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Analysis of Intracellular Drug (Ouabain) Sequestration as a Mechanism of Detoxification

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SUMMARY

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The cardiac glycoside ouabain, bound in a brief pulse to growing HeLa cells, is released over a period of days in a nonexponential manner that indicates heterogeneity of the dissociation processes. On release, the drug is chromatographically indistinguishable from authentic ouabain. We describe a model for release as the sum of three first-order processes: (a) simple dissociation from its membrane binding site [$(Na^+ + K^+)$-ATPase] into the medium; (b) sequestration or internalization of the drug, possibly together with its binding site, as part of membrane turnover; and (c) externalization of the internalized drug. Processes (a) and (b) lead to physiological recovery, and together occur substantially more rapidly than the sum of processes (a) and (c), which lead to release of drug from the cell. A program devised by Chandler *et al.* [(1972) *Comp. Biomed. Res.*, 5, 515–534] is used to compute from drug release data the optimum least-squares fit as well as the compartment sizes and rate constants for the three processes. The analyses, based on data from intact cells, yield results that closely resemble those from data obtained from more disruptive cell fractionation procedures. These results show that ouabain is neither degraded nor stored in the cell. It can be found free in the cytoplasmic fraction, but it is not cytotoxic in that location. It is eventually released intact. Thus ouabain is not converted to an innocuous form by the cell, but rather is translocated to an innocuous location within the cell, with concomitant physiological recovery of normal functions at the cell surface prior to drug release.

INTRODUCTION

Cellular recovery from the action of drugs commonly involves either simple dissociation of the drug from the cell or detoxification of the drug by its modification through cellular metabolism. In addition, many drugs are known to be taken up by cells through endocytosis and subsequently degraded in secondary lysosomes. This report presents a simple, quantitative description of an intermediate recovery mechanism in which a membrane-active drug is sequestered in the cell, possibly but not necessarily via endocytosis, at a site distinct from its site of action. The drug is not otherwise metabolized, and is even-

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tually released from the cell in its original form. The sequestration process is accompanied by physiological recovery, although the unmodified drug itself remains associated with the cell for a substantial period of time. This mechanism may be described by a three-compartment model which distinguishes recovery from drug release and accounts for the prolonged release of the membrane-active drug from the cells by its uptake and slow circulation through the cytoplasm.

We have observed this mechanism in the interaction of the cardiac glycoside ouabain with HeLa S_3 cells (1, 2). Ouabain binds to and inhibits the $(Na^+ + K^+)$-ATPase (EC 3.6.1.3), which is located almost exclusively in the plasma membrane of these cells (3–6). The following evidence suggests that under appropriate conditions ouabain binds solely to this enzyme: (a) the inhibition of K^+ (or Rb^+) influx or $(Na^+ + K^+)$-ATPase activity is proportional to the degree of ouabain binding (4, 7–9); (b) after rapid binding, ouabain is almost exclusively located in plasma membrane fractions of the cell (10); (c) the release of ouabain from purified plasma membranes or purified enzyme is commonly observed to be first-order (11, 12); (d) there is a limited number of ouabain binding sites, which correlates well with the estimated number of ATP-phosphorylated enzyme molecules (9, 13–15); (e) the dissociation constant of ouabain for HeLa cells is approximately 20 nM, which corresponds well with the K_i for inhibition of the alkali cation transport system (9, 16, 17); and (f) in wild type HeLa cells the Scatchard plot for binding may be described by a single straight line (18).

Ouabain binds to HeLa cells over a span of minutes at concentrations of 0.2–1 μM (4, 9, 10), but the drug is released from the cells very slowly in a nonexponential manner with a half-time of many hours (8, 14, 17–19). Nevertheless, when cells are treated with the drug to an extent such that the transport system is only partially blocked, and the cells are then placed in ouabain-free medium, physiological recovery is observed long before the cells are cleared of the drug (9, 17). The experimental results of this study indicate that the loss of ouabain from the plasma membrane correlates with recovery, and the ouabain remaining with the cells is sequestered in the cytoplasm, where it inhibits neither alkali cation transport nor cell growth. Other results (10) indicate that the uptake of ouabain into the cytoplasm is an energy-dependent process; this uptake may be regarded as a defense mechanism of the cells against toxic compounds.

Preliminary accounts of this work have been published (10, 20).

MATERIALS AND METHODS

Materials. [*G*-^{3}H]Ouabain and ^{86}RbCl were purchased from New England Nuclear Corporation. Paper chromatography of the glycoside in several solvent systems (Table 1) indicated that more than 98% of the radioactivity migrated in the same fashion as unlabeled ouabain. Powdered Eagle's minimal essential medium with spinner salts was purchased from Grand Island Biological Company; gentamicin, from Microbiological Associates; heat-inactivated (56° for 30 min) fetal calf serum, from Kansas City Biological Company; and HEPES,[3] from Sigma Chemical Company. All other chemicals were of reagent grade or better.

