Biochemical Pharmacology, Vol. 35, No. 20, pp. 3571–3581, 1986.
Printed in Great Britain.

0006–2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

THE RATE OF UPTAKE OF CARDIAC GLYCOSIDES INTO HUMAN CULTURED CELLS AND THE EFFECTS OF CHLOROQUINE ON IT

NAGI ALGHARABLY, DAVID OWLER and JOSEPH F. LAMB

Department of Physiology and Pharmacology, University of St Andrews, St Andrews, Fife, KY16 9TS, Scotland

(*Received* 11 *December* 1985; *accepted* 30 *April* 1986)

Abstract—HeLa cells grown on Petri dishes were either pulse labelled with various cardiac glycosides or grown in low concentrations of them for up to 2 days; either in the presence of chloroquine or not. The cells were then homogenised and the cell free homogenate layered on a continuous sucrose gradient; and the glycoside content and that of various markers measured. In another series of experiments HeLa cells were grown on plastic beads under the above conditions and then the content of glycosides and of some marker enzymes measured. The rate of internalisation of ouabain, digoxin and digitoxin from the plasma membrane preparation produced by the bead method is at 9% hr^{-1}, similar to the rate of loss of digoxin and digitoxin from whole cells but much faster than that of ouabain. In the sucrose gradient experiments it was found that [^{3}H]ouabain, digoxin and digitoxin all initially co-distribute with the plasma membrane marker, 5′-nucleotidase, and then leave this fraction of the homogenate at a fast rate when kept at 37°, to co-distribute with the lysosomal marker, β-hexosaminidase. At 2° the ouabain remains co-distributed with the plasma membrane marker. The rate of transfer is estimated to be some 90% hr^{-1}, much faster than previously thought. Chloroquine causes an increased retention of digoxin and digitoxin in the lysosomal fraction of the homogenate. These results are best explained by supposing that the sodium pump–glycoside complex rapidly enters a region of the peripheral cytoplasm, and that this region then controls the subsequent exit of digoxin and digitoxin from the cell. The main barrier for ouabain occurs at a stage later than this. The consequences of this model on other aspects of pump activity is discussed.

Sjoerdsma and Fisher [1] first showed that rabbit hearts perfused with radioactive digitoxin accumulated large concentrations of it. Subsequently many workers studied the uptake of various glycosides into tissue fractions (often rat heart) but the mechanism was unclear [2]. Recently it has been shown that sodium pumps are continually being internalized and replaced in the cell membrane [3] and it has been suggested that the mechanism for uptake of glycosides is in association with the sodium pump [4].

A main aim of the present experiments was to measure the rate at which glycosides are taken up into the various compartments of the cell from the plasma membrane. The current view based on experiments by Cook [3] and ourselves [5] suggested that this rate was at about 9% hr^{-1}, but some of our earlier autoradiography experiments suggested a much faster rate [6], and earlier experiments with perfused hearts [2] also suggested a rapid rate of uptake; these latter experiments had the disadvantage, however, that the concentration of glycoside used was high and so the route of uptake was unclear. The difference is important, for a rapid uptake is consistent with a fast cycling of the pumps at about the same rate as receptor mediated endocytosis [7], whereas a slower rate of uptake is consistent with a synthesis driven turnover. We have used two techniques to study this further, either producing a plasma membrane preparation by a bead method [8] or by a continuous sucrose gradient [3]. The results show that glycosides leave the plasma membrane at a fast rate and that the rate limiting step is in a region near the plasma membrane. In a further series of experiments we show that chloroquine slows the processing of digoxin through these cells at a lysosomal step, and hence at a stage later than this. Preliminary accounts of these results have appeared [9].

MATERIALS AND METHODS

HeLa cells, supplied by Flow, were cultured in BME with Earle's salts in a monolayer on 120 ml Roux bottles and subcultured onto 5 cm Petri dishes for the experiments. The cells were either pulse labelled with the radioactive glycoside in order to examine the excretory process [5] or were grown in low concentrations of the radioactive glycoside to study accumulation [4]. Digoxin and digitoxin bind to HeLa cells in an exchangeable and non-exchangeable way, whereas ouabain only binds non-exchangeably, the difference being due to the lipid solubility [4]. In all experiments involving glycoside incubation, the cells were washed for 15 m at 2° to remove this exchangeable fraction before further measurement.

The cells were fractioned on a linear sucrose gradient according to [3] (Fig. 1). Figure 2a shows the distribution patterns of the subcellular marker used.

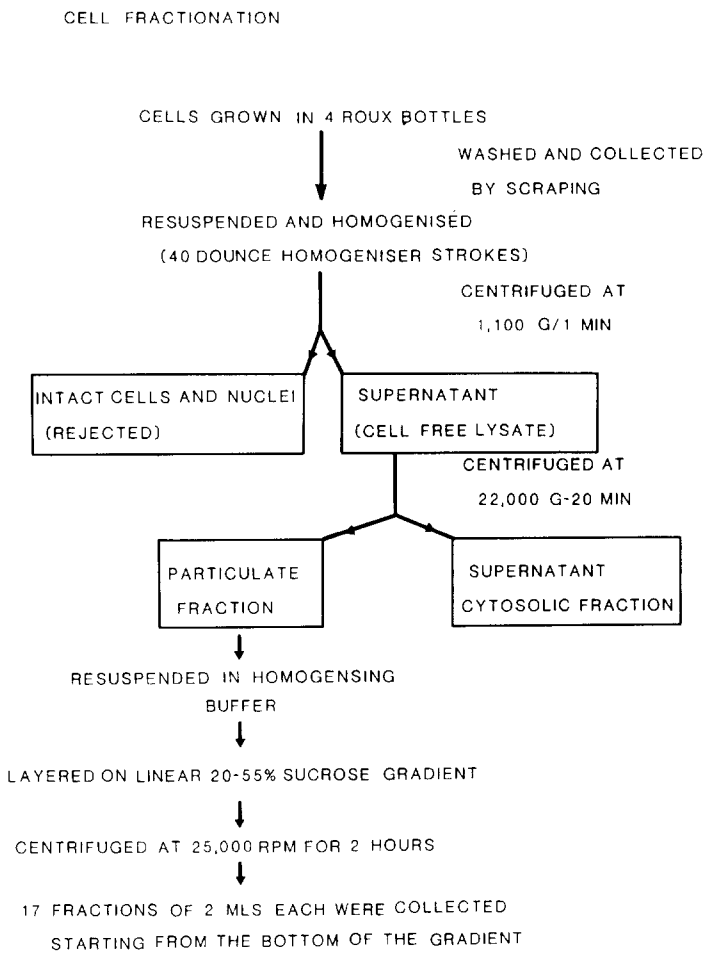

Fig. 1. Cell fractionation.

