Biochimica et Biophysica Acta, 436 (1976) 475–488

BBA 77341

MONITORING MEMBRANE POTENTIALS IN EHRLICH ASCITES TUMOR CELLS BY MEANS OF A FLUORESCENT DYE

PHILIP C. LARIS, HARRIHAR A. PERSHADSINGH* and ROSE M. JOHNSTONE

Department of Biological Sciences, University of California, Santa Barbara, Calif. (U.S.A.) and Department of Biochemistry, McGill University, Montreal, Quebec (Canada)

(Received December 2nd, 1975)

SUMMARY

1. The fluorescent intensity of the dye 3,3'-dipropylthiodicarbocyanine iodide was measured in suspensions of Ehrlich ascites tumor cells in an attempt to monitor their membrane potentials under a variety of different ionic and metabolic conditions.

2. In the presence of valinomycin, fluorescent intensity is dependent on log $[K^+]_{medium}$ (the fluorescent intensity increased with increasing $[K^+]_{medium}$) where K^+ replaced Na^+ in the medium. Cellular K^+ content also influenced fluorescent intensity in the presence of valinomycin. With lower cellular K^+, fluorescent intensity in the presence of valinomycin for any given concentration was increased.

3. In the presence of gramicidin fluorescent intensity was highest in Krebs-Ringer and decreased with the substitution of choline$^+$ for Na^+.

4. The observations with ionophores are consistent with the hypothesis that the dye monitors membrane potential in these cells with an increase in fluorescence indicating membrane depolarization (internal becomes more positive).

5. The estimated membrane potentials were influenced by the way in which the cells were treated. Upon dilution of the cells from 1 in 20 to 1 in 300 the initial estimations were between −50 and −60 mV. With incubation at 1 in 300 dilution for 1 h at room temperature or a 37 °C, the membrane potentials ranged from −18 to −42 mV.

6. Estimations of membrane potential on the basis of chloride distribution ($Cl^-_{cell}/Cl^-_{medium}$) in equilibrated cells ranged from −13 to −32 mV.

7. Addition of glucose to cells equilibrated at 37 °C for 30 min in the presence of rotenone led to a decrease in fluorescent intensity indicating hyperpolarization. Addition of ouabain in turn led to a 70 to 100 % reversal of fluorescent intensity. This hyperpolarization is therefore probably due to the electrogenic activity of the sodium pump.

8. The addition of amino acids known to require external Na^+ for transport

---

Abbreviations: MOPS, morpholinopropane sulfonic acid; diS-C_3-(5), 3,3'-dipropylthiodicarbocyanine iodide.

* Present address: Division of Hematology; Royal Victoria Hospital, Montreal, Quebec, Canada.

increased fluorescent intensity (depolarization) reaching a maximum at higher concentrations of amino acids. Plots of $1/\Delta$fluorescence vs. 1/[glycine] were linear with an apparent K_m of 2–3 mM. The increase in fluorescence with amino acids always required external Na^+. Plots of 1/fluorescence vs. $1/[Na^+]_{medium}$ were also linear with an apparent K_m of 29 mM. These apparent K_m values compare favorably with those derived from amino acid transport studies using tracers. These data indicate that the Na^+-dependent transport of amino acids in these cells is electrogenic.

INTRODUCTION

The membrane potential of Ehrlich ascites tumor cells has been estimated by a number of investigators using three different methods, direct measurements with electrophysiological techniques, measurements of the $^{36}Cl^-$ distribution and ^3H-labelled lipid-soluble cation distribution across the membrane. The results of direct measurements with microelectrodes fall into 2 ranges: (a) −8 to −12 mV [1–4], and (b) −20 to −40 mV [5, 6]. The estimation of membrane potential from measurements of chloride distribution depend on the assumption that the chloride is at equilibrium and also fall into 2 ranges: (a) −20 to −30 mV [7], and (b) −40 or above [8]. Estimation of the membrane potential with [^{3}H]dibenzyldimethylammonium ion gave values in agreement with Cl^- distribution values [9]. The problem with using Cl^- distribution as an estimate of the membrane potential arises from the fact that it is not known whether Cl^- is (a) equally distributed throughout cell water, or (b) sequestered preferentially in the nuclei, or (c) whether $^{36}Cl^-$ has achieved a steady state distribution after short periods of incubation. Since precise knowledge of the value of the membrane potential is important for any study in which the electrochemical gradients of ions must be known (i.e., ion or amino acid transport), we sought to estimate the magnitude of the potential using the fluorometric techniques, a system whereby changes in membrane potential could be rapidly monitored. A preliminary account of this work has already been presented [10].

METHODS

The procedure for preparation of Ehrlich ascites cells has been previously described [11]. After the cells were washed, they were packed and diluted 1 to 20 with Na^+-Ringer and stored at room temperature until use. The cells used in measurements of fluorescence were handled in 2 ways: (1) they were diluted 1 to 300 with various media and dye was added immediately, or (2) they were diluted 1 to 300 (to cuvette dilution condition) and incubated for various periods of time prior to the addition of dye. Na^+-Ringer contained 154 mM NaCl, 6 mM KCl, 1.5 mM $MgSO_4$ and 10 mM sodium phosphate buffer at pH 7.4. In K^+-free Ringer, NaCl was substituted for KCl. K^+-Ringer and choline$^+$-Ringer were prepared by substituting equivalent concentrations of KCl or choline chloride for NaCl. In some experiments the buffer employed was 10 mM MOPS (morpholinopropane sulfonic acid) brought to pH 7.4 with Tris hydroxide.

