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Species and ionic influences on the accumulation of digitalis glycosides by isolated perfused hearts

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Summary

1. The ability of isolated perfused guinea-pig (digitalis-sensitive species) and rat (digitalis-resistant species) hearts to accumulate radio-labelled digitalis glycosides was studied in relation to the ionic composition of the perfusion medium.

2. It was observed that in both species much less digoxin was accumulated than was digitoxin or proscillaridin.

3. The accumulation of digoxin was markedly inhibited in a low sodium or in high potassium medium. These effects were similar, but relatively less marked, with digitoxin and proscillaridin. Calcium and magnesium removal had relatively smaller effects on the accumulation of both polar and non-polar glycosides.

4. The low accumulation of all digitaloids by the rat heart in comparison to the guinea-pig heart may be due to the formation of unstable complexes between the cellular membranes in the rat heart and the various digitaloids used in this study. Although digitaloids have a reduced affinity for rat hearts and rat heart membranes in comparison to guinea-pigs, the order of the accumulation of different glycosides in both species is the same, i.e. much less with polar glycosides than with non-polar glycosides.

5. It was concluded that non-polar glycosides such as digitoxin and proscillaridin demonstrate the same ion-dependent accumulation mechanism as do the more polar glycosides such as digoxin and ouabain. In addition, the non-polar glycosides possess high capacity for ion-independent binding presumably due to lipophilic interactions with membranes.

Introduction

We have shown previously that the accumulation of ouabain by the isolated perfused guinea-pig heart is directly dependent on the concentration of sodium ions in the perfusing medium and is inversely related to the concentration of potassium ions in the extracellular fluid. The absence of magnesium had no significant effect on the accumulation of ouabain by the perfused heart (Dutta & Marks, 1969). In recent years, the effects of ionic composition of the medium on the electrical and mechanical responses of cardiac tissue to digitaloids have been well documented by various studies. Toda & West (1966) observed that the rate of onset of toxicity to ouabain in the spontaneously beating rabbit atrial preparation was directly related to the extracellular concentration of sodium and calcium.

In their study, low $[Na]_o$ delayed the onset of toxicity but did not influence or reverse an established toxicity. Studies by Caprio & Farah (1967) on the response of rabbit ventricular strips to ouabain revealed that changes in calcium, potassium or sodium concentrations in the medium influence the positive inotropic effects of ouabain. Sodium dependency of ouabain in inducing a positive inotropic effect has been observed in ventricular strips from *Rana temporaria* (Talbot, 1968). Clinically, intravenous administration of potassium has been recognized as a method of reversing certain digitalis-induced cardiac arrhythmias since its introduction for this purpose in 1943 by Sampson, Alberton & Kondo (1943). On the other hand, it is also known that hypokalemia, often caused by diuretics, can induce digitalis toxicity.

In view of the above mentioned observations, it is reasonable to expect that the ion dependency of ouabain accumulation such as we have observed in the guinea-pig heart may have some pharmacological implications. However, since there are marked quantitative differences in cardiac accumulation of various glycosides by the guinea-pig heart (Dutta, Goswami, Datta, Lindower & Marks, 1968b; Kuschinsky, Lullmann & Van Zwieten, 1968), we felt that it might be interesting to characterize further the differences between glycosides in their relationship to cardiac uptake. In this paper we describe the effect of ionic changes in the medium upon the accumulation and distribution of various glycosides, and also compare the species difference in the uptake of various glycosides by guinea-pig and rat hearts, known to represent sensitive and resistant species, respectively (Chen, 1963).

