Characterization of 6α-hydroxylation of taurochenodeoxycholic acid in pig liver

Hans Boström

Department of Pharmaceutical Biochemistry, University of Uppsala, Box 578, S-751 23 Uppsala, Sweden

Abstract The properties of the species-specific 6α-hydroxylation of taurochenodeoxycholic acid were studied in subcellular fractions from pig liver. The hydroxylation was observed in microsomes but not in mitochondria. A partially purified cytochrome P-450 fraction in the presence of NADPH-cytochrome P-450 reductase, NADPH, and phospholipid catalyzed 6α-hydroxylation of taurochenodeoxycholic acid at a 160-fold higher rate than the microsomes. This cytochrome P-450 fraction did not catalyze 6α-hydroxylation of 5β-cholestane-3α,7α-diol or testosterone, nor did it catalyze 7α-hydroxylation of cholesterol.— **Boström, H.** Characterization of 6α-hydroxylation of taurochenodeoxycholic acid in pig liver. *J. Lipid Res.* 1986. **27:** 807–812.

Supplementary key words cytochrome P-450

Cholic acid and chenodeoxycholic acid are the main primary bile acids in most mammals. There are some bile acids that can be considered species-specific even when they may occur in very small amounts in other species. Examples of species-specific bile acids in mammals are the muricholic acids in the mouse and the rat and hyocholic acid in the pig (1). It has been shown in in vivo experiments that hyocholic acid is formed by a 6α-hydroxylation of chenodeoxycholic acid or its conjugates (2). This hydroxylation occurs virtually only in the pig. The ancestors of the pig probably made cholic acid as the related warthog now does, but, at present, hyocholic acid is the only trihydroxylated bile acid in pig bile. It has been claimed that, in pigs, a 6α-hydroxylase with chenodeoxycholic acid or its conjugates as substrate was preferred during evolution to a reactivation of cholic acid biosynthesis (1).

The biosynthesis of cholic acid and chenodeoxycholic acid involves hydroxylations of the steroid nucleus as well as the side chain. These hydroxylations have been shown to be catalyzed by cytochrome P-450-dependent systems in mitochondria and microsomes (3). There is no information on the intracellular localization and properties of the enzyme catalyzing 6α-hydroxylation in the pig.

The present communication reports characterization and partial purification of 6α-hydroxylase in pig liver.

MATERIALS AND METHODS

Materials

[4-^{14}C]Cholesterol (61 Ci/mol) and [4-^{14}C]testosterone (50 Ci/mol) were obtained from the Radiochemical Centre, Amersham, England. The labeled cholesterol was purified by chromatography on aluminum oxide, grade III. 5β[7β-^{3}H]Cholestane-3α,7α-diol (500 Ci/mol) was prepared by reduction of the corresponding 7-keto-steroid (4) with tritium-labeled sodium borohydride (NEN, Dreieich, F.R.G.) Tauro[24-^{14}C]chenodeoxycholic acid (3 Ci/mol) was synthesized according to Norman (5). Octylamine-Sepharose 4B was prepared (6, 7) and materials were obtained as described previously (6). Untreated, castrated male pigs were used.

Enzyme purifications

All buffers were at pH 7.4 and contained 20% glycerol and 0.1 mM EDTA. Phosphate was buffer used as the potassium salt. The methods used for the preparation of microsomes (6, 8) and mitochondria (9) were the same as have previously been described. In a typical preparation of cytochrome P-450 fractions, 1 kg of liver obtained from three different pigs was used. Liver microsomes were solubilized with sodium cholate (3 mg per mg of protein) and precipitated with 8–15% (w/v) polyethylene glycol (10). The precipitate was dissolved in 100 mM phosphate

Nomenclature: The trivial names used are: α-muricholic acid, 3α, 6β, 7α-trihydroxy-5β-cholanoic acid; β-muricholic acid, 3α, 6β, 7β-trihydroxy-5β-cholanoic acid; hyocholic acid, 3α, 6α, 7α-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; cholic acid, 3α, 7α, 12α-trihydroxy-5β-cholanoic acid.

