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INTERACTIONS OF CARDIAC GLYCOSIDES WITH CULTURED CARDIAC CELLS

II. BIOCHEMICAL AND ELECTRON MICROSCOPIC STUDIES ON THE EFFECTS OF OUABAIN ON MUSCLE AND NON-MUSCLE CELLS

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Summary

Electron microscopic and biochemical studies revealed a salient difference in the response to toxic doses of ouabain by cultured cardiac muscle and non-muscle cells from neonatal rats. Progressive cellular injury in myocytes incubated with $1 \cdot 10^{-4}$—$1 \cdot 10^{-3}$ M ouabain ultimately leads to swelling and necrosis. The morphological damage in myocytes was accompanied by a drastic decrease in $^{14}CO_2$ formation from ^{14}C-labeled stearate or acetate but not glucose. Neither morphological nor biochemical impairments were observed in non-muscle cells. The interaction between ouabain and the cultured cells, using therapeutic doses of ouabain (i.e., $<1 \cdot 10^{-7}$ M), was characterized. Two binding sites were described in both classes of cells, one site is a saturable K^+-sensitive site whereas the other is non-saturable and K^+-insensitive. The complexes formed between the sarcolemma receptor(s) and ouabain, at low concentrations of the drug (e.g., $7.52 \cdot 10^{-9}$ M), had K_d values of $8.9 \cdot 10^{-8}$ and $2.3 \cdot 10^{-8}$ M for muscle and non-muscle cells, respectively. The formation and dissociation of the complexes were affected by temperature and potassium ions.

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Abbreviation: CDC, critical digitalis concentration.

Introduction

Mechanisms regulating cellular volume in animal cells require that Na^+ should be kept in the extracellular medium in order to balance colloid osmotic pressure [1]. This is achieved by an active extrusion of sodium from the cells, hence the role of the sodium pump is of utmost importance. Changes in cellular volume or in the water content of the cell could be eventually reflected in changes in the volume of subcellular particles such as mitochondria, endoplasmic reticulum and the nucleus in addition to the volume changes of the cytoplasm [2].

Partial inhibition of the sodium pump in cardiac cells by low concentrations of cardiac glycosides, which is manifested as positive inotropy, caused only limited alterations in the distribution of cellular cations [3—7]. Progressive inhibition of the active pumping by toxic concentrations of the drug is expected to cause substantial changes in the distribution of the cations. Indeed, the intracellular concentrations of K^+ were markedly reduced and those of Na^+ elevated [8]. Increased doses of cardiac glycosides lead to typical clinical symptoms caused by disturbances of conduction and excitability of the heart [9].

No direct effect of cardiac glycosides on intracellularly-located organelles or enzymatic systems has been demonstrated [3]. On the contrary, most evidence points to the sarcolemmal ($Na^+ + K^+$)-ATPase as the sole known target of the drug [10—12]. Consequently, any influence of digitalis on intracellular metabolic processes may be considered to be secondary to the inhibition of the sodium pump. Such conclusions are based also on studies done with HeLa cells, which were shown to 'internalize' membrane-bound ouabain molecules in an energy-dependent process. The 'internalized' drug was detected in lysosomes and was finally released unaltered and without causing any cytotoxic damage [13—14]. Hence, impairment of biochemical events could be caused merely by the alteration of cellular concentrations of the ions manifested ultimately as intracellular damage and redistribution of other small molecules or ions, e.g., Ca^{2+} [15,16].

Most eukaryotic cells contain a sodium pump, hence inhibition of this pump by cardiac glycosides is expected to result in cellular swelling. However, the rate of water movement is not necessarily the same for all cells or alternative transport systems may exist, and therefore the damage may develop rapidly in one type of cell, slowly in others, or not at all. In certain cells, the process of swelling due to accumulation of sodium may be depressed by the loss of potassium from the cell.

Such possibilities were presented as premises for our study on the effects of therapeutic and toxic doses of ouabain on cultured cells from neonatal rat hearts. The cells are 'digitalis-sensitive', provide a mixed population of muscle and non-muscle cells, and the morphological and biochemical data gathered clearly indicate a distinct difference in the response of these two types of cells to cardiac glycosides.

Materials and Methods

Preparation and separation of cells. Cardiac cells in monolayer cultures were prepared from hearts of newborn rats, 1- to 3-days old, according to the method of Pinson and Padieu [17] or that of Harary et al. [18], and were studied after 3 or 5 days in culture. Enrichment of the culture with respect to either muscle or non-muscle cells was achieved by the pre-plating technique according to Polinger [19].