Methods

The usual Langendorff perfusion and cardiac performance recording technique was used for guinea-pig and rat hearts equilibrated with Krebs-Henseleit (K-H) solution at an inflow rate of 4 ml/min and at a temperature of 28° C (Dutta, Goswami, Lindower & Marks, 1968a). Krebs-Henseleit solution contained in millimoles per litre: Na^+, 145·2; K^+, 5·8; Ca^{++}, 2·5; Mg^{++}, 1·2; Cl^-, 127·8; $SO_4^=$, 1·2; $H_2PO_4^-$, 1·0; HCO_3^-, 27·2; and glucose, 11·1. After a 30–45 min control period, the medium was changed to a K–H solution containing 10^{-7}M tritiated glycosides. The perfusion was continued for 64 min and terminated by changing back to normal K–H medium for 8 min of washout. The hearts were collected, cleaned and blotted and the ventricular tissue from each heart was homogenized with 9 volumes of sucrose-ethylene-diamine tetra-acetic acid (EDTA). By high speed centrifugation for one hour the homogenate was separated initially into supernatant and pellet fractions. At the end of this step, in guinea-pig experiments where the influence of ionic composition was studied, the pellet was resuspended in sucrose-EDTA and fractionally centrifuged to prepare nuclear (450 $\times g$ for 10 min), mitochondrial (12,000 $\times g$ for 15 min) and microsomal fractions (166,000 $\times g$ for 1 hour). Aliquots of the homogenate and of each fraction were used for scintillation counting and for protein determination, by the methods described earlier (Dutta *et al.*, 1968b). Since there is no evidence that the perfused heart is able to metabolize cardiac glycosides appreciably, it is assumed that the radioactivity is due to the parent compound studied.

In order to study the influence of reducing concentrations of sodium in the extracellular medium on the accumulation of H^3-digoxin, H^3-digitoxin and H^3-pros-

cillaridin, the K-H solution was modified to contain 27·2 mM sodium instead of 145·2 mM sodium present in the control K-H solution. Sucrose was added to maintain isotonicity of the low sodium K-H solution. Following equilibration with control K-H, each respective drug was perfused for 64 min in the low sodium K-H solution and washed for 8 min with regular K-H. Similarly, to determine the effect of low or high potassium, hearts were perfused with the respective cardiac glycoside in K-H solution modified to contain 1·0 or 15·4 mM potassium instead of 5·8 mM potassium, which was present in the control K-H. The effect of lack of Ca or Mg in the perfusion medium was determined for digoxin and digitoxin by perfusing these drugs for 64 min in calcium- or magnesium-free K-H solution. Randomly labelled H^3-digitoxin and H^3-ouabain were purchased from New England Nuclear Corp. Specifically labelled digoxin (12 α-H^3) was kindly supplied by Sandoz Ltd., Basel, Switzerland. Randomly labelled H^3-proscillaridin was supplied by Knoll Pharmaceutical Co.

Results

The effects of ion concentration on digitaloid accumulation by the guinea-pig heart

The amount of the various glycosides accumulated in guinea-pig hearts after 64 min of perfusion in normal K-H, low sodium K-H, low potassium K-H and high potassium K-H media is shown in Table 1. The control data demonstrate the marked quantitative differences seen in the accumulation of different glycosides by the heart (Dutta *et al.*, 1968b). The reduction of sodium concentration in the perfusion medium significantly reduced the cardiac uptake of all cardiac glycosides studied. However, the reduction of sodium concentration in the perfusion medium produced a greater proportional reduction in the cardiac uptake of digoxin than that of the glycosides digitoxin and proscillaridin. From Table 1 we see that myocardial cellular uptake in the low sodium medium is 18·7% of the control for digoxin, whereas under similar conditions the uptake of digitoxin and proscillaridin is 38·2% and 42·6% of the control.

