Biochemical Pharmacology, Vol. 20, pp. 437–446. Pergamon Press, 1971. Printed in Great Britain

METABOLISM *IN VITRO* OF DIGITOXIGENIN BY RAT LIVER HOMOGENATES

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(*Received* 22 *January* 1970; *accepted* 29 *May* 1970)

Abstract—Digitoxigenin is metabolized *in vitro* by rat liver homogenates and subcellular fractions. No metabolism by cardiac tissue homogenates was observed, with only limited conversion by kidney tissue homogenates. The primary product is 3-epidigitoxigenin, with smaller amounts of 3-dehydrodigitoxigenin and more polar metabolites being detected. The microsomal hydroxylation system can be induced by treatment of the animals with phenobarbital.

THE GENERAL pharmacology of digitalis and other cardiac glycosides has been reviewed in recent years by Modell,[1] Moe and Farah,[2] and Doherty.[3] The uptake, binding and exchange of various isotopically labeled cardiac glycosides by cardiac tissues[4–6] and plasma proteins[6] have been investigated, as has the distribution of radioactively labeled cardiac glycosides between various organs in a number of animal species.[5] The presence of digitalis receptor in cardiac microsomes has been proposed.[7]

The uptake and release of [^{3}H]-digitoxigenin by isolated electrically driven guinea-pig atria follow kinetics which are very similar to those of [^{3}H]-digitoxin. However, the discrepancy between kinetic and pharmacologic behavior is much larger for digitoxigenin than for digitoxin.[8]

The mechanism of action of cardiac glycosides at both the cellular and subcellular levels is not yet understood. West and Toda[9] have reviewed much of the present knowledge. Recently, several groups of investigators have implicated particulate fractions as the site of digitalis action. Matsui and Schwartz[10] have shown that a particulate ($Na^+ + K^+$)-activated ATPase is inhibited by digoxin, and that the binding of [^{3}H]-digoxin to this fraction is ATP dependent, being increased by Na^+ and decreased by K^+. Klaus and Lee[11] have found that cardiac glycosides increased the amount of free releasable Ca^{2+} from the sarcoplasmic reticular fraction of heart muscle, and that the effect was dose dependent.

The metabolic half-times and excretion rates of labelled cardiac glycosides have been shown to be related to their pharmacologic activity.[3] Digitoxin and digoxin and their metabolites are excreted primarily in the urine. The major metabolites of digitoxin are digitoxigenin and digoxin. Also found in lesser amounts are digoxigenin and the mono- and bis-digitoxosides of digitoxigenin and digoxigenin. The therapeutic activity of digitoxin may reside in its metabolites.[3] The majority of digoxin is excreted unchanged, with small amounts of digoxigenin and its mono- and di-digitoxoside, as well as 3-epidigoxigenin.[3] The role of the liver in the degradation of tritiated digoxin has previously been recognized.[12] Rat liver slices but not liver homogenates will metabolize [^{3}H]-digoxin, yielding essentially the same products as found excreted in the urine from administration *in vivo*.[13]

Since it has been suggested that the metabolites of cardiac glycosides may be responsible for the therapeutic activity,[3] we have undertaken a study *in vitro* of the metabolism of digitoxigenin by various rat tissues.

EXPERIMENTAL

Animals. Male weanling rats (Sasco, Inc., Omaha, Nebr.) were fed Purina Laboratory Chow and tap water *ad lib.* They were used experimentally after attaining a weight of approximately 300 g (12–14 weeks). All animals were fasted overnight prior to use.

