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Plasma Membrane Potential of Lettré Cells does not Depend on Cation Gradients but on Pumps

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Summary. The plasma membrane potential of Lettré cells has been determined with the optical indicator oxonol-V and found to be −57 mV at 37°C (range −20 to −80 mV depending on the physiological condition of the cells). Increasing extracellular K^+ does not depolarize cells: even in the presence of 155 mM K^+ the potential is −41 mV; membrane potential is also insensitive to the chemical gradient of Na^+, Mg^{2+}, Ca^{2+} or Cl^-. Ouabain depolarizes the cells; H^+ efflux from cells is stimulated by extracellular Na^+. We propose that in Lettré cells the plasma membrane potential is generated by electrogenic cation pumps. The balancing fluxes of Na^+ and K^+ are mainly through electroneutral cation exchanges (Na^+/K^+ and Na^+/H^+) and the magnitude of the potential is limited by organic anion leaks. Such a mechanism may operate in other biological membranes also.

Key Words oxonol-V · membrane potential · ion pumps · Lettré cell · organic anions

Introduction

The plasma membrane potential is an important determinant of cell function. In excitable cells, depolarization-repolarization events underlie nerve transmission, muscle contraction and endocrine secretion. Membrane potential contributes to the uptake of nutrients such as amino acids (Eddy & Philo, 1976; Philo & Eddy, 1978; Burckhardt & Pietrzyk, 1980; Hacking & Eddy, 1981) and an alteration in membrane potential is one of the earliest events associated with the mitogenic stimulation of quiescent cells (Kiefer, Blume & Kaback, 1980; Tsien, Pozzan & Rink, 1982).

The resting plasma membrane potential in excitable cells, which is generally recorded using intracellular electrodes, has been found to be set predominantly by the K^+ diffusion potential, with a small contribution from electrogenic mechanisms such as the Na^+ pump; its value may be calculated from the Goldman equation (Goldman, 1943; Hodgkin & Katz, 1949) modified to include a contribution from an electrogenic cation pump (Thomas, 1972; Lew, Ferreira & Moura, 1979). In frog muscles that have a membrane potential of −92 mV (Adrian, 1956), for example, −89 mV is caused by ionic diffusion, with a maximum of −3 mV contributed by electrogenic pumps when cells are in a steady state with regard to Na^+ and K^+ (Thomas, 1972).

Recording of membrane potential with an intracellular electrode is technically more difficult to achieve in small cells (*see*, for example, Ince, Ypey, van Furth & Verveen, 1983). The introduction of optical methods, such as the use of cyanine (Hoffman & Laris, 1974; Sims, Waggoner, Wang & Hoffman, 1974) and other (Cohen et al., 1974; Ross et al., 1977; Cohen & Salzberg, 1978; Bashford, 1981) dyes and of radioactive ion distribution methods, such as the use of tetraphenylphosphonium (Geck et al., 1980; Kiefer et al., 1980; Felber & Brand, 1982), have largely overcome this problem, and it is clear that many different cells have a plasma membrane potential of the same order of magnitude as that found in excitable cells (Laris, Pershadsingh & Johnstone, 1976; Philo & Eddy, 1978; Kiefer et al., 1980; Felber & Brand, 1982). Because of this similarity, and because the K^+ gradient across the plasma membrane of most cells is similar to the K^+ gradient across the plasma membrane of resting neurones (Williams, 1970), it has been generally assumed that the plasma membrane potential of cells is largely set by the K^+ diffusion potential (Williams, 1970). It may be wrong to assume that this is always the case. It has been argued (Thomas, 1972) that at steady state, a Na^+ pump having a $Na^+ : K^+$ ratio of 3 : 2 *cannot* contribute more than about 10 mV to the resting membrane potential. This is true only if the balancing Na^+ and K^+ fluxes are diffusional. The potential contributed by the pump could be much higher if at least part of the balancing Na^+ and K^+ fluxes are through electroneutral pathways (*see also* Lew et al., 1979). The experiments to be

described show that in Lettré cells a membrane potential of about −55 mV is generated mainly by electrogenic pumps and not by a diffusional mechanism (Alder et al., 1983*b*).

Materials and Methods

Lettré cells were grown by serial passage in the peritoneal cavity of Swiss mice, strain TO, and were harvested between seven and ten days of growth into a medium containing 150 mM NaCl, 5 mM KCl, 5 mM (N2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) and 1 mM $MgCl_2$ adjusted to pH 7.4 with NaOH (HEPES-buffered saline, HBS). On occasions heparin (5 units/ml) was included in the harvesting medium to prevent agglutination of Lettré cells. The cells were centrifuged at 700 × *g* for 3 min in an MSE Chilspin centrifuge and the cell pellet was resuspended with 4 volumes of HBS. Such a 20% (vol/vol) cell suspension was stored at room temperature with occasional aeration until required. Human peripheral blood mononuclear cells were prepared from fresh heparinized blood collected from healthy male volunteers using ficoll-hypaque (Boyum, 1968) and were stored at 37°C in Hams F10 medium supplemented with 3% foetal calf serum until required. Baby hamster kidney cells were grown as monolayer cultures in Eagle's minimal essential medium supplemented with 10% foetal calf serum in an atmosphere containing 95% air, 5% CO_2 at 37°C.

