EVIDENCE FOR THE GENETIC CONTROL OF THE SODIUM PUMP DENSITY IN HELA CELLS

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SUMMARY

1. HeLa cells were grown in normal and altered growth solutions; the ion contents, volumes, K sensitive ouabain binding, the Na-K-ATPase and the Na and K transport measured.

2. Cells grown in 1×10^{-4} M ethacrynate or low-K media for 24 hr have a raised $[Na]_i$, a decreased $[K]_i$, and an increased ouabain binding. Those grown in low-K also have an increased Na-K-ATPase activity.

3. When cells are put into low-K solutions the $[Na]_i$ initially rises to a high value, and then starts to fall some 8 hours later as the ouabain binding increases, suggesting that these additional sites represent working Na pumps. Flux measurements on low-K cells provide some support for this view.

4. Experiments in which sorbitol replaced $[Na]_o$ showed that the increased ouabain binding and Na-K-ATPase was related to the increase in $[Na]_i$ rather than the decrease in $[K]_i$ and was not due to a non-specific effect of $[K]_o$ change.

5. The protein synthesis inhibitors cycloheximide and puromycin stopped the effect of ethacrynate and low-K solutions on increased ouabain binding. They also decreased the ouabain binding and K influx in normal cells over 24 hr. Cycloheximide had similar effects on Na-K-ATPase in low-K treated and normal cells. These results suggest that protein synthesis is required for the appearance of more ouabain sensitive sites in the cell membrane, both in response to ethacrynate and low-K treatment and for normal replacement during the cell's life.

6. The RNA synthesis inhibitors actinomycin D (AMD) and cordycepin had complex effects on ouabain binding in fresh and ethacrynate

treated cells. These inhibitors increased the ouabain binding but decreased the K influx. This discrepancy was due to the appearance of ouabain binding sites with different characteristics from normal sites. A limited investigation of this phenomenon was carried out. Probably AMD stops the normal replacement of sites in the membrane.

7. These results are consistent with the hypothesis that HeLa cells have a system for controlling the number of Na pumps in their membranes. This system responds to the level of $[Na]_i$ within the cell and involves protein synthesis. It is not clear to what extent the nucleus is normally involved in this process.

INTRODUCTION

Over the past 20 years the detailed properties of Na pumping in erythrocytes, squid axons and some other tissues has been intensively studied. Very little information, however, is available about the mechanisms which must exist in cells for matching the number of Na pumping sites to the passive permeability of the cell membrane (Baker & Willis, 1972). One way of examining this point is to produce a mismatch between the active transport and the passive leak of a cell by reducing the total activity of the Na-K pumping system, and then measuring the response of the cell. In previous experiments (Lamb & McCall, 1971; Boardman, Lamb & McCall, 1972) we used low concentrations of ouabain to produce this partial reduction and showed that the cells then produced more ouabain binding sites (representing more Na pumps) and perhaps also decreased their passive leak. These experiments also showed that the number of new sites produced was closely related to the rise in $[Na]_i$ rather than the fall in $[K]_i$ produced by the partial ouabain block, suggesting a causal relationship.

The present paper is primarily concerned with a further examination of this problem in HeLa cells in which active transport was diminished by ethacrynate or by growth in low-K solutions. A start has also been made on the analysis of the cellular mechanisms involved in this process. The conclusion reached is that the rise in $[Na]_i$ produced by these blocking procedures switches on protein synthesis and that this then causes the appearance of new Na pumping sites. Preliminary reports of these experiments have appeared (Lamb & Newton, 1973; Lamb & Polson, 1973; Polson, 1973).

METHODS

HeLa cells (Clone S3; Gey, Coffman & Kubicek, 1952) obtained from Flow Laboratories, Irvine, were serially cultured in Roux flasks in the laboratory. For each experiment the cells were trypsinized and subcultured on 9 cm plastic Petri dishes (Falcon) or Roux flasks for 4–6 days in Eagles basal medium (BME) plus

glutamine (2 mM), calf serum (10 %) and gentamycin (2 μg/ml.). Addition of drugs or alteration of ion contents of the growth medium was generally done 1 day before the end of the experiment. To measure the ouabain uptake the cells were exposed to 2×10^{-7} M [³H]ouabain in K-free or 15 K Krebs for 1 hr. For Na, K and Li contents the cells were washed 4 times in 30 sec with ice-cold Ca-sorbitol buffered solution, then extracted in 5–10 ml. ion-free water for several hours and assayed against suitable standards on an EEL flame photometer. Further details of these techniques are given in Lamb & McKinnon (1971), Lamb & McCall (1972) and Boardman, Lamb & McCall (1972) and in the captions to the Figures.

ATPase assay procedures

Cells were grown in Roux flasks in the usual way. They were then harvested either by scraping off with a rubber policeman or by trypsin, and an aliquot counted for cell numbers. The further analysis was either carried out on the crude preparation or on the membrane fraction.

(1) *Crude cell preparation.* The cells were ruptured with 10 mM Tris and then washed $\times 3$, each time being spun down at 15,000 rev/min for 45 min, and the ATPase activity and protein content measured. The K sensitive and ouabain sensitive fractions were not significantly different and constituted about 30 % of the total. These measurements were averaged for statistical analysis.

(2) *The membrane fraction* was prepared by a technique based on that of Boone, Ford, Bond, Stuart & Lorenz (1969). In this the cells were washed with histidine buffered saline, trypsinized (0·025 %), neutralized and then spun at 300 g for 25 min, and resuspended in 50 ml. EDTA Tris at 0° C and then sonicated on ice for 3 min at 20 W. The nuclei and mitochondria were spun down at 1000 g for 15 min and the supernatant spun at 20,000 g for 30 min. The resultant pellet was re-suspended in 10 ml. Tris. Electron microscopic examination of this material confirmed the absence of mitochondria and nuclear material. This material was then used to assay ATPase activity and for protein analysis. Each ATPase assay was done in triplicate with a variability of individual values of 5 % from the mean. The ouabain sensitive ATPase values obtained were very similar to those of Wheeler & Whittam (1970).

The Na-K-ATPase had the following characteristics (1) a pH optimum of 7·5, a Mg^{2+} optimum of 1 mM and a temperature optimum of 37° C, (2) the K_m value for ATP was 0·6 mM and for K was 0·7 mM, (3) the optimal Na effect was obtained with concentrations between 20 and 100 mM, (4) excess Na or K concentrations caused a decreased activity.

ATPase assay. The cell fraction or membranes were incubated in a medium containing 1 mM-Mg^{2+}, 58 mM-Na, 10 mM-Cn^-, 0·1 mM-EDTA, 5 mM-K^+, 88 mM Tris and 1 mM-ATP. The Na-K-ATPase was taken to be the activity which was inhibited by 0·1 mM ouabain or lack of K^+ in the incubation medium. In the standard method of Bonting & Carravaggio (1963) for the estimation of P_1, the final colour increases due to instability of ATP in the presence of acid molybdate, therefore phosphomolybdate was extracted from ATP into ethylacetate as in the Wohler & Wollenberger (1958) method (see also Post & Sen, 1967). Tungstosilicic acid was added to minimize the absorption of phosphomolybdate to denatured protein.

Protein was measured by the Lowry technique (Lowry, Rosebrough, Farr & Randall, 1951). Preliminary experiments showed that the K, Na and pH sensitivities of the ATPase activity of these cells were similar to those from human erythrocytes and that the assay conditions were optimal.