Isotopes. Digitoxin-^{3}H (G) was obtained from New England Nuclear, Boston, Mass., and had a specific activity of 1 mc/μM. Digitoxigenin-^{3}H was prepared from the [^{3}H]-digitoxin. A 70% ethanol solution of [^{3}H]-digitoxin was refluxed with an equal volume of 0·20 N H_2SO_4 for 25 min. The solution was chilled in an ice-bath and extracted four times with an equal volume of methylene dichloride (CH_2Cl_2) using a separatory funnel. The pooled extracts were evaporated *in vacuo*. Less than 0·5 per cent of the radioactivity remained in the aqueous phase. The residue from the dried organic extract was dissolved in methylene dichloride–methanol (3:2), and chromatographed several times on silica gel H thin-layer plates (20 × 20 cm, 0·25 mm thick, E. Merck AG, Darmstadt), developing with methylene dichloride–methanol (9:1). In this system, digitoxin has an R_f of approximately 0·50 while the R_f of digitoxigenin is approximately 0·70. The zones corresponding to digitoxigenin were removed and were extracted for 8 hr in a Soxhlet apparatus using methylene dichloride. The extract was passed through a Millipore filter to remove small amounts of silica gel, evaporated to dryness *in vacuo*, and redissolved in methylene dichloride–methanol (3:2). Thin-layer chromatography on silica gel H plates using solvent systems of methylene dichloride–methanol (9:1) and chloroform–isopropanol (9:1) revealed that greater than 98 per cent of the radioactivity resided in the digitoxigenin.

3-*Dehydrodigitoxigenin* (*digitoxigenone*) *synthesis.* Digitoxigenin (100 mg) was dissolved in 15 ml of acetone, oxidized with 0·25 ml of Jones' reagent for 5·0 min at 0°, and the extraction carried out as described by El Masry *et al.*[14] The product was recrystallized from 70% ethanol. Its m.p. of 200–203° corresponded to that previously reported.[14,15] Its R_f values upon thin-layer chromatography (TLC) (see Table 1) agreed with the authentic material in all cases and complete oxidation of digitoxigenin was observed.

3-*Epidigitoxigenin synthesis.* The digitoxigenone was incubated with lithium-tri-*tert*-butoxy-aluminium hydride for 1 hr at 0° according to the method of Tamm.[16] The product was isolated as described[16] and further purified by recrystallization from 40% ethanol, giving 89 mg of fine needle-like crystals. The m.p. of 283–287° agreed with that previously reported,[16] and the R_f values were identical to those of authentic material upon TLC (see Table 1).

Phenobarbital-treated rats. To determine whether phenobarbital could induce enzymes capable of metabolizing [^{3}H]-digitoxigenin since no such reports could be found in the literature, rats weighing about 300 g were daily given phenobarbital sodium intraperitoneally (i.p.) in normal saline for 5 days at a dose of 100 mg/kg. After the last administration, the animals were fasted overnight and used experimentally. Control animals received only normal saline. Two levels of specific activity

of [^{3}H]-digitoxigenin were used as the substrate, one of 1 mc/μM ($3{\cdot}75 \times 10^{-4}$ mg/μc) and the other of 2·54 mc/mM (0·95 mg/μc). In each case 1 μc of radioactivity was added, providing two substrate concentrations.

Digitoxin-treated rats. To investigate the influence of high levels of administered digitoxin on the metabolism of [^{3}H]-digitoxigenin by isolated tissues, rats were given a solution of digitoxin (5 mg/ml) i.p. in 70% ethanol–propylene glycol (9:1). Each animal received 5 mg/kg on day one, 1 mg/kg on day three, and 1·75 mg/kg on days 4–7. The animals were fasted overnight and used experimentally. Control animals received an equivalent volume of the 70% ethanol–propylene glycol (9:1). Two substrate concentrations were employed as described above.

