Biochemical Pharmacology, Vol. 23, pp. 2817–2824. Pergamon Press, 1974. Printed in Great Britain.

SUBCELLULAR DISTRIBUTION OF K-STROPHANTHOSIDE (^{3}H) IN ISOLATED GUINEA-PIG HEARTS

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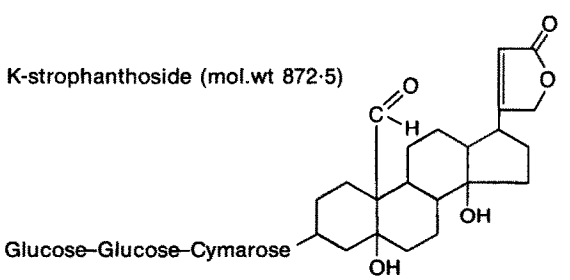
(*Received* 26 *November* 1973; *accepted* 6 *March* 1974)

Abstract—Guinea-pig hearts were perfused at a constant flow rate of 3 ml/min at 28° with the Krebs–Henseleit (K–H) medium for 30 min for equilibration, then for 4, 16, 32 or 64 min with K–H medium containing 10^{-7} M K-strophanthoside (^{3}H) and then with K–H medium alone for 8 min to wash the extracellular spaces. The incorporation of the drug into ventricles and atria increased from 4 to 64 min, with the ventricles invariably showing a higher degree of incorporation. The concentrations of K-strophanthoside (^{3}H) also increased with time in microsomes, mitochondria and nuclei, with microsomes in all cases showing the highest specific incorporation. K-Strophanthoside (^{3}H) in the supernatant fraction was not bound to any macromolecular structure.

THE PROBLEM of subcellular distribution of cardiac glycosides has been approached by autoradiography and electron microscopy *inter alia* by Smith and Fozzard,[1] Tubbs *et al.*,[2] Conrad and Baxter[3] and Fozzard and Smith[4] on the dog and frog hearts. These authors have observed most of the tritiated digoxin administered in the sarcomeres of the myofibril, principally overlying the A bands, with smaller amounts in the mitochondria and other structures, including the region of the Z bands.

A quantitative approach to this interesting problem by another technique, namely ultracentrifugation, was attempted by Harvey and Pieper[5] on the isolated guinea-pig heart and by Spratt and Okita[6] on *in situ* rat hearts, both authors using radioactive digitoxin. The most significant development in this field has however been achieved by Dutta *et al.*[7] who proposed a well-standardized method for studying the subcellular distribution of cardiac glycosides in perfused hearts, using the ultracentrifuge to separate subcellular fractions. Using this method, the same authors[8] investigated the



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subcellular distribution of ouabain, dihydro-ouabain, digoxin, digitoxin, convallatoxol and proscillaridin.

This paper reports quantitative data on the subcellular distribution of K-strophanthoside (^{3}H) in isolated perfused guinea-pig hearts, using the method proposed by Dutta *et al.*[7]

K-Strophanthoside is a short-acting polar cardiac glycoside, which is extracted from *Strophanthus kombé* together with smaller amounts of cymarin and K-strophanthin-β. Pharmacokinetic parameters of the drug have recently been obtained by us on guinea-pig[9,10] and human subjects.[11,12]

MATERIALS AND METHODS

Male guinea-pigs weighing 250–300 g were used. The animals were rendered unconscious by a sharp blow on the head and their hearts were quickly removed for perfusion through the aorta using a modified Langendorff technique.[13] The methods of perfusion, homogenization and centrifugation were those described by Dutta *et al.*;[7] they are briefly summarized as follows.

