7α-HYDROXYLATION OF TAURODEOXYCHOLIC ACID BY A RECONSTITUTED SYSTEM FROM RAT LIVER MICROSOMES

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SUMMARY

A reconstituted system from rat liver consisting of partially purified cytochrome P-450 and cytochrome P-450 reductase was found to catalyze an efficient 7α-hydroxylation of taurodeoxycholic acid in the presence of an NADPH-generating system. Addition of phosphatidyl choline stimulated the reaction slightly. The system had a low capacity to 6β-hydroxylate taurochenodeoxycholic acid and lithocholic acid.

The microsomal fraction of rat liver fortified with NADPH catalyzes 7α-hydroxylation of taurodeoxycholic acid and 6β-hydroxylation of taurochenodeoxycholic acid, taurolithocholic and lithocholic acid (1). Unconjugated deoxycholic and chenodeoxycholic acids are hydroxylated much less efficiently than the taurine conjugates whereas lithocholic acid is 6β-hydroxylated to about the same extent as taurolithocholic acid. Evidence has been presented that both the 7α-hydroxylation of taurodeoxycholic acid and the 6β-hydroxylation of taurochenodeoxycholic acid are catalyzed by cytochrome P-450-containing systems (2-5). Both reactions are stimulated by phenobarbital treatment and inhibited by carbon monoxide (2-4). Furthermore, taurodeoxycholic acid and taurochenodeoxycholic acid produce type I spectral changes when mixed with liver microsomes (5). Solubilization and partial purification of the 6β-hydroxylating system have been reported (6). Suspension of the microsomal fraction of rat liver in 1 M potassium phosphate buffer followed

by centrifugation at 100,000 x g yielded the major part of the 6β-hydroxylase activity in the supernatant. However, recent experiments indicate that no true solubilization is obtained under these conditions (7). The 1 M potassium phosphate buffer appears only to change the sedimentation properties of part of the microsomal membranes since dilution of the extract and subsequent centrifugation at 100,000 x g precipitated most of the 6β-hydroxylase activity (7).

Recently, methods for solubilization and partial purification of cytochrome P-450 and cytochrome P-450 reductase from rat liver have been reported by Coon, Lu and collaborators (8-10). These preparations have been shown to catalyze hydroxylation of various drugs, of lauric acid and of testosterone. As continuation of previous investigations on hydroxylation of steroids and bile acids in the liver, 7α-hydroxylation of taurodeoxycholic acid and 6β-hydroxylation of taurochenodeoxycholic acid and lithocholic acid by partially purified cytochrome P-450 and cytochrome P-450 reductase from rat liver have now been studied.

EXPERIMENTAL PROCEDURE

Materials. 24-^{14}C-Taurodeoxycholic acid (0.8 μC/mg) and tritium-labeled taurochenodeoxycholic acid (2 μC/mg) were synthesized (11) from 24-^{14}C-deoxycholic acid (ICN Chemical and Radioisotope Division, Irvine, Calif.) and tritium-labeled chenodeoxycholic acid (obtained by exposure of chenodeoxycholic acid to tritium gas according to Wilzbach (12)). 24-^{14}C-Lithocholic acid (3 μC/mg) was obtained from New England Nuclear, Boston, Mass. Dioleylglyceryl-3-phosporylcholine was obtained from Supelco, Bellefonte, Penn. The mixture of di- and monolauroylglyceryl-3-phosporylcholine was a generous gift from Dr. W.E.M.

Lands. This preparation was the same as the one used by Strobel et al. (9). Pyridine nucleotides, D,L-isocitric acid and isocitric acid dehydrogenase (Type IV) were obtained from Sigma Chemical Co., St. Louis, Mo.