The experimental procedures for Na^+ and K^+ determinations and wet and dry weight measurements have been described previously [12]. Chloride ratios (cell/medium) were determined by measuring the steady-state distribution of $^{36}Cl^-$. A

known volume of cells which had been incubated with $^{36}Cl^-$ was added to a tared tube containing [^{3}H]inulin. After mixing the sample was centrifuged, the supernatant decanted, and the tube swabbed dry and weighed to obtain the wet weight of the pellet. (The dry weight/wet weight ratio was predetermined from other samples.) Trichloroacetic acid was added to pellet and supernatant so that the final concentration was the same (5 %) in both cases. Aliquots of the pellet extracts and the supernatants were counted. The dry weight/wet weight ratio and [^{3}H]inulin distribution were used to determine the intracellular water compartment. The incubation mixture containing $^{36}Cl^-$ was sampled continuously until the last two showed no change in $^{36}Cl^-$ distribution (usually after 40 min). This was taken as the steady state condition.

The fluorescent dye, 3.3'-dipropylthiodicarbocyanine iodide (referred to as diS-C_3-(5) or simply as the dye) employed in these studies was obtained from Dr. Alan Waggoner of Amherst College. The fluorescent intensity of the dye in cell suspensions was measured as described previously [13]. The final concentration of dye in the cuvette was always $3.0 \cdot 10^{-6}$ M.

In the text measurements of fluorescence are given in "arbitrary units". Results given from different experiments in most cases are not directly comparable for no effort was made to standardize instrument settings from day to day. The cell suspensions on which the fluorescent measurements were made contained approximately 0.3 % cells. A number of substances were added from stock solutions in ethanol, i.e., valinomycin ($3.3 \cdot 10^{-4}$ M), gramicidin ($3.2 \cdot 10^{-5}$ M), rotenone (0.5 mg/ml) and dye (0.5 mg/ml).

Incubations were carried out in a gyratory water bath shaker model G76 (New Brunswick, N.J.) using air as gas phase.

RESULTS

In red cells [13] measurements of fluorescence using this type of dye have shown that the intensity of fluorescence is inversely related to the membrane potential and that estimates of the membrane potential by this method are in good agreement with values based on chloride distribution and in the case of Amphiuma erythrocytes electrical measurements. One useful approach derived from the above studies was that one could determine the medium K^+ concentration at which the K^+ distribution was at equilibrium. This method is based on the fact that fluorescence intensity will not be altered by the addition of valinomycin if K^+ is already at equilibrium distribution before the addition of valinomycin.

Estimations of membrane potential

Although the basis for the membrane potential in Ehrlich ascites tumor cells is not known, if the constant field theory is applicable, then according to Brading and Caldwell [14]

$$E_M = \frac{RT}{F} \ln \frac{P_K[K^+]_o + P_{Na}[Na^+]_o + P_{Cl}[Cl^-]_i + x}{P_K[K^+]_i + P_{Na}[Na^+]_i + P_{Cl}[Cl^-]_o + y} \quad (1)$$

where E_M is the membrane potential; R, the gas constant, P_K, P_{Na}, P_{Cl} the permeability constants for K, Na and Cl; $[K^+]_o$, $[Na^+]_o$, $[Cl^-]_o$ = K, Na and Cl concentrations of the medium and $[K^+]_i$, $[Na^+]_i$, $[Cl^-]_i$, concentrations of K, Na and Cl in the cell

water and x and y represent the contributions of other ions or electrogenic pumps. Assuming that x and y, the permeability coefficients of all ions other than K^+, and the ion concentrations remain constant in the presence of valinomycin for a short (30 s) period of time, then Eqn. 1 for our purposes can be reduced to:

$$E_M = \frac{RT}{F} \ln \frac{P_K[K^+]_o + c}{P_K[K^+]_i + d} \tag{2}$$

where $c = P_{Na}[Na^+]_o + P_{Cl}[Cl^-]_i + x$ and $d = P_{Na}[Na^+]_i + P_{Cl}[Cl^-]_o + y$. At an external K^+ concentration where there is no change in fluorescent intensity upon the addition of valinomycin (the "null point") then:

$$E_M = \frac{RT}{F} \ln \frac{P_K[K^+]_o + c}{P_K[K^+]_i + d} = \frac{RT}{F} \ln \frac{P_K^*[K^+]_o + c}{P_K^*[K^+]_i + d} \tag{3}$$

where P_K^* equals the permeability coefficient in the presence of valinomycin. From Eqn. 3 it can be shown that $[K^+]_o/[K^+]_i = c/d$. It can also be shown that at

these K^+ concentrations $\frac{P_K[K^+]_o + c}{P_K[K^+]_i + d} = \frac{[K^+]_o}{[K^+]_i}$ and

hence $E_M = \frac{RT}{F} \ln \frac{[K^+]_o}{[K^+]_i}$. (4)

Note that this relationship (Eqn. 4) is shown to be true at the null point only. Hence, this method allows one to calculate the potential difference only under these very special conditions.

