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EFFECT OF OUABAIN AND METABOLIC INHIBITORS ON THE Na AND K MOVEMENTS AND NUCLEOTIDE CONTENTS OF L CELLS

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SUMMARY

1. The general characteristics and Na and K movements of L cells (derived from mouse epithelium) have been measured. Both cells grown in suspension (LS cells) and as a monolayer (L cells) were used.

2. The volume of L cells was $1{\cdot}2 \times 10^{-9}$ cm^3 and of LS cells $3{\cdot}5 \times 10^{-9}$ cm^3; of this 82% was water.

3. Electron micrographs showed the presence of numerous protrusions (filopodia) from both forms of the cell. These had the effect of increasing the surface area of the cell by 2–4 times over smooth cells of the same volume. On changing from the flattened to the spherical shape during trypsinization, the filopodia altered to maintain a constant V/A ratio.

4. These cells contain K, about 170 m-mole/l. intracellular water and Na, 9 m-mole/l. intracellular water (L cells only) at 20° C. The K fluxes are 1·9 p-mole/cm^2 sec for LS cells and 0·8 p-mole/cm^2 sec for L cells and the Na fluxes are 1·8 p-mole/cm^2 sec for L cells (expressed as per total cell surface (including filopodia)). If expressed as p-mole/cell per sec then L and LS cells have the same K flux.

5. 10^{-4} M ouabain reduces the K influx to half, indicating an insensitivity to the glycosides common to the species. In the prolonged presence of ouabain the cells come into a new steady state with a $[K]_1$, of 140 and a $[Na]_1$ of 20–30 m-mole/l. intracellular water, but a constant $[Na + K]_1$.

6. Both DNP (10^{-3} M) and IAA (10^{-4} M) are required for maximum inhibition of K uptake, as both aerobic and anaerobic metabolic pathways may be used to drive the pump.

7. K removal decreases the Na efflux, and Na removal (eventually) decreases the K influx providing evidence for Na/K coupling.

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8. The cells contain 7·5 m-mole/litre intracellular water of ATP, a level some 15 times that of ADP.

9. The Na pump in these cells is very similar to that found in other tissues in that (*a*) it requires K to work, (*b*) it is blocked by ouabain and metabolic inhibitors and (*c*) it transports three molecules of Na for each two molecules of K.

INTRODUCTION

Almost all of the present detailed knowledge of ion distribution and membrane transport has been obtained in a few specialized cells, mainly R.B.C., squid axon and muscle cells (see Baker, 1966; Caldwell, 1968; and Whittam & Wheeler, 1970 for reviews). All have the advantage that the fractional exchanges of Na and K are low, either because the cells have low permeabilities or the cells are very large, and so the fluxes of these ions can be studied over a prolonged period. They have a disadvantage in that these cells are specialized in one way or another; thus squid axon and muscle cells are large cells with high membrane potentials; R.B.C.s are small, non-nucleated cells with low permeabilities. A further, more important disadvantage is that R.B.C.s and squid axons have no genetic material and so cannot adapt to their environment during the course of the experiment.

It is clear that cells in most tissues must have some form of Na pump, but it has proved difficult to obtain detailed information about Na pumping in tissues because (*a*) of the small size of the cells (leading to fast exchanges) and (*b*) of the complexity of the anatomical structure (leading to tortuous extracellular channels), so that it is widely believed that the kind of Na pump found in R.B.C.s and squid axon is generally applicable.

It has been found that the cells from various tissues can be cultured as a monolayer in cell culture, a state which allows of relatively easy measurement of Na pumping (Burrows & Lamb, 1962). The present experiments were designed to measure the Na and K transport properties in a detailed way in one such cell line (L cells derived from mouse subcutaneous tissue), primarily for comparison with the findings in R.B.C.s and squid axon, but also to provide a basis for work in progress on adaptation in cultured cells. The methods used are largely based on earlier work (Burrows & Lamb, 1962); preliminary accounts of the results have appeared (Lamb & MacKinnon, 1967).

METHODS

Suspensions of L cells clone 929 (Sanford, Earle & Likely, 1948) were obtained from Flow Laboratories, Irvine. In the first experiments these were in the LS form, adapted to grow in suspension in a low Ca medium; in later experiments, as L cells (which grew as a monolayer on a surface). The cells were subcultured for 4–6 days

and then used either as suspensions or as monolayers of cells on Petri dishes for the various experiments. At the end of each experiment the cells were counted and the diameter measured; the ion contents were determined and the results expressed as μ-mole/10^9 cells or as m-mole/l. cell water.

Culture methods

The methods used are outlined here, a more detailed account may be found in MacKinnon (1969).

LS cells. Cells were grown in stirrer cultures in a 1 l. aspirator bottle stirred with a Teflon magnet with an inlet and outlet for gassing and an inlet and outlet for feeding and withdrawing the cells (see Danes, 1957; McLimus, Davis, Glover & Rake, 1957). The medium used was Eagle's basal medium (BME) plus glutamine (1·6 mM), and calf serum 5 %, obtained from Flow Laboratories. Penicillin G (50,000 c.u./l.) was added to the growth solutions. This medium contains no added Ca. It was found necessary to keep the cells down to $< 1{\cdot}3 \times 10^6$/ml. to maintain logarithmic growth.

L cells. Cells were grown as a monolayer on plastic Petri dishes. To do so, 4 ml. of a trypsinized suspension of L cells at a concentration of 50,000 cells/ml. was added to each 5 cm Petri dish. The Petri dishes were then stacked in sandwich boxes, equilibrated with 95 % air 5 % CO_2, sealed and incubated at 37° C. Four days later each Petri dish had become covered with a monolayer containing some 500,000 cells. The medium used was BME plus glutamine (1·6 mM), foetal calf serum (10 %) and Penicillin G (50,000 c.u./l.). For a few experiments monolayers of L cells in plastic test tubes were bought from Flow Laboratories.

