in the cuvette, respectively, before the addition of cells. The amount of the dye in the extracellular medium (non cell-associated) is then:

$$\text{dye}_e = (1 - Q) \cdot c_0 \cdot v_e. \tag{2}$$

In a 1% albumin solution this quantity of dye is reversibly bound to the albumin.

The overall fluorescence signal F_s of a cell suspension is presumably the sum of two fractions: the fluorescence of the dye in the extracellular medium (F_e) and that of the cell-associated dye (F_c). F_e is proportional to the concentration of the non cell-associated dye, which is found in the supernatants:

$$F_e = F_1(1 - Q) \cdot c_0 \cdot \frac{v_e}{v_e + v_c} = F_{sn} \frac{v_e}{v_e + v_c}. \tag{3}$$

F_1 is the "micromolar fluorescence" (arbitrary units/μM), v_c is the volume of the added cells, and F_{sn} the fluorescence of the supernatants after spinning down the cells. If dilution of the dye in the extracellular medium by the added cells can be neglected, i.e. $v_c \ll v_e$, F_e is equal to F_{sn}.

The fluorescence F_c of the cell-associated dye should depend on its amount Q. Q can be found from Eqn. 3 as

$$Q = 1 - \frac{F_{sn}}{F_1 c_0} = 1 - \frac{F_{sn}}{F_b}.$$

F_b is the fluorescence of the buffer which is measured prior to the addition of cells. Cell-associated dye fluorescence F_c is experimentally determined as the difference between the fluorescences F_s of the cell suspension and F_e. A double logarithmic plot of F_c vs. Q visualizes the mathematical relationship (Fig. 2). This relationship is not linear, since the slope β in the double logarithmic plot $\neq 1$. The y-intercept gives the logarithm of the proportionality factor F_2.

$$F_c = F_s - F_e = F_2 \cdot Q^{\beta} \tag{4}$$

There is some incertainty in fitting the data in Fig. 2 by a straight line. Deviations may occur for small values of Q. In this range, however, the contribution of F_c to the overall signal F_s is small (see below) so that the use of Eqn. 4 can be justified.

As shown in Fig. 1, the major part of the overall signal is due to the non cell-associated dye when external potassium concentrations are high. Decreasing the extracellular K^+ concentration leads to an increasing contribution of the cell-associated dye. This is consistent with the idea of a potential-dependent partition of the dye, which can best be demonstrated by the fluorescence levels of the supernatants indicating lower concentrations of dye in the extracellular medium at low external potassium.