The other solutions used in these assays were: histidine buffered saline, 0·023 M-NaCl, 0·005 M histidine, pH 7·4; Tris magnesium buffer; 0·01 M Tris, 0·001 M-$MgCl_2$,

pH 7·4 adjusted with HCl; EDTA-Tris lysing solution; 0·01 M-EDTA, 0·02 M Tris, pH 7·4.

Measurement of cell numbers and volume. This was done in 'isoton' counting fluid in a Coulter counter, generally within 30 min of diluting the cell sample. In early experiments a model A was used for the cell number and the volume obtained by recounting 10 times at different volume thresholds. In later experiments a model ZF was used for cell numbers and the volume determined with a Coulter Channelyser C 1000 in one count. In each case the calibration of the instrument was checked regularly with plastic spheres. The size of these spheres was measured with a micrometer eyepiece at × 40 magnification (see Reed, Hughes, Taylor & Bruce, 1969 for a fuller discussion of the problem). Fig. 1 shows the type of display
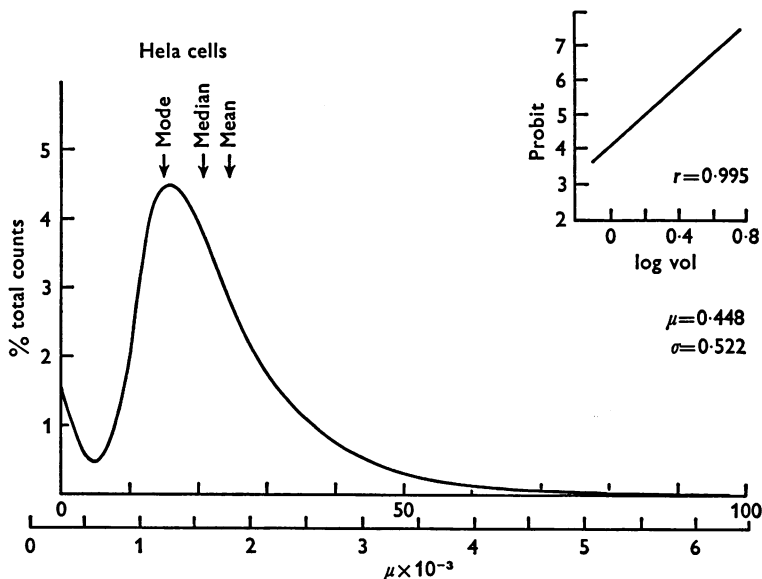

Fig. 1. Size distribution of HeLa cells. A Roux flask containing a monolayer of some 30×10^6 cells was trypsinized and the cells suspended in 'isoton'. A sample of about 30,000 cells was drawn through a Coulter counter (model ZF) and the resulting pulse heights displayed on a C-1000 pulse-height analyser. The main Figure shows a continuous curve drawn through the % counts in each of the 100 channels, the abscissa shows both the channel numbers and the volume. The inset figure shows a probit-log vol plot of the same data. It is clear that the volumes are distributed log-normally with (in this case) a μ of 0·448 and σ of 0·522. For routine use a curve such as that shown was integrated by taking 10 points only and using Simpson's Rule.

obtained on the C 1000 analyser, from which it is clear that the cells show a close fit to a log normal distribution with (in this case) a μ of 0·448 and σ of 0·522. (The standard deviation is calculated from

$$\text{S.D.} = \log_e \left[\frac{1}{2} \left(\frac{^x 50 \%}{^x 16 \%} + \frac{^x 84 \%}{^x 50 \%} \right) \right]$$

as given in Aitchison & Brown (1969)). The small peak of counts at low sizes is probably due to broken cells, for procedures which disrupt cells (e.g. severe pipetting) cause this to increase. For the routine counting and sizing of each sample the number of cells in channels 0, 10, 20 . . . 99 were noted and then the cell numbers and volumes computed with Simpson's rule of integration. The accuracy so achieved was within 1 % of that obtained by using the full curve. None of the effects expressed as 'per cell' are due to changes in cell size.

Flux measurements. [86]Rb was used to indicate the K influx in these cells, as preliminary experiments showed that Rb and K are handled similarly by the cells. [22]Na was used for the Na measurements.

Solutions. Cells were grown in Eagles basal medium (BME) with 10 % calf serum and glutamine 1·6 mM. Most of the experiments were done in Krebs containing (m-mole/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 and calf serum 5 or 10 %. In most experiments a Tris-maleate buffer was used. In later experiments this was changed to Tris-HCl because of the high cost of the maleate. We noticed no changes due to this.

Altered growth solutions were produced as follows: a zero-K solution was ordered from Flow Laboratories and sterile molar KCl added to vary the $[K]_o$ (and to produce the appropriate control); Na-free solutions were produced by making up an appropriate salt solution containing sorbitol instead of Na and then adding amino acids etc. Solutions of various $[Na]_o$ values were produced by mixing normal and low-Na media. Generally 10 % calf serum was added to growth media. In the low K experiments this was reduced to 5 % in order to reduce the K concentration contributed by the serum.

RESULTS

Procedures used to decrease active transport

As outlined in the introduction we wished to treat cells with procedures which would decrease Na/K transport, and so lead to a rise in $[Na]_i$ and fall in $[K]_i$ and then observe if the cell responds to this new situation. In preliminary experiments we tried blocking transport with ethacrynate (Bittar, Dick & Fry, 1968) and also with growth in a medium with a K sufficiently low to reduce Na pump activation. These low $[K]_o$ experiments led to the finding that serum concentration itself greatly influenced the Na pump density (Lamb *et al.* 1973) and delayed the low-K experiments described here so that some ethacrynate data was accumulated. These results with ethacrynate are less satisfactory than the low-K data but will be described as they provide supporting data for the other experiments.

Ethacrynate. Table 1 shows that the acute application of ethacrynate does not alter ouabain binding to HeLa cells but that growth in ethacrynate for 24 hr causes an increase in the specific ouabain binding of 39 % with a rise in $[Na]_i$ and fall in $[K]_i$ of about 50 mM. This concentration of ethacrynate does not decrease cell numbers and on removal of the ethacrynate the cells continue to grow normally. Higher concentrations of ethacrynate cause a greater rise in ouabain binding, a greater change in ion concentrations and a large reduction in cell numbers.

These ethacrynate results are therefore similar to the ouabain results

obtained earlier (Boardman *et al.* 1972) in that partial pump block leads to changes in ion levels and an increase in the specific ouabain binding sites. The actual Na, K and ouabain changes in ethacrynate are similar to those obtained earlier with ouabain.

TABLE 1. Effect of ethacrynate on ouabain binding

| | n | Ouabain bound (molecules $\times 10^{-6}$/ cell) | | Specific ouabain bound | $[Na]_i$ | $[K]_i$ |
		K-free	15K			
Fresh cells	8	$1\cdot63 \pm 0\cdot06$	$0\cdot22 \pm 0\cdot04$	$1\cdot40 \pm 0\cdot05$	13 ± 2	183 ± 3
Ethacrynate (10^{-4} M) for 24 hr	8	$2\cdot22 \pm 0\cdot04$	$0\cdot27 \pm 0\cdot04$	$1\cdot95 \pm 0\cdot06$	60 ± 5	131 ± 3
Fresh cells	7	$1\cdot11 \pm 0\cdot02$				
Ethacrynate (10^{-3} M) for 0–1 hr	7	$1\cdot10 \pm 0\cdot02$				

Plates of fresh HeLa cells or cells incubated in ethacrynate were used. Either the ouabain uptake was measured for 1 hr from 2×10^{-7} M [^{3}H]ouabain in 0 or 15K Krebs or the Na and K contents were measured. The results show that acute ethacrynate treatment does not affect the ouabain binding, but that 24 hr treatment does. The effect is to increase the specific ouabain binding (total in K free less that in 15K) by 39 % ($P < 0\cdot001$) with no significant change in the non-specific binding, and to raise $[Na]_i$ and decrease $[K]_i$ by about 50 mM. This ethacrynate concentration has no effect on cell numbers. The acute ethacrynate experiments were done on a different batch of cells from the chronic experiments. Error is S.E.