TABLE 1. THIN-LAYER CHROMATOGRAPHY*

	Color with 50% H_2SO_4 spray	Solvent system		
		CH_2Cl_2–CH_3OH (85:15)	$CHCl_3$–isopropanol (9:1)	CH_2Cl_2–CH_3OH–H_2O (84:15:1)
			R_f	
Digitoxigenin	Blue-green	0·81	0·39	0·75
3-Epidigitoxigenin	Blue-gray	0·78	0·32	0·72
3-Dehydrodigitoxigenin	Tan	0.90	0·55	0·84
Gitoxigenin	Purple	0·69	0·16	0·61
Digoxigenin	Yellow	0·60	0·12	0·53

* Silica gel H plates, to which the above standards were applied, were developed in the solvents listed in the table. The air-dried plates were sprayed with 50% H_2SO_4 and heated for 10–15 min at 100°.

Enzyme specificity. We desired to obtain information on the specificity of the enzyme systems involved in the metabolism of digitoxigenin. Each reaction flask contained 1 μc [^{3}H]-digitoxigenin with a specific activity of approximately 1 mc/μM ($3{\cdot}75 \times 10^{-4}$ mg/μc). To each was added 0·95 mg of nonradioactive digitoxigenin (an effective radioactivity dilution of 2530), digoxigenin, gitoxigenin or strophanthidin. The addition of 0·95 mg of nonradioactive digitoxigenin to the [^{3}H]-digitoxigenin gave a specific activity of approximately 2·54 mc/mM.

Homogenates and subcellular fractionation. The preparation of homogenates from heart, liver and kidney tissues and subcellular fractions from liver were accomplished by the conventional method of Schneider and Hogeboom.[17] Rats were killed by cervical dislocation and exsanguinated. The desired tissues were quickly excised and placed in an ice-cold solution containing 0·25 M sucrose plus TMN (0·05 M Tris chloride, pH 7·4, 0·005 M $MgCl_2$, and 0·010 M NaCl). A 10% homogenate of the appropriate tissue was prepared employing a Potter–Elvehjem homogenizer fitted with a Teflon pestle (A. H. Thomas Co.). This homogenate was either used as such or further fractionated. The crude nuclear-debris fraction was isolated by centrifuging the homogenate at 800 *g* for 10 min in a Sorvall RC2-B refrigerated centrifuge at 0–4°. The resulting supernatant fraction was centrifuged at 10,000 *g* for 10 min yielding the mitochondrial fraction. The 10,000 *g* supernatant fraction was centrifuged

in a Spinco Model L ultracentrifuge in a Titanium No. 50 rotor at 100,000 *g* for 60 min, giving the pelleted microsomal fraction and the cytoplasmic fraction. All pelleted fractions were washed once each. All fractions were employed for metabolic studies with 15 min of isolation.

Incubation mixtures. A homogenate of 0·75 g tissue or each subcellular fraction from this amount of tissue was incubated with 1 μc of [^{3}H]-digitoxigenin with a specific activity of 1 mc μM or 2·54 mc/mM, or 0·95 mg nonradioactive digitoxigenin in a total volume of 10·0 ml. In addition, the 25-ml Erlenmeyer flasks contained: 3·0 mg $NADP^+$, 14 mg glucose 6-phosphate, 5 units glucose 6-phosphate dehydrogenase, 0·005 M $MgCl_2$, 0·015 M KCl_2, 0·015 M nicotinamide, 0·10% bovine serum albumin, and 7·5 μM ATP. All tissues were added to the reaction flask in 7·5 ml of 0·25 M sucrose plus TMN. Incubations were normally carried out for 2 hr at 37° unless otherwise indicated. The flasks were gassed with 95% O_2–5% CO_2.

Extraction. The reactions were stopped by pouring the contents of each reaction flask into 40 ml CH_2Cl_2 in a glass-stoppered 250-ml Erlenmeyer flask. Each incubation flask was washed out with 3 ml of acetone which was added to the CH_2Cl_2. The flasks were stoppered and placed on a shaker for 1 hr to facilitate extraction of the substrate and its metabolites. The resulting emulsions were broken by centrifuging at 7000 *g* for 10 min. The upper aqueous phases were removed by pipet and discarded. The organic phases were dried over anhydrous sodium sulfate powder, filtered and evaporated to dryness *in vacuo*. The residues were transferred to 3-dram vials with three 1-ml portions of CH_2Cl_2–CH_3OH (3:2). From 80 to 90 per cent of the radioactivity was routinely recovered.

