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Cytochrome P450 Enzymes in the Metabolism of Cholesterol and Cholesterol Derivatives

BY

MARIA NORLIN

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ABSTRACT

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Cholesterol is metabolized to a variety of important biological products in the body including bile acids and vitamin D. The present investigation is focused on enzymes that catalyze 7α-hydroxylation or 27-hydroxylation in the metabolism of cholesterol, oxysterols (side chain-hydroxylated derivatives of cholesterol) and vitamin D_3. The enzymes studied belong to the cytochrome P450 enzyme families CYP7 and CYP27.

The study describes purification of a cytochrome P450 enzyme fraction active in 7α-hydroxylation of 25-hydroxycholesterol, 27-hydroxycholesterol, dehydroepiandrosterone and pregnenolone from pig liver microsomes. Peptide sequence analysis indicated that this enzyme fraction contains an enzyme belonging to the CYP7B subfamily. The purified enzyme was not active towards cholesterol or testosterone. Purification and inhibition experiments suggested that hepatic microsomal 7α-hydroxylation of 27-hydroxycholesterol and dehydroepiandrosterone involves at least two enzymes, probably closely related.

The study shows that recombinantly expressed human and rat cholesterol 7α-hydroxylase (CYP7A) and partially purified pig liver cholesterol 7α-hydroxylase are active towards 20(*S*)-, 24-, 25- and 27-hydroxycholesterol. CYP7A was previously considered specific for cholesterol and cholestanol. The 7α-hydroxylation of 20(*S*)-, 25-, and 27-hydroxycholesterol in rat liver was significantly increased by treatment with cholestyramine, an inducer of CYP7A. Cytochrome P450 of renal origin showed 7α-hydroxylase activity towards 25- and 27-hydroxycholesterol, dehydroepiandrosterone and pregnenolone but not towards 20(*S*)-, 24-hydroxycholesterol or cholesterol. The results indicate a physiological role for CYP7A as an oxysterol 7α-hydroxylase, in addition to the previously known human oxysterol 7α-hydroxylase CYP7B.

The role of renal sterol 27-hydroxylase (CYP27A) in the bioactivation of vitamin D_3 was studied with cytochrome P450 fractions purified from pig kidney mitochondria. Purification and inhibition experiments and experiments with a monoclonal antibody against CYP27A indicated that CYP27A plays a role in renal 25-hydroxyvitamin D_3 1α-hydroxylation.

The expression of CYP7A, CYP7B and CYP27A during development was studied. The levels of CYP27A were similar in livers of newborn and six months old pigs whereas the levels of CYP7A increased. The expression of CYP7B varied depending on the tissue. The expression of CYP7B increased with age in the liver whereas the CYP7B levels in kidney showed a marked age-dependent decrease.

Maria Norlin, Division of Biochemistry, Department of Pharmaceutical Biosciences, University of Uppsala, Biomedical Centre, Box 578, SE-751 23 Uppsala, Sweden

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This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Araya, Z., Norlin, M., and Postlind, H.
A possible role for CYP27 as a major renal mitochondrial 25-hydroxyvitamin D_3 1α-hydroxylase
FEBS Lett. **390**, 10-14 (1996)

II. Norlin, M., and Wikvall, K.
Biochemical characterization of the 7α-hydroxylase activities towards 27-hydroxycholesterol and dehydroepiandrosterone in pig liver microsomes
Biochim. Biophys. Acta **1390,** 269-281 (1998)

III. Norlin, M., Toll, A., Björkhem, I., and Wikvall, K.
24-Hydroxycholesterol is a substrate for hepatic cholesterol 7α-hydroxylase (CYP7A)
J. Lipid Res. **41,** 1629-1639 (2000)

IV. Norlin, M., Andersson, U., Björkhem, I., and Wikvall, K.
Oxysterol 7α-Hydroxylase Activity by Cholesterol 7α-Hydroxylase (CYP7A)
J. Biol. Chem. **275**, 34046-34053 (2000)

V. Norlin, M.
Expression of CYP7A, CYP7B and CYP27A during development
Manuscript

CONTENTS

NOMENCLATURE

The following trivial names and abbreviations are used:

chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid;
cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid;
hyocholic acid, 3α,6α,7α-trihydroxy-5β-cholanoic acid;
deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid;
lithocholic acid, 3α-hydroxy-5β-cholanoic acid;
7α-hydroxycholesterol, 5-cholestene-3β,7α-diol;
27-hydroxycholesterol, 5-cholestene-3β,27-diol;
7α,27-dihydroxycholesterol, 5-cholestene-3β,7α,27-triol;
25-hydroxycholesterol, 5-cholestene-3β,25-diol;
24-hydroxycholesterol, 5-cholestene-3β,24-diol;
22-hydroxycholesterol, 5-cholestene-3β,22-diol;
20-hydroxycholesterol, 5-cholestene-3β,20-diol;
dehydroepiandrosterone, 5-androsten-3β-ol-17-one;
pregnenolone, 5-pregnen-3β-ol-20-one;
7-dehydrocholesterol, 5,7-cholestadien- 3β-ol;
vitamin D_3, cholecalciferol;
7-oxocholesterol, 5-cholesten-3β-ol-7-one;
HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A;
LDL, low density lipoprotein;
HDL, high density lipoprotein;
LXR, liver X receptor;
CYP, cytochrome P450;
POEL, polyoxyethylene 10 lauryl ether;
GC/MS, gas chromatography-mass spectrometry;
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

INTRODUCTION AND BACKGROUND

Cholesterol and cholesterol metabolism

Cholesterol is an important molecule in the body. It is an essential structural component of all cellular and intracellular membranes and serves as a precursor for biosynthesis of a variety of products with specific biological activities (Fig. 1) [1-3]. Bile acids are formed from cholesterol in the liver and act as detergents in the intestine to emulsify dietary lipids. Bile acids facilitate the absorption of lipids and lipid-soluble vitamins. Other compounds formed from cholesterol are the steroid hormones, including male and female sex hormones, produced in testes, ovaries, and placenta, and cortisol and aldosterone, formed in the adrenal gland, which are important for normal glucose metabolism and salt excretion. Cholesterol is also a precursor for vitamin D_3 which is formed in the skin from 7-dehydrocholesterol under the influence of ultraviolet irradiation. Vitamin D_3, which may also be obtained in the diet, is further metabolized by enzymes in liver and kidney to its biologically active form, 1α,25-dihydroxyvitamin D_3. 1α,25-Dihydroxyvitamin D_3 functions as a hormone regulating the uptake of calcium in the intestine and the release and deposition of bone calcium and phosphate [4]. Deficiency of vitamin D_3 leads to defective bone formation resulting in diseases such as rickets or osteomalacia.