Growth in low-K medium

Ouabain binding. The external site of the K uptake system in these cells is half-maximally activated at a $[K]_o$ value of about 1 mM in the presence of Na, a value similar to that in erythrocytes (Glynn, 1956). It was therefore anticipated that the $[K]_o$ would need to be reduced below this to produce much effect on the ion contents of the cell. In order to do so the serum concentration was reduced from the usual 10 to 5 % in all these low-K experiments and thus only contributed 0·25 mM to the growth medium. Fig. 2 shows a preliminary experiment in which cells were grown for 24 hr in various levels of $[K]_o$. It is clear that as $[K]_o$ is dropped below 1 mM there is a marked rise in $[Na]_i$, a fall in $[K]_i$ and a rise in the specific ouabain bound. This result is similar to our past results obtained with partial ouabain block (see also Vaughan & Cook, 1972), and with the ethacrynate block of Table 1. The striking difference however is that the change in the intracellular ion concentrations after 24 hr in the presence of this increase in ouabain binding is much less than that obtained with the blocking agents ouabain or ethacrynate. An explanation was that the 'sites' produced in low-K could work whereas those

produced in ouabain and ethacrynate solutions would continue to be blocked by the drugs present.

To investigate this further we followed the time course of the changes which occurred when fresh cells were treated with low-K medium. Fig. 3 shows the collected results of several such experiments. Over the initial 8 hr of this experiment the Na rises and the K falls but the ouabain binding does not change. After this the ouabain binding increases and the $[Na]_i$ and $[K]_i$ start to return towards their normal values. It seems that the initial ion changes produced by this level of low $[K]_o$ is similar to that in ouabain or ethacrynate but that the cells then show a recovery towards a lower level of $[Na]_i$ and a higher level of $[K]_i$ and that this is accompanied by a rise in ouabain binding. Although the first points shown are at 2 hr after entering low K, other experiments showed that the ion changes occurred within 30 min. The initial values of $[Na]_i$ reached as a result of decreasing the activation of the Na pump were close to those expected from calculations based on Caldwell's (1970) model of the Na pump.

The simplest explanation of these results is to suppose that the increased ouabain binding sites represent an increase in the number of active Na/K pumps and that these have caused a decrease in the $[Na]_i$ and a rise in the $[K]_i$. Other possible explanations which we considered were (1) a rise in $[K]_o$ due to the K leaving the cells and so increasing the activation of the Na pump – measurements showed that the $[K]_o$ rose in the first 30 min and then remained constant at 0·4 to 0·6 mM for the rest of the time; (2) a change in the 'affinity' of the external site to K – in two experiments we found no difference between that in low K or in normal cells but an increased V_{max} in the low K cells (we were not able to use this increased V_{max} as an argument for an increased number of sites as with the higher $[Na]_i$ of these cells a greater activity would be expected. This general problem will be considered later in the paper); (3) if the cell reduced its leak then the same pumping rate would suffice to maintain greater ion gradients. We could not devise a way of testing this possibility as the 'passive' fluxes do not obey constant field equations and the cells are too small to measure resistance electrically; (4) kinetics of ouabain binding. The rate of binding of ouabain* to cells grown in low K, the rate of removal of ouabain from the cells and the ouabain bound in 15K, were not different from those parameters in normal cells. From this it was concluded that the ouabain binding sites in low-K cells had similar characteristics to those in normal cells.

Na-K-ATPase. If cells grown in low-K solutions have more Na pumps, then they ought to have more Na-K-ATPase activity per cell. To test this, cells were grown in low-K medium for 24 hr and their specific ouabain binding and Na-K-ATPase measured.

In order to measure their Na-K-ATPase activity cells are usually broken up and their membranes separated. During the preparation of the membrane fraction most of the plasma membrane is lost (see Boone *et al.* 1969 for HeLa cells and Wallach & Lin, 1973 for a general discussion) together with most of the Na-K-ATPase activity. As we were interested in a quantitative assessment of the Na-K-ATPase activity of control and treated
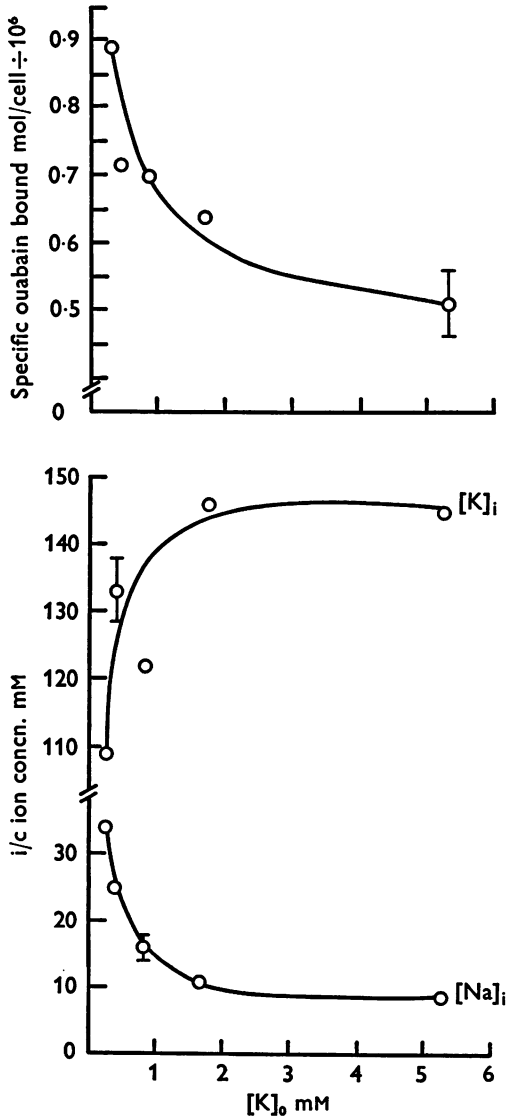

Fig. 2. For legend see facing page.

cells we decided to measure such activity both in crude cell preparations (in which all the cell constituents were still present) and in isolated plasma membrane preparations. Most of the work was done with the crude preparations and the conclusions checked with isolated membrane preparations.

The results of a typical experiment are shown in Fig. 4. The main point is that cells grown in low-K medium have an increased capacity to bind ouabain and split a greater amount of ATP per cell (the ATP split is the increase in the presence of optimal amounts of Na and K over that in K-free or ouabain treated membranes). In this as in two other experiments in which it was tested the increase in ouabain binding and in ATPase activity were not significantly different. In a total of seven such experiments there was an increase of 53 % in Na-K-ATPase per cell ($P < 0.01$ by analysis of variance). These experiments therefore show that growth in low-K medium causes an increased Na-K-ATPase activity per cell of the same order as the increase in ouabain binding. This is consistent with the hypothesis that there are more Na pumps present in the low-K cells.

