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Species Differences in the Toxicity and Cytochrome P450 IIIA-Dependent Metabolism of Digitoxin

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SUMMARY

In rats, cytochrome P450 (P450) IIIA enzymes are an important determinant of digitoxin toxicity. Induction of these liver microsomal enzymes decreases the toxicity of digitoxin by increasing its oxidative cleavage to digitoxigenin bis- and monodigitoxoside (dt_2 and dt_1). The present study shows that the susceptibility of different mammalian species to digitoxin toxicity is inversely related to liver microsomal P450 IIIA activity (measured as testosterone 6β-hydroxylase activity). Based on this correlation, we correctly predicted that hamsters, which have the highest P450 IIIA activity, are extremely resistant to digitoxin toxicity. To further examine the relationship between digitoxin toxicity and P450 IIIA activity, the pathways of digitoxin metabolism catalyzed by liver microsomes from nine mammalian species were examined by high performance liquid chromatography. The overall rate of digitoxin metabolism varied ~90-fold and followed the rank order: hamster > rat > guinea pig > dog > mouse ≈ monkey > rabbit ≈ cat > human. The qualitative differences in digitoxin metabolism were as striking as the quantitative differences. Formation of 16- and/or 17-hydroxydigitoxin was the major pathway of digitoxin oxidation catalyzed by liver microsomes from hamster, guinea pig, rabbit, cat, dog, and cynomolgus monkey. Guinea pig and, to a lesser extent, hamster liver microsomes also converted digitoxin to an unknown metabolite, the formation of which was catalyzed by P450. None of the species examined catalyzed the 12-hydroxylation of digitoxin to digoxin at a high rate. Similarly, none of the species examined catalyzed a high rate of conversion of digitoxin to dt_2, with the notable exception of the rat. However, dt_2 formation was the major pathway of digitoxin metabolism catalyzed by human liver microsomes, although humans were much less active (~2%) than rats in this regard. The rate of dt_2 formation varied ~41-fold among 22 samples of human liver microsomes, which was highly correlated ($r = 0.841$) with the rate of testosterone 6β-hydroxylation. Antibody against rat P450 IIIA1 inhibited the high rate of dt_2 formation by rat liver microsomes and the low rate catalyzed by mouse, guinea pig, dog, monkey, and human liver microsomes. In contrast, anti-P450 IIIA1 did not inhibit the 12-, 16-, or 17-hydroxylation of digitoxin (or the formation of the unknown metabolite), despite the fact that anti-P450 IIIA1 strongly inhibited (>70%) the 6β-hydroxylation of testosterone by liver microsomes from each of the species examined (except rabbit liver microsomes, which were inhibited only ~30%). Compared with the other species examined, rats were unique in their ability to catalyze a high rate of conversion of digitoxin to dt_2. Consequently, the observed correlation between digitoxin toxicity and P450 IIIA activity is apparently fortuitous, because species differences in the conversion of testosterone to 6β-hydroxytestosterone do not reflect species differences in the conversion of digitoxin to dt_2 (or any other metabolite). Formation of dt_2 was the only pathway of digitoxin metabolism inhibited by antibody against P450 IIIA1, ruling out the possibility that a second P450 IIIA-catalyzed pathway of digitoxin oxidation might contribute to the apparent relationship between P450 IIIA activity and digitoxin toxicity. Whereas all mammalian species appear to contain P450 IIIA enzymes that catalyze the 6β-hydroxylation of testosterone, we propose that rats contain a P450 IIIA enzyme that is unique in its ability to catalyze the oxidative cleavage of digitoxin to dt_2 at a high rate.

Digitoxin is a cardiac glycoside that has been used clinically in the treatment of congestive heart conditions for more than 200 years (1–4). In rats, the metabolism of digitoxin involves sequential oxidative cleavage of two sugar residues (digitoxosides) by P450 IIIA to give dt_2 and dt_1, followed by glucuronidation of dt_1 (5–10). The toxicity of digitoxin in rats is influenced by the levels of P450 IIIA, which is a subfamily of liver microsomal P450 enzymes.[3] Induction of P450 IIIA by treatment of rats with catatoxic steroids (such as pregnenolone-16α-carbonitrile and dexamethasone) increases the oxidative metabolism and decreases the toxicity of digitoxin (14–19). The

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[3]The term P450 IIIA refers to a subfamily of closely related P450 enzymes. P450 IIIA1 and IIIA2 are rat enzymes, P450 IIIA3, IIIA4, IIIA5, and IIIA7 are

ABBREVIATIONS: P450, cytochrome P450; HPLC, high performance liquid chromatography; dt_2, digitoxigenin bisdigitoxoside; dt_1, digitoxigenin monodigitoxoside; dt_0, digitoxigenin.

protective effect of catatoxic steroids can be abolished by simultaneously treating rats with troleandomycin, which causes a marked and sustained inhibition of P450 IIIA (19). The constitutive levels of P450 IIIA in liver microsomes are greater in adult male than adult female rats (20–22). This sex difference in P450 IIIA levels affects the rate of conversion of digitoxin to dt_2 and dt_1 (male > female) (19) and apparently influences the toxicity of digitoxin, which is approximately twice as toxic to adult female rats (LD_{50} = 56.2 mg/kg orally or 8.9 mg/kg intravenously) than adult male rats (LD_{50} = 93.9 mg/kg orally or 15.4 mg/kg intravenously) (23).

Rat P450 IIIA also catalyzes several other reactions, including the 2β-, 6β-, and 15β-hydroxylation of testosterone (21–27). These pathways of testosterone oxidation (as well as several minor pathways) are catalyzed by purified IIIA1 and IIIA2 (24–27) and are inhibited up to 95% when rat liver microsomes are incubated with antibody against IIIA1 or IIIA2 (21, 24, 26, 27). The 6β-hydroxylation of testosterone is a major pathway of testosterone oxidation catalyzed by liver microsomes from numerous mammalian species. We have shown that the overall rate of testosterone 6β-hydroxylation by liver microsomes from nine mammalian species varies ~65-fold and follows the rank order cat < rabbit < guinea pig < dog < human < rat < cynomolgus monkey < mouse < hamster (28). With one exception, the 6β-hydroxylation of testosterone by liver microsomes from these mammalian species is inhibited >70% by antibody against rat IIIA1 (29). The exception is liver microsomes from rabbits, which are inhibited only 30% by anti-IIIA1. Rabbit liver microsomes are also atypical in that they do not catalyze the 2β- and 15β-hydroxylation of testosterone. The testosterone 6β-hydroxylase activity that can be inhibited by anti-IIIA1 varies ~76-fold among the aforementioned species and follows the same rank order described above. These antibody-inhibition experiments suggest that P450 IIIA is the liver microsomal enzyme that catalyzes the 6β-hydroxylation of testosterone in most mammalian species, which implies that P450 IIIA activity can vary more than 65-fold among different mammalian species.