Binding and wash-out of [^{3}H]ouabain. 3-day old cultures were used: the growth medium was discarded and the plates rinsed with a Tyrode salt solution with or without K^+. 0.3 μCi [^{3}H]ouabain/plate and increasing amounts of non-labeled glycoside were added in Tyrode solutions to measure dose response. The amount of drug bound was determined after 30 min at 37°C with gentle constant shaking of the plate. The conditions were close to equilibrium (designated here as 'approach-to-equilibrium'). Incubation was terminated by placing the plates on ice, the medium dicarded and the cells rinsed several times with ice-cold Tyrode solutions [20]. Subsequently, the cells were taken up in 1 ml water with a rubber policeman and were ultrasonically irradiated for 5 s using a Braun Sonic 300 Homogenizator (Rochester, NY) at a setting of 60.

Aliquots were removed for determination of protein in the presence of 0.0075% sodium dodecyl sulfate and the remainder was taken for counting in Insta gel (Packard) using a TriCarb liquid scintillation spectrometer (model 3385, Packard). The binding was measured in the absence of K^+ ('total binding') and the period of incubation in the absence of K^+ did not cause damage to the cells (beating was resumed after addition of K^+): the binding in the presence of K^+ ('potassium-insensitive') was subtracted from the 'total' to obtain the 'potassium-sensitive' component of binding. Neither of the sites is related to the inulin space as determined by measuring the radioactivity in the cells' pellet which had been incubated with [^{3}H]ouabain and [^{14}C]inulin simultaneously. Non-specific binding was determined by adding a very large excess of unlabeled ouabain (e.g., $8 \cdot 10^{-3}$ M). Wash-out experiments were done to measure the rates of dissociation of bound ouabain from its complex with the cells in an ouabain-free Tyrode solution in the absence or presence of 9 mM K^+ either at 0—2 or at 37°C.

Incubation of cells with ouabain. (A) For electron microscopic studies 4- to 6-day old cultures were used. The growth medium was replaced by a fresh medium containing $1 \cdot 10^{-7}$ to $1 \cdot 10^{-3}$ M ouabain. The plates were then reincubated at 37°C for periods ranging from 30 min to 24 h. They were then prepared for electron microscopy as outlined below.

(B) Oxidation of glucose, stearate or acetate: in order to study the effects of ouabain on metabolic processes, plates with either mixed cell population or enriched with respect to muscle or non-muscle cells were incubated for 60 to 180 min with concentrations of ouabain ranging from $1 \cdot 10^{-7}$ to $1 \cdot 10^{-3}$ M. Following rinsing of the plates, the cells were reincubated with complete medium containing either [U^{14}C]glucose (6 mM, 1 μCi/plate), [1-^{14}C]stearate (0.2 mM, 1 μCi/plate) or [1-^{14}C]acetate (0.3—0.4 mM, 1 μCi/plate). During the oxidation of these substrates, ouabain was present in concentrations identical with those present earlier. The plates were placed in a device having parallel

channels specifically designed for measuring the simultaneous release of $^{14}CO_2$ in several Petri dishes [21]. The incubation was continued for 90 min at 37°C. The total amount of $^{14}CO_2$ formed, which includes the dissolved carbonates, was collected into hyamine hydroxide and counted in a TriCarb liquid scintillation spectrometer, model 2650 (Packard Instruments Co., Downers Grove, IL) which yields dpm directly.

Electron microscopy. For transmission electron microscopy, the cells were processed as previously described [22]. 10 ml of cold 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) were added to the culture medium and kept at room temperature for 5 min. The fixative was then removed, fresh 3% glutaraldehyde added and the plates stored for 25 min at 4°C, followed by post-fixation for 90 min with 2% OsO_4 in 0.1 M cacodylate buffer, pH 7.4, at room temperature. The cultures were dehydrated in a graded series of ethanol solutions, 70%, 90%, 95% and finally 100%, for 5 min each: the 100% ethanol contained 2% uranyl acetate. The cultured cells were detached from the plates by adding 2 ml of propylene oxide. The monolayers were then transferred, each to polypropylene tubes, washed several times with propylene oxide and infiltrated for at least 4 h with an equivolume mixture of Araldite/Epon/propylene oxide. Infiltration in Epon/Araldite alone was continued overnight. The cells were then transferred to conical 'Beem' capsules, centrifuged for 30 min at 10000 rev./min (swing-out rotor, RC-2 B fitted with a specially-designed holder) and the pellet was then hardened at 60°C for 3 days. Thin sections were cut and stained with lead citrate for 5 min and examined in a Philips 300 EM.

Results

Electron microscopic studies

Two types of cell exist in cultures obtained from neonatal rat hearts, one type beats and therefore is classified as 'muscle cell' (myocytes) and the other type fails to beat and hence was termed 'non-muscle cell' (fibroblasts). A morphological distinction between these two types of cell is illustrated in the electron micrograph shown in Fig. 1. The presence of myofibriles in muscle cells and their complete absence from non-muscle cells allows a microscopic identification. Under the conditions of pre-plating and culturing, plates enriched with non-muscle cells contained more than 90% of this type of cell and those enriched with muscle cells containg at least 80% myocytes.

