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EFFECT OF PROLONGED OUABAIN TREATMENT ON Na, K, Cl AND Ca CONCENTRATION AND FLUXES IN CULTURED HUMAN CELLS

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SUMMARY

1. Girardi and Hela cells (derived from human heart and cervix respectively) were grown as monolayer cultures in B.M.E. (Eagles basal medium) containing concentrations of ouabain up to 5×10^{-8} M for periods ranging up to 5 days. The cell sizes, numbers, Na, K, Cl, and Ca concentrations and fluxes were then measured.

2. Twenty-four hours incubation in ouabain concentrations equal to or less than 5×10^{-8} M caused a rise in $[Na]_i$ and an almost equal fall in $[K]_i$ to new steady levels. The concentrations so reached were linearly related to the ouabain concentrations, such that in 5×10^{-8} M ouabain $[Na]_i$ rose to 124 m-mole/l. intracellular water and $[K]_i$ fell to 55 m-mole/l. i.c. water in Girardi cells. In Hela cells the changes were smaller at any particular ouabain concentration. These levels were maintained constant for at least 5 days.

3. In cells in the logarithmic phase of growth, raising $[Na]_i$ and lowering $[K]_i$ by ouabain caused a slowing of growth rate proportional to the ouabain concentration used. In cells in the stationary phase there was no change in the cell numbers over 24 hr. The volume of the cells was not directly affected by the treatment.

4. Reducing $[K]_o$ from the normal value of 5·4 to 2·5 mM increased the effect of any ouabain concentration, whereas increasing $[K]_o$ to 7·5 decreased the effect of ouabain.

5. Reduction of $[K]_o$ to 2·5 mM had no effect on the $[K]_i$ or $[Na]_i$ but halved the cell numbers, probably by a reduction in the growth rate. The mechanism of this effect is obscure.

6. In Girardi cells raising $[Na]_i$ and lowering $[K]_i$ by prolonged treatment increases the total Na fluxes and decreases the total K fluxes but

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keeps the total Na + K flux constant. High-Na, low-K cells had a reduced Na:K exchange compared to fresh cells and also had a Na:K pumped ratio nearer 4:1 than the 3:2 normally found.

7. These cells also show ouabain-sensitive and ouabain-insensitive Na:Na exchanges. In high-Na, low-K cells the ouabain sensitive Na:Na exchange is the same as in fresh cells. The effect of treatment on the ouabain insensitive Na:Na exchanges has not been elucidated.

8. The Cl content and fluxes are not altered by prolonged ouabain treatment. From this it is inferred that the membrane potential in high-Na, low-K cells is the same as in normal cells.

9. High-Na, low-K cells have the same calcium content and fluxes as fresh cells. From this it is concluded that there is no Na:Ca coupling in these cells.

INTRODUCTION

Schatzman (1953) showed that the cardiac glycoside ouabain inhibited active Na and K transport in red cells. Since then the considerable amount of work done has shown that the acute application of cardiac glycosides specifically blocks active Na and K transport in most tissues (Glynn, 1964). The drugs bind to pump sites and inhibit the transport ATPase predominantly at the terminal dephosphorylation stage of the pump cycle (Glynn, 1968). Most work so far has been concerned with the acute application of digitalis; the main aim of the present work was to measure the effects on the ionic levels and fluxes in cultured cells following prolonged treatment with low concentrations of ouabain.

Work on guinea-pig atrium (Reuter & Seitz, 1968) and squid axon (Baker, Blaustein, Hodgkin & Steinhardt, 1969) has suggested that the therapeutic action of these drugs on the heart might be due to an increased Ca exchange secondary to an increased intracellular Na. A secondary aim of the present experiments was to look at whether the changes in intracellular Na resulting from ouabain treatment affected the Ca movements in cultured cells, since if this were so then this would be a suitable preparation in which to further examine this hypothesis.

METHODS

Girardi heart cells (derived from human atrium, Girardi, Warren, Goldman & Jeffries, 1958) or Hela cells (derived from human cervix, Gey, Coffman & Kubicek, 1952) obtained from Flow Laboratories, Irvine, as a freshly trypsinized suspension were grown on 5 cm plastic Petri dishes. After 4–6 days growth each plate was covered with a monolayer of about 1×10^6 cells. These plates of cells were used to measure the Na, K, Cl and Ca contents and fluxes under different experimental conditions, at 37° C. The methods used are outlined in Lamb & MacKinnon (1971*a*) and detailed in McCall (1971). Cells were grown in Eagles basal medium (B.M.E.)

plus glutamine (1·6 mM), foetal bovine serum (10 %) and penicillin G (50,000 iu./l.). Most flux experiments were done in Krebs containing 5 % calf serum.

Ouabain-sensitive fluxes

Preliminary experiments were carried out to determine the sensitivity of the Na/K transport system in Girardi cells to ouabain. The degree of inhibition of the K influx produced by a range of ouabain concentrations from 10^{-9} to 10^{-3} M was measured, and a dose–response curve plotted (Fig. 1). Half-maximal inhibition of the sensitive fraction of the influx occurs with a ouabain concentration of 10^{-7} M in normal Krebs, the cells therefore having a sensitivity to the glycoside similar to that of squid axon (Baker, Blaustein, Keynes, Manil, Shaw & Steinhardt, 1969) and human

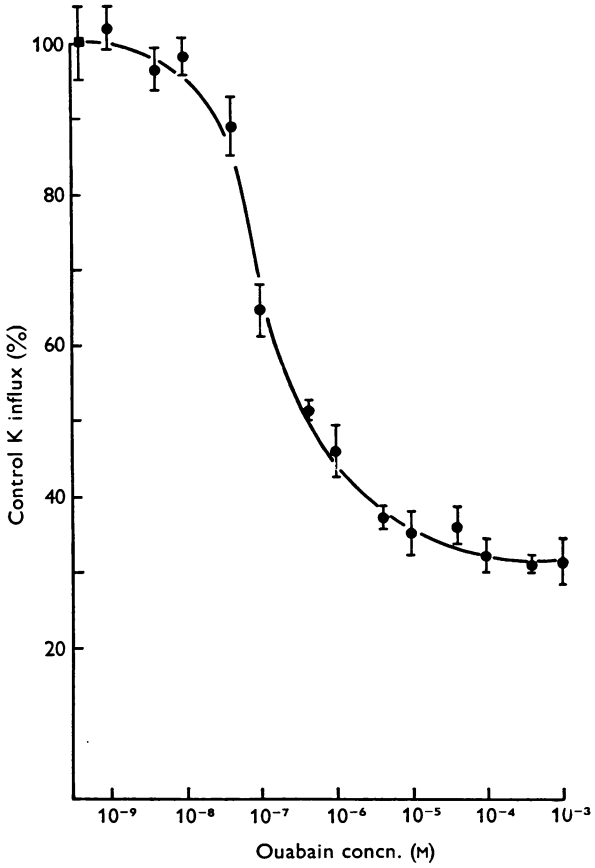

Fig. 1. Dose–response curve of the K influx in Girardi cells in the presence of ouabain (37° C). Abscissa: log ouabain concentration. Ordinate: influx as a % of the control influx. Ouabain applied for 10 min and then the influx measured over a subsequent 10 min period. Inhibition is half-maximal at 10^{-7} M ouabain and maximal at around 10^{-5}–10^{-4} M ouabain. Each point is the mean of five observations; bars are ± 1 S.E.