Fluorescence and absorbance measurements were made with a Johnson Research Foundation Compensated Fluorimeter/Spectrophotometer as described previously (Bashford et al., 1981, 1983*a*). Cell cations were measured using atomic absorption spectroscopy as described previously (Bashford et al., 1983*a*) after cells had been pelleted through oil (cell suspensions) or after extraction with 2% triton X-100 (cell monolayers). Concentrations of cell cations were determined from the amount of cation divided by the water content of the cell pellet (wet weight-dry weight).

Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), valinomycin and ouabain were from Sigma Chemical Co., oxonol-V was a gift from Dr. B. Chance, Johnson Research Foundation, University of Pennsylvania, Philadelphia, and nigericin (Lot 189-380B-171-A) was a gift from Miss V. Mason, Lilly Research Centre Ltd. Dye, ionophores and inhibitors were stored as stock solutions in ethanol or dimethylsulfoxide and diluted 1 : 1000 into the cell suspensions used for the experiments. Other chemicals were of the highest purity commercially available. Sendai virus, of the late-harvest, 'three-day' type, was grown as described by Impraim et al. (1980).

Results and Discussion

Validation of the Use of Oxonol-V to Measure Plasma Membrane Potential

The use of optical indicators to measure the electrical potential across membranes has been extensively reviewed (Cohen & Salzberg, 1978; Bashford & Smith, 1979; Waggoner, 1979; Bashford, 1981). Positively charged indicators such as the cyanines (Cohen et al., 1981; Johnson et al., 1981) are rapidly taken up by respiring mitochondria, and their use to measure *plasma* membrane potential requires the presence of inhibitors of mitochondrial respiration and uncouplers of oxidative phosphorylation in order to abolish *mitochondrial* membrane potential (Philo & Eddy, 1978). A mixture of inhibitors such as dinitrophenol, antimycin A and oligomycin was thought to be acceptable for mouse ascites cells because none of them appeared to affect plasma membrane potential (Philo & Eddy, 1978). However oligomycin can inhibit the plasma membrane (Na^+/K^+)-ATPase (Lichtman, Jackson & Peck, 1972; Felber & Brand, 1982), and FCCP, which has been used in conjunction with a different oxonol dye in a study of the plasma membrane potential of pig mesenteric lymphocytes (Felber & Brand, 1982), depolarizes Ehrlich ascites cells (Burckhardt & Pietrzyk, 1980) and, as documented below, directly affects the plasma membrane potential of Lettré cells in a manner dependent on the extracellular pH. The advantages of oxonol-V (Smith, Russ, Cooperman & Chance, 1976; Bashford, Chance & Prince, 1979*a*), used in the present investigations are (a) that it is negatively charged and therefore not taken up by mitochondria and (b) that it can readily be measured by its absorbance (e.g., A_{630}–A_{590}) (Bashford et al., 1979*a*,*b*), rather than by its fluorescence which may be quenched both by mechanisms not associated with membrane potential and by the presence of ionophores (Heytler, 1979) used in such studies.

Our method for determining the plasma membrane potential of Lettré cells, a cell line which is derived from a mouse ascites tumor, is shown in Fig. 1. The absorbance of cells treated with oxonol-V is monitored at 630 *minus* 590 nm and the reading is calibrated by using ionophores to modify the membrane permeability for a specific cation. In the presence of such an ionophore, and on the reasonable assumption that the ionophore increases the permeability only of the ion in question (Philo & Eddy, 1978), the membrane potential (V) approaches that defined by the Nernst relationship:

$$V = \frac{RT}{nF} \ln \frac{[\text{ion}]\ \text{medium}}{[\text{ion}]\ \text{cytoplasm}}.$$

Valinomycin, a K^+ specific ionophore (Harris & Pressman, 1967; Henderson, McGivan & Chappell, 1969), hyperpolarizes Lettré cells (suspended in a medium containing 5 mM K^+), which is registered by a decrease in absorbance at 630 *minus* 590 nm (Fig. 1, continuous trace). The subsequent addition of K^+ to the medium depolarizes the cells; the concentration of K^+ required to restore the absorbance to the value recorded before the addition of ionophore can be obtained by interpolation. In contrast the proton-specific ionophore FCCP (Harris & Pressman, 1967; Henderson et al., 1969) depolar-

izes Lettré cells, which are subsequently repolarized by the addition of base (Fig. 1, dashed trace). In each case the membrane potential before the addition of ionophore can be calculated by solving the above equation, using that concentration of H^+ or K^+ in the medium which restored the absorbance to its resting level (Rink et al., 1980). For the experiments illustrated in Fig. 1, values for V of −49 and −54 mV, respectively, were obtained. From other experiments carried out at this temperature (33°C), the mean membrane potential was found to be −51 ± 3.0 mV (SEM, $n = 36$). The fact that titration with K^+ in the presence of valinomycin, and with H^+ in the presence of FCCP, give the same value for membrane potential (Akerman & Wikstrom, 1976), makes it unlikely that interactions between dye and ionophore (Rink et al., 1980) seriously affect our measurement of membrane potential. Furthermore our observation that valinomycin hyperpolarizes normal cells suspended in a conventional medium (Fig. 1) but depolarizes such cells when the transmembrane K^+ gradient is diminished by nigericin (Fig. 4*B*), argues strongly for our contention that the dye signal is not due to complex formation between valinomycin and oxonol-V, but solely to the effect of valinomycin on the K^+ conductance.