Addition of relatively high concentrations of ouabain (e.g., $1 \cdot 10^{-5}$ to $1 \cdot 10^{-3}$ M) to the culture medium affected the beating of the myocytes which was observed under the phase-contrast light microscope. These concentrations of the drug are considered highly toxic for man and for digitalis-sensitive species [23].

The first signs of abnormal cellular response were in an increased rate of beating, followed by fibrillations. When followed by the electron microscope, the myocytes exhibited gradual changes quite similar to those obtained following the exposure of the cells to the specific anti-heart serum in the presence of complement [22]. After 30 min of incubation with ouabain, one could observe mitochondrial swelling, clumping of nuclear chromatin, some dilatation of endo-

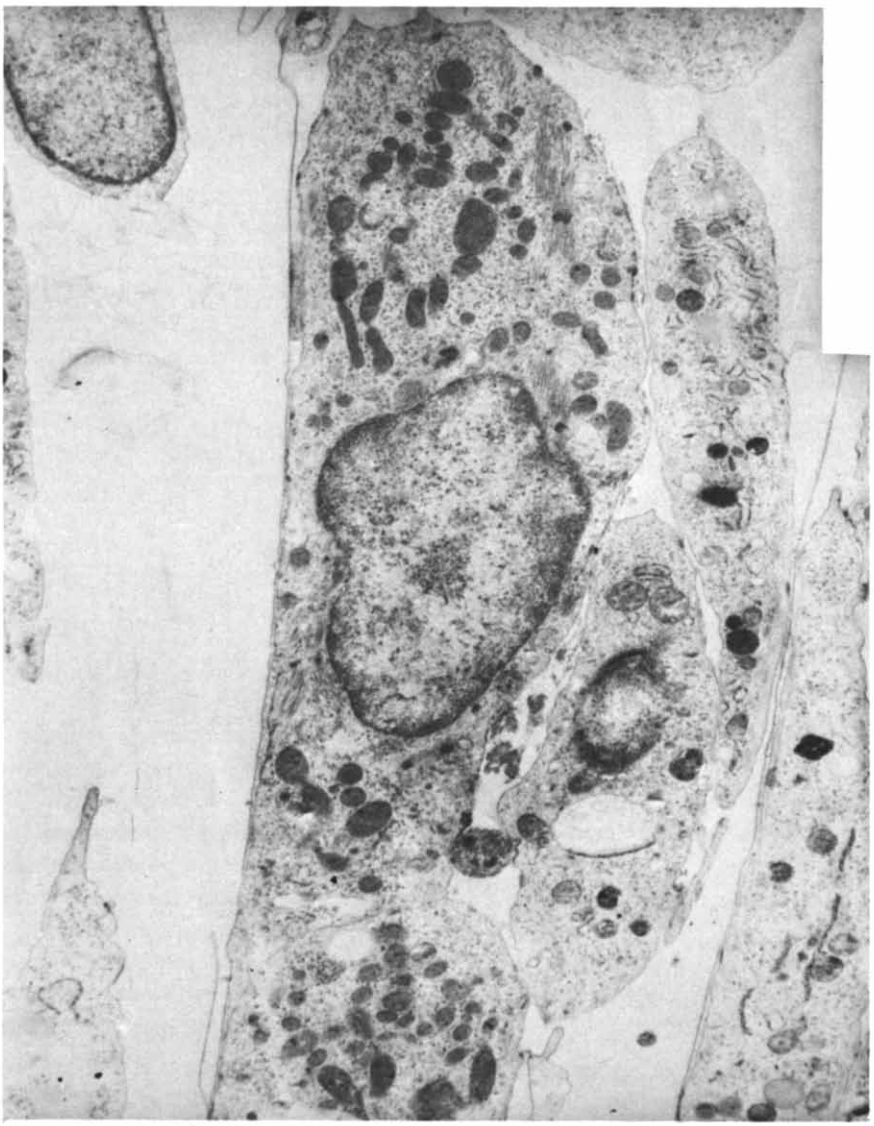


Fig. 1. Electron micrograph of cultured cardiac muscle and non-muscle cells. Magnification, ×5400. The muscle cells are characterized by the presence of myofibriles.

plasmic (sarcoplasmic) reticulum and opacity of the myoplasm. The non-muscle cells appeared without noticeable change (Fig. 2). These changes within the myocytes became much more pronounced after 60 to 120 min and by that time the mitochondria became markedly swollen, the matrix disappeared and the cristae were badly disrupted. Furthermore, after 60 min incubation, dense material appeared in the damaged mitochondria and the precipitation of this material increased with time (Fig. 3). After 24 h incubation, the myocytes were completely necrotic whereas the non-muscle cells retained the appearance of normal cells, except for occasional dilatations of endoplasmic reticulum.

