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METABOLITES AND SOME CHARACTERISTICS OF THE METABOLISM OF ^{3}H-DIGOXIGENIN BY RAT LIVER HOMOGENATES

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Abstract—^{3}H-digoxigenin is metabolized by rat liver homogenates to ^{3}H-3-epidigoxigenin and ^{3}H-3-dehydrodigoxigenin. Maximum conversion occurs after approximately 80 min of incubation. The amount of each metabolite which accumulates depends on the amount of $NADP^+$ added to the incubation mixture. No formation of more polar metabolites was observed, indicating that a microsomal hydroxylating system may not be operative in the metabolism of digoxigenin.

IN RECENT years, Modell,[1] Moe and Farah,[2] Doherty[3] and Noack[4] have reviewed the general pharmacology of cardiac glycosides, as well as much of the present knowledge concerning the metabolism of these compounds. The principal difference in the metabolism of digitoxin and digoxin is that the former is largely (70 per cent) converted to metabolic products whereas much of the latter is excreted unchanged.[3] Thus, it has been suggested that much of the therapeutic activity of digitoxin may reside in its metabolites, which include digitoxigenin, digoxin and lesser amounts of digoxigenin, and the mono- and bis-digitoxosides of digitoxigenin and digoxigenin.[3] The previously reported metabolic products of digoxin (15–20 per cent of the total administered dose) include digoxigenin, the mono- and bis-digitoxosides of digoxigenin, and 3-epidigoxigenin.[3] The present knowledge of the subcellular basis for the mechanism of cardiac glycoside action has most recently been reviewed by Lee and Klaus.[5]

Kuschinsky *et al.*[6] have noted the discrepancy between the kinetic and pharmacologic properties of ^{3}H-digitoxigenin and have suggested that extracardial factors, such as metabolism in the liver, may account for the observed difference in clinical action. Selye *et al.*[7] have reported that spironolactone can prevent the toxic manifestations of digitoxin but not of digitoxigenin.

Stohs *et al.*[8] have investigated the metabolism *in vitro* of ^{3}H-digitoxigenin and have observed that 60 per cent of the aglycone is converted to 3-epidigitoxigenin in 2 hr. Although the epimerization *in vivo* of ^{3}H-digoxigenin has been reported,[9] the reaction has not been studied quantitatively, nor has the overall metabolism of ^{3}H-digoxigenin investigated in detail. To these ends, we have undertaken the study of the meta- *in vitro* of ^{3}H-digoxigenin by rat liver homogenates.

EXPERIMENTAL

Animals. Male weanling rats derived from the Holtzmann strain were obtained from Sasco, Inc., Omaha, Nebr. They were maintained on a diet of Purina Laboratory Chow and tap water *ad lib.* and used experimentally after attaining a weight of approximately 150 g. Animals were fasted overnight prior to use.

^{3}H-digoxigenin. A generous supply of randomly labeled ^{3}H-digoxin, having a specific activity of 144 μc/mg, was provided by Burroughs–Wellcome. ^{3}H-digoxigenin was prepared by acid hydrolysis of ^{3}H-digoxin. ^{3}H-digoxin (144 μc) in 1 ml of 95% ethanol was added to 5 ml of 70% methanol. Carrier digoxigenin (5·0 mg) was dissolved in this solution and 60 μl of concentrated H_2SO_4 was added. The solution was refluxed for 5 min, cooled in ice and extracted three times with an equal volume of methylene dichloride. The organic extracts were pooled, concentrated to dryness *in vacuo*, and redissolved in methylene dichloride–methanol (3:2). The aglycone was isolated and purified by preparative thin-layer chromatography (TLC) on Silica gel H plates (20 × 20 cm, 0·375 mm thick; E. Merck AG, Darmstadt). The plates were developed three times in a solvent chamber containing chloroform–isopropanol (9:1). In this system, digoxigenin and digoxin have R_f values of approximately 0·44 and 0·33 respectively. The bands corresponding to digoxigenin were located by exposing the plates to I_2 vapors for 5 min. After allowing I_2 to evaporate from the plates overnight, the digoxigenin bands were removed and the gel was extracted 12 hr with benzene–acetone (4:1) in a Soxhlet apparatus. An aliquot of the digoxigenin was tested by TLC in the chloroform–isopropanol (9:1) system, and was found to have a radiochemical purity of 98·7 per cent.

