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SUBCELLULAR DISTRIBUTION OF DESLANATOSIDE C-^{3}H IN ISOLATED GUINEA PIG HEARTS. INTERACTION OF OTHER DRUGS

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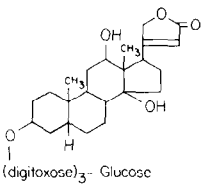
Abstract—Guinea-pig hearts were perfused with Krebs–Henseleit (K–H) medium 30 min for equilibration, then 4, 16, 32 or 64 min with Deslanatoside C-^{3}H 10^{-7} M in K–H medium and then again with K–H medium alone to wash extracellular spaces at a constant flow of 3 ml/min at 28° C. Deslanatoside C incorporated into the ventricles and atria increased from 4 to 64 min, the ventricles invariably showing a higher degree of incorporation. The concentration of Deslanatoside C also increased from 4 to 64 min in microsome, mitochondrial and nuclear fractions, the microsome fraction invariably showing the highest specific concentration. Glycoside in the supernatant fraction was not tightly bound to any macromolecular structure. A β-adrenergic blocking agent SD 1601 10^{-7} M, and Creatinol *O*-phosphate 10^{-5} M both increased myocardial uptake of Deslanatoside C-^{3}H and its incorporation into the pellet fractions. Daunomycin 10^{-5} M increased the myocardial uptake of the labelled glycoside and its concentration in both supernatant and pellet fractions. Neither phenobarbital sodium 10^{-6} M nor Ouabain 10^{-7} M affected uptake or the subcellular distribution of Deslanatoside C. Ouabain 10^{-6} M decreased both uptake by the heart and the subcellular concentrations of Deslanatoside C.

Deslanatoside C is the cardiac glycoside obtained from desacetylation of Lanatoside C, which is extracted from Digitalis lanata. It is used in the treatment of heart failure [1].

Deslanatoside C, MW=943



(digitoxose)$_3$– Glucose

This paper reports the subcellular distribution of this glycoside in the isolated, perfused guinea-pig heart and modifications of subcellular distribution in the presence of 5 other drugs which show activity on the myocardium, some of which are frequently administered combined with digitalis. Their main pharmacological properties are as follows: Ouabain is a well-known short-acting polar cardioactive glycoside which is taken up by the guinea pig heart partly via an active (saturable) transport mechanism. Ouabain inhibits the active myocardial uptake of other cardiac glycosides [2–5]. Phenobarbital sodium is a well-known barbiturate derivative. Like the similar drug Adriamycin, Daunomycin is an anticancer agent [6] which has marked cardiac toxicity. Both these drugs *inter alia* cause cardiac arrhythmias, markedly decrease myocardial sensitivity to digitalis and possess a negative inotropic effect. Therapy with both drugs is normally associated with digitalis treatment [7, 8].

SD 1601 is a new β-blocking agent [9] which has proved to antagonize ouabain-induced arrhythmias in the dog [10]. SD 1601, propranolol and generally all β-blocking agents are used in treating digitalis intoxication [11].

Creatinol *O*-phosphate (COP) [12] decreases the toxic effects of digitalis in human subjects, such as nausea, vomiting, arrhythmias and S-T ECG changes [13, 14]. An antagonizing effect of COP on ouabain-induced dose-dependent arrhythmias was observed by Musso *et al.* [15] on the isolated guinea-pig ventricles. An antagonizing effect of COP on aconitine-induced arrhythmias has also been observed by Ferrini and Miragoli (unpublished data) in guinea pig atria. In addition, COP had proved able to increase contractility in both normal and experimentally damaged rat and rabbit hearts [16]. COP also showed positive dose-dependent inotropic effect similar to Ouabain on the isolated frog heart [17].