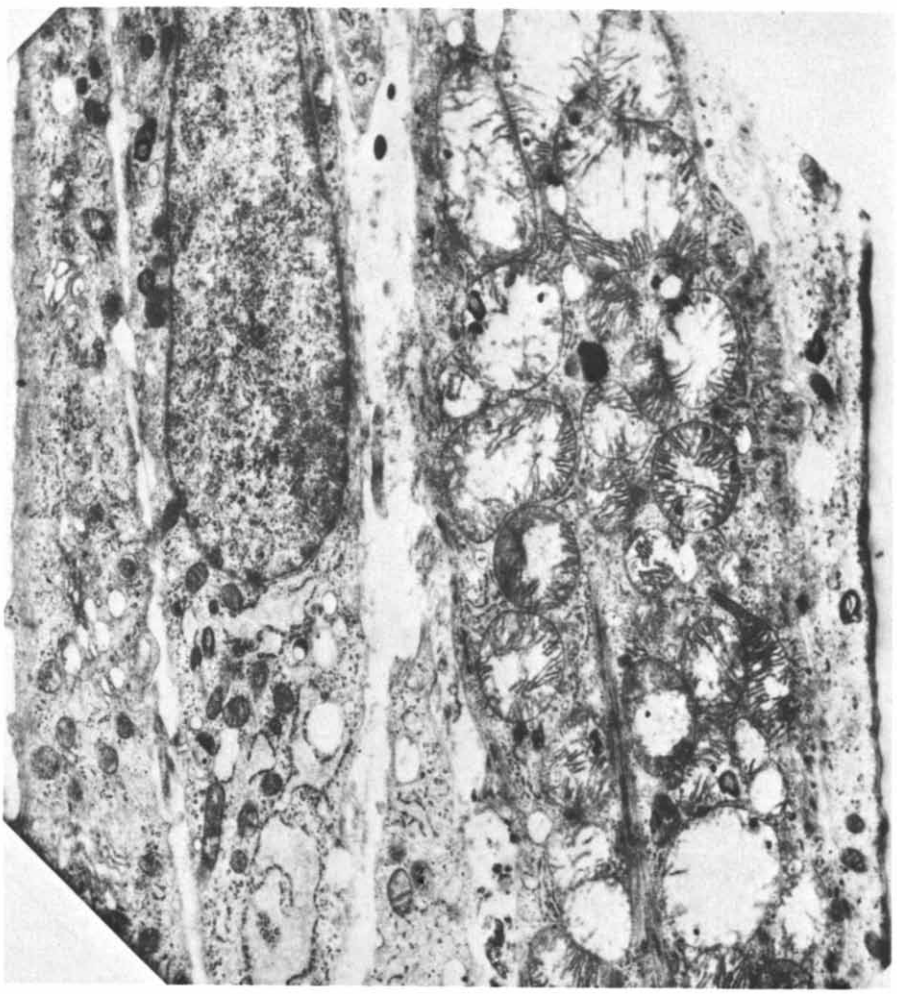


Fig. 2. Electron micrograph of cultured cardiac cells exposed to $2 \cdot 10^{-3}$ M ouabain for 30 min. Magnification, ×5400.

Although most obvious and immediate signs of cellular damage were observed at high concentrations of the cardiac glycoside (i.e., $1 \cdot 10^{-3}$ M), similar alterations were noticed with lower concentrations of the drug, only that the onset was delayed to 4 h and 24 h with $1 \cdot 10^{-4}$ and $1 \cdot 10^{-5}$ M ouabain, respectively.

Interactions of ouabain with cardiac cells at pharmacologically therapeutic doses

Physiological concentrations of K^+ present in the external medium interfered with the action as well as with the binding of ouabain with the cells. At higher concentrations (e.g., 5 to 10 mM), K^+ abolished the saturation binding of this drug to the cells when measured under equilibrium conditions of binding. We may refer to these binding sites as potassium-sensitive sites. This phenomenon was observed in both muscle and non-muscle cells. A dose-response curve

