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The effects of chloroquine and other weak bases on the accumulation and efflux of digoxin and ouabain in HeLa cells

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1 We have studied the effects of the weak bases chloroquine, NH_4Cl and amantadine on the handling of certain cardiac glycosides by HeLa cells.

2 When these weak bases are applied acutely to HeLa cells they have only minor effects on the binding of cardiac glycosides to the sodium pumps and on the recovery of pump function following block.

3 When cells are grown in these weak bases there is a variable (10–30%) reduction in pump numbers. This effect is additive to that of chronic treatment with cardiac glycosides.

4 If all sodium pumps are blocked with ouabain, digoxin or digitoxin then recovery of function recovers with a $T_{\frac{1}{2}}$ of about 7 h (10% h^{-1}); digoxin and digitoxin molecules are excreted at a similar rate but ouabain excretion occurs at a much slower rate (3% h^{-1}).

5 These weak bases greatly slow (× 3) the rate of excretion of digoxin and digitoxin but do not alter that of ouabain. The process affected by chloroquine was estimated to have a $T_{\frac{1}{2}}$ of 8 h.

6 Cells grown in the presence of cardiac glycosides accumulate large numbers of glycoside molecules; chloroquine, NH_4Cl and amantadine increase the accumulation of digoxin and digitoxin and may decrease that of ouabain.

7 Quantitatively these results fit a model whereby cardiac glycosides are accumulated by HeLa cells bound to the sodium pumps, are processed by the lysosomes and then excreted.

8 The results are consistent with a process of internalisation and renewal of sodium pumps by HeLa cells.

Introduction

There is a body of evidence to show that the sodium pumps of cells are continually being 'turned over', i.e. inserted into and removed from the plasma membrane. The most direct evidence for this was obtained by Lo & Edelman (1976) who showed that the large subunit of the pump in rat kidney was turning over at about 0.6% h^{-1}. Pollack, Tate & Cook (1981) found a much larger figure of 11% h^{-1} for the turnover of sodium pumps in HeLa cells using a heavy isotope technique. We obtained less direct evidence for pump turnover in human cultured cells by measuring the net changes in pump numbers in various conditions. In cells grown in the protein synthesis inhibitor cycloheximide, the number of pumps decline at a rate of 2% h^{-1} after a 3 h lag (Lamb, Boardman, Newton & Aiton, 1973; Aiton, 1976; Lamb & Ogden, 1982a): in cells grown in low K or high serum concentrations, a cycloheximide-sensitive increase in pump numbers occurs at rates of 5 and 10% h^{-1} respectively (Boardman, Huett, Lamb, Newton & Polson, 1974; Aiton, 1976): in cells grown in ouabain and then returned to normal medium a cycloheximide-sensitive recovery starts at 6 h and occurs at a rate of 7% h^{-1} (Aiton, Lamb & Ogden, 1981). Similar results on net changes in other cells have been reported by Chan & Sanslone (1969) in rat red blood cells, and by Fletcher, Stainer & Holmes (1967) in duck nasal glands. Membrane proteins in liver cells turnover at rates of about 2% h^{-1} (Dean, 1978).

Turnover of sodium pumps ought to affect the reaction of whole cells with cardiac glycosides. For example, recovery from a complete block ought to be partly due to pump renewal, as well as dissociation of cardiac glycosides from the pumps. Both Cook & Brake (1978) and Lamb & Ogden (1977, 1982a) have presented evidence for this in HeLa cells. When pumps are internalised it would be expected that

cardiac glycosides bound to them would also be internalised. Cook & Brake (1978) showed that this was a likely explanation for their results in HeLa cells. This process would also account for the accumulation of cardiac glycosides which occurs in hearts (Sjoerdsma & Fischer, 1951) and in cultured cells (Boardman, Lamb & McCall, 1972) exposed to cardiac glycosides (see Dutta, 1981 for a review), particularly as this accumulation is sensitive to $[K]_o$ (Boardman *et al.*, 1972).

The process of internalisation of cardiac glycoside in association with the sodium pump has similarities to 'receptor mediated endocytosis' (RME) in which a ligand is internalised with a membrane protein (Goldstein, Anderson & Brown, 1979). In such systems the lysosomes, by separating the ligand from the membrane component, play an important role in the process. Thus low density lipoprotein (LDL) is split into cholesterol subunits (Goldstein *et al.*, 1979) and viruses have their coats removed (Helenius, Kartenbeck, Simons & Fries, 1980), both actions occurring in lysosomal vesicles.

Chloroquine, amantadine and NH_4Cl all interfere with many receptor-mediated endocytotic processes. They do so either by blocking the internalisation step (Sando, Titus-Dillon, Hall & Neufeld, 1979; Fitzgerald, Morris & Saelinger, 1980) or the lysosomal processing step (Goldstein *et al.*, 1979; Helenius *et al.*, 1980) of RME.

Table 1 Washout of digoxin from HeLa cells

Treatment	*30 min [^{3}H]-digoxin*	*30 min [^{3}H]-digoxin + 15 min Krebs wash*
Digoxin 2×10^{-7} M in 0K Krebs	907 ± 21	542 ± 44
Digoxin 2×10^{-7} M in 15 K Krebs	421 ± 0	27 ± 8
'Specific' binding	486 ± 21	516 ± 44

All cells were soaked in [^{3}H]-digoxin at 2×10^{-7} M for 30 min either in 0 mM K or 15 mM K Krebs. The cells were then rapidly rinsed with ice-cold Krebs and either analysed or given a further wash of warm Krebs for 15 min and then analysed. It is clear that the warm Krebs wash removes almost all the digoxin uptake which occurred from 15 K Krebs and a similar amount from that which occurred in 0K Krebs. There is no significant difference between the [^{3}H]-digoxin uptake result obtained by subtracting 15K from 0K in the 2 conditions. Results are expressed as molecules of digoxin per cell ÷ 1000 ± s.e. $n = 3$.

The present experiments were designed to test the effect of a number of such agents on the handling of cardiac glycosides by HeLa cells, and so to provide evidence on the hypothesis that cardiac glycosides are internalised in a way similar to the general mechanism of macromolecular uptake systems. A preliminary report has been given to the Physiological Society (Lamb & Ogden, 1982b).

Methods

The main methods used have been detailed in earlier papers (see Aiton, Lamb & Ogden, 1981; Lamb & Ogden, 1982a). HeLa cells were subcultured on 5 cm Sterilin plastic petri dishes for 3–5 days in Eagles' Basal Medium (BME) supplemented with 10% calf serum at 37°C in a 5% CO_2/95% air atmosphere. For long term experiments this medium was added to or altered as indicated later. Several procedures, e.g. acute ouabain binding, were done in Krebs solution with added 1% serum on a bench incubator kept at 37°C.

