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Neither digitoxigenin and epidigitoxigenin nor their water soluble conjugation products can be found in the bile of rats within 4 hr after i.v. injection of digitoxin [1]. The major portion of conjugates consists of glucuronides and sulfates of digitoxigenin monodigitoxoside [1, 2]. Therefore it is necessary for the study of microsomal digitoxin metabolism *in vitro* to use the native glycoside as a substrate instead of digitoxigenin investigated previously [3, 4, 5].

Liver microsomes of male Wistar rats (200 ± 10 g body wt) were prepared by the method of Kutt and Fouts [6]. Microsomal protein was determined by the method of Lowry *et al.* [7] with bovine serum albumin as the standard. For a complete system the incubation volume of 2·0 ml consisted of 0·15 M KCl, 50 mM $MgCl_2$, 10 mM succinate, 0·2 mg bovine serum albumin (Behring-Werke), 10 mM isocitrate, 1 mM NADP, 0·3 mM NADH, 200 mU isocitrate dehydrogenase, 3·5 mg microsomal protein, and 7 μM $^3H_{21-22}$-digitoxin (660 Ci/mole) prepared by the method of Haberland and Merten [8]. The mixtures were incubated at 37° with shaking. Controls were obtained by the omission of either pyridine nucleotides and NADPH regenerating system or microsomes or incubation at 37°. After the indicated incubation periods (see Table 1 and Fig. 2) 0·2 ml of the incubation mixture was transferred to 0·2 ml ice-cold methanol and centrifuged for 3 min at 5000 *g*. The pellet was washed with 0·5 ml methanol and centrifuged again. Reference compounds were added to the combined supernatants and subjected to thin-layer chromatography on precoated silicagel 60 F254 aluminium sheets (Merck). After development three times in solvent system I ($CHCl_3$–CH_3OH, 92:8, v/v) the plates were scanned for radioactivity (Dünnschicht-Scanner II, Berthold-Friesecke). Four zones of radioactivity were detected corresponding to the origin, digoxigenin glycosides, digitoxigenin glycosides, and an 'apolar' fraction (R_F relative to digitoxigenin 1·15). They were scraped off and eluted with $CHCl_3$–CH_3OH (1:2, v/v) for scintillation counting (Tricarb, Packard). Fractions containing the digoxigenin- and the digitoxigenin glycosides were rechromatographed in solvent system II (ethylacetate or ethylacetate–acetic acid–$CHCl_3$, 90:5:5, by vol., 3 developments) achieving a separation of glycosides according to the number of digitoxoses per molecule. The separated glycosides were scanned, eluted, and counted again. The recovery of radioactivity eluted from the plates amounted to more than 94% after development in system I. After chromatography in system II the recovery was in the range of 92–97% for the digitoxigenin glycosides and 87–94% for the digoxigenin glycosides.

To confirm the structure of the aglycone all fractions

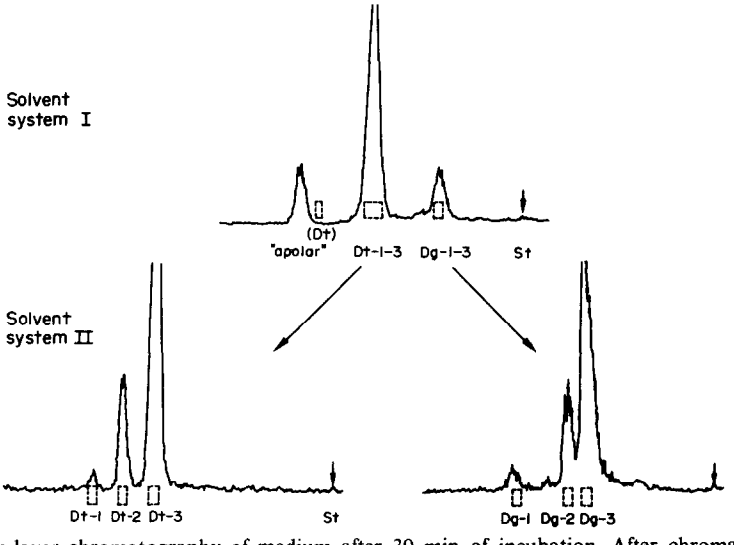

Fig. 1. Thin-layer chromatography of medium after 30 min of incubation. After chromatography in system I ($CHCl_3$–CH_3OH, 92:8, v/v) the zones corresponding to digitoxigenin- and digoxigenin digitoxosides were eluted and rechromatographed in system II (ethylacetate–$CHCl_3$–acetic acid, 90:5:5, by vol.) as described in the text. St = origin, Dg-1(2,3) = digoxigenin mono (bis, tri) digitoxoside, Dt-1(2,3) = digitoxigenin mono (bis, tri) digitoxoside, Dt = digitoxigenin.