red cells (Glynn, 1964). As 10^{-3} M ouabain is between 10 and 100 times more concentrated than that required for maximal inhibition, it was assumed that such a concentration of ouabain would completely block the sensitive component of the flux under all experimental conditions.

Solutions used

The basic experimental solution was 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, plus phenol red 0·0002 % and calf serum 5 %. A Tris-citrate (16 and 5 mM respectively) buffer was used. Washing was carried out using a Na and K free isotonic Ca sorbitol solution.

Drugs used

The 10^{-3} M ouabain solutions were made up as required using ouabain from Ouabaine Glucoside (Laboratory Naturelle Ltd.). To prepare the solutions containing low concentrations of ouabain, ampoules of sterile Ouabain injection (Arnaud Laboratories) containing 0·25 mg in 1 ml. H_2O were used. Serial dilutions of this ouabain solution in sterile distilled water were made to provide stock solutions such that the addition of 0·7 ml. of the appropriate stock solution to B.M.E. or Krebs gave the desired final concentration. By using this ouabain preparation it was possible to maintain the sterility of the growth medium.

RESULTS

General characteristics of Girardi and Hela cells

Table 1 shows the mean volume, surface area and V/A ratio of the cells used. The figures shown for surface area do not take account of any 'filopodia' present on the cell surface (Lamb & MacKinnon, 1971*a*), but do assume that both surfaces of the cells on the monolayer were available for ion transfer (Burrows & Lamb, 1962). As in previous work it was found that trypsinization did not alter the volume of the cells. The cell water was found to be 79 % of the cell volume (measured by osmotic means; Lamb & MacKinnon, 1971*a*).

TABLE 1. Mean volume and surface area of Girardi and Hela cells. Volumes were obtained by measurement of the diameter of trypsinized cells in suspension and the surface area by planimetry of the cells flattened as a monolayer. Measurements were made on each experiment and the value so obtained used to compute the results of that experiment. The Hela and Girardi measurements are significantly different at $P < 0·001$

Type of cell	N	Volume ($\times 10^{-9}$ cm^3)	Apparent surface area ($\times 10^{-5}$ cm^2)	V/A ($\times 10^{-4}$ cm)
Girardi	1635	2·24 ± 0·04	1·59 ± 0·07	1·41
HeLa	375	3·08 ± 0·06	2·11 ± 0·04	1·46

Intracellular ion concentrations in cells grown in ouabain

Extracellular K concentration at a value of 5·4 *m*M. In preliminary experiments with Girardi cells it was found that cells did not survive for 24 hr in ouabain concentrations exceeding 5×10^{-8} M, and so all experiments were done in ouabain concentrations equal to or less than this. Fig. 2 shows the results of growing cells for 5 days in normal medium and then incubating them for 24 or 36 hr in the ouabain concentration shown. It can be

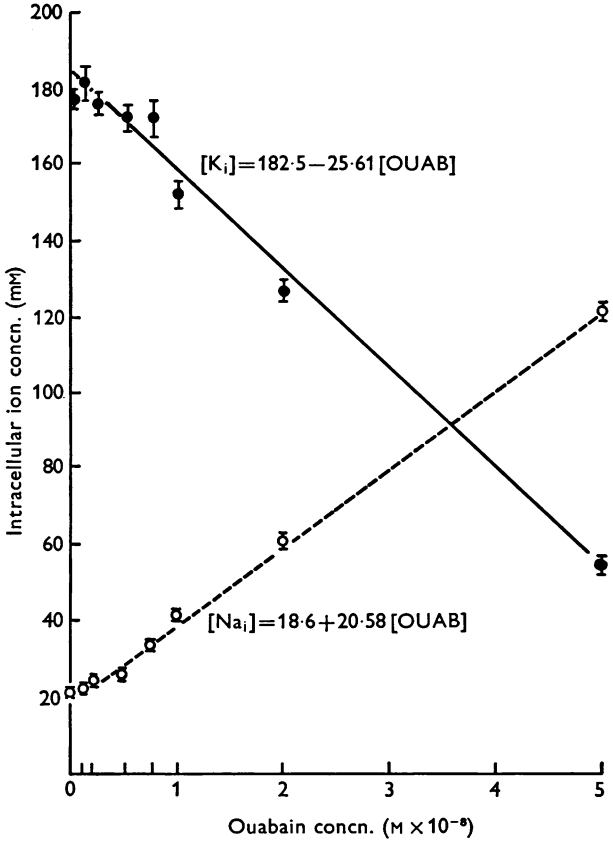

Fig. 2. Na and K concentrations of Girardi cells following prolonged ouabain treatment. Abscissa: ouabain concentrations on a linear scale. Ordinate: intracellular Na or K concentrations. The values shown are the means of the 24 and 36 hr observations, which did not differ significantly and therefore provide evidence that the cells were in a steady state. The lines are the calculated regression equations (with 10^{-8} ouabain taken as unity), both have a significance of $P < 0{\cdot}001$ and correlation coefficient of 0·99 and −0·98 for Na and K respectively. Bars are ± 1 S.E. Each point is the mean of twenty to forty observations.

seen that the cells reach a new steady state of $[Na]_i$ and $[K]_i$ which is dependent on and linearly related to the ouabain concentrations added. At 5×10^{-8} M ouabain the $[Na]_i$ exceeds the $[K]_i$. In these experiments neither the cell volume nor the cell numbers varied over the ouabain concentration range used; in some but not all individual experiments at the maximum ouabain concentration used the sum of the Na and K per cell was some 10% less than in control cells and so the calculated $[Na]_i + [K]_i$ was also 10% less. It is unlikely that this observation means that the