3-Dehydrodigoxigenin synthesis. Digoxigenin (150 mg) was converted to 3-dehydrodigoxigenin by a modification of the platinum oxidation method of Tamm and Gubbler.[10] A mixture of 95% O_2 to 5% CO_2 was bubbled through the reaction mixture in a 37° water bath. Aliquots were periodically tested by TLC using Silica gel H plates developed once in chloroform–isopropanol (7:1), until the reaction had gone to completion in approximately 36 hr. The product was then isolated as described[10] and recrystallized from 50% ethanol. Yield: 120 mg, m.p. 253–255°; reported m.p. 248–253°.[10]

3-Epidigoxigenin synthesis. 3-Dehydrodigoxigenin (35 mg) was converted to 3-epidigoxigenin by $NaBH_4$ reduction, as described by Tamm and Gubbler,[10] although the reaction time was extended to 16 hr. The product was isolated and recrystallized as described.[10] Yield: 27 mg, m.p. 255–260°; reported m.p. 249–262°.[10]

Liver homogenates. Animals were sacrificed and 10 per cent liver homogenates were prepared in 0·25 M sucrose plus TMN (0·05 M Tris chloride, pH 7·4, 0·005 M $MgCl_2$ and 0·010 M NaCl) according to the method of Stohs *et al.*[8]

Incubation media. Unless otherwise indicated, the following NADPH-generating system was employed: 1·25 ml of 0·25 M sucrose plus TMN, which contained 16 μmoles $NADP^+$, 14 mg glucose 6-phosphate, 5 units glucose 6-phosphate dehydrogenase, 0·015 M nicotinamide and 0·1% bovine serum albumin. An amount equivalent to 0·5 μc ^{3}H-digoxigenin having a specific activity of approximately 20 μc/mg was withdrawn from the refrigerated benzene–acetone stock solution, evaporated to dryness with N_2, redissolved in 0·40 ml of 70% ethanol along with 1·0 mg of carrier digoxigenin, and added to the medium. Liver homogenate (10 ml) was then added to each 125-ml Erlenmeyer flask containing the incubation medium and substrate. The flasks were shaken for 2 hr in an Eberbach water bath shaker at 37° and gassed with 95% O_2 to 5% CO_2 throughout the incubation.

Subcellular fractionation. Metabolism of ^{3}H-digoxigenin by rat liver subcellular fractions was studied. The nuclei-cell debris, mitochondrial, microsomal and cytoplasmic fractions were isolated according to the procedure previously described.[8]

These fractions were washed once each, resuspended in 0·25 M sucrose plus TMN, and added to the incubation media described above within 10 min of isolation.

Extraction. The reactions were stopped by pouring the reaction mixtures into 40 ml of methylene dichloride in separate 250-ml glass-stoppered Erlenmeyer flasks. The reaction flasks were rinsed several times with distilled water. The flasks were placed on a shaker for 2 hr at room temperature. The resulting emulsions were broken by centrifuging at 7000 *g* for 10 min. The upper aqueous phases were carefully removed by pipette and discarded. The organic phases were filtered through anhydrous sodium sulfate and evaporated to dryness *in vacuo*. The residues were transferred to 3-dram vials with 2 ml of methylene dichloride–methanol (3:2) divided in three portions. This procedure routinely yielded 90–95 per cent recovery of the radioactivity.

Chromatography. Aliquots of the extracts providing approximately 10,000 dis./min per sample were applied to 20 × 20 cm, 0·25 mm thick Silica gel H plates, scored into 2-cm wide columns. Unless otherwise indicated, the plates were developed twice in chloroform–isopropanol (7:1). Approximately 10 μl of standard digoxigenin, 3-dehydrodigoxigenin and 3-epidigoxigenin solutions were also applied to each plate. Addition of tissue extracts to these standards did not alter their R_f values. The standards were visualized with I_2 and the bands corresponding to the standards were marked on each column. The remainder of each 2-cm wide column was divided into 1-cm areas. Each such area, as well as the regions corresponding to the standards, was scraped into a liquid scintillation counting vial. A 20% absolute ethanol in toluene counting solution, containing 3·0 g 2,5-diphenyloxazole and 100 mg dimethyl 1,4-bis-2-(4-methyl 5-phenyloxazolyl)benzene per liter of solution, was added to each vial. The addition of the ethanol was necessary for complete elution of the substrate and its metabolites from the gel. Each sample was routinely counted for 20 min in a Beckman LS-100 liquid scintillation counter. The results are expressed as per cent total radioactivity in each extract ± the standard deviation for four to six individual determinations.