The measurements made were:

(1) *Estimation of the number of sodium pumps on HeLa cells*

Free sodium pumps were generally measured with [^{3}H]-ouabain at 2×10^{-7} M for 15–30 min (^{3}H-glycoside will be shown as glycoside* throughout.) The specific binding was usually taken as the difference between that bound from 0 mM K Krebs and that from 15 mM K Krebs ('0K' and '15K' respectively). The 15K binding, which was usually less than 10% of the total, was taken as the non-specific binding (Lamb & Ogden, 1982a).

Similar experiments with digoxin or digitoxin gives very large binding from 15K Krebs, making it seem less justified to use this as the non-specific binding. However, we found that such binding was readily washed out, whereas the binding from 0K Krebs was not (Table 1). This, combined with the previous finding that binding from 15K Krebs has a low activation energy (Lamb & Ogden, 1982a), justifies the use of 15K binding as being non-specific. Additional points found are: (a) pump numbers measured with ouabain or digoxin were not significantly different; (b) both ouabain and digoxin at 2×10^{-7} M for 30 min K-free block all glycoside sensitive transport; (c) once a glycoside molecule is bound from 0K Krebs it could not be displaced by another; (d) the larger non-specific digoxin uptake compared to ouabain uptake in HeLa cells is similar to that in RBCs (Gardner, Kuno, Swartz & Butler (1973). It was previously shown by Baker & Willis (1972) and Aiton (1976; Figure. 1.23) and Lamb & Ogden

Table 2 Comparison of Coulter counter and 3-O-methyl glucose methods for the estimation of cellular water

Medium osmolarity (mosmol)	*Plate water (μl)* *Coulter Counter*	*[^{14}C]-3-O-methylglucose*
210	1.10 ± .02	1.08 ± .04
310	0.98 ± .04	0.98 ± .03
410	0.90 ± .02	0.86 ± .02

HeLa cells were incubated for 1 h in Krebs containing 0.5 μCi ml^{-1} of [^{14}C]-3-O-methylglucose. The plates were then rinsed x5 with ice-cold Krebs of the same osmolarity, trypsinised, and aliquots counted for ^{14}C and on the Coulter counter. The osmolarity of the Coulter counting fluid was the same as that for the experiment. Osmolarity was varied by adding mannitol to Krebs containing 80% normal NaCl. (Values are means ± s.e.; $n = 5$). Results are from an unpublished experiment by J.F. Aiton and N.L. Simmons.

(1982a) that a measurement of pump numbers gives a good measure of pump function. Where we measured this in the present work this relation also held.

(2) *Cell number and volume*

This was done on a Coulter channelyser after trypsinising the cells (Boardman *et al.*, 1972). We think that the cell volume measured in this way is a good indication of that on the plate (Burrows & Lamb, 1962). Table 2 confirms this.

(3) *Na and K contents of the cells*

This involved rapid rinsing of the plates with a Ca sorbitol buffer solution and flame photometry.

(4) *^{86}Rb influx measurements as an index of K influx*

Single point estimates were made at 2, 5 or 10 min after application of ^{86}Rb Krebs to the cells.

(5) *Thin layer chromatography*

Thin layer chromatography (t.l.c) of the activity recovered from the plates and medium was carried out on alcohol extracts according to the protocols described by Carvalhas & Figueira (1973). Alcohol was found to extract all the activity. The solvent systems used were ethyl acetate-chloroform-acetic acid (90:5:5), one development of 15 cm and cyclohexane-acetone-acetic acid (49:49:2) two developments.

(6) *Statistical analysis*

This was done mostly on the University computer (Vax) using the Glim Package (Generalised Linear Interactive Modelling; release 3) of the Royal Statistical Society. Various error structures were used to test the robustness of the conclusions. The errors shown in the paper are ± s.e. unless otherwise stated.

Michaelis-Menten type of curves were generally fitted by the direct linear method suggested by Cornish-Bowden (1981). This involves calculating the K_m and V_{max} of all possible pairs of experimental points and using the median values of the K_ms and V_{max}s so obtained.

Results

In a previous paper we dealt extensively with the handling of ouabain by HeLa cells and suggested, on preliminary data, that digoxin was handled similarly. In the current work it soon became clear that digoxin and digitoxin were handled differently from ouabain; so the initial part of this paper is concerned with these differences.

The reaction between cardiac glycosides and cells can be regarded as occurring in two (related) ways:

(1) *Cardiac glycosides on sodium pump function*

The half time of pump occupancy from K-free Krebs solutions containing 2×10^{-7} M ouabain was approx. 3 min. From the relation that $K_1 = 1/(T_{\frac{1}{2}} \times \text{conc.})$ this gives a K_1 of 2.8×10^4 l mol^{-1} s^{-1} a figure similar to that reported by Baker & Willis (1972) for HeLa cells in K-free Krebs solution. The occupancy rate of digoxin was somewhat slower.

The time course of recovery of pump function after a complete block with ouabain or digoxin was previously shown to have a $T_{\frac{1}{2}}$ of about 7 h (i.e. 10% pump recovery per hour) and to be the same for ouabain and digoxin. In a further experiment with digitoxin we found that function recovered with a $T_{\frac{1}{2}}$ of 6 h, i.e. at a rate of 11% per h.

(2) *Uptake and loss of cardiac glycosides by the cell*

The cell can be regarded as a compartment (or compartments) which accumulates cardiac glycosides; so that a cardiac glycoside influx, efflux and accumulation can be measured. To obtain quantitative information for this model we measured the fluxes and accumulation of ouabain and digoxin.

Influx We have no measure of the appearance of the cardiac glycoside within the cells but have measured

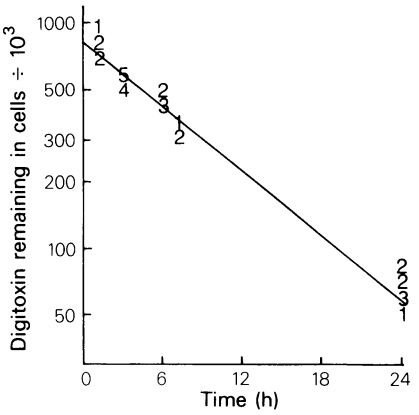

Figure 1 Efflux of digitoxin. Cells were incubated in 0K Krebs containing 3×10^{-8} M digitoxin* for 30 min, rinsed and returned to BME for up to 24 h. At the times shown plates were removed rinsed and analysed for cell number, volume and [^{3}H]-digitoxin content. Line fitted by Glim package. Results are molecules per cell ÷ 1000. Points shown are the experimental ones, 2 points coinciding shown by 2, etc.

the rate of binding to the cell under physiological conditions; the first step in internalisation. To do so, cells were incubated in normal growth medium (i.e. $[K]_o = 5$ mM) ± chloroquine with ouabain or digoxin at 10^{-7} M for periods up to 60 min. The rate constant of binding for ouabain was $1.10 \pm 0.12\ h^{-1}$ and that for digoxin was $0.34 \pm 0.05\ h^{-1}$; chloroquine had no effect on these rates. The on rate constants are thus 4.4×10^3 and 1.3×10^3 $1\ mol^{-1}\ s^{-1}$ for ouabain and digoxin respectively. The ouabain on rate constant is similar to that of 2.8×10^3 $1\ mol^{-1}\ s^{-1}$ found by Baker & Willis (1972) in HeLa cells in 6 mM Krebs solution.