Methods. HeLa S_3 cells from Dr. J. D. Regan (Biology Division, Oak Ridge National Laboratory) were cultured in suspension using established techniques (9, 21) with Eagle's medium (22) supplemented with 5–10% (v/v) fetal calf serum, 25 μg/ml of gentamicin, 20 mM HEPES (pH 7.3), and 2 mM L-glutamine (growth medium). Periodic examination using accepted procedures (21, 23, 24) indicated that the cells were free of contaminating bacteria, fungi, or mycoplasma. These cells grew with a generation time of about 27 hr, although growth was sometimes temporarily slowed in populations heavily treated with ouabain (see refs. 9 and 17 and below).

Cells were bound with [^{3}H]ouabain in growth medium at 37° for a sufficient period of time to achieve the desired degree

[3] The abbreviation used is: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

TABLE 1

Chromatogrdphic analysis of authentic [^{3}H]ouabain and radioactive drug released by or extracted from HeLa cells

Conditions	R_F		
	Butanol–acetic acid–water (80:12:30)	Water	$(NH_4)_2SO_4$ (2 M)
Authentic ouabain[a]	0.56	0.83	0.56
Extract from cells grown 2 days in 10 nM ouabain[a]	0.55	0.82	0.56
Supernatant from above[a]	0.56	0.82	0.57
Authentic ouabain[b]	0.49		
2-Propanol–water extract of lyophilized medium containing only radioactivity released from cells 20–47 hr after pulse label[b]	0.49		
Authentic ouabain plus heat-killed cells[b]	0.39		0.58
Authentic ouabain plus medium[b]	0.37		0.50
Cells pulsed-labeled with [^{3}H]ouabain, washed, and harvested 47 hr later[b]	0.40		0.57
Medium from cells above	0.41		0.55

[a] Chromatographed on Whatman No. 1 paper, descending.

[b] Chromatographed on Whatman No. 3MM paper, descending. The third part of the table, compared with the second part, reflects the retardation of ouabain in butanol–acetic acid–water when spotted in the presence of salts and protein.

of labeling (see figure legends for details). The labeled cells were then harvested by centrifugation at 500 × *g* for 6 min at 0–4°, washed once with cold, ouabain-free growth medium, and finally suspended in fresh growth medium at 37° without ouabain. Cell concentrations were monitored using a model B Coulter counter, making adjustments when necessary for cell volume changes and coincidence errors (25). Periodically a known number of cells was harvested at 3000 × *g* for 4 min at 0–4° and suspended in 1 ml of water or 0.1 N NaOH. In the latter case the base was neutralized with HCl before counting. Radioactivity was measured with a Nuclear-Chicago mark II scintillation counter after the cell homogenates or other samples had been dissolved in a toluene–Triton X-100 scintillation solution (26). Corrections for quenching were performed by the net external standard channels-ratio method (27).

Paper chromatography of ouabain and radioactive material from cell cultures was performed using the solvent systems in Table 1. Labeled material from cells was extracted by sonication in ethanol or water; more than 95% of the cell-associated radioactivity was recovered in the extracts. In certain experiments (see RESULTS) the radioactivity released from the cells into the medium was recovered by flash-evaporating the medium and extracting the residue with 2-propanol–water (5:1); more than 85% of the radioactivity was recovered by this method. Authentic ouabain or extracts were applied to paper strips, which were air-dried at room temperature and developed in the various solvent systems. Radioactivity was determined by cutting the strips into 1-cm segments, eluting the label with water, and counting as described above. In every case more than 90% of the radioactivity was located in a single peak at the R_F listed.

K^+ transport was determined as the ouabain-sensitive uptake of ^{86}Rb, measured in growth medium. Quadruplicate 5-ml samples of the appropriate suspension were made 0.1 mM in nonisotopic ouabain, a concentration which yields almost immediate and complete inhibition of transport. About 1 min later, these samples and a quadruplicate set of experimental samples were each brought to 1 μCi/ml with

^{86}RbCl. After 10 min at 37°, the samples were all rapidly chilled in an ice bath and centrifuged at 3000 × *g* for 3 min at 0°. The supernatants were removed and assayed for radioactivity; the tubes were drained and the walls wiped clean; and the cell pellets were dissolved in 1 ml of 0.1 N NaOH, neutralized, and assayed for radioactivity as described above. Since the specific activity (^{86}Rb:K^+) of the medium and the number of cells in each sample was known, transport was calculated from the difference in ^{86}Rb uptake between the experimental and 0.1 mM ouabain-treated cells. Experimentally, an uptake of 40 μEq/10^8 cells/hr (see Fig. 4) represents about 4500 cpm/sample. The ouabain-insensitive influx of ^{86}Rb into control cells is typically 25% of the total (9).