Increase or decrease of potassium in the perfusion medium had opposite effects on the uptake of all three cardiac glycosides by the guinea-pig heart. By reducing the extracellular potassium to 1·0 mM, there was an approximately equivalent absolute increase of uptake of all glycosides although the proportional increase was greatest for digoxin. On the other hand, increase of potassium concentration in the extracellular medium to 15·4 mM reduced the uptake of all glycosides by the heart. The accumulation of digoxin in high potassium medium was reduced more markedly than was the accumulation of digitoxin and proscillaridin. In the presence of the high potassium medium digitoxin uptake was 49·4% and proscillaridin uptake was 80·5% of the control. In contrast, digoxin had only 22·4% of the control uptake under similar conditions. The inverse relationship between potassium concentration and glycoside uptake is shown in Figure 1. If it is assumed that the glycoside uptake in high potassium medium is accumulated nonspecifically, then it can be said that the nonspecific accumulation of digitoxin is about 10 times and proscillaridin 30 times that of digoxin.

In Table 1 the influence of the changes in sodium and potassium ion concentration of the perfusion medium on the microsomal uptake of the various cardiac glycosides is also shown. Previous studies have shown that the content of digitalis glycosides is higher in the microsomal fraction (light membrane fraction) than in

any other cardiac subcellular fraction. The ionic changes in sodium and potassium affected the microsomal uptake of the three glycosides in a manner parallel to the effects described for whole heart uptake. The per cent change of uptake with alteration of sodium or potassium ion concentration of the medium was again much greater for digoxin than it was for digitoxin or proscillaridin.

TABLE 1. *Effect of sodium and potassium on the uptake of three cardiac glycosides by the isolated perfused guinea-pig heart**

	Homogenate (pmol/g)			
	Control K-H 149 mM Na^+ 5·8 mM K^+	Low sodium K-H 27·2 mM Na^+	High potassium K-H 15·4 mM K^+	Low potassium K-H 1·0 mM K^+
Digoxin	139±9·68 (10)†	26±2·07 (4) $P<0·001$‡	31·3±2·76 (3) $P<0·001$	330±6·70 (3) $P<0·001$
Digitoxin	594±44·7 (5)	227±12·7 (5) $P<0·001$	294±25.7 (5) $P<0·001$	823±28·7 (3) $P<0·005$
Proscillaridin	1,040±45·6 (4)	444±91·1 (3) $P<0·005$	838±81·5 (3) N.S.	1,160±63·0 (4) N.S.
	Microsomal fraction (pmol/mg protein)			
Digoxin	1·49±0·1 (4)	0·23±0·02 (4) $P<0·001$	0·32±0·03 (3) $P<0·001$	2·79±0·43 (3) $P<0·05$
Digitoxin	5·05±0·69 (5)	1·54±0·16 (5) $P<0·005$	1·76±0·13 (5) $P<0·005$	7·79±1·05 (3) N.S.
Proscillaridin	13·9 ±1·54 (4)	5·45±1·86 (3) $P<0·025$	8·86±0·55 (3) $P<0·025$	12·9±2·22 (4) N.S.

* Glycosides perfused in 10^{-7}M solution for 64 min, hearts washed out for 8 min by perfusion with normal K-H medium. Flow rate 4 ml/min, temperature 28° C. † Mean±standard error. Number of experiments in parentheses. ‡ *P* values are for comparison of corresponding control observations. Not significant (N.S.).

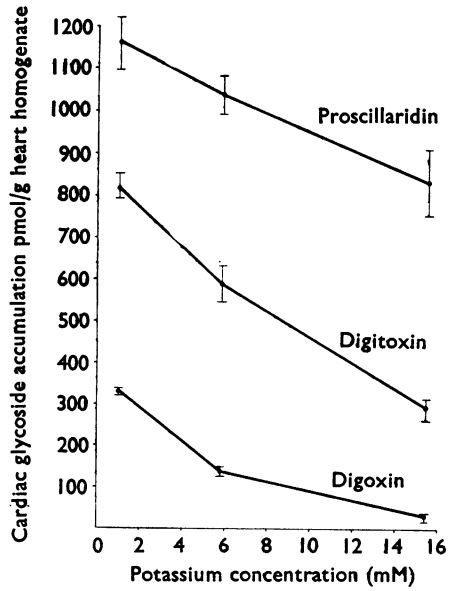

FIG. 1. The relationship of potassium concentration in the perfusion medium to the accumulation of H^3-digoxin, H^3-digitoxin and H^3-proscillaridin by the isolated guinea-pig heart. Perfusion concentration of digitaloids, 10^{-7}M; Time 64 min. Bars represent S.E.M.