Addition of K^+ and valinomycin

The intensity of fluorescence of the dye diS-C_3-(5) attained a constant level approximately 3–5 min after its addition to cells suspended in Na^+-Ringer solution. The same level of intensity was also reached in K^+-Ringer and mixtures of Na^+-Ringer and K^+-Ringer. Upon the addition of valinomycin (final concentration equals $1 \cdot 10^{-6}$ M) to cells suspended in various mixtures of Na^+-Ringer and K^+-Ringer, the changes in fluorescence observed were dependent on the external K^+ concentration (Fig. 1 insert). The measurements given in Fig. 1 were made on cells to which dye was added immediately after dilution (method 1 of handling cells in Methods). The constant level of fluorescence attained in the presence of valinomycin was also found to be dependent upon internal K^+ content (Fig. 2). The results of Figs. 1 and 2 are in keeping with the interpretation that the fluorescent intensity of the dye does monitor membrane potential. A value of approximately −50 mV was obtained for the cells used in Fig. 1 when the K^+ distribution at the "null point" was used to compute their membrane potential.

The non-linearity of the response to K_o^+ may reflect either one or a combination of the following possibilities: (1) the membrane potential in the presence of valinomycin is not equal to the K^+ equilibrium potential* and (2) the membrane potential is not directly proportional to fluorescent intensity at all levels of potential.

* It can be shown that the fluorescence is directly proportional to log (K_o^+ + constant) as predicated by Eqn. 2.

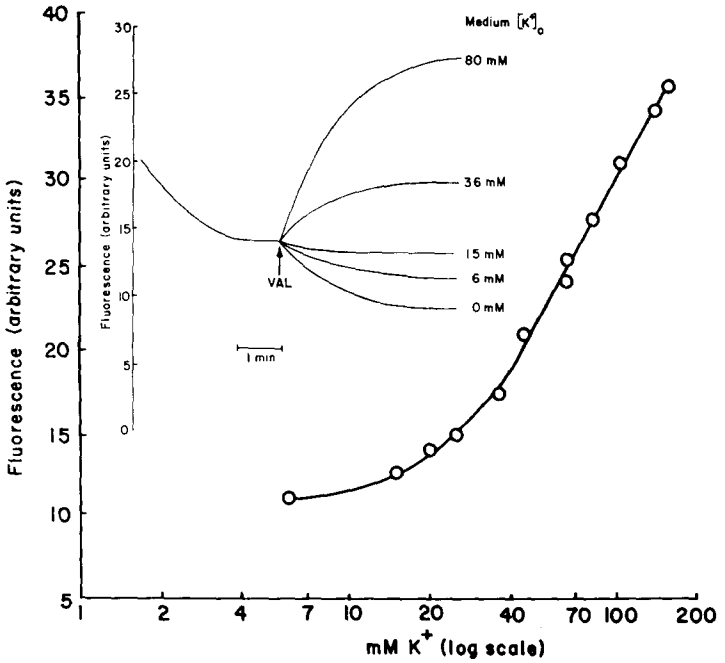


Fig. 1. Constant level of fluorescent intensity of dye ($3.0 \cdot 10^{-6}$ M) attained in the presence of $1 \cdot 10^{-6}$ M valinomycin as a function of log K^+ concentration of the medium. Insert. Fluorescent intensity of the dye with time in a 0.3 % suspension of Ehrlich ascites tumor cells in mixtures of Na^+-Ringer and K^+-Ringer to give the external K^+ concentration shown on the figure. Valinomycin (final concentration $1 \cdot 10^{-6}$ M) added where indicated (VAL).

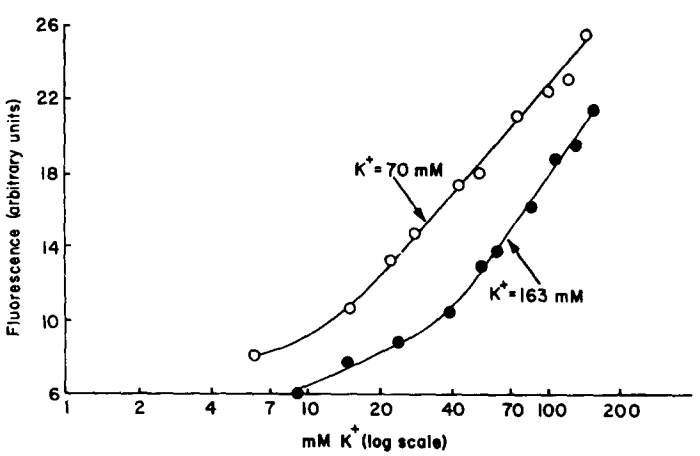


Fig. 2. Constant level of fluorescent intensity of dye in cell suspensions in various mixtures of Na^+-Ringer and K^+-Ringer containing 32 ng/ml rotenone and $1 \cdot 10^{-6}$ M valinomycin. The cells were preincubated in either K^+-Ringer (●–●) or in K^+-free Na^+-Ringer (○–○) containing 32 ng/ml rotenone at a 1 in 20 dilution for 2h at 37 °C. Cells incubated in K^+-Ringer contained 163 mmol K^+/L cell H_2O; in K^+-free medium, 70 mmol K^+/L cell H_2O.

Since fluorescent intensity is directly proportional to K_o^+ in the presence of valinomycin in the range 30–160 mM, the potential difference may be equivalent to the K^+ equilibrium potential only in this range of K_o^+. In this regard it is of interest to note that cells with identical levels of fluorescent intensity in Fig. 2 have similar K^+ equilibrium potentials. For example, note that the cell/medium K^+ ratio is approximately unity for both types of cells when the fluorescent intensity is 22 arbitratry units.

