The Membrane Potential of Mouse Ascites-Tumour Cells Studied with the Fluorescent Probe 3,3'-Dipropylloxadibocyanine

AMPLITUDE OF THE DEPOLARIZATION CAUSED BY AMINO ACIDS

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1. The magnitude of the K^+ gradient across the plasma membrane, which was in equilibrium with the membrane potential (E) of the tumour cells, was determined by the 'null point' procedure of Hoffman & Laris (1974) [*J. Physiol. (London)* **239**, 519–552] in which the fluorescence of the dye serves as an indicator of changes in the magnitude of E . 2. A mixture of oligomycin, 2,4-dinitrophenol and antimycin was used to stop the mitochondria from interfering with the fluorescence signal. Transport functions at the plasmalemma were maintained under these conditions in the presence of glucose. 3. Physiological circumstances were found in which incubation with glycine or with glucose changed the 'null point' value of E within the range -20 mV to -100 mV. The fluorescence intensity at the 'null point' was an approximately linear function of E over that range. The procedure enabled E to be inferred from the fluorescence intensity in circumstances where titration to the 'null point' was not feasible. 4. The rapid depolarization caused by L-methionine or glycine was shown in this way to have a maximum amplitude of about 60 mV. A mathematical model of this process was devised. 5. The electrogenic Na^+ pump hyperpolarized the cells up to about -80 mV when the cellular and extracellular concentrations of K^+ were roughly equal. 6. The observations show that the factors generating the membrane potential represent a major source of energy available for the transport of amino acids in this system.

Work with vertebrate tissues, with derived preparations of plasmalemma vesicles and with isolated cells (such as those of the mouse ascites tumour used in the present investigation) has provided qualitative support for the view that the electrochemical gradient of Na^+ acting across the plasmalemma serves as the energy source driving the accumulation of the amino acids and carbohydrates that these systems concentrate (Eddy, 1977; Crane, 1977). The feasibility of the Na^+ -gradient hypothesis is thus not in doubt. However, lack of information about the quantitative relations between the Na^+ gradient ($\Delta\mu^{Na}$) and the amino acid or carbohydrate gradient, in various physiological circumstances, has hindered acceptance of the hypothesis (Blaustein & King, 1976; Christensen, 1977; Schafer, 1977; Lever, 1977; Smith & Adams, 1977).

The magnitude of the Na^+ gradient is given by:

$$\Delta\mu^{Na} = RT \ln ([\text{extracellular } Na^+]/[\text{intracellular } Na^+]) - EF \quad (1)$$

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

where E is the membrane potential and RT and F have their conventional significance. The ratio $[\text{extracellular } Na^+]/[\text{intracellular } Na^+] = [Na_o]/[Na_i]$ is known approximately and in the present investigation we have developed a procedure (Hoffman & Laris, 1974; Philo & Eddy, 1975; Eddy *et al.*, 1977) for using the fluorescent probe 3,3'-dipropylloxadibocyanine iodide [1-(3-propylbenzoxazolidin-2-ylidene)-5-(3-propylbenzoxazol-2-ylonium)penta-1,3-dien-5-ylidene iodide] to assay the magnitude of E in preparations of the mouse ascites-tumour cells. Work with two other carbocyanine dyes had a similar objective (Laris *et al.*, 1976; Burckhardt, 1977). The dyes carry a delocalized positive charge, they penetrate lipid membranes readily and are concentrated within the lipid boundary in response to the membrane potential. This process is amplified by the subsequent binding of the dye on the membrane itself or on cellular proteins (Sims *et al.*, 1974; Hladky & Rink, 1976; Kimmich *et al.*, 1977).

We studied the fluorescence of the cell suspensions containing the dye under various conditions in which the glycine or methionine gradients formed in the presence of selected concentrations of Na^+ and K^+ have been assayed in a number of laboratories

(Schafer & Heinz, 1971; Johnstone, 1972; Reid *et al.*, 1974; Schafer, 1977). Gibb & Eddy (1972) suggested that the membrane potential of the mouse ascites tumour could become at least 40 mV more hyperpolarized than is indicated by the typical values of up to -24 mV that have been determined by impaling the cells with microelectrodes (Lassen *et al.*, 1971; Smith & Levinson, 1975). The observations described below support this supposition by showing that the membrane potential assayed with the fluorescent probe varies from about zero to about -100 mV in various circumstances. This is apparently the result of changes in the activity of the electrogenic Na⁺ pump that are superimposed on a large potential generated by the passive diffusion of Na⁺, K⁺ and Cl⁻ across the plasmalemma. The absorption of the amino acid itself is shown markedly to lower the membrane potential.

Materials and Methods

Cell preparations

Mouse ascites-tumour-cell suspensions were prepared as described by Morville *et al.* (1973). Thus, after collection and washing, the tumour cells were incubated for 30 min at 37°C in the standard Ringer solution. The cells were then collected in the bottom of a graduated centrifuge tube of 10 ml capacity by centrifugation at about 1500g for 1 min. The volume of packed cells (1–2 ml) was noted. Tumour cells that were to be loaded with Na⁺ were subsequently incubated for a further 30 min at 37°C in an Na⁺/Ringer solution from which K⁺ was omitted.