These are 5′-nucleotidase (a plasma membrane marker [10]) β-hexoaminidase (a lysosomal marker [11]), succinate dehydrogenase (a mitochondrial inner membrane marker) and lactate dehydrogenase (a cytosolic marker). In preliminary experiments cathepsin B was found to distribute in the same way as β-hexosaminidase. Figure 2b shows the recovered activity of these markers. In control experiments ouabain* or digoxin* was added to the particulate fraction and then layered on the gradient. All the activity remained in the overlay and none entered the gradient, showing that redistribution did not occur during preparation.

Assays. Protein was assayed by Lowry's method [12]; 5′-nucleotidase according to [13]. β-Hexosaminidase was assayed [12] in the presence of *p*-nitrophenyl-*N*-acetyl-*B*-glucosaminide as substrate, succinate dehydrogenase according to [14] and lactate dehydrogenase according to [15].

Total lipids were extracted from the particulate fraction [16], and total lipid phosphorus was determined [17].

Microcarrier cell culture. HeLa cells (15 million) were obtained from a rapidly dividing Roux culture and mixed with 0.5 g Superbead microcarriers in a 500 ml spinner culture vessel. The cells and beads were incubated in a final volume of 200 ml BME supplemented with 10% (v/v) new-born calf serum. The growth medium was gassed with a 95% air/5% CO_2 mixture and changed every 2–3 days. HeLa cells growing on microcarrier beads were harvested after 5–7 days.

Materials. The microcarrier culture stirring system MCS-104 and 500 ml spinner flasks were obtained from Techne (Cambridge) Ltd. Superbead microcarriers were obtained from Flow Laboratories (Scotland).

Plasma membrane isolation on microcarrier culture beads. This method was adapted from techniques of [18] and [8]. A 10 ml sample from a microcarrier culture with cells at or near confluence on the microcarrier beads, was collected into a sterile universal tube (Sterilin). This sample was washed 4 times with cold Tris buffered saline (TBS) (0.9% NaCl, 5 mM Tris). Between each wash the microcarrier beads were allowed to settle out before the supernatant was removed. Usually this took 3 min. The sample was then incubated with ice-cold hypotonic solution (1 mM Tris, HCl pH 8.0) usually for 30 min and then washed twice in ice-cold 10 mM Tris–HCl pH 8.0. A

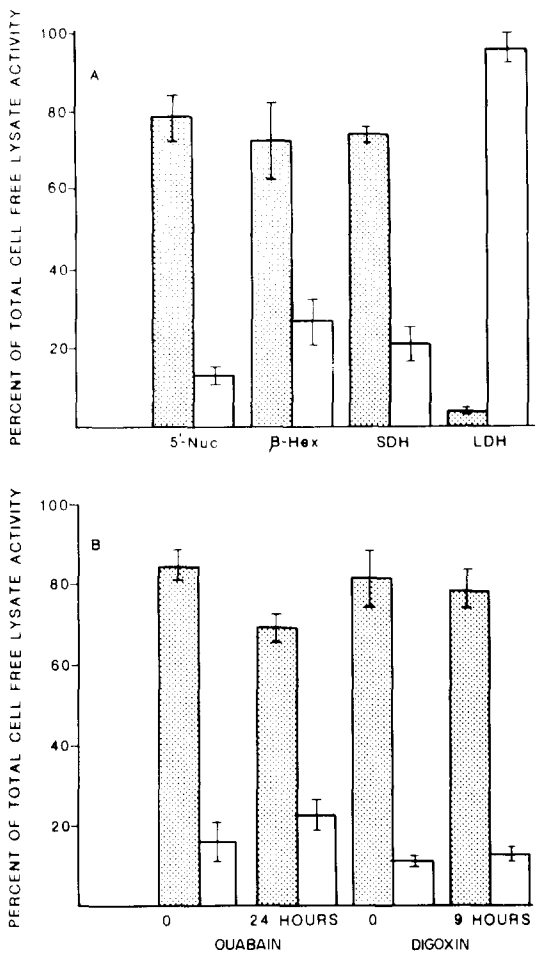

Fig. 2. The amount of enzymes, ouabain and digoxin activities recovered in the particulate fraction. The activity of the relevant markers was measured in the particulate fraction (dotted column) and in the cytosolic fraction (clear column). The recovered activity in each fraction is plotted as the percent of total cell free lysate activity. (A) shows the result of 5'-nucleotidase (5'-nuc), β-hexosaminidase (B-hex), succinate dehydrogenase (SDH) and lactate dehydrogenase (LDH). (B) shows that ouabain activity recovered in the particulate fraction was decreased after internalization and that remaining in the cytosolic fraction was increased. Digoxin activity did not change. The results are the mean of three experiments ± S.D.

10 ml aliquot of ice-cold 10 mM Tris–HCl pH 8.0 was added to the cells on beads and the suspension vortexed for 10 sec, resulting in complete cell lysis. The microcarrier beads were allowed to settle and then washed twice more in ice-cold 10 mM Tris–HCl pH 8.0. The supernatant was removed and enough ice-cold 10 mM Tris–HCl pH 8.0 added to give approximately a 50% suspension of beads. This suspension was then sonicated for 10 sec at 20 W in a Sonifier cell disruptor (model W 185) and then washed twice more in ice-cold buffer. All procedures were performed on ice. Analysis of isolated membranes on beads showed that about 25–30% of the total activity of plasma membrane markers was recovered in membranes on beads. The degree of purity of isolated HeLa plasma membranes on beads compared favourably with other isolation procedures tried.

Measurement of the rate of loss of cardiac glycosides from HeLa plasma membranes. HeLa cells growing in a microcarrier culture for 4–6 days were specifically labelled with [^{3}H]glycoside by methods previously described [4] and then returned to fresh, warm growth medium. At various time intervals samples were removed from the microcarrier culture, plasma membranes on beads prepared and the [^{3}H]glycoside bound to the plasma membrane determined.

RESULTS

Experiments with glycosides in HeLa cells grown on beads

HeLa cells were grown on plastic beads and then pulse labelled with glycosides in a zero K salt solution and returned to various growth media. Samples taken initially, and at various times thereafter were analysed for the amount of radioactivity in the part of the cell left on the bead after lysis and in the whole cell. Figure 3 shows the exponential rates of loss of ouabain in these different circumstances. The fastest rate of loss is of ouabain from the bead fraction at 37° (9% hr^{-1}), followed by that from whole cells also at 37° (4% hr^{-1}) and then that from the bead fraction at 4° (0.5% hr^{-1}). In similar experiments with digoxin the rates of loss of the glycoside from the bead fraction and from the whole cells was the same at around 9% hr^{-1}.