The absence of calcium from the perfusion medium affected digoxin uptake by whole heart tissue more than digitoxin. The absence of magnesium, on the other hand, had no effect on either glycoside (Table 2). The same effects were also seen in microsomal fractions, in which the digoxin and digitoxin uptake in calcium free medium were reduced to approximately half the control value. Magnesium-free perfusion medium had minimal effects, again being most marked in the reduction of digoxin uptake.

Comparison of cardiac glycoside accumulation in guinea-pig and rat hearts

In Table 3, the accumulation of four cardiac glycosides in ventricle muscle and in the total particulate (pellet) and supernatant fraction of guinea-pig and rat hearts are compared. It is clear that with all four drugs there was greater digitaloid accumulation in the digitalis-sensitive guinea-pig heart than in the digitalis-refractory rat heart. In previous experiments in which randomly labelled tritiated digoxin was used (Dutta *et al.*, 1968a) the accumulation of digoxin in the guinea-pig heart was found to be twice that of the rat heart. In this study we confirm our earlier observation with the use of specifically labelled digoxin tritiated in the 12 α position. In addition we found that, as in the guinea-pig heart, the accumula-

TABLE 2. *Effect of the absence of calcium or magnesium in the perfusing medium on the uptake of two cardiac glycosides by isolated perfused guinea-pig hearts**

	Homogenate				Microsomes			
	Without Ca^{++}		Without Mg^{++}		Without Ca^{++}		Without Mg^{++}	
Drugs	(pmol/g)	(% control)	(pmol/g)	(% control)	(pmol/mg protein)	(% control)	(pmol/mg protein)	(% control)
Digoxin	93·9±14·5 (4)† $P<0·025$	68	115±24·7 (3) N.S.	83	0·96±0·15 (4) $P<0·025$	64	1·27±0·10 (3) N.S.	85
Digitoxin	471±75·6 (3) N.S.	79	592±59·8 (3) N.S.	100	2·46±0·24 (3) $P<0·025$	49	5·17±0·81 N.S.	102

* Conditions of the experiments and control values for homogenate and microsomes are shown in Table 1. † Mean±standard error. Number of experiments in parentheses. *P* values are for comparison of corresponding control observations in Table 1.

TABLE 3. *Distribution of the cardiac glycosides in various fractions prepared from the perfused guinea-pig and rat hearts*

Drugs in K-H medium*	Homogenate (pmol/g)	Supernatant (pmol/g)	Pellet (pmol/g)	Supernatant to pellet ratio
Guinea-pig				
Ouabain	122± 2·97 (5)†	20·9± 1·38 (5)	94·1± 5·76 (5)	0·25
Digoxin 12 H^3	154±13·6 (5)	62 ±13·5 (3)	104 ±12·4 (3)	0·60
Digitoxin	604±39·2 (5)	240 ±24·0 (5)	362 ±36·8 (5)	0·65
Proscillaridin	1,040±45·6 (4)	131 ± 7·69 (4)	911 ±39·5 (4)	0·14
Rat				
Ouabain	57·6±8·65 (3) $P<0·001$	18·5± 4·38 (3)	42·0± 7·30 (3)	0·43 N.S.
Digoxin 12 H^3	82±3·49 (3) $P<0·001$	34 ± 7·93 (3)	45 ± 1·07 (3)	0·8 N.S.
Digitoxin	253±11·4 (3) $P<0·001$	154 ±22·6 (3)	104 ± 8·14 (3)	1·49 $P<0·05$
Proscillaridin	431±47·8 (3) $P<0·001$	141 ±25·8 (3)	279 ±19·4 (3)	0·5 $P<0·001$