Efflux We found a $T_{\frac{1}{2}}$ of approx. 20 h which gives a rate of loss of ouabain of about $3.5\%\ h^{-1}$; a value similar to that found previously (Cook & Brake, 1978; Lamb & Ogden, 1982a). The efflux rate of digoxin and digitoxin is always much faster, usually with a $T_{\frac{1}{2}}$ of the order of 6–9 h. Thus, Figure 1 shows the mean efflux for digitoxin efflux over 24 h in two experiments. (Figure 4 shows a similar result for digoxin.)

The $T_{\frac{1}{2}}$ is 6.5 h, giving a rate of loss of $11\%\ h^{-1}$. This means that for digoxin and digitoxin the rate of excretion of the glycoside is similar to the rate of recovery of pump function; for ouabain it is much slower.

Cycloheximide on digoxin efflux Cycloheximide and other protein synthesis inhibitors have no effect on the efflux of ouabain from HeLa cells. Table 3 shows however that cycloheximide does slow the efflux of digoxin and digitoxin (see later) from these cells.

Accumulation It has been known for many years that cells accumulate cardiac glycosides, the amount varying somewhat with the species, the cardiac glycoside and the tissue (see Dutta 1981). A later figure (Figure 10) shows the steady state accumulation of digoxin and ouabain by HeLa cells from growth medium containing around 1×10^{-8} M cardiac glycoside and a $[K]_o$ of about 5 mM. (This concentration of cardiac glycoside was chosen as one which gives a measurable accumulation without too great a reduction in free pumps in the cells. Higher concentrations cause large decreases in pump numbers, leading to large changes in ion concentration within the cells which might then interfere with cellular metabolism). The results are expressed as molecules of glycoside accumulated as a % of the normal pumps present. The figure shows that under these conditions digoxin is accumulated to around 30% and ouabain to more than 100% of the normal pump numbers. The ouabain result confirms an earlier result (Boardman *et al.*, 1972). The larger accumulation of ouabain compared to digoxin is expected qualitatively on the basis that the ouabain influx is larger and the efflux is much slower than that

Table 3 Cycloheximide on digoxin efflux from HeLa cells

	Cell nos. at 24 h (% initial)	*Mean % loss of digoxin h^{-1} over 24 h*	*Digoxin remaining at 24 h* (% initial value)
Control	149 ± 5	7.2 ± 1.2	19 ± 1
Cycloheximide	95 ± 6	5.1 ± 0.6	31 ± 1

Cells were blocked with a pulse of digoxin (2×10^{-7} M, K-free for 30 min) and returned to growth medium ± cycloheximide for 24 h. At various times plates of cells were removed and analysed. Cycloheximide stops cell growth and slows excretion of digoxin. The rate constant for digoxin loss is based on all points (15) and shows no significant difference. The digoxin remaining is based on 3 plates each and has $P < 0.01$.

Table 4 Effect of lysosomal inhibitors on various parameters of HeLa cells

	Cell no.	*Cell Vol.*	*Pump sites*	$[Na]_i$	$[K]_i$
				mM lcw	
Chloroquine 1×10^{-4} M	89±8	92±3	92±6	18±2	176±2
				15±1	185±11
NH_4Cl 10^{-2} M	71±14	—	88±4	19±0	222±6
				20±3	183±5
Amantadine 10^{-3} M	91±4	99±1	108±7	19±1	204±5
				14±1	195±5

Cells were grown for 1 day (NH_4Cl) or 2 days (chloroquine and amantadine) in growth medium ± drug, then the indicated measurements made. The results are expressed as % of control or as ion concentrations per litre cell water (lcw) (both control and drug values shown). $n = 3-5$. Data for each drug from separate experiments.

of digoxin. As the accumulation of ouabain exceeds the initial (and final) number of pumps on the plasma membrane this accumulation cannot be accounted for solely on the basis of pump occupancy – pump turnover is also required.

Effects of chloroquine, amantadine and NH4Cl on HeLa cells

(1) *Cell growth* In order to check if these agents affect cell health (Cohen & Yielding, 1965), we examined the effects of these drugs on cell number, volume, sodium pump numbers and ion contents and K influx. As most of these experiments were done during the log-growth phase of the cells, the cell numbers and volume are good indicators of cell 'health'.

Table 4 shows data obtained from separate experiments on chloroquine, NH_4Cl and amantadine and Table 5 mainly flux data all from one experiment. The effects of the drugs are relatively slight; all reduce the cell numbers by about 10% and have a small effect on cell volume. Both chloroquine and NH_4Cl reduce the number of pump sites by 10–15%. This effect takes some hours to become apparent so that it is not seen at 5 h but is present at 18–24 h (see Figure 3 and Table 5). The K influx is not reduced correspondingly but this may be because the Na_i rose slightly (Table 5); the results are small so that it was not worth calculating further. Chloroquine and amantadine seem to increase the cotransport component of the influx (i.e. furosemide sensitive component; Aiton, Chipperfield, Lamb, Ogden & Simmons, 1981).

The conclusion from these experiments is that these 'lysosomal inhibitors' do not greatly affect these parameters of the cells. Results with cycloheximide, which can abolish almost all protein synthesis,

Table 5 The K influx in cells treated with lysosomal inhibitors for 20 h

		Control	*Chloroquine*	*NH_4Cl*	*Amantadine*
Cell no. $\times 10^{-6}$ cells/plate		2.22±.05	2.1±.06	1.94±.05	1.53±.12
Cell vol. u^3		2394±31	2442±46	2401±27	2664±49
$[Na]_i$	mM lcw^{-1}	21±2	23±1	23±2	20±1
$[K]_i$		185±5	208±2	177±1	256±7
	Total	5.7±0.1	7.8±0.5	3.8±0.1	7.0±0.4
K influx (mM lcw min^{-1})	Ouabain-sensitive	2.8±0.3	3.5±0.4	2.2±0.1	2.9±0.4
	Furosemide-sensitive	1.5±0.3	3.5±0.4	1.0±0.1	3.2±0.4
	Remainder	1.2±0.1	0.90.2	0.7±0.02	0.9±0.1

Cells were grown for 20 hours in the conditions shown, 3 plates were taken for each measurement i.e. to measure total flux, + ouabain (10^{-3} M), + furosemide (10^{-4} M), ouabain + furosemide and Na + K contents. All results obtained in one experiment ± s.e. ($n = 4$ per condition). The ouabain and furosemide-sensitive fluxes were the mean of ± the other drug.