Solutions

The standard Ringer solution contained 155 mM-Na⁺, 8 mM-K⁺, 131 mM-Cl⁻, 16 mM-orthophosphate and 1.2 mM-MgSO₄. Na⁺/Ringer solution was of similar composition, except that it contained 163 mM-Na⁺ and no K⁺. K⁺/Ringer solution contained 163 mM-K⁺ and no Na⁺. Some preliminary work was carried out in a solution containing 300 mM-sucrose and 10 mM-Hepes, adjusted to pH 7.4 with Tris base.

Fluorimetry

The fluorescence of solutions of 3,3'-dipropylloxadicarbocyanine iodide was measured in an EEL-Corning 244 fluorimeter (Corning-Medical, Halstead, Essex CO9 2DX, U.K.). Fluorescence was excited with a mercury lamp, the 577 nm and 579 nm lines being selected with an Ilford spectrum yellow filter (no. 606). Emitted light was passed through an Ilford spec-

trum orange filter (no. 607) to the fluorimeter continuous wedge filter, which was set to 620 nm (13 nm half-bandwidth). The stirred cuvette was thermostatically maintained at 37°C. Assays were carried out with cell suspensions at 1.3 mg dry wt. in 4 ml to which 4 µg of dye in 40 µl of ethanol had been added. To standardize the fluorescence scale before each series of experiments, the instrument reading at 620 nm was adjusted to 100% (instrument sensitivity setting at 7) when the cuvette contained 2.5 µg of the dye dissolved in 4 ml of ethanol.

K⁺-titration 'null point' experiments

The intracellular K⁺ concentrations for the valinomycin K⁺-titration experiments were determined as follows. Packed cells (1 vol.) were suspended in the standard Ringer solution (5 vol.) and the suspension was split into 1 ml samples and stored on ice. Each 1 ml sample, containing approx. 26 mg dry wt. of the tumour cells, was added to 80 ml of Ringer solution of the required K⁺ concentration ([Na⁺] + [K⁺] was 163 mM in all cases). The solution also contained 60 µM-2,4-dinitrophenol, 6 ng of oligomycin/ml, 1 µg of antimycin/ml and glucose (2 mg/ml) as required. It was then incubated for an appropriate interval (10 or 30 min) at 37°C. A portion (80 µg) of the dye in 40 µl of ethanol was next added and 4 ml of the mixed suspension placed in the fluorimeter cuvette. About 70 ml of the remaining suspension was centrifuged at about 1000g for 1 min and the pellet resuspended in the remaining 6 ml. This suspension was added to a weighed tube containing 1 µCi of ³H-labelled inulin and the cellular K⁺ concentration determined as described by Morville *et al.* (1973). The addition of the valinomycin (10 µg) to the cell suspension in the fluorimeter coincided with the end of the first centrifugation step of the K⁺ assay. This was carried out 4–5 min after the addition of the dye.

Cell respiration

The tumour cells were prepared in the Na⁺/Ringer solution and stored at a density of 1 ml of packed cells/2 ml of suspension at 0°C for up to 2 h before use. Assays were carried out in a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambs., U.K.) at 37°C by addition of 0.2 ml of the stock cell suspension to 4 ml of the Na⁺/Ringer solution to which 5 mM-KCl had been added.

Chemicals

3,3'-Dipropylloxadicarbocyanine iodide was prepared as described by Sims *et al.* (1974), except that

the initial crystallization by addition of hot KI solution did not work in our hands. Instead the pyridine was removed from the reaction mixture by repeatedly adding and distilling off ethanol. The dye was crystallized from the resulting ethanolic solution and recrystallized from ethanol. Oligomycin was obtained from Sigma Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and valinomycin were obtained from Boehringer Corp. (London), Lewes, East Sussex BN7 1LG, U.K. ^3H -labelled inulin and [^{14}C]methionine were obtained from The Radiochemical Centre, Amersham, Bucks. HP7 0YB, U.K.

Mathematical Methods

Mathematical model of the Na^+ -dependent amino acid-transport system

(1) *Flux through the transport system.* Consider the scheme (Morville *et al.*, 1973):

where the subscripts o and i refer to the extracellular and intracellular media respectively. Let k_{+2} , k_{-2} (eqn. 3) and k_{+4} , k_{-4} (eqn. 5) be small compared with the other rate constants. Further, let the system be symmetrical so that $k_{+4} = k_{-4} = k_4$, $k_{+1} = k_{-3}$, $k_{-1} = k_{+3}$ (eqns. 2 and 4) and let $K_A = 2k_{-1}/k_{+1}$ and the total carrier concentration be $[\text{C}]_t$. The net rate of amino acid uptake is given by:

$$v = \frac{(k_{+2}[\text{Na}^+]_o[\text{A}]_o - k_{-2}[\text{Na}^+]_i[\text{A}]_i) [\text{C}]_t}{[\text{Na}^+]_o[\text{A}]_o \left(1 + \frac{k_{+2}}{k_4}\right) + [\text{Na}^+]_i[\text{A}]_i \left(1 + \frac{k_{-2}}{k_4}\right) + \frac{2[\text{Na}^+]_o[\text{A}]_o [\text{Na}^+]_i[\text{A}]_i (k_{+2} + k_{-2})}{K_A} + K_A} \quad (6)$$

for an electroneutral carrier system.