Measurement of the cell characteristics

Electron microscopy. Both LS and L cells were examined. The sections were prepared by methods similar to those used by Robbins & Jentzsch (1967). LS cells were spun down, fixed in 3 % gluteraldehyde and 3 % sucrose at pH 7·2 for 30 min, washed in 3 % sucrose and post-fixed in 1 % osmium tetroxide at pH 7·2 for 30 min. The cells were then dehydrated, embedded in Araldite (CIBA Ltd.), and sections of 500 μm made. L cells were grown on No. 0 Chance coverslips placed in a Petri dish. They were then treated in the same way as the LS cells, except that the Araldite used for embedding was held in gelatin capsules, inverted over the coverslips. After curing, the cover-slip was shattered with a few drops of liquid nitrogen, leaving the cells on the exposed end of the block.

Cell numbers. These were measured on a Vickers J 12 Cell Counter in a solution of NaCl (119 mM) and citric acid (50 mM). Frequent checks on the instrument were made by counting the cells on a haemocytometer.

Cell viability. A small sample of cell suspension was mixed with 1–2 drops of 1 % Naphthalene Black in Krebs on a cover-slip. Dead cells stain, so that the viability was taken as the % of cell nuclei stained after 1 min. For flux experiments the results are expressed as per viable LS cells. Similar checks on L cells showed few stained cells. This is probably because dead cells do not remain adherent to the plates and so are washed off.

Measurement of the volume and surface area of cells. Initially these were calculated from the diameter of the LS cells or from the diameter of trypsinized L cells in suspension. The diameters of twenty-five to forty cells were measured in a Neubauer haemocytometer with a calibrated micrometer eyepiece, and the cell volume, and volume/surface area (V/A) calculated (these values were then averaged to give mean values for each experiment).

For L cells, this method of measuring cell volume depends on the assumption that trypsinization does not greatly alter the volume of the cell but merely the shape; this problem has been discussed in an earlier paper (Burrows & Lamb, 1962). To check this assumption, the cell volume was also measured by considering L cells as 'right prisms' and as 'oblate spheroids' (i.e. before trypsinization).

Calculation of cell water. The proportion of the total cell occupied by cell water was obtained from experiments in which the external osmotic pressure was raised by KCl and the volume changes in the cells observed 5 min later, the intracellular water being calculated from

$$W = \frac{V_1 - V_2}{1 - \pi_1/\pi_2}, \tag{1}$$

where W is the intracellular water, V_1 the cell volume (from the diameter) in solution with osmotic pressure π_1 and V_2 that in π_2. The cell water in three experiments (in LS cells) was 0·97, 0·93 and $1{\cdot}02 \times 10^{-9}$ cm^3 which gave ratios of cell water/total volume of 0·82, 0·85 and 0·78; a mean value of 0·82. This value was used both in L and LS cells. Attempts to measure cell water directly, by means of extracellular labels, failed.

To measure the effect of the filopodia (see Fig. 1) on the cell V/A a morphometric analysis (Chalkley, Cornfield & Park, 1949) was done. This consists of projecting a grid of short lines of known length on to an E.M. picture, and counting the number of occasions a line end-point fell on the cell or pseudopod and the number of times the lines intersected the cell or pseudopod boundary. From this the ratio of volume to surface ratio of the whole cell can be estimated.

Ion flux measurements

Influx measurements were made by exposing cells to the radioactive form of the ion for periods short compared to the $t_{\frac{1}{2}}$ of exchange and calculated by

$$m_{in} = \frac{d[C_1]^*}{dt} \cdot V/A, \tag{2}$$

where m_{in} is the influx in p-mole/cm^2 sec, C_1^* the concentration of ion entering in time dt. Efflux was measured by loading cells with the isotope and then observing its loss into inactive solution, either by collecting the effluent or by measuring the amount of isotope left in the cells at various times. The efflux was then calculated from the relation

$$m_{out} = \frac{\ln 2}{t_{\frac{1}{2}}} \cdot \frac{V}{A} \cdot [C_1]^*. \tag{3}$$

This equation was derived for steady-state conditions (Keynes & Lewis, 1951). On occasions the intracellular concentration of the ion we were studying was changing with time, and as we did not know the relation between flux and concentration, strictly this equation could not be applied. We did however use this equation if the rate of change of concentration was small compared to the resting flux. Under all experimental conditions blank plates containing no cells were run. These blank values were then subtracted from the experimental values.

LS cells. The methods used were similar to those for R.B.C.s (Glynn, 1956) and involved centrifugation and resuspension of the cells. After loading with isotope, three washes were carried out to clear the extracellular activity; each took 3 min. The first was at the experimental temperature, the others at 0° C. The time for the first centrifugation was added to the influx time, as experiment showed this to be neces-

sary. Because the time resolution of the method was quite long and Na movements were fast (see later), no Na was ever detected in these cells.

L cells. The methods were similar to those detailed by Burrows & Lamb (1962) except that the cells were grown as a monolayer on plastic Petri dishes (5 or 9 cm diameter). To change solution the supernatant was poured off, the remaining few drops sucked off and fresh solution poured on. In this way six fluid changes could be made in 30 sec. To measure Na contents these washes were done with ice-cold Krebs. Control experiments showed that probably less than 5 % of the intracellular Na was lost in this time.

All radioactivity measurements were made in a Tricarb. Scintillation Counter against similarly prepared standards; ^{24}Na and ^{42}K were generally counted by the Cerenkov effect (Garrahan & Glynn, 1966).