buffer containing 0.7% (w/v) sodium cholate and subjected to octylamine-Sepharose chromatography (column 4 × 50 cm) as described by Imai and Sato (7). The column was washed with the equilibrating buffer containing 0.46% (w/v) sodium cholate. The main part of cytochrome P-450 was eluted with the equilibrating buffer containing 0.37% sodium cholate and 0.06% (w/v) Emulgen 913. The eluted cytochrome P-450 was diluted fourfold and applied to a hydroxylapatite column (3 × 40 cm) equilibrated with a buffer containing 10 mM phosphate. Cytochrome P-450 was eluted from the hydroxylapatite column stepwise with the equilibrating buffer containing 0.2% Emulgen and 35 mM, 120 mM, and 300 mM phosphate. The different hydroxylapatite fractions were diluted severalfold with 20% glycerol and adsorbed on hydroxylapatite columns. The columns were washed with 10 mM phosphate until no absorption at 276 nm was detectable. The cytochrome P-450 fractions were eluted with 500 mM phosphate and finally dialyzed against 50 volumes of 100 mM phosphate.

Cytochrome P-450 was determined as described by Omura and Sato (11) and protein as described by Lowry et al. (12).

NADPH-cytochrome P-450 reductase was prepared from liver microsomes of phenobarbital-treated rats as described by Yasukochi and Masters (13).

Gel electrophoresis was performed in the presence of sodium dodecyl sulfate as described by Laemmli (14). The gels were silver-stained as described by Wray et al. (15).

Optical spectra were measured as described by Haugen and Coon (10) using a Shimadzu multipurpose recording spectrophotometer.

Incubation procedures

When studying the hydroxylase activities in microsomes or in purified microsomal fractions, the substrates were incubated for 10 min with microsomal protein corresponding to 0.25 nmol of cytochrome P-450 or with cytochrome P-450 (0.1–0.25 nmol), NADPH-cytochrome P-450 reductase (2.0–4.0 units), and dilauroylglycero-3-phosphocholine (15 μg) together with 2 μmol of NADPH in a total volume of 1 ml of 50 mM Tris–acetate buffer (cholesterol and testosterone) or 150 mM phosphate buffer (taurochenodeoxycholic acid and 5β-cholestane-3α,7α-diol), pH 7.4, containing 20% glycerol and 0.1 mM EDTA. Incubations with cholesterol contained 5 mmol of dithiothreitol and 0.05% Triton X-100. In incubations with taurochenodeoxycholic acid, glycerol was omitted in the buffers. When studying the hydroxylase activities in mitochondria, the substrates were incubated with 10 mg of mitochondrial protein, 4.6 μmol of isocitrate, and 30 μmol of $MgCl_2$ in a total volume of 3 ml of 50 mM Tris–acetate buffer.

Analysis of incubations

Incubations with taurochenodeoxycholic acid were terminated with 3 ml of ethanol and hydrolyzed with 2 M KOH in 50% (v/v) aqueous ethanol for 12 hr at 110°C. The hydrolysis mixture was diluted with water, acidified, and extracted with ether. The ether extracts were washed with water until neutral. The residues of the ether extracts were subjected to thin-layer chromatography. The developing system was trimethylpentane–ethyl acetate–acetic acid 50:50:15. Incubations with taurochenodeoxycholic acid were also anlayzed according to the following procedure. Protein precipitates were filtered off on a sintered-glass Büchner funnel. The samples were concentrated on a rotary evaporator until only water remained and then passed through a Sep-Pak C_{18} cartridge. The bile acids were recovered by elution with methanol and hydrolyzed with cholyglycine hydrolase (16). The hydrolyzed bile acids were again extracted with a Sep-Pak cartridge and the unconjugated bile acid fraction was isolated by chromatography on a Lipidex-DEAP column (17). After methylation with diazomethane, the compounds in this fraction were analyzed as trimethylsilyl ethers by gas–liquid chromatography and mass spectrometry using a Finnigan 1020 instrument, equipped with a fused-silica column, coated with SE-30 (18). Gas–liquid chromatography with radioactivity detection of the compounds was performed using a Packard 894 instrument equipped with a 3% QF-1 column at 235°C (4). Incubations with testosterone were analyzed by HPLC–chromatography using authentic standards. Additional incubation procedures and analyses of the incubation mixtures were the same as those described previously (9, 19).