MATERIALS AND METHODS

Male guinea-pigs (250–300 g), were stunned by a sharp blow on the head and their hearts were rapidly removed for perfusion through the aorta, using the Langendorff technique. The methods of perfusion, homogenization and centrifugation were those described by Dutta *et al.* [2], slightly modified as regards centrifugation procedures. They are briefly summarized as follows. Two perfusion media were used, one being the modified Krebs–Henseleit Ringer (I) [18] and the other K–H medium plus Deslanatoside C-^{3}H (II) 10^{-7} M with a specific activity of 1000 mCi/mM*. Both media were preoxygenated with a flow of 95% O_2–5%

* New England Nuclear Corporation, 575 Albany Street, Boston, Massachesetts 02118-USA. The drug was labelled randomly by catalytic ion exchange and purified by chromatographic processes. Its chemical and radiochemical purity was ascertained by the t.l.c. technique.

CO_2. The perfusion temperature was 28°C and flow rate 3 ml/min. The hearts were perfused initially with K–H medium (I) for equilibration, and thereafter with Medium II for 4, 16, 32 or 64 min, and then again with Medium I for 8 min to wash out the labelled glycoside from the extracellular spaces. Concentrations of the glycoside in the perfusate did not significantly differ from the 4th to the 64th min.

Concentrations of the glycoside in the perfusate 5–8 min after the washout were very low and constant, and again did not differ to any statistically significant degree. Thus, according to Dutta *et al.* [2, 3] and Marzo and Ghirardi [19] who worked with other cardiac glycosides, it was assumed that the extracellular space was completely free of Deslanatoside C-^{3}H after 8 min of washout.

A second series of experiments was carried out in order to obtain the subcellular distribution of Deslanatoside C-^{3}H in the presence of the other drugs listed in the previous section. In this case, both K–H Mediums I and II contained Ouabain 10^{-7} M, or Ouabain 10^{-6} M, or Phenobarbital sodium 10^{-6} M, or Daunomycin 10^{-5} M, or SD 1601 10^{-7} M, or Creatinol *O*-phosphate 10^{-5} M. The concentration of each drug was selected at values between 10^{-5} and 10^{-7} M based on a comparison of activity levels and doses in previous *in vivo* and *in vitro* experiments. Perfusion with the labelled glycoside was in all cases carried out for a period of 64 min. The other conditions were the same as those described above. The hearts were not driven electrically. The mean heart rate observed after the pretreatment with Ringer I was 123 ± 5 (SE) beats/min while after pretreatment with the other five drugs it was: Ouabain 10^{-7} M 109 ± 13, Ouabain 10^{-6} M 86 ± 9, Phenobarbital sodium 10^{-6} M 63 ± 18, Daunomycin 10^{-5} M 132 ± 10, SD/1601 10^{-7} M 117 ± 10, and Creatinol *O*-phosphate 10^{-5} M 110 ± 5.

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) were taken from each heart, solubilized and counted in the liquid scintillation spectrometer. The remaining portions of the hearts were pooled in groups of 2 hearts each, weighed, minced and homogenized in 9 vols of sucrose 0·32 M, EDTA(Ethylenediamino tetraacetic acid) 10^{-6} M, $MgSO_4$ 10^{-6} M and Tris (Trihydroxymethylaminomethane) $5{\cdot}10^{-5}$ M buffered at pH 7·2 (III) according to De Robertis *et al.* [20]. The homogenate was filtered through a sheet of surgical gauze. The filtered homogenate was centrifuged for 10 min at 900 *g*. The nuclear pellet obtained was resuspended in Medium III and recentrifuged for 10 min at 900 *g*. The latter washing operation was then repeated. The pooled supernates were centrifuged for 20 min at 12,000 *g*. The mitochondrial pellet was again twice resuspended in Medium III and recentrifuged for 20 min at 12,000 *g*. The pooled supernates were centrifuged for 1 hr at 166,000 *g*. The microsome pellet was resuspended in III and recentrifuged for 1 hr at 166,000 *g*. Aliquots of the filtered homogenate, the final supernate of 166,000 *g* (supernate) and each of the three resuspended pellets (nuclear, mitochondrial, and microsomal) were taken to evaluate radioactivity content, as described previously, and protein content according to Lowry *et al.* [21].