Plasma membrane ghosts were prepared by a modification of the method of Atkinson and Summers (28). Briefly, this involves gentle (6–10 strokes) homogenization with a size B Dounce homogenizer (29) of cells swollen in a hypotonic medium (15 mM NaCl, 0.5 mM KCl, 0.3 mM $MgCl_2$, and 10 mM Tris, pH 7.4). Immediately after homogenization, 0.1 volume of 50% (w/w) sucrose in 1.5 M NaCl, 50 mM KCl, 30 mM $MgCl_2$, and 10 mM Tris (pH 7.4) was added and mixed thoroughly. The nuclei were removed by centrifugation at 1000 × *g* for 30 sec. The membrane-enriched supernatant was layered on a discontinuous sucrose gradient composed of 7 ml of 45% sucrose and 15 ml of 30% (w/w) sucrose made up in 0.15 M NaCl, 5 mM KCl, 3 mM $MgCl_2$, and 10 mM Tris (pH 7.4). During a 20-min centrifugation at 8000 × *g*, a fraction enriched 10–15-fold in plasma membranes collects at the 30%/45% sucrose interface. Fractions of the gradients were assayed for radioactivity, enzyme activity, and, in certain cases, protein (30). The activity of 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) was measured by the method of Avruch and Wallach (31) at pH 7.0–7.4 in the presence of 0.15 M NaCl, 5 mM KCl, 3 mM $MgCl_2$, and 10 mM Tris and 33 or 100 μM 5'-AMP (K_m approximately 10μM).

Computations were performed on an IBM 360/91 computer.

RESULTS

Drug release from whole cells. Figure 1 is a set of drug release curves, plotted on semilogarithmic coordinates, for cells treated to different extents with [^{3}H]-ouabain. It is known from separate measurements (data not shown) that the cells used in this experiment had a total of about 9 × 10^5 ouabain binding sites per cell. The binding sites were either about two-thirds saturated (open circles) or one-fifth saturated (solid circles) with ouabain at the time the cells were resuspended in normal growth medium from binding medium. Earlier work (9, 17) has shown that under these conditions the number of binding sites titratable with a second saturating challenge returns to control levels in 5–8 hr, and that the transport capacity and K^+ content of the cells are restored to control levels within the same time period, even though most of the drug bound in the initial challenge is still cell-associated. Points of particular interest to this study are that the drug release curve of Fig. 1 is not linear on semilogarithmic coordinates,

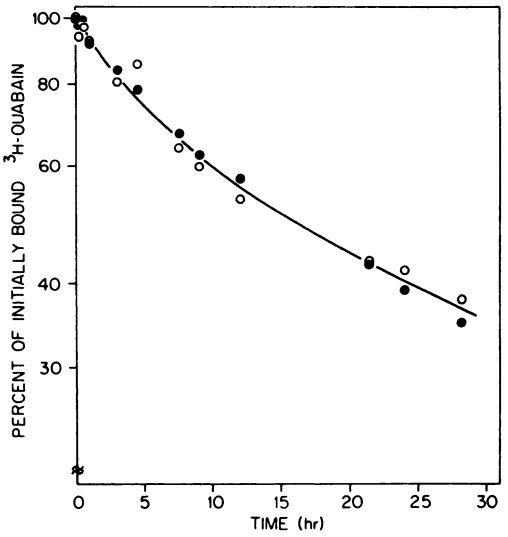

FIG. 1. *Ouabain release from HeLa cells*

[^{3}H]Ouabain was bound to different extents to two populations of cells, which were then returned to their normal growth medium; drug release was followed for the next 30 hr. Control cells (not shown) had 9 × 10^5 ouabain binding sites per cell. ●, 6.5 × 10^5 sites/cell bound at zero time; ○, 1.7 × 10^5 sites/cell bound at zero time.

indicating heterogeneity of the release process, and that although the absolute values are very different, the two sets of data may be adequately fitted to a single curve starting from a common zero-time point. Therefore the proceesses underlying drug release can be treated as independent of the extent of initial binding.

The finding that cells recover from the effects of ouabain while radioactivity is still associated with them might be due to metabolism of the drug to an inactive but still labeled product. This possibility was tested by chromatographing the radioactive material (a) extracted from cells 47 hr after the start of the drug release process, (b) released into the medium from the cells in the interval between 20 and 47 hr after binding, or (c) extracted from cells grown continuously in low concentrations of ouabain for 72 hr. The chromatography was performed with a number of solvent systems (Table 1). In every case the material found at the R_F listed was in a single peak that comprised more than 90% of the radioactivity on the entire chromatogram, including the origin and front. Since a chemical alteration of ouabain would be expected to change its partitioning in at least one of these solvent systems, and since no such change was observed, it is concluded that the labeled material associated with the cells and that released by the cells into the medium is authentic ouabain.

Three-compartment model for drug release. Using earlier observations (9, 17) and drug release curves similar to that in Fig. 1, we have represented the handling of ouabain by the cells in terms of a three-compartment model (Fig. 2). The upper compartments represent the cell membrane and cytoplasm; together these comprise the whole cell. The third compartment is the medium in which the cells are suspended. After the cells are briefly challenged with ouabain, then washed free of radioactive medium and any loosely associated drug, all of the radioactivity is bound to the $(Na^+ + K^+)$-ATPase, presumably the catalytic subunit, on the external surface of the cell (32–34). The labeled drug, corresponding to inactivated subunits, is represented by the × in the diagram. Since ouabain does not bind covalently to the $(Na^+ + K^+)$-ATPase, any radioactive material on the surface may be expected to dissociate and return to the medium with a characteristic rate constant k_{diss}. Simultaneously a second, parallel pathway operates for removal of ouabain from the surface by some active cellular process that takes the drug into the cytoplasmic compartment. This is represented in the model as an internalization process that proceeds with the characteristic rate constant k_{int}. The model requires no explicit assumptions as to the nature of this process. It could be, for example, a mechanism that removes only the ouabain from the surface, leaving an active $(Na^+ + K^+)$-ATPase. Alternatively, the entire enzyme subunit, with the ouabain bound to it, may be internalized as part of a membrane turnover process in which the internalized protein is replaced by a new, fully active molecule. What is required, in order to account for all the data, is that the internalization be associated with recovery of function at the surface. The internalization of ouabain increases the amount of drug in the cytoplasmic compartment. Once there, the drug eventually escapes back into the medium. This latter process appears very complex, but it may be characterized simply as externalization with a rate coefficient k_{ext} for the rate-limiting process.