Two perfusion media were used, one being Krebs–Henseleit (K–H) Ringer alone, and the other K–H medium plus 10^{-7} M K-strophanthoside (^{3}H) with a sp. act. of 985 mCi/mM.* The perfusion temperature was 28°C and the flow rate was 3 ml/min. The hearts were perfused initially for 30 min with K–H medium for equilibration, and thereafter with K–H medium + K-strophanthoside (^{3}H) for 4, 16, 32 or 64 min, and then again with K–H medium alone for 8 min to wash out the extracellular spaces. Samples of perfusate were taken during the first 10 min, at the 64th min and during the 8th min of the final washout and counted in a liquid scintillation spectrometer. The concentrations of K-strophanthoside in the perfusate 4 min and 64 min after perfusion did not differ significantly. Concentrations of the drug from 5 to 8 min of the washout did not differ to any statistically significant degree. The low and constant concentration of the drug after 8 min of the washout allows us to assume, in line with the findings of Dutta *et al.*[7,8] obtained with six other cardiac glycosides, that the extracellular space was completely free of K-strophanthoside after this period (Fig. 1). At the end of the washout period a small sample of the left and right atria and another of the left and right ventricles (50–100 mg each) were taken from each heart, and were solubilized and counted in a liquid scintillation spectrometer. The remainder of the heart was weighed, minced and homogenized in 10 volumes of sucrose (0·33 M) EDTA (10^{-3} M) solution at 4°. The homogenate was centrifuged at 4° at 166,000 *g* for 1 hr. The total pellet was resuspended in cold sucrose–EDTA solution and centrifuged at 450 *g* for 10 min to obtain the nuclear fraction, after which the supernatant was centrifuged at 12,000 *g* for 15 min to obtain the mitochondrial fraction. The last supernatant was then centrifuged at 166,000 *g* for 1 hr to obtain the microsomal fraction and the final supernatant. Aliquots of homogenate, initial and final supernatant, and of each resuspended pellet were taken to measure protein content, according to Lowry *et al.*[14] and radioactivity.

A few samples of each particulate fraction were fixed in buffered 2% glutaraldehyde,

* New England Nuclear Corporation. The drug was labelled randomly by catalytic ion exchange and purified by chromatographic processes. Its chemical and radiochemical purity was ascertained by the TCL technique as described previously.[9]

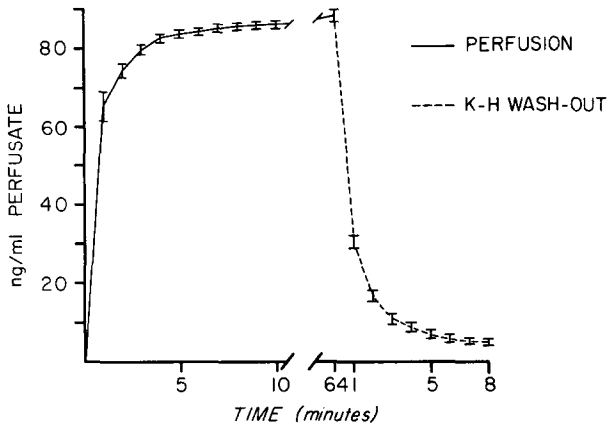

FIG. 1. K-Strophanthoside (^{3}H) concentrations in perfusate of guinea-pig heart during perfusion with K-strophanthoside 10^{-7} M and during washout with Krebs–Henseleit medium alone, at a constant flow of 3 ml/min at 28°. Mean values of twelve determinations ±S.E.

postfixed in osmium tetroxide, dehydrated in a series of alcohols and then embedded in epoxy resin. Thin sections were post-stained with uranyl acetate and lead citrate for electron microscope observation. Only the microsomal fraction was uncontaminated by mitochondria or nuclei (Fig. 2). Both mitochondrial and nuclear fractions were consistently contaminated by each other and cell fragments.

In order to evaluate whether any intracellular distribution of the drug had occurred during the experimental manipulations, K-strophanthoside (^{3}H) was added to each of four homogenates of guinea-pig hearts in sucrose–EDTA in an amount as far as possible the same as in other homogenates. The homogenates were centrifugated at 166,000 *g* for 1 hr to obtain total pellets and supernatant. A further four guinea-pig hearts were homogenated in K–H Ringer after addition of K-strophanthoside (^{3}H). The homogenates were incubated at 28° in a flow of 95% O_2–5% CO_2. After 64 min the homogenates were centrifuged at 166,000 *g* for 1 hr to obtain pellet and supernatant.