In two experiments in which the cell membrane fraction was isolated and assayed the mean ouabain sensitive ATPase activity was 0.12 μM-P_1/mg protein.hr in control cells and rose to 0.50 μM-P_1/mg protein.hr in cells treated with low K for 24 hr. In these experiments the mean membrane protein per cell was 9.9 pg/cell and did not differ significantly between control and low K treated cells.

If all the ouabain sensitive ATPase activity of these cells was measured in the crude cell extracts then the average splitting of ATP per site was 32 sec^{-1} in the normal cells of Fig. 4, and about the same in low K cells.

Fig. 2. Effect of growth in various low-K media on ouabain binding and ion contents. Normal growth medium and K-free growth medium were mixed in various proportions and cells grown in the mixtures for 24 hr. At the end of this period the specific ouabain binding and the Na and K contents were measured as usual and the Na and K concentration of the medium analysed. The Figure shows the ouabain bound, and the cellular Na and K concentrations on the ordinate plotted against the final K in the growth medium (abscissa). It is clear that as $[K]_o$ is lowered the cellular Na rises, K falls and the ouabain bound increases. A plot of $[Na]_i$ against $[Na]_i$/specific ouabain bound gave a linear correlation of $r = 0.98$ ($P < 0.001$) giving a V_{max} of 1.08×10^6 molecules/cell and a K_m of 9.1 mM. In comparison with the ethacrynate results it is evident that the increased ouabain binding is accompanied by a much smaller increase in $[Na]_i$. In this experiment there was a significant decrease in cell numbers (of 18 %, $P < 0.001$) but no decrease in cell volume as the $[K]_o$ was reduced. Each point is the mean of two observations. The bars are typical errors ($\pm$ S.E.). Lines fitted by eye. All observations carried out in same experiment.

This figure is of the same order as that calculated for the turnover rate of the Na pump in these cells from a measurement of the Na and K fluxes and the number of sites (Boardman *et al.* 1972 and unpublished observations). In two experiments in which we measured the O_2 consumption of normal HeLa cells in the presence and absence of 10^{-3} M ouabain we obtained a difference equivalent to a phosphate splitting rate of 40 sec^{-1} per site using a P/O ratio of 3 (see Baker, 1965). In these experiments the actual O_2 consumption was about $3 \times 10^{-6} \mu l/\text{cell.hr}$, a normal figure
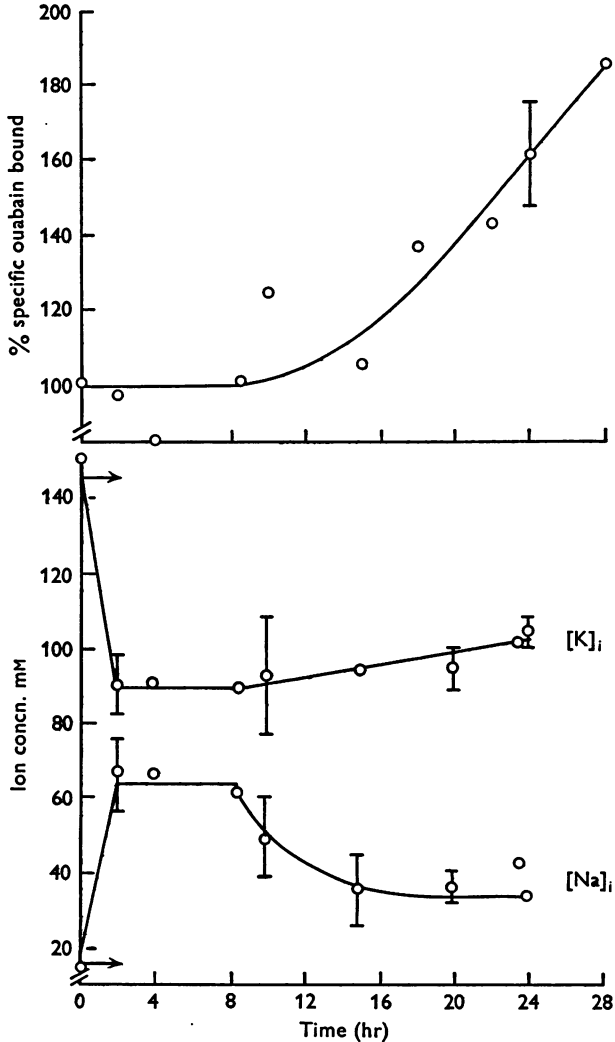
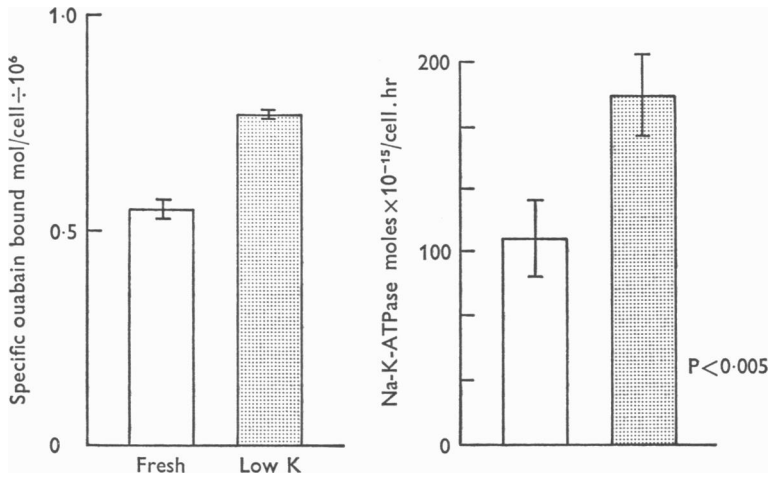

Fig. 3. For legend see facing page.

Fig. 4. Effects of growth in low K on ouabain binding and Na-K-ATPase. HeLa cells grown on plates were used to measure cell volumes and ouabain uptake per cell; those grown in Roux flasks for ATPase activity and membrane protein content. The figure shows that 24 hr in low K medium caused (left) a rise in specific ouabain binding of 40 % ($P < 0.001$) and (right) a 69 % rise in the Na-K sensitive ATP split, per cell ($P < 0.005$). The rise in ouabain binding was not significantly different from that in ATPase activity. Therefore growth in low K increases the specific ouabain binding and Na-K-ATPase per cell. Na-K-ATPase activity measured as the total ATPase activity in broken cell preparations less the mean of K in sensitive and ouabain blocked activity. $n = 3$ per column. The protein present was 190 ± 9 pg/cell over the whole series (s.e.; $n = 26$). Bars are $\pm$ s.e. All experiments on same day with same batch of cells.