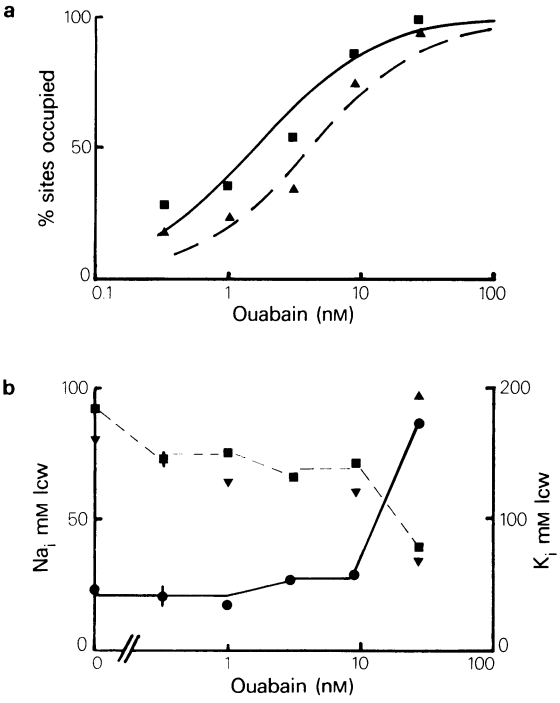

Figure 2 Effects of chloroquine (1×10^{-4} M) and ouabain for 2 days on pump numbers and Na and K concentrations. (a) Shows the pump numbers occupied by ouabain and (b) the $[Na]_i$ and $[K]_i$. The total pump sites in control and chloroquine were $936{,}000 \pm 140{,}000$ and $599{,}000 \pm 21{,}000$ per cell respectively. $[K]_i$ falls at 0.33 nM ouabain and $[Na]_i$ rises at 3 nM ouabain ($P < 0.05$). Lines fitted by Cornish-Brown procedure. $n = 3$ for sites; 4 for ions. In (a) (▲) chloroquine; (■) control. In this experiment the free pump numbers at the end of the initial 2 days ouabain treatment was measured by [^{3}H]-ouabain at 2×10^{-7} M as usual. (a) is calculated as the total control sites less that remaining after 2 days growth in ouabain. In (b) (■)K; (▼)K in chloroquine; (●)Na; (▲) Na in chloroquine.

may be contrasted with these results. When applied to growing cells all further increase in cell numbers stops and the pump numbers decrease steadily (see Figure 9, Lamb & Ogden, 1982a; and Table 3).

Cells treated chronically with cardiac glycosides show large reductions in their ability to pump ions (Lamb & McCall, 1972). Treatment with chloroquine also reduces pump numbers. Are these effects additive or do they interact with each other? In several such experiments we found that the effects were additive so that cells in cardiac glycosides and chloroquine have less pumps than in either alone; there was no interaction between the effects (by analysis of variance). Figure 2 shows the results of growing the cells for two days in a range of ouabain concentrations with or without chloroquine. The total sites in this experiment were markedly reduced by chloroquine (to 64% of normal). Figure 2a shows that the affinity of ouabain as a blocking agent is, if anything, marginally reduced by chloroquine, a calculated K_d of 4 instead of 2 nM. Figure 2b shows the Na and K concentration in the cells, from which it can be seen that 0.3 nM of ouabain reduces the $[K]_i$, whereas 3 nM is required to increase $[Na]_i$. Chloroquine has little effect on these ion concentrations; we think that this may be due to a concomitant reduction in 'leak' but did not further investigate this.

Comparison of Tables 4 and 5 and Figure 2 shows that the longer term response to chloroquine on pump sites is rather variable between experiments. We did not do sufficient experiments to characterize this, but think that it may be due to variations in the serum used in the growth medium.

(2) *On the recovery of function following glycoside block* Figure 3 shows one complete experiment in which we measured the recovery of free pump sites after a complete block with ouabain or digoxin (see Lamb & Ogden, 1982a for details) and the effect of chloroquine on this recovery. It is clear that the recovery after ouabain or digoxin is much the same (about 30%), the actual rate for ouabain agrees with our earlier results (Lamb & Ogden, 1982a; Figure 3). Chloroquine decreases the recovery by about 15%, with no significant difference between ouabain or digoxin. In a number of experiments we measured

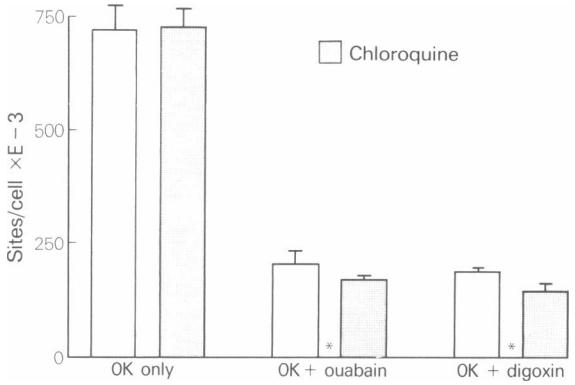

Figure 3 Chloroquine (shaded columns) on the recovery of pump sites following a complete glycoside block. Cells were treated with 0K Krebs alone or with 10^{-6} M ouabain or digoxin for 10 min, rinsed and returned to growth medium for 5 h. The number of free pump sites was then estimated with ouabain* in the usual way. Chloroquine significantly reduces recovery following digoxin or ouabain block, and the recovery following ouabain or digoxin block is not significantly different. (by analysis of variance). All plates from same experiment, $n = 3$.

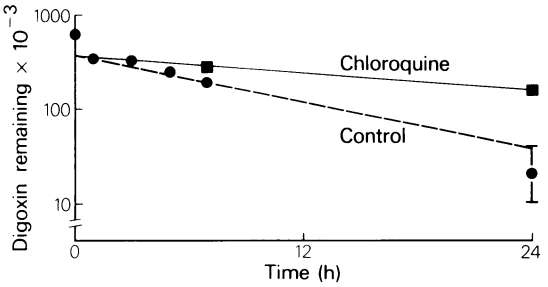

Figure 4 Chloroquine on the efflux of digoxin*. Cells were loaded with digoxin* by 25 min treatment in 2×10^{-7} M K-free Krebs, rinsed and returned to growth medium ± chloroquine 10^{-4} M. At each time point thereafter 3 control and 3 treated plates were removed and analysed for radioactivity and cell numbers. Initial experiments showed that this digoxin concentration blocked all pumping. The best fit lines (1–24 h) gave the same intercept and different slope (Glim package, normal error). (k of 0.095 and 0.038 h^{-1} for control and chloroquine respectively; difference of 0.056 ± 0.015 h^{-1}). The large drop in activity during the first hour corresponds to the non-specific loss of Table 1. The digoxin left at 24 h was 3 ± 3% and 25 ± 2% for control and chloroquine-treated respectively; expressed as a % of the initial value. Several other experiments gave similar results, although more digoxin* was usually present at 24 h.