Ion contents

Flame photometer. Total K content was measured by washing cells with ice-cold Ca-sorbitol solution, extracting in distilled water and measuring on an E.E.L. flame photometer against appropriate standards. In L cells with raised $[Na]_i$ this method could also be used for Na measurement.

Isotope. All ion contents were also measured by soaking in radioactive Krebs to equilibrium, then washing in ice-cold inactive Krebs and counting the resulting activity left in the cells. Preliminary experiments showed that the K results so obtained were not significantly different from those obtained by flame photometry, indicating complete K exchange.

Estimation of nucleotides in L cells

The method used was based on that of Wilson (1968) and Wilson & Thompson (1969). Approximately 200×10^6 cells were used for each nucleotide estimation, this quantity being grown in six Roux flasks. The cells in each flask were removed with Trypsin for 3 min and thereafter handled at 0° C. After precipitation with perchloric acid the nucleotides were separated by ion-exchange chromatography and extinction readings taken against air at 260 mμ on a Hilger H 700 Uvispek spectrophotometer. Inorganic phosphate was measured by the method of Fiske & Subbarrow (1925), which uses the blue colour which develops when reduced phosphomolybdate is formed. This was measured at 675 mμ in the Uvispek. A correction was applied for extracellular phosphate (measured by using [^{14}C]sucrose as a label); this was about 15 % of the total.

Solutions used

The basic experimental solution was Krebs containing (mmole/l.) Na^+ 136·58; K^+ 5·65; Ca^{2+} 2·8; Mg^{2+} 1·17; Cl^- 146·96; PO_4^{2-} 0·58; SO_4^{2-} 1·17; glucose 10·98; plus Phenol Red 0·0002 % and calf serum 5 %. Initially attempts were made to use a bicarbonate buffer; this was abandoned and a Tris buffer used. Most experiments were done in Tris citrate (16 and 5 mM respectively). Direct experiment with a frog's ventricle showed that the presence of citrate reduced the ionized Ca^{2+} to 0·8 m-mole/l. from a total (free and bound) of 2·8 m-mole/l.

Drugs used

These were made up as required. Ouabain from ouabaine glucoside (Laboratory Nativelle, Ltd). DNP as the sodium salt of 2,4-dinitrophenol (B.D.H. Ltd). Iodoacetic acid from B.D.H. Ltd. All concentrations refer to the salt. The pH was always checked and adjusted to 7·4 as necessary.

RESULTS

General characteristics of L and LS cells

Volume. The average volume of LS cells was $1{\cdot}20 \times 10^{-9}$ cm^3 per cell (Table 1). The volume of L cells measured in the three ways was in the range of $3\text{–}4 \times 10^{-9}$ cm^3 per cell (Table 1) and, as these volumes were not significantly different from each other, a mean value of $3{\cdot}49 \times 10^{-9}$ cm^3

TABLE 1. Volume and surface areas of LS and L cells. These values were obtained from LS cells or trypsinized L cells by measurement of the diameters; in L cells by measurement of the dimensions of the cells growing on a surface and in L and LS cells by a morphometric analysis of E.M. pictures. *N* indicates the number of observations; the errors shown are the S.E. of the means

Cell type	Method of measurement	*N*	Volume ($\times 10^{-9}$ cm^3)	Apparent surface area ($\times 10^{-6}$ cm^2)	*V*/*A* ($\times 10^{-4}$ cm)
LS cells	Diameter measured	1575	$1{\cdot}20 \pm 0{\cdot}12$	$5{\cdot}40 \pm 0{\cdot}51$	2·30
	Morphometric analysis of E.M. pictures	6	—	—	$1{\cdot}13 \pm 0{\cdot}11$
L cells	Trypsinized then diameter measured	157	$3{\cdot}07 \pm 0{\cdot}30$	$10{\cdot}35 \pm 0{\cdot}60$	$2{\cdot}91 \pm 0{\cdot}10$
	Cells as right prisms	76	$3{\cdot}70 \pm 0{\cdot}19$	$32{\cdot}96 \pm 0{\cdot}80$	$1{\cdot}12 \pm 0{\cdot}03$
	Cells as oblate spheroids	76	$3{\cdot}76 \pm 0{\cdot}34$	$36{\cdot}85 \pm 3{\cdot}70$	$1{\cdot}02 \pm$
	Morphometric analysis of E.M. pictures (trypsinized)	3	—	—	$0{\cdot}51 \pm 0{\cdot}09$
	(monolayer)	3	—	—	$0{\cdot}55 \pm 0{\cdot}12$

was used in these experiments as the L cell volume. It therefore appears (1) that L cells are three times the volume of LS cells under the conditions of our experiments and (2) trypsinization does not increase the volume significantly.

Surface area. The methods used for volume estimation also gave values for the apparent surface area of LS and L cells and hence allowed calculation of V/A (Table 1). These results suggested that change of L cells from the flattened to the spherical state was accompanied by a considerable decrease in surface area. However, when electron micrographs of these cells were studied (Fig. 1) it became apparent that both types of cell had many fine hair-like processes protruding from them. These 'filopodia' had the effect of greatly increasing the surface area (roughly $\times 2$) with only a small increase in volume ($< 5\%$). From a morphometric analysis of these sections (Chalkley *et al.* 1949) a V/A of $1{\cdot}13\ \mu^{-1}$ was obtained for

LS cells and a V/A of $0{\cdot}53\ \mu^{-1}$ for L cells (Table 1). The following points may therefore be made. (1) If it is considered that the surface membrane of the filopodia take part in ion exchanges, then the V/A calculated from light microscopy is too large, by a factor of about 2. There is no direct