A few samples of each particulate fraction were fixed in buffered 2% gluteraldehyde, postfixed in osmium tetroxide, dehydrated in a series of alcohols and then embedded in epoxy resin. Thin sections were poststained with uranyl acetate and lead citrate for electron microscope observation. Fragments of the endoplasmatic reticulum and ribosomes were seen in the microsome fraction together with marginally lesser amounts of mitochondrial fragments. On the other hand, both the mitochondrial and nuclear fractions were contaminated by each other and cell fragments. Similar findings have been obtained previously by Dutta *et al.* [2] and Marzo *et al.* [19].

Two kinds of experiment were carried out in order to rule out the possibility of any intracellular redistribution of Deslanatoside C occurring during experimental manipulation. In the first type, the glycoside was added to each of 4 homogenates of guinea-pig hearts in Medium III in an amount nearly the same as that found in the homogenates of perfused hearts. The homogenates were centrifuged at 166,000 *g* for 1 hr to obtain the supernate and the total pellets. In the second experiment a further 4 guinea-pig hearts were homogenated in K–H Medium after addition of the labelled glycoside. The homogenates were incubated at 28°C 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 supernate and total pellet fractions.

Samples for radioactivity counts were obtained and counted as described previously [19]. Several checks by t.l.c. on heart perfusate and on supernate of perfused hearts showed no detectable amounts of metabolites of Deslanatoside C-^{3}H. Thus, following previous findings of other authors [2, 19, 22–25] who worked with other glycosides, it was assumed that radioactivity in guinea pig hearts represents Deslanatoside C alone. The mean recovery of radioactivity calculated in all the experiments (44 cases) was 103 ± 2% (SE); the mean recovery of the protein in the same 44 cases was 92 ± 1% (SE). For this evaluation, radioactivity and the protein contents in the filtered homogenate were assumed to be 100%.

RESULTS

Concentrations of Deslanatoside C-^{3}H in atria and ventricles of guinea-pig hearts perfused with the labelled glycoside rose in time, more rapidly from 4 to 32 min, thereafter slowing down from 32 to 64 min, indicating continuous uptake of the glycoside by the heart (Fig. 1). Concentrations of Deslanatoside C-^{3}H in the ventricles were higher than those in the atria at all experimental times, in the atria being about 60% of those in the ventricles at the 64th min. Specific concentrations of Deslanatoside C-^{3}H (ng/mg protein) in the three particulate fractions rose with perfusion time from 4 to 64 min, the microsomal concentration being at all times higher than in the nuclear or mitochondrial fractions, and showing the highest rate of increase (Fig. 2).

When the hearts were perfused for 64 min with Deslanatoside C-^{3}H in the presence of the other drugs, myocardial uptake of the labelled glycoside was not affected by Phenobarbital sodium 10^{-6} M

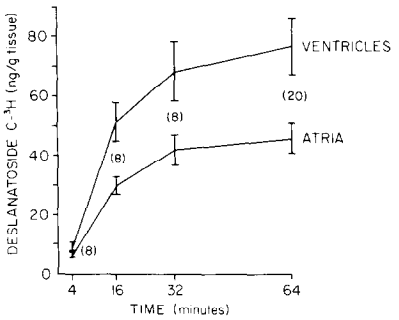


Fig. 1. Concentrations of Deslanatoside C-^{3}H in ventricles and atria of guinea-pig hearts perfused with the glycoside 10^{-7} M for 4, 16, 32 and 64 min. The number of findings is reported in parentheses. Mean values ± standard error.