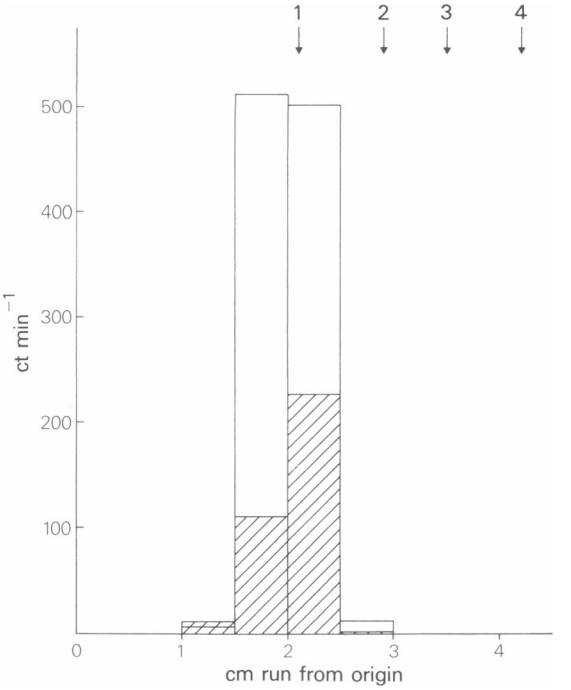

Figure 5 Thin layer chromatography (t.l.c.) of digoxin* remaining in HeLa cells. HeLa cells were treated as described in Figure 1, except that 7 plates were removed for control and test conditions at each point. Three plates were counted as before, the other 4 plates were taken in pairs, extracted in alcohol and dried down under nitrogen. The samples were resuspended in chloroform-methanol and spotted on t.l.c. plates. These samples were run with various controls in the ethylacetate or cyclohexane systems, dried, the plates cut with a scalpel and the strips put into counting vials. The results for the cyclohexane system at 24 h are shown. Hatched columns are control, open columns + chloroquine. The arrows indicate the centre of the spots for (1) digoxin, (2) digoxigenin bisdigitoxoside, (3) digoxigenin monodigitoxoside, and (4) digoxigenin, respectively. In another experiment the % digoxin remaining at 24 h was 16 and 39 for control and chloroquine-treated respectively, and 7 and 24 for the corresponding total radioactivity.

the pump activity directly by measuring the ouabain-sensitive K influx and the Na and K contents of the cells at 5 h after glycoside block. These usually showed a decrease in the ouabain-sensitive K influx and a small rise in Na_i and fall in K_i. The results were not impressive nor significant but were consistent with the small changes in binding shown in Figure 3.

The reduction in the recovery of pump numbers due to chloroquine following a glycoside block is similar to that seen in the long term experiment detailed earlier. This is not seen in the control cells at 5 h probably because there is no 'repair' process going on then. If recovery is allowed to proceed for much longer, 18 to 24 h, then both cells blocked with glycosides and controls not blocked show similar declines in pump numbers.

Chloroquine has a larger effect than NH_4Cl or amantadine on glycoside efflux (see later) and has only minor effects on pumps recovery. We did not, therefore, measure the effect of NH_4Cl or amantadine on pump recovery after an acute block, but did check their long term effects.

The efflux of cardiac glycosides following pulse labelling

Chloroquine effects Figure 4 shows the efflux of digoxin* from HeLa cells in normal growth medium and that containing chloroquine. The chloroquine effects start to become apparent on the efflux at 7 h and by 24 h there is more digoxin left in the treated cells than in the controls. The control efflux $T_{\frac{1}{2}}$ is 7 h, that in chloroquine 18 h. Figure 5 shows another experiment in which we checked by t.l.c. that the label was still attached to digoxin. In another experiment the $T_{\frac{1}{2}}$ of efflux was increased from 7 to 29 h as measured on total counts and from 8 to 21 h on the t.l.c. counts recovered from the ethylacetate or cyclohexane systems. We do not regard the difference as

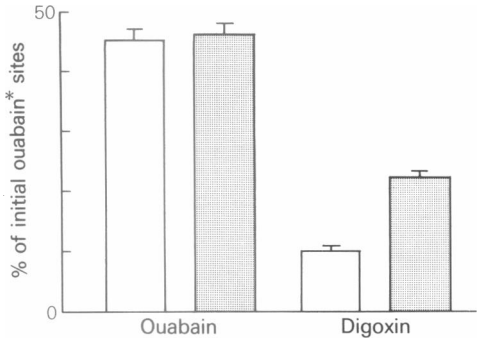

Figure 6 The effect of chloroquine (shaded columns) on the efflux of ouabain* and digoxin*. The results are expressed as % of the initial load remaining after 24 h of efflux. The efflux of ouabain is slow ($T_{\frac{1}{2}}$ of 21 h) and is not affected by chloroquine; that of digoxin is fast and is slowed by chloroquine ($T_{\frac{1}{2}}$ of 7 and 12 h respectively; $P = 0.05$). ($n = 3$ to 4 per column).

important, for the t.l.c. data were obtained on plates whose cell numbers were unknown. Clearly these results show that chloroquine slows the efflux of digoxin.

These experiments were repeated with ouabain and with digoxin. Typical results are shown in Figure 6 and Table 6.

These may be summarised: (1) the efflux of ouabain* is very slow, with a $T_{\frac{1}{2}}$ of around 20 h as previously shown (Cook & Brake, 1978; Lamb & Ogden, 1982a) so that some 40% of the original ouabain is still present on the cells after 24 h efflux. Chloroquine has no effect on delaying this efflux and even on some occasions slightly increased it. (2) Digitoxin* is normally excreted at about the same rate as digoxin and its excretion is significantly slowed by chloroquine.

Ammonium chloride and amantadine effects Table 6 also shows that NH_4Cl and amantadine slows the efflux of digoxin* from HeLa cells. In both cases the normal amount of digoxin left at 24 h is about 10–20% as in other experiments.

The order of effectiveness of these agents on efflux

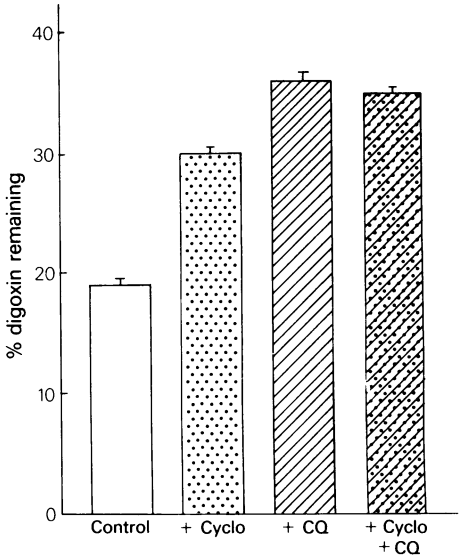

Figure 7 Interactions of chloroquine (CQ) and cycloheximide (Cyclo) on digoxin efflux. Cells were incubated in 0K Krebs containing 2×10^{-7} M digoxin for 30 min, rinsed for 15 min in Krebs and then returned to BME alone or BME containing cycloheximide (100 $\mu g\,ml^{-1}$), chloroquine (10^{-4} M) or both cycloheximide and chloroquine for 24 h. The amount of digoxin* remaining at 24 h was measured. The results are expressed as a % of the amount of digoxin bound initially. All results are significant with respect to the control. $n = 3$, s.e. mean shown by vertical lines.

