7α-HYDROXYLATION OF CHOLESTEROL BY RECONSTITUTED SYSTEMS FROM RAT LIVER MICROSOMES

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SUMMARY

A reconstituted system from rat liver microsomes, consisting of partially purified fractions of cytochrome P-450 and NADPH-cytochrome P-450 reductase was shown to catalyze 7α-hydroxylation of cholesterol in the presence of NADPH and a synthetic phosphatidylcholine. The rate of 7α-hydroxylation of added [4-^{14}C] cholesterol was linear with the concentration of cytochrome P-450 and increased with the concentration of NADPH-cytochrome P-450 reductase up to a certain level and then remained constant. Omission of phosphatidylcholine resulted only in a 20% decrease in cholesterol 7α-hydroxylase activity of the system. The rate of 7α-hydroxylation was 2-3 times higher in reconstituted systems with cytochrome P-450 from cholestyramine-treated rats than in those with cytochrome P-450 from untreated rats.

The 7α-hydroxylation of cholesterol is the first step in the major pathways for the conversion of cholesterol into bile acids and several lines of evidence indicate that the reaction is rate limiting in the overall biosynthesis of bile acids (for a review, see ref. 1). The 7α-hydroxylation of cholesterol is catalyzed by the microsomal fraction of liver homogenate fortified with NADPH. The reaction is inhibited by carbon monoxide and by an antibody against NADPH-cytochrome c reductase, suggesting the participation of cytochrome P-450 and NADPH-cytochrome P-450 reductase (2-4). The 7α-hydroxylation of cholesterol differs in several respects from most other cytochrome P-450-catalyzed hydroxylations. An important difference is the marked stimulatory effect of biliary drainage through a bili-

ary fistula or by feeding the anion exchanger cholestyramine (3,4). Biliary drainage has no effect on a number of other microsomal hydroxylations and results in no change or a slight reduction of total cytochrome P-450 content (2,5-7). In view of the role of 7α-hydroxylation of cholesterol in bile acid biosynthesis there has been an increasing interest in studying the reaction under various physiological and pathological conditions. Assay of cholesterol 7α-hydroxylase activity meets with certain problems related to the presence of large amounts of endogenous cholesterol in the microsomal fraction (8,9). It is apparent that an important advancement in the study of the 7α-hydroxylation of cholesterol would be if the reaction could be shown in soluble systems. Recent work in this laboratory has shown that reconstituted systems from rat liver microsomes catalyze several hydroxylations in the biosynthesis and metabolism of bile acids (10,11). In the present communication, the 7α-hydroxylation of cholesterol by reconstituted systems from rat liver microsomes is reported.

EXPERIMENTAL PROCEDURE

Materials. [4-^{14}C] Cholesterol (61 μCi/μmole) was obtained from the Radiochemical Centre, Amersham, England. The labeled cholesterol was purified by chromatography on a column of aluminum oxide, grade III. Cholestyramine (Cuemid^{R}) was obtained from Merck, Sharp & Dohme, West Point, Pa. Tween 80 and cofactors were obtained from Sigma Chemical Co., St. Louis, Mo. The mixture of synthetic mono- and dilauroylglycero-3-phosphorylcholine was a generous gift from Dr. W.E.M. Lands and was the same as used in previous reports.

Methods. Male rats of the Sprague-Dawley strain, weighing about 200 g, were used. In some experiments, the rats were fed a diet