Addition of Na^+ and gramicidin

Studies with a second ionophore, gramicidin, also support the hypothesis that the dye (fluorescent intensity) monitors potential. Gramicidin induces rapid movements of both K^+ and Na^+ but not choline$^+$ [15] and Johnstone, unpublished observations). Hence one would expect gramicidin to cause rapid depolarization of cells in Na^+-Ringer but not in choline$^+$-Ringer. These predictions were verified by the data shown in Fig. 3, where the constant levels of fluorescent intensity attained in mixtures of Na^+-Ringer and choline$^+$-Ringer are shown as a function of log external Na^+.

Influence of the cellular environment on fluorescence

Although there is a general relationship between the fluorescent intensity and membrane potential, precise determinations of the potential are hampered by the fact that fluorescent intensity and hence estimates of the potential difference vary with the way the cells are handled. If cells are kept at room temperature at 1 to 20 dilution and diluted to cuvette conditions (0.2 ml of 1/20 cell suspension+3.0 ml medium, a 1/300 dilution) with Na^+-Ringer just before addition of dye, the steady level of fluorescence

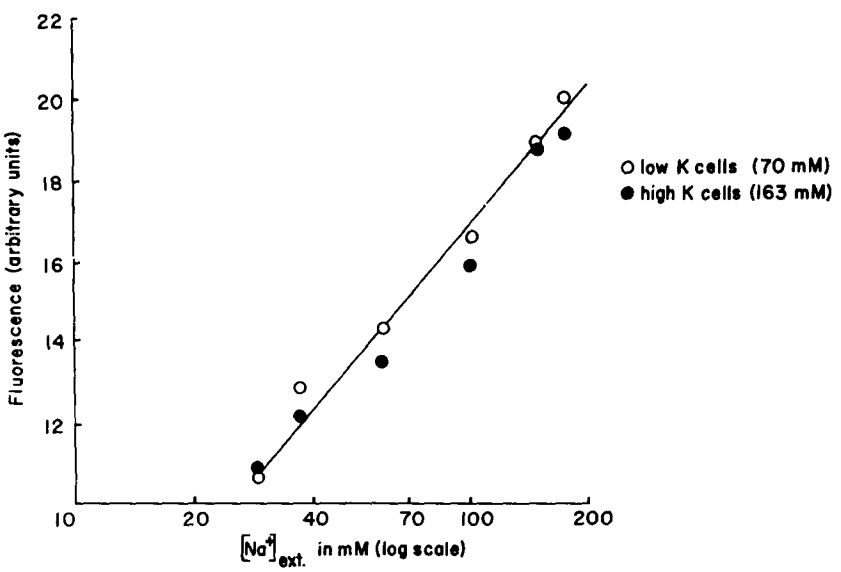


Fig. 3. Constant level of fluorescent intensity of dye in cell suspensions in various mixtures of Na^+-Ringer and choline$^+$-Ringer containing 32 ng/ml rotenone and $2 \cdot 10^{-7}$ M gramicidin. The cells used are those described in Fig. 2 which should be consulted for details.

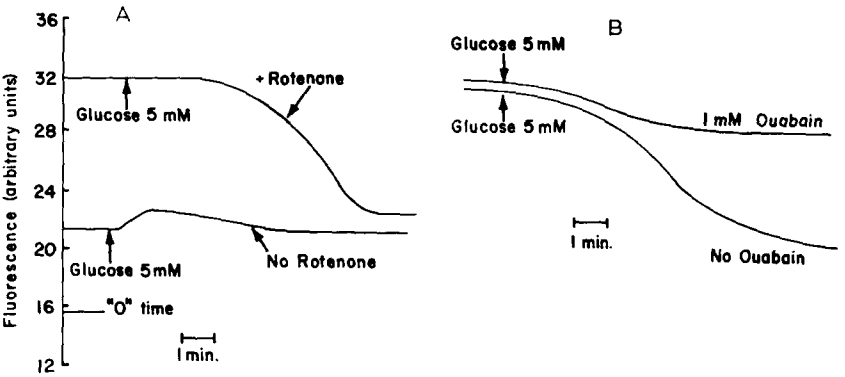


Fig. 4. (A), Levels of fluorescent intensity of dye with time in 0.3 % suspensions of cells in Na^+-Ringer. Line marked "0" time indicates level of fluorescent intensity 4–5 min after dilution of cells to 0.3 % and addition of dye. In line labelled No Rotenone, cells were diluted to 0.3 %, incubated at 37 °C for 30 min prior to the addition of dye. In line marked + Rotenone, the cells were treated the same as the control except that the incubation medium contained 32 ng/ml rotenone. Glucose was added where indicated. (B), A 0.3 % suspension of cells in Na^+-Ringer+rotenone (32 ng/ml suspension) was incubated at 37 °C for 30 min. 3.2 ml aliquots were removed, centrifuged and resuspended in Na^+-Ringer+rotenone+dye with and without ouabain (final concentration 1 mM) for 5 min. Glucose (final concentration 5 mM) was added where indicated.

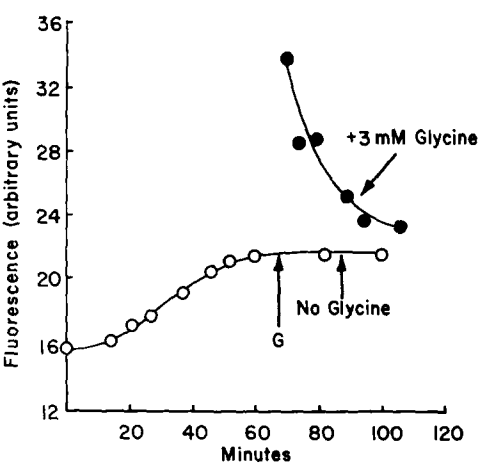


Fig. 5. Constant level of fluorescent intensity of dye with time for 0.3 % suspensions of cells in Na^+-Ringer incubated at room temperature. At 0 min, cells were diluted to 0.3 % and at the times indicated above, dye was added to 3.2 ml aliquots withdrawn from the cell suspension (○-○). The constant levels of fluorescent intensity are presented above. At time marked G, glycine was added (final concentration equals 3 mM) to a portion of the incubation mixture and the sampling was continued (●-●).