The new information these experiments shows is that both ouabain and digoxin move at the same rate from the plasma membrane fraction produced by the bead method into the rest of the cell. This means (a) that the slow release of ouabain from the whole cell occurs at a later stage within the cell but that (b) the rate limiting step for the loss of digoxin is in this fraction at or near the plasma membrane.

Table 1 summarizes the results of a large number of experiments on this preparation. The main points are that (a) reducing external potassium concentration from the normal 5 mM to about 0.2 mM increases the specific glycoside binding to the cells [6], and reduces the glycoside efflux [6]. The potassium effect on efflux occurs and disappears within the time course of the efflux, i.e. the first 6 hr. Calculation from the present result on the model given later suggests that the entire explanation may be due to this direct effect; (b) serum interacts with this effect of potassium in that at high serum concentrations it is not present; (c) a high external potassium also slows efflux to about half, perhaps by a reduced membrane potential; (d) the drugs chloroquine, cycloheximide and monensin all slow the efflux of digitoxin from cells, but not from the bead preparation. This means that they act at a stage later than the periphery of the cytoplasm. Many other drugs tried had no effect.

These experiments appear to confirm earlier results in showing that the rate of movement from the plasma membrane to the rest of the cell occurs at a rate of about 9% hr^{-1}. There are, however, some problems with this interpretation. Thus cells

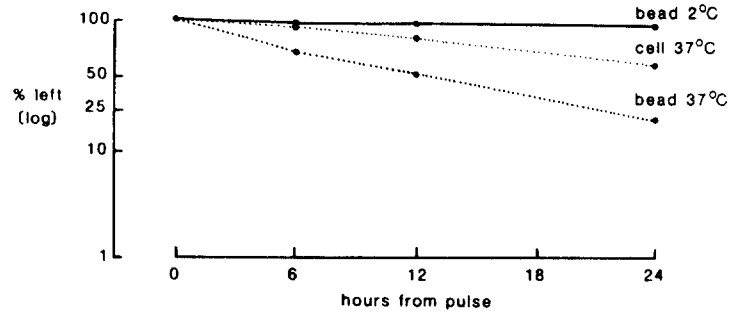

Fig. 3. The efflux of ouabain from HeLa cells under various conditions. HeLa cells were pulsed with 2×10^{-7} M [^{3}H]ouabain in K-free Krebs, washed for 15 min in 5 mM K Krebs and returned to growth medium. At 6, 12 and 24 hr the cells were sampled and the [^{3}H]ouabain activity, cell numbers, etc. measured, and the result expressed as a % of that initially present. The results are shown for three conditions. The activity present in the bead fraction of the cells decays with a rate constant of 0.005 hr^{-1} at 2°, at 0.09 hr^{-1} at 37° but in the whole cell it decays at a rate constant of 0.04 hr^{-1}. The results are typical experiments on separate occasions.

grown on beads become very firmly attached, probably because the microvilli grow into the interstices of the bead. When such cells are then lysed by exposure to low tonicity fluid about 25% of the plasma membrane remains together with a thin rim of the adjacent region of the cell and the contents of the microvilli. So it is not only a pure plasma membrane preparation, for analysis of the material left on the beads shows that it contains both plasma membrane and other constituents of the cell. Thus though the plasma membrane marker 5′-nucleotidase was enriched by 5–7-fold the lysosomal marker β-hexosaminidase was still present in reduced amounts. No other markers were present (Table 2).

It is not possible, therefore, to be sure that these results prove that the rate limiting step for internalization is at the plasma membrane; the results are also consistent with a movement of the glycoside

Table 1. Effect of diverse conditions on the specific activity and efflux of cardiac glycosides from HeLa cells

Conditions		Specific activity (pmoles per mg protein)	Efflux rate constant (hr^{-1})
(a) Ouabain and digoxin on cells and beads			
Ouabain pulse	Beads	20.6 ± 0.9	9.4 ± 0.2
	Cells	2.04 ± 0.24	4.4 ± 0.3
Digoxin pulse	Beads	19.5 ± 2.8	9.8 ± 0.1
	Cells	1.87 ± 0.38	9.6 ± 0.1
(b) Varying K in growth medium on ouabain in bead preparation			
Ouabain control on beads		21.3 ± 1.8	9.0 ± 0.1
24 hr low K growth low K efflux		29.2 ± 1.6†	3.9 ± 0.5†
24 hr low K growth 5 K efflux		27.6† ± 0.7	9.1 ± 0.3
Normal growth low K efflux		19.7 ± 3.0	4.6 ± 0.4†
2% serum		15.5 ± 0.5	3.9 ± 0.5†
10% serum			9.3 ± 0.5
20% serum			9.8 ± 0.7
135 mM K, 12 mm Na 2% serum		27.3 ± 4.3	5.3 ± 0.5†
(c) Drugs on digoxin efflux in whole cells			
Control		0.77 ± 0.20	9.4 ± 0.2
Chloroquine 100 μM			6.3 ± 0.4†
Cyclohexamide			8.0 ± 0.4*
Monensin			6.7 ± 0.4†

Part a shows the overall control values for ouabain and digoxin on the content of the plasma membrane fraction from the bead preparation and in whole cells. Part b shows the effect of growing in low K medium (around 0.2 mM) on the specific activity (increases by 37%) and on the ouabain efflux (decreases to 43%). This effect on efflux occurs as soon as low K medium is applied and goes as soon as the normal 5 K medium is re-applied. High external K also reduces the ouabain efflux.

Part c shows that chloroquine, cycloheximide and monensin all decrease the digoxin efflux from whole cells. These drugs had no effect on the cardiac glycosides retained by the bead preparation. Cytochalasin b, nocadazole, monodansylcadaverine and trifluoroperizene had no effect on ouabain or digoxin on either preparation.

Means and S.E. shown, N was 3–8 per value. Effluxes were fitted by the Glim package on Vax. * and † shows significance at the 0.05 and 0.01 levels respectively, when compared to their own controls.

Table 2. Concentration of some markers in whole HeLa cells and in the bead preparation

Markers	Site	Whole cell	Beads	Ratio
5′-Nucleotidase (a)	Plasma membrane	1133 ± 110	5477 ± 460	4.8
Adenylate cyclase (b)		769 ± 44	4042 ± 220	5.3
Ouabain binding (c)		2.413 ± 0.197	18.96 ± 0.54	7.9
β-Hexosaminidase (d)	Endosomes Lysosomes	3.9 ± 0.08	2.8 ± 0.02	0.7

The units for each are: (a) nmoles hydrolysed per 1 per mg protein; (b) pmoles cAMP per 1 per mg protein; (c) pmoles ouabain bound per mg protein; (d) μmoles p-nitrophenol per 1 per mg protein.