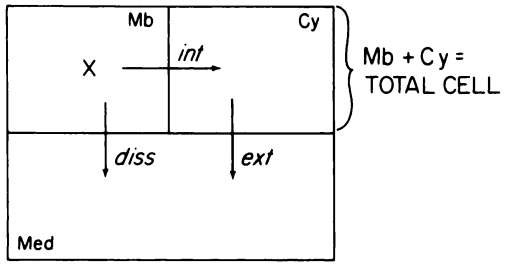

FIG. 2. *Model of release of ouabain from HeLa cells*

×, [^{3}H]ouabain-labeled sites on the plasma membrane (Mb). The arrows describe the movement of the label into the cytoplasmic compartment (Cy) and the medium (Med) by either internalization (int), dissociation (diss), or externalization (ext).

In testing this model, our primary con-

cern is the number of molecules in a given volume of cell suspension that move from one compartment to another. Such movements should appropriately be described by their first-order rate coefficients, since the latter are a measure of the probability that a given molecule will move within a fixed time period. It is thus necessary to be concerned only with the number of molecules within a compartment and not, for the first analysis, with growth-associated changes in size of the compartment. In other words, to compute the number of membrane-bound ouabain molecules in 1 ml of suspension, it is not necessary to correct for the concomitant cell growth. In a subsequent analysis, concerned with the density or concentration of ouabain molecules on the cell surface, growth will have to be taken into account.

The rate at which ouabain molecules are lost from the cell surface is the sum of the dissociation and internalization processes:

$$\frac{dX_{Mb}}{dt} = -(k_{diss}X_{Mb}) - (k_{int}X_{Mb}) \quad (1)$$

$$= -(k_{diss} + k_{int})X_{Mb} \quad (1a)$$

where X_{Mb} is the number of membrane-bound ouabain molecules in a given volume of cell suspension. From the assumptions given, it is clear that the negative of this expression is a statement of the physiological recovery rate of the initially inactivated enzyme molecules.

The movement of ouabain through the cytoplasmic compartment may be described as

$$\frac{dX_{Cy}}{dt} = +(k_{int}X_{Mb}) - (k_{ext}X_{Cy}) \quad (2)$$

where X_{Cy} is the number of ouabain molecules associated with the cells but not on the cell surface. The sum of Eqs. 1 and 2,

$$\frac{dX_{(Mb + Cy)}}{dt} = -(k_{diss}X_{Mb}) - (k_{ext}X_{Cy}) \quad (3)$$

describes the loss of ouabain from the cells. Its integral, with the boundary condition that $X_{Cy,t=0} = 0$, is the drug release curve:

$$\frac{X_{(Mb + Cy),\,t}}{X_{(Mb + Cy),\,t=0}} = \frac{X_{(Mb),\,t=0}}{(k_{diss} + k_{int}) - k_{ext}} \cdot\{(k_{diss} - k_{ext}) \exp[-(k_{diss} + k_{int})t] + k_{int} \exp(-k_{ext}t)\} \quad (3a)$$

This result is in the form of the sum of two decaying exponentials, the experimental variables being time and the amount of ouabain on the membrane at zero time. As observed in Fig. 1, if we normalize this initial value to 1.0, drug release data starting at very different absolute values can be described by a single curve.

To fit the drug release data to Eq. 3 it is necessary to compute k_{int} and to estimate X_{Cy} at the various time points. These estimations, as well as the over-all fitting of Eq. 3 to the drug release data, have been facilitated by the use of a program (CRICF) originally written by Chandler *et al.* (35) for the solution of sets of stiff differential equations describing parallel and sequential enzymatic reactions. This program fits, by the least-squares method, sets of drug release data to the model in Fig. 2, and in so doing makes a "best" estimate of the three rate coefficients and the number of ouabain molecules in the three compartments as a function of time. The results of such an analysis are illustrated in Fig. 3A, and the computed rate constants are given in Table 2. It is apparent that the curvilinearity of drug release from intact cells may be described by a set of statements: The drug dissociates directly from the surface into the medium, but the uptake of the drug into the cell is a somewhat faster process. Conversely, the loss of ouabain from the cell cytoplasm is very slow. After 12 hr of recovery, therefore, most of the drug is still associated with the cell even though very little is on the surface and physiological recovery is nearly complete. At still longer times, virtually all of the loss takes place by the slowest pathway, that of externalization. In other words, $k_{int} > k_{diss} \gg k_{ext}$.