That it is *plasma* membrane potential that is being measured by oxonol-V in our studies is indicated by the following observations. Haemolytic Sendai virus completely depolarizes Lettré cells within 2 min at 37°C (Impraim et al., 1980) presumably because of its known action of making cells permeable to ions and low molecular weight compounds (Pasternak & Micklem, 1973, 1981; Poste & Pasternak, 1978); the permeabilizing action of Sendai virus has been shown to be due to fusion between viral envelope and cell plasma membrane (Knutton, 1978), and viral envelope components can be detected at the cell surface at this time (Knutton & Bachi, 1980). Although viral nucleocapsid enters cells, this cannot be the cause of the depolarization, since depolarization (and permeability changes) can be completely prevented by extracellular Ca^{2+} (Impraim et al., 1980), whereas fusion, and hence entry of nucleocapsid, are insensitive to extracellular Ca^{2+} (Wyke et al., 1980). The potential that is being measured is not mitochondrial, since valinomycin and FCCP each depolarizes mitochondria *in situ* (Johnson et al., 1981), yet have opposite effects on oxonol absorbance in Lettré cells (Fig. 1); on the other hand gramicidin, which does not enter cells (Rink et al., 1980), depolarizes Lettré cells (*see* Fig. 4*A*). We conclude from such experiments with ionophores specific for either K^+ or H^+ that, irrespective of whether it is external K^+ or external H^+ that is altering, oxonol-V absorbance measures the membrane potential as defined by the Nernst equation.

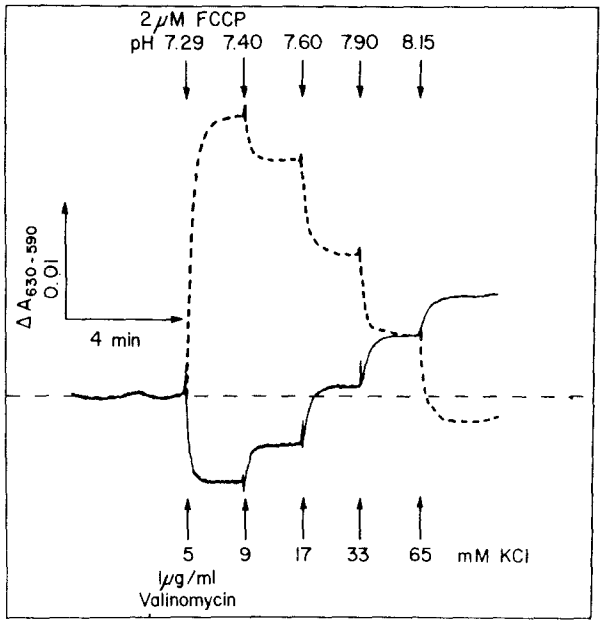

Fig. 1. Use of oxonol-V to measure membrane potential. The absorbance of 2 μM oxonol-V was followed in a stirred suspension of Lettré cells (2.5 to 5 × 10^6 cells/ml) in 5 mM HEPES-buffered isotonic saline (HBS), pH 7.3. FCCP and NaOH (dashed trace) or valinomycin and KCl (continuous trace) were added to cells at 33°C to give the final concentrations indicated. The concentration of H^+ or K^+ at which the trace crosses the base line was obtained by interpolation. From these 'null-points' (Hoffman & Laris, 1974) and values for K_i^+ (105 mM) and H_i^+ (0.6 × 10^{-7} M), the membrane potential was calculated to be −49 and −54 mV, respectively

It should be stressed that the values of plasma membrane potential of Lettré cells and human peripheral lymphocytes (*see* Table 2) obtained by us with the use of oxonol-V agree well with the values obtained by others using cyanines (Laris et al., 1976; Philo & Eddy, 1978), other oxonols (Rink et al., 1980; Felber & Brand, 1982) or the radioactive ion distribution method (Kiefer et al., 1980; Felber & Brand, 1982). They are higher than values found by impalement of the cells with electrodes (Williams, 1970), presumably because of membrane damage and other artefacts inherent in the application of this method (Hoffman, Simonsen & Sjoholm, 1979; Ince et al., 1983).

Plasma Membrane Potential of Lettré Cells is Unaffected by Changing the Gradients of Inorganic Ions

Increasing extracellular K^+ has little effect on the membrane potential of Lettré cells unless the K^+ ionophore valinomycin is added (Fig. 2*A*). This shows that the membrane potential cannot have