The membrane potential can be approximated according to the Nernst equa-

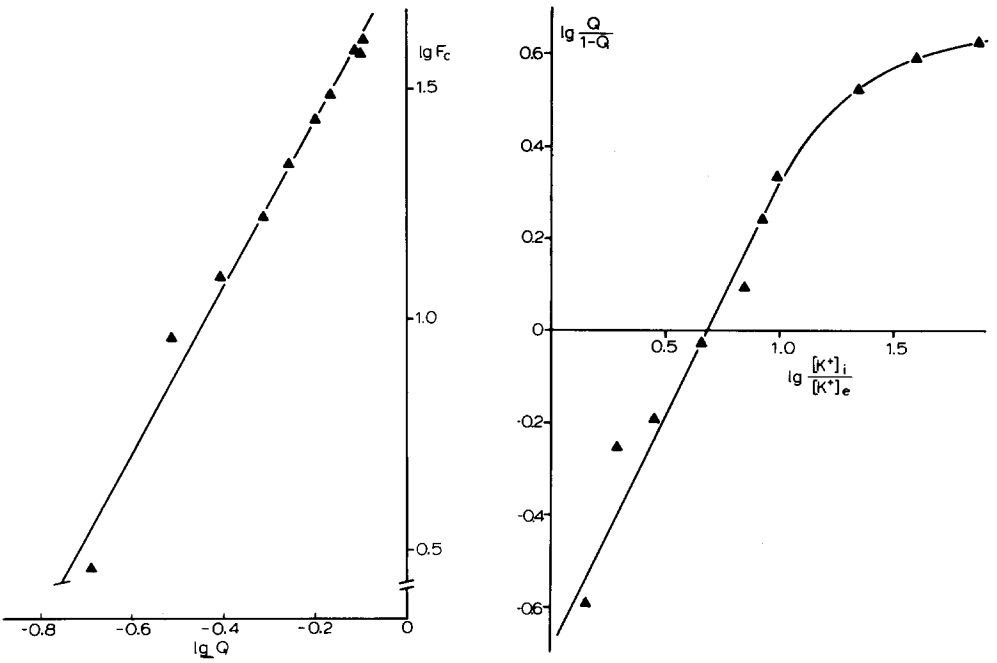


Fig. 2. Fluorescence F_c of the cell-associated dye as a function of the bound fraction Q of the total dye present in the cuvette. F_c is the difference of cell suspension fluorescence F_s and fluorescence of the dye in the medium F_e (data from Fig. 1 for valinomycin = 18.6 μM). Q is given by $1 - F_{sn}/F_b$, where F_b is the fluorescence of the cell free buffer, F_{sn} that of supernatants. The line is constructed by the method of least squares; the slope gives $\beta = 1.8$, the y-intercept $\log F_2$; $F_2 = 61.14$ (for details see text).

Fig. 3. The partition of the dye in dependence on the potassium gradient with 18.6 μM valinomycin present. Q is the fraction of cell-associated dye (cf. Fig. 2), $(1 - Q)$ represents the fraction of dye in the extracellular medium. Both values are calculated from Fig. 1. $[K^+]_i$ and $[K^+]_e$ are the concentrations found at the end of the fluorescence determinations. Regression line for $\log ([K^+]_i/[K^+]_e) < 1$: slope 1.005, y-intercept = $\log a = -0.689$; $a = 0.205$.

tion from the potassium gradient, if potassium permeability is increased by valinomycin:

$$F\Psi = -RT \ln \frac{[K^+]_i}{[K^+]_e} \tag{5}$$

It has been found that in the presence of 1% albumin valinomycin concentrations as high as 18.6 μM have to be used to increase potassium permeability of Ehrlich cells sufficiently (Geck, personal communication).

In Fig. 3 a double logarithmic plot of the dye partition $Q/(1 - Q)$ vs. $[K^+]_i/[K^+]_o$ was constructed to find out the relationship between the partition and the K^+ gradient. For values of $\log ([K^+]_i/[K^+]_e) < 1$ the relationship is approximately linear with a slope of 1. In this range the following empirical equation holds:

$$\frac{Q}{1-Q} = a \frac{[K^+]_i}{[K^+]_e} \tag{6}$$

The proportionality factor a is evaluated by the y-intercept in Fig. 3. The devia-

tions for small $[K^+]_e$ are probably due to the influence of $[Na^+]_e$ on the membrane potential. Note that in Eqn. 6 the partition of dye amounts and not of dye activities is related to the potassium gradient. This is only valid if dye activity is proportional to amount of dye in the applied concentration range. Factors relating dye activity to dye amount and to K^+ activities will be involved in the empirical proportionality factor a. A basic physical explanation for a, however, cannot be given at this time.

The fluorescence F_s of a cell suspension is the sum of F_e and F_c:

$$F_s = F_e + F_c = F_1(1 - Q)\, c_0 \frac{v_e}{v_e + v_c} + F_2 Q^\beta$$

$$F_s = F_1 c_0 \frac{v_e}{v_e + v_c} \frac{1}{1 + a \cdot e^{-(F/RT)\Psi}} + F_2 \left[\frac{a \cdot e^{-(F/RT)\Psi}}{1 + a \cdot e^{-(F/RT)\Psi}}\right]^\beta \tag{7}$$

In Eqn. 7 Q is replaced by using Eqns. 5 and 6. Eqn. 7 gives the relationship between the fluorescence of a cell suspension (F_s) and the membrane potential ψ. F_s is not a linear function of ψ.

Fig. 4 is the theoretical curve given by Eqn. 7 with the constants evaluated from Figs. 2 and 3. In the range of −40 to +20 mV F_s is approximately proportional to the membrane potential. For higher and lower potentials a saturation can be seen.

Fig. 5 shows the shape of the calibration curve as modified by F_2, all other factors being the same as in Fig. 4. Increasing F_2 leads to an absolute decrease in the "linear" part of the curve. Because $\beta > 1$ a minimum is developed for higher F_2. Higher values of F_2 indicate a smaller quenching of the fluorescence of cell-associated dye. The curve for $F_2 = 0$ is the fluorescence of dye in the medium without any contribution by the cell-associated dye. Even in this case F_s is not proportional to ψ over the whole range. The changes in F_s, however, are much greater than for cell suspensions with $F_2 > 0$, which can also be seen in Fig. 1.