TABLE I

MEMBRANE POTENTIALS CALCULATED FROM NERNST EQUATION

$E_M = \frac{RT}{F} \ln \frac{[K^+]_o}{[K^+]_i}$ at "null point" or from $E_M = \frac{RT}{F} \ln \frac{[Cl^-]_i}{[Cl^-]_o}$.

membrane potential (mV)	Number of cell samples*	
	Based on K^+ ratio	Based on Cl^- ratio
below −13	0	0
−13 to −17	0	6
−18 to −24	7	11
−25 to −30	1	2
−31 to −36	6	10
−37 to −42	2	0
above −42	0	0

* Each cell sample recorded represents an estimation performed on cells from a different mouse.

is relatively low and the potential difference indicated by "null point" analysis is in the range of −50 to −60 mV. If, on the other hand, the cells were diluted as they would be in the cuvette (method 2 of handling cells) and incubated at room temperature or at 37 °C for various periods of time before adding dye, then with incubation the final levels of fluorescence were seen to rise (Figs. 4 and 5). To find the "null point" for cells thus diluted and incubated, aliquots of the cell suspension were centrifuged, the supernatant fluids removed, replaced with various mixtures of K^+-Ringer and Na^+-Ringer, and dye added. The constant level of fluorescent intensity obtained was the same as that seen before centrifugation and was not altered by changes in external K^+/Na^+ ratios. As before valinomycin was added to determine the Na^+-Ringer/K^+-Ringer combination which described the "null point". According to the null point method the membrane potentials of the diluted cells were in the range of −18 to −42 mV (Table I). The reason for the difference observed between diluted and undiluted cells is unknown. The possibility that dilution and incubation resulted in the accumulation in the medium of substances capable of increasing fluorescent intensity was eliminated for two reasons. First, if the medium in which cells were incubated was replaced by fresh medium, there was no change in the intensity (it remained elevated). Second, if undiluted cells were suspended in medium in which cells had been incubated, the fluorescent intensity was identical to that of undiluted cells in fresh medium.

Dye fluorescence and Cl^- distribution as measures of the membrane potential

On the whole, measurements of the membrane potential using dye fluorescence are somewhat higher than those calculated from the Cl^- distribution as illustrated in Table I. Nonetheless 13 of the 16 values of the membrane potential calculated for diluted cells fall well within the range estimated from $^{36}Cl^-$ distribution ratios.

Metabolic aspects

Cell suspensions incubated with rotenone (16 or 32 ng/ml) attained higher

levels of fluorescent intensity after incubation for 30 min at 37 °C than did cells incubated without rotenone (Fig. 4). Again it was demonstrated that substances capable of altering the fluorescent intensity of the dye were not accumulating in the medium and hence it was concluded that the cells were depolarized to a greater extent than were the controls. The addition of D-glucose but not the non-metabolizable sugars, L-glucose or D-ribose (final concentration equals 5 mM), led after some delay (1–4 min) to a decrease in fluorescent intensity which reached a low point in approximately 5 min. This decrease was not observed in the presence of 10 mM NaF. The fluorescent intensity of control cell suspensions generally showed a small transient rise upon the addition of D-glucose.

The increase in fluorescent intensity with rotenone and the decrease on addition of D-glucose (which is rapidly glycolyzed) suggest that the membrane potential is affected by metabolic activity. One possible reason for such dependence on metabolic activity would be the presence of a $(Na^+ + K^+)$-ATPase whose operation is electrogenic under these conditions. To test this possibility the effect of 1 mM ouabain on the response to glucose was examined. The data in Fig. 4 show that there was a 47 % change in fluorescent intensity on the addition of glucose to rotenone treated cells in the absence of 1 mM ouabain as compared to a 14 % change in its presence. The influence of ouabain on rotenone treated cells was seen more directly by the addition of solid ouabain to bring its final concentration to 1 mM in the cuvette. This addition led to a reversal (70–100 % effective) on the effects of glucose within 3–4 min. No change in fluorescent intensity was seen upon the addition of ouabain to rotenone-treated cells in the absence of glucose or to control cells.

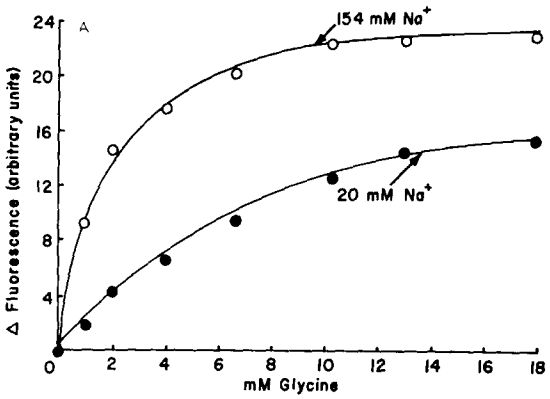
The decrease in fluorescent intensity upon the addition of glucose to cells treated with rotenone was also influenced by cellular cationic composition. The addition of glucose led to a 30 % decrease in fluorescent intensity if cells were pre-incubated with rotenone in Na^+-Ringer (cell Na^+ content approximately 40 mmol/L cell H_2O) and to a decrease of 4 % or less if cells were pre-incubated with rotenone in K^+-Ringer (cell Na^+ content less than 20 mmol/L cell H_2O).