Chromatography. Thin-layer plates of silica gel H (E. Merck AG) were routinely employed. The plates were 20 × 20 cm and had an adsorbent thickness of 0·375 mm. The extracts and standards were each applied as individual spots in 50 μl amounts, which provided approximately 10,000–15,000 counts/min. The addition of tissue extracts to standards did not significantly affect their mobility or the chromatographic separation of digitoxigenin from 3-epidigitoxigenin. The thin-layer plates were developed in $CHCl_3$–isopropanol (9:1) to the top of the plates. The developed extracts and control were each cut into 20 1 cm × 2 cm areas with a razor blade, each of which was placed in a liquid scintillation counting vial and toluene counting solution added [3·0 g 2,5-diphenyl oxazole (PPO) and 100 mg dimethyl POPOP (1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)-benzene) per liter of toluene]. Each fraction was routinely counted for 20 min in a Beckman LS-100 liquid scintillation counter, with low counting samples being counted for longer periods of time. The results are expressed as per cent conversion with the standard deviations unless otherwise indicated. Each value represents the average of 3–6 individual sample determinations.

Isolation of [^{3}H]-3-*epidigitoxigenin.* The extracts from approximately 40 incubations were pooled and chromatographed on thick (0·50 mm) silica gel H plates. Digitoxigenin and 3-epidigitoxigenin were applied as standards on each plate. Each plate was developed twice in $CHCl_3$–isopropanol (9:1). The 3-epidigitoxigenin bands were located by spraying each plate with Rhodamine 6G and examination under u.v. light. The desired areas were removed from the plates and the product removed from the silica gel by Soxhlet extraction with CH_2Cl_2 for 12 hr. The [^{3}H]-3-epidigitoxigenin was repurified twice more by TLC and subsequently shown to have a radiochromatographic purity of greater than 99 per cent.

RESULTS AND DISCUSSION

In Table 1 are given the R_f values of a series of standard compounds that were chromatographed in several solvent systems. The colors that developed upon spraying with 50% H_2SO_4, followed by heating for 10–15 min at 100°, are also given. In addition, sarmentogenin (11α-hydroxydigitoxigenin), acovenosigenin (1β-hydroxydigitoxigenin), periplogenin (5β-hydroxydigitoxigenin) and 7β-hydroxydigitoxigenin all have R_f values that tend to overlap with digoxigenin (12β-hydroxydigitoxigenin) and gitoxigenin (16β-hydroxydigitoxigenin).

The metabolism of [^{3}H]-digitoxigenin by rat heart, liver and kidney homogenates, after 2-hr incubations, is given in Table 2. As can be seen, no metabolites were detected when incubating digitoxigenin with heart homogenates. This agrees with the work of Kuschinsky *et al.*,[8] who reported similar results. Liver homogenates resulted in the production of three radioactive loci distinct from [^{3}H]-digitoxigenin upon TLC. 3-Epidigitoxigenin and 3-dehydrodigitoxigenin were readily identified on the basis of

TABLE 2. METABOLISM OF [^{3}H]-DIGITOXIGENIN BY RAT HEART, KIDNEY AND LIVER HOMOGENATES*

	Control (No homogenate)	Homogenate		
		Heart %	Kidney %	Liver %
3-Epidigitoxigenin	0	0	0	58·7 ± 2·2
Digitoxigenin	98·7	94·7 ± 4·6	90·9 ± 0·3	26·6 ± 4·2
3-Dehydrodigitoxigenin	0	0	9·0 ± 0·2	8·9 ± 0·4
Polar metabolites	0	0	0	3·0 ± 0·2