Table 1. NADPH dependent digitoxin metabolism by rat liver microsomes

Variation of complete system	Metabolites (pmoles/3·5 mg microsomal protein/30 min)			
	Digoxigenin digitoxosides	'Apolar' fraction	Digitoxigenin mono- digitoxosides	Digitoxigenin bis- digitoxosides
	2661 ± 338	2443 ± 587	399 ± 133	1693 ± 456
−Isocitrate −ICDH −NADH	448 ± 125	149 ± 102	76 ± 54	297 ± 125
−NADP	403 ± 123	245 ± 120	71 ± 12	201 ± 53
−NADP, −NADH + 0·3 mM NAD	415 ± 41	92 ± 11	74 ± 41	202 ± 83
−NADP, −NADH	229 ± 35	138 ± 90	78 ± 56	203 ± 74
$-O_2$, +CO	509 ± 131	197 ± 137	103 ± 32	261 ± 41
+0·1 mM SKF 525A	1730 ± 176	654 ± 192	122 ± 37	340 ± 55
No incubation at 37°	201 ± 59	161 ± 109	76 ± 27	159 ± 95

For composition of the complete incubation system containing NADP, NADH, isocitrate (IC), isocitrate dehydrogenase (ICDH), microsomes, and 7 μM ^{3}H-digitoxin see text. Values are means ±S.D. of 3–5 rats.

were hydrolyzed in 0·05 N H_2SO_4 in 50% CH_3OH and rechromatographed in system I. The reduction of the 'apolar' fraction was performed in dimethylformamide with $NaBH_4$ by a method to be described elsewhere [9].

As shown in Fig. 1, the microsomal metabolism of digitoxin includes both the hydroxylation of the genine part forming digoxigenin glycosides and the cleavage of glycoside bonds forming bis- and monodigitoxosides of digitoxigenin and digoxigenin. The kinetic time course of metabolism is given in Fig. 2. As can be seen the reaction velocity

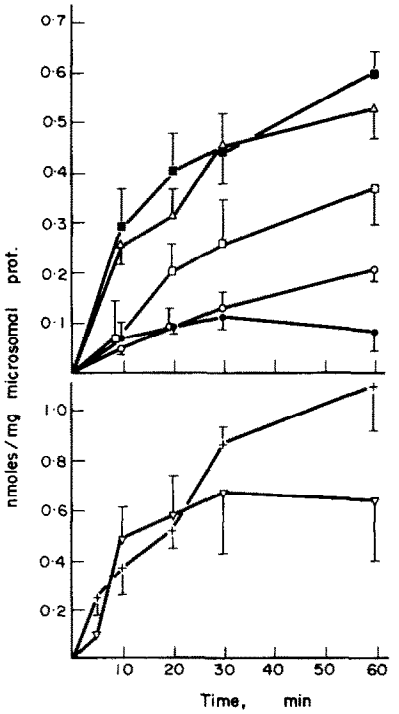

Fig. 2. Time course of microsomal digitoxin metabolism. After indicated periods aliquots of the incubation mixture were analyzed by t.l.c. and scintillation counting. Results are means ±S.D. for 3–5 rats. ▽——▽, 'apolar' metabolite, +——+ digoxigenin digitoxosides, ●——● digitoxigenin monodigitoxoside, ■——■ digitoxigenin bisdigitoxoside, ○——○ digoxigenin monodigitoxoside, □——□ digoxigenin bisdigitoxoside, △——△ digoxin.

was constant for about 30 min. After 60 min of incubation the total of metabolites formed by 3·5 mg microsomal protein amounted to 55% of the starting digitoxin (14 nmoles).

No hydroxylation products corresponding to digoxigenin digitoxosides could be found in the presence of NADP (without NADPH regenerating system), NAD or NADH. Furthermore, the hydroxylation is inhibited by SKF 525A and carbon monoxide. From this experiment it may be concluded that the microsomal mixed function oxidases are involved in the C_{12}-hydroxylation (Table 1). Unexpectedly, also the digitoxosides of digitoxigenin were cleaved only in the presence of NADPH. The cleavage was inhibited by SKF 525A and carbon monoxide. Apparently the cleavage of digitoxin forming digitoxigenin bis- and monodigitoxoside is not a simple hydrolysis but a consequence of a previous oxidation mediated by microsomal NADPH dependent enzymes.

Free genins, ketogenins and epigenins were not detected. According to experiments *in vivo* the 'apolar' fraction (Fig. 1) did not correspond to these compounds but mainly consisted of an oxidation product of digitoxigenin bis-digitoxoside [1]. The glycosidic nature of this fraction was shown by acid hydrolysis, after which the radioactivity ran like digitoxigenin. Probably this metabolite was formed by NADPH dependent oxidation of one or more hydroxyl groups of the sugar chain to carbonyl groups (presumably at the diol structure at the C_3''' and C_4'''). This proposed mechanism is supported by the following experiments: (i) reduction of the 'apolar' fraction by $NaBH_4$ yielded digitoxigenin bisdigitoxoside, (ii) in solvent system I the 'apolar' metabolite ran like a chemically oxidized digitoxin (3'''- and/or 4'''-dehydrodigitoxin), (iii) after reaction with dinitrophenylhydrazine the radioactivity of the 'apolar' fraction ran like the dinitrophenylhydrazone of dehydrodigitoxin (two developments in benzene–ethylacetate (1:1, v/v)).