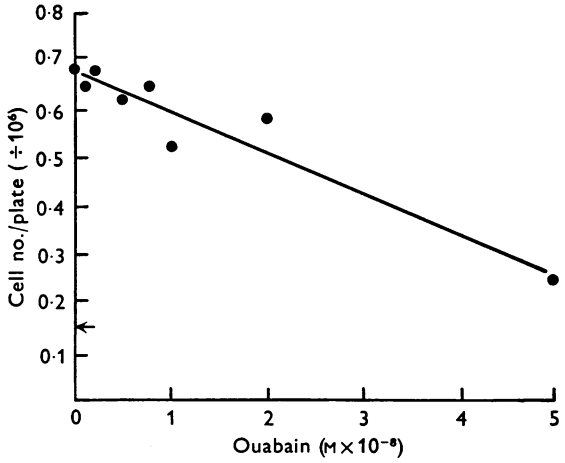

Fig. 3. The effect of 5 days treatment with low concentrations of ouabain on the growth rate in Girardi cells. Abscissa: ouabain concentration on a linear scale. Ordinate: cell numbers per plate after 5 days growth. Line is the regression equation, with $r = -0{\cdot}88$, $P < 0{\cdot}001$. Arrow indicates the cell number originally applied to the plates. Each point is the mean of eight observations.

osmotic pressure of the cells altered and the result may simply be due to an alteration in the binding of Na or K or to an error in the volume measurement. We were unable to decide between these possibilities. We interpret the observation that the cell numbers remain constant to mean that the cells once grown do not die at a faster rate when the intracellular Na and K gradients are reversed.

In a further series of experiments the same range of ouabain concentration was added to the cells 1 day after plating out and left for a further 5 days. The intracellular Na and K values reached were not significantly different from those in Fig. 2 confirming that the cells had reached a new steady state in 24 hr. Fig. 3 shows that the number of cells recovered from the plates at the end of 5 days was dependent on the ouabain concentration used. The number of dead cells per plate (estimated by eye) did not appear

to be affected by ouabain and so it was supposed that ouabain diminished the growth rate of the cells rather than the death rate. It was presumed that this was due to the change in $[K]_i$ and/or $[Na]_i$ rather than a direct effect of ouabain. In these experiments the cell volume in high ouabain was greater than that in control cells; this was expected as the cell number was lower in high ouabain and this itself increases the cell volume (Fig. 2, Lamb & MacKinnon, 1971*a*; McCall, 1971).

In these experiments it was found that the rate at which the new steady state was reached was dependent on the ouabain concentration used (Fig. 4).

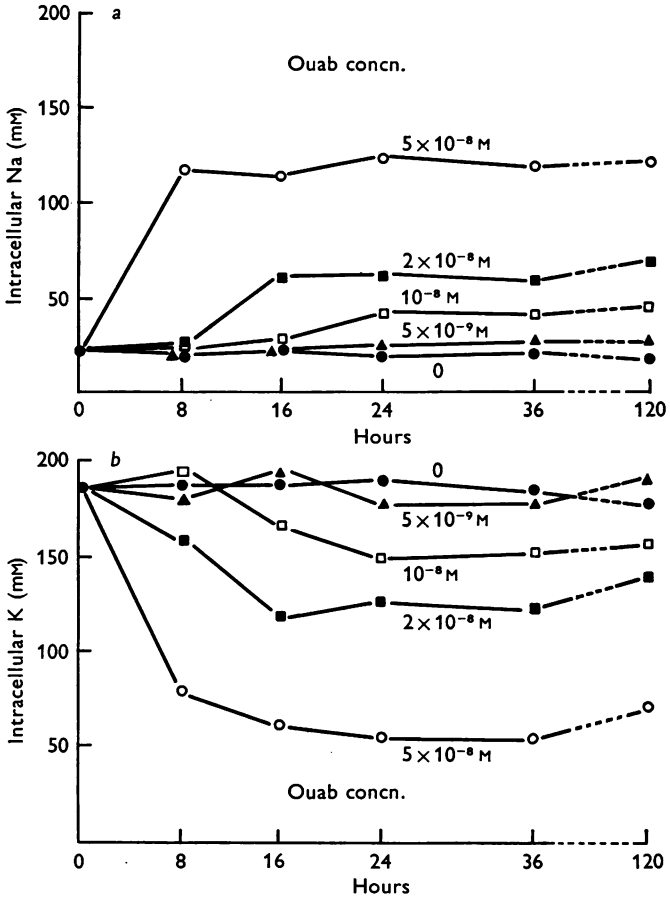

Fig. 4. Corresponding $[Na]_i$ and $[K]_i$, values in Girardi cells after various times in low concentrations of ouabain. The Figure shows that new steady values for $[Na]_i$ and $[K]_i$ are reached in 8 hr in 5×10^{-8} M ouabain, 16 hr in 2×10^{-8} M ouabain and by 24 hr in the lower concentrations. Thereafter the levels are maintained for 5 days. $n = 5$ for each point. Typical S.E. was Na ± 0·70, K ± 2·40.

Thus at 5×10^{-8} M the new steady states were reached in about 8 hr whereas 24 hr is required at 10^{-8} M. Thereafter it was found that the intracellular concentrations stayed constant over the next 4 days.

In similar experiments with Hela cells the same general result was obtained but it was found that at any ouabain concentration the ion levels changed to a smaller extent. Thus 5×10^{-8} M ouabain causes the intracellular Na to rise to 124 m-mole/l. in Girardi cells but only to 63 mM in Hela cells.