The samples for radioactivity measurement were obtained by dissolving tissues, homogenates or pellets in Packard Soluene TM 100 at 45°. The solutions so obtained were added to 15 ml of scintillation fluid prepared by dissolving 5 g of PPO* and 300 mg of dimethyl-POPOP† in 1 l. of toluene. The supernatants (200 μl) were added straight to 15 ml of scintillator. All the samples thus obtained were counted in a Packard Liquid Scintillation Spectrometer Tri-Carb Model 3320 on two counting channels. The efficiency of each count was evaluated from a linear relationship between channel ratio and efficiency previously obtained.[9]

Several checks by TLC on heart perfusate and on supernatant of perfused heart homogenates at no time showed any detectable amounts of metabolized K-strophanthoside (^{3}H). Thus, following Marcus *et al.*,[15] Kuschinsky *et al.*,[16] Lage and Spratt[17] and Dutta *et al.*[7,8] working with digoxin, it was assumed that radioactivity in guinea-pig hearts represents K-strophanthoside alone.

* PPO = 2,5 diphenyloxazole.
† Dimethyl-POPOP = 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene.

RESULTS

Table 1 shows the K-strophanthoside (^{3}H) found in the initial supernatant, total pellet and final supernatant, as a percentage of that present in the homogenate which was assumed to be 100 per cent in the group of heart perfused with the glycoside for 64 min. The percentage recovery was 98·55 per cent.

TABLE 1. RECOVERY OF K-STROPHANTHOSIDE FROM FRACTIONS OF 8 GUINEA-PIG HEARTS PERFUSED FOR 64 MIN WITH 10^{-7} M K-STROPHANTHOSIDE (^{3}H)

	%
Homogenate	100
Supernatant	29·15
Pellet	66·50
Final Supernatant	2·90
Recovery	98·55

The uptake of K-strophanthoside by the ventricles increased with time from 16 ng/g at 4 min to 108 ng/g at 64 min, indicating continuous uptake of the drug by the heart. Concentrations in the atria were however at all times lower than those observed in the ventricles (Fig. 3).

Figure 4 shows the supernatant pellet ratios observed in heart homogenates which decrease quickly and to a statistically significant degree ($P < 0{\cdot}001$) from 4 min (when most of the drug was soluble) to 32 and 64 min when most of the drug was particulate.

Figure 5 shows the specific concentrations of K-strophanthoside (^{3}H) as ng/mg of proteins in the nuclear, mitochondrial and microsomal fractions from 4 to 64 min. In all three fractions the concentration of the drug rose in time, invariably to the highest degree in microsomes and the lowest in mitochondria. At the 64th min the concentration in mitochondria was, on average, 25 per cent and in the nuclei 64 per cent of the microsomal mean value. These results have been expressed in terms of specific concentrations (ng/mg of protein) since the size of the three particulate pellets differed substantially from each other, while the total amount of radioactivity did not differ so much between the three fractions.

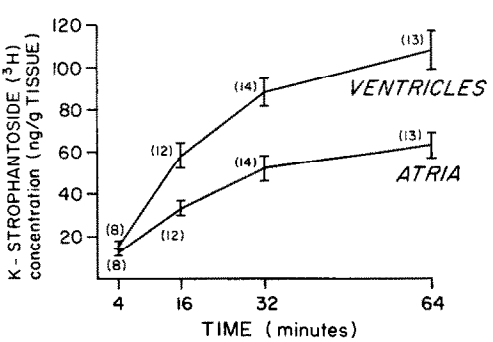

FIG. 3. K-Strophanthoside (^{3}H) concentrations in guinea-pig ventricles and atria after 4, 16, 32 and 64 min of perfusion with the drug. Mean values ± S.E. Number of determinations in parentheses.