However, we shall suppose that the loaded form of the carrier bears a net charge of +1, whereas the unloaded form bears no net charge. Eqn. 6 then has the form (Morville *et al.*, 1973):

$$v = V_m \left[\frac{-[\text{Na}^+]_o[\text{A}]_o EF}{RT(1 - e^{EF/RT})} + \frac{[\text{Na}^+]_i[\text{A}]_i EF}{RT(e^{-EF/RT} - 1)} \right] / D \quad (7)$$

$$\begin{aligned} \text{where } D = & [\text{Na}^+]_o[\text{A}]_o \left(1 - \frac{k_2}{k_4} \frac{EF}{RT(1 - e^{EF/RT})}\right) \\ & + [\text{Na}^+]_i[\text{A}]_i \left(1 - \frac{k_2}{k_4} \frac{EF}{RT(e^{-EF/RT} - 1)}\right) \\ & - [\text{Na}^+]_o[\text{A}]_o[\text{Na}^+]_i[\text{A}]_i \frac{2}{K_A} \frac{k_2}{k_4} \left(\frac{EF(1 + e^{EF/RT})}{RT(1 - e^{EF/RT})}\right) \\ & + K_A \end{aligned}$$

In eqn. (7), E is the membrane potential, R is the gas constant, F the Faraday, T the temperature, also $k_{+2} = k_{-2} = k_2$ when $E = 0$ and $V_m = k_2/[\text{C}]_t$. Eqn. (7) is based on the constant-field assumption (Goldman, 1943).

Now there is also (Morville *et al.*, 1973) a passive amino acid flux:

$$L = P_A([\text{A}]_o - [\text{A}]_i) \quad (8)$$

and in the steady state:

$$L + v = 0 \quad (9)$$

Hence the steady-state value of $[\text{A}]_i$ can be calculated by substituting eqns. (7) and (8) into eqn. (9), provided that the values of $[\text{Na}^+]_o$, $[\text{A}]_o$, $[\text{Na}^+]_i$, E , V_m , K_A and P_A are known. We assumed for this purpose that $k_2 = k_4$.

(2) *Membrane potential in the presence of amino acid.* The passive fluxes of K^+ ($\bar{m}_K$), Na^+ ($\bar{m}_{\text{Na}}$) and Cl^- ($\bar{m}_{\text{Cl}}$) and the Na^+ flux ($\bar{i}_{\text{Na}}$) through the amino acid pump are related, in the absence of other pumped fluxes, by the equation:

$$\bar{m}_{\text{Na}} + \bar{m}_K + \bar{i}_{\text{Na}} - \bar{m}_{\text{Cl}} = 0 \quad (10)$$

$\bar{i}_{\text{Na}}$ is given by eqn. (7) above and on the constant-field assumption:

$$\bar{m}_i = - \frac{P_i ([\text{I}]_o - [\text{I}]_i) e^{nEF/RT} nEF}{RT(1 - e^{nEF/RT})} \quad (11)$$

where P_i is the permeability coefficient of the ion I and n is the charge on the ion.

Substitution of eqns. (7) and (11) into eqn. (10) gives:

$$E = \frac{RT}{F} \ln \left(\frac{P_{\text{Na}}[\text{Na}^+]_o + P_{\text{K}}[\text{K}^+]_o + \frac{P_{\text{Cl}}[\text{Cl}^-]_i + V_m[\text{Na}^+]_o[\text{A}]_o/D}{P_{\text{Na}}[\text{Na}^+]_i + P_{\text{K}}[\text{K}^+]_i + \frac{P_{\text{Cl}}[\text{Cl}^-]_o + V_m[\text{Na}^+]_i[\text{A}]_i/D}}{P_{\text{Cl}}[\text{Cl}^-]_o + V_m[\text{Na}^+]_i[\text{A}]_i/D}} \right) \quad (12)$$

Numerical solutions to eqn. (12) were obtained by using the routine COSAAF. Given the values of $[\text{Na}^+]_o$, $[\text{K}^+]_o$, $[\text{Cl}^-]_o$, $[\text{A}]_o$, $[\text{Na}^+]_i$, $[\text{K}^+]_i$, $[\text{Cl}^-]_i$, P_A ,

V_m , P_{Na} , P_{Cl} and P_K and that $k_2/k_4 = 1$, eqns. (9) and (12) can be solved to obtain the corresponding steady-state values of A_1 and E . Again this was carried out with the routine CO5AAF.

(3) *Amplitude of depolarization caused by the amino acid.* The amino acid lowers the membrane potential from E_1 to E_2 where:

$$E_1 = E_2 - \frac{RT}{F} \ln \left(1 + \alpha \left[\frac{K_A}{[Na^+]_o[A]_o} + 1 - \frac{E_2 F}{RT(1 - e^{E_2 F/RT})} \right]^{-1} \right) \quad (13)$$

and $\alpha = V_m (P_{Na} [Na^+]_o + P_K [K^+]_o + P_{Cl} [Cl^-]_i)^{-1}$. We have supposed for this purpose that $k_2/k_4 = 1$.