Thus these data indicate that the metabolism of the native glycoside digitoxin by rat liver microsomes includes: (i) C_{12}-hydroxylation, (ii) cleavage of tridigitoxosides up to monodigitoxosides, (iii) formation of an 'apolar' metabolite probably consisting of dehydrobisdigitoxoside(s) of digitoxigenin. All the metabolic reactions require NADPH and can be inhibited by carbon monoxide and SKF 525A.

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Evidence against multiple forms of reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase in rat liver microsomes

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The mixed-function oxidase system of the liver endoplasmic reticulum (microsomes) catalyzes the hydroxylation of a wide variety of lipophilic compounds including steroids, fatty acids, drugs, pesticides, carcinogens and other xenobiotics [1, 2]. An intriguing property of this electron transport system is its apparent non-specificity with regard to the substrates it hydroxylates. This property has led to the hypothesis that multiple mixed-function oxidase activities are present in liver microsomes [3–7], and currently there is much interest in the mechanism by which multiple activities might be attained. The two known enzymatic components of the system present in rat liver microsomes are a flavoprotein, NADPH-cytochrome c reductase and the terminal, substrate-binding oxidase, cytochrome P-450 [1–3]. Any mechanism which might be proposed for attaining different hydroxylation activities would have to include multiplicity at the level of the cytochrome component since this binds the substrate, and indeed there is spectral [1, 2], catalytic [8–10], and SDS-polyacrylamide gel electrophoretic [11, 12] evidence which is consistent with this proposal. It is not known, however, if the multiple hydroxylation activities are due solely to the existence of multiple forms of the cytochrome component, or if completely different electron transport chains are present in microsomes and different forms of NADPH-cytochrome c reductase might also be present in this membrane. To investigate this question, the properties of the NADPH-cytochrome c reductase enzymes present in the liver microsomes of control rats might be compared with those present in the liver microsomes from rats pretreated with phenobarbital (PB) or 3-methylcholanthrene (3-MC), two compounds known to induce different hydroxylation activities in the microsomes [1, 2]. Since methods have not yet been developed by which the reductase enzyme can be isolated from rat liver microsomes without the use of proteases [13], however, such comparisons have been difficult to perform.

Kuriyama *et al.* [14] have shown that the trypsin-solubilized NADPH-cytochrome c reductase enzymes from the liver microsomes of control and PB-pretreated rats behave similarly during chromatography on Sephadex G-100 and on DEAE-cellulose. In addition, they appear immunologically identical on the basis of Ouchterlony double diffusion analysis against antibody prepared to the trypsin-solubilized reductase from the microsomes of PB-pretreated rats. These observations cannot be considered proof that the reductase enzymes present in these microsomes are the same, however, since these studies were performed on protease-solubilized enzymes and proteases undoubtedly modify the catalytic properties of the reductase, as suggested by the inability of such enzymes to reconstitute microsomal hydroxylations [15, 16].

Antibody prepared to protease-solubilized NADPH-cytochrome c reductase enzymes present in the liver microsomes of PB-pretreated rats has also been shown to inhibit, to the same extent, the reductase enzymes present in the microsomes from control and PB-pretreated rats [14, 17] and in the microsomes from 3-MC-pretreated rats [17]. While this property too suggests that the reductases found in all three types of microsomes are immunologically similar, it also cannot be taken as proof that they are the same, since a similar antibody is capable of inhibiting the reductase from adrenal microsomes, and the enzymes from liver and adrenal microsomes are not immunologically identical on the basis of Ouchterlony double diffusion analysis [18].

More recently, Lu *et al.* [9] have attempted to study the existence of multiple forms of the reductase by using partially purified detergent-solubilized NADPH-cytochrome c reductase fractions from the liver microsomes isolated from PB- and 3-MC-pretreated rats to reconstitute hydroxylation activity with partially purified cytochrome P-450 fractions also isolated from these liver microsomes. The results of these studies suggested that, for benzphetamine *N*-demethylation, the reductase fractions play a role in the ability of the reconstituted system to metabolize this compound. This suggests that different reductases may be present in the liver microsomes isolated from 3-MC- and PB-pretreated rats and emphasizes the necessity of pursuing studies on the physical properties of the "native" reductases present in these different microsomes.

We have recently described a technique which could be used to determine the molecular weight of the "native" reductase present in the microsomes isolated from PB-pretreated rats [19], and in this communication describe the use of this technique for the comparison of the molecular weights of the NADPH-cytochrome c reductase enzymes present in the rat liver microsomes from control and PB- or 3-MC-pretreated rats. This method employs the use of an antibody prepared to a protease (bromelain)-solubilized fragment of NADPH-cytochrome c reductase enzyme to immunoprecipitate the "native" form of this enzyme from sodium deoxycholate-solubilized ^{125}I-labeled microsomes. The molecular weight of the "native" form of the reductase enzyme was then determined by electrophoresing the immunoprecipitate on sodium dodecyl sulfate (SDS)-polyacrylamide gels and scanning the gels for ^{125}I to determine the migration position of the "native" reductase on the