RESULTS

Table 1 summarizes the catalytic activities of pig liver mitochondria, microsomes, and purified microsomal enzyme fractions towards substrates in bile acid biosynthesis. The mitochondrial fraction catalyzed 26-hydroxylation of 5β-cholestane-3α,7α-diol but was inactive in 6α-hydroxylation of taurochenodeoxycholic acid and 7α-hydroxylation of cholesterol. The microsomal fraction was inactive in 26-hydroxylation but catalyzed the 6α- and 7α-hydroxylations. When free chenodeoxycholic acid was used as substrate instead of the taurine conjugate, the rate of 6α-hydroxylation fell by a factor of five.

Since it seemed probable that the 6α-hydroxylation of taurochenodeoxycholic acid was catalyzed by a cytochrome P-450, the microsomal fraction was solubilized and purified according to procedures used in preparation of cytochrome P-450 fractions active on substrates in bile acids biosynthesis. The microsomal fraction was solubilized with sodium

TABLE 1. Hydroxylase activities of microsomes, mitochondria, and purified microsomal enzyme fractions from pig liver

Fraction	Cytochrome P-450 (Specific Content)	Hydroxylation of: Taurochenodeoxycholic acid 6α	Cholesterol 7α	5β-Cholestane-3α,7α-diol 26
	nmol/mg protein	*pmol/nmol cytochrome P-450 × min*		
Mitochondria	ND[a]	≤2[b]	≤2[b]	20[b]
Microsomes	0.6	25	15	≤5
35 mM Fraction	2.0	4,100	≤5	≤5
300 mM Fraction	6.0	≤5	2,000	≤5

The incubation procedures are described under Experimental Procedures. The following amounts of substrate were used: cholesterol, 25 nmol; taurochenodeoxycholic acid, 300 nmol; 5β-cholestane-3α,7α-diol, 100 nmol.
[a]Not determined.
[b]These values are expressed as pmol/mg protein × min.

cholate and chromatographed on octylamine-Sepharose and hydroxylapatite columns. The hydroxylapatite column was eluted with 35, 120, and 300 mM potassium phosphate buffer. In the presence of NADPH-cytochrome P-450 reductase, NADPH, and phospholipid, the 35-mM fraction catalyzed 6α-hydroxylation of taurochenodeoxycholic acid but not 7α-hydroxylation of cholesterol. Conversely, the 300-mM fraction catalyzed 7α-hydroxylation but not 6α-hydroxylation. To ascertain that the different substrate specificity of the two cytochrome P-450 fractions was not due to the presence in the fractions of remaining detergent such as sodium cholate or Emulgen, a series of experiments was performed with addition of one fraction to the other. No changes in catalytic activity of the respective cytochrome P-450 fractions were observed in these mixing experiments. This finding shows that the difference in substrate specificity of the two cytochrome P-450 fractions was not due to the presence of stimulatory or inhibitory factors in the fraction such as detergent, endogenous cholesterol, or phospholipid. The 120-mM fraction showed very low 6α- and 7α-hydroxylase activities. None of the fractions had any detectable 26-hydroxylase activity towards 5β-cholestane-3α,7α-diol. This is to be expected in view of the lack of 26-hydroxylase activity in the microsomes.