* Concentration of each drug in the medium was 1×10^{-7}M. † Mean±standard error. Number of experiments in parentheses. *P* values are for comparison of corresponding rat and guinea-pig data.

tion of various cardiac glycosides in the rat heart varies greatly. In both species ouabain accumulated to the least and proscillaridin to the greatest extent. The supernatant to pellet ratio (S/P), a figure inversely related to the digitaloid binding to membraneous elements, shows significantly higher values for digitoxin and proscillaridin in the rat than in the guinea-pig. This higher S/P ratio in the case of the rat indicates that rat membranes probably have less affinity for binding these cardiac glycosides than guinea-pig membranes.

It might be noted that in comparison to its concentration in K-H solution (100 pmol/ml), six-fold more digitoxin is taken up per g of guinea-pig heart in normal K-H solution. The digitoxin concentration in the soluble supernatant fraction of guinea-pig heart tissue is several times higher than the perfusion medium concentration (Table 3). Since the extracellular space of these hearts had been washed by perfusion with non-radioactive K-H medium, this soluble supernatant fraction ostensibly represents the digitaloid content of the intracellular water compartment alone. In order to determine whether digitoxin, which appears to be transported against a concentration gradient, is free or bound to a soluble protein or other macromolecules in the soluble supernatant fractions, 0·5 ml of the supernatant fraction mixed with blue dextran as a marker was placed on a Sephadex G-75 column, which was then fractionally eluted with 0·9% sodium chloride solution. The blue dextran (M.W. 2,000,000) was eluted in a void volume of 50–60 ml. Digitoxin of the soluble supernatant fraction came off the column with a peak at 140 ml (average value of two experiments range being 130–150 ml), similar to the elution pattern obtained with standard H^3-digitoxin in 0·5 ml K-H solution. No digitoxin came off the column in association with the major protein peak at the void volume. This suggested that the digitoxin in the supernatant was not firmly protein bound, and that there was, in fact, a substantial concentration gradient of free digitoxin between the intra- and extracellular space.

Discussion

These studies indicate that as was observed with ouabain (Dutta & Marks, 1969), the external sodium and potassium ion concentrations greatly affect the uptake of digoxin, digitoxin and proscillaridin in the guinea-pig heart. The behaviour of digoxin quantitatively resembles closely that reported earlier for ouabain. The extent of cardiac accumulation of cardiac glycosides appears to be directly related to the concentration of sodium ions in the perfusing medium and inversely related to the concentration of potassium ions. Recently, Baker & Willis (1970) have shown the inverse relationship of the extracellular potassium to the binding of H^3-ouabain by HeLa cells and cells cultured from the human heart. In their study, concentration of the glycoside in the medium varied from 10^{-9} to 10^{-6}M. They noted that with ouabain concentrations higher than 10^{-6}M the uptake was no longer affected by potassium. It has been suggested that the potassium sensitivity of the H^3-ouabain uptake which saturates at low concentration of the glycoside is due to competition between extracellular potassium ions and ouabain for some binding site at the cell surface (Dutta & Marks, 1969). The behaviour of digoxin in the present studies is compatible with this view. On the other hand, digitoxin and proscillaridin uptake are proportionally much less influenced by changes in sodium and potassium concentrations in the outside medium. The difference between ouabain and digoxin, on the one hand, and proscillaridin and digitoxin

on the other, arises primarily because the latter glycosides possess a large component of ion-insensitive binding. In addition to this (non-specific) binding, digitoxin and proscillaridin also demonstrate the normal specific binding which is characterized by conventional ion sensitivity. This difference in membrane binding may explain the difference in the effects on membrane potential produced in guinea-pig hearts by these drugs (Ito, Hollander, Marks & Dutta, 1969).