Fig. 3. Time course of low-K on HeLa cells. Abscissa (above) specific ouabain bound (as % of control), (below) intracellular Na and K concentrations per litre water. Ordinate time after applying low K medium. Arrows on ordinate, indicate intracellular K (upper) and Na (lower) values of fresh cells. After 4 days growth in normal BME with 10 % serum the medium was changed for BME containing either 5 or 0.25 mM-K with 5 % serum. At various times thereafter plates were removed for analysis of Na and K and ouabain binding (see Table 1 for details) in the usual way. The Figure shows that the Na rises and K falls within the first 2 hours and then remains steady for the next 6 hr ($P > 0.05$), thereafter the Na shows a steady decline and the K a slower rise. The ouabain binding remains constant for 8 hr and then rises at about the same time as the ions change, to reach a value about twice the normal in 28 hr. Measurement of the actual $[K]_o$ throughout the experiment showed that it rose to 0.4–0.6 within the first $\frac{1}{2}$ hr (due to residual K in and between the cells) and then stayed constant over the rest of the experiment. Typical errors are $\pm$ s.e. Lines fitted by eye. Each point represents four to six values collected from several experiments. Cell numbers increased by 51 % over the 24 hr with no difference between normal and low-K media.

(Davis & Paul, 1961). These results are all fairly consistent and suggest that the turnover rate of the Na pumps in these cells is in the range of 20–40 sec^{-1}.

Analysis of the mechanism of the effects of partial transport block

When the active Na/K transport of a cultured cell is partially blocked the cell responds by increasing the number of specific ouabain binding sites and the Na-K-ATPase activity of its membrane. On general grounds it would be expected that this response would be mediated through the genetic material of the cell. This expectation is supported to some extent by the observation that human erythrocytes do not alter their Na-K-ATPase when incubated in low-K media (L. J. Boardman & J. F. Lamb, unpublished observations). If this is so then there must be an afferent pathway to the genetic material and an efferent pathway from the genetic material to the cell membrane. The purpose of the present group of experiments was to start an analysis of these pathways.

Afferent pathway. Partial blockage of active transport causes a rise in the intracellular Na concentration and a fall in the intracellular K concentration. Either of these could be a signal for the genetic material of the cell or they could simply be associated phenomena. Previous results (Boardman *et al.* 1972) showed that the change in [Na]$_i$ was more closely related to the change in ouabain binding but did not give any evidence of a causal relationship. In order to test this point more directly we grew cells in a solution which would cause a drop in the [K]$_i$ without a rise in [Na]$_i$, in addition to the normal low-K solutions which decrease [K]$_i$ and cause a rise in [Na]$_i$. Preliminary experiments showed that this condition could be achieved by substituting about 40 mM of the external Na with sorbitol.

The fall in [Na]$_i$ produced by this moderate reduction in [Na]$_o$ is probably the result of two effects, (a) the reduction in Na influx caused by the smaller driving force and (b) a greater activation of the external K site of the Na pumps by a reduced competition from Na (Baker, Blaustein, Keynes, Manil, Shaw & Steinhardt, 1969, Fig. 5). This latter effect was particularly clearly shown in one experiment in which a reduction of [Na$_o$] caused a rise in [K]$_i$ despite a very low [K]$_o$.

The results of a number of experiments of this kind are given in Table 2, which shows that the normal increase in [Na]$_i$, ouabain binding and Na-K-ATPase activity in low-K growth medium are all abolished by a reduction in [Na]$_o$, although the [K]$_i$ still remains low. This experiment therefore shows that the low-K growth solution has no direct effect on ouabain binding and ATPase activity unless the internal Na concentration rises. This provides some evidence for a causal relationship and suggests that a

rise in [Na]ᵢ somehow switches on the mechanism responsible for the appearance of new Na pump sites.

Efferent pathway. Genetic control in cells usually occurs either at the stage of RNA production from the DNA in the nucleus (transcription) or at the production of protein at the ribosomes controlled by the RNA (translation). To test these steps directly is not easy and as a preliminary investigation we have used drugs which are thought to act either at the transcription or translational levels. (Cycloheximide, Gautschi & Kern,

TABLE 2. Effect of replacing Na by sorbitol on ouabain binding and Na-K-ATPase activity in low K medium

Growth medium	$[Na]_i$	$[K]_i$	Specific ouabain bound (molecules × 10^{-5}/cell)	Na-K-ATPase × 10^{-15} mole/ cell sec^{-1}
135 Na 5K	$12 \cdot 7 \pm 0 \cdot 5$	172	$0 \cdot 46 \pm 0 \cdot 01$	172 ± 21
135 Na 0·5K	$24 \cdot 0 \pm 3 \cdot 5$	145 ± 2	$0 \cdot 73 \pm 0 \cdot 01$	290 ± 2
85 Na 5K	$13 \cdot 6 \pm 0 \cdot 7$	172 ± 3	$0 \cdot 44 \pm 0 \cdot 01$	140 ± 35
85 Na 0·5K	$16 \cdot 0 \pm 2 \cdot 0$	152 ± 2	$0 \cdot 52 \pm 0 \cdot 02$	158 ± 4

Cells grown for 24 hr in one of the four solutions produced by reducing [Na]₀ from 135 to 85 mM (replaced by sorbitol) with [K]₀ at 5 or 0·5 mM. Low K normal Na solution causes the usual rise in [Na]ᵢ, fall in [K]ᵢ and rise in ouabain binding and Na-K-ATPase (line 2). If the [Na]ᵢ rise is stopped by growth in low K low Na solution there is no rise on the ouabain bound or Na-K-ATPase, but there is still a significant fall in [K]ᵢ. Ouabain binding and Na-K analysis on one batch of cells ($n = 2–4$), Na-K-ATPase on a separate batch ($n = 3$). The K value without an error is a single observation.

1973; puromycin, Weiss, 1968; actinomycin D (AMD), Clark, Love, Studzinski & Ellem, 1966; cordycepin, Penman, Rosbash & Penman, 1970. All these results were obtained on HeLa cells. Gale, Cundliffe, Reynolds, Richmond & Waring (1972) give a general description of these and other drugs.) The design of these experiments was to induce new ouabain binding sites in the usual way in the presence or absence of various inhibitors.

Protein synthesis inhibitors. Cycloheximide and puromycin both stop protein synthesis in HeLa cells, but their actions are at different parts of the protein assembly on the ribosomes. When used at concentrations which suppress most protein synthesis we found that both drugs stopped the induction of extra ouabain binding sites. Fig. 5 shows these effects for cyclohexamide on the induction produced by growth in low K and etha-crynate. A complication of these experiments is that cycloheximide affects both the ion gradients and ouabain binding of otherwise normal cells. A