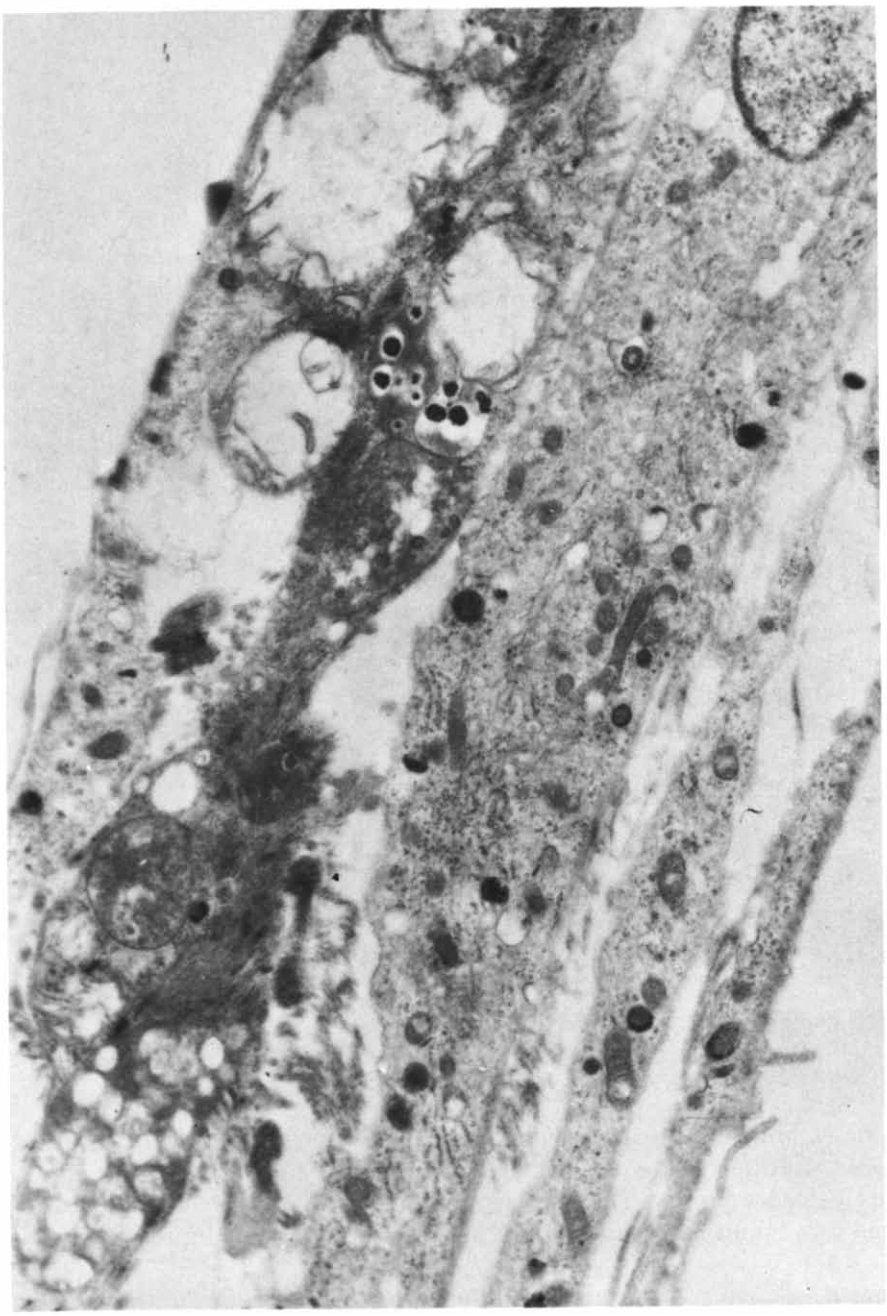


Fig. 3. Electron micrograph of cultured cardiac cells exposed to $2 \cdot 10^{-3}$ M ouabain for 120 min. Magnification, ×5400.

describing the binding of ouabain in the absence and presence of 2.68 mM K^+ to both types of cells using concentrations of the drug ranging from $7.5 \cdot 10^{-9}$ to $1 \cdot 10^{-5}$ M is shown in Fig. 4. These relatively short periods of exposure to a medium free of K^+ did not harm the cells as was ascertained by regeneration of