In addition to the curvilinearity of over-all drug release, the solution to this model predicts (a) that after 12 hr approximately half the initially bound ouabain

will be in the cytoplasmic fraction, (b) that ouabain will be lost from the membrane fraction according to first-order kinetics with a half-time of about 4 hr, and (c) that the cells will show approximately 85% recovery of transport in 12 hr. Prediction (c) is derived from prediction (b) and the assumption that every ouabain molecule lost from the membrane represents either directly or indirectly the recovery of the transport-enzyme activity that it had inhibited.

To test these predictions quantitatively, we isolated plasma membranes and measured the membrane-bound drug compared with the total cell-associated drug.[4] In such experiments HeLa cells were bound with ouabain to the desired extent, and the cells were then washed free of unbound drug and returned to growth medium. A sample was taken immediately to measure the number of drug molecules per cell. At the same time a parallel sample was taken and chilled to prevent both dissociation and internalization, and a membrane-rich fraction was prepared as described in MATERIALS AND METHODS. This preparation required approximately 45 min, during which time less than 1% of the initially bound ouabain dissociated. The ratio of [^{3}H]ouabain to the activity of 5′-nucleotidase was taken as a quantitative measure of the concentration of ouabain on the cell surface. Similar samples were taken after the cells had been incubated for some hours in the growth medium. In these samples the distribution of 5′-nucleotidase in the sucrose gradients did not change appreciably from the initial pattern (10), but the cell-associated ouabain moved from the membrane-rich fraction on the lower shelf of the gradient into the upper parts of the gradient, where the cytosol enzymes and virtually all other organelles of the cell (except nuclei) are found.

In these experiments the cells grow with a generation time τ; the growth rate

[4] An account of the turnover of ouabain binding sites and the subcellular locations of internalized ouabain, based on cell fractionation studies, will be published in detail elsewhere.

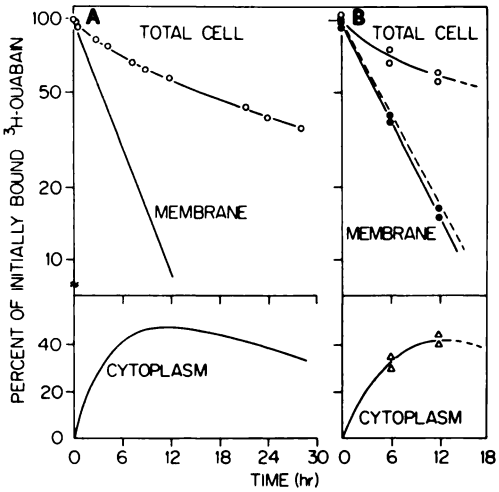

FIG. 3. *Analysis of drug release curve (upper curves: "total cell"), separting loss from membranes and gain followed by loss in a nonmembrane cell compartment ("cytoplasm")*

Note that the upper curves are plotted on semilogarithmic coordinates, and the lower curves, on arithmetic coordinates. A. Analysis from whole cell data (points same as ● in Fig. 1) according to the model in Fig. 2, showing computer-calculated, time-dependent quantities of ouabain expected in the membrane and cytoplasm compartments. B. Experimentally determined distribution of [^{3}H]ouabain among the same compartments. The content in the membrane compartment was measured as the ratio of [^{3}H]ouabain to 5′-nucleotidase as described in the text. - - -, correction for cell growth (see Eq. 5), giving the fractional quantity of initially bound ouabain in the membrane compartment.

depends on experimental conditions and is monitored in each case. With growth, 5′-nucleotidase is constantly being inserted into the growing membranes, and a decreasing ratio of [^{3}H]ouabain to 5′-nucleotidase reflects not only the process described in the model but dilution with new 5′-nucleotidase as well. The amount of marker enzyme on the surface membranes of the cells in 1 ml of the suspension may be estimated by the usual growth equation:

$$N = N_{t=0}2^{t/\tau} \quad \text{or} \quad \ln\left(\frac{N}{N_{t=0}}\right) = \left(\frac{\ln 2}{\tau}\right)t \quad (4)$$

Since the integral of Eq. 1a is

$$\ln\left(\frac{X_{Mb}}{X_{Mb,t=0}}\right) = -(K_{diss} + k_{int})t \quad (1b)$$

the specific activity in a growing population may be obtained by dividing Eq. 1b by Eq. 4:

$$\ln\left(\frac{X_{Mb}/N}{X_{Mb,t=0}/N_{t=0}}\right) = -\left(k_{diss} + k_{int} + \frac{\ln 2}{\tau}\right)t \quad (5)$$

If the growth rate is known, the third term in parentheses can be computed, and the sum of the other two terms can be estimated from the experimentally determined rates of change in the [^{3}H]ouabain to 5′-nucleotidase ratio in the membranes.