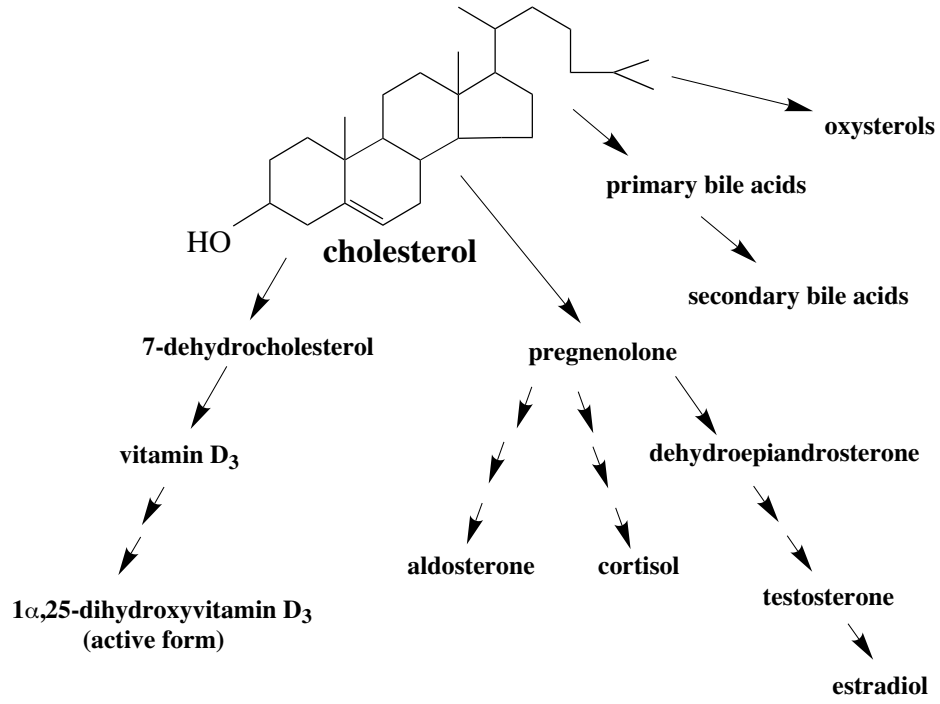

Fig. 1. Cholesterol metabolism

Notwithstanding the essential role of cholesterol in multiple physiological processes, accumulation of this steroid is a risk factor for the development of disease. Excess cholesterol in the blood circulation may accumulate in macrophages and vascular cells and lead to atherosclerosis [5]. Increased amount of cholesterol in the bile may result in gallstones [6]. The maintenance of adequate cholesterol levels in various tissues and cells requires the complex interaction of a number of physiological factors [1,2]. The mechanisms for regulation of cholesterol homeostasis include effects on biosynthesis, uptake, esterification, and metabolism.

Cholesterol homeostasis

Cholesterol may be obtained in the diet or synthesized in the body from acetate [1]. Biosynthesis of cholesterol which involves approximately 30 enzymatic steps can take place in most cell types. Absorption of cholesterol from the intestine requires solubilization in micelles containing bile acids, phospholipids, fatty acids, and glycerides. The absorbed cholesterol is incorporated in chylomicrons, large lipoprotein particles which are secreted in the lymph vessels and enter the bloodstream via the thoracic duct [7]. Increase of dietary cholesterol increases plasma cholesterol levels in most humans.

About 50-75% of the total cholesterol is synthesized endogenously [1]. A large part of the endogenously formed cholesterol is synthesized in the liver. The rate-limiting step is the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonic acid catalyzed by HMG-CoA reductase [1,2]. The activity of this enzyme is regulated by negative feed-back mechanisms. Compounds reported to down-regulate HMG-CoA reductase include mevalonic acid and oxysterols (hydroxylated derivatives of cholesterol) such as 25-hydroxycholesterol and 27-hydroxycholesterol [1,8]. Transcriptional down-regulation of HMG-CoA reductase by oxysterols occurs via sterol regulatory element binding proteins (SREBPs). Disturbed cholesterol biosynthesis during embryogenesis may lead to organ defects or in severe cases to intrauterine or neonatal death [9]. Cholesterol can be stored in the cells as cholesteryl esters after esterification by acyl coenzyme A:cholesterol acyltransferase (ACAT) [1,2].

About 7% of the body cholesterol circulates in plasma incorporated in different types of lipoproteins [1,2]. The lipoproteins consist of a hydrophobic core of triglycerides and cholesteryl esters and a hydrophilic surface of phospholipids, unesterified cholesterol and apolipoproteins [10]. The dominating cholesterol carrier in fasting plasma is low-density

lipoprotein (LDL). Binding of LDL to specific receptors on the cell surface leads to cellular uptake of LDL cholesterol. The number of LDL receptors adjusts to cellular cholesterol demand [1]. The uptake of LDL cholesterol by the cell induces a negative feed-back signal which leads to down-regulation of the synthesis of LDL receptors and suppression of HMG-CoA reductase. Consequently the LDL receptors play an important role in cholesterol homeostasis. Excess cholesterol can be removed from extrahepatic cells by interaction with high-density lipoprotein (HDL) particles and transported as HDL to the liver where it is taken up and metabolized [11]. An additional mechanism for removal of cholesterol from extrahepatic tissues, involving conversion of cholesterol into 27-hydroxycholesterol, has been described [12,13]. 27-Hydroxycholesterol and other 27-oxygenated steroids are more polar than cholesterol and can be transported from the cells more efficiently than cholesterol. 27-Oxygenated steroids are taken up and metabolized by the liver.