TABLE II

THE CHANGE IN FLUORESCENT INTENSITY OF DYE IN 0.3 % CELL SUSPENSIONS (Na^+-RINGER) UPON THE ADDITION OF VARIOUS AMINO ACIDS

(Final concentrations either 2 or 12 mM). AIB, α-aminoisobutyric acid.

Amino acid	ΔF (arbitrary units)	
	2 mM	12 mM
Glycine	10.8	19
Alanine	18	20.3
AIB	12.5	18
Arginine	2.0	3.6
Threonine	7.1	15.9
Proline	19.1	24.3
Glutamic acid	1.3	7.2
Methionine	13.8	17.9
N,N-Dimethylglycine	0	< 1
ε-Amino-*n*-caproic acid	0	< 1



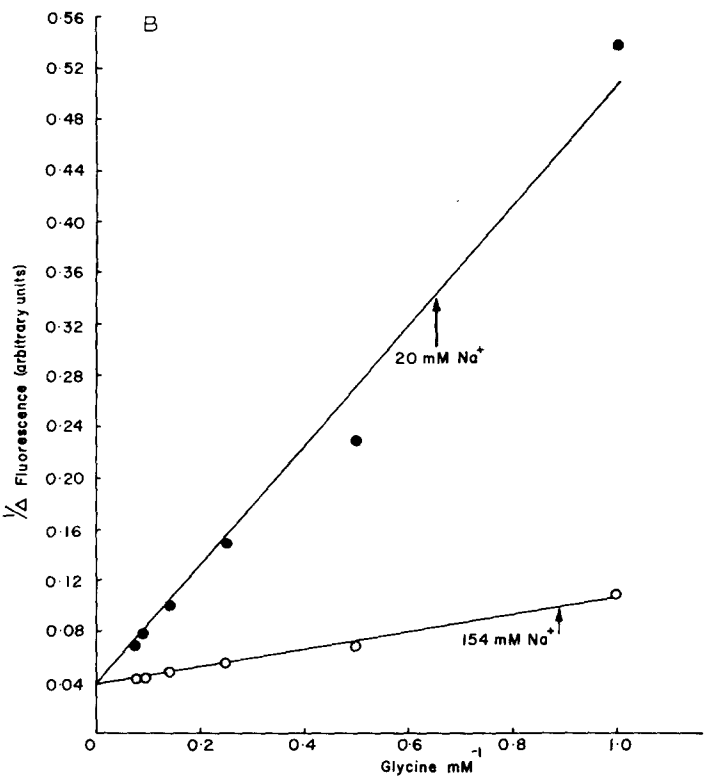


Fig. 6. (a) The maximum change in fluorescent intensity of dye upon the addition of glycine to 0.3 % suspensions of cells in media containing either 154 mM NaCl, 6 mM KCl, 1.5 mM $MgSO_4$ and 10 mM Tris/MOPS buffer at pH 7.4 (○–○) or 20 mM NaCl, 140 mM KCl, 1.5 mM $MgSO_4$ and 10 mM Tris/MOPS buffer at pH 7.4 (●–●). The cells used in this experiment were washed and resuspended in a medium containing 154 mM choline Cl, 6 mM KCl, 1.5 mM $MgSO_4$ and 10 mM Tris/MOPS at pH 7.4. (b) Double reciprocal plot of the data shown in (a).

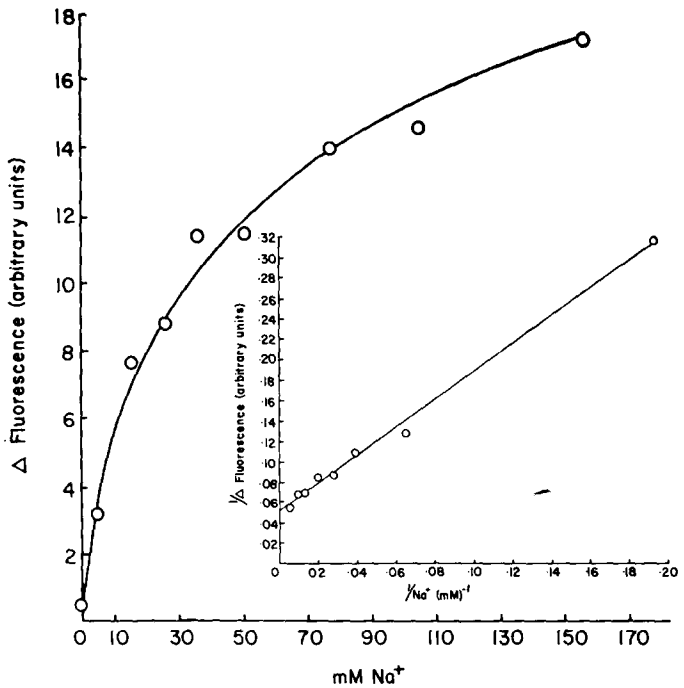


Fig. 7. The change in fluorescent intensity of dye upon the addition of glycine (final concentration 7.3 mM) to 0.3 % suspensions of cells in mixture of a solution containing 154 mM NaCl, 6 mM KCl, 1.5 mM $MgSO_4$ and 10 mM Tris/MOPS and another containing 160 mM KCl, 1.5 mM $MgSO_4$ and 10 mM Tris/MOPS, both at pH 7.4, to give the Na^+ concentrations indicated on the abscissa. Insert, double reciprocal plots of data.