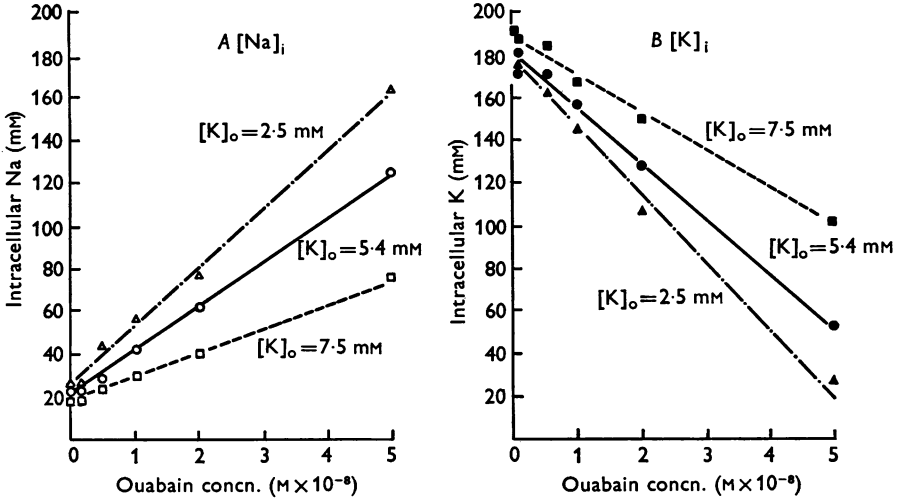

Fig. 5. Influence of $[K]_o$ on the effect of 24 hr ouabain treatment on the Na and K contents of Girardi cells. When $[K]_o$ = 5·4 mM (normal B.M.E.) results are identical to those in Fig. 2, but both reducing $[K]_o$ to 2·5 mM and increasing it to 7·5 mM significantly modifies the ouabain effect ($P < 0{\cdot}001$). Points are the means of five observations and the lines are the calculated regression lines. For each Na line $r > +0{\cdot}96$ and for each K line $> -0{\cdot}90$, all have $P < 0{\cdot}001$.

Effect of changes in the extracellular K concentration. It has been known clinically for many years that the effect of cardiac glycosides is to some extent dependent on the extracellular K concentration (Clark, 1912). More recently Glynn (1957) showed that the inhibition of active transport in the red cell by low concentrations of cardiac glycosides could be reduced or prevented by increasing the K concentration in the incubating medium. It was therefore of interest to measure the effect of altering the $[K]_o$ on the long term effect of ouabain in these cells. Fig. 5 shows the effect of $[K]_o$ values of 2·5, 5·4 and 7·5 mM on the intracellular Na and K levels in various ouabain concentrations. It is clear that raising the extracellular K decreases the ouabain effect and lowering the extracellular K increases the ouabain

effect. So these results are those expected on the pump competition effects observed by Glynn (1957) and Baker & Willis (1970). It will be noted that any particular $[Na]_i$ or $[K]_i$ value could be produced by a combination of different ouabain and $[K]_o$ values.

Reduction of $[K]_o$ to 2·5 mM in the absence of ouabain did not significantly alter the $[Na]_i$ and $[K]_i$ values of these cells over 24 hr. This result is to be expected on the basis that half-activation of the pump occurs at a $[K]_o$ of about 1 mM (L. J. Boardman and J. F. Lamb, unpublished observations) and so at 2·5 mM-$[K]_o$ there is still quite a considerable activation of the external pump site. Reduction of $[K]_i$ to 2·5 mM in the absence of ouabain did however influence the cell growth rate. Thus with Girardi cells it reduced the cell numbers to 66 % and in Hela cells to 52 %, of the numbers in 5·4 and 7·5 mM-$[K]_o$ B.M.E. This difference in cell numbers was maintained throughout the entire range of ouabain concentrations used (Tables 9*a* and *b*, McCall, 1971). This is a curious effect as it showed that $[K]_o$ can influence growth rate by a mechanism not dependent on the intracellular $[Na]_i$ and $[K]_i$ concentrations.

The membrane potential in cells treated with ouabain

In cultures L cells it has been shown that the membrane potential is low (−18 mV) and that Cl is passively distributed (Lamb & MacKinnon, 1971*b*). In some preliminary experiments (B. Murray, unpublished data) an E_m of about −20 mV was found for Girardi cells and the chloride content was consistent with a passive distribution. We have assumed that in these cells Cl is passively distributed, so that the Cl distribution may be used to measure the E_m in cells grown in different ouabain concentrations. Table 2 shows that there is no alteration in E_{Cl} as the ion concentrations change and, if it is assumed that $E_m = E_{Cl}$, then there is no change in E_m under these various conditions. In these experiments the Cl fluxes were also measured, giving a value of $46{\cdot}5 \pm 1{\cdot}6$ (S.E.; $n = 4$) p-mole/cm^2.sec, a value not altered by the immediate application of 10^{-3} M ouabain or by prolonged incubation in the ouabain concentrations shown in Table 2.

The Na and K fluxes in Girardi cells treated with ouabain

In these experiments the total and ouabain sensitive components of the Na and K fluxes in normal cells and in cells pre-treated with ouabain were measured. To do so fluxes were measured under the conditions in which the cells were grown and in 10^{-3} M ouabain. It was assumed that this high ouabain concentration would inhibit any remaining pumped Na and K movement. Fig. 6 shows a typical Na efflux. In this as in all such experiments a separate plate of cells was used for each point. This method, although less sensitive than collecting the efflux from each plate, avoids

TABLE 2. Na, K and chloride contents of Girardi cells grown in the various ouabain concentrations shown for 24 hr. Na measured by ^{24}Na, Cl by ^{36}Cl and K by flame photometry on separate plates in the same experiment. The calculated E_{Cl}'s do not differ significantly. $n = 6$ for Cl and 20–40 for Na and K; errors are ± S.E.

Ouabain concn. 24 hr treatment	Intracellular ion concn. (m-mole/l. cell water) Na	K	Cl	E_{Cl} (mV)
0	20·1 ± 0·4	180·3 ± 2·3	59·2 ± 2·0	−21·9
10^{-9} M	22·5 ± 0·8	186·6 ± 2·9	58·7 ± 2·2	−22·2
5×10^{-9} M	24·3 ± 1·1	170·5 ± 4·4	60·8 ± 0·8	−21·2
10^{-8} M	40·5 ± 0·6	150·9 ± 5·4	61·2 ± 1·3	−20·9
5×10^{-8} M	123·7 ± 3·2	55·8 ± 2·9	58·2 ± 2·2	−22·4