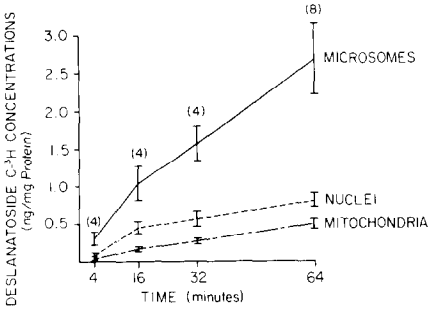


Fig. 2. Specific concentrations of Deslanatoside C-^{3}H (ng/mg of protein) in microsomal, nuclear and mitochondrial fractions of guinea-pig hearts perfused 4, 16, 32 and 64 min with the labelled glycoside 10^{-7} M. The number of findings is reported in parentheses. Mean values ± standard error.

or by Ouabain 10^{-7} M, was decreased in both ventricles and atria by Ouabain 10^{-6} ($P < 0.05$), and was increased by Daunomycin 10^{-5} M ($P < 0.001$), SD/1601 10^{-7} M ($P < 0.001$) and Creatinol *O*-phospate 10^{-5} M ($P < 0.01$) (Fig. 3). A comparison of the concentrations of Deslanatoside C-^{3}H in the supernate and in the total pellet, shows that the increase in myocardial uptake of the glycoside caused by SD/1601 and Creatinol *O*-phosphate was significant only in the pellet fraction, while the increase in the same parameter brought about by Daunomycin and the decrease brought about by Ouabain 10^{-6} M were significant in both the supernate and the pellet fractions (Fig. 4). These variations in Deslanatoside C-^{3}H concentration in the pellet fraction caused by SD/1601 10^{-7} M, Daunomycin 10^{-5} M, Creatinol *O*-phosphate 10^{-5} M, and Ouabain 10^{-6} M were also observed in each of the three particulate fractions, being more evident and significant in the microsome fraction (Fig. 5).

When the guinea-pig hearts were not perfused, Deslanatoside C-^{3}H was almost entirely in the supernate (94–96%) with a S/P ratio varying between 16 and 24. When the hearts were perfused, the labelled glycoside in the supernate was only 29% with a S/P ratio of 0·43.

The supernate of a pool of two guinea-pig hearts perfused for 64 min with Deslanatoside C-^{3}H was chromatographed on a Sephadex G25 column (2·5 × 30 cm) using NaCl 0·9% as the eluent. 200 ml of eluate were collected in fractions of 5 ml each. Transmittance at 280 mμ and the radioactivity contents of each fraction were measured. The same chromatographic process was repeated with Deslanatoside

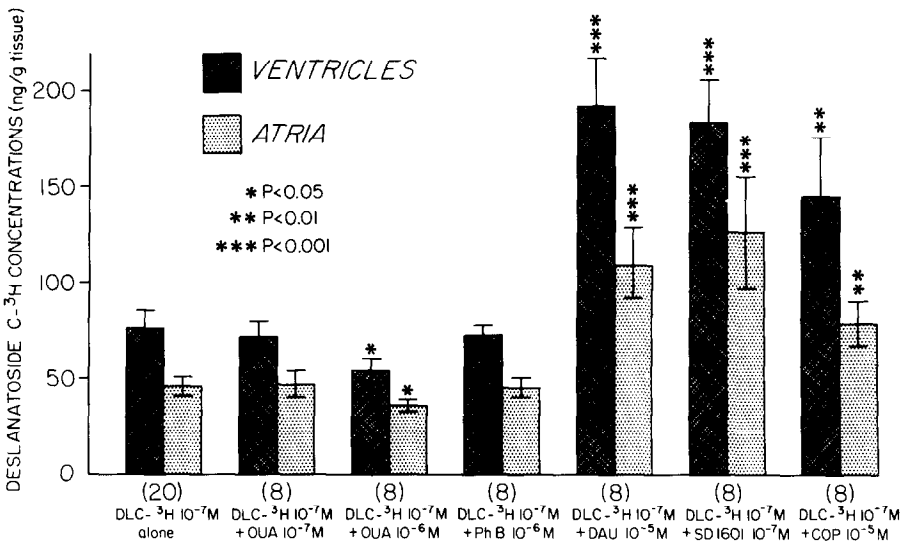


Fig. 3. Concentrations of Deslanatoside C-^{3}H in ventricles and atria of guinea-pig hearts perfused for 64 min with the labelled glycoside 10^{-7} M alone and in the presence of the other drugs. Mean values ± standard error. Number of findings in brackets Ps for statistical comparison were obtained with the Student test.