There was no detectable lactate dehydrogenase, succinic dehydrogenase or NAD-fenicyanide reductase in the bead preparation.

from the plasma membrane to vesicles near the membrane and a rate limiting step there [19]. A useful extension of these experiments would be to fraction the cell remains from the beads on a gradient, but we have not succeeded in this so far.

Experiments with continuous sucrose gradients

The markers used to identify the positions of the plasma membrane, the lysosomal and the mitochondrial fractions were 5′-nucleotidase, *b*-hexosaminidase and succinic dehydrogenase respectively. Although there was a considerable overlap of the distribution of these markers they showed clearly distinct peak activity in fractions 12, 10 and 8 corresponding to specific gravities of 1.127–1.132, 1.151–1.156 and 1.171–1.176, respectively (Fig. 4). The cytosolic marker (lactate dehydrogenase) appeared at the top of the gradient.

Ouabain and digoxin distributions with time and temperature

Cook and his colleagues [3] showed that when cells were pulsed with ouabain it initially co-distributed with the plasma membrane marker but 48 hr later co-distributed with the lysosomal marker. Our preliminary experiments gave similar results and in addition showed that this movement from plasma membrane to the lysosomal compartment was temperature sensitive. Figure 5a shows that when cells are pulsed with ouabain (2×10^{-7} M in K free salt solution) and then kept at 2° for 24 hr the ouabain remains co-distributed with the plasma membrane, whereas at 37° it moves to the lysosomal compartment. At 2° the total amount decreases by around 10%, whereas at 37° it decreases to half. This is the kind of behaviour expected for an active uptake process. In control experiments we found that pulsing with the same concentration of ouabain in a salt solution containing 15 mM K gave less than 10% uptake compared to K-free conditions, and no clear distribution of ouabain could be distinguished on the gradient. Attempts to load the cells at 2° gave similar negative results. These control experiments show that the uptake is "specific" via a receptor and not by simple diffusion. Much earlier data has identified this receptor as the sodium pump.

Figure 5a also shows that although ouabain and β-hexosaminidase both co-distribute by 24 hr at 37°, both show a large activity at the top of the gradient. We tried to increase the separation of the lysosomal and plasma membrane markers by growing the cells in dextran-500 which has been used for this purpose for *in vivo* experiments [20]. Figure 5b shows that while this does shift part of the distribution towards

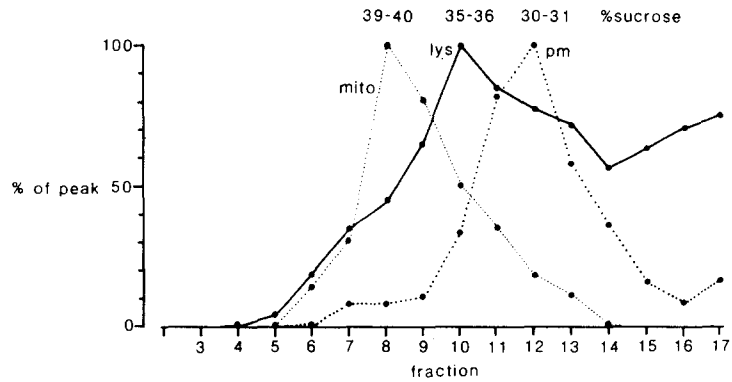

Fig. 4. The distribution patterns of subcellular markers. The particulate fraction was prepared and then layered on the linear sucrose gradient. The enzymes activities were detected in each gradient fraction and plotted as a percent of peak fraction. 5′-Nucleotidase peaked at 30–31% w/w sucrose and little activity was found in the overlay. β-hexosaminidase distributed broadly throughout the gradient and peaked at 35–36% w/w sucrose with a substantial amount of activity remaining in the overlay. All the succinate dehydrogenase pelleted activity entered the gradient and showed a limited distribution with a peak in the dense part of the gradient at 39–40% w/w sucrose. All the pelleted activity of the cytosolic marker lactate dehydrogenase remained in the overlay. The points are the mean of three gradients.

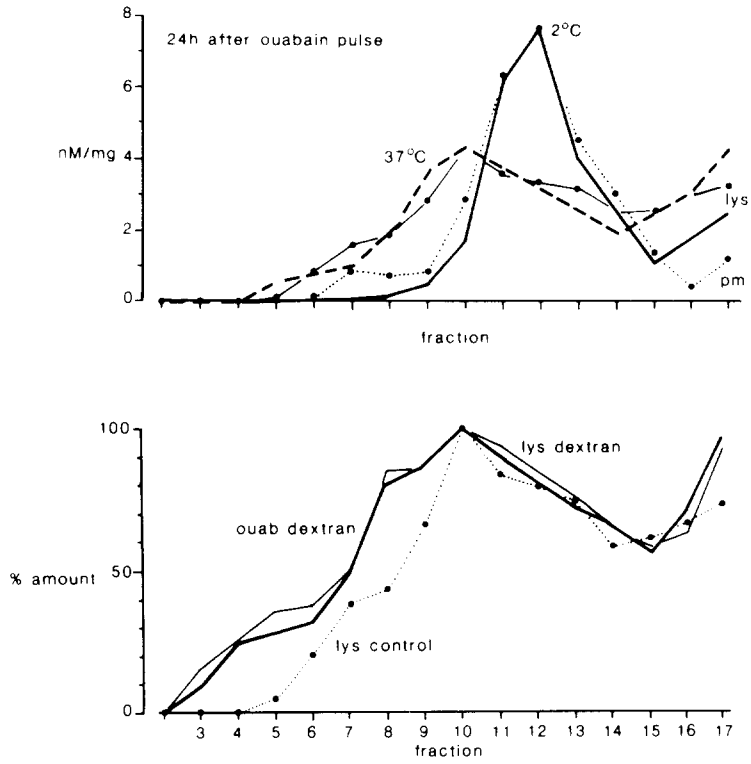

Fig. 5. Ouabain distribution pattern on a linear sucrose gradient 24 hr after pulse loading. HeLa cells were pulse labelled and returned to growth medium for 24 hr at 2° or 37°. The ouabain distribution in the cells kept at 2° (a) is close to that of the plasma membrane marker 5′-nucleotidase, and the amount remaining is only some 10% less than that present initially. The ouabain distribution in those cells kept at 37° is close to that of the lysosomal marker β-hexosaminidase, and the amount is about half of that originally present. Figure 5b shows the broadening of the distribution of β-hexosaminidase and of ouabain produced by growth in dextran-500; without the peak moving. In this and succeeding illustrations the distributions of the plasma membrane marker 5′-nucleotidase and the lysosomal marker, 5′-nucleotidase done with the same batch of cells is shown. The ouabain results are the mean of 3 experiments.

the denser end of the gradient it does not alter the mean peak position, nor reduce the activity at the top end of the gradient. Ouabain does continue to co-distribute with the β-hexosaminidase marker, as expected if it is associated with the lysosomes.