The apparent relationship between liver microsomal P450 IIIA activity and digitoxin toxicity in the rat, coupled with the aforementioned observations on species differences in testosterone 6β-hydroxylase activity, raises the possibility that species differences in liver microsomal P450 IIIA might contribute significantly to species differences in digitoxin toxicity. The studies described in this paper were designed to investigate this possibility, i.e., whether species differences in digitoxin toxicity correlate with species differences in the P450 IIIA-dependent metabolism of digitoxin.

Materials and Methods

Chemicals. Digitoxin, 12-hydroxydigitoxin (digoxin), estradiol-3-methyl ether, androstenedione, and dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Digitoxin was purchased from New England Nuclear (Boston, MA). 16-Hydroxydigitoxin (gigitoxin), dt_2, dt_1, and dt_0 were purchased from Atomergic Chemetals (Farmingdale, NY). 17-Hydroxydigitoxin and 17-hydroxy-dt_0 were generously provided by Dr. M. A. Figueira at the Gulbenkian Institute (Oeiras Codex, Portugal) or were synthesized according to published procedures (30–32).

Animals and preparation of liver microsomes. Unless otherwise indicated, the animals used in this study were 8–10-week-old males. Long Evans rats (n = 6) were purchased from Blue Spruce Farms (Altamont, NY); C3H/OuJ mice (n = 5) were from The Jackson Laboratory (Bar Harbor, ME); Golden Syrian hamsters (n = 5) and Hartley guinea pigs (n = 4) were from Sasco (Omaha, NE), and New Zealand White rabbits (n = 4) were from White Hare Rabbitry (Stark, MO). Animals were allowed free access to feed and water and were allowed to acclimate to a 12-hr diurnal light cycle. An 8–12-month-old cat (n = 1) was purchased from Brink Kennel (Paola, KS) and euthanized the day of arrival by lethal injection of T-61 euthanasia solution (American Hoechst Corp., Somerville, NJ). This procedure was performed by a trained Animal Care worker, in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Frozen liver samples from mature male cynomolgus monkeys (n = 4) were provided by Dr. William Bracken of Alcon Laboratories (Fort Worth, TX). Frozen liver samples from human organ donors (n = 22) were provided by the Stanford Research Institute (Menlo Park, CA). Liver microsomes were prepared by the method of Lu and Levin (33) and were stored at −80° as a concentrated suspension in 0.25 M sucrose. In addition to these microsomes, liver microsomes from mature male beagle dogs (n = 9) were generous gifts from Dr. James Halpert (University of Arizona) and Tammy Braun (Texas A & M University).

Metabolism of digitoxin. Liver microsomes (0.5–1.0 mg of protein/ml) were incubated at 37° in 1-ml incubation mixtures containing potassium phosphate buffer (100 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), NADP (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 unit/ml), and digitoxin (50 μM) or [^{3}H]digitoxin (50 μM, 4 μCi/ml). Reactions were initiated by addition of the NADPH-generating system and were terminated after 1–30 min by addition of 6 ml of dichloromethane. Estradiol-3-methyl ether (1.0 nmol in 100 μl of dichloromethane) was added to each sample as an internal standard. The samples were vigorously mixed for 2 min on a batch vortexer and were subjected to low speed centrifugation (1000 × g for 10–15 min). In some cases, an aliquot (200 μl) of the aqueous (upper) phase was mixed with 5 ml of scintillation fluid (BioSafe II; Research Products International, Mount Prospect, IL), and radioactivity was determined with a Packard Tri-Carb 3330 scintillation counter. An aliquot (4 ml) of the organic phase was transferred to a culture tube (12 × 75 mm) and evaporated in a vacuum concentrator (Savant SV-100 Speed Vac) or under a stream of nitrogen gas at 35°. The residue was dissolved in 200 μl of mobile phase (see below), and a 50-μl aliquot was analyzed by reverse phase HPLC.

HPLC. A modification of the HPLC method of Plum and Daldrup (34) was used in this study. Digitoxin, seven potential metabolites, and the internal standard estradiol-3-methyl ether were resolved on a Supelcosil C_{18} column (5 μm, 15 cm × 4.6 mm) with a Shimadzu LC-6A HPLC system equipped with an SIL-6A autosampler (Shimadzu Scientific Instruments, Columbia, MD). The column was preceded by a 2-cm LC-18 guard column (40 μm) (Supelco, Bellefonte, PA). Digitoxin and metabolites were eluted isocratically over ~30 min, at room temperature, with methanol/acetonitrile/water (30:30:40, v/v/v), at a flow rate of 1 ml/min. When nonradioactive digitoxin was used as substrate, metabolites and unreacted substrate were monitored at 220 nm, with a variable wavelength UV detector (Shimadzu SPD-6A), and quantified by comparison of their peak areas (measured with a Shimadzu C-R3A integrator) with those of authentic standards. In terms of rates of digitoxin oxidation, the average limit of detection of this analytical system was <0.5 pmol/mg of microsomal protein/min. Recovery was determined with the internal standard estradiol-3-methyl ether, which eluted after digitoxin. In most cases, androstenedione, which eluted before digitoxin, could be used as an alternative internal

human enzymes, and IIIA6 is a rabbit enzyme. Rat IIIA1 is also known as cytochrome PCN1, P450p, $P450_{6\beta\text{-}4}$, and PCNa. The closely related isozyme, IIIA2, is also known as PCN2 and is apparently identical to cytochrome $P450_{PCN\text{-}E}$, PB-2a, P450 PB-1, $P450_{6\beta\text{-}1}$ or $P450_{6\beta\text{-}3}$, and PCNb or PCNc. Human IIIA3, IIIA4, IIIA5, and IIIA7 are also known as HLp, $P450_{NF}$, HLp2, and HLp3, respectively. Rabbit IIIA6 is also known as P450 3c (11–13).

standard, which reduced the analysis time from ~30 min to ~19 min. A problem with using androstenedione as the internal standard was that it coeluted with what appears to be an unstable metabolite of digitoxin that was only observed when dt_2 was formed. Based on studies by Schmoldt and Rohloff (7), this unstable metabolite is probably 9′-dehydrodigitoxin. Certain microsomal samples (such as those from monkeys and humans) contained substances that coeluted with authentic standards (such as digoxin). This problem was circumvented by incubating liver microsomes with [^{3}H]digitoxin and quantifying the metabolites with a flow-through radioactivity detector (Ramona-LS, Raytest USA Inc., Pittsburgh, PA). The flow-through radioactivity detector was operated with a 1.8-ml flow cell and a 1:3 ratio of mobile phase to scintillation fluid (Flo-Scint III; Radiomatic Instruments and Chemical Co., Inc., Tampa, FL). Because an isocratic system was used, detection efficiency did not vary throughout the HPLC analysis. Recovery was determined from the sum of the radioactivity associated with all metabolites and unreacted substrate and was confirmed by measuring the radioactivity, by scintillation counting, in an aliquot (10 μl) of each sample analyzed by HPLC.