Methods. Male rats of the Sprague-Dawley strain weighing 50-100 g were injected intraperitoneally with phenobarbital (100 mg/kg body weight) once daily for 3 days. The animals were killed by decapitation 24 hours after the last injection. Liver microsomes were prepared from a 20% (w/v) homogenate in 0.25 M sucrose and stored under nitrogen for 1-3 days at -20°C in the homogenizing medium (about 30 mg microsomal protein/ml). The cytochrome P-450 and cytochrome P-450 reductase were prepared as described by Lu et al. (10). The fraction precipitating between 0 to 45% saturation with ammonium sulfate was used as source of cytochrome P-450 and the final preparation contained 1.0 nmoles of cytochrome P-450 per mg protein. Cytochrome P-450 was determined as described by Omura and Sato (13) and protein was determined as described by Lowry et al. (14). The cytochrome P-450 was contaminated by small amounts of cytochrome P-450 reductase, about 30 units per mg protein. The fresh preparation of cytochrome P-450 contained about 10% cytochrome P-420 but the amount of cytochrome P-420 increased after storage at 4°C for more than one week. The cytochrome P-450 reductase preparation had a specific activity of about 400 units per mg protein when assayed as described by Masters et al. (15). One unit corresponds to one nmole of cytochrome c reduced per min under the conditions of the assay (10). In the standard incubation procedure the labeled bile acid, 50 µg dissolved in 50 µl of modified Bucher medium (16), was added to 0.44 nmoles of cytochrome P-450 (0.45 mg of protein), 400 units of cytochrome

P-450 reductase (about 1.0 mg of protein), 50 µg of mono- and dilauroylglyceryl-3-phosporylcholine and an NADPH-generating system (3) in a total volume of 3 ml of modified Bucher medium. Incubations were performed at 37°C for 20 min and were terminated by addition of 95% (v/v) aqueous ethanol. The solutions were hydrolyzed with 1 M KOH for 12 hours at 110°C. The mixture was extracted with ethyl acetate after acidification. The washed and neutralized ethyl-acetate extract was subjected to thin-layer chromatography with solvent system S 11 (17). The extent of conversion was determined with a thin-layer scanner (Berthold, Karlsruhe, Germany). In some experiments the labeled cholic acid was eluted from the appropriate chromatographic zone with methanol and crystallized to constant spécific radioactivity after addition of authentic cholic acid.

RESULTS

The reconstituted system consisting of partially purified cytochrome P-450 and cytochrome P-450 reductase and a mixture of mono- and dilauroylglyceryl-3-phosphorylcholine was found to catalyze 7α-hydroxylation of taurodeoxycholic acid in the presence of an NADPH-generating system. Under the standard incubation conditions the conversion was 10-20%. The product, taurocholic acid, was identified after hydrolysis by crystallisation to constant specific radioactivity together with authentic cholic acid. The rate of 7α-hydroxylation of taurodeoxycholic acid was linear with the amount of cytochrome P-450 added up to about 1.5 nmoles and with time up to about 30 min (Fig. 1). The amount of cytochrome P-450 reductase needed for optimal conversion was about 400 units (Fig. 1). The cytochrome P-450 preparation had in itself a small capacity to 7α-hydroxylate taurodeoxycholic acid. This is probably due to contamination

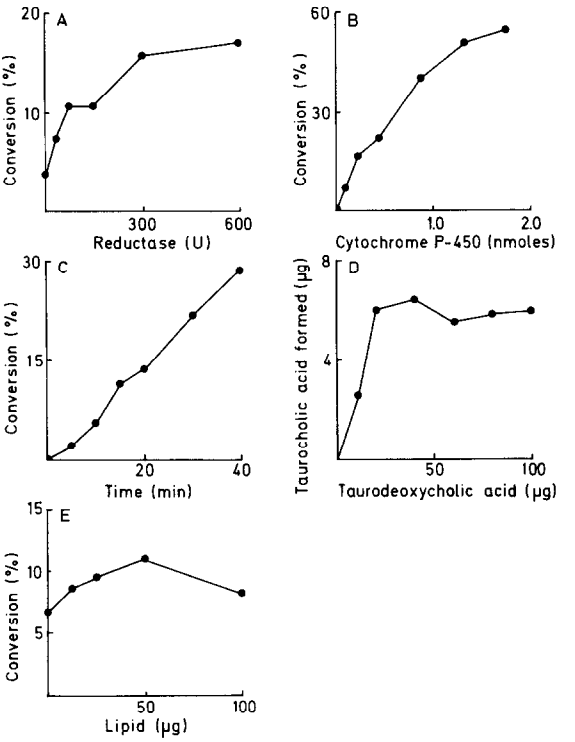

Fig. 1. Effect of cytochrome P-450 reductase concentration (A), cytochrome P-450 concentration (B), time (C), substrate concentration (D) and lipid concentration (E) on 7α-hydroxylation of taurodeoxycholic acid catalyzed by the reconstituted system. With the exception of the variable factor in each set of experiment the standard incubation conditions were used (cf. Methods).