* Ten per cent homogenates (0·75 g of each tissue) were incubated 2 hr with 1 μc [^{3}H]-digitoxigenin (0·95) mg at 37°. The contents of each flask were extracted with CH_2Cl_2. This extract was concentrated and examined by TLC, developing with $CHCl_3$–isopropanol (9:1). The radioactivity associated with each area was determined by liquid scintillation counting.

their R_f values in several solvent systems as well as their color upon treatment with 50% H_2SO_4 (see Table 1). These compounds were also Kedde positive, giving the same pink color as the standards. The Kedde reagent is specific for the unsaturated lactone ring system.[18] The [^{3}H]-epidigitoxigenin was isolated in a crystalline, radiochemically pure form. The synthesis of 3-dehydro- and 3-epidigitoxigenin and the recrystallization of isolated [^{3}H]-epidigitoxigenin to constant specific activity with synthesized 3-epidigitoxigenin from 40% ethanol (Table 3) further substantiate the identity of our two major metabolites.

Approximately 60 per cent of the digitoxigenin was converted into 3-epidigitoxigenin, with about 9 per cent of the 3-dehydro derivative being formed. Nozaki *et al.*[19] have shown that 3-dehydrodigitoxigenin is an intermediate in the conversion of digitoxigenin to 3-epidigitoxigenin by the fungus *Absidia orchidis*. The pharmacologic properties of 3-epidigitoxigenin[15] have been investigated, with this epimer being almost devoid of activity. A small broad radioactive area corresponded to the mobilities of digoxigenin, gitoxigenin and other hydroxylated derivatives of digitoxigenin, and accounted for only 3 per cent of radioactivity. The remaining radioactivity

TABLE 3. CRYSTALLIZATION OF [^{3}H]-EPIDIGITOXIGENIN BY ISOTOPE DILUTION TO CONSTANT SPECIFIC ACTIVITY*

Crystallization	Yield (mg)	counts/min/mg
First	22·8	1440 ± 110
Second	16·9	1505 ± 115
Third	10·7	1495 ± 40

* To 25 mg nonradioactive material was added approximately 40,000 counts/min [^{3}H]-epidigitoxigenin. The epidigitoxigenin was crystallized from 40% ethanol yielding fine needle-like crystals. The crystals were dried, weighed and dissolved in CH_2Cl_2–CH_3OH (3:2). Replicate aliquots were removed, placed in counting vials, dried, counting solution added, and the radioactivity of the samples counted.

was present as the unmetabolized [^{3}H]-digitoxigenin. The only metabolite produced by kidney homogenates was 3-dehydrodigitoxigenin, with a maximum yield of 9 per cent.

In order to determine the subcellular location of the enzymes involved in the metabolism of digitoxigenin, rat liver was fractionated and the fractions incubated with 0·95 mg [^{3}H]-digitoxigenin in the presence of an NADPH-generating system. The results are given in Table 4. Similar experiments were conducted with nonradioactive digitoxigenin and the relative intensities of the Kedde positive metabolites determined. There was very good agreement between the data using the two methods. The results indicate that the epimerase enzyme which produced 3-epidigitoxigenin is found primarily in the cytoplasm with lesser amounts in the nuclear, microsomal and mitochondrial fractions. The presence of this enzyme in these latter fractions might possibly be because of contamination with cytoplasm, although the fractions were each washed once. Surprisingly, no 3-dehydrodigitoxigenin was detected in any of the experiments involving mitochondria and the cytoplasm and very little in the microsomal systems. Mechanistically, it is conceivable that 3-dehydrodigitoxigenin is an intermediate in the conversion of digitoxigenin to 3-epidigitoxigenin in which we have a 3β-hydroxy to 3α-hydroxy inversion. It is possible that in these more highly purified