RESULTS AND DISCUSSION

Approximately 45 per cent of the ^{3}H-digoxigenin substrate was consistently converted to metabolites by rat liver homogenates within 2 hr. This radioactivity was localized in the bands corresponding to 3-dehydrodigoxigenin and 3-epidigoxigenin after TLC. Aliquots of each extract were co-chromatographed in three TLC systems: $CHCl_3$–isopropanol (7:1), developed twice; $CHCl_3$–acetone (5:2), developed twice; and CH_2Cl_2–CH_3OH–H_2O (84:15:2), developed once. The radioactivity in the digoxigenin, 3-epidigoxigenin and 3-dehydrodigoxigenin bands remained the same, independent of the system used.

The metabolites were further characterized by isotope dilution. Approximately 400,000 dis./min corresponding to the 3-epidigoxigenin band were purified and isolated by preparative TLC and Soxhlet extraction to greater than 95 per cent purity. Likewise, approximately 75,000 dis./min corresponding to the 3-dehydrodigoxigenin band were obtained. Chemically synthesized 3-dehydrodigoxigenin (15 ml) and 3-epidigoxigenin (20 mg) were added to the appropriate tritiated sample and each was recrystallized from 50% ethanol to constant specific activity.

Three replicate aliquots were counted after each of three crystallizations for each of the two metabolites. The specific activity of the 3-dehydrodigoxigenin fell within

the range 5070 ± 130 dis./min/mg after each of the recrystallizations. The specific activity of the 3-epidigoxigenin fell within the range 1,9230 ± 575 dis./min/mg after each of the recrystallizations.

TABLE 1. METABOLISM OF ^{3}H-DIGOXIGENIN BY UNDIALYZED RAT LIVER HOMOGENATES

Metabolite	Control (no homogenate)	Components of incubation medium*		
		$NADP^+$, G-6-P, G-6-PDH	NAD^+, G-6-P, G-6-PDH	$NADP^+$
		Extractable radioactivity (%)		
3-Epidigoxigenin	0	41·4 ± 4·2	3·4 ± 1·1	36·9 ± 2·0
Digoxigenin	98·7	56·1 ± 5·9	77·0 ± 5·5	57·2 ± 3·6
3-Dehydrodigoxigenin	0	2·6 ± 0·35	16·8 ± 3·2	3·7 ± 0·7

* Ten per cent liver homogenates (1·0 g tissue) were incubated 2 hr with 0·5 μc (1·0 mg) of ^{3}H-digoxigenin in NADPH-generating medium containing either 16 μmoles $NADP^+$ or NAD^+, or in medium containing $NADP^+$ but no exogenous glucose 6-phosphate (G-6-P) or glucose 6-phosphate dehydrogenase (G-6-PDH). The contents of each flask were extracted with CH_2Cl_2. This extract was concentrated and tested by TLC, developing with $CHCl_3$–isopropanol (7:1). The radioactivity associated with each area was determined by liquid scintillation counting.

The pyridine nucleotide coenzyme requirement of the two metabolic products was initially examined by performing experiments in which glucose 6-phosphate (G-6-P) and glucose 6-phosphate dehydrogenase (G-6-PDH) of the exogenously added NADPH-generating system were omitted. The results of these studies are given in Table 1. The accumulation of 3-epidigoxigenin was only slightly reduced in the experiments in which G-6-P and G-6-PDH had been omitted, suggesting that the formation of 3-dehydrodigoxigenin produced NADPH, which was then used to form 3-epidigoxigenin, regenerating the $NADP^+$. However, when an equivalent amount of NAD^+ was substituted for $NADP^+$ in the system (see Table 1), the major product was 3-dehydrodigoxigenin with little of the 3-epidigoxigenin being formed. These results suggest that NAD^+ favors the accumulation of 3-dehydrodigoxigenin and that the subsequent epimerization is NADPH-dependent.

To further investigate the pyridine nucleotide coenzyme requirements of the reactions, we performed experiments using homogenates which had been dialyzed 24 hr at 8–10° against 2 l. of 0·005 M Tris buffer, pH 7·4 (Table 2). Maximum digoxigenin metabolism was not as extensive by the dialyzed preparations as by the undialyzed homogenates. In order to obtain significant amounts of 3-epidigoxigenin, it was necessary to add both NAD^+ and $NADP^+$ to the incubation medium. When only NAD^+ was added in conjunction with G-6-P and G-6-PDH, significant amounts of 3-dehydrodigoxigenin accumulated. When only NAD^+ was added with G-6-P and G-6-PDH, the accumulation of the two metabolites was not greater than when no additions were made to the incubation flasks (Table 2). These results confirmed our original observation that NAD^+ is required for the formation of 3-epidigoxigenin (see Table 1). The epimer also accumulated when NAD^+ and $NADP^+$ were both added to an incubation medium containing dialyzed homogenate but no exogenous