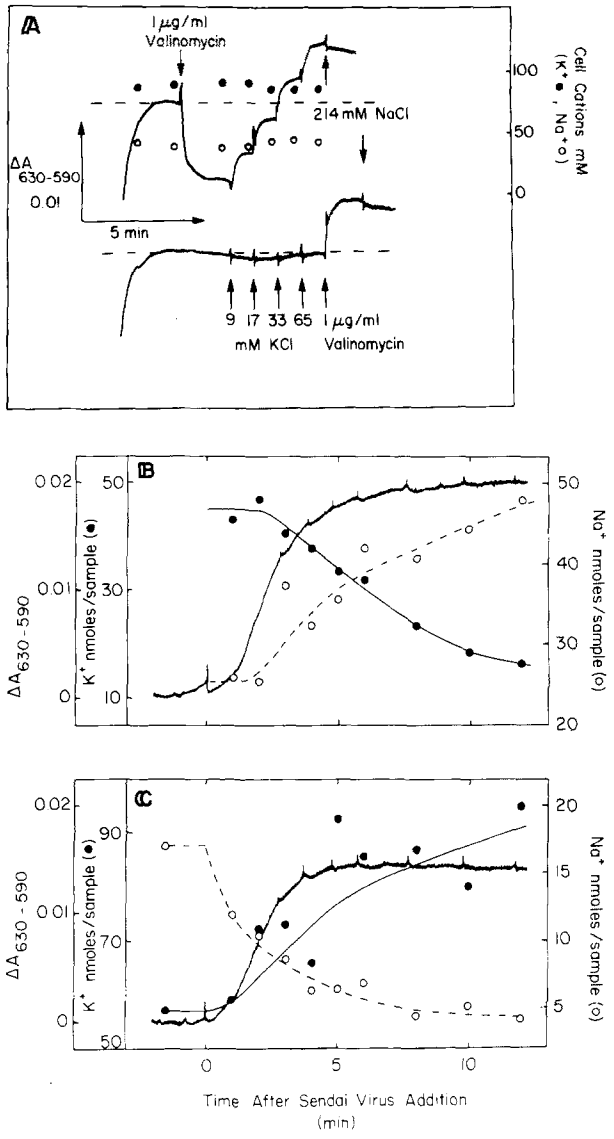

Fig. 2. Insensitivity of membrane potential of Lettré cells to KCl. The absorbance of 2 μM oxonol-V was measured in a stirred suspension of Lettré cells (5×10^6/ml) in media containing 5 mM HEPES and 1 mM $MgCl_2$, pH 7.3. (*A*) 150 mM NaCl, 5 mM KCl, 5 mM glucose and 1 mM Na-pyruvate at 37°C; valinomycin, further KCl and NaCl were added to give the final concentrations indicated; the membrane potential, calculated from the upper trace, is −39 mV. (*B*) 150 mM NaCl and 5 mM KCl. (*C*) 155 mM KCl. In (*B*) and (*C*) 2 HAU Sendai virus/ml (final concentration) was added to cells at 33°C as indicated and aliquots were removed at intervals for measurement of cell cations, which are expressed as nmoles found per 200-μl sample; membrane potential before the addition of virus was −55 mV (*B*) and −41 mV (*C*), as determined by titration with H^+ in the presence of FCCP

been generated by K^+ diffusion: if it had been, addition of K^+ would have depolarized cells. Similar results have previously been observed with Ehrlich ascites cells, using a cyanine dye (Laris et al., 1976; Philo & Eddy, 1978) and intracellular electrodes (Smith & Robinson, 1981*a*,*b*) to measure membrane potential. Indeed, Lettré cells suspended in 155 mM KCl and titrated with H^+ in the presence of FCCP have a membrane potential of −41 mV (*see* legend to Fig. 2). Yet in this situation, the gradient of K^+ across the plasma membrane is actually reversed, as is seen by the fact that addition of Sendai virus, which permeabilizes the cells, causes intracellular K^+ to *increase* (and intracellular Na^+ to decrease). In other words Sendai virus causes a depolarization of Lettré cells irrespective of whether intracellular K^+ is decreasing or increasing (Fig. 2*B* and *C*). These results are incompatible with the notion that membrane potential is generated by the diffusion of K^+; it also rules out the possibility that membrane potential is set by the Na^+ diffusion potential. Nigericin, which causes the internal $K^+ : Na^+$ ratio to reverse within 5 min by catalyzing electroneutral cation exchange (Harris & Pressman, 1967; Henderson et al., 1969), actually hyperpolarizes the cells (*see* Fig. 4*B* below).

The effect of other variations of the ionic composition of the medium on the membrane potential of Lettré cells is shown in Fig. 3. Increasing concentrations of H^+ depolarize cells by 23 mV per pH unit (Fig. 3*A*); K^+ depolarizes cells by 8 mV per decade (Fig. 3*B*); and Ca^{2+} depolarizes cells by 10 mV per decade (Fig. 3*C*); the scatter in panels *A* and *B* is due to the fact that all the experiments performed are included: for any one preparation of cells, the change in potential with increasing concentration is approximately that indicated by the line of best fit drawn through the points. Although there is some uncertainty about the precise concentration of Ca^{2+} in the cytosol, it is clear that at no concentration of H^+, K^+ or Ca^{2+} does the measured membrane potential correspond to the equilibrium diffusion potential for that cation and we conclude that diffusion of neither H^+, K^+ nor Ca^{2+} generates the resting potential of Lettré cells. Similarly, the possibility that Mg^{2+} generates the potential is excluded by the observation that the intracellular free Mg^{2+} concentration, calculated from the resonance frequencies of the phosphate groups of ATP in a ^{31}P nuclear magnetic resonance experiment (Gupta & Yushok, 1980), is approximately 0.5 mM (C.L. Bashford and A.N. Stevens, *unpublished observations*), a value close to that of Mg^{2+} in the medium (1 mM).