possible explanation for this is that cycloheximide is interfering with the normal replacement of Na pumps at the cell membrane, and therefore causing a deterioration of the ion gradients. If this explanation is correct then it would be expected that cycloheximide should have a smaller effect on normal cells at shorter times. This was confirmed when it was
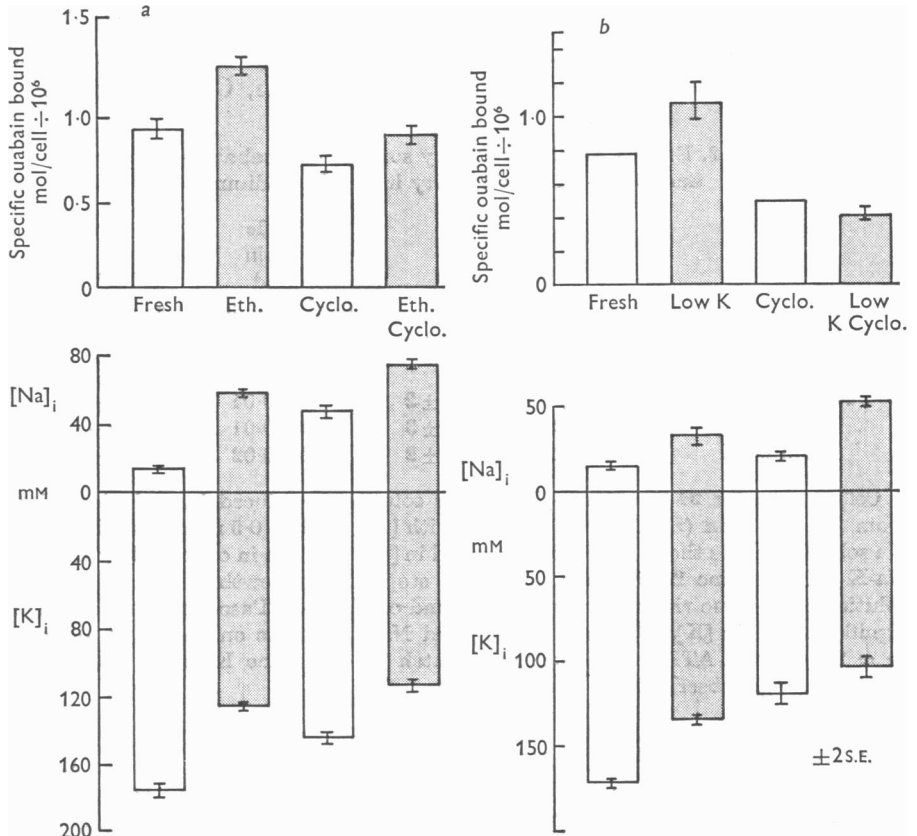

Fig. 5. Effect of cycloheximide on ouabain binding and ion levels in fresh and pre-treated cells. Pre-treated cells were either grown in 10^{-4} M ethacrynate (a) or in low K (b) for 24 hr and then the ouabain uptake and ion levels for these and for fresh cells were measured as usual. Half the fresh and pretreated cells also had cycloheximide (15 μg/ml.) present during the 24 hour growth period. Ethacrynate and low K both cause a rise in $[Na]_i$, a fall in $[K]_i$ and an increased specific ouabain binding $[P < 0.001]$. Cycloheximide by itself decreased ouabain binding (by 23 %, $P < 0.005$) and increased $[Na]_i$ and decreased $[K]_i$; when present with either ethacrynate or low-K it interacted so that the increased ouabain uptake is blocked and the ion gradient deteriorates further ($P < 0.01$) (this latter effect is most clearly seen in low-K cells). Ethacrynate and low-K experiments done at different times. In (b) cycloheximide reduced cell numbers to 55 % ($P < 0.001$) with no volume effect. $n = 8$ for Fig. a and 2 for Fig. b. Errors are (± 2 S.E.) for a and b.

TABLE 3. Effect of cycloheximide on the ouabain binding and internal sodium concentration of cells grown in normal or low K media

Condition	n	Specific ouabain bound (molecules $\times 10^{-6}$/cell		n	[Na]$_i$ m-mole/l. cell water	
		$-$ cyclo- heximide	$+$ cyclo- heximide		$-$ cyclo- heximide	$+$ cyclo- heximide
2K 8 hr	2	0.45 ± 0.02	0.49 ± 0.04	2	30 ± 4	30 ± 11
5K 14 hr	2	0.57 ± 0.03	0.39 ± 0.06	3	24 ± 3	39 ± 11
0.3K 8 hr	2	0.60 ± 0.04	0.40 ± 0.03	4	55 ± 2	67 ± 4
0.3K 14 hr	2	0.70 ± 0.05	0.31 ± 0.06	3	36 ± 2	88 ± 10

Mean 0.39 ± 0.03.

At zero time cells were put into 5 or 0.3 mM-K growth medium with or without cycloheximide (15 μg/ml.) then removed and analysed 8 or 14 hr later. The ouabain binding under the various conditions depends on whether cycloheximide is present or not ($P < 0.005$): in its absence growth in low K causes an increase ($+27\%$; $P < 0.005$) and time also causes an increase ($+22\%$; $P < 0.005$); in its presence both low K and time cause a decrease [of about 20%; $P = 0.05$–0.1; all significances obtained by analysis of variance]. The intracellular sodium concentrations also depend on whether cycloheximide is present or not; continued growth in low K without cycloheximide leads to a lower [Na]$_i$ at 14 hr compared to 8 hr ($P < 0.02$), and compared to 14 hr in low K with cycloheximide ($P < 0.05$; both by t test). All ouabain binding results and some of the Na results from the same experiment, the other Na results were taken from the succeeding experiment. Errors are $\pm$ S.E.

TABLE 4. Actinomycin D (AMD) and ethacrynate on ouabain uptake and ion concentrations in HeLa cells

	n	Control	AMD 1 μg/ml.	Ethacrynate 2×10^{-4} M	AMD 1 μg/ml.
Ouabain bound	4	0.67 ± 0.07	0.89 ± 0.03	1.05 ± 0.06	1.43 ± 0.14
[Na]$_i$	2	16.9 ± 0.2	50.9 ± 2.3	131 ± 1	154 ± 10
[K]$_i$	2	177 ± 1	151 ± 2	39.8 ± 1.6	21.3 ± 0.8

Cells grown in growth medium containing the combination of the drugs shown in the table for 24 hr and then the cell numbers and volume, ion contents and ouabain binding measured as usual. Ethacrynate at a concentration of 2×10^{-4} M has a similar but larger effect to that occurring at 1×10^{-4} M. AMD by itself raises [Na]$_i$ and lowers [K]$_i$ ($P < 0.005$) and also raises the ouabain bound ($P < 0.01$). Ethacrynate and AMD do not interact, i.e. their effects are additive (by analysis of variance). Both ethacrynate at this concentration and AMD greatly decrease cell numbers (to 36 and 24% respectively); together they decrease cell numbers to 8% of the control. These effects seem to be due to lack of adhesion to the plates rather than lack of growth. Cell volumes were not altered in any consistent way. All experiments done at same time.

found that cycloheximide abolished the rise in ouabain binding and recovery of $[Na]_i$ which occur in cells in low K after 8–12 hr with no significant effect on normal cells (Table 3).

We measured the ouabain sensitive ATPase activity of the membrane fraction of cells grown in control or low-K media in the presence or
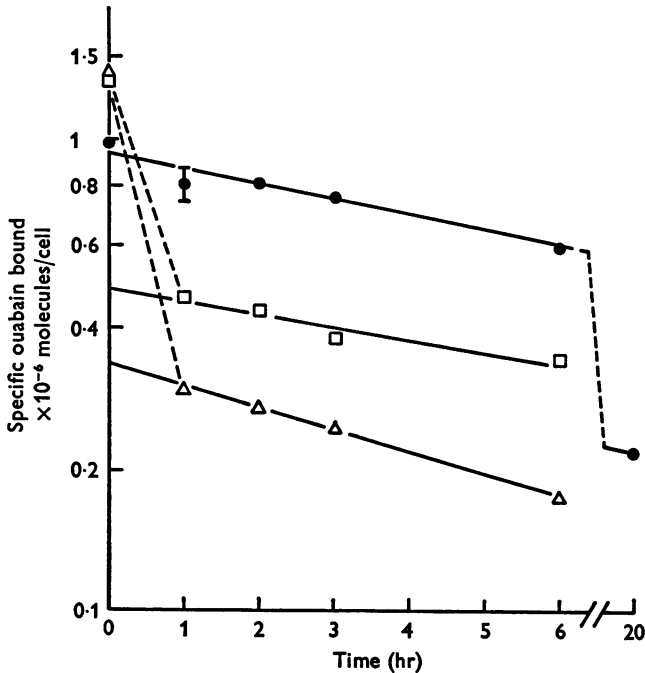