DLC = Deslanatoside C
OUA = Ouabain
PhB = Phenobarbital sodium
DAU = Daunomycin
COP = Creatinol *O*-phosphate

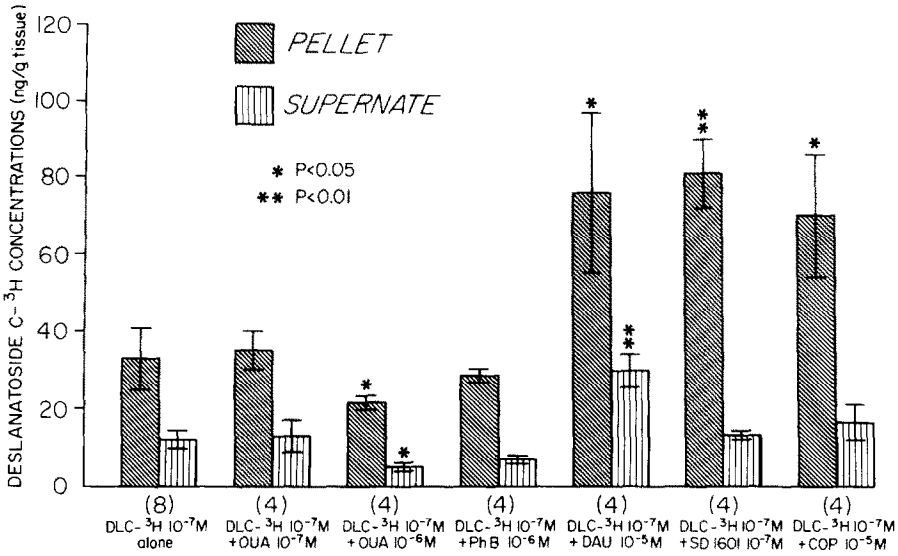


Fig. 4. Concentrations of Deslanatoside C-^{3}H in the pellet and supernate of guinea-pig hearts perfused for 64 min with the labelled glycosides 10^{-7} M alone and in the presence of other drugs. Mean values ± standard error. Number of pools tested in brackets. Each pool consists of two hearts. Ps for statistical comparison were obtained with the Student test. Legend as in Fig. 5.

C-^{3}H in NaCl 0·9% solution. The proteins of supernate came off the column from 40 to 60 ml, peaking at 50 ml, while the Deslanatoside C-^{3}H of both supernate and saline solution came off the column from 55 to 95 ml, peaking at 70 ml, thus indicating that Deslanatoside C in the supernate (166,000 *g*) of guinea-pig hearts was not bound, anyway tightly, to the proteins.

DISCUSSION

This data on subcellular distribution of Deslanatoside C may be compared with those of Marzo and Ghirardi relating to K-strophanthoside [19] and those of Dutta *et al.* [3] on ouabain, dihydroouabain (which is inactive), digoxin, digitoxin, convallatoxol and proscillaridin, all these 8 cardiac glycosides having been investigated with Dutta's method [2].