Test of predictions. Cells were briefly treated with [^{3}H]ouabain, returned to growth medium, and sampled as described at appropriate intervals. Owing to the number of manipulations required, a limited number of points could be taken in a single experiment, but these were adequate to test the argument (Fig. 3B). The upper curve depicts total cell-associated ouabain, decreasing in a curvilinear manner similar to that seen in Fig. 3A. Since virtually all the initially bound ouabain is on the surface of the cell, the zero-time points for cell-associated and membrane-associated drug are taken as equal. The [^{3}H]ouabain to 5′-nucleotidase ratio in the membrane falls rapidly, reaching 15% of its initial value in 12 hr. The negative slope of this line, with $t_{1/2}$ = 4.1 hr, is 0.016 hr^{-1}. Since these treated cells were growing with a generation time of about 40 hr during the experiment, the sum of (k_{int} + k_{diss}) may be calculated from Eq. 5 to be 0.15 hr^{-1}. The line with this negative slope is indicated by dashes in the figure. The difference between the upper curve and the dashed line corresponds to our formal definition for the quantity of cytoplasmic (i.e., associated with cells but not membranes) ouabain, and is indicated in the lower part of the figure. Note that at 12 hr this value was predicted to reach 48%, with a half-rise time of 2.4 hr. Experimentally the value is 41% at 12 hr, with a half-rise time of approximately 2.8 hr. Considering that these are separate experiments, done on different days, the correspondence between predictions (a) and (b) derived from Fig. 3A and the data obtained in Fig. 3B is qualitatively excellent and quantitatively satisfactory.

It is important to note that these half-rise times for the cytoplasmic fraction are not the same measure as the half-times for internalization given in Table 2. The latter assume a steady state and are calculated simply from $-\ln 0.5/k_{int}$. The former is a function of the interaction of all three translocations and is short because the membrane-bound ouabain available for internalization falls rapidly in the early hours of the observations.

Prediction (c) is substantiated in Fig. 4. The upper part of the figure again shows cell-associated and membrane-associated drug after pulse binding. The line drawn through the latter is first-order, with a half-time of 5.0 hr. In this case the ouabain

TABLE 2

Rate constants for ouabain dissociation, internalization, and externalization computed from drug release curves and measured in fractionation experiments

Values are separate determinations.

Translocation	Computed		Measured	
	k	$t_{1/2}$	k	$t_{1/2}$
	hr^{-1}	hr	hr^{-1}	hr
Dissociation from membrane	0.07, 0.07	10, 10		
Internalization	0.13, 0.13	5.2, 5.2	0.09, 0.10[a]	
Sum of dissociation + internalization	0.20	3.5	0.14, 0.15	4.6, 5.0
Externalization	0.015, 0.03	25, 50	0.01–0.03[b]	25, 70[b]

[a] Estimated from the measured initial rise in the cytoplasmic compartment, as in Fig. 3B (lower).

[b] Estimated from tails (after 15 hr) of drug release curves, as in Fig. 1.

binding sites were nearly saturated at the outset of the experiment and growth was markedly inhibited, to the point that no significant correction need be applied to estimate ($k_{diss} + k_{int}$). That value is in this case 0.14 hr^{-1}, compared with the computed prediction of 0.20 hr^{-1} (Table 2). The extensive binding was deliberately permitted to facilitate the simultaneous measurement of transport inhibition and its recovery (Fig. 4, lower). At zero time, ^{86}Rb transport was inhibited by the ouabain to less than 15% of the control value, but in 10 hr it recovered to a value equal to the initial control, with a half-time of 3.9 hr. As in the model, this recovery rate corresponds more closely to decrease of membrane-associated drug rather than to loss of cell-associated drug. That transport recovers somewhat more rapidly than ouabain is lost from the surface, eventually exceeding the initial control, is due to the facts that the severely inhibited cells lose K^+ and gain Na^+, and that the enhanced intracellular Na^+ is itself a stimulus to transport until new steady-state ion concentrations are achieved (9).

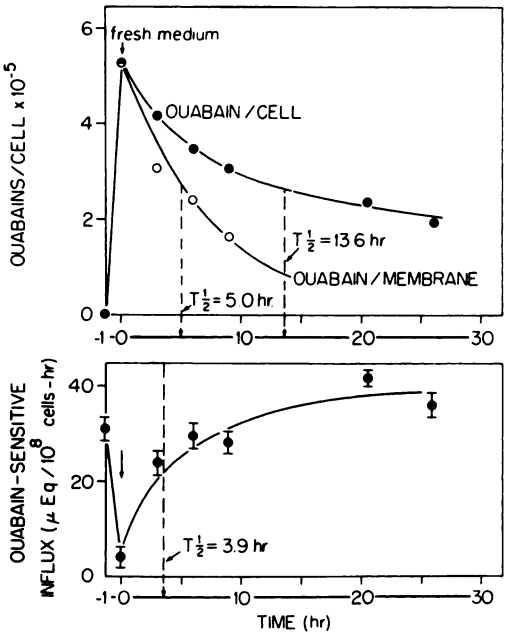

FIG. 4. *Ouabain release and recovery of alkali cation transport*

The upper curves show, on arithmetic coordinates, ouabain binding and release from whole cells and cell membranes; the latter curve as drawn is first-order. The lower curves depict ^{86}Rb transport in this same population of cells. Note that the half-time for transport recovery (3.9 hr) corresponds to that for drug loss from membranes (5.0 hr) but not from whole cells (13.6 hr).