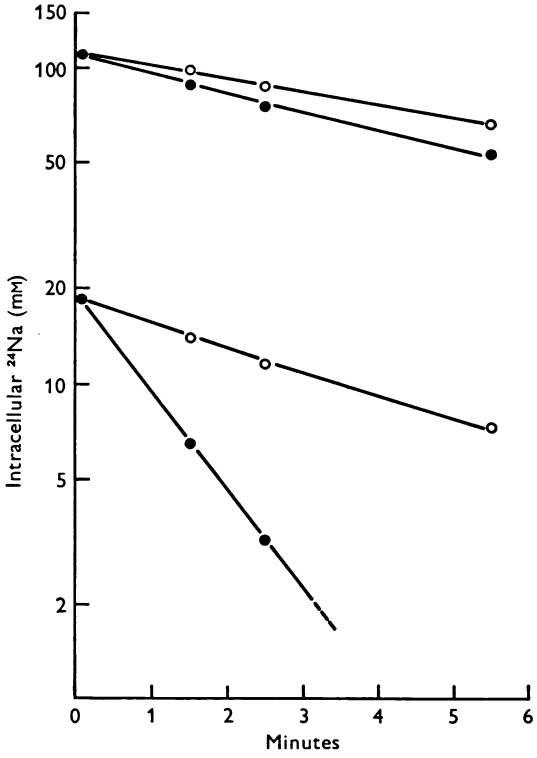

Fig. 6. Typical Na efflux experiments from cells with a normal $[Na]_i$ (19·5 mM) and with a high $[Na]_i$ (112 mM). Cells were previously equilibrated in ^{24}Na Krebs solution then washed in control Krebs or control Krebs containing 10^{-3} M ouabain (○) from time 0. Each point represents a single plate of some 1×10^6 cells. Lines fitted to points by eye. The high $[Na]_i$ was produced by incubation of the plates in 5×10^{-8} M ouabain for 24 hr; in this experiment the control Krebs referred to contained 5×10^{-8} M ouabain (●).

the possibility of errors due to cells leaving the plate during the efflux. In the fresh cells the efflux has a $t_{\frac{1}{2}}$ of about 1 min which rises to 5 min in cells with a high $[Na]_i$. It can be seen from this experiment that ouabain has a much smaller effect in the treated cells than in fresh cells.

Na fluxes. Table 3 shows the average results of all experiments. In all conditions (except perhaps Na in normal cells) the influx and efflux were not significantly different and so the cells were in equilibrium before application of 10^{-3} M ouabain. Treatment with ouabain for 24 hr (with a consequent rise of internal sodium concentration) leads (1) to an increased total exchange of Na, (2) to a diminution of the net ouabain-sensitive extrusion of Na from the cells, (3) to little change in the ouabain-sensitive influx.

These results on the net ouabain sensitive extrusions of Na with various levels of ouabain treatment are qualitatively as expected, for with a greater number of ouabain molecules present in the medium it is expected that more pumps would be blocked, with a consequent diminution in the active extrusion of Na from the cell. The diminution in the Na influx with 10^{-3} M ouabain is probably the result of blocking the Na:Na exchange by the Na pump with ouabain, a finding similar to that described by Garrahan & Glynn (1967*a*) in R.B.C.S (in R.B.C.S there is little Na:Na exchange at a $[K]_o$ of 5·4 mM). It therefore appears that treating cells with ouabain for 24 hr leads to a reduced net Na efflux but no change in the ouabain–sensitive component of the Na:Na exchange.

Table 3 also shows that the Na efflux remaining after 10^{-3} M ouabain is still substantial and rises as the internal Na rises. If this Na efflux is entirely passive and independent of influx then it should be related to the remaining Na influx according to the equation (Shaw, 1955).

$$\frac{M_o}{M_i} = \frac{[Na]_i}{[Na]_o}, \tag{1}$$

where M_o and M_i are the efflux and influx and Na_i and Na_o are the internal and external Na concentrations. In these cells the chloride ratio is 0·44 and $[Na]_o$ is 137 mM. Therefore the equation becomes

$$\frac{M_o}{M_i} = \frac{[Na]_i}{311}. \tag{2}$$

From this, the ratio of M_o/M_i for fresh cells for independent passive movements should be 0·062; the observed ratio of the residual ouabain insensitive fluxes is 0·30, a value considerably in excess of that expected.

This large residual efflux could mean that the cells are not obeying the independence relation of Ussing or that there is a Na:Na exchange not sensitive to ouabain. In some respects the present situation is similar to that described by Keynes & Steinhardt (1968) in frog muscle where a Na:Na exchange occurs which is ouabain-

TABLE 3. Na and K fluxes and intracellular concentrations in Girardi cells, either untreated or grown for 24 hr in ouabain. The flux differences shown are all significant ($P < 0.01$, except † which is just significant at $P < 0.05$). Ouabain at 10^{-3} M decreased all Na and K movements compared to controls ($P < 0.01$) except for the K efflux in 5×10^{-8} M ($P > 0.05$). Incubation in 5×10^{-8} M ouabain caused an increased total exchange of Na and a decreased total exchange of K ($P < 0.001$ by analysis of variance) but no change in the total Na + K exchange. $N = 5$ for each flux figure and 10 for the concentrations. Error is ± S.E.

Ouabain concn. in growth medium (M)	Ouabain concn. in expt. medium (M)	Sodium				Potassium			
		Intracellular concn. (m-mole/l.)	Influx (p-mole/cm².sec)	Efflux (p-mole/cm².sec)	Flux difference	Intracellular concn. (m-mole/l.)	Influx (p-mole/cm².sec)	Efflux (p-mole/cm².sec)	Flux difference
0	0	19·0 ± 0·3	25·00 ± 0·32	27·20 ± 0·60	2·20†	172 ± 2·0	13·78 ± 0·30	13·52 ± 0·27	—
	10^{-3}	—	18·58 ± 0·34	7·15 ± 0·31	11·43	—	4·60 ± 0·23	12·32 ± 0·16	7·72
10^{-8}	10^{-8}	37·4 ± 0·4	29·6 ± 0·69	28·48 ± 0·67	—	150 ± 2·0	8·60 ± 0·15	8·56 ± 0·21	—
	0	—	27·9 ± 0·76	29·86 ± 1·28	—	—	8·38 ± 0·18	8·56 ± 0·21	—
	10^{-3}	—	21·54 ± 0·69	11·52 ± 0·29	10·02	—	3·58 ± 0·21	6·48 ± 0·10	2·90
5×10^{-8}	5×10^{-8}	107·0 ± 1·4	35·56 ± 0·94	35·26 ± 0·72	—	74·4 ± 1·6	4·24 ± 0·25	3·72 ± 0·14	—
	0	—	34·98 ± 1·08	36·62 ± 1·16	—	—	4·10 ± 0·16	3·64 ± 0·13	—
	10^{-3}	—	29·02 ± 0·42	24·16 ± 0·19	4·86	—	2·14 ± 0·13	3·36 ± 0·17	1·22

insensitive and which increases as $[Na]_i$ does. To test between these possibilities we examined the Na efflux of normal and high Na Hela cells into Na free solutions. In order to show the movements we did these experiments at 20° C. Each efflux was carried out for 3 min only and the efflux lost over this time expressed as the fraction of the original Na content. In twelve experiments in the presence of ouabain 10^{-3} M, replacement of Na with choline reduced the residual efflux to half ($n = 6$ per treatment; $P < 0{\cdot}05$). Although this provides evidence for a substantial Na:Na exchange in fresh cells which is not sensitive to ouabain, quantitatively it is still not sufficient to bring the ratio of M_o/M_i to that expected for passive independent movement. In cells loaded with Na the situation was more complex as the Na efflux increased on removal of external Na. It seems possible that other substances are exchanging for Na in this situation.