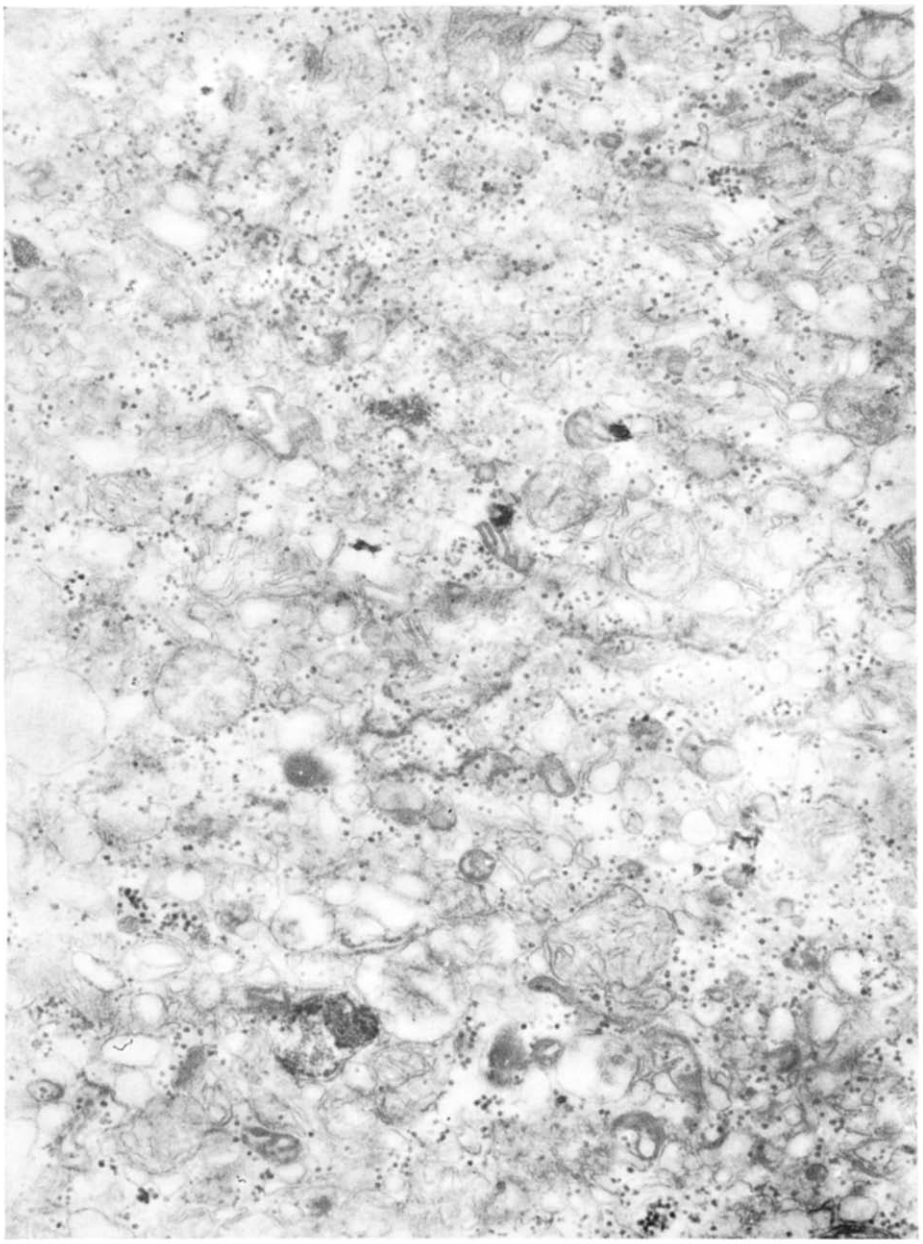

FIG. 2. Electron micrograph of the microsomal fraction of a heart perfused for 64 min. Fragments of the endoplasmic reticulum and ribosomes may be seen together with lesser amounts of mitochondrial fragments. Magnification × 20,600.

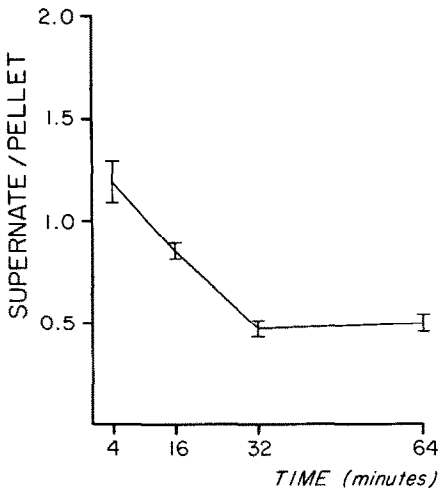

FIG. 4. Supernatant/pellet ratios in homogenates of guinea-pig hearts after 4, 16, 32 and 64 min of perfusion with K-strophanthoside (^{3}H). Heart homogenates were centrifuged at 166,000 g for 1 hr to obtain supernatant and total pellet. Mean values of eight determinations ± S.E.