Digoxin initially co-distributes with the plasma membrane marker (Fig. 6a), but with much less activity at the top of the gradient than ouabain. By 9 hr the amount on the gradient has decreased to about half and it now co-distributes with the lysosomal marker β-hexosaminidase (Fig. 6b), but again with very little free activity at the top of the gradient. The amount of activity left at 9 hr is that expected from earlier results, but the distribution of it is not, for little activity is associated with the plasma membrane marker 5′-nucleotidase.

The rate of internalization of glycosides

To get a better measure of the rate of uptake of digoxin we measured the distribution at 2 and 4 hr after pulsing. This showed that by 2 hr digoxin largely co-distributed with the plasma membrane marker. To show this and other data in a more abbreviated form, we have quantified the shifts by expressing them as the ratio of the plasma membrane to the lysosomal markers (Fig. 7). To do so we measured the activity in fractions 12 and 13, as a measure of plasma membrane marker activity, and in fractions 9 and 10, as a measure of lysosomal marker activity. The ratios of plasma membrane to lysosomal activity calculated in this way for ouabain, digoxin and digitoxin just after pulse labelling is around 4, similar to that of the plasma membrane marker 5′-nucleotidase. After the cells have been incubated for 2 hr at 37° the ratio for digoxin is less than 1, similar to that of the lysosomal marker β-hexosaminidase. Cells pulsed with ouabain and left for 24 hr at 2° had a ratio of over 5. From the digoxin result at 2 hr the minimum rate of movement of the glycoside away from the plasma membrane is 90% hr^{-1}, some 10-fold faster than the earlier "bead" estimate. This result fits our previous data obtained by the autographical localization of ouabain after pulse labelling and various periods of incubation, which showed that ouabain moved into the cell at a rate of at least 80% hr^{-1} [6].

Cook and his colleagues [21] showed that internalized ouabain and the lysosomal enzyme -β-hexosaminidase were similarly sensitive to shearing force and to osmotic shock applied to the particulate frac-

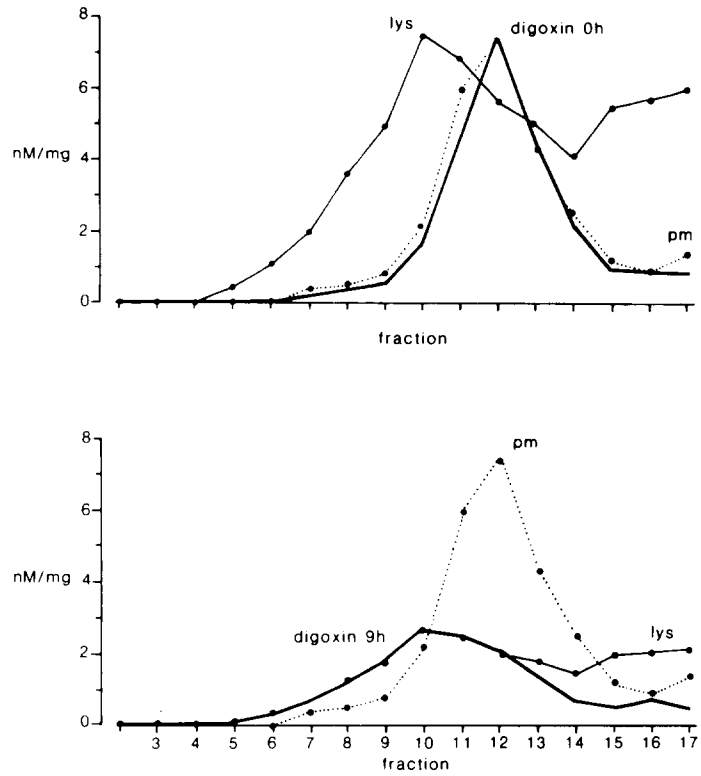

Fig. 6. The distribution pattern of digoxin activity (a) immediately after binding and (b) 9 hr later. Cells were labelled with [^{3}H]digoxin and prepared as in Fig. 5. Digoxin initially co-distributes with the plasma membrane marker 5′-nucleotidase (a) but by 9 hr it co-distributes with the lysosomal marker β-hexosaminidase (b); there was no digoxin activity in the overlay at 9 hr. Results are the mean of 3 separate experiments.

tion. In our experiments cells which were severely homogenized (100 strokes), released most of their β-hexosaminidase and internalized ouabain to the cell free lysate. When this was layered on the gradient most of the activities of β-hexosaminidase and ouabain remained at the top. Digoxin, however, remained in the same fraction after this treatment so that its sedimentability does not depend on lysosomal integrity (Fig. 8). This is consistent with the view that digoxin is tightly bound to the lysosomal membrane.

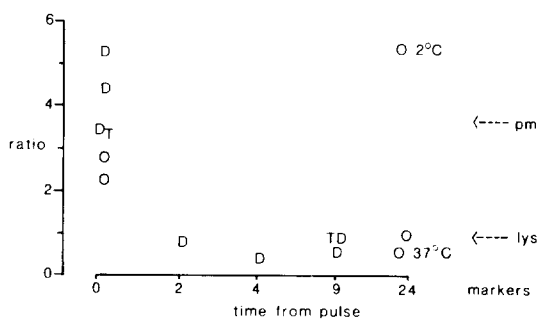

Fig. 7. The ratio of plasma membrane to lysosomal activities under different conditions. HeLa cells were labelled and run on the sucrose gradient as before. The figure shows the ratio of fractions (12 + 13) to (9 + 10) for the glycosides ouabain (O), digoxin (D) and digitoxin (T) at various times and at 2 or 37°. They all have an initial ratio similar to that of the plasma membrane marker 5′-nucleotidase, but within 2 hr at 37° have a ratio similar to that of the lysosomal marker β-hexosaminidase. When kept for 24 hr at 2° the ratio is at the upper end of the initial one.

Effects of chloroquine

In previous experiments we showed that chloroquine (and other weak bases) reduces the efflux of digoxin after a pulse label and increases its uptake in chronic growth experiments with HeLa cells, but has little effect on ouabain handling [4]. We have repeated these experiments with our present techniques. The actions of chloroquine on cells are complex, for it not only increases the pH in the lysosomal compartments, but has diverse effects on other parts of the endocytotic and other pathways [22]. In preliminary experiments we checked its effects in inhibiting lysosomal phospholipases [23, 24] and on β-hexosaminidase concentrations. Table 3 shows that chloroquine causes the accumulation of phospholipids in HeLa cells, and decreases the activity of β-hexosaminidase but has no effect on the lactate dehydrogenase activity. These are the expected results.