Testosterone oxidation. Liver microsomes (0.1–0.5 mg of protein/ml) were incubated at 37° in 1-ml incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), NADP (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 unit/ml), and testosterone (250 μM). Reactions were initiated by addition of the NADPH-generating system and were terminated after 10 min by addition of 6 ml of dichloromethane. Each sample was spiked with 11β-hydroxytestosterone (3 nmol in 100 μl of dichloromethane) as an internal standard. Metabolites were resolved and quantified by HPLC as described by Sonderfan *et al.* (22). Incubations also contained 2.5 μM 17β-*N*,*N*-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one (a steroid 5α-reductase inhibitor), as described by Sonderfan and Parkinson (35).

Antibody-inhibition experiments. Liver microsomes were incubated for 15 min at room temperature with rabbit polyclonal antibody against P450 IIIA1 (0–20 mg of IgG/mg of microsomal protein). The anti-IIIA1 had been subjected to immunoabsorption chromatography to remove antibodies that cross-reacted with enzymes belonging to other P450 subfamilies, as described by Halvorson *et al.* (26). The total amount of antibody in each incubation was adjusted to a constant concentration with IgG purified from preimmune rabbit serum. After 15 min, each sample was incubated with digitoxin or testosterone as described above. Reactions were terminated after 0, 10, or 15 min. Digitoxin and testosterone metabolites were resolved and quantified by HPLC, as described above.

Other assays. Protein concentration was measured by the method of Lowry *et al.* (36), with bovine serum albumin as standard. The concentration of P450 was determined by the method of Omura and Sato (37), from the carbon monoxide difference spectrum of dithionite-reduced microsomes, based on a extinction coefficient of 91 mM^{-1} cm^{-1}. To compensate for hemoglobin contamination, the concentration of P450 in monkey liver microsomes was determined by the method of Matsubara *et al.* (38), from the difference spectrum of dithionite-reduced versus oxidized microsomes gassed with carbon monoxide, based on an extinction coefficient of 104 mM^{-1} cm^{-1}.

Toxicity of digitoxin in hamsters. Eight-week-old-male hamsters (six/group) were treated intraperitoneally with 0, 100, 250, 500, 750, 1000, or 1250 mg/kg digitoxin (dissolved in dimethyl sulfoxide). Eight-week-old-male rats (n = 5) treated intraperitoneally with 10 mg/kg digitoxin (2 × LD_{50}) served as positive controls. Lethality was recorded for 24 hr, at which time the study was terminated (although some overtly moribund animals were euthanized throughout the experiment).

Results and Discussion

Species differences in digitoxin toxicity and P450 IIIA activity. The P450 IIIA-dependent oxidative cleavage of digitoxin to dt_2 is the major pathway of digitoxin metabolism catalyzed by rat liver microsomes (5, 7, 9, 10). P450 IIIA inducers, such as pregnenolone-16α-carbonitrile, dexamethasone, and spironolactone, stimulate this pathway of metabolism and protect rats against digitoxin toxicity (9, 10, 16–19, 39). These data suggest that P450 IIIA is an important determinant of digitoxin toxicity in rats (10), but the role of P450 IIIA in digitoxin metabolism by other mammalian species has not been determined. However, P450 IIIA levels are known to vary considerably among different species (20, 28), as does the susceptibility of different species to digitoxin toxicity (40–43). Species differences in P450 IIIA activity were found to correlate well with species differences in digitoxin toxicity, as shown in Fig. 1. Published or experimentally determined LD_{50} values were used to compare the acute toxicity of digitoxin in cats (44), dogs (45), rabbits (45), guinea pigs (44), mice (44), rats (44), and hamsters (present study). The activity of P450 IIIA represents liver microsomal testosterone 6β-hydroxylase activity that could be inhibited with antibody against rat P450 IIIA1 (although essentially the same results were obtained if total testosterone 6β-hydroxylase activity was plotted against digitoxin toxicity).

The results in Fig. 1 suggest that the susceptibility of mammals to digitoxin toxicity is inversely related to the activity of liver microsomal P450 IIIA. When this inverse relationship first became apparent, data on the toxicity of digitoxin in hamsters were not available. As indicated in Fig. 1, we correctly predicted that hamsters, which have the greatest P450 IIIA activity, are extremely resistant to the toxic effects of digitoxin (hamsters were 2 orders of magnitude more resistant to digitoxin toxicity than were rats, which are considered to be a relatively resistant species). This correct prediction added support to the hypothesis that species differences in the P450 IIIA activity contribute significantly to species differences in digitoxin toxicity. To test this hypothesis further, liver microsomes were incubated with digitoxin, to determine whether species differences in the rate of digitoxin metabolism correlated with species differences in digitoxin toxicity. We predicted that species differences in IIIA activity (measured as testosterone 6β-hydroxylase activity) would give rise to similar differences in the rate of conversion of digitoxin to dt_2, which is known to

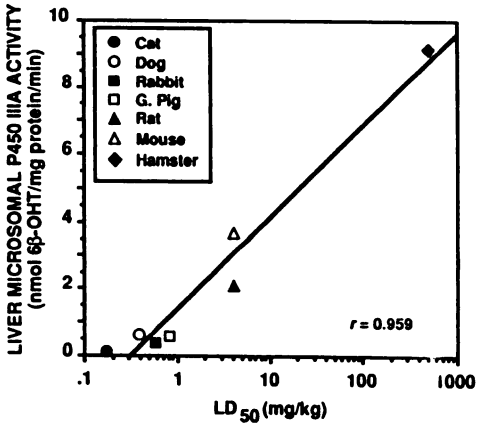

Fig. 1. Relationship between digitoxin toxicity and liver microsomal P450 IIIA activity. Published or experimentally determined LD_{50} values were used to compare the acute toxicity of digitoxin in cats, dogs, rabbits, guinea pigs, mice, rats, and hamsters (18, 40–45). The activity of P450 IIIA represents liver microsomal testosterone 6β-hydroxylase activity that was inhibited with antibody against rat P450 IIIA1 (29).

be the major digitoxin metabolite formed by rat P450 IIIA (7, 9, 19).

Species differences in digitoxin metabolism. The overall rate of digitoxin oxidation by liver microsomes from the nine mammalian species tested varied ~90-fold, as shown in Fig. 2. This variation was not correlated with the amount of liver microsomal P450, which varied ~5-fold. On a per milligram of protein basis, the overall rate of digitoxin oxidation was highest for hamster and was lowest for human (~340 versus 4 pmol/mg of protein/min). On a per nmole of P450 basis, the overall rate of digitoxin oxidation was highest for rat and was lowest for rabbit (~253 versus 6 pmol/nmol of P450/min). Water-soluble metabolites accounted for 3, 14, 22, 28, 30, 38, 41, 57, and 71% of the total digitoxin oxidation catalyzed by liver microsomes from the rat, rabbit, human, hamster, dog, monkey, mouse, guinea pig, and cat, respectively. The identity of the water-soluble metabolites was not determined, whereas the organic-soluble metabolites were resolved and quantified by reverse phase HPLC. The method described in Materials and Methods resolved digitoxin, seven potential metabolites of digitoxin, and the internal standard estradiol-3-methyl ether, as shown in Fig. 3. In all cases, the recovery of digitoxin and each of the seven available metabolites from mock incubation mixtures exceeded 85%.