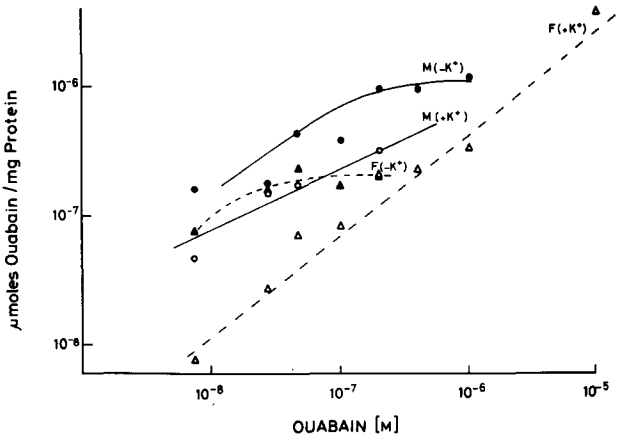


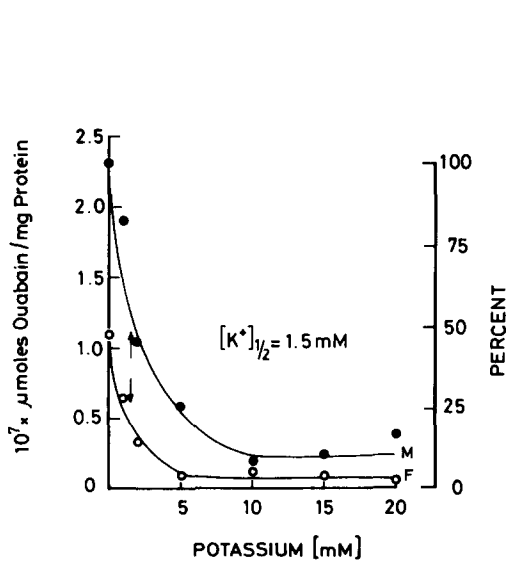
Fig. 4. Dose of ouabain binding to cultured cells enriched with either myocytes or non-muscle cells. Cultures enriched with respect to either muscle (M: ●, ○) or non-muscle (F: ▲, △) cells were incubated in Tyrode salt solution with ouabain ($7.5 \cdot 10^{-9}$ to $1 \cdot 10^{-5}$ M) for 30 min approaching equilibrium binding, in the absence or presence of 2.68 mM K^+: Following rinsing, the amount of radioactivity bound to the cells' pellet was determined. (Filled symbols, minus K^+: open symbols, plus K^+). The lines represent typical results obtained repeatedly.

beating following addition of K^+. Two types of binding are clearly observed in these cells: a saturable (potassium-sensitive) and non-saturable linear (potassium-insensitive) site, confirming our earlier observations with human erythrocytes [23]. The two lines coincided approximately at a concentration of ouabain ranging from $5 \cdot 10^{-7}$ to $1 \cdot 10^{-6}$ M. At higher concentrations of the drug only the linear component prevailed. The range of concentrations which allows the distinction between sensitive and non-sensitive sites was termed on a previous occasion, 'Critical Digitalis Concentration' (CDC [23]). The binding process approached equilibrium within 30 min at 37°C and at concentrations of ouabain below the CDC. The dissociation constants calculated from a Scatchard plot [24] for both myocytes and non-muscle cells were quite close, i.e., $8.9 \cdot 10^{-8}$ and $2.3 \cdot 10^{-8}$ M, respectively. Furthermore, at therapeutic concentrations below the CDC, i.e., $7.52 \cdot 10^{-9}$ M ouabain, in the medium myocytes and fibroblasts bound, at equilibrium, $2.84 \cdot 10^{-13}$ and $1.45 \cdot 10^{-13}$ mol ouabain/mg protein, respectively. These values correspond to approx. 11 000 and 2100 molecules ouabain bound/cell, respectively, for myocytes and fibroblasts at equilibrium. These values refer to the total number of sites, potassium-insensitive plus potassium-sensitive sites. At concentrations of the drug which saturate the potassium-sensitive sites (e.g., at $>3 \cdot 10^{-7}$ M ouabain), the total number of K^+-insensitive and K^+-sensitive sites, also calculated according to Scatchard [24], were 147 000 and 33 000 per cell, in myocytes and fibroblasts, respectively. With mixed populations of cells, intermediary values were calculated when a limited number of points was plotted. However, with large numbers of experimental data, a double saturation curve was obtained. The first curve, in the range $1 \cdot 10^{-8}$—$1 \cdot 10^{-7}$ M ouabain, yielded a calculated K_d value of $1.4 \cdot 10^{-8}$ M. The second curve ranged from $2 \cdot 10^{-7}$ to $1 \cdot 10^{-6}$ M and a calculated K_d of $8.33 \cdot 10^{-8}$ M. These values correspond well with those obtained in cells which were enriched by selective culturing. Hence, the careful

excecution of the experiments dose-response binding for ouabain enables one to distinguish between two cell populations having different binding characteristics without the need for a physical separation.

However, Fig. 5 indicates that the effect of K^+ on the binding of ouabain to myocytes or fibroblasts is quite similar: at 1.5 mM K^+ in the medium, the number of ouabain molecules bound/cell was 50% of the value as compared to that obtained in the absence of the cation. The time of exposure of the cells to ouabain, in the absence of K^+, was 30 min which is sufficient to reach binding values close enough to equilibrium, yet diminish the possible damage to cardiac myocytes due to lack of K^+.