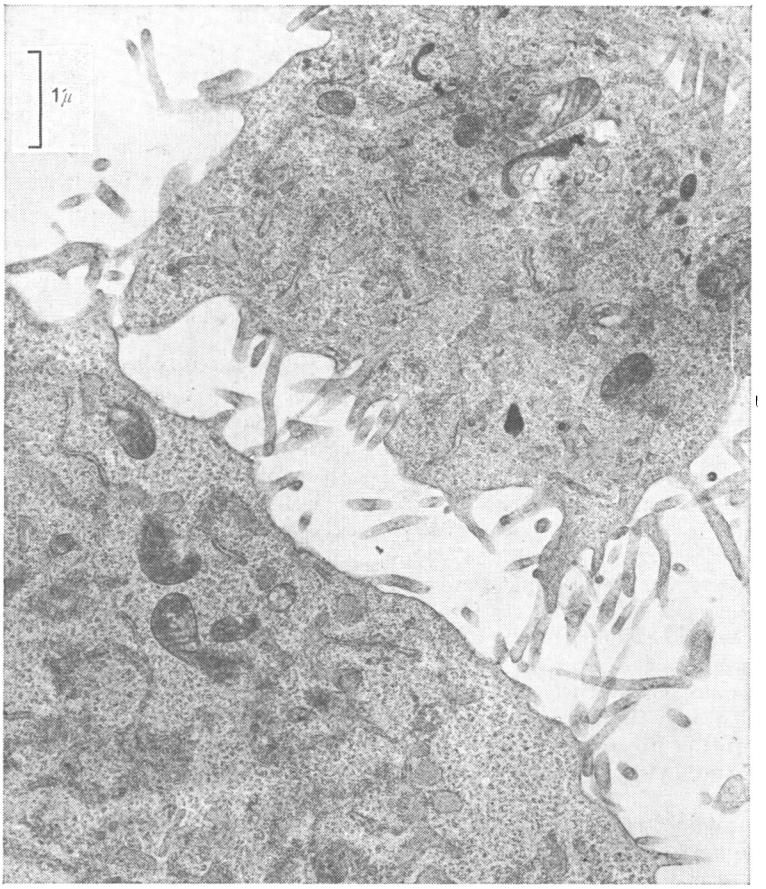

Fig. 1. An electron micrograph of L cells growing as a monolayer. Cells fixed in gluteraldehyde and then in osmium and embedded in Araldite. Sections cut at 500 μm. Note the numerous filopodia present.

evidence on this point, but calculations show that if each filopod is considered as a cylinder of length $1{\cdot}5\ \mu$ and diameter $0{\cdot}1\ \mu$, then diffusion of K within the cylinder ought to be some 1000 times faster than diffusion across the membrane of the filopod (MacKinnon, 1969; assuming that diffusion in the filopods was of the same order as in free solution) and therefore the filopod surface could be available for ion transfer. (2) When L

cells change from flattened to spherical shape on trypsinization then the V/A of the cells does not change. These cells apparently absorb the 'extra' surface membrane by forming more filopodia. This type of mechanism would mean that no change in membrane ionic properties would be required in undergoing a change in shape.

Area available for exchange. Burrows & Lamb (1962) calculated that the membrane of cells next to the supporting surface was available for ion transfer, but Borle (1969) has recently assumed this was not so. During the course of the present work, numerous E.M. pictures were taken of L cells showing the space between them and the supporting medium. This again showed that only about 5% of the cell membrane was within 1000 A° of the supporting surface. As the experiments were usually carried out with a cell density such that gaps existed between cells, it is probable that the whole membrane of the L cell is available for ion transfer. The figures in Table 1 have been calculated on this basis.

Volume changes associated with L cell growth and population density. L cells were grown in a standard way in these experiments; they were plated out on a Friday at a density of 50,000/ml. and used on the following Tuesday. In some later experiments, thicker cultures were grown for a longer period. We noticed that these cells were clearly smaller than usual. A limited investigation of this was undertaken, in order to exclude the possibility of this affecting our results.

Twenty 9 cm Petri dishes were plated thickly, and the cell numbers, cell volume and K content measured at 15, 43, 64 and 86 hr after the cells started growing. The first three of these measurements were during the logarithmic phase of growth, the last as the cells entered the stationary phase. Fig. 2 shows the cell volume plotted against cell density for this experiment (open circles). As the density (i.e. cells per square cm of plate) increases, the volume decreases ($r = -0{\cdot}99$, $P < 0{\cdot}001$). These results could also be due to 'ageing' of the culture, as the observations were made chronologically. However, results obtained in older cultures in plates (+) and in a thickly plated Roux flask (Δ) at other times during these experiments fit this relationship quite closely, suggesting that crowding is the cause of the decrease in cell volume.

Cell protein was also measured on two of the cultures, by the method of Lowry, Roseborough, Farr & Randall (1951). The results are shown in Fig. 2 (filled circles), and are seen to decrease in proportion to the cell volume. It was therefore assumed that as the cell volume decreases, both the dry weight and water content go down in proportion. The total K in these cells was also measured and the intracellular K concentration calculated. It was found that this remained constant at 180 m-mole/l. intracellular water over this range of cell size in this experiment.

Normal ion fluxes and contents

The experimental solutions were somewhat different from the growth solutions, due (*a*) to the difficulty of using a bicarbonate buffer under experimental conditions and (*b*) to the fact that the experiments were done at room temperatures whereas the cells were grown at 37° C. It was therefore found necessary to equilibrate cells in Krebs before starting the

various experiments. Preliminary experiments indicated that a time of 90 min was sufficient and this was adopted as the standard pre-incubation time.