Fig. 6. Ouabain* efflux from normal and AMD treated cells. Normal cells or those grown in AMD 1 μg/ml. for 24 hr were loaded with [³H]ouabain for 1 hr as usual and then the efflux into normal or AMD growth medium followed. Filled symbols fresh cells in plain solution; open symbols AMD in growth and ouabain loading media, $\triangle$, efflux in AMD; $\square$, efflux in plain solution. The ouabain loss from fresh cells was exponential ($r = -0.97$) with a $t_{\frac{1}{2}}$ of 12 hr, that from AMD treated cells had a fast initial component followed by an exponential loss with a $t_{\frac{1}{2}}$ not significantly different from fresh cells. The three extrapolated intercepts were significantly different at $P < 0.01$. These results suggest that AMD for 24 hr causes an increase in labile binding sites but a decrease in normal sites. Lines fitted by least squares method, typical error bars shown ($\pm$ S.E.). $n = 4$ for zero and 1 hr and 2 for other points.

absence of cycloheximide. In one experiment cycloheximide caused a reduction of the ouabain sensitive ATPase from 1·97 to 0·10 μmole P_1/mg protein.hr in cells grown in 5K. In another experiment it caused a reduction of the ouabain sensitive ATPase from 0·56 to 0·27 μmole P_1/mg protein.hr in cells grown in low K. The protein per cell was not altered by

cycloheximide in either case. In a further experiment the Na-K-ATPase activities of cells grown in the stated conditions were (μmole PO_4/mg.protein per hour) normal K (5 mM), 0·52; normal K + cycloheximide (5 μg/ml), 0·14; low K (0·3 mM), 1·11; and low K + cycloheximide, 0·51.

These experiments are consistent with the view that protein synthesis is required either to make new Na pumps or to uncover existing pumps already in the membrane.

Inhibitors of RNA production. Actinomycin D (AMD) and cordycepin are both drugs which stop RNA production from DNA, but again at different sites on the assembly process. When used in our experiments both of these drugs gave unexpected, but similar, results. Table 4 shows that AMD over 24 hr has the effect of increasing the specific ouabain bound, both in otherwise normal cells and in those treated with ethacrynate. A limited investigation of this phenomenon gave the following results.

(1) These new sites appear more quickly than normal ones, for within 3 hr of applying AMD to normal cells there was a 60 % increase in the specific ouabain bound with no change in $[Na]_i$ or $[K]_i$ levels in the cells.

(2) The ouabain bound to these new sites is more labile than that bound to normal sites (Fig. 6 shows this).

(3) On removal of AMD these new sites (*a*) persist in the membrane for a long time (as shown by loading with ouabain* at various times) and (*b*) are converted to normal sites quite quickly (this can be noticed on Fig. 6 lower two lines).

(4) These new sites do not work in that the K influx is decreased in cells treated with AMD (see later).

These results with AMD suggest that AMD blocks the production of replacement Na pumps in normal cells, but causes the appearance in the membrane of molecules with some of the characteristics of Na pumps and which may then be readily converted to Na pumps when AMD is removed. This kind of unco-ordinated synthesis would be consistent with an action of AMD on post-transcriptional control systems (Tomkins, Levinson, Baxter & Dethlepin, 1972; but see also Kenney, Lee, Stiles & Fritz, 1973 for opposing evidence). Less complete cordycepin experiments gave similar results.

These results with inhibitors are consistent with the view that protein synthesis is required for the increase in ouabain binding and Na-K-ATPase which follows an elevation of $[Na]_i$. The site of control of this process remains in doubt but the AMD results suggest that there is some degree of transcriptional control present.

Transport changes

The evidence so far shows that when the $[Na]_i$ in HeLa cells is raised then there is an increase in the number of ouabain binding sites and an increase in the Na-K-ATPase per cell. This is consistent with the hypothesis that raising $[Na]_i$ over a prolonged period causes the appearance of more Na pumps. Both ouabain binding and Na-K-ATPase measure the machinery for transport but do not show if it is functional, so that we then wished to test if more Na transport actually occurred in these cells.

The difficulty in making a measurement of transport changes in HeLa cells arise from two causes. Firstly, the cells are small, with consequently a large surface to volume ratio (say $5000:1$ cm^{-1}); and secondly, in these as in other cells the internal Na site is normally unsaturated so that when the $[Na]_i$ rises there is a steep increase in the Na and K active fluxes. It therefore follows that primary changes in flux are followed very rapidly by changes in ion contents, until the active and passive fluxes come into equilibrium again. For example, reducing $[K]_o$ from 5 to 0·5 mM has been found to cause $[Na]_i$ to rise from 16 to about 70 mM in a few minutes. Return to normal $[K]_o$ causes the extra Na to be pumped out again with a t_i of about 5 min. In general therefore it is seldom possible to measure fluxes in non-steady state conditions in these cells, for the internal ions adjust themselves very quickly to a new steady-state value. As the $[Na]_i$ changes, the activation of the Na pump changes, and so the fluxes can no longer be compared, unless the relationship between ion concentration and flux is known. Caldwell (1970) has calculated this relationship for squid axon erythrocytes and we found that under normal conditions his model provided a good fit for our cells. Thus on reducing $[K]_o$ from 5 to 0·5 mM the calculated rise of $[Na]_i$ is close to that actually found. Wherever possible we have used this model (with squid axon values) to calculate the expected flux under various conditions. A final technical difficulty with these cells is that the unidirectional Na fluxes are so fast that influx measurements need to be made over times of less than 20 sec to satisfy the normal criteria for such measurements (Keynes & Lewis, 1951). For this reason we usually measured K fluxes or net Na movements.

TABLE 5. Ouabain-sensitive K influx into HeLa cells treated in various ways

	$[Na]_i$	n	K influx (mole $\times 10^{-17}$ cell per sec)	Calculated influx	Observed calculated (%)
Control	14	4	$1·34 \pm 0·13$	1·34	100
Cycloheximide	48	4	$0·46 \pm 0·04$	2·36	19
Ethacrynate (1×10^{-4} M)	59	2	$1·78 \pm 0·46$	2·54	70
Ethacrynate + cycloheximide	75	2	$0·75 \pm 0·26$	2·77	27
Control	11	4	$1·67 \pm 0·23$	1·67	100
Low-K	25	4	$2·77 \pm 0·23$	2·06	123

Cells were grown for 24 hr in solutions shown, then the K influx measured over 10 min in these solutions in 5 mM $[K]_o$. In order to allow for changes in flux consequent on changes in $[Na]_i$, Caldwell's model of the Na pump for squid axon was used to

calculate the expected K influx with raised $[Na]_i$. The observed/calculated % gives the change in the flux on this model. Cycloheximide and AMD produce a significant decrease in the observed K influx (to 34 and 60 % respectively; $P < 0.01$) and a greater decrease when allowance is made for the rise in $[Na]_i$ which occurred. Ethacrynate treated cells show a significant rise in observed K influx compared to those not so treated ($+49\%$ $P < 0.01$ by analysis of variance of all data) but a fall in the expected influx when allowance is made for the rise in $[Na]_i$. Influx for low-K experiment measured on return to 5 mM solution over 10 min; $[Na]_i$ value given is the starting value, it was assumed this would decrease with a t_i of 5 min. Growth in low K gives an increased K influx even when allowance is made for the increased $[Na]_i$ K influx estimated by trace amounts of Rb. Data from different experiments with the same batch of cells for upper part of Table. Lower two lines from same experiment at different time.