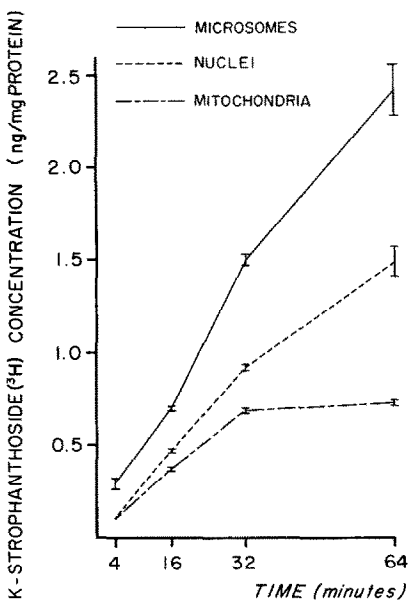

FIG. 5. K-Strophanthoside (^{3}H) specific concentrations (ng/mg of protein) in microsomal, nuclear and mitochondrial fractions of guinea-pig hearts perfused for 4, 16, 32 and 64 min with the drug. Mean values of eight determinations ± S.E.

Table 2 shows the amount of K-strophanthoside (^{3}H) present in the supernatant relative to that in the homogenate (assumed to be 100 per cent), and the supernatant/pellet ratios of the amounts of the drug, in the two control experiments (K-strophanthoside added to unperfused hearts) and in the group of hearts perfused with the glycoside for 64 min, as described in the Methods section. In the control experiments

TABLE 2. K-STROPHANTHOSIDE (^{3}H) IN THE SUPERNATANT OF CONTROL AND PERFUSED GUINEA-PIG HEARTS, AS PERCENTAGES OF THE DRUG PRESENT IN THE HOMOGENATE, ASSUMED TO BE 100%

	No.	Homogenate (%)	Supernatant (%)	Supernatant/ pellet ratio
Hearts homogenated in 0·33 M sucrose and 10^{-3} M EDTA (10 volumes) plus-K-strophanthoside (^{3}H) centrifuged immediately	4	100·0	96·00 ± 1·08	29·50 ± 7·58
Hearts homogenated in K–H Ringer (10 volumes), plus K-strophanthoside (^{3}H), incubated 64 min at 28° in O_2–CO_2 flow, and then centrifuged	4	100·0	93·75 ± 0·25	15·25 ± 0·75
Hearts perfused with K-strophanthoside (^{3}H) for 64 min, and treated as described in the text	8	100·0	33·04 ± 1·57	0·50 ± 0·04

Results are expressed as mean ± S.E.

the drug remained almost entirely in the supernatant, whereas in the perfusion experiments most of the drug was in the pellet.

The soluble fraction of a guinea-pig heart perfused for 64 min with K-strophanthoside (^{3}H) was chromatographed on a Sephadex G-25 column using a 0·9% NaCl solution as eluent. 150 ml of eluate were collected in fractions of 5 ml each. Transmittance at 280 nm and radioactivity contents of each fraction were measured. The same chromatographic process was repeated with K-strophanthoside (^{3}H) in 0·9% NaCl. The proteins of the supernatant were eluted with 35 to 65 ml, peaking at 50 ml. The K-strophanthoside (^{3}H) of the supernate as well as that in saline solution was eluted from the column with 75–115 ml, attaining a peak at 95 ml (Fig. 6).