DISCUSSION

An important advantage of the computer analysis described here, insofar as its conclusions can be independently substantiated, is that the drug release data are obtained with intact cells. The correspondence of these results to results obtained with the more disruptive cell fractionation methods mutually enhances the value of both approaches.

As is consistent with its size and low octanol/water partition coefficient (0.02 at 37°), ouabain does not enter the HeLa cell by passive permeability or as a consequence of its proximity to the surface when it dissociates from its membrane binding site. We find that the uptake of ouabain into the cytoplasmic compartment of the HeLa cell is an active, ATP-requiring process (data not shown; see footnote 4), although this result is at variance with the reported passive uptake of ouabain by Burkitt lymphoma cells (36). Once inside the cell, ouabain does not affect the transport of alkali cations at the plasma membrane, because the ouabain binding site is not internally exposed (5, 37, 38) and because the high intracellular concentration of free K^+ would in any case be an effective inhibitor of ouabain binding to the ($Na^+ + K^+$)-ATPase (10). That intracellular ouabain is ineffective as a cytotoxic drug is also attested by the finding that 12–15 hr after extensive binding to the cell surface, with concomitant inhibition of growth as well as transport, the intracellular concentration may exceed 0.1 μM and yet rates of both transport and growth will have returned to normal levels. The ouabain released from the cells does not again influence HeLa transport or metabolism, because the cellular compartment is relatively so small that the medium concentration does not again exceed approximately 0.1 nM, which is two orders of magnitude below the K_i of ouabain for these cells

Under conditions *in vivo*, however, it is conceivable that the loss of cardiac glycosides from cells could lead to a plasma concentration as high as 1 nM (39–41), which could be significant with respect to other tissues in which the K_i is very low, as is the case with the human red cell (42, 43), or under conditions in which these drugs are not metabolized (44).

It is well known that many drugs, especially membrane-reactive ones, are taken up via endocytosis into secondary lysosomes (45–48). Material so incorporated appears to undergo one of several fates. Nonmetabolizable materials like sucrose, dextran, or polystyrene latex are incorporated into phagolysosomes and can remain there indefinitely (49). More commonly, the membrane-reactive agent is taken into secondary lysosomes, degraded, and released as small molecules. The acetylcholine receptor-specific α-bungarotoxin is an example of this. [^{125}I]Bungarotoxin binds irreversibly to the acetylcholine receptor on the surface of muscle cells and, following internalization of the binding site, the radioactive label appears within a few minutes or hours as monomeric[^{125}I]-iodotyrosine in the medium (50). In an unusual case, the membrane ligand *Ricinus communis* agglutinin (RCA_{II}) binds to the surface of SV3T3 cells, is taken up in an endocytic vesicle, and is released, presumably by vesicle breakdown, into the cytoplasm without the formation of a secondary lysosome (51). The released ligand exerts its toxic effects by binding to cytoplasmic ribosomes, to which it has access by this uptake mechanism.

The handling of ouabain as described here differs from the above in that although ouabain is not degraded, it also is not stored; it can be found free in the cytoplasmic fractions but is not cytotoxic in that location; and it is eventually released intact.

The significance of this study is not in describing the precise mechanism by which the drug is removed from its site of action, but rather in defining the fact that it can be removed and sequestered. Thus it is not converted to an innocuous form but rather is translocated to an innocuous location within the cell, with the consequence of physiological recovery of normal functions at the cell surface.

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REFERENCES

1. Gey, G. O., Coffman, W. D. & Kubicek, M. T. (1952) *Cancer Res.*, 12, 264–265.
2. Puck, T. T., Marcus, P. I. & Cieiura, S. J. (1956) *J. Exp. Med.*, 103, 273–284.
3. Bosmann, H. B., Hagopian, A. & Eylar, E. H. (1968) *Arch. Biochem. Biophys.*, 128, 51–69.
4. Baker, P. F. & Willis, J. S. (1972) *J. Physiol (London)*, 224, 441–462.
5. Trams, E. G. & Lauter, C. J. (1974) *Biochim. Biophys. Acta*, 345, 180–197.
6. Boardman, L., Hume, S. P., Lamb, J. F., McCall, D., Newton, J. P. & Polson, J. M. (1975) in *Developmental and Physiological Correlates of Cardiac Muscle* (Lieberman, M. & Sano, T., eds.), pp. 127–138, Raven Press, New York.
7. Hansen, O. (1971) *Biochim. Biophys. Acta*, 233, 122–132.
8. Wilson, W. E., Sivitz, W. I. & Hanna, L. T. (1970) *Mol. Pharmacol.*, 6, 449–459.
9. Cook, J. S., Vaughan, G. L., Proctor, W. R. & Brake, E. T. (1975) *J. Cell. Physiol.*, 86, 59–70.
10. Cook, J. S., Will, P. C., Proctor, W. R. & Brake, E. T. (1976) in *Biogenesis and Turnover of Membrane Macromolecules* (Cook, J. S., ed.), pp. 15–36, Raven Press, New York.
11. Akera, T. & Brody, T. M. (1971) *J. Pharmacol. Exp. Ther.*, 176, 545–557.
12. Yoda, A. (1974) *Ann. N. Y. Acad. Sci.*, 242, 598–616.
13. Baker, P. F. & Willis, J. S. (1968) *Biochim. Biophys. Acta*, 183, 646–649.
14. Kyte, J. (1972) *J. Biol. Chem.*, 247, 7634–7641.
15. Skou, J. C. (1974) in *Perspectives in Membrane Biology* (Estrada-O., S. & Gitler, C., eds.), pp. 263–278, Academic Press, New York.
16. Baker, P. F. & Willis, J. S. (1970) *Nature*, 226, 521–523.
17. Vaughan, G. L. & Cook, J. S. (1972) *Proc. Natl. Acad. Sci. U.S.A.* 69, 2627–2631.
18. Baker, R. M. (1976) in *Biogenesis and Turnover of Membrane Macromolecules* (Cook, J. S., ed.), pp. 93–103, Raven Press, New York.
19. Cook, J. S., Will, P. C. & Tate, E. (1975) *Fed. Proc.*, 34, 249.
20. Cook, J. S., Brake, E. T. & Will, P. C. (1976) *Biophys. J.*, 16, 28a.