The most cholesterol-rich organ in the body is the brain where cholesterol plays an important role as a component in myelin [14]. Myelin is essential for the propagation of nerve impulses in myelinated nerve fibers. Cholesterol homeostasis in the brain is considered to involve enzymatic conversion of cholesterol to 24-hydroxycholesterol which is more easily transported through the brain-blood barrier than cholesterol [14].

The quantitatively most important metabolic pathway in elimination of cholesterol from the body is formation of bile acids in the liver [1,2,15,16]. The liver also secretes some unmetabolized cholesterol directly into the bile. Reported mechanisms for regulation of bile acid biosynthesis include enzyme induction by oxysterols and feed-back inhibition by bile acids.

Biosynthesis of bile acids

The primary bile acids in most mammalian species are cholic acid and chenodeoxycholic acid [15,16]. In the pig, hyocholic acid is synthesized instead of cholic acid [15]. The primary bile acids are conjugated with glycine and taurine before secretion into the bile which empties into the upper portion of the small intestine. In the intestine, primary bile acids are converted to secondary bile acids, *e. g.* deoxycholic acid and lithocholic acid, by microbial enzymes. Most of the bile acid pool is reabsorbed and returned to the liver via the portal vein to be used over again [1,2].

Biosynthesis of bile acids from cholesterol in the liver involves a number of different enzymes [2,16]. Two main pathways have been suggested, the ”neutral” and the ”acidic”

pathway (Fig. 2) [17]. The neutral pathway starts with 7α-hydroxylation of cholesterol by cholesterol 7α-hydroxylase (CYP7A), a microsomal cytochrome P450 enzyme. The 7α-hydroxylation of cholesterol is rate-limiting in the neutral pathway. 7α-Hydroxycholesterol is then converted to 7α-hydroxy-4-cholesten-3-one by a microsomal 3β-hydroxy-Δ^5-C_{27} steroid dehydrogenase/isomerase. The further metabolism, resulting in cholic acid and chenodeoxycholic acid, requires a variety of enzymes in endoplasmic reticulum, mitochondria, cytosol and peroxisomes. For the formation of cholic acid a 12α-hydroxyl group must be introduced in the steroid nucleus. 12α-Hydroxylation is catalyzed by the microsomal enzyme sterol 12α-hydroxylase (CYP8B). An important enzyme for bile acid formation is sterol 27-hydroxylase (CYP27A), a mitochondrial cytochrome P450 which introduces a hydroxyl group in the 27-position. Substrates for sterol 27-hydroxylase are for instance the intermediates 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol.

Sterol 27-hydroxylase is also able to catalyze 27-hydroxylation of cholesterol, the first step in the acidic pathway. The 27-hydroxylation step has been suggested to be rate-limiting for this pathway, at least in the rat. The formed 27-hydroxycholesterol may undergo oxidation to 3β-hydroxy-5-cholestenoic acid. In the acidic pathway 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid are further 7α-hydroxylated by a microsomal enzyme referred to as oxysterol 7α-hydroxylase or CYP7B. This enzyme does, however, not 7α-hydroxylate cholesterol. The dehydrogenase/isomerase active towards 7α-hydroxycholesterol in the neutral pathway is also able to oxidize 7α,27-dihydroxycholesterol into 7α,27-dihydroxy-4-cholesten-3-one. The major product of the acidic pathway is considered to be chenodeoxycholic acid.

The relative contribution of the neutral and acidic pathways for bile acid formation is not clear. The neutral pathway is considered to be the quantitatively most important pathway for formation of primary bile acids in humans. In rats, the contribution of the acidic pathway seems to be as much as 50% of total bile acid biosynthesis [16]. It appears that the acidic pathway can compensate and maintain bile acid formation under conditions where the neutral pathway is repressed [16,18,19]. Recent findings indicate that the acidic pathway may play an important role for human infants [20].

Regulation of bile acid biosynthesis

The enzymatic step considered to be the most important for the regulation of bile acid formation is the 7α-hydroxylation of cholesterol by cholesterol 7α-hydroxylase (CYP7A).