The product formed after incubation of [^{14}C]taurochenodeoxycholic acid with the 35-mM fraction was analyzed by combined gas–liquid chromatography–mass spectrometry and gas–liquid chromatography with radioactivity detection. The total ion current chromatogram obtained in the combined gas chromatographic–mass spectrometric analysis showed two peaks (**Fig. 1**). The compounds producing the peaks gave mass spectra identical to those

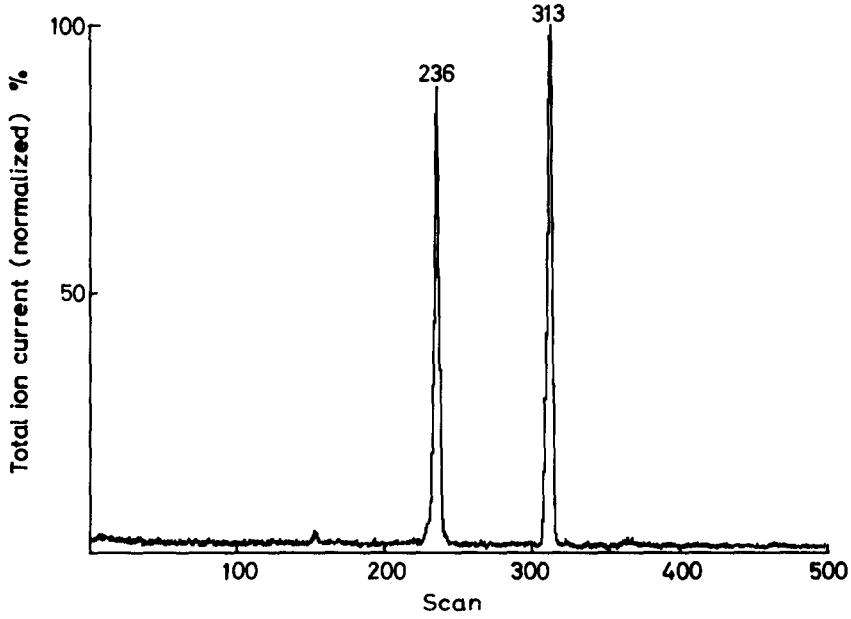

Fig. 1. Total ion current chromatogram obtained in the combined gas–liquid chromatographic–mass spectrometric analysis of trimethylsilyl ethers of hydrolyzed, methylated bile acid mixture isolated from incubation of [24-^{14}C]taurochenodeoxycholic acid with the 35 mM fraction. The ordinate represents consecutive mass scanning. Scan no. 236 corresponds to the cholic acid derivative and scan no. 313 to the hyocholic acid derivative.

given by the methyl ester trimethylsilyl ether derivatives of cholic and hyocholic acids, respectively (20). Gas–liquid chromatography with radioactivity detection showed that radioactivity was present only in hyocholic acid (**Fig. 2**). The unlabeled cholic acid originates from the cytochrome P-450 preparation. Sodium cholate is used in the purification procedures and the purified fractions contained up to 0.001% of this detergent.

As shown in **Table 2**, the 6α-hydroxylase activity in the 35-mM fraction from the hydroxylapatite column showed an absolute requirement for cytochrome P-450, NADPH-cytochrome P-450 reductase, and NADPH. Omission of phospholipid resulted in a 30% decrease of the activity. Limited requirements for phospholipid have also been observed for other hydroxylations in biosynthesis and metabolism of bile acids. Dithiothreitol increased the activity about 3-fold. The extent of purification of the 6α-hydroxylase activity was about 160-fold. The fraction had no detectable 6α-hydroxylase activity towards 5β-cholestane-3α,7α-diol or testosterone.