Fig. 4 shows the individual metabolites of digitoxin formed by liver microsomes from each of the species tested. The qualitative differences in digitoxin metabolism were as striking as the quantitative differences. As previously reported (7, 9, 19), rat liver microsomes primarily catalyzed the oxidative cleavage of digitoxin to dt_2, whereas 12-hydroxylation to form digoxin was a minor pathway of metabolism. Only a small amount of dt_1 was formed (presumably by further metabolism of dt_2) during the short incubation times used in this experiment. Mouse liver microsomes also formed dt_2 as a primary pathway of digitoxin metabolism but at ~1/14 the rate catalyzed by rat liver microsomes. Hamster liver microsomes, which catalyzed the highest overall rate of digitoxin oxidation, formed two primary metabolites, 16- and 17-hydroxydigitoxin. The 17-hydroxylation of digitoxin was also the major pathway of digitoxin metabolism catalyzed by guinea pig liver microsomes, as

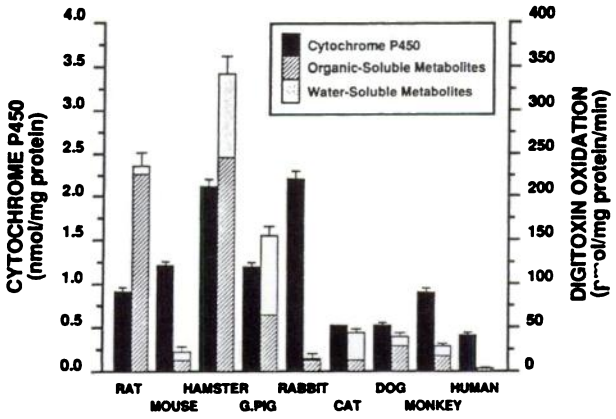

Fig. 2. Comparison of P450 levels and total digitoxin metabolism in liver microsomes from different mammalian species. Formation of water-soluble and organic-soluble metabolites of [^{3}H]digitoxin by liver microsomes was measured by scintillation counting and reverse phase HPLC analysis, respectively, as described in Materials and Methods. With the exception of the cat (n = 1) and human (n = 22), values represent the mean ± standard error of 4–6 determinations.

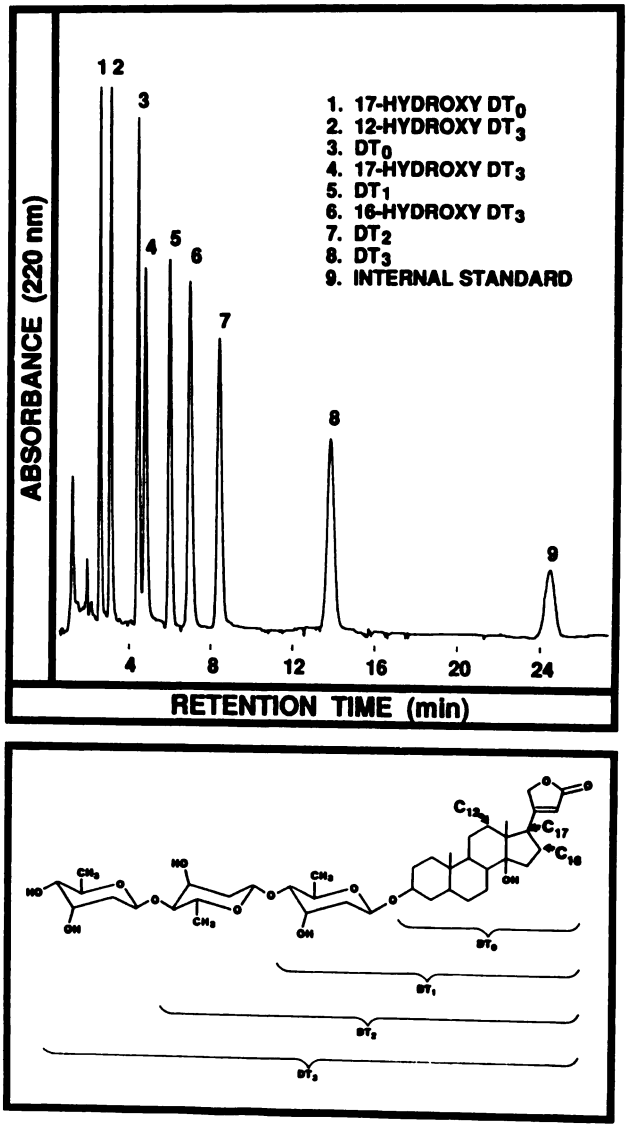

Fig. 3. Separation of digitoxin and its metabolites by reverse phase HPLC. A mixture of authentic standards and the internal standard estradiol-3-methyl ether were resolved by reverse phase HPLC, as described in Materials and Methods. dt_3, digitoxin.

previously reported by Carvalhas *et al.* (32). Oxidative cleavage of digitoxin to dt_2 was a minor pathway of metabolism catalyzed by guinea pig liver microsomes. In addition, liver microsomes from guinea pig and hamster converted digitoxin to a polar metabolite whose retention time (~3.7 min) failed to match any of the available standards (see below). Rabbit liver microsomes converted digitoxin to a single metabolite, 17-hydroxydigitoxin. Cat liver microsomes formed 16- and 17-hydroxydigitoxin at equal rates. Dog liver microsomes catalyzed the 16-hydroxylation of digitoxin and the oxidative cleavage to dt_2 in a 2:1 ratio. Likewise, monkey liver microsomes catalyzed these reactions and the 17-hydroxylation of digitoxin at approximately equal rates. Human liver microsomes, which had the lowest overall rate of digitoxin metabolism, catalyzed the oxidative cleavage of digitoxin to dt_2 at ~1/65 the rate catalyzed by rat liver microsomes.