Polynomial fitting

The routine EO2ABF was used where polynomials were to be fitted through sets of points (Nottingham Algorithms Group Subroutine Library, NAG Central Office, 13 Banbury Road, Oxford OX2 6NN, U.K.). Routine CO5AAF was also from this source.

Results

Role of the mitochondria in the fluorescence signal

Cyanine dyes are known to be taken up by isolated mitochondria, probably in response to the mitochondrial membrane potential (Laris *et al.*, 1975). This possibility complicates the interpretation of the fluorescence changes observed in intact cell preparations (Philo & Eddy, 1975; Eddy *et al.*, 1977; Burckhardt, 1977). We therefore determined both (1) the minimum concentration of oligomycin and antimycin required for maximum inhibition of respiration of the intact tumour cells, and (2) the minimum concentration of 2,4-dinitrophenol required for maximum stimulation of respiration after oligomycin inhibition (Table 1). We suggest that the presence of these concentrations of oligomycin, antimycin and 2,4-dinitrophenol is likely to prevent a mitochondrial membrane potential being generated by either the adenosine triphosphatase or by respiration. The fluorescence assays were therefore carried out in the presence of 6 ng of oligomycin, 1 μ g of antimycin and 60 nmol of 2,4-dinitrophenol per ml of cell suspension containing 0.32 mg dry wt. of the tumour cells and 1 μ g of the dye.

Toxicity of the dye. The presence of the amount of dye stipulated above, together with the mitochondrial inhibitors, had no significant effect on the amount of methionine the tumour cells accumulated during 30 min at 37°C from the standard Ringer solution containing glucose. The Na^+ pump was not inhibited, as was shown by the maintenance of $[Na^+]_i$ and

Table 1. *Effect of graded amounts of antimycin, oligomycin and 2,4-dinitrophenol on the endogenous respiration rate*

The tumour cells were suspended in the standard Na^+ /Ringer solution (50 mg dry wt. of cells/ml) and kept at 0°C for up to 2 h before use. For assay, 0.2 ml of this suspension was added to 3.8 ml of Ringer solution at 37°C containing 163 mM- Na^+ , 5 mM- K^+ and 4 mg of glucose. The metabolic inhibitors were added (10 μ l) as solutions in ethanol, except for 2,4-dinitrophenol, which was dissolved in water.

	Successive additions	Rate of O_2 uptake (nmol/min per mg dry wt.)
Expt. 1	Initial	2.6
	Oligomycin (10 ng)	2.6
	Oligomycin (10 ng)	2.6
	Oligomycin (10 ng)	1.8
	Oligomycin (10 ng)	1.0
	Oligomycin (10 ng)	0.72
	Oligomycin (10 ng)	0.72
Expt. 2	Initial	1.6
	Oligomycin (2 μ g)	0.8
	2,4-Dinitrophenol (50 nmol)	1.8
	2,4-Dinitrophenol (50 nmol)	1.8
	Carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone (500 pmol)	1.8
Expt. 3	Initial	3.0
	Antimycin (5 μ g)	0.4
	Antimycin (5 μ g)	0.4

$[K^+]_i$ at about 50 mM and 150 mM respectively, values like those observed in controls without the metabolic inhibitors. Raising the dye concentration 3-fold lowered the ratio [cellular methionine]/[extracellular methionine] by about 30%. Thus the preferred dye concentration appears to be just tolerated by the system provided glucose was added to maintain a supply of ATP. No evidence was obtained in the present investigation that this selected concentration of cyanine dye altered the permeability characteristics of the tumour cells towards methionine, Na^+ or K^+ , although such effects have been observed in human erythrocytes (Hoffman & Laris, 1974; Simons, 1976).

Relation between fluorescence and membrane potential (E). In the valinomycin 'null point' method for estimating membrane potential with cyanine dyes (Sims *et al.*, 1974; Hoffman & Laris, 1974) a concentration of K^+ in the Ringer solution is found such that the addition of valinomycin causes no change in fluorescence. It is assumed that under these conditions the cellular concentration of K^+ ($[K^+]_i$) and the extracellular concentration of K^+ ($[K^+]_o$) are in

equilibrium with the membrane potential. Thus $E = RT/F \ln ([K^+]_o/[K^+]_i)$. The method is valid only if valinomycin increases the permeability of the cell membrane to K^+ ions without affecting other parameters governing the membrane potential. The procedure outlined below is based on the same assumption.

In one such 'null point' assay (Fig. 1), the tumour cells were incubated for 10 min with the metabolic inhibitors in various Ringer solutions containing $[K^+]$ in the range 1–150 mM, and the dye was then added. The addition of valinomycin caused a graded change in the fluorescence of the various cell suspensions with a 'null point' corresponding to some intermediate value of $[K^+]_o$ (Fig. 1a). Fig. 1(b) shows how the fluorescence observed before and after the addition of valinomycin varied with $\log ([K^+]_o/[K^+]_i)$. The two solid lines were computed by fitting quadratic functions through the points. The intersection of these lines was taken to be the interpolated 'null point'. Paradoxically, the use of this procedure does not imply that K^+ ions were in reality strictly in equilibrium with the membrane potential at the 'null point' for an extended period of time, for this would only be the case if their absorption through the Na^+ pump had ceased. The validity of the method thus appears to depend on both the rapidity of the dye response and on the ability of valinomycin rapidly to increase the K^+ permeability to a marked extent.