K fluxes and contents. The K influx was measured by a 5 min exposure to [^{42}K]Krebs, the content by equilibration in [^{42}K]Krebs or by flame photometry. The K efflux was measured by first equilibrating the cells in

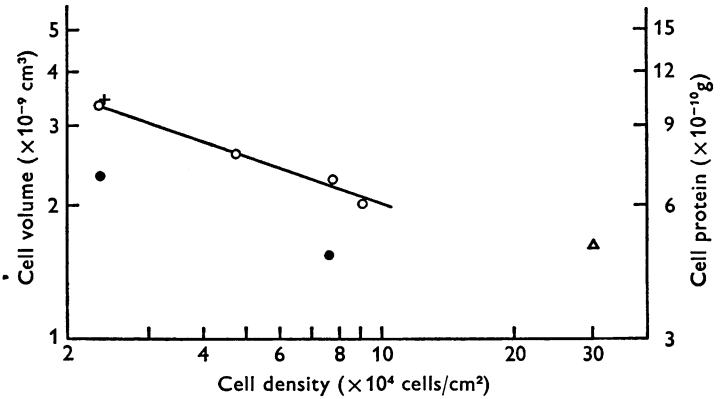

Fig. 2. Relationship between L cell density and cell volume or protein content. Circles show single observations made chronologically as the culture grew; open circles cell volume, filled circles cell protein. + and Δ show cell volume from other experiments. Log-log plots. Cell volume correlated to cell density ($r = -0{\cdot}99$, $P < 0{\cdot}001$).

TABLE 2. The Na and K contents and fluxes in normal Krebs. Contents as m-mole/l. intracellular water, fluxes as p-mole/cm² sec both ± S.E. with number of observations in brackets. * represents one experiment with five plates. The K fluxes in L cells are significantly lower than in LS cells ($P < 0{\cdot}001$), the other corresponding values are not significantly different

	$[K]_i$	K influx	K efflux
LS cells	176 ± 7 (23)	1·92 ± 0·08 (91)	1·95 ± 0·15 (5)
L cells	167 ± 5 (15)	0·82 ± 0·11 (30)	0·79*
	$[Na]_i$	Na influx	Na efflux
LS cells	—	—	—
L cell	8·60 ± 0·62 (41)	1·77 ± 0·3 (6)	1·83 ± 0·19 (13)

[^{42}K]Krebs and following the loss of K into inactive Krebs for 1–3 hr. Table 2 shows the results of these experiments. The K concentration did not differ significantly between L and LS cells at about 170 m-mole/l. cell water and did not change with time in Krebs. The influx and efflux are not significantly different, confirming that the cells are in a steady state. The fluxes in LS cells are about twice that in L cells when calculated per surface area. If, however, they are calculated per cell they are the same (this also applies to the ouabain sensitive influx – next section). These two

forms of the cell have similar genetic material and perhaps have similar capabilities of transferring K per nucleus.

Na fluxes and contents. These could not be measured in LS cells. This is probably explained by the observation that the $t_{\frac{1}{2}}$ of exchange in L cells was 2–5 min so that all the Na had left the cells by the end of the 10 min required for centrifugation to be complete. In L cells the intracellular Na concentration found was 8·6 m-mole/l. intracellular water and remained at this value over the 6–8 hr it was studied (Fig. 3*a*). The influxes and efflux were not significantly different from each other in L cells at a value of about 1·8 p-mole/cm² sec (Table 2).

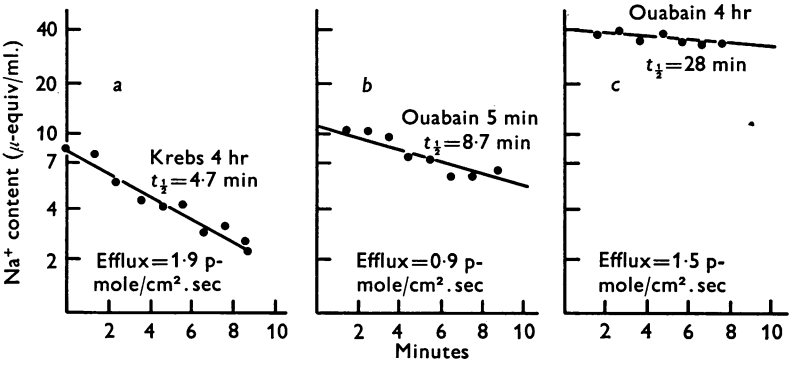

Fig. 3. Typical Na efflux experiments in (*a*) Krebs, and Krebs + 10^{-3} M ouabain after (*b*) 5 min and (*c*) 4 hr. Cells previously equilibrated in Na Krebs, then washed in inactive Krebs from time 0. Each point represents a single plate of some 1×10^6 cells. Lines fitted by method of least squares.

Agents acting on the fluxes and contents

Ouabain. The immediate effect of 10^{-3} M ouabain was to reduce the K influx and the Na efflux (Figs. 3 and 4) without altering the K efflux or Na influx. Therefore the cells gained Na and lost K. However, with continued exposure to ouabain we found that the Na efflux and K influx returned towards the control values (Fig. 4, upper) and the intracellular Na and K settled down at new steady-state values (Fig. 4, lower). The fact that the K efflux returns to 'normal' despite a lower intracellular concentration is due to a shorter $t_{\frac{1}{2}}$ of exchange of K (normally about 100 min). This is presumably due to an increased leakiness of the cell to potassium as the intracellular concentration falls.

In this new steady state, metabolic inhibitors still reduce the K influx and K removal still reduces the Na efflux as in normal cells (see next section), so that Na and K are still being pumped across the cell membrane. Initially we thought that this was a manifestation of 'desensitization' to

ouabain and as such would have been of considerable interest. Our current hypothesis is that this arises because L cells are rather insensitive to ouabain so that even in 10^{-3} M a residual fraction of the pump remains unblocked; as the $[Na]_i$ rises this fraction increases until it balances the inward Na leak and so the cell comes into a new steady state.