K fluxes. In control experiments K exchanges with a $t_{\frac{1}{2}}$ of about 20 min. Table 3 shows the average fluxes in fresh cells and those pre-incubated in 10^{-8} and 5×10^{-8} M ouabain. It is clear that pre-treatment with ouabain decreases all the components of the K fluxes. Thus the total flux and the net ouabain-sensitive influx both decrease. The component of the efflux which is ouabain-sensitive and may represent K:K exchange (Glynn, Lew & Luthi, 1970) is not altered in any clear way although it is smallest in the presence of 5×10^{-8} M ouabain. As with the Na fluxes there seems to be no close correlation between the magnitude of the Na/K and the K/K movements. An interesting feature of these fluxes is that the total Na + K exchanges remains constant despite the large changes in $[Na]_i$ and $[K]_i$ induced by ouabain pre-treatment.

Flux ratio. From the flux differences shown in Table 3 the ratio of the 'uphill' movements of Na to K was calculated (the small difference between the influx and efflux of Na in fresh cells was ignored; if used it reduces the Na/K ratio further). The Na/K ratios obtained were $1{\cdot}5 \pm 0{\cdot}1$, $3{\cdot}5 \pm 0{\cdot}4$ and $4{\cdot}0 \pm 0{\cdot}8$ for cells grown in 0, 10^{-8} and 5×10^{-8} M ouabain (both the ratios of cells grown in ouabain are significantly greater than that in normal cells at $P < 0{\cdot}01$ but are not different from each other). These results indicate that under these conditions the ratio of the Na/K pumped increases.

Recovery from ouabain treatment

Plates of cells treated in the usual way with 10^{-8} and 5×10^{-8} M ouabain for 24 hr were washed 4 times to remove extracellular glycoside and Krebs added to each plate. At 30-min intervals over the next 4 hr plates from each group were removed and analysed for their Na and K content (by flame photometer). Over this time period no recovery of content occurred. This is the expected result from experiments reported by Barve, Boardman, Lamb & McCall (1970), who showed that the efflux of [^{3}H]ouabain from Hela cells in similar experimental conditions occurs with a $t_{\frac{1}{2}}$ of about 12 hr. On the other hand Baker & Willis (1970) found an efflux of [^{3}H]ouabain with a $t_{\frac{1}{2}}$ of some 30–60 min in trypsinized Hela and Girardi cells, and if this

were occurring in our cells we would have expected a substantial recovery of $[Na]_i$ in 4 hr. It seems clear that the particular conditions of these experiments has a large effect on the wash-off of [^{3}H]ouabain.

Effect of ouabain pretreatment on Ca contents and fluxes. One of the objects of these experiments was to find out if this preparation of heart cells was a suitable model situation to study Na:Ca interactions. To see if change in intracellular Na concentration affected the Ca content or fluxes we examined the extreme circumstances of fresh cells with a $[Na]_i$ of about

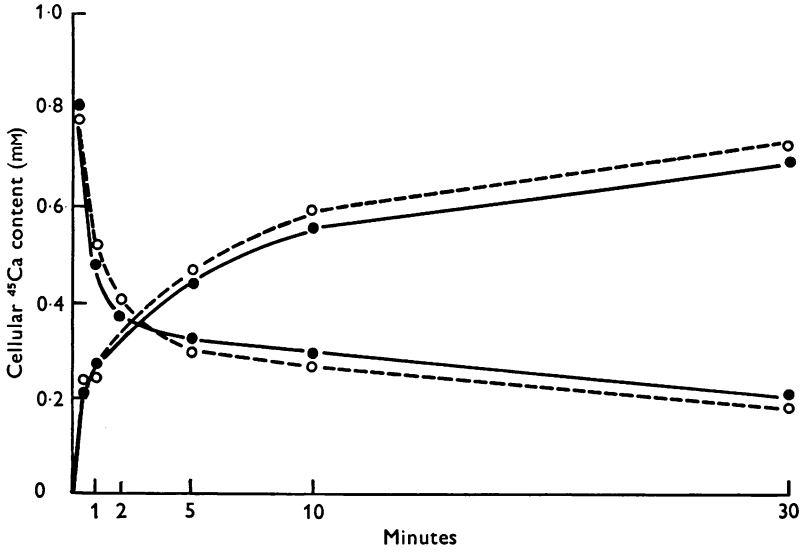

Fig. 7. ^{45}Ca efflux and uptake curves in Girardi cells, untreated (●) and treated with 5×10^{-8} M ouabain, 37° C (○). The efflux was obtained by equilibrating cells in ^{45}Ca B.M.E. ± ouabain for 24 hr then washing in inactive Krebs ± ouabain from time 0. Each point represents a single plate of approximately 1×10^6 cells. The uptake was obtained by adding to five plates of cells grown in inactive B.M.E. ± ouabain for 24 hr, ^{45}Ca Krebs ± ouabain. At various times thereafter a plate of cells washed six times with ice-cold inactive Krebs and the ^{45}Ca content determined. Each point represents one such plate. The lines were fitted to the points by eye.

20 m-mole/l. and cells equilibrated in 5×10^{-8} M ouabain which have a $[Na]_i$ of about 120 m-mole/l. To do so ^{45}Ca was used. The results (Fig. 7) show that under these conditions there is no alteration in the Ca content or fluxes with pre-incubation in 5×10^{-8} M ouabain and consequent changes in intracellular ionic levels. This means that this preparation of cells is not suitable for studying Na:Ca interactions. A more extended series of experiments (Lamb & Lindsay, 1971) has shown that in these cultured cells

the Ca efflux is driven by ATP and is not influenced by the Na gradient and so is similar to that described in R.B.C.S (Schatzmann & Vincenzi, 1969).