A limitation of the above procedure is that the membrane potential is eventually determined only under the ionic conditions at the 'null point', the dye fluorescence serving, as it were, merely as a titration indicator. To circumvent this limitation Eddy *et al.* (1977) suggested that a calibration curve might be constructed showing the relation between the fluorescence of the cell suspension at the 'null point' and the membrane potential (E) when both parameters varied with the physiological circumstances. This raised the question whether there was a unique relation between the fluorescence and the magnitude of E , or whether the effect of the membrane potential on the dye distribution would be obscured by variations in the binding of the dye to the plasmalemma under the different incubation conditions (Kimmich *et al.*, 1977). The choice of conditions was based on qualitative evidence that the presence of an amino acid depolarizes the plasmalemma, whereas the operation of the Na^+ pump tends to hyperpolarize it (Philo & Eddy, 1975; Heinz *et al.*, 1975; Laris *et al.*, 1976). A series of 31 assays like those illustrated in Fig. 1 was therefore carried out with cell preparations in which the magnitude of E was deliberately varied by incubating the tumour cells with methionine or glycine and glucose, for appropriate time intervals, as indicated in the legend to Fig. 2.

Fig. 2 shows that the fluorescence at the 'null point',

Fig. 1. Observations made in the course of a single 'null point' titration designed to determine the intensity of fluorescence at the K^+ equilibrium potential for that particular cell preparation

(a) Ten portions of a given preparation of the tumour cells were incubated for 10 min without glucose in Ringer solutions containing the mitochondrial inhibitors. $[Na^+] + [K^+]$ was 163 mM and $[K^+]$ was: A, 1.3 mM; B, 1.8 mM; C, 2.9 mM; D, 4.5 mM; E, 8.1 mM; F, 14 mM; G, 23 mM; H, 42 mM; I, 71 mM; J, 144 mM. The carbocyanine dye was then added and a portion (4 ml) of the cell suspension was put into the fluorimeter cuvette (see the Materials and Methods section). These traces show how the fluorescence intensity changed abruptly to a new value in each case within about 1 min after the addition of 10 μ g of valinomycin to the system. (b) Transformation of the observations shown in (a). The fluorescence intensity just before (●) and about 1 min after (○) the addition of valinomycin is shown as a function of $\log ([K^+]_o/[K^+]_i)$. The solid lines represent quadratic functions fitted to the observations.

assayed under the standard conditions, was roughly linearly related to the corresponding membrane potential. A regression analysis based on E as the dependent variable showed that the slope was 2.85 ± 0.17 , with an intercept of -183.9 ± 7.8 ($r = 0.951$; $P < 0.001$). Although such linearity clearly cannot be maintained over an indefinitely extended range of membrane potential, there are theoretical grounds for expecting approximate linearity over a restricted range of values of E

Fig. 2. Calibration curve showing the fluorescence intensity at the 'null point' as a function of the corresponding membrane potential in 31 cell preparations in which it was deliberately varied

Three series of assays were carried with cell preparations that initially contained at least 60 mM- Na^+ . Each assay involved a titration like that illustrated in Fig. 1 (see the text). In six such assays $[\text{Na}^+]$ was constant at 30 mM and choline chloride replaced KCl as $[\text{K}^+]$ was varied. The results fitted the same regression line as the other 25 assays. The solid lines depicted represent the regression of E on the fluorescence parameter together with the 95% confidence limits. In the first series $-E$ was varied from 22 to 45 mV. The Ringer solutions contained no glucose and the cell preparations were incubated for 10 min (four assays). Otherwise 1 mM-L-methionine was also present (three assays). In the second series $-E$ was varied from 33 to 52 mV. The cells were incubated with glucose for 30 min (three assays) and with 1 mM-methionine as well (three assays). In the third series $-E$ varied from 58 to 110 mV. The cells were incubated with glucose for 10 min (five assays), with 2.5 mM-glycine as well (seven assays), and with 1 mM-glycine (three assays) or with 1 mM-methionine as well (three assays).

(Burckhardt, 1977; Kimmich *et al.*, 1977). We conclude that there was a systematic relation between the fluorescence intensity assayed under the standard conditions and the magnitude of E . We have used this relation to estimate membrane potentials in conditions where the 'null point' method is inapplicable because K^+ ions are not in equilibrium with the membrane potential. For instance, when the tumour cells were incubated for 30 min in the standard Ringer solution containing glucose and 1 mM-methionine, the fluorescence intensity ($\pm$ S.E.M.) exhibited in the standard assay was 45.0 ± 0.6 units ($n = 3$). This corresponds to $E = -56 \pm 4$ mV. Similarly the mean of two determinations made in the absence of methionine was -55 mV.