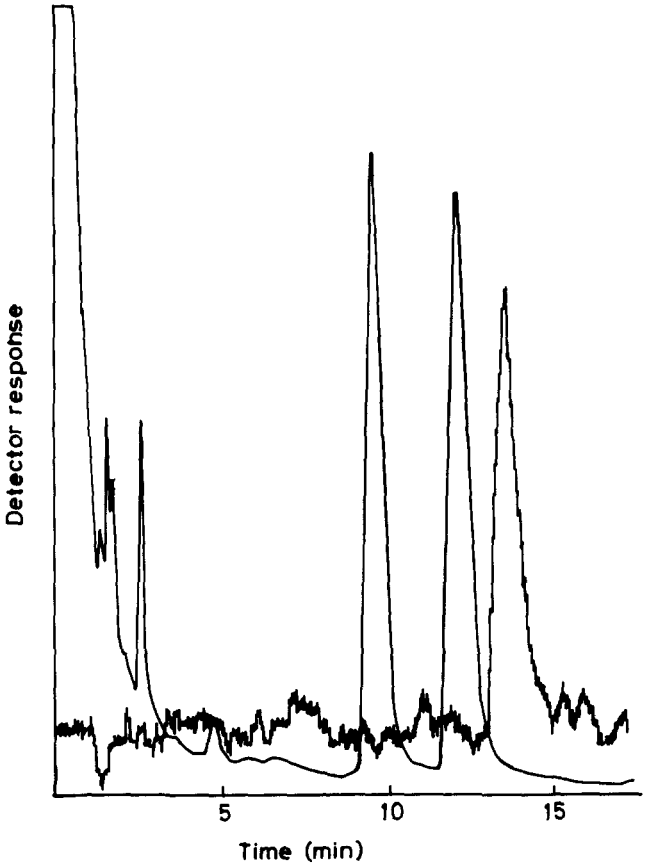

Fig. 2. Radio-gas-liquid chromatogram of trimethylsilyl ethers of hydrolyzed, methylated bile acid mixture isolated from incubation of [24-^{14}C]taurochenodeoxycholic acid with the 35 mM fraction. The smooth solid line represents mass and the jagged line represents radioactivity. The peak appearing at 9.5 min corresponds to the cholic acid derivative and that at 12.0 min to the hyocholic acid derivative. Only the latter derivative is labeled (note that there is a delay of 1.5 min before radioactivity is registered).

TABLE 2. Reconstitution conditions for 6α-hydroxylation of taurochenodeoxycholic acid

Components	Taurochenodeoxycholic Acid 6α
Complete system	4,100
– NADPH-cytochrome P-450 reductase	≤5
– Cytochrome P-450	≤5
– NADPH	≤5
– Phospholipid	2,870
+ Dithiothreitol (5 mmol)	11,200

Incubations were performed as described in Table 1 and in Experimental Procedures. The complete system contained: 35 mM fraction, 0.1 nmol; NADPH-cytochrome P-450 reductase, 2 units; phospholipid (dilauroylglycero-3-phosphocholine), 15 μg; and NADPH, 2 μmol.

The specific content of cytochrome P-450 in the 6α-hydroxylase fraction was 2 nmol per mg of protein. The fraction showed several protein bands upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate followed by silver staining of the protein. There were two major protein bands with apparent molecular weights of 48,000 and 57,000, respectively. With respect to the electrophoretic appearance fo the 6α-hydroxylating cytochrome P-450 fraction, it is not possible at present to identify any of the protein bands with the cytochrome P-450 catalyzing 6α-hydroxylation of taurochenodeoxycholic acid.

The carbon monoxide difference spectrum of the 6α-hydroxylating cytochrome P-450 fraction showed a maximum at 449 nm, and the absorbance at 420 nm was lower than that at 490 nm. The absolute spectrum of the fraction showed absorbance maxima at 410 nm, 530 nm, and 570 nm in the oxidized state. The reduced spectrum showed absorbance maxima at 420 nm and 550 nm. The reduced carbon monoxide complex showed absorbance maxima at 420 nm and 449 nm. The absorbance peak at 420 nm was higher than that at 449 nm.

DISCUSSION

Studies wtih several mammalian species have shown that cytochrome P-450 systems catalyze a number of hydroxylations in the biosynthesis of cholic acid and chenodeoxycholic acid (3, 6, 19). The hydroxylations of the steroid nucleus and the 25-hydroxylation of the side chain are catalyzed by microsomal cytochromes P-450, whereas 26-hydroxylation, the initial reaction in the main pathway for side chain degradation, is catalyzed predominantly by a mitochondrial cytochrome P-450 (9).