As mentioned above, guinea pig and hamster liver microsomes converted digitoxin to a polar metabolite that was chro-

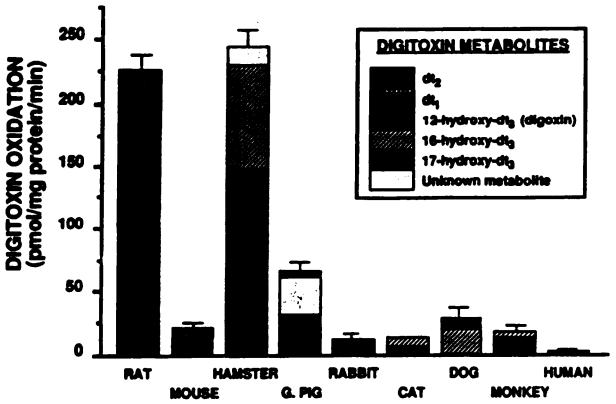

Fig. 4. Pathways of digitoxin oxidation by liver microsomes from different mammalian species. Liver microsomes (0.5–1.0 mg/ml) were incubated with [^{3}H]digitoxin (50 μM, 4 μCi/ml) for 1–30 min, as described in Materials and Methods. Digitoxin metabolites were resolved and quantified by reverse phase HPLC. With the exception of the cat (n = 1) and human (n = 22), values represent the mean ± standard error of 4–6 determinations.

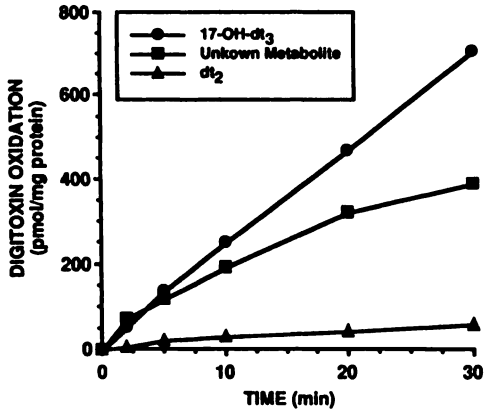

Fig. 5. Time course of digitoxin oxidation by guinea pig liver microsomes. Pooled guinea pig liver microsomes (1.0 mg/ml) were incubated with digitoxin (50 μM) for 1–30 min, as described in Materials and Methods. Digitoxin metabolites were resolved and quantified by reverse phase HPLC. Values represent the average of duplicate incubations, which varied <5%.

matographically distinct from the available standards. This metabolite accounted for 35–40% and 5–6% of the total digitoxin oxidation catalyzed by guinea pig and hamster liver microsomes, respectively. Formation of this metabolite required NADPH, was inhibited by antibody against rat NADPH-P450 reductase, and was inhibited by a 90:10 mixture of carbon monoxide and oxygen. These results implicate P450 in the formation of the unknown metabolite. Inasmuch as 17-hydroxydigitoxin and dt_2 were also formed by guinea pig microsomes, we considered the possibility that the unknown metabolite was formed either by 17-hydroxylation of dt_2 or by oxidative cleavage of 17-hydroxydigitoxin, both of which would form 17-hydroxy-dt_2. However, the unknown metabolite was not formed when guinea pig liver microsomes were incubated with either 17-hydroxydigitoxin or dt_2. Furthermore, a time-course study revealed no lag in the formation of the unknown metabolite, as shown in Fig. 5. These results indicate that the unknown compound is a primary metabolite of digitoxin formed by P450 and is not a secondary metabolite of either 17-hydroxydigitoxin or dt_2. However, the identify of this metabolite remains unknown.

Human liver microsomes primarily catalyzed the conversion of digitoxin to dt_2, but there was considerable sample to sample variation in the rate of this reaction, as previously reported (46, 47). Fig. 6 shows the rate of dt_2 formation and the 6β-hydroxylation of testosterone catalyzed by human liver microsomes from 22 different individuals (14 males and 8 females). Information on the individuals from whom these samples were obtained is summarized in Table 1. The rate at which liver microsomes from these individuals catalyzed the conversion of digitoxin to dt_2 varied ~41-fold (0.39–15.8 pmol/mg of protein/min), compared with an ~11-fold variation in the rate at which they catalyzed the 6β-hydroxylation of testosterone (1.2–13.7 nmol/mg of protein/min). There was, however, a fairly good correlation (r = 0.841) between the rate of dt_2 formation and the rate of testosterone 6β-hydroxylation (with few exceptions, the rate of dt_2 formation by rat liver microsomes also correlates well with the rate of testosterone 6β-hydroxylation). These data suggest that the same human enzyme or enzymes catalyze both of these reactions (see below). Fig. 7 shows a time course of digitoxin oxidation catalyzed by the most active human liver microsomes (sample H2, from a 39-year-old female). A decrease in the rate of dt_2 formation with time was accompanied by an increase in the rate of formation of dt_1. This lag in dt_1 formation suggests that dt_1 is not a primary metabolite of digitoxin but forms from the further metabolism of dt_2, as previously reported for rat liver microsomes (7). In addition to dt_2 and dt_1, some of the human liver microsomal samples also catalyzed the formation of digoxin as a very minor pathway of digitoxin oxidation. The conversion of digitoxin to digoxin has previously been reported to be quantitatively negligible in humans (48).

Species differences in dt_2 versus 6β-hydroxytestosterone formation. The results shown in Fig. 4 reveal major differences in the rate and/or ability of liver microsomes from the various species tested to catalyze the oxidative cleavage of digitoxin to dt_2. These data indicate that liver microsomes from some species, including those from the hamster (the species most resistant to digitoxin toxicity), do not catalyze the conversion of digitoxin to dt_2. In addition, the results in Fig. 4 suggest that rat liver microsomes are somewhat unique in their ability to catalyze the conversion of digitoxin to dt_2 at a high rate. As previously mentioned, we predicted that species differences in the rate of testosterone 6β-hydroxylation would give

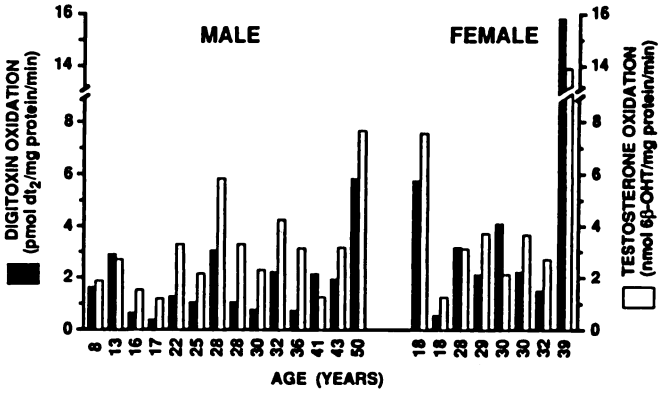

Fig. 6. Interindividual differences in digitoxin and testosterone oxidation catalyzed by human liver microsomes. Human liver microsomes (0.4–1.0 mg/ml) were incubated either with [^{3}H]digitoxin (50 μM, 4 μCi/ml) for 30 min or with testosterone (250 μM) for 10 min, as described in Materials and Methods. Digitoxin and testosterone metabolites were resolved and quantified by reverse phase HPLC. Values represent the average of duplicate incubations, which varied <5%.