Time course of the fluorescence changes

Effect of amino acid influx or efflux. The addition of valinomycin caused a change in the fluorescence of

Fig. 3. Increase in fluorescence intensity (depolarization) after the addition of glycine to a cell suspension in the standard Ringer solution containing glucose, the mitochondrial inhibitors and the carbocyanine dye at 37°C

The fluorescence was assayed as described in the Materials and Methods section. Traces (a) and (b) show how the fluorescence intensity varied immediately after the addition of 1 mM-glycine (trace a) or 9.8 mM-glycine (trace b). Controls to which a similar volume of either water or the Ringer solution was added exhibited no significant changes in fluorescence (results not shown). Traces (c) and (d), which correspond to roughly the same initial fluorescence intensity, show what happened when the experiment was continued for 40 min. For trace (c) the 2 mM-glycine was added at the start and the assay was continued until the fluorescence intensity was roughly constant. The control to which no addition of glycine was made is represented by trace (d).

the dye in the standard assay system that was completed in about 1 min (Fig. 1a). Likewise, the uptake of glycine, provided the Ringer solution contained Na^+ (Laris *et al.*, 1976; Eddy *et al.*, 1977), increased the fluorescence to a maximum value, reached in about 0.5 min (Fig. 3, trace a). The fluorescence intensity declined during the next 40 min (Fig. 3, trace c). The fluorescence intensity was then slightly larger than was observed in a parallel cell suspension (control) to which the amino acid was not added. The system was intact at that time because the addition of glycine to the control caused a marked increase in fluorescence (results not shown). We attribute the behaviour illustrated in Fig. 3(trace c) to the circumstance that the uptake of glycine reached a steady state by about 40 min and that the influx and efflux of glycine were both electrogenic. The inferred hyperpolarization accompanying the efflux of glycine was demonstrated more directly (Fig. 4). On being transferred to a Ringer solution without glycine, cell preparations that had previously accumulated glycine exhibited a somewhat lower fluorescence

Fig. 4. Lowering of the fluorescence intensity (hyperpolarization) associated with the efflux of glycine into two types of Ringer solutions

The tumour cells were incubated for 15 min in the standard Ringer solution. One portion was then incubated for 15 min at 37°C in the standard Ringer solution with 30 mM-glycine and 170 mM-glucose to load the cells with glycine. A further portion was incubated with a similar solution without glycine (controls). Each cell preparation was then collected and washed. At the indicated time, a portion (50 μ l) was put in the fluorimeter cuvette containing either 4 ml of the standard Ringer solution or 4 ml of the K⁺ Ringer solution lacking Na⁺, together with the mitochondrial inhibitors and 4 μ g of the carbocyanine dye. The fluorescence intensity was recorded for about 5 min in each instance. Trace (a) is the control for trace (b) and trace (c) is the control for trace (d). For (b) and (d) the cell preparations were loaded with glycine and for (a) and (c) they were not. Traces (a) and (b) were obtained by using the standard Ringer solution and traces (c) and (d) by using the K⁺/Ringer solution.

intensity than did those kept for a similar period in the absence of glycine. The effect was observed when the fluorescence assay was carried out in the standard Ringer solution or in one lacking Na⁺ ions (Fig. 4).

Role of the Na⁺ pump. Earlier work had shown (1) that the tumour-cell preparations that had accumulated Na⁺, at the expense of cellular K⁺, exhibited a relatively small fluorescence in the standard Ringer solution containing the cyanine dye, as though the plasmalemma was hyperpolarized. Since this behaviour depended on the presence of both extracellular K⁺ and glucose, and since it was blocked by ouabain (Philo & Eddy, 1975; Laris *et al.*, 1976; Eddy *et al.*, 1977), hyperpolarization appeared to be due to the accelerated working of the Na⁺ pump. (2) Gibb & Eddy (1972) and Heinz *et al.* (1975) suggested that the tumour cells loaded with Na⁺ were hyperpolarized in Ringer solutions containing about 30 mM- or

Fig. 5. Effects of early or late additions of ouabain or valinomycin on the fluorescence intensity exhibited by cell preparations in a Ringer solution containing 80 mM-Na⁺ and 150 mM-K⁺

The tumour cells were loaded with Na⁺ and transferred to the hyperosmotic Ringer solution (Gibb & Eddy, 1972) in the cuvette at the time indicated by the start (left-hand end) of the trace. The solution (4 ml) also contained 2 mg of glucose/ml, the mitochondrial inhibitors and 4 μ g of the dye. For trace (a) 0.25 mM-ouabain was added after about 5 min. For trace (b), which refers to the same scale of fluorescence intensity as trace (a), 0.25 mM-ouabain was added at 30 min. For trace (c) 10 μ g of valinomycin was added at 5 min. For trace (d), drawn on the same scale as trace (c), 10 μ g of valinomycin was added at 30 min.

80 mM-Na⁺ and 150 mM-K⁺. The addition of either 0.25 mM-ouabain or valinomycin (2.5 μ g/ml) to such preparations, within 15 min of the start of the incubation, caused a marked increase in fluorescence (Fig. 5). Our interpretation is that the Na⁺ pump initially hyperpolarized the cell preparations under these particular ionic conditions and that the pump action was blocked by ouabain and short-circuited by valinomycin. After 30 min, the distribution of Na⁺ and K⁺ would tend to reach a steady state in which the contribution of the Na⁺ pump to the membrane potential would be small (Thomas, 1972).