The present study shows that the species-specific 6α-hydroxylation of taurochenodeoxycholic acid in the pig (to form the major primary bile acid in this species, hyocholic acid) is catalyzed by the microsomal but not the mitochondrial fraction. It has further been found that high 6α-

hydroxylase activity can be shown in a reconstituted system consisting of a partially purified microsomal cytochrome P-450 fraction, NADPH-cytochrome P-450 reductase, NADPH, and phospholipid. A noteworthy property of the 6α-hydroxylating cytochrome P-450 fraction was the considerably stimulatory effect of dithiothreitol This finding indicates that sulfhydryl groups in the enzyme must be in the reduced state for maximal catalytic activity. A similar dependence on reduced sulfhydryl groups for maximal activity has been found for cholesterol 7α-hydroxylase (21, 22). Other cytochromes P-450 involved in bile acid biosynthesis do not show this dependence on sulfhydryl groups. The 6α-hydroxylating cytochrome P-450 fraction had no 6α-hydroxylase activity towards testosterone, nor did it show any 7α-hydroxylase activity towards cholesterol. Cholesterol 7α-hydroxylation, the main rate-limiting step in bile acid biosynthesis, was catalyzed by another cytochrome P-450 fraction. This fraction did not catalyze 6α-hydroxylation of taurochenodeoxycholic acid. These observations show that 7α-hydroxylation of cholesterol and 6α-hydroxylation of taurochenodeoxycholic acid are catalyzed by different enzymes.

The fact that the 6α-hydroxylating cytochrome P-450 fraction did not 6α-hydroxylate cholesterol or 5β-cholestane-3α,7α-diol has implications with respect to pathways for bile acid biosynthesis in the pig. In cholic acid formation, 12α-hydroxylation occurs at an early stage and the main substrates are 7α-hydroxy-4-cholesten-3-one and 5β-cholestane-3α,7α-diol (3, 23, 24). In analogy, the same compounds would be likely substrates if the 6α-hydroxylation in the formation of hyocholic acid occurred at an early stage. However, the lack of 6α-hydroxylase activity towards cholesterol and 5β-cholestane-3α,7α-diol in the 6α-hydroxylating cytochrome P-450 fraction strongly indicates that 6α-hydroxylation occurs subsequent to 7α-hydroxylation and side chain oxidation. Thus, the reaction appears to be the last step in the biosynthesis of hyocholic acid. In fact, the finding that conjugated chenodeoxycholic acid is a much better substrate for the enzyme than the unconjugated bile acid suggests that the 6α-hydroxylation occurs after taurine conjugation.

The present investigation has provided a basis for further studies of the species-specific 6α-hydroxylation of taurochenodeoxycholic acid. Such studies should be of interest from several points of view. 6α-Hydroxylation is the only hydroxylation in the biosynthesis and metabolism of bile acids in mammals that involves an equatorial position. The hydroxylation is also interesting from an evolutionary aspect. There is evidence that during evolution the pig lost the ability of its ancestors to synthesize cholic acid. It then developed 6α-hydroxylation to fill the requirement for a trihydroxy bile acid in bile (1). In consequence, purification of this cytochrome P-450 to homogeneity and comparisons of the primary structures of different cytochromes P-450 with that of taurochenodeoxycholic acid 6α-hydroxylase might provide insight into the evolution of these enzymes.

The skillful technical assistance of Ms. Angela Lannerbro and Ms. Britt-Marie Johansson is gratefully acknowledged. I also wish to express my gratitude to Professor Jan Sjövall, Dr. Magnus Axelsson, and Ms. Birgitta Mörk for performing the combined gas–liquid chromatography–mass spectrometry analyses. This work was supported by the Swedish Medical Research Council (project 03X-218).

Manuscript received 30 October 1985.