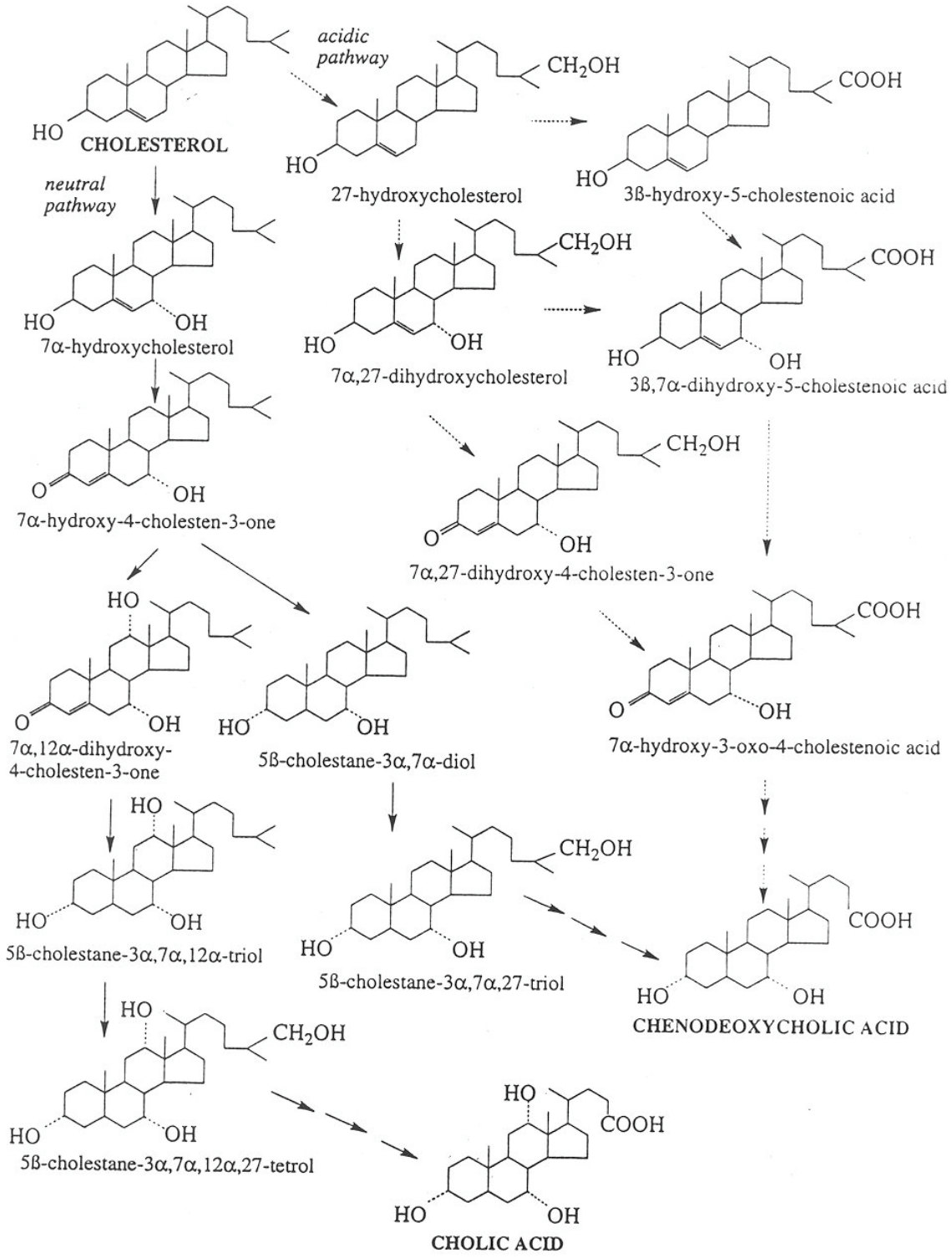

Fig. 2. Biosynthesis of primary bile acids from cholesterol

neutral pathway ——→

acidic pathway ------→

CYP7A is suppressed by hydrophobic bile acids at the transcriptional level [16,21]. Cholesterol feeding leads to an induction of cholesterol 7α-hydroxylase in rats and mice but not in monkeys, rabbits, and hamsters [16]. A number of oxysterols, including 20(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol, 24-hydroxycholesterol, 25-hydroxy-cholesterol, and 27-hydroxycholesterol (Fig. 3), have been shown to induce transcriptional activation of the *CYP7A* gene [22]. Cholesterol 7α-hydroxylase may also be a target for hormonal control. Hormones reported to influence the levels of this enzyme include glucocorticoids, thyroxine and insulin [2,16, 23].

Another enzyme which appears to be a target for regulation is sterol 27-hydroxylase (CYP27A), which is active in both the neutral and the acidic pathway [16]. The mechanisms for regulation of sterol 27-hydroxylase are however not well understood. The data obtained from a number of studies, mainly in the rat, are inconsistent. Compounds reported to affect the levels of CYP27A activity and/or mRNA include cholesterol, bile acids, and insulin. The effects of these compounds on CYP27A expression vary between different species.

Other bile acid forming enzymes that have been suggested as possible targets for regulation of bile acid formation are the 12α-hydroxylase (CYP8B) and the oxysterol 7α-hydroxylase (CYP7B). Little information is however available on regulatory mechanisms involving these enzymes [16, 24,25].