TABLE 4. METABOLISM OF [^{3}H]-DIGITOXIGENIN BY RAT LIVER SUBCELLULAR FRACTIONS*

	Control (%)	Total homogenate (%)	Fraction			
			Nuclei (%)	Mitochondria (%)	Cytoplasm (%)	Microsomes (%)
3-Epidigitoxigenin	0	61·4	56·7	63·5	82·4	69·3
Digitoxigenin	98·6	18·8	33·2	36·1	17·4	23·3
3-Dehydro-digitoxigenin	0	8·8	2·7	0	0	1·1
Polar metabolites	0	6·0	3·1	0	0	8·0

* Either 0·75 g of rat liver homogenate or the subcellular fractions from this amount of tissue was incubated with 1 μc [^{3}H]-digitoxigenin (0·95 mg) at 37° for 2 hr. The reaction mixtures were extracted with CH_2Cl_2. The CH_2Cl_2 extracts were concentrated *in vacuo* and examined by TLC using silica gel H plates. The TLC plates were developed in $CHCl_3$–isopropanol (9:1). The radioactivity associated with each compound was determined by liquid scintillation counting.

systems the 3-dehydro- intermediate does not accumulate but is rapidly converted to 3-epidigitoxigenin. From these experiments it is apparent that the hydroxylating enzymes reside in microsomes as is to be expected, giving rise to such possible products as gitoxigenin, digoxigenin and related compounds. Some hydroxylating activity apparently resides in the nuclear fraction as well. Whether the products are identical to those of microsomal origin is still unknown.

A kinetic or time-course study of the metabolism of [^{3}H]-digitoxigenin by rat liver homogenates was undertaken. The results in Table 5 are expressed as nanomoles of metabolite formed per gram of wet weight of liver and indicate that the conversion of [^{3}H]-digitoxigenin to 3-epidigitoxigenin and 3-dehydrodigitoxigenin does not reach a maximum until 2 to 3 hr after the initiation of incubation. On the other hand, no significant increase in the production of polar metabolites appears to occur after 60 min. Tissue binding may make the substrate unavailable to the oxidative enzymes.

TABLE 5. TIME-COURSE OF [^{3}H]-DIGITOXIGENIN METABOLISM BY RAT LIVER HOMOGENATE*

	Incubation time (min)						
	15	30	45	60	90	120	180
	(nmoles product formed/g liver)						
3-Epidigitoxigenin	2·0	4·8	8·2	11·0	16·6	25·2	21·3
3-Dehydrodigitoxigenin	1·0	1·1	1·9	2·4	3·0	3·3	4·2
Polar metabolites	1·4	1·9	1·8	2·1	2·2	2·0	2·1

* Three g of rat liver as a homogenate was incubated with 4·0 μc [^{3}H]-digitoxigenin (3·80 mg) in a total volume of 40 ml of buffered solution at 37°. Aliquots (5·0 ml) were removed at the indicated times and extracted with CH_2Cl_2. The CH_2Cl_2 extracts were concentrated *in vacuo* and the metabolites separated by TLC using silica gel H plates with $CHCl_3$–isopropanol (9:1) as the developing solvent. The radioactivity was determined by liquid scintillation counting. At the end of 180 min, 98·0 per cent of the recovered radioactivity from the control flasks was still present as digitoxigenin, while 61·8 per cent from the experimental flasks was present as 3-epidigitoxigenin.

Since phenobarbital is known to induce microsomal hydroxylating enzymes,[20] we investigated the effect of phenobarbital on the metabolism of [^{3}H]-digitoxigenin by rat liver homogenates. As can be seen in Table 6, two levels of specific activity of [^{3}H]-digitoxigenin were used by adding nonradioactive material to the initial product which had a specific activity of approximately 1·0 mc/μM. In both cases, the same number of counts per minute were added to the reaction flask. With a specific activity of 1·0 mc/μM, less than 0·01 mg of digitoxigenin was added per flask, while at the lower specific activity of 2·54 mc/mM approximately 0·95 mg of digitoxigenin was added to each reaction flask. In both cases, the polar metabolites as digoxigenin, gitoxigenin and related compounds are the major conversion products from the phenobarbital-treated rats as compared with the controls. The identification of these compounds is not known and is presently under investigation. Quantitative differences do exist when one compares the two substrate levels. The use of barbiturates by patients on digitalis preparations may produce an increased rate of metabolism of the cardiac glycosides and a subsequent decrease in their duration of action. Whether such a phenomenon occurs *in vivo* is not known.