DISCUSSION

In therapeutic use ouabain and other digitalis compounds are used for prolonged periods; only very seldom are they used acutely. Yet most of the information about their action has of necessity been obtained in acute experiments. The main aim of the present experiments was to examine their chronic effects on cells for comparison with their already well known acute effects.

The preliminary experiments showed that the sensitivity of these cells to ouabain was similar to that of freshly drawn R.B.C.S (Glynn, 1964), so that these cells had retained their typical high species sensitivity (Straub, 1924) during prolonged culture. A similar result was reported in L cells (Lamb & MacKinnon, 1971). Although the general order of sensitivity of the two cell lines studied was similar, Girardi cells showed a larger response than Hela cells to any ouabain concentration. Thus to produce a $[Na]_i$ of 120 m-mole/l. Girardi cells required a ouabain concentration of 5×10^{-8} M but Hela cells 10^{-7} M. The reason for this difference in sensitivity is not clear.

The results of prolonged ouabain incubation on the Na and K levels are as expected. With each ouabain concentration at or less than 5×10^{-8} M a new steady-state of raised $[Na]_i$ and lowered $[K]_i$ is reached and thereafter maintained for at least 5 days. The rate at which this new state is reached depends on the actual concentration and varies from 8 to 24 hr. Once this steady state is reached there is no evidence for a 'desensitization' of the cells to that ouabain concentration. The cells gain Na and lose K in almost equal amounts, but there is no change in $[Cl]_i$ so that probably there is no change in E_m. Reducing $[K]_o$ enhances and increasing $[K]_o$ reduces the effect of any ouabain concentration. This observation is consistent with the recent experiments of Baker & Willis (1970) who showed that the $[K]_o$ level affects the number of molecules of ouabain taken on to the cell membrane, and hence the eventual steady-state level of ions in the cells.

The large changes in Na and K levels in these cells did not cause them to swell or to die more quickly, but it did slow down the growth rate markedly. There is no evidence in the present work on the mechanism of this effect but it is probably related to a reduced rate of amino acid and other substrate uptake; for in these cells as in others a reduced $[Na]_o/[Na]_i$ gradient produced by a rise in $[Na]_i$ decreases the amino-acid uptake (Hume, 1971; Lubin, 1967). An unexpected observation which was made was that reduction of $[K]_o$ to 2·5 mM reduced the number of cells on the

plates without there being any change in $[Na]_i$ or $[K]_i$. This was again probably a result of a decreased growth rate of the cells. The mechanism of this effect is obscure; it could possibly be due to some alteration in surface properties of the cell or perhaps an effect on the transport of some other substrate.

The flux changes which occur in Girardi cells on prolonged incubation with ouabain are quite complex and have not been entirely elucidated. The following points may be made. (*a*) The total Na + K exchanges across the membrane remain remarkably constant despite large alterations in the component parts of the fluxes. (*b*) The 'downhill' movements of both Na and K with their respective electrochemical gradients are reduced by 10^{-3} M ouabain. By analogy with R.B.C.S (Garrahan & Glynn, 1967*a*; Glynn *et al.* 1970) it has been assumed that this represents exchange diffusion of these ions across the membrane. If so then it shows two differences from R.B.C.S: (1) that it occurs in the presence of external $[K]_o$ and (2) is unaltered by a rise in internal $[Na]_i$. An attempt was made to examine these points in more detail on Hela cells after most of the experiments in this paper were completed but several detailed differences between Hela and Girardi cell fluxes became evident, so that the results obtained could not be applied to Girardi cells. (*c*) The 'uphill' movements of both Na and K (calculated as the net ouabain sensitive change) showed a progressive decline with increasing ouabain in the incubating medium. The ratio of the Na efflux to the K influx also changed, from 3:2 normally (as in R.B.C.S; see Garrahan & Glynn, 1967*b*) to approximately 4:1 in 5×10^{-8} M ouabain. This would indicate that the stoicheiometry of the pump changes under these conditions so that more Na is pumped with less K. (*d*) The residual 'uphill' movements of Na in the presence of 10^{-3} M ouabain in normal cells is still greater than would be expected for independent passive movements and this fraction increases greatly in loaded cells. One common explanation for this phenomenon is that there is an exchange diffusion of the ion concerned occurring. In normal Hela cells this has been shown to be so but no evidence is available for Girardi cells or for high Na/low K cells of either kind.

The most curious features of these results is (1) that the ouabain-sensitive 'downhill' movements of Na is not altered by incubation in low ouabain concentrations despite a large uptake of ouabain on to the Na:K pumps, and (2) that as the Na:K movement across the cell membrane is blocked an increasing Na:Na movement occurs so that the total ion exchange remains constant. The mechanism of this Na:Na exchange had not been elucidated but it would seem to be consistent with the idea that ouabain incubation is converting Na:K sites to Na:Na ones.

In cells incubated in ouabain with a consequent reduction in pumping,

the new steady-state ionic values reached will depend both on the residual pumping capacity and on the passive leak of the membrane. The next paper is concerned with the pumping capacity of the membrane, here we wish to consider the passive leak. In these experiments this leak could only be assessed by calculation from the Goldman equation as the cells are too small for resistance measurements using micro-electrodes. This raises the difficulty that the passive component of the Na and K movement across the membrane does not obey the Ussing relationship and so the justification for using the Goldman equation is poor. If it is assumed that the passive component of the Na and K movement equals the pumped movement (Lamb & MacKinnon, 1971*b*) then the P_{Na} and P_K of normal cells is $6{\cdot}5 \times 10^{-8}$ cm/sec and $1{\cdot}15 \times 10^{-7}$ cm/sec respectively and these fall to $2{\cdot}32 \times 10^{-8}$ cm/sec and $0{\cdot}72 \times 10^{-7}$ cm/sec after incubation in 5×10^{-8} M ouabain for 24 hr. If this argument is justified then prolonged ouabain incubation leads to a reduced Na and K passive leak.

Ouabain is used clinically but no information is available on the plasma levels which are effective. Measurement of plasma digoxin levels, however, by radio-immunoassay (Smith & Haver, 1969; Chamberlain, White, Howard & Smith, 1970; Evered, Chapman & Hayter, 1970) and bio-assay (Grahame-Smith & Everest, 1969; Shapiro, 1969) in patients receiving therapeutic dosage of the drug, have shown that the levels are in the range of 10^{-9}–10^{-8} M. If the effective ouabain concentrations are of the same order then 10^{-8} M ouabain in Girardi cells doubled the internal Na concentration, reduced the active Na efflux to 65% and the active K influx to 40% and hence altered the pumping ratio of Na/K from 3:2 to 4:1 approximately. For many years attempts have been made to determine if ouabain and other glycosides in therapeutic concentrations do alter the steady-state ion levels in heart muscle. The results of these experiments have been very variable (see Müller, 1965). The explanation may lie in the rather small changes shown in the ion concentration (at therapeutic concentrations of the glycosides) in the present experiments. In whole tissues these might be difficult to detect against the background of a large and variable extracellular space.