An artifact in incubations of 30 min duration. Preliminary work had shown that a fraction of the order of 10% of the dye present in the cuvette containing the cell suspension progressively became attached to the wall of the vessel during an assay of 30 min duration. The fluorescence reached at that time therefore corresponds to a different membrane potential than would be predicted on the basis of Fig. 2. For this reason the observations shown in Fig. 3 (traces c and d) and Fig. 5 provide only a rough guide to the magnitude of the membrane potential at any particular stage of the assay. Further quantitative work involved a standard incubation with the dye for about 5 min (Fig. 2). It was shown in that way that

Fig. 6. Eqn. (13) and the increase in fluorescence intensity caused by selected concentrations of L-methionine. The assays were carried out at 37°C in the standard Ringer solution containing glucose. The increase in fluorescence intensity was assayed as illustrated in traces (a) and (b) of Fig. 3. The fluorescence change caused by a given concentration of the amino acid was extrapolated to the time the addition was made. The magnitude of the change determined in that way is illustrated as a function of the methionine concentration. The solid line was computed from eqn. (13) with $\alpha = 33.6$ and $K_A/[Na^+]_0 = 2.74$ mM. The magnitude of E was inferred from the fluorescence intensity by means of the calibration curve (Fig. 2).

the mean value $\pm$ S.E.M. of E after incubation of the tumour cells with glucose for 8 min in a Ringer solution containing 80 mM- Na^+ , 150 mM- K^+ and 1 mM-L-methionine was -92 ± 6 mV (5). Clearly these cell preparations were hyperpolarized as compared with cells kept for 30 min in the standard Ringer solution (see above).

Magnitude of the depolarization caused by amino acid absorption

As was also observed with glycine (Fig. 3, trace a), the addition of methionine caused an increase in the fluorescence exhibited in the standard assay system. The magnitude of the change, determined by extrapolation to zero time, increased with the methionine concentration up to a maximum value of about 25 units of fluorescence intensity (Fig. 6), or about 70 mV (Fig. 2). The observations roughly fitted a rectangular hyperbola, with a K_m of about 0.3 mM for methionine. Similar behaviour was reported by Laris *et al.* (1976) for glycine. However, they observed an apparent K_m of 2.5 mM for that amino acid, which is larger than the value of about 0.75 mM we have now obtained (Table 2).

Laris *et al.* (1976) also reported rough quantitative agreement between the apparent K_m for glycine of 1.5 mM observed in transport studies and the above value based on the fluorescence assay. Our obser-

Table 2. Parameters deduced from the increase in fluorescence (cellular depolarization) caused by glycine or L-methionine

In both instances the observations obtained in three independent assays, like that illustrated in Fig. 6, were pooled. The maximum increase in fluorescence and apparent K_m were deduced by expressing the fluorescence increase as a hyperbolic function of the amino acid concentration.

Function	Glycine	L-Methionine
(1) Apparent K_m with respect to amino acid concentration	0.75 mM	0.40 mM
(2) Maximum increase in fluorescence (arbitrary units)	26	20
(3) Corresponding voltage change inferred from Fig. 2	74 mV	56 mV
(4) $K_A/[Na]_0$ of eqn. (13)	9 mM	3.1 mM
(5) α of eqn. (13)	48	23
(6) K_m from velocity of amino acid uptake (Eddy <i>et al.</i> , 1967; Morville <i>et al.</i> , 1973)	4 mM	0.96 mM

vations, summarized in Table 2, indicate that the apparent K_m values obtained by the two methods are probably different. Indeed the fact that one assay is based on changes in the membrane potential E whereas the other monitors the amino acid flux means that the same value of K_m would not necessarily apply in both instances.

Application of the mathematical model. The amplitude of the Na^+ -dependent depolarization caused by these amino acids showed that a relatively large change in Na^+ permeability had taken place. Moreover, the net uptake of Na^+ with glycine was accompanied by the expulsion of less than an equivalent amount of K^+ , possibly because Cl^- was also absorbed (Eddy, 1968). Thus the relative magnitudes of the flux of Na^+ through the amino acid carrier and the fluxes through the various channels conducting Na^+ , K^+ and Cl^- would govern the membrane potential (eqns. 2–13).

In practice the values of α and $K_A/[Na]_0$ shown in Table 2 were obtained by fitting the observations illustrated in Fig. 6 to eqn. (13). The position of the solid line depicted in Fig. 6 shows that a satisfactory fit was obtained. This procedure thus leads (1) to a value of the dissociation constant K_A of the ternary complex $ANaC$, and (2) to a value of the complex parameter α , defined as the ratio of V_m for the amino acid carrier to a second function based on the permeability coefficients for Na^+ , K^+ and Cl^- . The further development of the mathematical model is described by Philo & Eddy (1978).

Discussion

Although the membrane potential of the strain of tumour cells used in the present investigation has not been studied by conventional electrophysiological techniques, work with the related mouse Ehrlich ascites tumour has given results either in the vicinity of -11 mV (Hempling, 1962; Aull, 1967; Smith & Levinson, 1975) or in the range from -20 mV to -40 mV (Johnstone, 1959; Lassen *et al.*, 1971). Lassen *et al.* (1971) found that, after insertion of the glass electrode, the apparent membrane potential decayed rapidly with time, from a value in the forementioned upper range to one in the lower range. This behaviour was attributed to the failure of the plasmalemma to form an insulated seal around the inserted electrode. Moreover, work with HeLa cells, with fibroblasts and with excitable cultured cells of neural or myocytic origin has led to estimates of the membrane potential varying from about -40 mV to -60 mV (Sachs & McDonald, 1972; Okada *et al.*, 1973). Thus the conclusion reached by Williams (1970), on the basis of earlier literature, that the 'resting' membrane potential in non-excitabile cells is much lower than in nerve and muscle does not hold in general and may not apply to the mouse ascites-tumour cells.