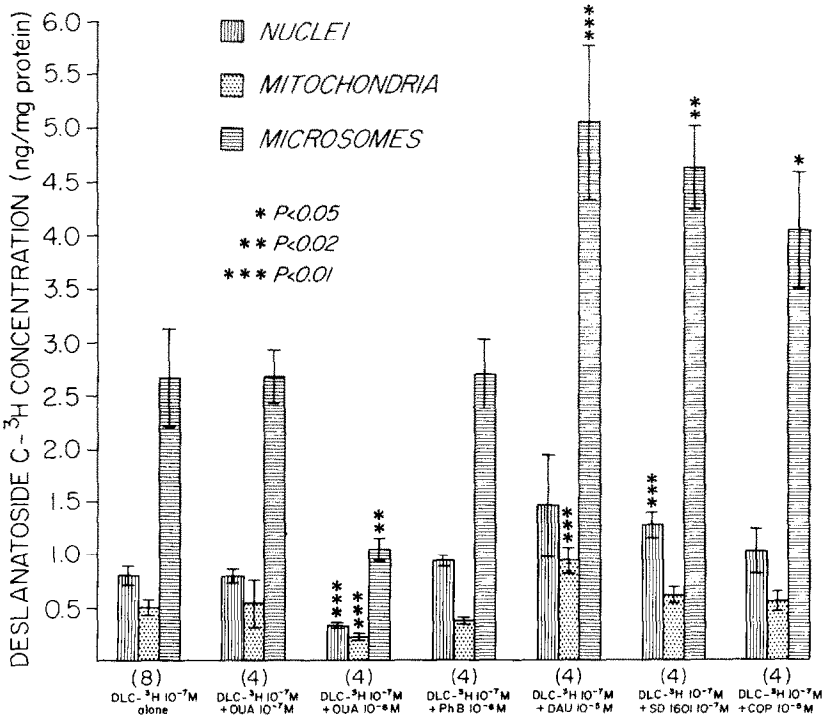


Fig. 5. Specific concentrations of Deslanatoside C-^{3}H in guinea-pig hearts perfused 64 min with the labelled glycoside 10^{-7} M alone and in the presence of the other drugs. Mean values ± standard error. Number of pools tested in brackets. Each pool consists of two hearts. Ps for statistical comparison were obtained with the Student test. Legend as in Fig. 5.

In guinea-pig hearts perfused for 64 min with Deslanatoside C-^{3}H the ventricle concentration of the drug i.e., 77 ng/g (82 pmol/g), is in the range of the other 7 glycosides which showed the following pmol/g values respectively: dihydroouabain 10, K-strophanthoside 134, ouabain and digoxin about 150–200 and values between 700 and 1000 in the case of convallatoxol, digitoxin and proscillaridin. This parameter does in fact show a marked variation, from 10 to 1000 pmol/g. The tissue/medium ratio was about 0·1 with dihydroouabain, about 1 with Deslanatoside C and K-strophanthoside, 1·5–2 with ouabain and digoxin, and higher than 7 with digitoxin, proscillaridin and convallatoxol. All 8 cardiac glycosides invariably showed higher concentrations in the ventricles than in the atria. This difference does not depend on the technique used in perfusing the hearts, since concentrations in the ventricle higher than in the atria were also observed by Dutta and Marks [26] in the hearts of rats and guinea-pigs treated i.v. with ouabain and digoxin.