The dependence of the curve on factor a, which relates dye accumulation to potassium gradient, is given in Fig. 6. Higher dye accumulation at a given potassium gradient for instance by mitochondrial dye uptake or sticking to membrane structures shifts the curves parallel to more positive potentials. The percent change of fluorescence remains constant for a given potential change in the "linear" part of the curves. This shift could be a reason, why dyes with different lipophilicity give different answers to membrane potential changes.

Figs. 5 and 6 show that estimating membrane potentials by a curve constructed with results from valinomycin-treated cells (Fig. 4) leads to wrong results, if the factors a and F_2 are altered by valinomycin. As in the absence of valinomycin also other ions than potassium and presumably electrogenic pumps can influence the membrane potential, Eqn. 5 does not hold. Therefore it is not possible to evaluate a by plotting $Q/(1 - Q)$ vs. $[K^+]_i/[K^+]_e$. For reasons given below a is considered not to be altered by valinomycin. Factor F_2 can be obtained for cells without valinomycin by a plot given in Fig. 2. Unfortunately, F_2 for cells without valinomycin is different from that of valinomycin-treated cells. Membrane potentials are therefore calculated from the fluorescences of the supernatants F_{sn}, which are independent of F_2 as can be seen in

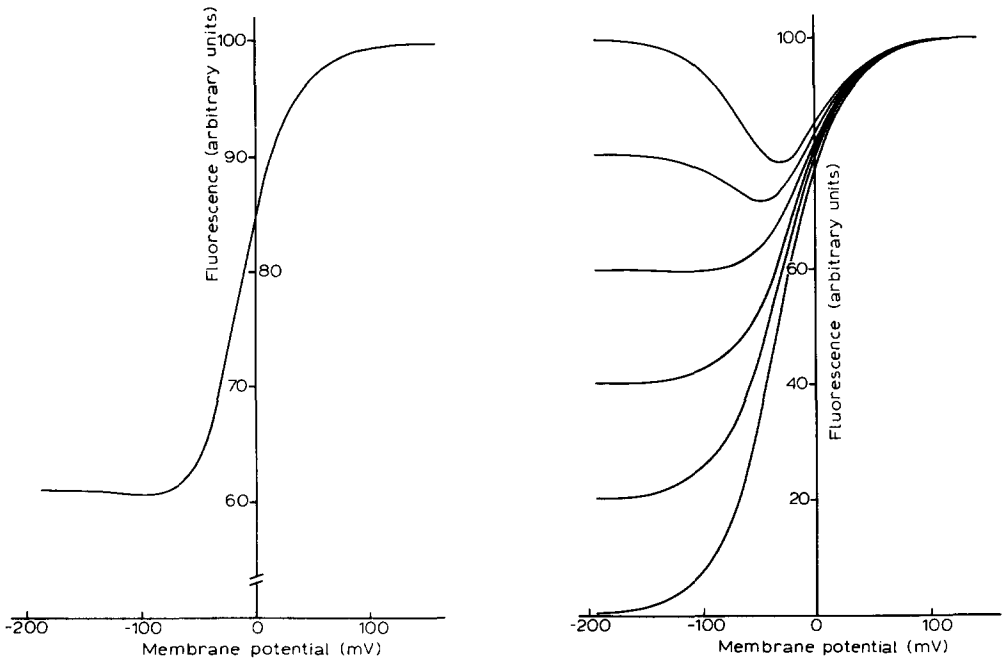


Fig. 4. The cell suspension fluorescence as a function of the membrane potential calculated by Eqn. 7. The factors F_2, a, and β are obtained by Figs. 2 and 3. $F_1 = 100$, $c_0 = 1$ μM, $v_e = 3$ ml, $v_c = 0.01$ ml, $F_2 = 61.14$, $a = 0.205$, $\beta = 1.8$. Fluorescence is given in arbitrary units, membrane potential in mV, sign with respect to the cell interior.