REFERENCES

1. Haslewood, G. A. D. 1967. Bile salt evolution. *J. Lipid Res.* **8:** 535–550.
2. Bergström, S., H. Danielsson, and A. Göransson. 1959. On the bile acid metabolism in the pig. *Acta Chem. Scand.* **13:** 776–783.
3. Danielsson, H., and J. Sjövall. 1975. Bile acid metabolism. *Annu. Rev. Biochem.* **44:** 233–253.
4. Björkhem, I., and J. Gustafsson. 1973. ω-Hydroxylation of steroid side-chain in biosynthesis of bile acids. *Eur. J. Biochem.* **36:** 201–212.
5. Norman, A. 1955. Preparation of conjugated bile acids using mixed carboxylic acid anhydrides. *Ark. Kemi.* **8:** 331–342.
6. Hansson, R., and K. Wikvall. 1980. Catalytic properties of different forms of cytochrome P-450. *J. Biol. Chem.* **255:** 1643–1649.
7. Imai, Y., and R. Sato. 1974. A gel-electrophoretically homogeneous preparation of cytochrome P-450 from liver microsomes of phenobarbital-pretreated rabbits. *Biochem. Biophys. Res. Commun.* **60:** 8–14.
8. Björkhem, I., H. Danielsson, and K. Wikvall. 1974. Hydroxylations of bile acids by reconstituted systems from rat liver microsomes. *J. Biol. Chem.* **244:** 6439–6445.
9. Wikvall, K. 1984. Isolation of cytochrome P-450 from rabbit liver mitochondria catalyzing 26-hydroxylation of C_{27}-steroids. *J. Biol. Chem.* **259:** 3800–3804.
10. Haugen, D. A., and M. J. Coon. 1976. Properties of electrophoretically homogenous phenobarbital-inducible and β-naftoflavone-inducible forms of liver microsomal cytochrome P-450. *J. Biol. Chem.* **251:** 7929–7939.
11. Omura, T., and R. Sato. 1964. The carbon monoxide-binding pigment of liver microsomes. *J. Biol. Chem.* **239:** 2379–2385.
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
13. Yasukochi, Y., and B. S. S. Masters. 1976. Some properties of a detergent-solubilized NADPH-cytochrome c (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J. Biol. Chem.* **251:** 5337–5344.
14. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227:** 680–685.
15. Wray, W. T., T. Boulikas, V. P. Wray and R. Hancock. 1981. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118:** 197–203.
16. Setchell, K. D. R., A. M. Lawson, N. Tanida, and J. Sjövall. 1983. General methods for the analysis of metabolic profiles of bile acids and related compounds in feces. *J. Lipid Res.* **24:** 1085–1100.

17. Almé, B., A. Bremmelgaard, J. Sjövall, and P. Thomassen. 1977. Analysis of metabolic profiles of bile acids in urine using a lipophilic anion exchanger and computerized gas–liquid chromatography–mass spectrometry. *J. Lipid Res.* **18:** 339–362.
18. Tandon, R., M. Axelson, and J. Sjövall. 1984. Selective liquid chromatographic isolation and gas chromatographic–mass spectrometric analysis of ketonic bile acids in faeces. *J. Chromatogr.* **302:** 1–14.
19. Hansson, R., and K. Wikvall. 1979. Properties of reconstituted cholesterol 7α-hydroxylase system from rat and rabbit liver microsomes. *Eur. J. Biochem.* **93:** 419–426.
20. Sjövall, J., P. Eneroth, and R. Ryhage. 1971. Mass spectra of bile acids. *In* The Bile Acids. Vol. 1. P. P. Nair and D. Kritchevsky, editors. Plenum Press, New York. 209–248.
21. Kalles, I., and K. Wikvall. 1981. Role of sulfhydryl groups in catalytic activity of purified cholesterol 7α-hydroxylase system from rabbit and rat liver microsomes. *Biochem. Biophys. Res. Commun.* **100:** 1361–1369.
22. Andersson, S., and H. Boström. 1984. Effect of disulfiram on rat liver cholesterol 7α-hydroxylase. *Biochem. Pharmacol.* **33:** 2930–2932.
23. Einarsson, K. 1968. On the properties of the 12α-hydroxylase in cholic acid biosynthesis. *Eur. J. Biochem.* **5:** 101–108.
24. Hansson, R., and K. Wikvall. 1982. Cytochrome P-450 LM_4 and 12α-hydroxylation of 5β-cholestane-3α,7α-diol. *Eur. J. Biochem.* **125:** 423–429.