The S/P ratio of 0·43 observed in guinea pig hearts perfused for 64 min with Deslanatoside C is in the range of the other 7 glycosides, which showed the following values respectively: proscillaridin 0·17, ouabain 0·22, dihydroouabain 0·43, K-strophanthoside 0·50, digoxin 0·61, digitoxin 0·73 and convallatoxol 0·81. In any event, this value was lower than 1 with all 8 glycosides. Deslanatoside C in the supernate of the guinea-pig hearts perfused 64 min with the glycoside was not bound, anyway tightly, to any macromolecular structure, in line with the observations of Dutta *et al.* [2], Kim *et al.* [25], who worked with digoxin, and Marzo and Ghirardi [19], working with K-strophanthoside. The microsome concentration of Deslanatoside C-^{3}H after 64 min of perfusion, i.e. 2·67 ng/mg protein (2·84 pmol/mg protein), is in the range of the other 7 glycosides, which showed the following values in pmol/mg protein respectively; dihydroouabain 0·11, digoxin and convallatoxol 1·70, ouabain 2·20, K-strophanthoside 2·64, digitoxin 2·97 and proscillaridin 13·50. In any event, in all the 8 cardiac glycosides, microsome concentration was the highest, mitochondrial and nuclear concentrations being lower. In the case of Deslanatoside C, nuclear and mitochondrial specific concentrations were 19 and 30% respectively as compared with the microsomal concentration, assumed to be 100%. In the case of the other 7 cardiac glycosides, a similar evaluation leads to specific concentrations in nuclear and mitochondrial fractions varying from 30 to 60% as compared with the concentration in the microsomal fraction. We thus feel that filtration of the homogenate and recentrifugation of each pellet fraction introduced by us in the case of Deslanatoside C may possibly have lessened the degree of contamination of the nuclear and mitochondrial fractions by the microsomal fraction and consequently may have caused some enrichment of the glycoside concentration in the microsomal fraction. Of all the 8 glycosides considered, only dihydroouabain is almost entirely inactive on myocardial contractility. Myocardial uptake and microsomal specific concentrations seem to reflect the inactivity of dihydroouabain rather than its S/P ratio. In effect the S/P ratio of dihydrooabain (0·43) is virtually the same as that of Deslanatoside C (0·43) and nearly the same as that of K-strophanthoside (0·50) whereas the ventricle uptake of these two active glycosides was around 10 times higher and their microsomal specific concentration 26 times higher than the values observed with dihydroouabain.

Reciprocal inhibition in myocardial uptake of ouabain and digitoxin has been observed by Godfraind and Lesne [5] in isolated guinea-pig atria. These authors observed two different transport mechanisms of ouabain and digitoxin, one being passive (or diffusional). Its contribution to total transport becomes greater than the other (active or saturable transport) at a concentration of ouabain higher than 10^{-6} M. These authors observed only a passive non-saturable transport mechanism in dihydroouabain. Both a saturable and a non-saturable mechanism for digoxin uptake by isolated guinea-pig atria were also encountered by Kuchinsky *et al.* [23]. A reduction in cardiac uptake of ouabain-^{3}H in the presence of digitoxin was also observed by Dutta and Marks [4] in the isolated perfused guinea-pig heart, confirming the presence of a saturable component in the uptake of ouabain by the heart. Moreover, the latter authors observed an increment of this inhibitory effect when the ratio of digitoxin to ouabain in the perfusion medium increased. The reduction in myocardial uptake of Deslanatoside C by ouabain obtained by us when the ratio of ouabain to Deslanatoside C in the perfusion medium (mole/l) was increased from $10^{-7}/10^{-7}$ to $10^{-6}/10^{-7}$ appears to be in agreement with previous data [4, 5, 23] and also indicates the presence of an active (saturable) component in the uptake of Deslanatoside C by the heart. SD/1601, Creatinol *O*-phosphate and Daunomycin all increase the myocardial uptake of Deslanatoside C. SD/1601 and Creatinol *O*-phosphate, however, increase glycoside concentration only in the pellet fractions, whereas Daunomycin increases both pellet and supernate concentrations of the glycoside.

The fact that drugs such as SD/1601, Creatinol *O*-phosphate and Daunomycin, which decrease the myocardial toxicity of digitalis glycoside, can cause a higher degree of incorporation of digitalis glycosides, at any rate Deslanatoside C, is an interesting observation which may at first sight appear to be a contradiction.

Our data do not clarify the mechanism involved in the higher cardiac uptake of Deslanatoside C brought about by SD/1601, Creatinol *O*-phosphate and Daunomycin. They do however indicate that: a) drugs which decrease the myocardial toxicity of digitalis glycosides can cause a higher degree of incorporation of digitalis glycosides, at any rate Deslanatoside C; b) while the myocardial uptake of cardiac glycosides may be inhibited by other cardiac glycosides, drugs also exist which increase the myocardial uptake of cardiac glycosides.

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