It has been reported by Albers, Koval & Siegel (1968) that the rate of onset of inhibition of *Electrophorus* electric organs Na-K-ATPase is significantly more rapid with digitoxin and scillaren A than with digoxin and ouabain. Because of this observation they have suggested that the rate of interaction of cardiac glycosides with the transport enzyme is inversely related to the number of hydroxyl and sugar substitutes and may be lipophilic in nature. It is interesting that a similar trend is noted in the binding of glycosides by both guinea-pig and rat hearts. Decreased ring hydroxylation in the case of digitoxin and proscillaridin may be related to increased accumulation by the heart of these non-polar cardiac glycosides relative to the more polar glycosides ouabain and digoxin. The lipophilic nature of the binding was also seen with *in vitro* binding studies of various glycosides with the sarcoplasmic reticulum membraneous fragments (SRF) isolated from beef heart. This demonstrated that the magnitude of uptake of glycosides by SRF was similar for ouabain and digoxin, while digitoxin and proscillaridin showed three- to four-fold greater binding (Dutta *et al.*, 1968b).

With conditions of low $[Na]_0$ or high $[K]_0$, the heart content of ouabain, as reported earlier (Dutta & Marks, 1969), or digoxin, in this study is only one-fifth of the medium concentration, while under similar conditions the cardiac tissue to medium ratio is as high as 3·0 in the case of digitoxin and 8·4 in the case of proscillaridin. In normal K-H, the tissue/medium ratio is 6 for digitoxin and 10 for proscillaridin. Digitoxin demonstrates particularly high concentrations in the intracellular water compartment, approximated as the soluble supernatant fraction. Though fractionation of the soluble supernatant fraction on a Sephadex column may disturb the binding equilibrium and tend to increase the concentration of free digitoxin, the elution pattern of the supernatant fraction suggests that digitoxin in this fraction may be entirely free, not even partially associated with macromolecules. This may indicate that digitoxin is actively transported into the intracellular space against a concentration gradient, and subsequently equilibrates with intracellular membraneous elements. This may account for the excessive accumulation and significantly high supernatant to pellet ratio of digitoxin as compared with polar glycosides. The characteristic feature of the association of digitoxin and proscillaridin with membranes is that it is largely independent of the sodium and potassium ion concentration of the extracellular medium. This is in sharp contrast to the behaviour of digoxin and ouabain.

In general, the absence of calcium in the medium lowered the microsomal binding of digoxin and digitoxin. In comparison to alteration of $[Na]_0$ the reduction of uptake of these two cardiac glycosides by the microsomal fraction due to the absence of calcium was only moderate ($P<0·025$). Further, there was no systematic difference of effect of $[Ca]_0$ between the polar and non-polar glycosides.

Our observations upon the rat heart confirm an earlier report (Dutta *et al.*, 1968a) that the rat exhibits very poor digoxin uptake, the concentration in the heart homogenate being approximately one-half that found for the guinea-pig.

The rat also exhibits a high supernatant/pellet ratio for digitoxin and proscillaridin which indicates that its membranes have a lesser affinity for binding non-polar digitaloids than those of the guinea-pig. Allen & Schwartz (1969) have shown that rat Na-K-ATPase takes up two-fold more H^3-ouabain than dog or beef enzymes but the complex formed between rat enzyme and H^3-ouabain is less stable and therefore more easily dissociable. Since we have washed these hearts, low values of digitaloid uptake by the rat heart in comparison to the guinea-pig heart may indicate the formation of an unstable complex between membranes and the various digitaloids used in this study. However, it should be pointed out that although the digitaloid complex may be less stable, the rat heart membranes seem to share some important properties with the guinea-pig heart membranes. For example, the order of the accumulation of different glycosides in the rat heart and the guinea-pig heart appears to be the same, with ouabain being least bound and proscillaridin highest in both species. These findings perhaps indicate that the digitaloid binding sites in rat and guinea-pig hearts have similar structural dependence and mechanism, although different affinity characteristics.

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