The membrane potential of Lettré cells is unaffected when Cl^- in the medium is replaced by SO_4^{2-} (Fig. 3*D*), indicating that diffusion of neither of these anions generates the potential; this observation confirms an electrophysiological study (Hoffman et al., 1979) which showed that >90% of the

Cl^- flux in Ehrlich ascites cells is electroneutral. However, when the Cl^- in the medium is replaced by organic anions such as HCO_3^- or lactate (Fig. 3*E,F*), a different response of membrane potential is observed. At concentrations of organic anion below 15 mM the cells are slightly hyperpolarized while at concentrations above 15 mM the anions depolarize cells by 60 mV per decade. These observations suggest that the diffusion of organic anions such as HCO_3^- and lactate may have an important role in the setting of membrane potential in Lettré cells.

It might be argued that in Lettré cells, the presence of nondiffusible anions, i.e. nucleic acids, proteins and phosphorylated low molecular weight compounds, contributes substantially to the membrane potential. This can be discounted by the observation that Sendai virus collapses the membrane potential entirely within 5 min at 33°C. Not only do nucleic acids and proteins not leak out of Lettré cells under these conditions (Pasternak & Micklem, 1973, 1981; Poste & Pasternak, 1978), but low molecular weight phosphorylated compounds do not begin to leak out until *after* the membrane potential has already collapsed (Bashford, Micklem & Pasternak, 1983*b*).

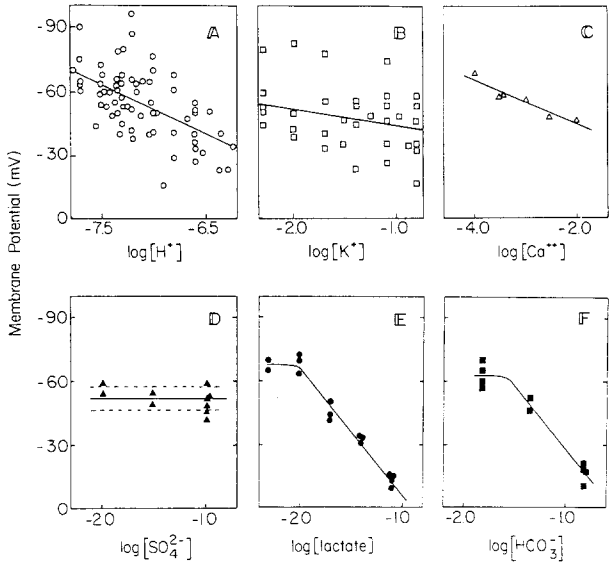

Fig. 3. Effect of ions on plasma membrane potential of Lettré cells. Membrane potential of Lettré cells (5×10^6/ml) at 33°C suspended in HBS or a similar medium containing 0.5 mM HEPES, pH adjusted to 7.3 or as otherwise indicated, was determined by 'null point' titrations similar to those shown in Fig. 1. (*A*) Dependence of membrane potential on H^+ concentration: (*B*) effect of replacing NaCl with KCl; (*C*) effect of $CaCl_2$; effect of replacing NaCl with Na_2SO_4 (*D*), Na lactate (*E*), $NaHCO_3$ (*F*)

Evidence that Electrogenic Pumps Generate Plasma Membrane Potential of Lettré Cells

A role for the Na^+ pump (Na^+, K^+-ATPase) in generating membrane potential is indicated by the fact that stimulation of the pump increases membrane potential (i.e. hyperpolarizes cells) and that inhibition of the pump decreases membrane potential. Thus addition of K^+ to cells suspended in K^+-free medium, which stimulates the Na^+ pump, decreases $A_{630-590}$ (increase in potential), as shown in Fig. 4*A* (*i*); such an effect has been postulated for Ehrlich cells (Pietrzyk, Geck & Heinz, 1978) and observed by electrophysiological means with liver cells *in vivo* (Williams, Withrow & Woodbury, 1971). Similarly, elevation of internal Na^+ (Henderson et al., 1969; Laris, Bootman, Pershadsingh & Johnstone, 1978; Philo & Eddy, 1978; Pietrzyk et al., 1978) (Fig. 4*B*) brought about by addition of the $Na^+/K^+/H^+$ ionophore nigericin (Harris & Pressman, 1967; Pressman, 1968; Henderson et al., 1969) decreases $A_{630-590}$ (Fig. 4*B*). Ouabain (1 mM) increases $A_{630-590}$ (i.e. depolarizes) in cells suspended in conventional medium (Fig. 4*A* (*ii*)); in cells suspended in K^+-free medium, which have a membrane potential of <-20 mV, ouabain has little effect; moreover it inhibits the K^+-stimulated hyperpolarization (Fig. 4*A* (*i*)). It also depolarizes cells hyperpolarized by K^+ or by nigericin (Fig. 4*B*). Others have failed to observe such an effect of ouabain on Ehrlich ascites cells (Heinz, Sachs & Schafer, 1981*a*; Heinz et al., 1981*b*; but *see* Laris et al., 1978; Philo & Eddy, 1978), possibly because insufficient ouabain was added: the Na^+ pump is known to have differing affinities for ouabain (Charnock & Simonson, 1977; Kaplan, 1978) in different cells and under different conditions, particularly in Ca^{2+}-free media (Mansier & Lelievre, 1982). We have observed considerable variation in the ouabain sensitivity of Lettré cells, which might suggest that the Na^+ pump is variably activated in these cell preparations. In no case does ouabain depolarize cells entirely, as is shown, for example, by further depolarization caused by the addition of gramicidin (Fig. 4*A* (*i*) and (*ii*)), an ionophore that is known to depolarize cells by greatly increasing the permeability to Na^+ and K^+ (Rink et al., 1980). The extent to which the failure of ouabain to depolarize cells completely is due to the presence of another electrogenic pump (*see below*), or to the relative insensitivity of the Na^+ pump of rodent cells to ouabain, remains to be assessed.