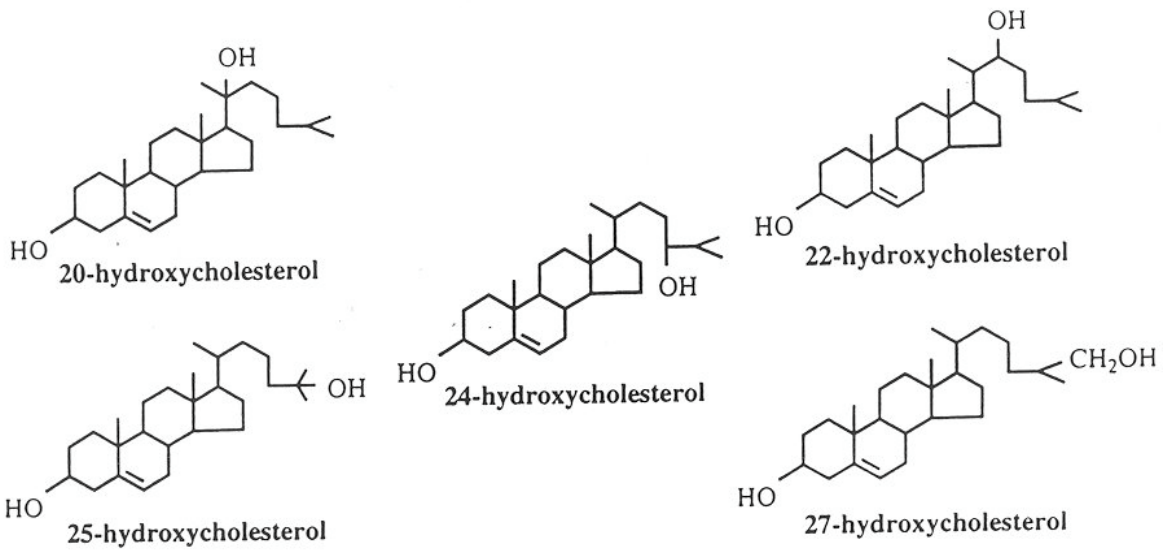

Fig. 3. Some oxysterols

Bioactivation of vitamin D

Vitamin D_3 is either supplied in the diet or formed in the skin from 7-dehydrocholesterol by ultraviolet irradiation. To become biologically active vitamin D_3 has to undergo metabolic activation (Fig. 4) [4,26,27]. The initial metabolic step is a 25-hydroxylation which is considered to take place mainly in the liver. 25-Hydroxylase activity towards vitamin D_3 has also been detected in other tissues *e. g.* in the kidney [27,28]. 25-Hydroxylase activity has been found in both microsomes and mitochondria. The mitochondrial enzyme responsible for this reaction is considered to be sterol 27-hydroxylase (CYP27A). Recently, a cDNA for a microsomal 25-hydroxylase, CYP2D25, was isolated from pig liver [29]. 25-Hydroxyvitamin D_3, which is the major circulating form of vitamin D_3, is subsequently converted to 1α,25-dihydroxyvitamin D_3 in the kidney (Fig. 4). The highest 1α-hydroxylase activity is localized in kidney mitochondria. 1α,25-Dihydroxyvitamin D_3 regulates the calcium and phosphate levels in the body together with parathyroid hormone (PTH). In addition, 1α,25-dihydroxyvitamin D_3 is considered to play a role in cellular differentiation. 25-Hydroxyvitamin D_3 may also undergo hydroxylation in the 23-, 24-, 26- and 27-positions. A cDNA encoding a mitochondrial cytochrome P450 enzyme which catalyzes 24-hydroxylation of 25-dihydroxyvitamin D_3 has been isolated and designated CYP24. The physiological significance of the 23-, 24-, 26- and 27-hydroxylated metabolites is not clear.

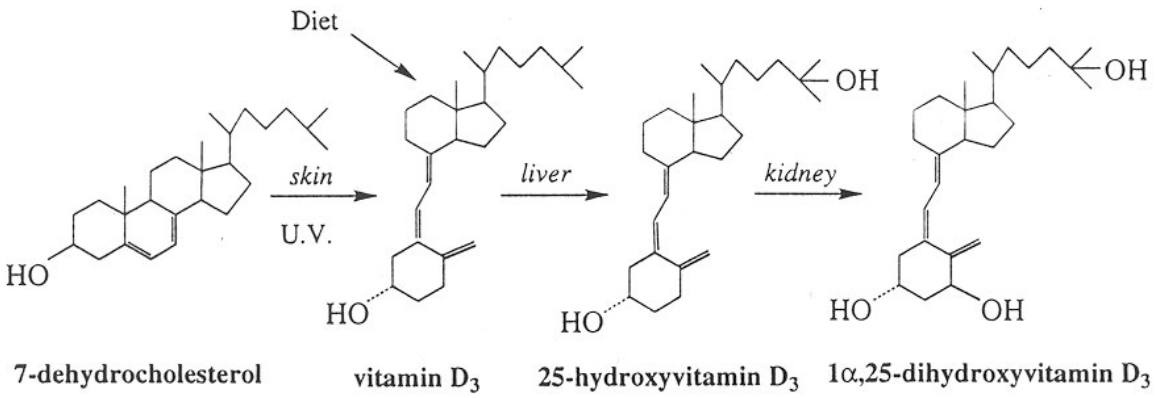

Fig. 4. Bioactivation of vitamin D

Cytochrome P450 monooxygenase system

The cytochrome P450 enzyme system was discovered in the 1950s as a pigment in liver microsomes which in its reduced state bound carbon monoxide and showed a characteristic absorbance peak at 450 nm [30]. Omura and Sato [31] characterized this CO-binding pigment as a heme protein and suggested the name P450. Cytochrome P450 (CYP) has turned out to be a large group of heme-containing proteins catalyzing monooxygenase reactions [32-34].

The cytochrome P450 enzymes are classified into families and subfamilies based on their sequence similarity [35]. Enzymes that are more than 40% identical at the amino acid level belong to the same family, indicated by an Arabic number (*e. g.* CYP1). If sequences are more than 55% identical, the enzymes belong to the same subfamily, indicated by addition of a capital letter (*e. g.* CYP1A). Each individual enzyme within the subfamily is then designated with an additional Arabic number (*e. g.* CYP1A1, CYP1A2).

Cytochrome P450 enzymes are found in virtually all living organisms. Eukaryotic cytochrome P450s are membrane bound and act on numerous substrates, both exogenous and endogenous [33,34]. A number of cytochrome P450 enzymes function to convert foreign chemicals, including drugs, into more polar compounds to facilitate secretion of these chemicals from the body. In some cases, however, the substrates may be converted into toxic metabolites with mutagenic or carcinogenic properties. Many cytochrome P450s act on endogenous substrates, catalyzing physiologically important reactions in for instance steroid hormone biosynthesis, bile acid formation and bioactivation of vitamin D. Many cytochrome P450s are known to be induced by different classes of compounds such as phenobarbital, polycyclic aromatic hydrocarbons and ethanol. In mammals, cytochrome P450 enzymes have been found in all tissues examined, predominantly in the mitochondria and the endoplasmic reticulum (microsomes).