With these difficulties in mind we made a limited investigation of transport changes (*a*) during the various conditions used and (*b*) after cells had been treated with low K for 24 hr. Table 5 shows the ouabain sensitive K influx under the various conditions studied. Both cycloheximide and AMD reduce the ouabain sensitive influx below the actual control value and therefore below that expected for the new ion levels, which is consistent with a reduction in the number of pumps active in the membrane. Ethacrynate increases the K influx but when allowance is made for the increase in $[Na]_i$ the influx is less than expected. When ethacrynate and cycloheximide or AMD are present together the fluxes are greater than in the absence of ethacrynate. In all these conditions the cells were in a steady state compared to the time scale of the measurements and so it is presumed that the passive leaks to the ions showed similar changes to those shown for the active transport.

When cells grown in low-K for 24 hr are returned to normal medium there is still a difference of ouabain binding for some hours, even though the ion levels have returned to normal. These cells show a larger actual and calculated influx than controls.

DISCUSSION

The problem studied in this paper is whether cells have a system for the control of the number of Na pump sites in their outer membranes. Suggestive evidence that such a system exists is given in Table 6 of Baker & Willis (1972), which shows nearly a 1000-fold change in Na-K-ATPase per mg membrane protein and ouabain binding between different kinds of cells with only at most a × 10 change in the turnover rate of the pumps. This suggests that transport changes occur by altering the number of sites at the membranes rather than by an increase in the turnover rate of each site. This perhaps means that Na pumps have a most efficient rate of working. Recent evidence on uridine and amino acid uptake systems of

cultured cells show that the membrane functions are genetically controlled (Hare, 1972; Gazzola, Franchi, Saibone, Ronchi & Guidotti, 1972; Franchi-Gazzola, Gazzola, Ronchi, Saibone & Guidotti, 1973; Hume & Lamb, 1974) and can adapt to altered environmental conditions. It would therefore be not unexpected if the Na pump system was similarly controlled.

One way of studying this system is to cause a mismatch between the passive permeability of the cell and the sodium pumping and then to follow the cell's response to this. In the present and in past experiments we have done this by decreasing Na pumping, either by inhibitor substances or by growing in low K media. To measure if the cells have responded to this alteration we have used three measures of the sodium pumping properties of the cell membrane: (1) the K-sensitive ouabain binding to the cells; (2) the total Na-K-ATPase of broken cells and the ouabain sensitive ATPase of the cell membrane and (3) the Na and K transport across the cell membrane. (1) and (2) measure the presence of structures in the membrane normally associated with Na pumping whereas (3) measures the function of the structures.

The present experiments show that partial pump blockage causes both an increased K sensitive ouabain binding and an increased Na-K-ATPase in treated cells. The measurement of transport across the cell membranes gives a less clear result. Probably the best evidence for this is from the time course of the effect of low K (Fig. 3). When the cells are first exposed to low K the $[Na]_i$ rises to about 60 mM, a value similar to that with 1×10^{-4} methacrynate (Table 1). After some hours this high $[Na]_i$ starts to decrease as the ouabain binding increases and it eventually settles down at about half its initial value. This is the result expected if the new ouabain binding sites represented working Na pumps.

The above evidence is consistent with the hypothesis that when the normal pump activity of HeLa cells is decreased the cell responds by producing more Na pumps. This could occur either by an increase in the density of Na pumps in the membrane, or by an increase in the surface area of the cell with a constant pump density per unit membrane area. It is clear that the apparent surface area of the cells does not change sufficiently to account for the increased pump density but it would still be possible for there to be an increased actual surface area due to the appearance of more filopodia in the treated cells. If this were so there ought to be an increased membrane protein per cell, but the evidence given shows that this is not so. It seems most likely then that the cells are increasing the density of Na pumps in their membranes rather than the total surface area.

The second main point dealt with in this paper is the mechanism by

which this occurs. On *a priori* grounds it seems most likely that any such system would involve the genetic material of the cell. This is supported by the experiments with erythrocytes which show that low-K treatment does not affect the Na-K-ATPase of these anuclear cells. The most obvious afferent signal to the genetic material is the level of the intracellular $[Na]_i$ or $[K]_i$. The evidence (Table 2) suggests that a rise in $[Na]_i$ rather than a fall in $[K]_i$ is the causal agent.

The efferent pathway to the cell membrane could involve the cell nucleus and ribosomes or only the ribosomes. We have investigated this indirectly by the use of blocking agents which are thought to act at various sites of the pathway from the nucleus to protein synthesis. The two agents which stop protein synthesis (cycloheximide and puromycin) stopped the inductive effects of ethacrynate and low K on ouabain binding. The two agents acting on the transcription of DNA to RNA (actinomycin D and cordycepin) caused the appearance of aberrant ouabain binding sites but a decrease in the normal binding sites. This evidence suggests that protein synthesis is necessary for the cellular response to raised $[Na]_i$ but does not settle whether nuclear involvement is also necessary. The delay in the appearance of new Na pumps in these cells is longer than that necessary for new proteins to appear at the cell surface of paramecium (15 min; Sommerville, 1970) or for increased transport of amino acids in these (2 hr; Hume & Lamb, 1974) or other cells (Gazzola *et al.* 1972; Franchi-Gazzola *et al.* 1973), though the ouabain binding itself may appear much more quickly when AMD is present. This delay may be related to the complexity of the structures involved. The present experiments provide no evidence for whether the protein synthesis is used to make new pumps for insertion into the membrane, or for molecules which then uncover or modify structures already present in the cell membrane.

As erythrocytes age the amount of Na-K-ATPase per cell decreases (Yunis & Arimura, 1966) and the ion levels 'run-down' (Prankerd, 1958; Bernstein, 1959; see also Bolis & Gumperts, 1972), evidence consistent with the idea that Na pumps have a certain life and are not then replaced in erythrocytes. In the present experiments both cycloheximide and AMD caused a decrease in ouabain binding (of about 25 % per day) and an increase in $[Na]_i$ and $[K]_i$. This also suggests that there is a 'turnover' of Na pumps in the membranes of HeLa cells so that these blocking agents cause a decrease in the total number by decreasing the production rate. It would also be possible to alter the total number by a decrease in the destruction rate of pumps. We have no evidence for such a process in the present experiments but have found on other occasions that in the presence of either dialysed serum or low serum concentrations and low K that the number of ouabain binding sites falls very markedly in a few hours,

suggesting a rapid removal of Na pumps. So far the conditions necessary for this have not been adequately defined.

The evidence given in this paper is consistent with the hypothesis that when the $[Na]_i$ of HeLa cells is raised for a prolonged period, protein synthesis is stimulated and new Na pumps appear in the cell membrane. These new pumps then cause the intracellular Na to fall. The direct consequences of such a mechanism is that it provides a means of matching the Na pumping activity of a membrane to the passive Na leak of that membrane, at the same turnover rate of the pumps in the membrane. Perhaps a wider significance of such a mechanism could be that it provides a way for $[Na]_i$ to control protein synthesis more generally. Thus increased electrical activity in a cell could control the synthesis of proteins via the $[Na]_i$ of the cell, perhaps in learning.

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