TABLE 2. METABOLISM OF ^{3}H-DIGOXIGENIN BY DIALYZED RAT LIVER HOMOGENATES

	Components of incubation medium*				
Metabolite	$NADP^+$, G-6-P, G-6-PDH	NAD^+, G-6-P, G-6-PDH	NAD^+, $NADP^+$, G-6-P, G-6-PDH	NAD^+ $NADP^+$	No additions
	Extractable radioactivity (%)				
3-Epidigoxigenin	4·4	5·1	24·1	17·0	7·6
Digoxigenin	94·8	90·6	74·8	80·8	91·7
3-Dehydrodigoxigenin	0·8	4·1	0·9	1·9	0·6

* Twenty per cent homogenates were dialyzed against 2 l. of 0·005 M Tris buffer, pH 7·4, at 8–10° for 24 hr, changing the buffer once. The dialyzed homogenates were resuspended, then diluted with sufficient 0·25 M sucrose + TMN to yield 10% suspensions. The homogenates so prepared were added to NADPH-generating incubation media containing either no pyridine nucleotide coenzyme, or 16 μmoles of either $NADP^+$, NAD^+ or both nucleotides, or to a medium containing 16 μmoles of both coenzymes but no G-6-P or exogenous G-6-PDH. Incubations were carried out and results were obtained by the procedure described in Experimental.

G-6-P or G-6-PDH (Table 2). Apparently NAD^+, not $NADP^+$, is reduced in the formation of 3-dehydrodigoxigenin.

The time course of the metabolism of ^{3}H-digoxigenin to ^{3}H-3-epidigoxigenin was followed. The results, presented in Table 3, indicate that very little further conversion occurs after 80 min under our experimental conditions. A similar time course was reported by Stohs *et al.*[8] for the epimerization of ^{3}H-digitoxigenin by rat liver homogenates, using a similar quantity of substrate. They noted a final conversion of about 60 per cent, approximately 20 per cent greater than the conversion seen here for digoxigenin.

TABLE 3. TIME COURSE OF THE METABOLISM OF ^{3}H-DIGOXIGENIN TO ^{3}H-3-EPIDIGOXIGENIN*

Incubation time (min)	Product formed (nmoles/g liver)	Extractable radioactivity (%) as ^{3}H-3-epidigoxigenin
20	489·1	18·1
40	948·7	29·6
60	1208·3	37·7
80	1285·2	40·1
100	1291·6	40·3

* ^{3}H-digoxigenin (38 μc, 5·0 mg) was incubated in 40 ml of 10% homogenate buffered incubation medium containing 64 μmoles $NADP^+$. Aliquots (8 ml) were withdrawn at the times indicated above and extracted with CH_2Cl_2; the metabolites were separated by TLC and radioactivity was determined by liquid scintillation counting.

No localized radioactivity in regions with R_f values less than that of 3-epidigoxigenin was observed in these experiments, indicating that compounds more polar, such as hydroxylation products of 3-epidigoxigenin, were not formed. In contrast, the metabolism of digitoxigenin by liver homogenates results in the accumulation of small amounts of unresolved "polar metabolites".[8] To examine this difference more closely as well as determine the subcellular location of the enzymes involved in digoxigenin metabolism, incubations were done with rat liver nuclei, mitochondria, microsomes and cytosol. The results, reported in Table 4, agree with those reported

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

Metabolite	Extractable radioactivity (%)				
	Whole homogenate	Nuclei	Mito-chondria	Micro-somes	Cytosol
3-Epidigoxigenin	49·6	5·0	0·0	4·1	43·0
Digoxigenin	48·2	92·8	98·0	95·0	52·9
3-Dehydrodigoxigenin	1·5	1·7	0·5	0·5	1·3

* Either 1·0 g of 10% rat liver homogenate or the subcellular fractions derived from this amount of tissue were incubated for 2 hr at 37° with 0·5 μc (1·0 mg) of ^{3}H-digoxigenin in NADPH-generating media containing 16·0 μmoles $NADP^+$. The reaction mixtures were extracted, the metabolites were separated by TLC, and the radioactivity was determined by liquid scintillation counting.

for digitoxigenin in that most of the epimerase activity is found in cytosol.[8] However, no production of polar metabolites by the microsomes was noted. Therefore, it seems probable that the microsomal hydroxylating system is not operative in the metabolism of digoxigenin. Further studies involving the effects of known microsomal inducers on ^{3}H-digoxigenin metabolism and investigations on the interactions of digitoxigenin and digoxigenin with cytochrome P-450 may explain this difference.

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