21. Paul, J. (1970) *Cell and Tissue Culture*, Ed. 4, Livingstone, Edinburgh.
22. Eagle, H. (1959) *Science*, **130**, 432–433.
23. Ludovici, P. P. & Holmgren, N. B. (1973) *Methods Cell Biol.*, **6**, 143–208.
24. Schneider, E. L., Stanbridge, E. J. & Epstein, C. J. (1974) *Exp. Cell Res.*, **84**, 311–318.
25. Cook, J. S. (1967) *J. Lab. Clin. Med.*, **70**, 849–856.
26. Patterson, M. S. & Greene, R. C. (1965) *Anal. Chem.*, **37**, 854–857.
27. Hash, J. H. (1972) *Methods Microbiol.*, **6B**, 109–155.
28. Atkinson, P. H. & Summers, D. F. (1971) *J. Biol. Chem.*, **246**, 5162–5175.
29. Atkinson, P. H. (1973) *Methods Cell Biol.*, **7**, 157–188.
30. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265–275.
31. Avruch, J. & Wallach, D. F. H. (1971) *Biochim. Biophys. Acta*, **233**, 334–347.
32. Ruoho, A. & Kyte, J. (1974) *Proc. Natl. Acad. Sci. U. S. A.*, **71**, 2352–2356.
33. Hegyvary, C. (1975) *Mol. Pharmacol.*, **11**, 588–594.
34. Lane, L. K. (1976) *FEBS Lett.*, **64**, 375–379.
35. Chandler, J. P., Hill, D. E. & Spivey, H. O. (1972) *Comp. Biomed. Res.*, **5**, 515–534.
36. Turi, J. S., Ho, N. F. H., Higuchi, W. I. & Shipman, C. (1975) *J. Pharm. Sci.*, **64**, 631–639.
37. Caldwell, P. C. & Keynes, R. D. (1959) *J. Physiol. (London)*, **148**, 8–9.
38. Hoffman, J. F. (1966) *Am. J. Med.*, **41**, 666–680.
39. Basalt, R. C., Wright, J. A. & Cravey, R. H. (1975) *Clin. Chem.*, **21**, 44–62.
40. Jusko, W. J., Gerbracht, L., Golden, L. H. & Koup, J. R. (1975) *Res. Commun. Chem. Pathol. Pharmacol.*, **10**, 189–192.
41. Klaassen, C. D. (1975) *Biochem. Pharmacol.*, **24**, 923–925.
42. Hoffman, J. F. (1969) *J. Gen. Physiol.*, **54**, 343S–347S.
43. Gardner, J. D. & Conlon, T. P. (1972) *J. Gen. Physiol.*, **60**, 609–629.
44. Klaassen, C. D. (1972) *J. Pharmacol. Exp. Ther.*, **183**, 520–526.
45. DeDuve, C. (1969) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Fell, H. B., eds.), Vol. 1, pp. 3–40, North-Holland Publishing Co., Amsterdam.
46. Allison, A. C. & Young, M. R. (1969) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Fell, H. B., eds.), Vol. 2, pp 600–628, North-Holland Publishing Co., Amsterdam.
47. Slater, T. F. (1969) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Fell, H. B., eds.), Vol. 1, pp. 469–492, North-Holland Publishing Co., Amsterdam.
48. Irvin, J. E. & Mellors, A. (1975) *Biochem. Pharmacol.*, **24**, 305–307.
49. Werb, Z. & Cohn, Z. A. (1971) *J. Exp. Med.*, **134**, 1570–1590.
50. Devreotes, P. N. & Fambrough, D. M. (1976) in *Biogenesis and Turnover of Membrane Macromolecules* (Cook, J. S., ed.), pp. 123–141, Raven Press, New York.
51. Nicolson, G. L., Lacorbiere, M. & Hunter, T. R. (1975) *Cancer Res.*, **35**, 144–155.