Addition of amino acids

It has been established that the transport of some amino acids in these cells is dependent on Na^+ and that Na^+ uptake is increased in the presence of these amino acids [16]. Such a phenomenon could lead to a decrease in the membrane potential. The data in Table II show that this prediction is correct. Amino acids known to be transported by Na^+-dependent routes, gave rise to an increase in fluorescence in a Na^+ but not a choline$^+$ or K^+ medium. Solutes not dependent on Na^+ such as *N,N*-dimethylglycine and ε-amino-*n*-caproic acid were without effect. In a rough way, the percentage change in fluorescence was proportionate to the extent of transport of the particular amino acid.

The increase in fluorescence caused by an amino acid is transient (Fig. 5) and dependent on the Na^+ concentration in the medium (Fig. 6a) being smaller with 20 mM Na^+ than 154 mM Na^+ and almost absent with no external Na^+. Plots of 1/change in fluorescent intensity vs. 1/glycine concentration are linear (Fig. 6b) and indicate that Na^+ influences the apparent K_m of glycine but not the maximal change in fluorescent intensity.

The influence of Na^+ on the change in fluorescence with glycine also approached saturation with higher levels of Na^+ (Fig. 7). A plot of 1/change in fluorescent intensity vs. 1/Na^+ concentration was linear with an apparent K_m constant for Na^+ equal to 29 mM (Fig. 7 insert).

DISCUSSION

The evidence presented above demonstrates that the fluorescent intensity of diS-C_3-(5) is dependent upon the membrane potential in the Ehrlich ascites cell and that the relationship is similar to that reported for a number of different preparations [13, 17, 18]. In all of these studies and with the ascites cell, a decrease in intensity was observed whenever the potential was expected to become more negative internally whereas an increase was seen as it became more positive. Hence it appeared that the dye diS-C_3-(5) could be used to determine the membrane potential in ascites cells and to monitor rapid changes in this potential difference.

The membrane potential determined for these cells varied consistently with conditions of incubation. When cells were incubated at a dilution of 0.3 % in Na^+-Ringer, the value of the potential (calculated from the null point) decreased from a range of −50 to −60 mV seen initially to the range of −18 to −42 mV estimated for cells after 30 min incubation at 37 °C. The potential then remained in this lower range for some time (1–2 h). Values for the membrane potential calculated from the Nernst equation, assuming that chloride is in equilibrium, also fall in this lower range as do the direct electrophysiological measurements made by Lassen et al. [5] and Johnstone [6]. The reason for the apparent shift in membrane potential with dilution and incubation remains unknown. In any event, our values for membrane potential are more negative than those obtained by a number of other investigators who have employed electrophysiological techniques (approximately −11 mV). The possibility that the potentials recorded by these investigators represent junction potentials between damaged cells and their environments has been suggested by Lassen et al. [5].

There was a rapid increase in fluorescent intensity, indicating depolarization, upon the addition of certain amino acids. This depolarization required the presence of Na^+ and was specific for amino acids which exhibit Na^+-dependent active transport. Quantitative agreement was observed between the apparent K_m for glycine, 2.5 mM, and Na^+, 29 mM, measured here and the apparent K_m for glycine, 1.5 mM, and Na^+, 31 mM, measured in transport studies [12]. These observations indicate that entrance of Na^+ coupled to the entrance of amino acids is electrogenic. Similar observations have been obtained with electrophysiological techniques for Na^+-dependent transport of organic solutes in other cells (viz., rabbit ileum-epithelium [19], bullfrog small intestine epithelium [20], and newt kidney proximal tubule cells [21]).

Previous studies have indicated that active $Na^+ + K^+$ fluxes require an input of metabolic energy supplied by ATP or by some high energy intermediate of oxidative phosphorylation [22, 23]. Observations reported here demonstrate that membrane potential is also dependent on membrane energy. Inhibition of electron transport by rotenone resulted in a depletion of cellular ATP [24] and a depolarization of the membrane. When glucose was added and the ATP level restored, a substantial hyperpolarization was observed provided that the cellular Na^+/K^+ ratio was increased. This hyperpolarization was inhibited by ouabain suggesting that the activity of the $Na^+ + K^+$ dependent ATPase was involved in the hyperpolarization.

Since ouabain had no effect on the potential difference in control cells (not treated with rotenone), these data indicate that in the control cells the activity of the Na^+ pump does not directly influence membrane potential. When the cellular Na^+/

K^+ ratio is altered, however, the pump activity is increased and/or the stoichiometry of the pump is changed such that it now has a marked influence on the potential difference. An increase in Na^+ pump rate in response to an increase in Na^+/K^+ ratio has been reported for other cells [25–27] and the stoichiometry of the Na^+ pump in ascites cells has been reported to be variable [28, 29]. It is interesting to note that the membrane potential in control cells is not influenced by the addition of ouabain even though a depolarization is observed with an inhibition of electron transport and a fall in cellular ATP. There is the possibility that components of the oxidative phosphorylation system are part of the cell membrane [23] and that these components influence potential difference directly. Studies of the influence of electron transport and ATP on the membrane potential are currently in progress.