Mechanism of cytochrome P450-dependent hydroxylations

The microsomal cytochrome P450 system consists of two protein components, cytochrome P450 and NADPH-cytochrome P450 reductase [36]. The mitochondrial system involves three components, cytochrome P450, ferredoxin (an iron sulfur protein), and ferredoxin reductase (a flavoprotein) [36]. Both cytochrome P450 systems are dependent on NADPH (Fig. 5). The first step in the cytochrome P450 reaction cycle is binding of the substrate to the oxidized form of cytochrome P450. The formed cytochrome P450-substrate

complex is then reduced by an electron transferred from NADPH via NADPH-cytochrome P450 reductase (microsomal system) or via ferredoxin and ferredoxin reductase (mitochondrial system). Next, molecular oxygen binds to the reduced P450-substrate complex after which a second electron is transferred from NADPH via the microsomal or mitochondrial electron transferring components. The final reaction steps involve splitting of the oxygen-oxygen bond and insertion of one molecule of oxygen into the substrate. The hydroxylated substrate and one molecule of H_2O leave the cytochrome P450 which is thereby restored to the oxidized state.

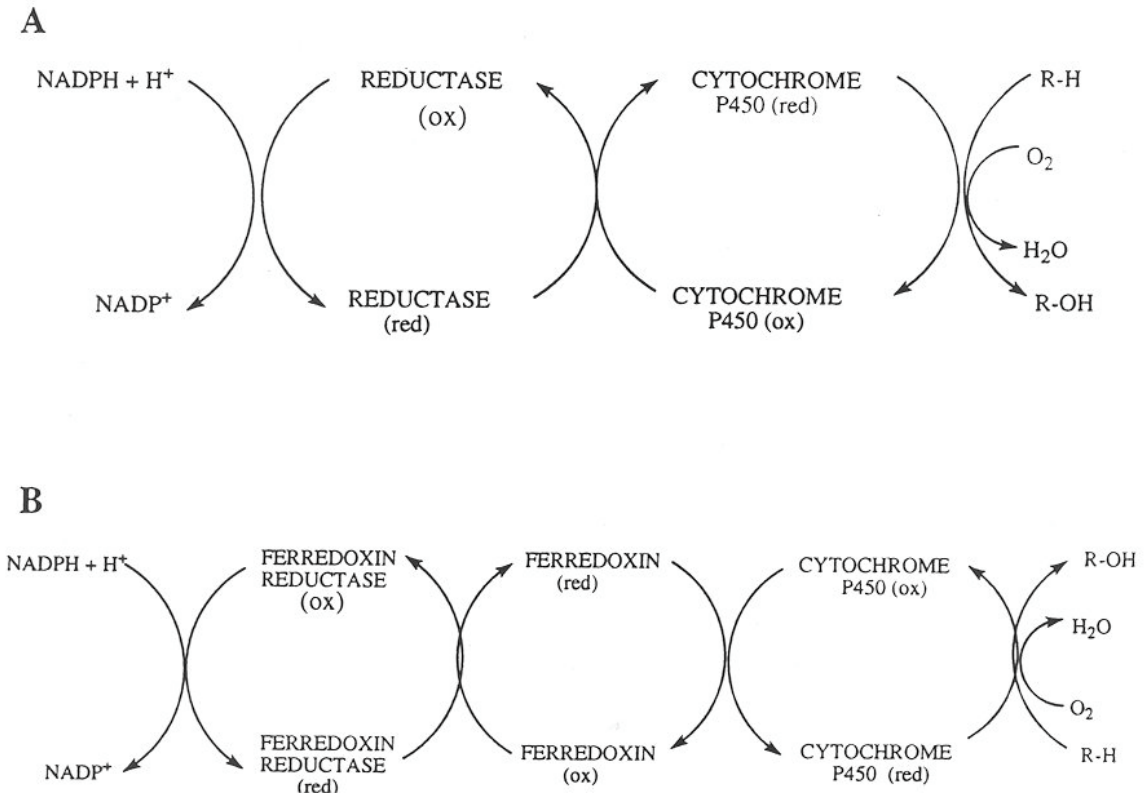

Fig. 5. Mechanism of microsomal (A) and mitochondrial (B) cytochrome P450-dependent hydroxylations

Previous work on 27-hydroxylating and 7α-hydroxylating cytochrome P450 enzymes

Sterol 27-hydroxylase (CYP27A)

Sterol 27-hydroxylase participates in several different metabolic reactions [2,16,26,37]. This mitochondrial cytochrome P450 enzyme catalyzes 27-hydroxylation of intermediates in

the neutral pathway of bile acid biosynthesis, as well as 27-hydroxylation of cholesterol in the acidic pathway. Sterol 27-hydroxylase has been suggested to be rate-limiting in the acidic pathway. The enzyme is also known to catalyze formation of 27-hydroxycholesterol in extrahepatic cells [12,13]. Furthermore, sterol 27-hydroxylase performs 25-hydroxylation of vitamin D_3, the first step of vitamin D_3 bioactivation, as well as 25-hydroxylation of 1α-hydroxyvitamin D_3, a nonphysiological compound which is used as a drug in treatment of diseases caused by disturbances in calcium metabolism. Hepatic sterol 27-hydroxylase has also been shown to catalyze both 1α- and 27-hydroxylation of 25-hydroxyvitamin D_3 [38,39]

Sterol 27-hydroxylase has been purified from livers of several species and from pig kidney [40-44]. cDNA encoding for sterol 27-hydroxylase has been isolated from rabbit [45], rat [46,47], human [48] and pig [49]. CYP27A mRNA has been found in numerous tissues, including liver, kidney, brain, and vascular endothelium [37,45,50].

The function of sterol 27-hydroxylase in extrahepatic tissues is believed to be related to the role of oxysterols in the maintenance of cholesterol homeostasis in various cells. 27-Hydroxycholesterol suppresses the rate-limiting enzyme in cholesterol biosynthesis, HMG-CoA reductase [8,51]. In addition, 27-hydroxycholesterol and other 27-oxygenated products are excreted from the cell more efficiently than cholesterol. 27-Oxygenated products formed in extrahepatic tissues can be transported to the liver and eliminated through conversion to bile acids [13]. The function of sterol 27-hydroxylase in extrahepatic tissues may also be related to the role of LDL. It is reported that the suppressive effect of LDL on HMG-CoA reductase is decreased when 27-hydroxylation of LDL cholesterol in fibroblasts is inhibited [52].