TABLE 6. METABOLISM OF [^{3}H]-DIGITOXIGENIN BY LIVER HOMOGENATES OF PHENOBARBITAL-TREATED RATS*

	Control (No homogenate)	Control animals		Phenobarbital-treated animals	
	(%)	1·0 mc/μM) (%)	2·54 mc/mM (%)	1·0 mc/μM (%)	2·54 mc/mM (%)
3-Epidigitoxigenin	0	68·3 ± 1·2	62·5 ± 2·5	4·4 ± 0·5	31·4 ± 0·9
Digitoxigenin	98·6	12·4 ± 0·5	15·8 ± 1·4	4·6 ± 1·1	13·7 ± 2·6
3-Dehydrodigitoxigenin	0	7·4 ± 0·4	9·9 ± 1·3	11·3 ± 1·3	10·0 ± 2·8
Polar metabolites	0	16·6 ± 0·9	3·8 ± 0·2	74·1 ± 1·3	45·3 ± 0·7

* Rats were given 100 mg/kg phenobarbital i.p. in normal saline for 5 days. Control animals received normal saline. [^{3}H]-digitoxigenin (1 μc) at two specific activities, 1·0 mc/μM ($3·75 \times 10^{-4}$ mg) and 2·54 mc/mM (0·95 mg), was incubated with 0·75 g of homogenized rat liver in a total volume of 10·0 ml. The reaction mixtures were incubated 2 hr at 37° and extracted with CH_2Cl_2. The CH_2Cl_2 extracts were concentrated, the metabolites separated by TLC as previously described, and the radioactivity associated with each metabolite was determined by liquid scintillation counting.

We determined the effect of giving large doses of digitoxin to rats on the metabolism *in vitro* of [^{3}H]-digitoxigenin. These rats were given a total digitoxin dose of 13·0 mg/kg over a 1-week period of time prior to their use experimentally. Again two levels of specific activity were used for [^{3}H]-digitoxigenin. As can be seen in Table 7, for both substrate levels, significantly less [^{3}H]-digitoxigenin was metabolized by the digitoxin-treated animals as compared to the controls. Also in each case, less 3-epidigitoxigenin and 3-dehydrodigitoxigenin are produced. No effect was noted on the production of polar metabolites. The treatment of rats with digitoxin therefore had no apparent effect on the hydroxylase enzyme complex, but caused a small inhibition of the epimerase enzyme system. Whether the action occurs by indirect or direct inhibition, or simply through dilution of the [^{3}H]-digitoxigenin, is not known.

TABLE 7. METABOLISM OF [^{3}H]-DIGITOXIGENIN BY LIVER HOMOGENATES OF DIGITOXIN-TREATED RATS*

	Control (No homogenate)	Control animals		Digitoxin-treated animals	
	(%)	1·0 mc/μM (%)	2·54 mc/mM (%)	1·0 mc/μM (%)	2·64 mc/mM (%)
3-Epidigitoxigenin	0	71·5 ± 1·5	64·8 ± 5·1	60·7 ± 4·7	47·8 ± 5·8
Digitoxigenin	98·4 ± 0·4	6·2 ± 0·3	17·4 ± 6·9	19·9 ± 3·4	36·9 ± 5·0
3-Dehydrodigitoxigenin	0	9·8 ± 1·2	11·6 ± 0·7	6·4 ± 2·8	6·7 ± 1·5
Polar metabolites	0	12·0 ± 0·4	4·1 ± 1·2	11·7 ± 0·6	4·8 ± 0·6