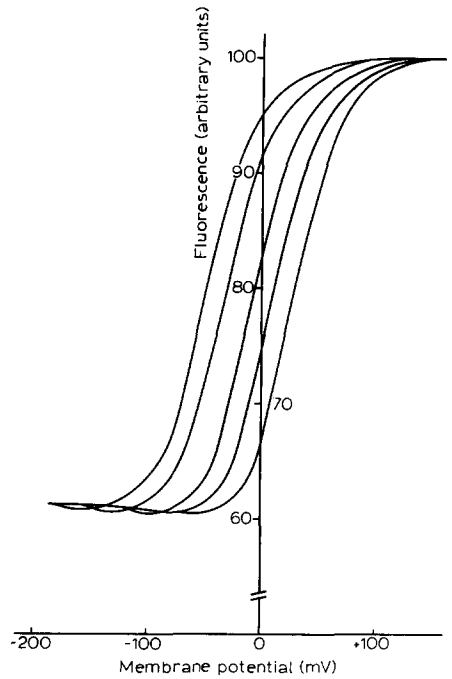
Fig. 5. The cell suspension fluorescence as a function of the membrane potential (Eqn. 7). Influence of F_2. All other factors are given in Fig. 4. $F_2 = 100; 80; 60; 40; 20; 0$ (beginning at top curve).

Eqn. 3. Inserting Eqns. 5 and 6 into 3 and rearranging yields:

$$\ln \frac{F_1 c_0 - F_{sn}}{F_{sn}} = \ln \frac{F_b - F_{sn}}{F_{sn}} = -\frac{F}{RT}\Psi + \ln a \tag{8}$$

Fig. 7 summarizes the experimental data obtained under four different conditions: two dye concentrations (1 and 2 μM) and with 0 and 0.186 μM valinomycin present. Valinomycin in this concentration range is thought to depolarize the mitochondria but to have a negligible effect on the potassium permeability of the cell membrane (Geck, personal communication). There is no significant change of fluorescence upon addition of 0.186 μM valinomycin indicating that mitochondrial dye accumulation after 30 min incubation does not alter the fluorescence markedly. Incubation with dye for less than 15 min exhibits an enhancement after adding 0.186 μM valinomycin (not shown). Assuming that a is not changed by mitochondrial dye accumulation after 30 min, the potentials in Fig. 7 were calculated with the same factor a as evaluated for cells with saturating valinomycin concentrations. In contrast to findings by Laris et al. [3], the potentials are not independent of $[K^+]_e$. They cover a range of −19 to −52 mV at room temperature.

Membrane potentials for cells with 18.6 μM valinomycin are given in Fig. 8. For very small extracellular potassium concentrations there is a deviation from



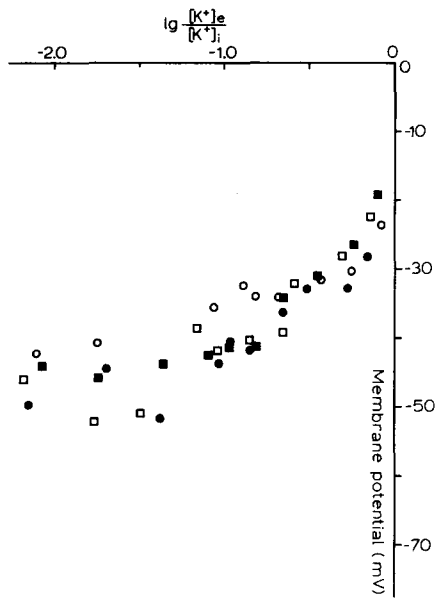


Fig. 6. The influence of the factor a on the potential-dependent cell suspension fluorescence (Eqn. 7). a = 0.05; 0.1; 0.25; 0.5; 1.0 (beginning at the left curve). All other factors are given in the legend of Fig. 4.

Fig. 7. The membrane potential of Ehrlich ascites cells at room temperature as a function of the potassium gradient. The potentials were calculated according to Eqn. 8. All fluorescence measurements were done 30 min after addition of cells to dye containing buffer solutions of various K^+ concentrations. The sum of $[K^+]_e$ and $[Na^+]_e$ remained constant (154.6 mM). $[K^+]_e$ and $[K^+]_i$ are the concentrations found at the end of the fluorescence measurements. Dye concentrations: 1 μM (■,●) and 2 μM (□,○); valinomycin: 0 (○,●) and 0.186 μM (□,■).