Mutations in the *CYP27A* gene result in the disease cerebrotendinous xanthomatosis (CTX), a rare autosomal gene defect manifested by symptoms such as xanthomas, cholesterol accumulation in various tissues, development of premature atherosclerosis and progressive neurological dysfunction [53].

Most of the studies on regulation of sterol 27-hydroxylase have been performed with the rat as a model. Bile acids and cholesterol appear to affect this enzyme differently in different species. Sterol 27-hydroxylase activity and mRNA levels are decreased by bile acids in the rat [54] but unaffected in the rabbit [55,56]. Cholesterol is reported to increase sterol 27-hydroxylase levels in the rabbit [55] but not in the rat [54]. An inhibitory effect of insulin on CYP27A transcription has been reported in studies with rat hepatocytes [57].

Cholesterol 7α-hydroxylase (CYP7A)

Cholesterol 7α-hydroxylase catalyzes the 7α-hydroxylation of cholesterol, the first and rate-limiting step in the neutral pathway of bile acid biosynthesis [2]. This enzyme is believed to have a narrow substrate specificity, active only towards cholesterol and its 5α-saturated analogue cholestanol. Cholesterol 7α-hydroxylase is a liver-specific microsomal enzyme. The enzyme has been purified from rat liver [58,59] and cDNA encoding for CYP7A has been isolated from rat, mouse, hamster, and human [60-65].

Studies on mice with a disruption in the *Cyp7a* gene [18] demonstrated a crucial role for this enzyme in bile acid biosynthesis. Most Cyp7a-deficient animals died within the first 18 days of life if not treated with vitamins and cholic acid due to liver failure and deficiencies of fat-soluble vitamins.

Several mechanisms for regulation of cholesterol 7α-hydroxylase have been described. The transcription of the *CYP7A* gene is down-regulated by bile acids via negative feedback control. Regulation of CYP7A by bile acids involves the nuclear receptor FXR (farnesyl X receptor) which binds to bile acid response elements in the *CYP7A* gene and suppresses promoter activity [21,66]. Cholestyramine, a drug used in the treatment of hyperlipoproteinaemia, increases the levels of cholesterol 7α-hydroxylase by binding to bile acids in the intestine and preventing their reabsorption to the liver [59,67]. Another mechanism proposed for regulation of cholesterol 7α-hydroxylase involves the nuclear receptor LXRα (liver X receptor alpha) [22,68]. This receptor mediates induction of *CYP7A* gene expression by oxysterols. A critical role for LXRα in induction of cholesterol 7α-hydroxylase is supported by results obtained with mice with a disruption in the LXRα gene [69]. LXRα-deficient mice were defective in cholesterol 7α-hydroxylase induction mechanisms and accumulated very large amounts of cholesterol in the liver. An increase of dietary cholesterol is reported to increase transcription of cholesterol 7α-hydroxylase in some species but decrease the transcription in others [16]. Since oxysterols can be formed from cholesterol, the induction of cholesterol 7α-hydroxylase by cholesterol feeding may be mediated via the LXRα regulatory mechanism. Cholesterol 7α-hydroxylase levels are also influenced by hormones [16,23]. Experiments with hypophysectomized rats showed a stimulating effect by thyroxine. Thyroxine alone did not stimulate cholesterol 7α-hydroxylase expression in experiments with primary cultures of rat hepatocytes. However, supplementation of culture media with both thyroxine and glucocorticoids resulted in a synergistic effect on cholesterol 7α-hydroxylase transcription. Glucocorticoids are reported to stimulate the promoter of the rat *CYP7A* gene whereas insulin inhibits promoter activity.

Some evidence for a posttranscriptional regulation of cholesterol 7α-hydroxylase has been reported, but most of the data available suggest that the regulation is predominantly on a transcriptional level [16,70,71].

Oxysterol 7α-hydroxylase (CYP7B)

Oxysterol 7α-hydroxylase, sometimes called 27-hydroxycholesterol 7α-hydroxylase, catalyzes 7α-hydroxylation of 25-hydroxycholesterol, 27-hydroxycholesterol, 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid but is not active towards cholesterol [72-76]. 27-Hydroxycholesterol 7α-hydroxylase activity has been found in both microsomes and mitochondria. 7α-Hydroxylase activity towards 25-hydroxycholesterol and 27-hydroxycholesterol is present in many tissues and cells including liver, brain, ovaries and fibroblasts [77-80]. In the liver, this enzyme catalyzes the second step in the acidic pathway of bile acid biosynthesis [16]. The role of 27-hydroxycholesterol 7α-hydroxylase in extrahepatic tissues remains unclear.

It has been suggested that 7α-hydroxylation of 27-hydroxycholesterol could play a role in modulating the effects of oxysterols [81,82]. According to some reports 7α-hydroxylation of 25-hydroxycholesterol and 27-hydroxycholesterol alters the regulatory effects of these compounds on cholesterol biosynthesis [81,83]. It has also been reported that 7α,25-dihydroxycholesterol is a several-fold less potent ligand than 25-hydroxycholesterol for LXRα, the nuclear receptor involved in oxysterol-mediated induction of CYP7A [22].

Several other steroids may be 7α-hydroxylated in the liver and other tissues, for instance dehydroepiandrosterone and pregnenolone which are intermediates in the steroid hormone biosynthesis (Fig. 1) [84-87]. The 7α-hydroxylated derivatives of dehydroepiandrosterone and pregnenolone are, however, not converted to steroid hormones. The role of these 7α-hydroxyderivatives is not clear. It has been suggested that dehydroepiandrosterone and pregnenolone and/or their 7α-hydroxylated derivatives may be important for brain function [86,87] and in the immune system [88]. A cDNA has been isolated from mouse and human encoding a cytochrome P450 enzyme called CYP7B which is expressed in a number of tissues including brain, liver, and kidney [24,87,89,90]. This enzyme catalyzes 7α-hydroxylation of 25-hydroxycholesterol, 27-hydroxycholesterol, dehydroepiandrosterone, and pregnenolone.