Our previous results with the "bead" method suggested that chloroquine slowed the rate at which ouabain and digoxin left the plasma membrane region [4], but when all the results are examined this effect is not significant (Table 1). This means that the slowing of the digoxin efflux by chloroquine

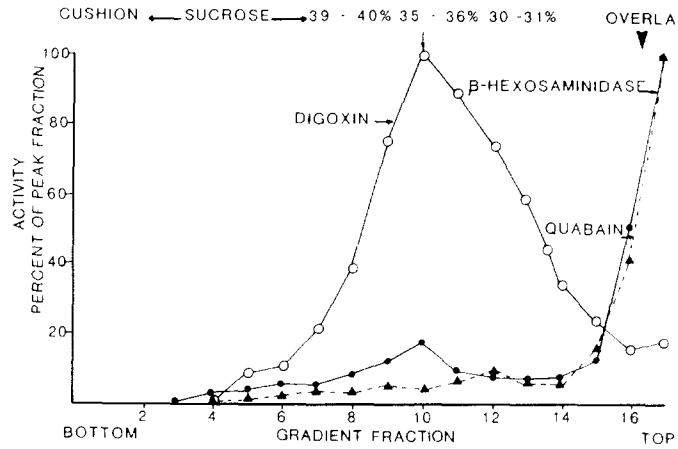

Fig. 8. The effect of severe homogenisation on the release of β-hexosaminidase, ouabain and digoxin activities and their distribution patterns. Cells were loaded with ouabain and digoxin, washed and returned to normal growth medium for 24 hr (ouabain) or 10 hr (digoxin). The cell free lysate was prepared after applying severe homogenization (100 strokes), to the cell suspension. Then it was layered on the linear sucrose gradient, and both β-hexosaminidase and ouabain activities in each gradient fraction were detected. The distribution patterns show that β-hexosaminidase and ouabain were solubilized by severe homogenization and most of β-hexosaminidase and ouabain activities remained in the top of the gradient giving a different distribution pattern from those obtained with normal homogenization. The digoxin distribution after 100 strokes was similar to that obtained with normal homogenization. The results are the mean of three gradients plotted as the percent of peak activity.

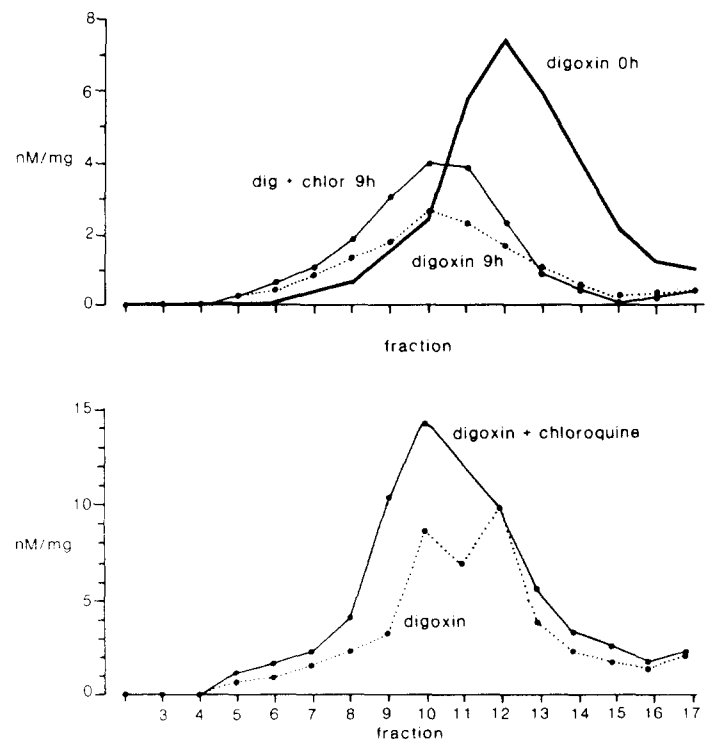

Fig. 9. The effect of chloroquine on the excretion and accumulation of digoxin. (a) Cells were loaded with digoxin, washed and returned to normal growth medium ± 1X1E-04 M chloroquine for up to 9 hr. Chloroquine-treated cells retained more digoxin 9 hr after pulse labelling than untreated cells. The retained activity caused by chloroquine showed a peak of activity similar to that of the lysosomal marker but different from that obtained immediately after binding. The rate of decrease of digoxin was 12% hr^{-1} normally and 7% hr^{-1} after chloroquine. (b) Cells were grown in the presence of 1XE-08 M digoxin ± 11XE-04 M chloroquine for 48 hr. The distribution pattern of the particulate fraction prepared from chloroquine untreated cells showed two peaks of activity, in position similar to that of the lysosomal marker and the plasma membrane marker. Very little soluble activity was found in the overlay. Chloroquine treated cells showed only one sharp peak at the lysosomal peak fraction with a large increase in the accumulated amount of digoxin.

Table 3. The effect of chloroquine on the amount of phospholipids β-hexosaminidase and lactate dehydrogenase in HeLa cells

	μg Phosphorus/mg protein	β-Hexosaminidase (μmol/culture)	Lactate dehydrogenase (μmol/culture)
Control	0.275 ± 0.083	1.15 ± 0.07	0.79 ± 0.06
10 μM Chloroquine	0.511 ± 0.071		
100 μM Chloroquine	0.663 ± 0.067	0.70 ± 0.4	0.81 ± 0.07

The phospholipid results are from cells grown for 48 hr in chloroquine; those for the others are after 5 hr treatment. Although the amount of *b*-hexosaminidase released per hour was increased by chloroquine it was not significant at the 5% level. Each result is the mean of 4 experiments.

occurs at a stage later than this, and is not due to a slowing of internalization.

Figure 9a shows that the slowing of efflux in cells pulse labelled with digoxin and then returned to growth medium ("acute experiments") is due to an increased retention of glycoside in the lysosomal compartment of the cell; the amount of this retention (+35%) is similar to that reported before [4] for whole cells. Taken with the "bead" data this means that the slowing effect of chloroquine on the efflux is somewhere along the lysosomal chain, later than in the region of the cell membrane. It could be either on the pathway to the lysosomes or in them. In similar experiments with ouabain we found no effect of chloroquine, either using the "bead" method or the sucrose gradient. This confirms earlier findings and is possibly because the excretion of ouabain from the cell is very slow due to its low fat solubility, thus hindering its exit across the various membranes within or bounding the cell.