Table 6 The collected results of the effect of various agents on the efflux of digoxin, digitoxin and ouabain

	Control	*Chloroquine*	*NH_4Cl*	*Amantadine*	*Cycloheximide*
Digoxin 1×10^{-7} M	70 ± 8	149 ± 12	125 ± 3	115 ± 5	104 ± 9
Digitoxin 2×10^{-7} M	219 ± 15	359 ± 6	305 ± 9	273 ± 22	251 ± 5
Ouabain 1×10^{-6} M	45 ± 2	39 ± 1	—	43 ± 2*	—

Cells were incubated in 0K Krebs containing digoxin or digitoxin for 30 min or ouabain for 10 min and rinsed in warm Krebs. In the digitoxin experiment (and digoxin marked*) a preliminary efflux in BME only was done for 1 h to remove non-specific digitoxin. The efflux was then continued for a total of 24 h (18 h for ouabain) in BME alone or that containg chloroquine (10^{-4} M), NH_4Cl (10^{-2}), amantadine (10^{-3} M) or cycloheximide (100 $\mu g\,ml^{-1}$). The results are expressed as molecules retained per cell ÷ 10^3 for digoxin and digitoxin and as % of initial sites for ouabain. The digoxin, digitoxin and ouabain results are from separate experiments and the results marked* are from a separate experiment. $n = 4 \pm$ s.e.

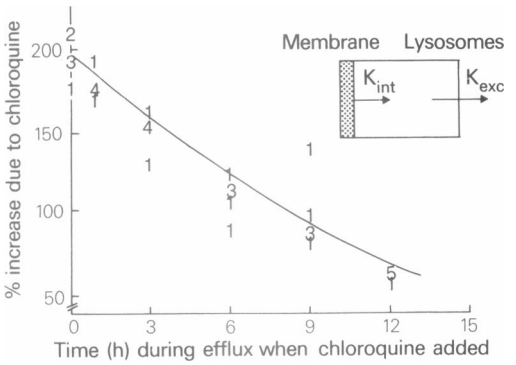

Figure 8 Effect of adding chloroquine at various times during digoxin* efflux. Cells were blocked with 1×10^{-6} M digoxin* in K-free Krebs for 10 min; rinsed and returned to growth medium. At the indicated times, 8 plates were drained and replaced by fresh medium + chloroquine. At 24 h the plates were rinsed, etc. and remaining digoxin* counted. The fitted line was computed for a compartment which lost digoxin with a $T_{\frac{1}{2}}$ of 8 h normally and 16 h in the presence of chloroquine (K exc on inset). The regression line fitted to the data had a slope of 8 h ($t = 4.66$, $P < 0.01$).

is chloroquine, NH_4Cl, amantadine, similar to that on preventing viral infectivity (Helenius *et al.*, 1980).

Interactions of cycloheximide and chloroquine on digoxin-efflux*

Both chloroquine and cycloheximide slow the efflux of digoxin* from HeLa cells, although neither has this effect on ouabain* efflux (Lamb & Ogden, 1982a). One explanation of this is that cycloheximide reduces the supply of lysosomal enzymes and so interferes with digoxin release from the sodium pump or other membrane component. If this were so then the effects of cycloheximide and chloroquine should interact, e.g. in the presence of chloroquine cycloheximide should have no effect. Figure 7 shows this; cycloheximide has a smaller effect than chloroquine and has no further effect in its presence.

Lysosomal inhibition at various times after adding digoxin

Chloroquine acts quickly on receptor mediated endocytotic processes (Fitzgerald *et al.*, 1980; Helenius *et al.*, 1980) and so can be used to time the progress of the internalisation process. For these experiments we loaded the cells with a short (10 min) pulse of digoxin* and at various times thereafter the medium was removed and replaced with that containing chloroquine or NH_4Cl. The amount of digoxin remaining at 24 h was used as a measure of total digoxin efflux. Figure 8 shows that the effectiveness of chloroquine in slowing efflux is less the later the chloroquine is added. A single experiment using NH_4Cl instead of chloroquine gave similar results.

This result was examined by computing the model shown in the inset to Figure 8, assuming that chloroquine (and NH_4Cl) only acted on the dominant process determining efflux. The line fitted to the experimental points in Figure 8 (a $T_{\frac{1}{2}}$ of 8 h or 9% h^{-1}) confirms this interpretation.

The form in which cardiac glycosides are excreted by HeLa cells

To examine this we collected the cardiac glycosides appearing in the growth medium following a pulse label of digoxin or digitoxin and ran them on an appropriate t.l.c. system, with controls consisting of the starting material and breakdown products. The results of 2 such experiments (Figure 9) show that these glycosides are apparently excreted unchanged.

Effect of the agents on the accumulation of cardiac glycosides

Chloroquine, NH_4Cl and amantadine all reduce digoxin* efflux and have either no effect or marginally increase the ouabain* efflux. Figure 10 shows that these agents all increase the accumulation of digoxin and decrease that of ouabain; results expected on the model introduced earlier.

Discussion

The results in this paper will be discussed in relation to the model that sodium pumps in the plasma membrane of HeLa cells 'turnover' continuously, i.e. are being inserted and removed from the plasma membrane at a steady rate.

(1) *Recovery of pump function after cardiac glycosides*

We have shown that the excretion of digitoxin and digoxin by HeLa cells is much faster than that of ouabain, yet the recovery of pump function following block by any of these cardiac glycosides occurs at much the same rate. This dissociation of recovery and excretion of the blocking molecule is expected on the internalisation model outlined above, but not on a dissociation model.

(2) *Accumulation of cardiac glycosides*

Although it has been known for many years that cardiac glycosides are accumulated by heart and