It might be thought paradoxical that nigericin, gramicidin and Sendai virus cause similar changes in cation movement, yet their effects on membrane potential are different (*cf.* Fig. 4*B* with Figs. 4*A* and

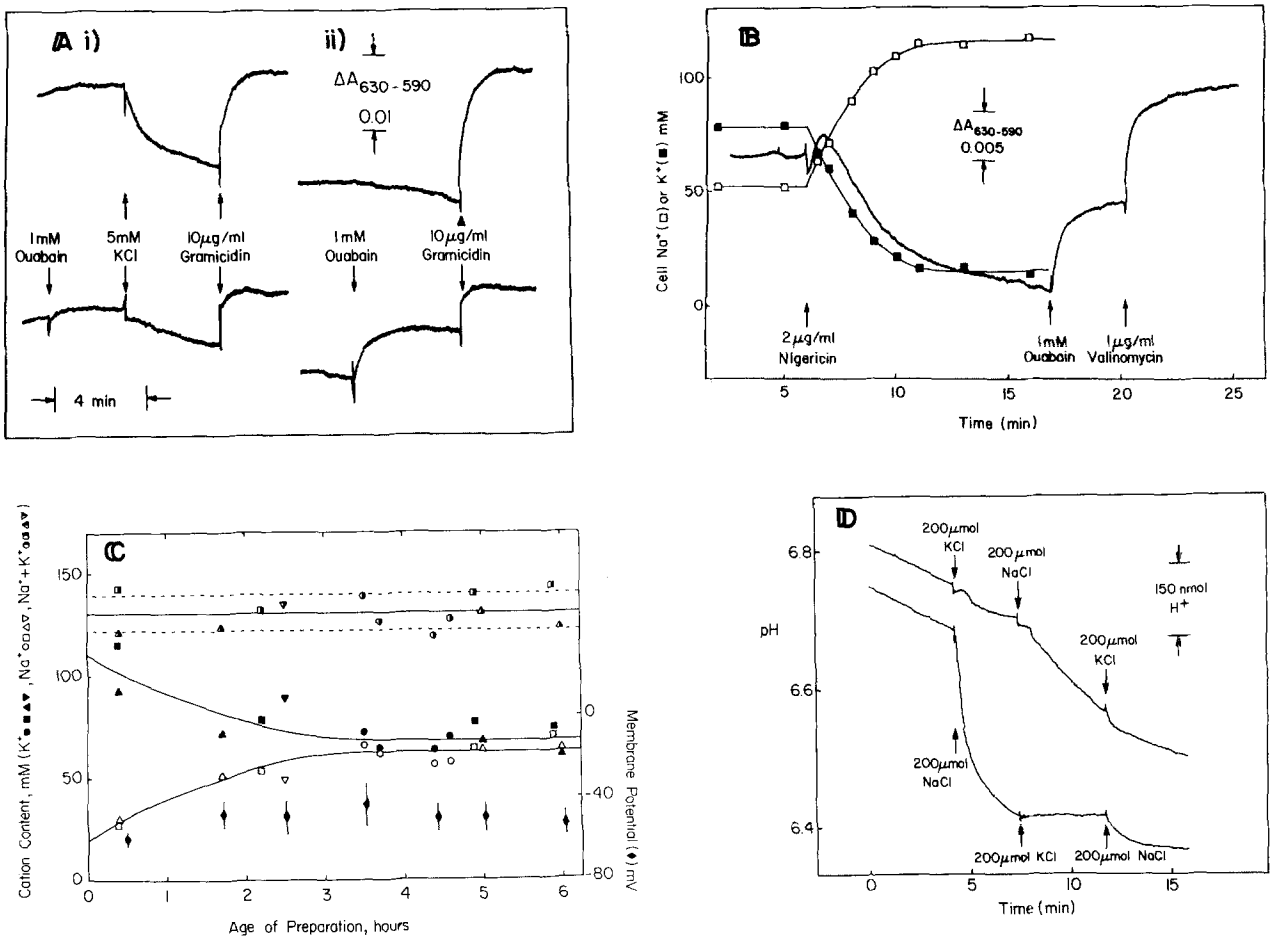

Fig. 4. Effect of activators and inhibitors of the Na^+ pump on membrane potential of, and proton extrusion by, Lettré cells. (*A–C*) The absorbance of 2 μM oxonol-V was measured in a stirred suspension of Lettré cells (5×10^6/ml) at 33°C in HBS, pH 7.3 ± 5 mM KCl. (*A*) Cells suspended in K^+-free medium (*i*) or K^+-containing medium (*ii*), each containing 1 mM glucose: ouabain ((*i*) and (*ii*), lower traces only), K^+ (*i*) and gramicidin ((*i*) and (*ii*)) were added to give the final concentrations indicated. The membrane potential before any additions was <-20 mV (*i*) and -55 mV (*ii*). (*B*) Nigericin, ouabain and valinomycin were added to a cell suspension containing 5 mM KCl and 5 mM glucose to give the final concentrations indicated. (*C*) Cells (2×10^8/ml) were kept at room temperature (21 to 23°C) in HBS, pH 7.3, for the times indicated. Samples were diluted into similar medium and membrane potential calculated from 'null point' titrations (Fig. 1). (*D*) The extrusion of H^+ (i.e. change in extracellular pH) from a stirred suspension (3.5 ml) of Lettré cells (10^7 cells/ml) at 32°C in a medium containing 250 mM sucrose, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM HEPES, pH adjusted to 6.8 with NaOH, was measured with a Corning semi-micro combination electrode. KCl and NaCl were added as indicated; at the end of each experiment NaOH was added in order to quantitate the number of protons extruded, as shown by the double-arrowed inset. Note that the rate of H^+ extrusion by NaCl is diminished in the presence of 62 mM KCl

2*B* and *C*). The reason is that nigericin catalyzes only a 1 : 1 cation exchange (Harris & Pressman, 1967; Henderson et al., 1969), with no change in membrane resistance; this is true neither of Sendai virus, which induces a nonspecific leakiness in cells such that all ions move down their concentration gradients with accompanying entry of water (Poste & Pasternak, 1978) and a decrease in membrane resistance (Forda et al., 1982), nor of gramicidin (Rink et al., 1980).