TABLE 1

History of human liver samples

All liver samples were obtained from the Stanford Research Institute, International (Menlo Park, CA), under contracts NIEHS ES-55109 and NIDDC N01-DK-8-2235. All samples tested negative for human immunodeficiency virus. Several were tested and found to be negative for hepatitis B, syphilis, and cytomegalovirus. Samples H-21 and H-24 tested positive for cytomegalovirus.

ID	Age	Sex	Race	Case history
	years			
H-21	8	M	Caucasian	Gunshot to head
H-16	13	M	Unknown	Cerebral hemorrhage from bicycle-automobile accident; pentobarbital 4 days before death; Tiurett syndrome
H-6	16	M	Caucasian	Gunshot to head
H-15	17	M	Unknown	Motor vehicle accident
H-37	22	M	Unknown	Gunshot to head
H-23	25	M	Caucasian	Motorcycle accident
H-8	28	M	Unknown	Gunshot to head
H-9	28	M	Unknown	Gunshot to head; cocaine and intravenous drug user
H-4	30	M	Caucasian	Pedestrian hit by car
H-38	32	M	Unknown	Alcohol and crack abuse
H-19	36	M	Caucasian	Subarachnoid hemorrhage
H-18	41	M	Caucasian	Motorcycle accident
H-7	43	M	Caucasian	Intracranial hemorrhage
H-35	50	M	Unknown	Cerebral infarct with brain stem hernia; history of alcohol abuse
H-10	18	F	Unknown	Stroke
H-26	18	F	Oriental	No case history
H-31	28	F	Unknown	No case history
H-24	29	F	Unknown	Automobile accident; light smoker
H-3	30	F	Caucasian	Glioblastoma; 25 weeks pregnant
H-17	30	F	Caucasian	Motorcycle accident
H-5	32	F	Caucasian	Suicide by carbon monoxide poisoning
H-2	39	F	Caucasian	Subarachnoid hemorrhage

rise to similar differences in the rate of conversion of digitoxin to dt_2. However, as indicated in Fig. 8, there was no correlation ($r = 0.004$) between the rate of these two reactions catalyzed by liver microsomes from the nine species tested. Consequently, species differences in digitoxin toxicity do not correlate with differences in the rate of digitoxin metabolism. In other words, the correlation between digitoxin toxicity (LD_{50} values) and P450 IIIA activity (measured as testosterone 6β-hydroxylase activity) among different species is apparently fortuitous (see Fig. 1). The lack of correlation between dt_2 formation and testosterone 6β-hydroxylation raises the question: does P450 IIIA even play a role in digitoxin metabolism by liver microsomes from species other than the rat?

Role of P450 IIIA in digitoxin metabolism. The role of P450 IIIA in digitoxin metabolism by liver microsomes was assessed by antibody-inhibition experiments. Liver microsomes from all nine species were incubated with antibody against rat P450 IIIA1 (0–2 mg of IgG/mg of microsomal protein) for 15 min at room temperature, after which digitoxin metabolism was measured as described in Materials and Methods. We have

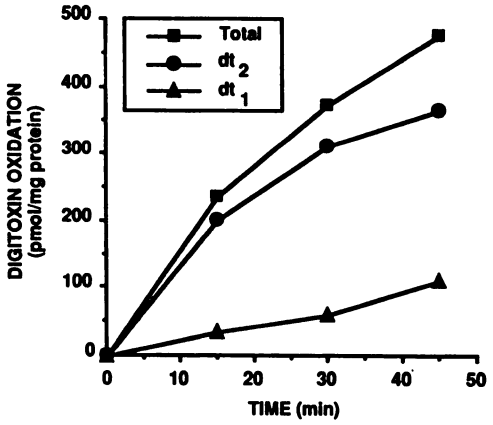

Fig. 7. Time course of digitoxin oxidation by human liver microsomes. Human liver microsomes (1.0 mg/ml from sample H2) were incubated with [^{3}H]digitoxin (50 μM, 4 μCi/ml) for 0–45 min, as described in Materials and Methods. Digitoxin metabolites were resolved and quantified by reverse phase HPLC. Values represent the average of duplicate incubations, which varied <5%.

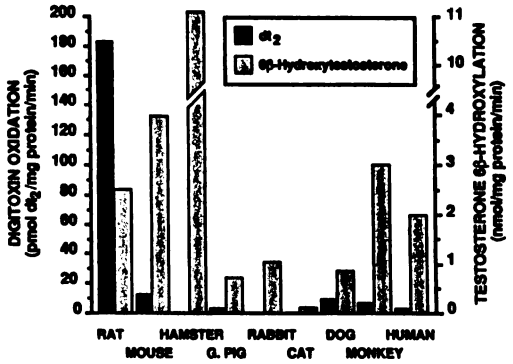

Fig. 8. Comparison of dt_2 and 6β-hydroxytestosterone formation by liver microsomes from different mammalian species. Liver microsomes (0.5–1.0 mg/ml) were incubated with [^{3}H]digitoxin (50 μM, 4 μCi/ml) for 1–30 min, as described in Materials and Methods. Digitoxin metabolites were resolved and quantified by reverse phase HPLC. Testosterone oxidation represents the amount of 6β-hydroxylase activity that could be inhibited by anti-P450 IIIA1 (29).

previously shown that, with the exception of rabbit liver microsomes, the 6β-hydroxylation of testosterone by liver microsomes from these same species is inhibited >70% by anti-P450 IIIA1 (29). With rat liver microsomes, anti-P450 IIIA1 inhibited the conversion of digitoxin to dt_2 by ~95%, but it did not inhibit the formation of digoxin, as shown in Fig. 9. These results confirm previous reports that the oxidative cleavage of digitoxin by rat liver microsomes is catalyzed by P450 IIIA, whereas the 12-hydroxylation of digitoxin to digoxin is catalyzed by another, as yet unidentified, form of P450 (19, 49). Under these conditions, anti-P450 IIIA1 also inhibited the low rate of dt_2 formation by liver microsomes from the mouse, guinea pig, dog, monkey, and human. Anti-P450 IIIA1 had little or no effect (<10%) on other pathways of digitoxin oxidation catalyzed by liver microsomes from the various species examined (results not shown). These results suggest that, for all of the species examined, the conversion of digitoxin to dt_2 is catalyzed predominately by P450 IIIA, whereas enzymes other than IIIA catalyze the 12-, 16-, and 17-hydroxylation of digitoxin (as well as formation of the unknown metabolites by guinea pig and hamster liver microsomes).