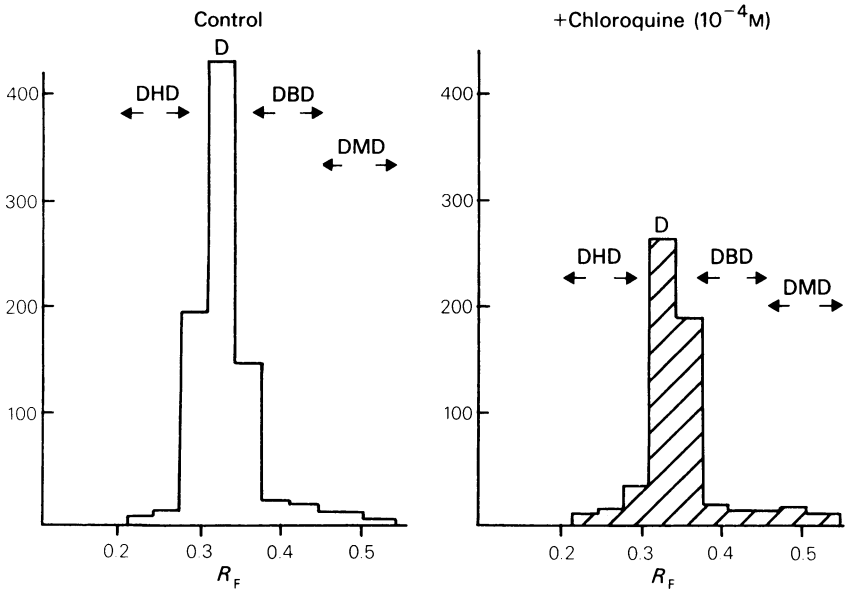

Figure 9 Thin layer chromatography (t.l.c.) of digoxin excreted by HeLa cells in presence and absence of chloroquine (10^{-4} M). Cells were pulse labelled with digoxin*, washed and replaced in growth medium; 24 h later the growth medium was removed, ethanol added and mixed. Chloroform was then added, the mixture centrifuged, and aqueous phase discarded and the organic phase dried down under nitrogen. The dried samples were resuspended in chloroform-methanol and run on the cyclohexane: actone: acetic acid system together with [^{3}H]-digoxin and various metabolites. The chromatograms of the medium extracts resembled that of the native digoxin rather than the metabolites. D = digoxin + [^{3}H]-digoxin; DHD = dihydrodigoxin, DMD = digoxigenin monodigitoxoside; DBD = digoxigenin bisdigitoxoside.

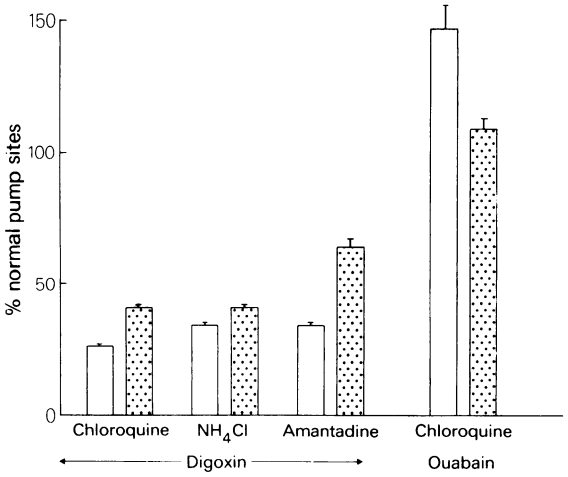

Figure 10 The effect of lysosomal inhibitors on the accumulation of digoxin and ouabain by HeLa cells. Cells were grown for up to two days in growth medium ± drug and approx. 1×10^{-8} M glycoside. The results are shown as molecules retained, expressed as a % of the pump sites present initially. Spotted columns are drug-treated, open column are controls. Glycoside concentration in chloroquine experiments was 1×10^{-8} M, others were 1.2×10^{-8} M. Time of growth 48 h for chloroquine and amantadine; 24 h for NH_4Cl. All results are from different experiments.

other cells and appear in the microsomal fraction of the cells, this has not usually been explained as being related to the blocking action of the glycosides. In the acute loading experiments in this paper we show that most of the accumulation occurs following binding to the sodium pump and not by diffusion. The evidence for this is that conditions which block the pump with glycoside* leads to a glycoside uptake by the cells which is then lost very slowly; whereas conditions which protect the pump i.e. high K or previous blocking with non-radioactive glycoside, leads to an uptake by the cells which is much smaller and is readily lost from the cells.

In the chronic accumulation experiments described in this paper and observed in whole animals, the evidence for accumulation following attachment to the sodium pump is less clear, nevertheless we believe that most of it also occurs by this route. The most direct evidence is that the K-sensitivity of ouabain* accumulation by these cells (Boardman *et al.*, 1972; Figure 3) is similar to that of ouabain* binding to the pump itself (Baker & Willis, 1972). Similar evidence has been produced by Dutta (1981). The relative uptakes of ouabain via the pump and by diffusion can be calculated approximately from data such as that given by Baker & Willis (1972, Figure 1) which shows the relative uptake into HeLa cells via the pump and by diffusion. At a ouabain concentra-

tion of 1×10^{-8} M in 5 mM K solution the ratio of pump/non-specific uptake is about 10/1. If the internalisation process is rapid compared to the rate of binding to the pump, then about 90% of the uptake would be via the sodium pump. For digoxin the ratio may be less due to a greater non-specific binding. It is not certain, of course, that non-specific uptake of cardiac glycosides can obtain entry to the lysosomal apparatus of the cell.

Model calculations We have computed the expected amount of accumulation for growth in cardiac glycosides on two models of the process. Model 1 assumes that the glycoside binds to the sodium pump on the cell surface and eventually dissociates, i.e. no internalisation occurs. Model 2 assumes that the initial step involves a similar surface binding but that the glycoside/pump complex is then internalised and some replacement of the surface pump occurs. For simplicity the cell in this model is considered as a single compartment with an influx, efflux and accumulation occurring between it and the bathing medium. The influx probably involves several steps but could not be faster than the initial rate of binding to the pumps.

The expected results for these models may be computed under equilibrium conditions:–

Model 1

$$\frac{x}{n} = \frac{k}{(k_1 + k_2)} \qquad (1)$$

where x is the number of the initial pumps (N) blocked at equilibrium and k_1 and k_2 are the on and off rate constants.

Model 2

$$\frac{x}{N} = \frac{k_1}{k_2} \qquad (2)$$

where x is the accumulation of glycoside molecules, N is the number of free sites at equilibrium and k_1 and k_2 are the on and off rate constants.

To compute these relationships the influx rate at the glycoside concentration used for accumulation (1×10^{-8} M) is required. This cannot readily be measured, for it is so low, but if the influx is first order (Baker & Willis, 1972; Boardman *et al.*, 1972) then the rate at 1×10^{-8} M should be 1/10 of that measured at 1×10^{-7} M and given earlier.

Figure 11 shows a comparison between the expected accumulation of ouabain on the 2 models and that observed, expressed as a ratio. It is clear that model 2 more closely describes the observed results. It should be noted that one of the implications of model 2 (i.e. internalisation) is that little dissociation of the cardiac glycosides occurs from surface pumps. Ouabain rather than digoxin or digitoxin was chosen for this comparison because it gives the clearest distinction between the models; to use digoxin or digitoxin we would need to grow the cells in a greater glycoside concentration.