Mills and Tupper [30] have recently suggested that the membrane potential of these cells is determined primarily by the passive movements of Na^+ and K^+. Their argument is based on the agreement between a potential (−18 mV) calculated from their estimates of the permeability constants for K^+ and Na^+ using the constant field equation and the potential calculated from the Cl^- ratio (−21 mV). This suggestion is at variance with our work since in our experiment the substitution of K^+ or choline$^+$ for Na^+ in the medium had little or no influence on the fluorescent intensity of celll suspensions. If the potential difference was the result of the passive movements of ions, removal or substitution of these ions would markedly influence the potential difference. The finding that external K^+ does not effect internal K^+ over a large range of concentrations [31] is consistent with our observations.

Most recently, Smith and Levinson [4] have measured membrane potential differences in Ehrlich ascites tumor cells by microelectrode impalement. Their values for control (untreated) cells (−12 mV, inside negative) differ from ours. In fact, in their experiments valinomycin had no effect on this value. The same authors have reported previously that exposure of the cells to lanthanum (La^{3+}) affected their permeability properties [32, 33]. As part of their methodology they use La^{3+} (20 μM) to immobilize the cells thus facilitating easy impalement. Smith and Levinson [4] argue that since application of these same methods to Amphiuma erythrocytes yields measurements of potential differences compatible with other procedures, these methods must also be valid for the Ehrlich ascites tumor cell. We suggest that this might not be the case.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to Thomas Mickel and Janet Larsen for their excellent technical assistance. This work was sponsored by P. H. S. Grant HL-16417 to P. C. Laris and Medical Research Council of Canada Grant MA 1984 to R. M. Johnstone.

REFERENCES

1 Hempling, H. G. (1962) J. Cell. Comp. Physiol. 60, 181–198
2 Aull, F. (1967) J. Cell. Physiol. 69, 21–32
3 Bernhardt, J. and Pauly, H. (1967) Biophysik 4, 101–112
4 Smith, T. C. and Levinson, C. (1975) J. Membrane Biol. 23, 349–366

5 Lassen, U. V., Nielsen, A-M. T., Pape, L. and Simonsen, L. O. (1971) J. Membrane Biol. 6, 269–288
6 Johnstone, B. M. (1959) Nature 183, 411
7 Simonsen, L. O. and Nielsen, A. (1971) Biochim. Biophys. Acta 241, 522–527
8 Pietrzyk, C. and Heinz, E. (1974) Biochim. Biophys. Acta 352, 397–411
9 Cespedes, C. D. and Christensen, H. N. (1974) Biochim. Biophys. Acta 339, 139–145
10 Laris, P. C., Pershadsingh, H. A. and Johnstone, R. M. (1975) Abstracts 29th Meeting of the Society of General Physiologists, Woods Hole, Mass., p. 14a
11 Johnstone, R. M. and Scholefield, P. G. (1961) J. Biol. Chem. 236, 1419–1424
12 Potashner, S. J. and Johnstone, R. M. (1971) Biochim. Biophys. Acta 233, 91–103
13 Hoffman, J. F. and Laris, P. C. (1974) J. Physiol. Lond. 239, 519–552
14 Brading, A. F. and Caldwell, P. C. (1971) J. Physiol. Lond. 217, 605–624
15 Chappell, J. B. and Crofts, A. R. (1966) in Biochim. Biophys. Acta Library, Regulation of Metabolic Processes in Mitochondria (Tager, J. M. et al. eds.) Vol. 7, pp. 293–314, Elsevier, Amsterdam
16 Christensen, H. N. (1970) in Membranes and Ion Transport, Linked Ion and Amino Acid Transport (Bittar, E. E., ed.) Vol. 1, pp. 365–394, Wiley-Interscience, New York
17 Laris, P. C. and Pershadsingh, H. A. (1974) Biochem. Biophys. Res. Commun. 57, 620–626
18 Laris, P. C., Bahr, D. P. and Chaffee, R. R. J. (1975) Biochim. Biophys. Acta 376, 415–425
19 Rose, R. C. and Schultz, S. G. (1971) J. Gen. Physiol. 57, 639–663
20 Maruyama, T. and Hoski, T. (1972) Biochim. Biophys. Acta 282, 214–225
21 White, J. F. and Armstrong, W. McD. (1971) Am. J. Physiol. 221, 194–201
22 Weinstein, S. W. and Hempling, H. G. (1964) Biochim. Biophys. Acta 79, 329–336
23 Levinson, C. and Hempling, H. G. (1967) Biochim. Biophys. Acta 135, 306–318
24 Johnstone, R. M. (1974). Biochim. Biophys. Acta 356, 319–330
25 Post, R. L. and Jolly, P. C. (1957). Biochim. Biophys. Acta 25, 118–128
26 Brinley, F. J. and Mullins, L. J. (1974) N.Y. Acad. Sci. Ann. 242, 406–432
27 Harris, E. J. (1965) J. Physiol. Lond. 177, 355–376
28 Hempling, H. G. (1958) J. Gen. Physiol. 41, 565–583
29 Hempling, H. G. (1972) in Transport and Accumulation in Biological Systems (Harris, E. J., ed.), 3rd edn., pp. 271–288, University Park Press, Baltimore
30 Mills, B. and Tupper, J. T. (1975) J. Membrane Biol. 20, 75–97
31 Hempling, H. G. (1962) J. Cell. Comp. Physiol. 60, 181–198
32 Smith, T. C., Mikiten, T. M. and Levinson, C. (1972) J. Cell. Physiol. 79, 117–126
33 Levinson, C., Mikiten, T. M. and Smith, T. C. (1972) J. Cell. Physiol. 79, 299–308