The effects of anti-P450 IIIA1 on both digitoxin and testos-

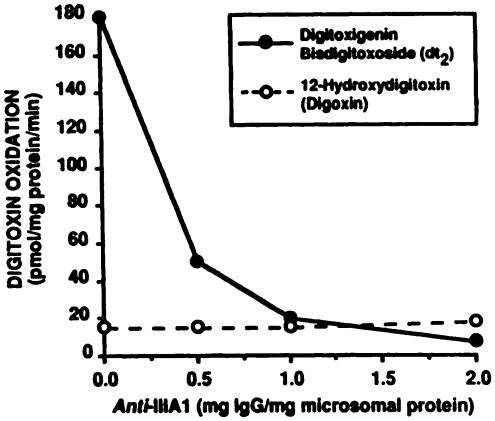

Fig. 9. Effects of antibody against rat P450 IIIA1 on digitoxin oxidation by rat liver microsomes. Rat liver microsomes (1.0 mg of protein/ml) were incubated at room temperature with either 0, 0.5, 1.0, or 2.0 mg/ml anti-P450 IIIA1. The total amount of IgG in each incubation was adjusted to 2.0 mg/ml with an IgG fraction purified from preimmune rabbit antiserum. After 15 min, each sample was incubated at 37° with digitoxin (50 μM), as described in Materials and Methods. Digitoxin metabolites were resolved and quantified by reverse phase HPLC. Values represent the average of duplicate incubations, which varied <5%.

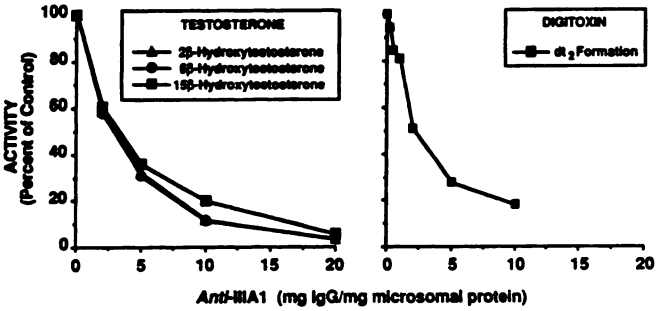

Fig. 10. Effects of antibody against rat P450 IIIA1 on testosterone and digitoxin oxidation by human liver microsomes. *Left*, human liver microsomes (0.1 mg/ml from sample H2) were incubated at room temperature with 0, 0.2, 0.5, 1.0, or 2.0 mg/ml anti-P450 IIIA1. The total amount of IgG in each incubation was adjusted to 2.0 mg/ml with IgG purified from preimmune antiserum. After 15 min, each sample was incubated at 37° for 15 min with testosterone (250 μM), as described in Materials and Methods. Testosterone metabolites were resolved and quantified by reverse phase HPLC. Values represent the average of duplicate incubations, which varied <5%. The absolute rates of testosterone 2β-, 6β-, and 15β-hydroxylation were 2.2, 25, and 0.94 nmol/mg of microsomal protein/min, respectively. *Right*, human liver microsomes (1.0 mg/ml from sample H2) were incubated at room temperature with 0, 0.2, 0.5, 1.0, 2.0, 5.0, or 10.0 mg/ml anti-P450 IIIA1. The total amount of IgG in each incubation was adjusted to 10 mg/ml with IgG purified from preimmune antiserum. After 15 min, each sample was incubated at 37° for 15 min with digitoxin (50 μM), as described in Materials and Methods. Digitoxin metabolites were resolved and quantified by reverse phase HPLC. Values represent the average of duplicate incubations, which varied <5%. The absolute rate of dt_2 formation was 16 pmol/mg of microsomal protein/min.

terone oxidation by human liver microsomes (sample H2) are shown in Fig. 10. The rates of testosterone 2β-, 6β-, and 15β-hydroxylation and the conversion of digitoxin to dt_2 were inhibited in a similar fashion by anti-P450 IIIA1. In incubations containing 10 mg of anti-P450 IIIA1/mg of microsomal protein, the rates of testosterone 2β-, 6β-, and 15β-hydroxylation and the conversion of digitoxin to dt_2 were inhibited 88, 88, 80, and 82%, respectively. Comparable inhibition of human liver microsomal testosterone 2β-, 6β-, and 15β-hydroxylase activity by 10 mg of anti-P450 IIIA2/mg of microsomal protein has previously been reported by Waxman *et al.* (50). In our studies, all three pathways of testosterone oxidation were further inhibited (by 96, 96, and 94%) when the ratio of anti-P450 IIIA1 was increased to 20 mg of IgG/mg of microsomal protein. The influence of anti-P450 IIIA1 on digitoxin oxidation was not evaluated at such high ratios because quantitation of the low rates of dt_2 formation required relatively high concentrations of microsomal protein. Nevertheless, these antibody-inhibition experiments strongly suggest that the 2β-, 6β-, and 15β-hydroxylation of testosterone and the oxidative cleavage of digitoxin to dt_2 are catalyzed by human liver microsomal P450 IIIA.

We have previously shown that the conversion of digitoxin to dt_2 by rat liver microsomes is inhibited ~46% by 100 μM testosterone, which suggests that digitoxin and testosterone are substrates for the same enzyme (51). The effects of testosterone on the oxidation of digitoxin by human liver microsomes (sample H2) are shown in Fig. 11. When human liver microsomes were simultaneously incubated with digitoxin and testosterone, testosterone caused a progressive inhibition of dt_2 formation at concentrations above 100 μM. These data suggest that, like the rat enzyme, testosterone is a substrate for the human enzyme that catalyzes the oxidative cleavage of digitoxin. Fig. 11 also shows the effects of increasing concentrations of α-naphthoflavone or potassium phosphate on digitoxin oxidation catalyzed by human liver microsomes. α-Naphthoflavone and high ionic strength buffers have previously been shown to stimulate reactions catalyzed by rat P450 IIIA (52). The results in Fig. 11 show that, as in the case of rat liver microsomes, the oxidative cleavage of digitoxin to dt_2 by human liver microsomes was stimulated by α-naphthoflavone and high ionic strength. Inhibition by anti-P450 IIIA1 and testosterone and stimulation by α-naphthoflavone and high ionic strength, all suggest that the conversion of digitoxin to dt_2 by human liver microsomes is catalyzed by P450 IIIA, as it is in rat liver microsomes.

Species differences in P450 IIIA function. Compared with the other species examined in this study, rats are unique in their ability to catalyze the conversion of digitoxin to dt_2 at a high rate (Figs. 4 and 8). This finding was unexpected, because antibody-inhibition experiments indicate that the 6β-hydrox-

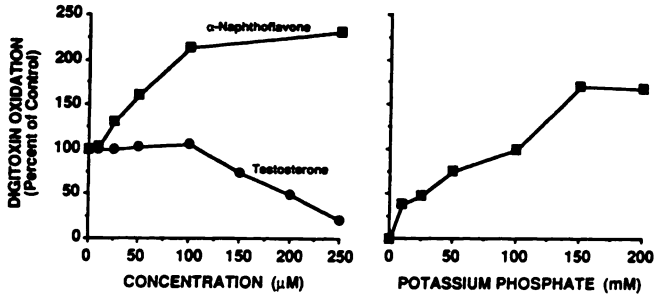

Fig. 11. Effects of testosterone, α-naphthoflavone, and potassium phosphate on digitoxin oxidation by human liver microsomes. *Left*, human liver microsomes (1.0 mg/ml from sample H2) were incubated for 15 min with digitoxin (50 μM) in the presence or absence of 0–250 μM testosterone or 0–250 μM α-naphthoflavone, as described in Materials and Methods. *Right*, human liver microsomes (1.0 mg/ml from sample H2) were incubated for 15 min with digitoxin (50 μM) in 0–200 mM potassium phosphate buffer, pH 7.25, as described in Materials and Methods. Digitoxin metabolites were resolved and quantified by reverse phase HPLC. Values represent the average of duplicate incubations, which varied <5%. Absolute rates of dt_2 formation in the absence of testosterone or α-naphthoflavone and in the presence of 100 μM potassium phosphate were ~15 pmol/mg of protein/min.