Figure 4*C* shows that at steady state, achieved after some 4 hr of suspension of Lettré cells in isotonic saline at room temperature, membrane potential is -55 mV. If this potential is generated mainly by the Na-pump it must be assumed that there is some electroneutral mechanism, such as a Na^+/H^+ exchange (Mitchell, 1968; Murer, Hopfer & Kinne, 1976) by which Na^+ ions pumped out electrogenically are returned to cells. That Lettré cells may possess such an activity is suggested by our observation that addition of NaCl, but not KCl, to cells suspended in isotonic sucrose causes an increased

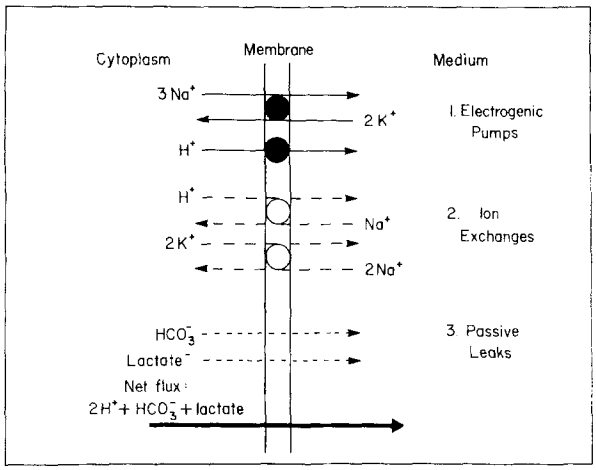

Fig. 5. Model for setting of plasma membrane potential in Lettré cells. 1 Electrogenic pumps. 2. Electroneutral exchange processes. 3. Passive leaks. Electroneutral movements not involved in setting the potential, such as the furosemide-sensitive Na^-, K^- 2 Cl^- co-transport system (Geck et al., 1980), or the lactate-H^- cotransport system (Spencer & Lehninger, 1976), have been omitted for simplicity. The combination of the Na^- pump (1) and the electroneutral exchanges (2) (i.e. one proton extruded) accounts for the ouabain-sensitive component of membrane potential at steady-state for Na^+ and K^+; the proton pump (1) is postulated to represent the ouabain-insensitive component (Heinz et al., 1981*a,b*); HCO_3^- and lactate provide the leak currents necessary to maintain the resting potential

Table 1. Plasma membrane potential of Lettré cells at different temperatures

Temp. (°C)	Membrane potential[a] (mV)
37	−57 ± 3.0 (SEM; n = 34)
33	−51 ± 3.0 (SEM; n = 36)
28	−56 ± 7.0 (SEM; n = 7)
22	−53 ± 5.9 (SEM; n = 6)
16	−52 ± 5.7 (SEM; n = 5)

[a] The membrane potential of cells in HBS pH 7.3 was measured by null point titrations as in Fig. 1.

amount of H^+ to appear in the medium (Fig. 4*D*); since KCl alone has little effect, the stimulation by NaCl must be due to an effect of Na^+, not Cl^-.