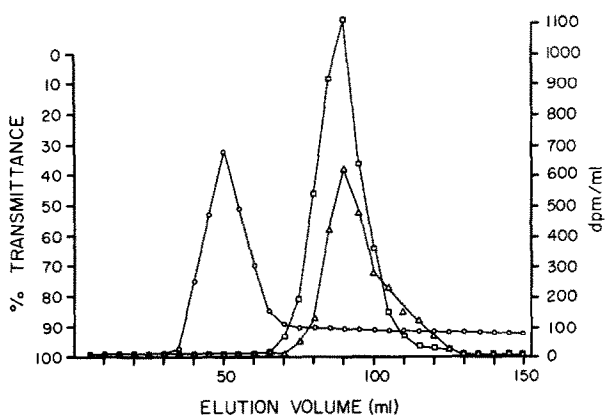

FIG. 6. Elution diagram of K-strophanthoside (dpm/ml) and protein (% transmittance at 280 mμ) obtained on a 2·5 by 30 cm Sephadex G-25 column, using 0·9% NaCl as eluent. □——□, K-strophanthoside (^{3}H) concentrations of the eluate when the cardiac glycoside was placed in the column in NaCl solution. △——△, K-strophanthoside (^{3}H) concentrations in the column eluate when the supernatant of the perfused guinea-pig heart was placed in the column. ○——○, protein content of the eluate when the supernatant of the guinea-pig heart perfused with K-strophanthoside (^{3}H) was placed in the column.

DISCUSSION

In guinea-pig hearts perfused 64 min with ouabain Dutta *et al.*[8] found a ventricle concentration of about 105 μg/g (180 pmoles/g) which is near the value of 108 ng/g (124 pmoles/g) observed by us with K-strophanthoside; dihydroouabain showed a very low value, while the other four glycosides studied by Dutta showed higher values. K-Strophanthoside and Dutta's six other glycosides all showed higher concentrations in the ventricles than in the atria. All the glycosides showed considerable variations in uptake by the heart tissue. In the opinion of Dutta this might be due to differing specific affinities of each glycoside with Na^+, K^+-ATPase, which seems to be a releasing factor for the active transport of cardiac glycosides across the cell membrane. In effect, the active transport of cardiac glycosides is the rate-limiting step in the accumulation and binding of the glycosides in the heart. Specific binding of cardiac glycosides assuming the integrity of the cell's metabolism has also been described by Godfraind and Lesne.[19] These authors did however encounter an unsaturable uptake as well.

The supernatant/pellet ratio of 0·5 found in homogenates of hearts perfused for 64 min with K-strophanthoside is near the 0·61 found by Dutta for digoxin, whereas proscillaridin, ouabain and dihydroouabain showed lower values and digitoxin and convallotoxol higher values. In any event, this value was lower than 1 in all 7 glycosides. K-Strophanthoside in the supernatant of perfused hearts was not bound to any macromolecular structure (Fig. 6). This is in line with the observation of Dutta *et al.*[7] and Kim *et al.*[18] on digoxin.

K-Strophanthoside shows a specific concentration in the microsomal fraction of 2·42 ng/mg of protein (2·64 pmoles/mg protein). This is an intermediate value between 2·20 pmoles/mg protein for ouabain and 2·97 for digitoxin. Dihydroouabain, digoxin and convallatoxol all showed lower values, whereas proscillaridin showed the highest value. All 7 cardiac glycosides show their highest specific concentration in the microsome fraction, the mitochondrial and nuclear fractions being lower. This leads to the conclusion that the receptor (or receptors) of the cardiac glycosides must lie on this light fraction, and in particular on the sarcoplasmatic reticulum as shown by Dutta *et al.*[8] An excellent demonstration of this hypothesis has recently been made by Kim *et al.*,[18] who have shown a significant correlation between the positive inotropic effect and the contents of tritiated digoxin in a crude microsomal fraction of electrically-driven, isolated and perfused guinea-pig hearts.

Our data on K-strophanthoside show that when the cell membrane was disrupted the glycoside did not accumulate in the pellet in agreement with the findings of Dutta *et al.*[8] with digoxin. This leads to the conclusion that the distribution of K-strophanthoside cannot be accounted for by a simple physico-chemical exchange or redistribution during or after homogenization, but seems to arise from a specific uptake process. This confirms the view that the cell membrane is involved in this specific uptake process of active cardiac glycosides. The fact that K-strophanthoside accumulates in the microsomal fraction suggests that the "receptors" or "binding sites" of active cardiac glycosides may be present in this light fraction, in good agreement with the opinion of both Dutta *et al.*[8] and Kim *et al.*[18]

Acknowledgements—We are most grateful to Dr. G. Tettamanti of the Institute of Biochemistry at Milan University for his help in centrifuging and subfractionating, and to Mr. G. Meroni and Mr. G. Mascheroni for their assistance in the laboratory throughout the experiments.

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