of the cytochrome P-450 fraction with cytochrome P-450 reductase. The enzyme was saturated with 20 μg of substrate (Fig. 1). It has been reported that various types of phosphatidyl cholines stimulate hydroxylations of drugs and fatty acids by a reconstituted system from rat liver (9,10) and that dioleylglyceryl-3-phosphorylcholine and a mixture of mono- and dilauroylglyceryl-3-phosphorylcholine are most efficient (9). These lipids were tested in the present system and a mixture of mono- and dilauroylglyceryl-3-phosphorylcholine was found to be the more efficient. The amount of this lipid required for optimal conversion was about 50 μg (Fig. 1). It should be pointed out

that the degree of stimulation was very much less than that reported with lauric acid as substrate. The 7α-hydroxylation of taurodeoxycholic acid by the reconstituted system was highly sensitive to carbon monoxide and the reaction was inhibited by more than 90% when the incubation was carried out in an atmosphere consisting of 40% CO, 56% N_2 and 4% O_2.

When the reconstituted system with the optimal amounts of the different constituents was incubated with taurochenodeoxycholic acid, the conversion into a product with the chromatographic properties of 3α,6β,7α-trihydroxy-5β-cholanoic acid was 1% or less. Incubation with lithocholic acid resulted in conversion of about 4% into a product with the chromatographic properties of 3α,6β-dihydroxy-5β-cholanoic acid. Due to the small extent of conversion, no further identification of the products formed in incubations of taurochenodeoxycholic acid and lithocholic acid was made.

DISCUSSION

The present work confirms that 7α-hydroxylation of taurodeoxycholic acid is catalyzed by a cytochrome P-450-containing system. The reconstituted system used in the present investigation was the one described by Lu *et al.* (10). In addition to 7α-hydroxylation of taurodeoxycholic acid, the system was found to catalyze hydroxylation of testosterone in several positions, hydroxylation of lauric acid in the ω1- and ω2-positions as well as demethylation of aminopyrine (*cf.* ref. 9 and 10). It is noteworthy that the stimulatory effect of phospholipid was much less with taurodeoxycholic acid as substrate for the reconstituted system than with testosterone, lauric acid and aminopyrine as substrates. It is possible that the requirement for phospholipid is more pronounced with substrates that are more

lipophilic than taurodeoxycholic acid. Taurine conjugates are soluble in water in contrast to the steroids and drugs that have been previously used as substrates for the system (10).

Several reports indicate strongly that 6β-hydroxylation of taurochenodeoxycholic acid is cytochrome P-450 dependent (2,3,5,6). Taurochenodeoxycholic acid and taurodeoxycholic acid are hydroxylated to about the same extent by microsomal fraction of liver of rats pretreated with phenobarbital (3). Yet, in the reconstituted system taurochenodeoxycholic acid was hydroxylated to a much lower extent than taurodeoxycholic acid. Apparently, the purification of the system has led to loss of one or more components required for optimal 6β-hydroxylation of taurochenodeoxycholic acid and work is in progress to attempt to define this (these) component(s). It should be mentioned that previous work has indicated differences between 7α-hydroxylation of taurodeoxycholic acid and 6β-hydroxylation of taurochenodeoxycholic acid. The 7α-hydroxylation of 7α-^{3}H-taurodeoxycholic acid is accompanied by an isotope effect whereas 6β-hydroxylation of 6β-^{3}H-taurochenodeoxycholic acid is not indicating different rate-limiting steps in the two hydroxylations (18).

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