Model

Possible mechanisms involved in the generation of plasma membrane potential in steady-state Lettré cells are shown in Fig. 5. We propose that there is an electrogenic sodium pump which can account for at least 50% of the measured potential. Under conditions of zero net flux of Na^+ and K^+, such as is achieved after 4 hr at room temperature (Fig. 4*C*), the flux due to the pump is balanced by Na^+/H^+ as well as by Na^+/K^+ exchange: in other words the Na^+ pump works effectively as a proton pump. The plasma membrane appears to possess additional proton pump(s) which are ouabain insensitive (Heinz et al., 1981*a,b*) and which may account for the ouabain-insensitive component of the potential. The accompanying anions in each case are bicarbonate, lactate or other organic anions. These arguments suppose that steady-state Lettré cells have sufficient metabolism to generate H_2CO_3 (from H_2O and CO_2) or lactic acid. The fact that Lettré cells suspended in isotonic saline maintain ATP levels in excess of 1 mM, as determined by ^{31}P NMR (Bashford et al., 1983*a*), for several hours suggests that this assumption is a reasonable one. In Lettré cells the balance between pump-generated fluxes and passive ionic conductances is achieved at membrane potentials of about −55 mV. Reversal of the transmembrane chemical gradient of Na^- or K^+ (Fig. 2*C*), or replacing chloride in the medium with sulfate (Fig. 3*D*), has little effect on the steady potential which makes it unlikely that the 'leak' current is carried predominantly by any of these ions. Low concentrations of organic ions, on the other hand, hyperpolarize cells; at other concentrations they depolarize (Fig. 3*E,F*), presumably by entering the cells as undissociated acids (Spencer & Lehninger, 1976) and leaving such cells as dissociated anions.

In many respects the generation of plasma membrane potential in Lettré cells resembles the generation of the potential across the inner mitochondrial membrane (Mitchell, 1968) or across the plasma membrane of *Neurospora* (Sanders, Hansen & Slayman, 1981). In each case there is an outwardly directed, electrogenic proton pump whose flux is sustained by electroneutral exchange processes and whose potential is limited by the conductance of the membrane to accompanying anions and to protons.

If efflux of H^+ plus HCO_3^- and lactate is the major mechanism by which the membrane potential of Lettré cells is set then it should prove possible to detect an acidification of the medium in which the cells are suspended. This has previously been reported for Ehrlich ascites tumor cells (Heinz et al., 1981*a,b*) suspended in a glucose-containing medium at pH 7.2, and it is shown for Lettré cells suspended in weakly buffered isotonic media in Fig. 4*D*. Since in this experiment the external pH was initially 6.8, while the cytosolic pH was 7.0 (Alder, Bashford & Pasternak, 1983*a*; Bashford et al., 1983*a*), the acidi-

Table 2. Plasma membrane potential of some nonexcitable cells[a]

Cells	Membrane potential (mV)	Membrane potential in 80 mM KCl (mV)	Membrane potential in 1 mM ouabain (mV)
Lettré	−57 ± 3.0 (SEM; n = 34)	−53 ± 87 (SEM; n = 34)	−28 ± 3.1 (SEM; n = 24)
Human lymphocytes	−55 ± 2.3 (SEM; n = 14)	−18[b]	−44
BHK	−83 ± 1.8 (SEM; n = 6)	−48	ND

[a] Lymphocytes were prepared from fresh heparinized blood by the Ficoll-Hypaque method (Boyum, 1968) and membrane potential measured as described in Fig. 1. BHK-21 cells were grown in monolayer and membrane potential measured as previously described (Bashford et al., 1981). All measurements were carried out at 37°C.

[b] Note that this value is close to the equilibrium potassium diffusion potential calculated according to the Nernst equation.

fication reflects either the activity of a proton *pump* or the leak of undissociated acids. It cannot be due to a proton *leak*, because acidification occurs against both the chemical gradient of protons and the negative membrane potential. At 37°C, pH 7.4, the proton efflux has a magnitude of 4.2 ± 0.2 (SEM; n = 8) nmol $H^+/10^6$ cells/min.

It might be expected that, since the plasma membrane potential of Lettré cells appears to be generated by one or more metabolic reactions, it should be more temperature-sensitive than if it were set by diffusion of ions down their concentration gradients. The data in Table 1, however, show that there is no significant change of membrane potential between 16 and 37°C, which confirms a previous observation (Hoffman et al., 1979), and suggests that both the pumps and the leaks have similar, compensating, temperature dependences. It is clear, however, that at no temperature is membrane potential of Lettré cells set by the K^+ diffusion potential: indeed cells at 20°C are less sensitive to alteration of external K^+ than are cells at 37°C and a substantial fraction of the membrane potential is ouabain-sensitive at all temperatures.

Plasma Membrane Potential of Other Cells

The demonstration that Lettré cells can generate a membrane potential of some −55 mV by a mechanism that is independent of inorganic ion gradients poses the question of whether this property is shared by other cells. Table 2 shows that human peripheral lymphocytes in suspension, or BHK cells in monolayer culture, are each partially sensitive to alteration of external K^+, and partially sensitive to ouabain, as previously observed by others (Kiefer et al., 1980; Felber & Brand, 1982). That is, their membrane potential is generated partially by the diffusion of ions down their concentration gradients, and partially by electrogenic pump(s).

The picture that emerges is that a surface membrane potential of around −60 mV can be generated either by diffusion of ions down their chemical gradient, or by electrogenic pump(s), or by a combination of the two. Neurons are clearly at one end of the spectrum, and Lettré cells are at the other. If membrane potential in the latter cells proves to be more than a consequence of pumps that have some other functions, it becomes important to elucidate the cellular controls that operate to limit it at a particular value. Optical methods employing oxonol-V would seem to provide a suitable methodology for monitoring qualitatively and quantitatively, changes of plasma membrane potential in a variety of experimental circumstances.

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