The fluorescence intensity exhibited by the tumour cells incubated for 30 min in the standard Ringer solution with glucose corresponded to a membrane potential of about -55 mV. Comparable assays with another carbocyanine dye and the Ehrlich ascites tumour in the absence of glucose gave values near -40 mV, in a system that was calibrated on the basis of the assumption that the membrane potential was equal to the K^+ equilibrium potential in the presence of $18.6 \mu\text{M}$ -valinomycin (Burckhardt, 1977). Similarly Laris *et al.* (1976) inferred values ranging from -18 mV to -42 mV by using a different carbocyanine dye as an indicator in a 'null point' titration with valinomycin. Both of the above estimates depend on the further assumption that the addition of valinomycin caused a change in fluorescence intensity that was not due to the redistribution of the dye between the mitochondria and the cytosol. Bearing in mind the very different procedures used by Burckhardt (1977) and Laris *et al.* (1976) their observations are similar to the upper range of estimates of the membrane potential obtained by inserting microelectrodes into the Ehrlich cells.

The marked hyperpolarization up to about -100 mV that took place when the Na^+ pump was expelling Na^+ rapidly from the tumour cells (Figs. 2 and 5) corresponds to values much larger than the K^+ equilibrium potential. Similar phenomena have been observed by implanting glass electrodes in other mammalian cells (see Sachs & McDonald, 1972). On the other hand, the magnitude of the depolarization

of up to 70 mV caused by the addition of the amino acids (Fig. 6) is larger than has been detected by microelectrode techniques in either the kidney or the intestine. This is possibly because the methods used only detected the voltage changes in these last-named systems in an attenuated form, owing to the presence of a low-resistance shunt (Maruyama & Hoshi, 1972; Rose & Schultz, 1971). It is noteworthy in this connection that the proton-dependent absorption of glucose by *Neurospora* depolarized the hyphae by up to 100 mV (Slayman & Slayman, 1974).

Smith & Levinson (1975) have concluded that the Ehrlich ascites-tumour-cell membrane is atypical in being unable to support valinomycin-mediated K^+ transport. These workers nevertheless observed that valinomycin caused a greater loss of cellular K^+ and influx of cellular Na^+ than the accompanying depletion of the cellular ATP content was itself expected to cause. Reid *et al.* (1974) attributed the similar behaviour they observed, with the strain of tumour cells used in the present investigation, to the increase in K^+ permeability (P_K) caused by valinomycin. This increase would be expected to raise the membrane potential (E). However, Smith & Levinson (1975) found that the membrane potential of -12 mV recorded by microelectrode impalement of the Ehrlich cells was scarcely affected by the presence of valinomycin. We suggest that the changes in the ionic composition of the tumour cells observed by Smith & Levinson (1975) are consistent with the hypothesis of Reid *et al.* (1974) and that the method the former workers used for assaying the membrane potential, which involves the introduction of La^{3+} into the system, may not be sound (see Laris *et al.*, 1976). This view that valinomycin increases P_K and thereby changes E is indeed consistent with other phenomena.

- (1) It explains the effect of valinomycin and of other ionophores on the accumulation of amino acids in cell preparations depleted of ATP (Reid *et al.*, 1974).
- (2) It accounts for the ability of valinomycin to affect the cellular accumulation of the carbocyanine dyes (Fig. 2; Laris *et al.*, 1975; Burckhardt, 1977) and other lipophilic cations (Cespedes & Christensen, 1974).
- (3) It explains why a mixture of nigericin and a proton conductor mimicked the effect of valinomycin on the dye fluorescence exhibited by suspensions of the tumour cells (R. Philo & A. A. Eddy, unpublished work; cf. Kimmich *et al.*, 1977).

It is useful to enumerate the assumptions involved in using Fig. 2 to assay the membrane potential. First, there is the supposition that the changes in the fluorescence signal, measured under the standard conditions, varied in a systematic way with the magnitude of E as inferred from the K^+ equilibrium potential at the relevant 'null point'. The latter inference in turn depends on two further assumptions.

- (1) Valinomycin affected only P_K among the factors

governing the membrane potential. (2) Changes in the distribution of the dye and hence the fluorescence of the cell suspension were governed by only one independent variable, namely the magnitude of E . The very fact that the change in K^+ equilibrium potential correlated with a major fraction of the variance associated with the fluorescence intensity (Fig. 2) encourages us to believe that the above assumptions are valid. The effect of various ionophores on the dye distribution shows, moreover, that it is the membrane potential and not the ratio $[K]_i/[K]_o$ that is the significant variable (Fig. 1a; Laris *et al.*, 1976; Burckhardt, 1977; Kimmich *et al.*, 1977).

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