The stability of the complex between the membrane's receptor and the drug was examined by wash-out, measuring the rate of ouabain released under various conditions. The kinetics of the ouabain wash-out are demonstrated in Fig. 6. The complex seems to be quite stable at low temperature and although only a short period is given here, experience indicates that ouabain dissociated from its complex at low temperature only very slowly. In the absence of K^+ in the medium, the rate of ouabain release from the complex was approx. 40% per h at 37°C. This rate decreased to half its value (i.e., 20% per h) when K^+ was added to the medium. The effect of K^+ on the lower rate of ouabain dissocia-



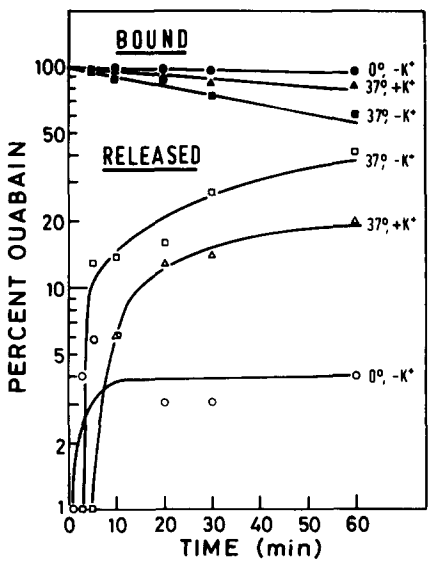


Fig. 5. Effect of potassium concentrations on the binding of ouabain to cultured cardiac cells. The details of the experiment are similar to those of Fig. 4 except that the concentration of ouabain was kept constant (i.e., $7.52 \cdot 10^{-9}$ M) and the concentration of K^+ varied from 0 to 20 mM. ●, muscle cells (M); ○, non-muscle cells (F). The arrows point to the $[K^+]_{1/2}$ values.

Fig. 6. Wash-out kinetics of ouabain bound to cultured cardiac cells. A mixed population, incubated with $7.52 \cdot 10^{-9}$ M ouabain at 37° C for 30 min: the cells were rinsed with cold medium and the monolayer covered again with a ouabain-free medium, in the absence or presence of 9 mM K^+. The plates were incubated at 0—2 or 37°C and at intervals, aliquots of the medium were withdrawn for counting. The results are expressed as percent release or percent bound in which the value of 100% is that obtained for the amount of ouabain originally bound. Filled symbols indicate the bound drug; open symbols, the released drug. ●, ○, incubations at 0—2° C in absence of K^+; ▲, △, incubations at 37° C in presence of K^+; ■, □, incubations at 37° C in absence of K^+.

tion from its complex can be due to stabilizing effect of K^+ on the complexes, hence delaying ouabain release. It should be stressed that the concentrations of ouabain used in the binding were below the CDC. These concentrations are known to elicit therapeutic consequences (i.e., inotropic effects).

Effects of toxic doses of ouabain on $^{14}CO_2$ formation from radioactive substrates

Complete inhibition of the sodium pump by high toxic doses of cardiac glycosides is expected to alter, indirectly, normal metabolic processes. Consequently, we have attempted to distinguish between the possible sites of lesions by following the production of $^{14}CO_2$ from different substrates. CO_2 may evolve from the oxidation of glucose either through the glucose-6-phosphogluconate dehydrogenase (EC 1.1.1.43) reaction or the tricarboxylic acid cycle or both. However, acetate or stearate can be oxidized only via the tricarboxylic acid cycle. The myocytes draw their energetic needs primarily from the oxidation of fatty acids, however, partial mitochondrial injury can be manifested as an impairment of the 'β-oxidation complex', the tricarboxylic acid cycle or both. Hence, effects on $^{14}CO_2$ formation from either acetate or stearate may be used as approximate criteria for such a distinction.

Mixed populations of cultured cells or, alternatively, cultures enriched with respect to non-muscle cells (i.e., fibroblasts) were only slightly affected biochemically following prolonged exposure to high concentrations of ouabain (e.g. $1 \cdot 10^{-3}$ M). On the other hand, enriched cultures of cardiac muscle cells, which contain only a small proportion of non-muscle cells, were very sensitive to toxic concentrations of the drug, although no effect was observed at therapeutic concentrations of ouabain. The drastic decrease in the rate of

TABLE I

EFFECT OF OUABAIN ON $^{14}CO_2$ FORMATION

5-day old monolayers of mixed cell population (M + F), highly enriched non-muscle cells (F) or muscle cells (M) were incubated for 60 to 180 m in a 0, $1 \cdot 10^{-7}$, $1 \cdot 10^{-5}$ or $1 \cdot 10^{-3}$ M ouabain-containing medium. The results obtained represent mean values and are expressed as percent formation relative to the amounts in controls totally devoid of ouabain (the numbers in brackets in the first column indicate the number of experiments).