ylation of testosterone and the oxidative cleavage of digitoxin to dt_2 are both catalyzed by P450 IIIA enzymes (Figs. 8, 9, and 10). Liver microsomes from several species, including hamster, mouse, monkey, and human, catalyze rates of testosterone 6β-hydroxylation that are greater than or equal to those catalyzed by rat liver microsomes. In contrast, microsomes from these same species either are unable to catalyze the conversion of digitoxin to dt_2, or do so at only a fraction of the rate catalyzed by rat liver microsomes (Fig. 8). These results raise the question: why can rat P450 IIIA catalyze both the 6β-hydroxylation of testosterone and the conversion of digitoxin to dt_2 when, for all practical purposes, P450 IIIA from other species only catalyzes the 6β-hydroxylation of testosterone?

We recently reported that, under appropriate reconstitution conditions, purified rat P450 IIIA1 can catalyze the 6β-hydroxylation of testosterone but not the conversion of digitoxin to dt_2. Similar results were obtained with partially purified P450 IIIA2 (26). These results suggest that the ability of P450 IIIA enzymes to convert digitoxin to dt_2 is not a property of all rat P450 IIIA enzymes. The particular P450 IIIA enzyme responsible for converting digitoxin to dt_2 has not been identified. However, we have presented evidence that this reaction is catalyzed by a 50-kDa P450 IIIA enzyme, which is electrophoretically distinct from P450 IIIA1 and P450 IIIA2 (both of which migrate as 51-kDa proteins in sodium dodecyl sulfate-polyacrylamide gels) (51, 53). The 50-kDa enzyme is regulated in a similar but distinct manner from P450 IIIA1 and P450 IIIA2, and it may correspond to a P450 IIIA enzyme, designated $P450_{6\beta\text{-}2}$, that was recently purified by Nagata *et al.* (27). If this interpretation is correct, the available data would suggest that $P450_{6\beta\text{-}2}$ has undergone structural changes that enable it to convert digitoxin to dt_2 without losing its ability to catalyze the 6β-hydroxylation of testosterone. What these structural changes in $P450_{6\beta\text{-}2}$ might be is not known, but presumably they have not occurred to the same extent in other P450 IIIA enzymes, including those in the rat (i.e., P450 IIIA1 and P450 IIIA2), as well as those in other species. There is precedence for minor structural changes having a dramatic effect on the substrate specificity of P450 enzymes. For example, Lindberg and Negishi (54) have shown that a single amino acid substitution is sufficient to confer high testosterone 15α-hydroxylase activity on mouse $P450_{coh}$ (P450 IIA5).

There is also precedence for rat P450 IIIA enzymes being able to catalyze reactions that are not catalyzed efficiently by P450 IIIA enzymes in other species. For example, rat P450 IIIA catalyzes the 4-hydroxylation of (*S*)-mephenytoin, but this same reaction is not catalyzed by human P450 IIIA enzymes. In terms of species differences, however, there is an important difference between the P450 IIIA-dependent metabolism of digitoxin and the P450 IIIA-dependent metabolism of (*S*)-mephenytoin. In the case of digitoxin, the reaction catalyzed by rat P450 IIIA (i.e., oxidative cleavage to dt_2) is barely catalyzed by human liver microsomes. In the case of (*S*)-mephenytoin, however, the reaction catalyzed by rat P450 IIIA *is* catalyzed by human liver microsomes, but it is catalyzed by $P450_{MP}$, which belongs to the P450 IIC not the P450 IIIA gene family (55, 56). Whether the conversion of digitoxin to dt_2 and the 4-hydroxylation of (*S*)-mephenytoin are catalyzed by the same rat P450 IIIA enzyme is not known. However, the results of the present study preclude the possibility that the human P450 IIC enzyme or enzymes responsible for (*s*)-mephenytoin 4-hydroxylation also convert digitoxin to dt_2.

P450 IIIA and species differences in digitoxin toxicity. These studies were initiated to test the hypothesis that previously reported species differences in digitoxin toxicity are related to differences in P450 IIIA activity. A correlation between digitoxin toxicity (LD_{50} values) and P450 IIIA activity (measured as testosterone 6β-hydroxylase activity) among different species was observed, as shown in Fig. 1. However, this correlation is apparently fortuitous, because species differences in testosterone 6β-hydroxylase activity do not reflect differences in the rate of conversion of digitoxin to dt_2 (or any other metabolite); hence, there is no correlation between species differences in digitoxin toxicity and digitoxin metabolism (Figs. 1, 4, and 8). In fact, despite being the species most resistant to digitoxin toxicity and the one with the highest testosterone 6β-hydroxylase activity, hamsters do not catalyze the conversion of digitoxin to dt_2 (<0.5 pmol/mg of protein/min). Formation of dt_2 was the only pathway of digitoxin oxidation inhibited by anti-P450 IIIA, ruling out the possibility that a second P450 IIIA-catalyzed pathway of digitoxin oxidation might contribute to the apparent relationship between P450 IIIA levels and digitoxin toxicity. The results of these experiments suggest that species differences in digitoxin toxicity cannot be simply ascribed to species differences in P450 IIIA activity.

Whether pharmacokinetic differences in the metabolism of digitoxin contribute at all to species differences in digitoxin toxicity remains unanswered. Most of the available evidence indicates that species differences in toxicity are related to species differences in the sensitivity of the cation-transport enzyme Na^+/K^+ ATPase to inhibition by digitoxin (and other cardiac glycosides) (40–43). In other words, Na^+/K^+ ATPase in resistant species, such as the rat, mouse, and hamster, is less susceptible to the inhibitory effects of digitoxin than Na^+/K^+ ATPase in sensitive species, such as the cat, dog, monkey, and human. This explanation for species differences in digitoxin toxicity is supported by differences in the specific lethal effects of digitoxin and other cardiac glycosides in resistant versus susceptible species. In sensitive species, death is usually attributed to cardiotoxic effects, whereas neurotoxicity is normally the cause of death in resistant species. However, the large qualitative and quantitative differences in liver microsomal digitoxin oxidation make it difficult to eliminate a contributing role for metabolism in species differences in susceptibility to digitoxin toxicity.

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