In support of this hypothesis, it was found that half maximal inhibition of pumping in L cells occurs at 10^{-4} M ouabain, a reduction in sensitivity of about a hundredfold compared to human cultured cells (J. F. Lamb &

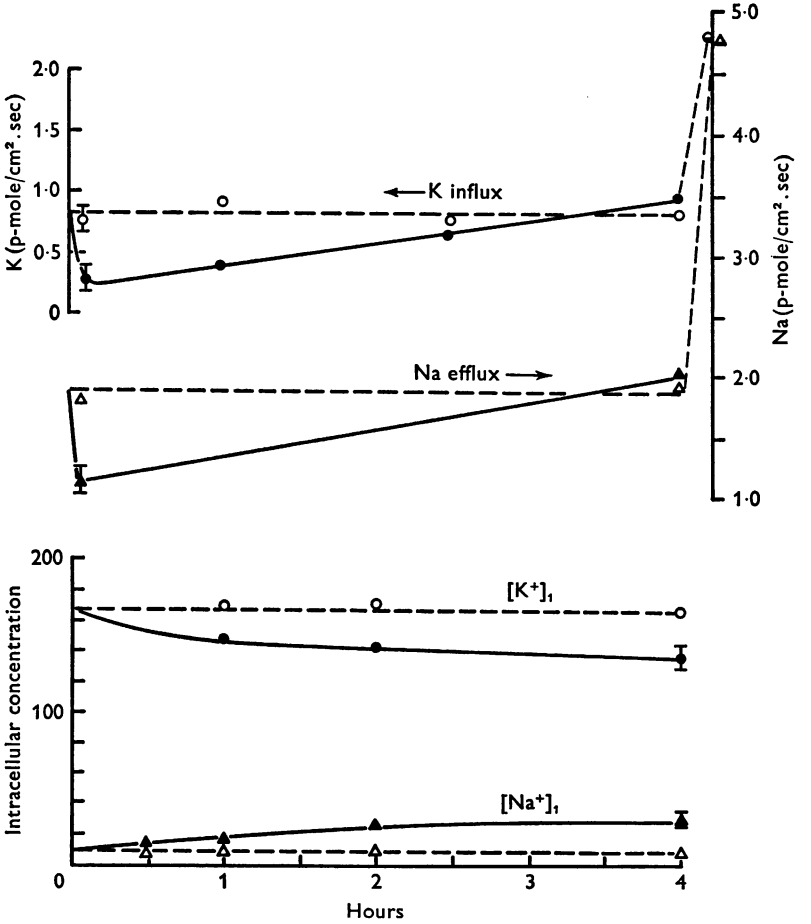

Fig. 4. The effect of prolonged ouabain treatment on the K influx, the Na efflux and the Na and K concentrations in L cells. K, circles; Na, triangles. Open symbols in Krebs; filled symbols in Krebs + ouabain 10^{-3} M; semi-filled symbols recovery in Krebs after ouabain. Each point represents the mean of six to twenty single observations. Typical errors (± 2 S.E.) are shown. All corresponding mean values significantly different ($P < 0{\cdot}01$) except for K influx at $2\frac{1}{2}$ and 4 hr and Na efflux at 4 hr.

D. McCall, unpublished observations) and to squid axon (Baker, Blaustein, Keynes, Manil, Shaw & Steinhardt, 1969). To test this hypothesis further we loaded L cells with various Na concentrations by pre-incubation in K-free Krebs and then measured the Na fluxes under various conditions. Fig. 5 shows the results. It can be seen that (1) Na efflux rises both with and without ouabain but with a different slope and (2) Na influx also rises

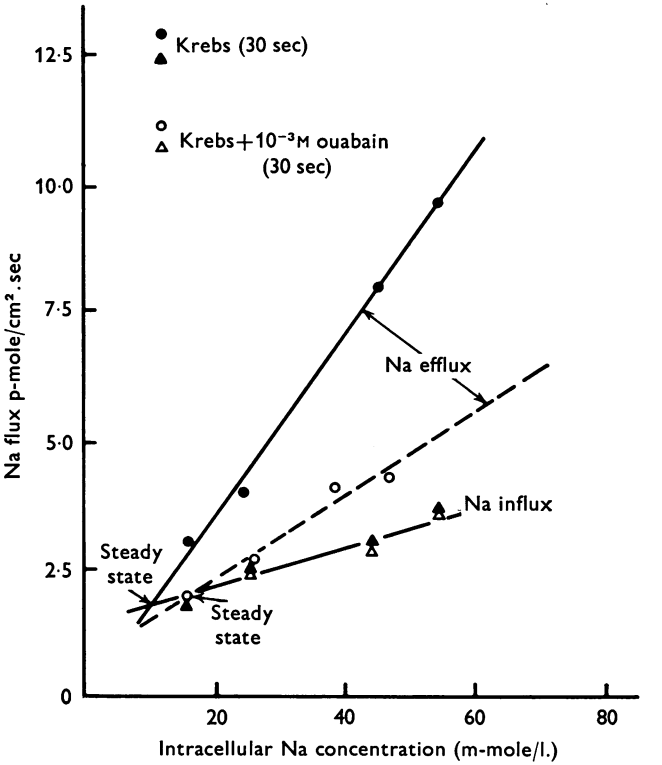

Fig. 5. The Na fluxes at various intracellular Na concentrations in the presence and absence of ouabain. $[Na]_i$ raised by prior incubation in K-free Krebs, followed by 30 sec washing in Krebs or Krebs + ouabain 10^{-3} M. Circles, efflux, triangles influx, filled symbols Krebs, open symbols ouabain 10^{-3} M. Each efflux point obtained from four to five plates (as in Fig. 3), each influx point from one plate. Lines fitted by method of least squares. The effect of ouabain is to move the steady state $[Na]_i$ to a higher value.

but the rise is not affected by ouabain. These results show that there are two values of $[Na]_i$ at which the cells are in a steady state: (*a*) about 8 m-mole/l. intracellular water in Krebs and (*b*) about 20 m-mole/l. in Krebs + ouabain. This experiment therefore is in agreement with the hypothesis proposed above; it also shows that there is no 'desensitization' to ouabain, as cells loaded with Na in K-free conditions and then trans-

ferred to ouabain have fluxes similar to other cells equilibrated in ouabain but with the same $[Na]_i$.