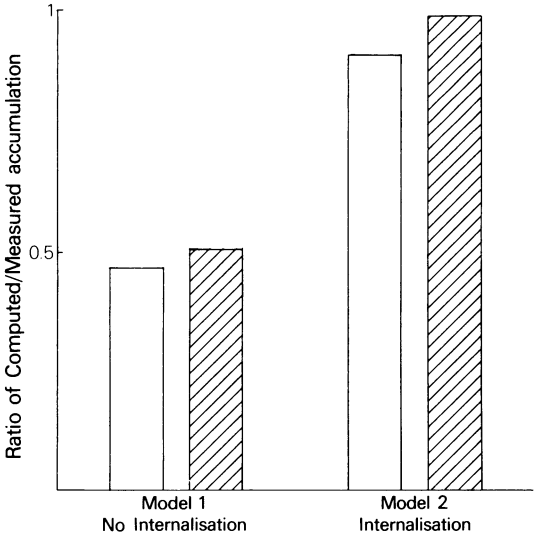

Figure 11 Comparison of the measured accumulation of ouabain with that expected on internalisation or no internalisation. The results are expressed as computed measured for model 1 and 2. The accumulation of ouabain is computed from the steady state in 10^{-8} M ouabain using data given in this paper or Lamb & Ogden (1982a). The hatched columns are the accumulations in the presence of chloroquine.

(3) *Effects of chloroquine, NH_4Cl and amantadine*

These agents have been shown to interfere with the handling of various substances by cells, and we show here that they also interfere with the excretion of digoxin or digitoxin by HeLa cells. We have as yet no direct evidence on the site of action of these substances on glycoside handling; it could either be at the internalisation step or at the lysosomal step of the process. If at the internalisation step then the glycoside will accumulate on the cell surface; if at the lysosomal step it will accumulate in the lysosomes. We are now testing these predictions. Because cells grown in chloroquine accumulate large quantities of glycoside, a situation similar to increased cholesterol accumulation in chloroquine-treated cells (Goldstein *et al.*, 1979), we believe that chloroquine decreases lysosomal release of glycosides from ingested sodium pumps.

A scheme which explains the lack of effect of chloroquine on ouabain excretion is to suppose that lysosomal activity releases digoxin and ouabain from the pump and that both then diffuse across the cell

membrane. Digoxin has a high lipid solubility and so the main determinant of excretion rate is the rate of lysosomal activity; once this is inhibited the overall excretion rate is decreased. Ouabain has a low lipid solubility and so the rate of diffusion out of the cell is the main determinant of the excretion rate; inhibiting lysosomal activity therefore does not much affect the overall excretion rate. The time course of the action of chloroquine and NH_4Cl is, however, much slower than that in other systems (e.g. viral uptake), hours rather than minutes.

(4) *Consequences of cardiac glycoside action on turnover of Na pumps*

There are certain consequences of this model on the interaction of cardiac glycosides with sodium pumps in whole cells which may not be obvious.
(1) The 'classical' reaction which occurs between the sodium pump and cardiac glycosides in membrane preparations is

$$O + E \rightleftharpoons O.E. \qquad (3)$$

where O is the cardiac glycoside, E the pump and O.E. the pump glycoside combination. It has generally been assumed that this will also occur in whole cells. Our evidence shows that this is incorrect for HeLa cells at least and that the reaction should be represented as

$$O + E \rightleftharpoons O.E. \quad \text{internalised} \qquad (4)$$
$$E \leftarrow \text{pump insertion}$$

the new pump inserted into the membrane may be from synthesis, from a store or by internal stripping of the glycoside from the pump.

The degree to which reaction 4 predominates over reaction 3 in any situation will depend primarily on the relative rate of the off reaction of the glycoside used to the rate of internalisation. In rat cells, which are relatively insensitive to cardiac glycosides due to a high off reaction, we would expect that some of reaction 3 would still occur in whole cells. In human cells, with their high glycoside sensitivity, reaction 4 mainly occurs with ouabain, digoxin and digitoxin but with dihydro-oubain (which has a high off rate) reaction 3 mainly occurs (unpublished observations).

Caution is thus required in extrapolation from the results of one cardiac glycoside in one cell species to another. In particular, results with rodents may not be directly transferred to man.
(2) The equilibrium position of reaction 4 (above) will depend both on the association rate constant of the cardiac glycoside for the pump and on the rate of insertion of the pumps into the membrane. If the latter is designated as K'_2 then the equilibrium state K'_d will be given by K'_2/K_1.

It will be seen that this equilibrium state therefore depends on the metabolic processes of the cell which ultimately drive internalisation synthesis, etc. It cannot be assumed that K_d' will be independent of these processes.
(3) We think that the internalisation of cardiac glycosides can be satisfactorily explained by a sequence in which the glycoside is bound to the pump, the pump/glycoside complex is internalised and accumulated by the cells and therafter excreted.
(4) Cells respond to cardiac glycosides by activating a repair process which detoxifies the drug. The steady state result which ensues is an interaction between this repair process and the concentration of drug added.

These results are consistent with a scheme as follows: the sodium pumps in HeLa cells are turning over normally at about 2% h^{-1}; when synthesis is maximally stimulated e.g. by serum, glycoside block, etc. this rate can increase to about 10% h^{-1}. Normally the turnover of pumps is controlled by a pool of mRNA for it is sensitive to cycloheximide (a protein synthesis inhibitor) but not to actinomycin D (a mRNA synthesis inhibitor). When the cell is stimulated to produce more pumps, nuclear involvement is required, for this stimulation is sensitive to both cycloheximide and to actinomycin D (Aiton, 1976).

Appendix Model calculations

I *Mathematical solutions*

Model 1 Let there be a total of N sites on the plasma membrane initially and x (t) sites bound after influx for time t.

k_1 and k_2 are the on and off rate constants.

then $x(t + dt) = x(t) + k_1[N - x(t)]\,dt - k_2 x(t)\,dt$

$$\frac{dx(t)}{dt} = k_1 N - (k_1 + k_2)\,x(t)$$

$$\int \frac{(k_1 + k_2)\,dx}{k_1 N - k_2 x} = (k_1 + k_2)\,t$$

$$\frac{x}{N} = \frac{k_1}{(k_1 + k_2)} \left[1 - e^{-[k_1 + k_2]t} \right]$$

Model 2 In this case the sites bound are internalised and replaced by fresh sites, N represents the free sites in the steady state and x represents the total load of glycoside; k_1 and k_2 the influx and efflux rate constants.
If it is supposed that most of the accumulated

glycoside is intracellular then at equilibrium the influx = efflux, so that

$$N \times k_1 = x \times k_2$$

$$\frac{x}{N} = \frac{k_1}{k_2}$$

II *Iterative solutions*

These models were also calculated iteratively using time intervals of 0.5 h; the steady state results obtained were not different from the mathematical solutions obtained above. The advantage of this latter method is that intermediate results with variable N profiles could readily be calculated.

We (with David Owler) have recently measured the rate of loss of cardiac glycosides from HeLa cell membranes using the bead technique of Gotlib (1982). Preliminary results show that ouabain and digoxin both leave the membrane at a rate of $8.9 \pm 0.2\%\ h^{-1}$ ($n = 3$); chloroquine slowed both by about 20% (NS). The $\times 3$ slowing of digoxin efflux by chloroquine is not due to a purely membrane effect; the decrease in ouabain accumulation by chloroquine may be due to a decrease in internalisation.

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