	Cell type	Preincubation with ouabain (min)	Substrate	Ouabain during oxidation	$^{14}CO_2$ formation at given concentration of ouabain (M), (percent of controls)		
					$1 \cdot 10^{-7}$	$1 \cdot 10^{-5}$	$1 \cdot 10^{-3}$
A	F + M (2)	180	glucose	+	85	95	90
	F (1)	60	glucose	+	100	100	100
	F (1)	180	glucose	+	100	—	100
B	F + M (3)	60	stearate	—	88	92	92
	F + M (4)	180	stearate	—	95	96	100
	F (1)	90	stearate	+	100	—	91
	M (1)	60	stearate	+	100	26	17
	M (1)	180	stearate	+	94	—	17
C	F (1)	180	acetate	+	100	—	100
	M (1)	180	acetate	+	88	—	26

$^{14}CO_2$ production from both [^{14}C]acetate and [^{14}C]stearate points to serious damage to mitochondrial performance which is reflected in an impairment of the integrity of the tricarboxylic acid cycle and probably damage to the complex of the β oxidation as well (Table I).

Discussion

The volume of living cells is regulated by their ionic content, therefore understanding the process of ion regulation requires studies on the transport of cellular ions as well as water movement. Errors, defects or poisoning of the regulatory processes of cell volume lead to cell swelling and ultimately to lysis. Addition of cations and anions to the cell's cytoplasm increases the total cellular solute concentration, consequently reducing the activity of the cell's water. Since the plasma membrane is highly permeable to water, due to the Gibbs-Donnan equilibrium, any impairment of active pumping mechanisms will cause inward movement of water, resulting in an increased cell volume. The active transport system of monovalent cations is controlled by the sodium pump which is specifically inhibited by cardiac glycosides. A large body of evidence points to this pump and its biochemical expression, the $(Na^+ + K^+)$-ATPase, as the site of cardiac glycoside action at the molecular level [3,10,12].

Cardiac glycosides exert positive inotropic effects on normal or failing heart muscles, but not on skeletal muscles [25,26]. At therapeutic doses of digitalis, which are rather low (in the range $1 \cdot 10^{-9}$—$1 \cdot 10^{-8}$ M), the pump or the enzymatic activity is only partially inhibited leading, apparently, to a limited gross alteration in cellular content of ions, although allowing certain non-electrogenic exchanges such as an Na^+-Ca^{2+} exchange [27]. Such concentrations inhibited the enzymatic activity of $(Na^+ + K^+)$-ATPase in vitro only to a maximal extent of 30% [27]. At higher concentrations of the drug which caused an inhibition of 60% or more of the enzymatic activity, the pump failed to extrude a significant portion of intracellular Na^+, hence, concentrations of which increased in the myocardium [8].

Changes in the osmoregulatory mechanism of renal tubular cells lead to lysis which was related to the inhibition of the sodium pump by cardiac glycosides: this has been divided, morphologically, into five stages [29]. Similar changes were also observed in ischemic conditions developed in canine myocardium [30]. At a later stage of the damage, deposits of electron-dense precipitates were observed in small and large mitochondria which were described as calcium deposits due to an intense transport of Ca^{2+} during the early stages of the inhibition [31] which becomes only evident, however, at later stages of the injury (cf. Fig. 3 and Refs. 22, 31—33).

Cardiac glycosides have been shown also to affect certain aspects of metabolism in intact tissues which resulted in alterations in the cellular capacity to carry out several reactions as was also demonstrated in the present study. However, isolated subcellular organelles or enzyme systems involved in energy-producing pathways such as glycolysis, the oxidative part of the pentose shunt or the mitochondrial oxidation via the 'β-oxidase complex' and/or the Krebs cycle, are not influenced by the drug in vitro [3,8]. In the present study, the differential injury observed in the electron micrograph of muscle cells, but not

in the non-muscle cells, was corroborated by biochemical oxidation studies done with appropriately selected substrates to assist in locating the possible sites of intracellular damage. The formation of $^{14}CO_2$ from glucose, acetate or stearate was used in the attempts to distinguish biochemically between the muscle and non-muscle cells, since muscle cells draw their energy requirements primarily from the oxidation of fatty acids and the non-muscle cells react differently. The latter cells were almost unaffected by the high concentrations of ouabain, both morphologically and biochemically. This was rather surprising because these cells do contain a sodium pump which binds [^{3}H]ouabain and is probably inhibited by it, hence alternative mechanisms of volume regulation might exist in these cells or the difference in the structure or composition of their plasma membrane versus the sarcolemma of the myocytes is the appropriate explanation. The fact that the muscle cells are 'excitable', whereas the non-muscle cells are not, could be the basis for such differences. This is also reflected in the number of sodium-pumping sites per cell which has been roughly estimated to be 5-fold higher in myocytes than in non-muscle cells [34]. Furthermore, both classes of cell exhibited the two types of glycoside receptor, which were termed K^+-sensitive and K^+-insensitive sites in the present communication. These sites may coincide with the two populations of sodium-pumping sites described by Godfraind and Ghysel-Burton [35] in guinea-pig atria. These two sites also exhibited high and low affinities for ouabain in a range up to $1 \cdot 10^{-8}$ and $1 \cdot 10^{-4}$ M, respectively.

Acknowledgements

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