In another series of experiments cells were grown for 1–2 days in low concentrations of digoxin or ouabain in normal growth medium in the presence or absence of chloroquine ("chronic growth"). Figure 9b shows that in the absence of chloroquine digoxin is present both in the plasma membrane and the lysosomal compartments, giving a ratio of 1.2 as calculated for Fig. 7. This is the (qualitative) result expected for steady state conditions where digoxin is continually being bound to sodium pumps on the plasma membrane, and then being internalized by the cell. When chronic growth occurs in the presence of chloroquine, the total amount of digoxin on the gradient increases by 56%, with a larger increase in the lysosomal fraction than in the plasma membrane fraction. This result is quantitatively consistent with the results obtained in whole cell experiments described previously [4], and is also consistent with the acute experiments described using the bead and fractionation methods above. It can be explained by a mechanism in which digoxin is retained in the bulk of the lysosomes due to the increased pH reducing the activity of the lysosomal enzymes. Similar experiments with ouabain showed no effect on the amount or distribution of the glycoside, a result the same as that found previously [4].

DISCUSSION

The main finding of these experiments is that the two different methods of preparing a plasma membrane fraction to measure the rates of movement of the cardiac glycosides ouabain and digoxin into the cells give different results. When we use the "bead"

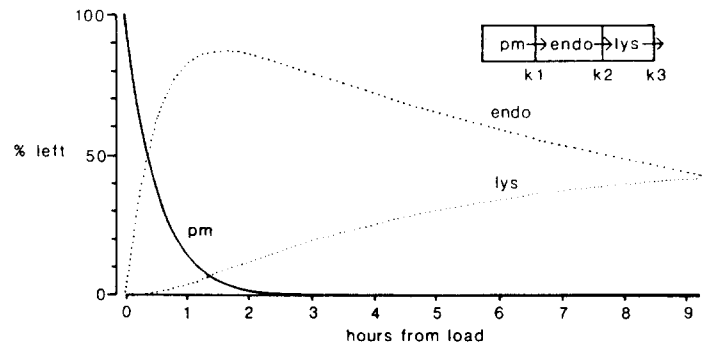

Fig. 10. Model of glycoside internalisation in HeLa cells, following a pulse load. The model was calculated using rate constants of 2, 0.1 and 0.07 hr^{-1} for K_1, K_2 and K_3, representing plasma membrane to endosomes, endosomes to lysosomes, and lysosomes to the outside of the cell, respectively. The rate limiting step for ouabain release is K_3, so that a large amount of ouabain is retained in the lysosomes; while that for digoxin is K_2, for K_3 becomes very large and thus there is virtually no digoxin in the lysosomal compartment. Chloroquine acts on K_3 so retaining digoxin in the lysosomes, an appropriate value for K^3 to explain this is 0.4 hr^{-1}. When cells are grown in digoxin most is held in the endosomes rather than the plasma membrane, due to K_1 being much larger than K_2. The model shows the movement of the glycoside ligand between the compartments. The movement of the label is only in one direction, from plasma membrane to lysosomes, as much backflow would not fit the observation that the plasma membrane is cleared of digoxin following pulse labelling. It is possible that unlabelled sodium pumps move in both directions between the plasma membrane and lysosomal compartments.

method we get rates of around 9% hr^{-1} for both glycosides; consistent with earlier results; but when we use a sucrose gradient to identify the plasma membrane we get a rate some ten times faster. There are two kinds of explanation for this result:

(1) The simplest one is that the sucrose gradient does not give a sufficient discrimination to tell if the glycosides are on the plasma membrane or elsewhere. This seems unlikely in that if cells are kept for 24 hr at 2° then the glycoside remains very close to the plasma membrane marker 5′-nucleotidase, whereas in 2 hr at 37° it has moved to co-distribute with the lysosomal marker β-hexosaminidase (Fig. 7).

(2) The more complex explanation is that both the experimental results are correct, but that the "bead" method gives a preparation which contains both plasma membrane and a part of the lysosomal system closely associated with it, and that the glycosides are moving rapidly from the plasma membrane to this part of the lysosomal system.

Previous results with autoradiography experiments showed that ouabain moves into the cell away from the plasma membrane at a fast rate [6], so that this second explanation fits better. Figure 10 shows a schematic diagram of this model with rate constants chosen to fit the data from these results. In this model we have called the vesicles near to the plasma membrane endosomes [19]. Digoxin moves from the plasma membrane to the endosomes with a rate constant of about 1 hr^{-1}, and from the endosomes to the lysosomes with a rate constant of about 0.1 hr^{-1}; it is then rapidly processed there and leaves the cell. The rate limiting step for digoxin (and digitoxin) is from the endosomes to the lysosomes. Ouabain follows a similar route to the lysosomes but is then greatly delayed in its release from the cell (to about 0.07 hr^{-1}). A suggested reason for this comes from the present observations that rupture of the lysosomal membranes releases all the ouabain to the top of the gradient, whereas this does not happen with digoxin, so ouabain seems to be free within the lysosomes. The explanation then is that ouabain is slow to leave the cell because of its low lipid solubility, whereas digoxin leaves rapidly as soon as it is free.

This model implies that there is a rapid internalization of glycosides into the cell, similar in rate to receptor mediated endocytosis of LDL and other substances [7], and that the overall rate limiting step is in a region close to the plasma membrane. There is no direct evidence that the receptor for this internalization, i.e. the sodium pump, is handled in the same way for we have not measured it directly. It might be considered likely, however, in that in comparison with other endocytotic schemes we could suppose that the pump-glycoside complex is internalized, the glycoside split off in a low pH environment [25] and then the pump recycled to the plasma membrane. There are several pieces of circumstantial evidence that the sodium-pump does follow the same route at the same rate.

(a) Cook [11] found that the turnover rate of sodium pumps in HeLa cells as measured by heavy amino-acids was with a rate constant of about that of our endosomes to lysosome rate, i.e. 1.2 hr^{-1}. This implies that the pumps are in our endosome fraction.

(b) We find that digoxin is tightly bound to the lysosomal membrane until released by the enzymes. This means that either the digoxin molecule is still bound to the pump or has been transferred to another molecule until splitting.

(c) All the sodium pumps on the plasma membrane of HeLa cells are not available for blocking at 2° but become available when the cells are heated back to 37° [26]. This is consistent with a model of sodium pumps held on the plasma membrane and in a pool just beneath it.

The experiments with low potassium give a rate constant for digoxin loss (K_2) of about 4 hr^{-1} compared to 9 hr^{-1} in normal potassium. If this is the only effect of low potassium it is sufficient to account for the "upregulation" observed. Further work needs to be done to compare this change in rate constant with changes in internal sodium. The interaction between serum and low K fits that observed earlier [6].