A subsidiary aim of the present experiments was to look for Na/Ca interactions in these cells. The results show quite clearly that there is no interaction of the type shown in squid axon and therefore these cells are not suitable for studying this problem. A similar conclusion was recently reached for L cells (Lamb & Lindsay, 1971).

REFERENCES

BAKER, P. F., BLAUSTEIN, M. P., HODGKIN, A. L. & STEINHARDT, R. A. (1969). The influence of calcium on the sodium efflux in squid axons. *J. Physiol.* **200**, 431–458.

BAKER, P. F., BLAUSTEIN, M. P., KEYNES, R. D., MANIL, J., SHAW, T. I. & STEINHARDT, R. A. (1969). The ouabain-sensitive fluxes of sodium and potassium in squid giant axons. *J. Physiol.* **200**, 459–496.

BAKER, P. F. & WILLIS, J. S. (1970). Potassium ions and the binding of cardiac glycosides to mammalian cells. *Nature, Lond.* **226**, 521–523.

BARVE, ANGELI, BOARDMAN, LESLEY, J., LAMB, J. F. & MCCALL, D. (1970). The uptake of tritiated ouabain by monolayers of cultured cells under various conditions. *J. Physiol.* **211**, 12–13*P*.

BURROWS, R. & LAMB, J. F. (1962). Sodium and potassium fluxes in cells cultured from chick embryo heart muscle. *J. Physiol.* **162**, 510–531.

CHAMBERLAIN, D. A., WHITE, R. J., HOWARD, M. R. & SMITH, T. W. (1970). Plasma digoxin concentrations in patients with atrial fibrillation. *Br. med. J.* **iii**, 429–432.

CLARK, A. J. (1912). The influence of ions upon the action of digitalis. *Proc. R. Soc. Med.* **5**, 181–197.

EVERED, D. C., CHAPMAN, C. & HAYTER, C. J. (1970). Measurement of plasma digoxin concentration by radioimmunoassay. *Br. med. J.* **iii**, 427–428.

GARRAHAN, P. J. & GLYNN, I. M. (1967*a*). The behaviour of the sodium pump in red cells in the absence of external potassium. *J. Physiol.* **192**, 159–174.

GARRAHAN, P. J. & GLYNN, I. M. (1967*b*). The stoichiometry of the sodium pump. *J. Physiol.* **192**, 217–235.

GEY, G. O., COFFMAN, W. D. & KUBICEK, M. T. (1952). Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res.* **12**, 264–265.

GIRARDI, A. J., WARREN, J., GOLDMAN, C. & JEFFRIES, B. (1958). Growth and C.F. antigenicity of measles virus in cells derived from human heart. *Proc. Soc. exp. Biol. Med.* **98**, 18–23.

GLYNN, I. M. (1957). The action of cardiac glycosides on sodium and potassium movements in human red cells. *J. Physiol.* **136**, 148–173.

GLYNN, I. M. (1964). The action of cardiac glycosides on ion movements. *Pharmac. Rev.* **16**, 381–407.

GLYNN, I. M. (1968). Membrane adenosine-triphosphatase and cation transport. *Br. med. Bull.* **24**, 165–169.

GLYNN, I. M., LEW, V. L. & LUTHI, U. (1970). Reversal of the potassium entry mechanism with and without reversal of the entire pump cycle. *J. Physiol.* **207**, 371–391.

GRAHAME-SMITH, D. G. & EVEREST, M. S. (1969). Measurement of digoxin in plasma and its use in the diagnosis of digoxin intoxication. *Br. med. J.* **i**, 286–289.

HUME, SUSAN, P. (1971). A study of glycine transport in Hela cells. B.Sc. Thesis, University of St Andrews, Fife.

KEYNES, R. D. & STEINHARDT, R. A. (1968). The components of the sodium efflux in frog muscle. *J. Physiol.* **198**, 581–599.

LAMB, J. F. & LINDSAY, R. (1971). Effect of Na, metabolic inhibitors and ATP on Ca movements in L cells. *J. Physiol.* **218**, 691–708.

LAMB, J. F. & MACKINNON, M. G. A. (1971*a*). Effect of ouabain and metabolic inhibitors on the Na and K movements and nucleotide contents of L cells. *J. Physiol.* **213**, 665–682.

LAMB, J. F. & MACKINNON, M. G. A. (1971*b*). The membrane potential and permeabilities of the L cell membrane to Na, K and chloride. *J. Physiol.* **213**, 683–689.

LUBIN, L. (1967). Intracellular potassium and macromolecular synthesis in mammalian cells. *Nature, Lond.* **213**, 451–453.

MCCALL, D. (1971). The long-term effects of ouabain on the ionic contents and fluxes in cultured cells. Ph.D. Thesis, University of Glasgow.

MÜLLER, P. (1965). Ouabain effects on cardiac contraction, action potential, and cellular potassium. *Circulation Res.* **17**, 46–56.

REUTER, H. & SEITZ, N. (1968). The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. *J. Physiol.* **195**, 451–470.

SCHATZMAN, H. J. (1953). Herzglykoside als Hemmstoffe für den aktiven Kalium and Natrium Transport durch die Erythrocytenmembran. *Helv. physiol. pharmac. Acta* **11**, 356–364.

SCHATZMAN, H. J. & VINCENZI, F. F. (1969). Calcium movements across the membrane of human red cells. *J. Physiol.* **201**, 369–395.

SHAPIRO, W. (1969). Bioassay of digitalis in plasma. *Circulation* **40**, suppl. III, 184–187.

SHAW, T. I. (1955). Potassium movements in washed erythrocytes. *J. Physiol.* **129**, 464–475.

SMITH, T. W. & HAVER, E. (1969). Radioimmunoassay of serum digoxin and digitoxin in toxicity and malabsorption states. *Circulation* **40**, suppl. III, 188–192.

STRAUB, W. (1924). Die Digitalisgruppe. In *Handbuch der experimentellen Pharmakologie*, Bd. II, 2, pp. 1355–1452, ed. HIFFTER, A. Berlin: Springer.