Recovery from ouabain. On returning cells to Krebs from ouabain 10^{-3} M, the K influx and Na efflux both increased very markedly within 5 min (Fig. 4). The Na efflux measured under these conditions was similar to that measured in cells where $[Na]_i$ was raised to a similar level by incubating in K-free Krebs and then returning to Krebs (Fig. 5). This evidence is interpreted to mean that the ouabain effect is rapidly and completely reversible.

Effect of metabolic inhibitors. The inhibitors DNP and IAA were applied to LS cells for periods greater than 30 min and the K influx measured.

TABLE 3. The combined and separate effect of DNP and IAA on K influx in LS cells. Each result is the influx in p-mole/cm² sec in one plate. DNP and/or IAA applied for 30 min before influx measured. Analysis of variance showed that the DNP and IAA effects are significant at $P < 0{\cdot}001$ and are additive so that a maximum inhibition (90 %) is obtained with both present

Krebs	DNP (10^{-3} M)	IAA (10^{-4} M)	DNP 10^{-3} M IAA 10^{-4} M
2·67	0·77	2·03	0·21
2·91	0·86	2·07	0·34

In preliminary experiments it was found that increasing amounts of DNP produced increasing inhibition up to a maximum effect at 10^{-3} M; similarly IAA produced a maximum effect at 10^{-4} M. In a further eight experiments the effects of 10^{-3} M-DNP and 10^{-4} M-IAA for 30 min were measured. These results are shown in Table 3. It can be seen that both DNP and IAA inhibit K influx, but that a maximum inhibition (of 90 %) is obtained when both are present. In other experiments these inhibitors produced the expected effects on the $[K]_i$ in LS cells. No measurements of inhibitors on Na movements were made. The presence of both aerobic and anaerobic inhibitors are required because L cells can produce ATP both by aerobic and anaerobic pathways (Danes & Paul, 1961).

Interactions between Na and K

K on Na efflux. Removal of external K led to a decrease in the efflux of Na in L cells. The average efflux in K-free Krebs was $0{\cdot}96 \pm 0{\cdot}06$ ($n = 6$) p-mole/cm² sec compared to a control of $1{\cdot}81 \pm 0{\cdot}25$ ($n = 5$) p-mole/cm² sec, a reduction of 0·85 p-mole/cm² sec (53 % $P < 0{\cdot}01$). This reduction was not significantly greater than that caused by the immediate addition of ouabain. It was found however, that in the experiments in which $[Na]_i$ was raised (Fig. 5), the Na efflux into K-free Krebs was consistently less than into Krebs containing ouabain 10^{-3} M ($P < 0{\cdot}01$, Fig. 57 MacKinnon, 1969). We therefore consider that K-free Krebs causes a greater inhibition than ouabain at all levels of $[Na]_i$. In cells equilibrated in 10^{-3} M ouabain,

K removal caused a reduction in Na efflux from $2{\cdot}06 \pm 0{\cdot}16$ ($n = 6$) to $1{\cdot}27 \pm 0{\cdot}22$ ($n = 4$) p-mole/cm^2.sec, a reduction to 62% ($P < 0{\cdot}02$), showing that external K was still necessary for the Na efflux in this condition.

Na on K influx. In these experiments external Na was replaced isosmotically by sorbitol or choline chloride, the cell Na allowed to equilibrate (10 min) and then the K influx measured. Fig. 6 shows the results. It can

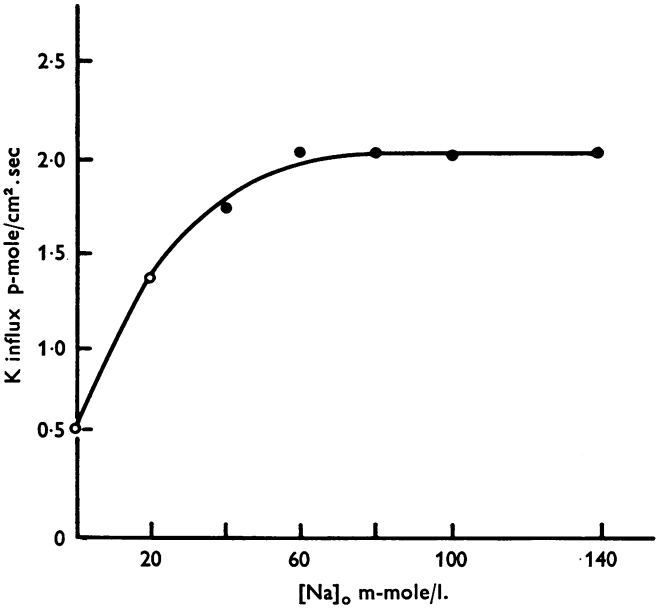

Fig. 6. The effect of $[Na]_o$ variation on the K influx in LS cells. $[Na]_o$ replaced isosmotically by sorbitol or choline chloride for 30 min before the influx measured. Each point represents the average of five experiments except for the '0' Na value which represents eighteen experiments. The open circles are significantly different from the control value ($P < 0{\cdot}01$) the filled circles not.

be seen that decrease in $[Na]_o$ leads to a decline in K influx, so that at Na levels < 1 mM, the influx is $0{\cdot}49 \pm 0{\cdot}03$ (17) compared to a control of $2{\cdot}05 \pm 0{\cdot}70$ (16) a decrease to 24% ($P < 0{\cdot}001$). This is presumably largely a reflexion of a decrease in the intracellular sodium which leads to a diminished pumping rate. This effect was still present in cells equilibrated in ouabain 10^{-3} M.