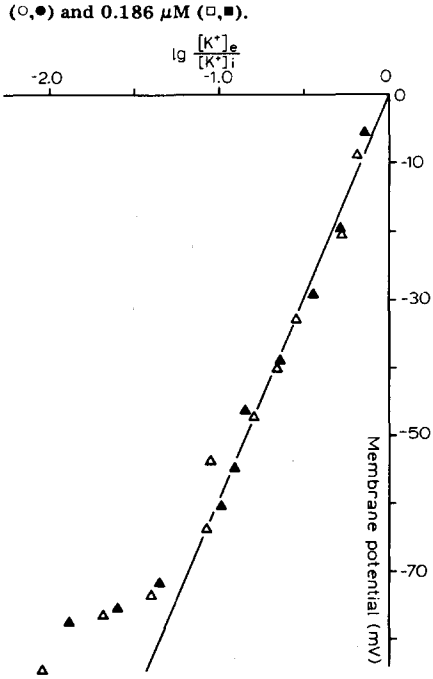


Fig. 8. Membrane potential of Ehrlich ascites cells at room temperature as a function of the potassium gradient. Valinomycin concentration 18.6 μM, dye concentrations 1 μM (▲) and 2 μM (△). The potentials were calculated as in Fig. 7. The line gives the theoretical potentials for a potassium electrode: $\psi = (RT/F) \ln ([K^+]_e/[K^+]_i)$.

the linearity probably due to Na^+ influencing the membrane potential. For higher external potassium the cells behave like a potassium electrode. The potentials go from —84.3 to —5.2 mV.

Discussion

The experiments with the dye DiO-C_6-(3) show that the fluorescence of an Ehrlich ascites cell suspension is not a linear function of the membrane potential. Plots of fluorescence vs. ψ give S-shaped curves, as was also found by Laris and Pershadsingh [4] with *Streptococcus faecalis*. The saturation effects are due to the limitation of the amount of dye and can be expected to occur always with systems in which the partition between two phases leads to marked concentration changes in both phases. A calibration curve can be constructed (Fig. 4) based on the measurements of fluorescence in the buffer solution, the cell suspension, and the supernatant. Plots as given by Figs. 2 and 3 give the factors F_2, a, β, which may be different from experiment to experiment, but must be kept constant within the same experiment.

Membrane potential determinations were often done under the assumption of a linear relationship between fluorescence and membrane potential. This leads to approximately correct results only in a limited region of fluorescence values. Table I gives the deviations from the true potential, if calculations are based on an approximation of the calibration curve in Fig. 4 by a straight line passing through the fluorescence values for —40 and 0 mV. The deviations are less than ±5 mV in the range of —55 to +20 mV. This corresponds to fluorescence values between 63.4 and 91.9 arbitrary units (cell free buffer with dye: 100 arbitrary units). The given potential and fluorescence limits of this region of approximate proportionality are dependent on F_2, a, and β and have to be

TABLE I

APPROXIMATION OF MEMBRANE POTENTIALS BY A LINEAR EQUATION AND THEIR DEVIATION FROM THE TRUE POTENTIALS

ψ is the given membrane potential. For every ψ the fluorescence F_S is calculated by Eqn. 7 with factors given in Figure 4. The resulting curve F_S vs. ψ (Fig. 4) is approximated by a straight line passing through the fluorescence values for ψ = —40 mV and ψ = 0 mV: $F_S = 0.439\ \psi_{lin} + 85.28$. The third column gives the deviation of ψ_{lin} from the true potential in mV.

ψ (mV)	ψ_{lin} (mV)	$\psi_{lin} - \psi$ (mV)
30	20.10	—9.89
25	17.74	—7.26
20	14.98	—5.02
15	11.86	—3.14
10	8.31	—1.69
0	0	0
—10	—9.77	+0.23
—20	—20.38	—0.38
—30	—30.81	—0.81
—40	—40	0
—45	—43.85	1.15
—50	—47.11	2.89
—55	—49.8	5.2
—60	—51.89	8.11

tested in every single experiment, for instance by plotting cell suspension fluorescence in the presence of valinomycin vs. log $[K^+]_e$ ($[K^+]_i$ = const.) or vs. log ($[K^+]_e/[K^+]_i$). For higher $[K^+]_e$ the cells behave like a potassium electrode and membrane potentials can be calculated from the K^+ distribution. Only fluorescence values falling on an apparently straight line in this plot are approximately proportional to membrane potential. Fig. 9 shows the dependence of the cell suspension fluorescence F_s on the extracellular K^+ concentration predicted under the assumption that only Na^+ and K^+ influence the membrane potential according to the Goldman-Hodgkin-Katz equation [5,6]

$$\Psi = -\frac{RT}{F} \ln \frac{P_K[K^+]_i + P_{Na}[Na^+]_i}{P_K[K^+]_e + P_{Na}[Na^+]_e} = -\frac{RT}{F} \ln \frac{[K^+]_i + \alpha[Na^+]_i}{[K^+]_e + \alpha[Na^+]_e} \tag{9}$$