* Rats were given a total of 13·0 mg/kg digitoxin i.p. over a 7-day period. Control animals received the ethanol–propylene glycol solvent. [^{3}H] digitoxigenin (1 μc) at two specific activities, 1·0 mc/μM ($3·75 \times 10^{-4}$ mg) and 2·54 mc/mM (0·95 mg), was incubated with 0·75 g of homogenized rat liver in a total volume of 10·0 ml. The reaction mixtures were incubated 2 hr at 37° and extracted with CH_2Cl_2. The CH_2Cl_2 extracts were concentrated, the metabolites separated by TLC, and the radioactivity associated with each metabolite was determined by liquid scintillation counting.

Information on the specificity of the two enzyme systems involved in the metabolism of digitoxigenin was obtained. The results of adding 0·95 mg of nonradioactive digitoxigenin, digoxigenin, gitoxigenin or strophanthidin to each reaction flask are presented in Table 8. The addition of 0·95 mg cold digitoxigenin gives a radioactivity dilution of 2530. The results confirm those previously obtained (see Tables 6 and 7), indicating that there is a significant reduction in the per cent of polar metabolites formed at the higher digitoxigenin substrate concentration. A small but significant

TABLE 8. INFLUENCE OF OTHER CARDENOLIDES ON THE METABOLISM OF [^{3}H]-DIGITOXIGENIN BY RAT LIVER HOMOGENATES*

	Control (No homogenate) (%)	0·95 mg of Cardenolide added				
		None (%)	Digitoxigenin (%)	Digoxigenin (%)	Gitoxigenin (%)	Strophanthidin (%)
3-Epidigitoxigenin	0	53·8 ± 1·4	54·9 ± 3·9	59·3 ± 3·4	52·3 ± 7·2	61·4 ± 0·5
Digitoxigenin	98·4	21·8 ± 2·8	26·7 ± 3·0	24·6 ± 4·4	30·7 ± 8·5	21·7 ± 1·1
3-Dihydrodigitoxigenin	0	8·8 ± 0·5	12·4 ± 0·2	7·1 ± 0·7	12·6 ± 0·7	7·5 ± 1·0
Polar metabolites	0	13·2 ± 1·3	4·2 ± 0·3	7·1 ± 0·6	4·5 ± 0·2	8·5 ± 1·0

* [^{3}H]-digitoxigenin (1 μc), with a specific activity of 1·0 mc/μM, was incubated with 0·75 g homogenized rat liver in a total volume of 10·0 ml. To appropriate flasks, 0·95 mg of either digitoxigenin, digoxigenin, gitoxigenin, or strophanthidin was added. The reaction mixtures were incubated 2 hr at 37° and extracted with CH_2Cl_2. The CH_2Cl_2 extracts were concentrated, the metabolites separated by TLC, and the radioactivity associated with each was determined by liquid scintillation counting.

increase in the accumulation of 3-dehydrodigitoxigenin also was observed. It is noteworthy that the addition of nonradioactive gitoxigenin (16β-hydroxydigitoxigenin) gives results identical to those of added nonradioactive digitoxigenin, indicating that gitoxigenin can equally replace digitoxigenin as the substrate. Both digoxigenin (12β-hydroxydigitoxigenin) and strophanthidin only partially decrease the production of polar metabolites and produce a small increase in the formation of 3-epidigitoxigenin. The significance of this increase is not known. Strophanthidin differs from digitoxigenin in that it is oxygenated at positions 5 and 19. From these results it can be concluded that the substrate specificity of the hydroxylating enzyme complex is as follows: digitoxigenin = gitoxigenin > digoxigenin > strophanthidin.

Acknowledgements—This work was supported in part by a grant from the Nebraska Heart Association, Omaha, Nebr. The authors express their gratitude to Miss Sandra Khem and Miss Janice Fisher for excellent technical assistance.

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