containing 3% cholestyramine or injected intraperitoneally with phenobarbital (100 mg/kg body weight) for 5 days. Liver homogenates, 33% (w/v), were prepared in 0.25 M sucrose containing 1 mM EDTA. The microsomal fraction was prepared and stored as described previously (10,11). The cytochrome P-450 preparations were made from 2,000-4,000 mg of microsomal protein according to Lu et al. (12) as described previously (10,11). The cytochrome P-450 preparations from untreated and cholestyramine-treated rats contained 0.7 and 0.8 nmoles of cytochrome P-450 per mg of protein, respectively when assayed according to Omura and Sato (13). Preparations of NADPH-cytochrome P-450 reductase were made from 2,000-4,000 mg of microsomal protein according to Lu et al. (12) as described previously (10,11). The preparations of NADPH-cytochrome P-450 reductase from untreated, cholestyramine-treated and phenobarbital-treated rats contained 150, 200 and 300 units per mg protein, respectively, when assayed according to Masters et al. (14) and expressed in units as described by Lu et al. (12). Protein was determined according to Lowry et al. (15). In the standard incubation procedure, 0.03 μmoles of the labeled cholesterol in a suspension with 3 mg of Tween 80 was incubated for 15 min at 37°C with 2 nmoles of cytochrome P-450 from cholestyramine-treated rats, 600 units of NADPH cytochrome P-450 reductase from phenobarbital-treated rats, 50 μg of the mixture of mono- and dilalauroyl-glycero-3-phosphorylcholine and 3 μmoles of NADPH in a final volume of 3 ml 0.1 M potassium phosphate buffer, pH 7.0. Incubations were terminated by the addition of chloroform-methanol (2:1, v/v) and 0.2 volumes of 0.9% (w/v) sodium chloride solution were then added. The chloroform phase was collected and after evaporation subjected to thin-layer chromatography

with benzene-ethyl acetate, 2:3 (v/v) as solvent. Conversion of [4-^{14}C]cholesterol into [4-^{14}C]5-cholesten-3β,7α-diol was determined by radioscanning of the chromatoplate using a Berthold Dünnschichtscanner II. The product was identified by combined gas chromatography-mass spectrometry.

RESULTS

Incubation of [4-^{14}C]cholesterol with partially purified cytochrome P-450, NADPH-cytochrome P-450 reductase, a mixture of mono- and dilauroylglycero-3-phosphorylcholine and NADPH resulted in a significant conversion into 5-cholestene-3β,7α-diol. The extent of conversion was 0.6-0.8% of added labeled cholesterol under standard incubation conditions with cytochrome P-450 from cholestyramine-treated rats. When either cytochrome P-450 or NADPH-cytochrome P-450 reductase was omitted, the extent of conversion was below 0.15% (Fig. 1). Omission of the phosphatidylcholine resulted in a 20% decrease in conversion. The rate of 7α-hydroxylation of [4-^{14}C]cholesterol was linear with time up to 15 min and with cytochrome P-450 concentration up to 4 nmoles (Fig. 1). The 7α-hydroxylation in the presence of 2 nmoles of cytochrome P-450 was stimulated by increasing amounts of NADPH-cytochrome P-450 reductase up to about 500 units. Reductase from phenobarbital-treated rats was up to twice as efficient as reductase from cholestyramine-treated or untreated rats (Fig. 1). In combination experiments (Table 1), cytochrome P-450 from cholestyramine-treated rats had two to three times higher catalytic activity than cytochrome P-450 from untreated rats.

DISCUSSION

The present results can be considered to provide further evidence that cytochrome P-450 is the terminal oxidase for the

7α-hydroxylation of cholesterol and that electrons from NADPH are transported by NADPH-cytochrome P-450 reductase. However, in view of the differences between the 7α-hydroxylation of cholesterol and most other cytochrome P-450-catalyzed hydroxylations it can not be ruled out that the cytochrome P-450 fraction contains an unknown, terminal oxidase different from cytochrome P-450 catalyzing the 7α-hydroxylation of cholesterol. The stimulatory effect of cholestyramine treatment on the reaction was shown to reside in the cytochrome P-450 fraction. In previous reports (10,11) it has been shown that the stimulatory effects of starvation on 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one and of phenobarbital treatment on 7α-hydroxylation