$\alpha = P_{Na}/P_K$. Assuming that the sum of external K^+ and Na^+ is always 155 mM, and the intracellular concentrations of these ions are constant, the influence of α on the shape of the curve can be predicted (Fig. 9). At $\alpha = 0$ the calibration curve represents that of a K^+ electrode and corresponds experimentally to cells treated with saturating valinomycin concentrations. Fluorescence values above the dashed line are approximately proportional to membrane potential (deviation < +5 mV). With the factors F_2, a, β, given in Fig. 4, all values for $\alpha > 0.15$ fall into the range of approximate proportionality between F_s and ψ. In this

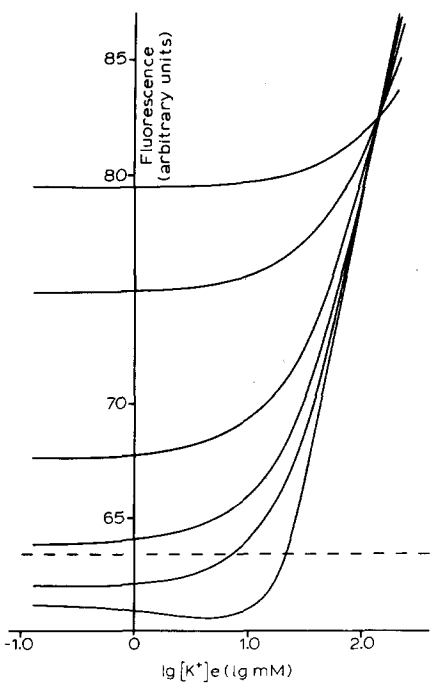


Fig. 9. The fluorescence of a cell suspension as a function of the extracellular potassium concentration and the influence of $\alpha = P_{Na}/P_K$ (see text). The membrane potentials are calculated by the Goldman-Hodgkin-Katz Eqn. 9 with $[Na^+]_i$ = 20 mM; $[K^+]_i$ = 180 mM; $[Na^+]_e + [K^+]_e$ = 155 mM and inserted in Eqn. 7. The other factors are given in the legend of Fig. 4. Fluorescence values above the dotted line are approximately proportional to membrane potential with a deviation <+5 mV from the true potential. α = 0.75; 0.5; 0.25; 0.15; 0.1; 0 (beginning at top curve).

range α can be evaluated by fitting theoretical fluorescence curves calculated according to Eqn. 9, the fluorescence being assumed as a linear function of ψ [7]. Approximation of α by this method is not possible for $\alpha < 0.15$, because the curves are not proportional to the membrane potential in the range of lower $[K^+]_e$. This could explain the discrepancy between α-values for K^+-loaded and -depleted cells found by Hoffman and Laris [7]. Membrane potentials for $\alpha < 0.15$ and low $[K^+]_e$ can only be found by construction of a calibration curve F_s (or F_{sn}) vs. ψ after evaluation of F_2, a, and β.

All curves in Fig. 9 intersect at $[K^+]_e = 139.5$ mM. At this point fluorescence and membrane potential are independent of α, which is only valid for the Donnan-equilibrium, where $[K^+]_e/[K^+]_i = [Na^+]_e/[Na^+]_i$. In Fig. 1 the intersection point is shifted to lower $[K^+]_e$ than expected from the Donnan-equilibrium. There are two explanations for this discrepancy. (1) Valinomycin decreases not only α by increasing K^+-permeability but also depolarizes mitochondria thus reducing mitochondrial dye accumulation (factor a in Eqn. 5 gets smaller). This shifts the curve F_s vs. log $[K^+]_e$ for valinomycin-treated cells and the intersection point between the two curves to lower $[K^+]_e$ (cf. Fig. 6). (2) In the absence of valinomycin there are more ions than Na^+ and K^+ and/or electrogenic pumps influencing membrane potential towards more negative values. This would shift the curve for non valinomycin-treated cells to lower fluorescence values than expected from Eqns. 7 and 9, and again move the intersection point to lower $[K^+]_e$. The lacking effect of mitochondria depolarizing valinomycin concentrations (Fig. 7) and the increasing evidence of the existence of electrogenic pumps [8,9] are in favour of the second explanation.

Acknowledgements

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