The intracellular concentrations of ATP, ADP, P and other nucleotides

Table 4 shows the results of three experiments in which these nucleotides were measured at 20° C in Krebs, K-free Krebs or Krebs + 10^{-3} M ouabain.

This experiment shows that ATP and UDP Co-enzyme are present in high concentrations compared to ADP and AMP, giving a ratio of [ATP]/[ADP] of 15/1. K-free Krebs and ouabain had no effect on these levels. It is odd that ouabain and K removal have no effect on the nucleotide levels. This may be because the pumped fluxes here are too small.

TABLE 4. The nucleotide concentrations in L cells. Means are concentrations in intracellular water and are themselves each derived from three experiments in Krebs, Krebs + ouabain, and K-free Krebs for 4 hr. As there was no significant differences between these treatments, the results have been averaged

	Concentration (m-mole/l. H_2O)	
Nucleotide	Mean	S.E.
UDP-Co	8·638	± 1·594
ATP	7·521	± 0·419
ADP	0·533	± 0·146
AMP	0·276	± 0·099
GTP	1·852	± 0·283
UTP	1·552	± 0·081
NAD	1·046	± 0·137
GDP	0·829	± 0·128
P	5·394	± 0·587

DISCUSSION

The surface of these cells is complex, due to the presence of numerous processes. The primary function of these is not clear, but one use to which they are put is to 'store' membrane required when the cells change shape. Calculations suggest that from a diffusional point of view these filopodia could behave in the same way as the rest of the cell surface; for this reason they have been included in the estimate of cell surface. This possibility should, however, be tested more directly.

Both forms of cell studied have similar genetic material but have been cultured to grow on a surface (L) or in suspension (LS). Our results show that the K flux per unit apparent area for these cells is different by a factor of 2. However, when these results are expressed as flux per cell they are identical. Similar results were obtained for cells of different sizes due (*a*) to crowding and (*b*) to osmotic treatment (these results were less complete in that no information is available on the E.M. appearances of the cell membranes under these conditions). These results all show that the K flux per nucleus is constant under a variety of conditions. One attractive hypothesis would be to suppose that the number of pump sites and the passive leak of the cell membrane was genetically determined and remained constant despite changes in the cell size.

It is well known (see Baker *et al.* 1969) that no one method may be used

to define the active transport of Na and K across a cell's membrane. The present work also shows this. Thus when various inhibitors are used for short periods of time, so that the intracellular Na and K concentrations do not change much, then in LS cells the K influx is decreased to 10% by DNP + IAA, to 24% by the removal of Na and to 33% by ouabain 10^{-3} M; in L cells the Na efflux is reduced to 52% by K removal and to 65% by ouabain. The general difficulty in interpretation is that none of these treatments acts specifically and completely on the Na/K pump. Nevertheless, the present results show that ouabain, at the high concentration of 10^{-3} M, does not completely block the Na/K pump. This is based on the finding that the cells come into a new steady state with raised $[Na]_i$ and a lowered $[K]_i$ in the presence of 10^{-3} ouabain, and yet are still pumping Na and K across their membranes. At first the present results were confusing because the Na efflux and K influx return to normal in ouabain after an initial period of inhibition. It is now clear that this is due to the rise in the $[Na]_i$ with time in ouabain which increases the absolute Na and K pumping. In retrospect, therefore, it is apparent that L cells were not the best choice for these experiments, as their sensitivity to ouabain is low and hence the Na/K pump is not completely blocked. It is of some interest that their insensitivity to ouabain is similar to that of the species (mouse) from which they were derived some 25 years ago (Straub, 1924). Therefore the whole animal insensitivity is reflected in a membrane insensitivity and this persists through many generations in isolation.

The immediate effect of ouabain application is to decrease the Na efflux by 0·68 p-mole/cm^2 sec and the K influx by 0·46 p-mole/cm^2 sec with no change in Na influx or the K efflux. If each ouabain molecule binds to a pump site and inactivates it then these pumps transfer three molecules of Na and two molecules of K, a figure similar to that found in R.B.C.s and nerve (Garrahan & Glynn, 1967; Baker *et al.* 1969).

Na removal stops K pumping in these cells. We think this is another effect due to the high fractional exchanges occurring, for within 5–10 min of entering a Na-free medium, no intracellular Na is left and so no K pumping is possible. In large cells the intracellular sodium does not change markedly in this time and so interactions of Na and K at the outside of the membrane may then be studied.

Increases in initial intracellular $[Na]_i$ (by prior incubation in K-free medium) results in approximately proportionate increases in all the Na exchanges. At the maximum $[Na]_i$ reached (60 m-mole/l. cell water) in these experiments, there was no evidence of a saturation of the Na efflux, a result similar to that in squid axon (Baker *et al.* 1969). The increase in passive exchange is curious in that this does not occur in most other cells studied, and it is not inhibited by ouabain. A possible explanation for

both these observations would be to suppose that as the intracellular Na rises, more pump sites are mobilized and these also increase the leakiness of the cell membrane.

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