The slowing effects of high K are new findings. We did these experiments because it has been found [27] that potassium manipulations had an effect on LDL internalization, although the precise effects were difficult to entangle in these experiments. The present results are consistent with an effect of membrane potential on internalization.

The effect of chloroquine

Chloroquine and other weak bases interfere with various functions of cultured and other cells. The most obvious action, known for many years, is that they produce vacuolation due to an expansion of the vesicular system. This was shown by de Duve [28] to be due to a large accumulation of the weak bases within the acidic vesicles in the cell, with a consequent rise in the pH of these vesicles. Further work has shown that chloroquine and the other weak bases also have effects on the various vesicular pathways within cells, and on the fusion of vesicles as well as its effect in increasing the pH of lysosomes and endosomes [22].

Griffiths *et al.* [4] showed that chloroquine, amantadine and ammonium chloride decreased the excretion rate and increased the accumulation of digoxin and digitoxin by HeLa cells, and also "down-regulated" the number of sodium pumps in long term experiments. The present experiments suggest that these effects of chloroquine are not due to changes in the internalization of the pump-glycoside complex but occur at a stage after the peripheral cytoplasm on the path from endosomes to lysosomes, although whether it is due to altered fusion or a direct effect of a change in pH is not clear. These results are consistent with our previous evidence that the chloroquine effects were occurring at a stage with a rate constant of around 9 hr^{-1} [4]. If this interferes with the ligand–sodium pump splitting, due perhaps to the reduced pH in the endosomes or lysosomes splitting off the glycoside [25], then a reduced recycling of the pump might result. This may partly explain the marked down regulation of pump numbers which occurs in cells grown in chloroquine. Other possible explanations for this effect are inhibition of membrane fusion and a decrease in protein synthesis [22].

Overview of the action of cardiac glycoside on the heart

There is an extensive literature on attempts at relating the accumulation and handling of cardiac glycosides, as studied here, with the therapeutic actions of these substances [2]. We think that a likely overview of the scheme is the following.

1. The cardiac glycoside binds to the sodium pump on the plasma membrane of heart cells and so reduces the pumping capacity of the cell; this eventually leads to a rise in internal sodium with consequent sodium-calcium interactions and a positive inotropic action on the heart.
2. The pump–ligand complex on the plasma membrane is rapidly internalized, enters the endosome/lysosome system where the glycoside is split from the pump, and the pump may be re-cycled to the plasma membrane. There may also be a cycling of unblocked pumps to the plasma membrane to replace those blocked by the glycoside. Both these mechanisms have the effect of opposing the blocking actions of the cardiac glycosides.
3. The inotropic effect and the accumulation of glycosides within the cells thus follow divergent pathways after the initial binding (and blocking) event, so that they do not have any simple relationship; thus the many attempts to show such a relationship give very variable results [2].

Acknowledgements—This work was supported by a British Heart Foundation grant to Professor Lamb, an MRC studentship to David Owler and a training grant from Saudi Arabia to Nagi Algharably.

REFERENCES

1. A. Sjoerdsma and M. D. Fischer, *Circulation* **4**, 100 (1951).
2. S. Dutta, in *Cardiac Glycosides*, Vol. II (Ed. K. Greiff), pp. 141–168. Springer, Berlin (1981).
3. J. S. Cook and E. T. Brake, *Cell Membrane Receptors for Drugs and Hormones* (Eds. R. W. Straub and L. Bolis), pp. 343–351. Raven Press, New York (1978).
4. N. Griffiths, J. F. Lamb and P. Ogden, *Br. J. Pharmac.* **79**, 877 (1983).
5. J. F. Lamb and P. Ogden, *Quart. J. exp. Physiol.* **67**, 105 (1983).
6. N. Algharably, J. F. Lamb, P. Ogden, D. Owler and E. M. Tenang, in *The Sodium Pump* (Eds. I. Glynn and C. Ellory), Company of Biologists Ltd. (1985).
7. R. G. W. Anderson, M. S. Brown and J. L. Goldstein, *Cell* **10**, 351 (1977).
16. J. Folch, M. Lees and G. H. Sloanestanley, *J. Biol.* **226**, 497 (1957).
17. G. Rouser, S. Fleischer and A. Yamamoto, *Lipids* **5**, 494 (1969).
18. C. M. Cohen, D. I. Kalish, B. S. Jacobson and D. Branton, *J. Cell. Biol.* **75**, 119 (1977).
19. T. Saermark, N. Flint and W. H. Evans, *Biochem. J.* **225**, 51 (1985).
20. H. Beaufay, in *Lysosomes, A Laboratory Handbook* (Ed. J. T. Dingle), pp. 1–32. North-Holland, Amsterdam (1972).
21. J. S. Cook, E. H. Tate and C. Shaffer, *J. Cell. Physiol.* **110**, 84 (1982).
22. R. T. Dean, W. Jessup and C. V. Roberts, *J. Biochem.* **217**, 27 (1984).
23. Y. Matsuzawa and K. Y. Hostetler, *J. biol. Chem.* **255**, 5190 (1980).
8. L. J. Gotlib, *Biochim. biophys. Acta* **685**, 21 (1982).
9. N. Algharably, Ph.D. Thesis, University of St Andrews (1985).
10. J. D. Gardner, D. R. Kiino, T. J. Swartz and V. P. Butler, Jr., *J. clin. Invest.* **52**, 1820 (1973).
11. A. J. Barret, in *A Laboratory Handbook* (Ed. J. T. Dingle), pp. 1–32. North-Holland, Amsterdam (1972).
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
13. G. Cramb and J. W. Dow, *Biochim. biophys. Acta* **736**, 99 (1983).
14. R. J. Pennington, *Biochem. J.* **80**, 649 (1961).
15. H. U. Bergmeyer and E. Brent, in *Methods of Enzymatic Analysis*, Vol. 2 (Ed. H. U. Bergmeyer), pp. 574–579. Academic Press, New York (1974).
24. K. Y. Hostetler and D. Richman, *Biochem. Pharmac.* **31**, 3795 (1982).
25. P. F. Baker and J. S. Willis, *J. Physiol.* **224**, 441 (1972).
26. J. F. Lamb, P. Ogden and J. Singh, *Q. J. exp. Physiol.* **71**, 381 (1986).
27. J. M. Larkin, M. S. Brown, J. L. Goldstein and R. G. W. Anderson, *Cell* **33**, 273 (1983).
28. C. de Duve, T. de Barsay, B. Poole, A. Trouet, P. Tulkens and F. van Hoof, *Biochem. Pharmac.* **23**, 2495 (1974).