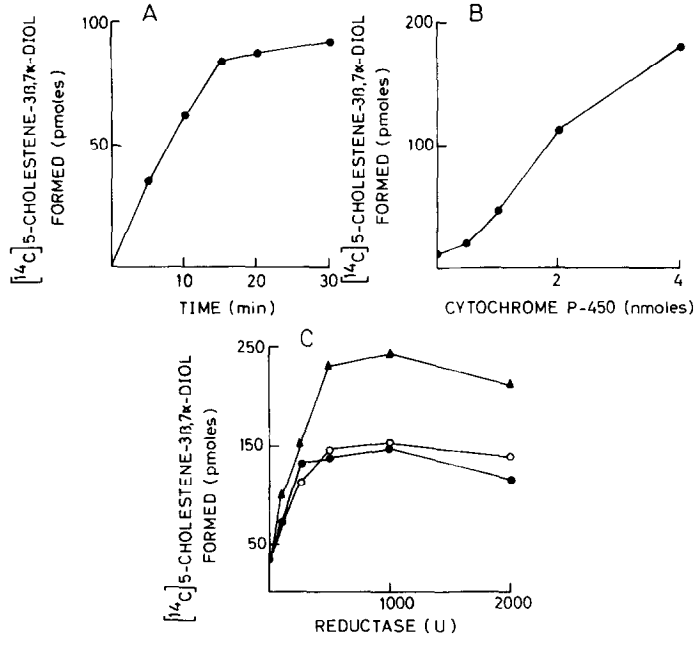

Fig. 1. Effect of time (A), cytochrome P-450 concentration (B), and NADPH-cytochrome P-450 reductase concentration (C) on 7α-hydroxylation of cholesterol. With the exception of the variable factor in each set of experiments, standard incubation conditions were used (cf. Methods). In the experiments with increasing concentrations of NADPH-cytochrome P-450 reductase (C), preparations from untreated rats (●—●), cholestyramine-treated rats (○—○) or phenobarbital-treated rats (▲—▲) were used.

Table 1

7α-Hydroxylation of cholesterol by different reconstituted systems from rat liver microsomes. Irrespective of source, 2 nmoles of cytochrome P-450 and 600 units of NADPH-cytochrome P-450 reductase were used and were incubated with 10 μg of [4-^{14}C] cholesterol under standard conditions.

System	[4-^{14}C] 5-Cholestene-3β,7α-diol formed
	picomoles
Cytochrome P-450 from untreated rats + NADPH-cytochrome P-450 reductase from untreated rats	50
Cytochrome P-450 from untreated rats + NADPH-cytochrome P-450 reductase from phenobarbital-treated rats	99
Cytochrome P-450 from untreated rats + NADPH-cytochrome P-450 reductase from cholestyramine-treated rats	74
Cytochrome P-450 from cholestyramine-treated rats + NADPH-cytochrome P-450 reductase from untreated rats	99
Cytochrome P-450 from cholestyramine-treated rats + NADPH-cytochrome P-450 reductase from phenobarbital-treated rats	248
Cytochrome P-450 from cholestyramine-treated rats + NADPH-cytochrome P-450 reductase from cholestyramine-treated rats	174

of taurodeoxycholic acid and 6β-hydroxylation of taurochenodeoxycholic acid and lithocholic acid also reside in the cytochrome P-450 fraction. A noteworthy finding was that there was a significant difference in extent of stimulation of the 7α-hydroxylation of cholesterol between NADPH-cytochrome P-450 reductase from phenobarbital-treated rats and reductase from untreated or cholestyramine-treated rats. No such marked difference between reductase preparations from various sources has been observed in the hydroxylations studied previously (10,11).

No explanation can be offered at present and it should be mentioned that in the strain of rats used phenobarbital treatment has a slight inhibitory effect on the 7α-hydroxylation of cholesterol (3). It might also be mentioned that differences in protein concentrations between the different reductase preparations were not responsible since addition of reductase from phenobarbital-treated rats to incubations with reductase from untreated or cholestyramine-treated rats resulted in a marked stimulation of the 7α-hydroxylation of cholesterol.

In all experiments assays of cholesterol 7α-hydroxylase activity of the reconstituted system were based on extent of conversion of [4-^{14}C]cholesterol. The preparations contained cholesterol in excess of the added [4-^{14}C]cholesterol but the finding of an increasing extent of conversion of [4-^{14}C]cholesterol with increasing concentrations of cytochrome P-450 (Fig. 1) indicates a preferential utilization of exogenous cholesterol. Work is in progress to study the degree of utilization of endogenous and exogenous cholesterol of the reconstituted system by analysis of the ratios between ^{12}C- and ^{14}C-labeled molecules in total cholesterol and 7α-hydroxycholesterol in the incubations.

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