Experiments with Cyp7a-deficient mice indicate that in situations where the neutral pathway is inactive, due to suppression of cholesterol 7α-hydroxylase, the acidic pathway may compensate for this by producing 7α-hydroxylated bile acids via the oxysterol 7α-

hydroxylase [18,19]. These experiments showed that 90% of the mice with a disruption in the *Cyp7a* gene died within the first three weeks of life, but in those that survived the pathological symptoms resolved, coinciding with an upregulation of oxysterol 7α-hydroxylase. The role of the 7α-hydroxylating enzymes in bile acid formation may vary in different species and in different ages. Recently, Setchell et al [20] described a newborn child with severe neonatal cholestasis with a mutation in the *CYP7B* gene. Although this patient had a normal *CYP7A* gene, cholesterol 7α-hydroxylase activity was not detectable in the liver samples. Control samples from the livers of normal infants (< 1 year of age) also lacked cholesterol 7α-hydroxylase activity. These results indicate that CYP7B is critical for bile acid biosynthesis in infancy.

AIMS OF THE PRESENT INVESTIGATION

The aims of the present investigation were:

* to purify and characterize pig liver microsomal 27-hydroxycholesterol 7α-hydroxylase (oxysterol 7α-hydroxylase)

* to study if 20(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol and 24-hydroxycholesterol are 7α-hydroxylated in liver and kidney and, if so, to investigate the roles of known 7α-hydroxylating cytochrome P450 enzymes in these reactions

* to study the role for CYP27A in 25-hydroxyvitamin D_3 1α-hydroxylation in the kidney

* to study the developmental variation of 7α-hydroxylating and 27-hydroxylating cytochromes P450 (CYP7 and CYP27).

EXPERIMENTAL PROCEDURES

Materials

24-Hydroxycholesterol, 24-[3α-^{3}H]hydroxycholesterol, 7α,24-dihydroxycholesterol, 7α,20(*S*)-dihydroxycholesterol and 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol were synthesized as described [14,91-94]. 27-Hydroxycholesterol, prepared from kryptogenin [95], was a kind gift from Dr L. Tökes, Syntex, Palo Alto, CA, USA. 1α-Hydroxyvitamin D_3 was a kind gift from Dr. Lise Binderup, Leo, Copenhagen, Denmark. Octylamine-Sepharose was prepared as described [96]. Cholestyramine was obtained from Bristol Laboratories. The pJLH7α1.5 vector containing human CYP7A cDNA [97] and the pCMV-hCYP7B1 vector containing human CYP7B cDNA were generous gifts from Dr. J. Y. L. Chiang, Northeastern Ohio Universities, and Dr. D. W. Russell, University of Texas, USA, respectively. The pSVL simian-virus-40 eukaryotic expression vector containing cDNA for human CYP7A [65] and the plasmid clone p7α-11 [60] containing rat CYP7A cDNA inserted in a pBluescript vector were kind gifts from Dr. K. Okuda, Miyazaki Medical College, Miyazaki, Japan. The pSVL expression vector containing rat CYP7A cDNA was constructed by inserting the 2.2 kB *Xho*I-*Xho*I fragment into pSVL vector as described [60]. Remaining compounds were purchased from commercial sources. All chemicals were of analytical grade.

Animals

The livers and kidneys from six months old male pigs were obtained from the local slaughterhouse. Livers and kidneys from pigs aged from a few days to three months were obtained from the Funbo-Lövsta Research Centre, Department of Animal Breeding and Genetics, Swedish University of Animal Sciences, Ultuna. All pigs used were castrated except those five days old or younger.

In paper IV, female Sprague-Dawley rats (M&B, Ry, Denmark) weighing about 250 g were fed a chow diet (R36, Lactamin AB, Vadstena, Sweden) supplemented with cholestyramine. The animals were housed together (3 or 4 in each cage) under controlled conditions (22.4°C, 50% air humidity, 12 h light cycle) and were acclimatized for one week before the experiment started. The animals had free access to food and water during the experiment.

Purification of enzymes from porcine tissues

Purification of 7α-hydroxylating cytochrome P450 from microsomes of pig liver and kidney

Preparation of pig liver cytochrome P450 active in 7α-hydroxylation of 27-hydroxy-cholesterol and dehydroepiandrosterone (oxysterol 7α-hydroxylase) was performed as described in Paper II. The purification procedure is summarized below:

Pig liver microsomes
↓
Sodium cholate solubilization
↓
Polyethylene glycol precipitation
↓
DEAE-Sepharose chromatography
↓
Hydroxylapatite chromatography
↓
High performance S-Sepharose chromatography 1
(with 0.1% sodium cholate and 0.05% POEL)
↓
High performance Q-Sepharose chromatography
↓
High performance S-Sepharose chromatography 2
(with 0.4% POEL, no sodium cholate)

Preparation of pig liver microsomal cytochrome P450 active in 7α-hydroxylation of cholesterol was performed as previously described by Toll et al [72].

A cytochrome P450 extract catalyzing the 7α-hydroxylation of cholesterol, 27-hydroxycholesterol and dehydroepiandrosterone was partially purified from liver microsomes from newborn (five days old) and six months old pigs as described in Paper V. The microsomal preparations used were mixtures of liver tissue from three individuals of each age. The methodology for these preparations was identical with the first four purification steps in the preparation of oxysterol 7α-hydroxylase (see above).

Preparation of pig kidney cytochrome P450 active in 7α-hydroxylation of 27-hydroxycholesterol and dehydroepiandrosterone was performed as described in Paper IV. The purification procedure is summarized below:

Pig kidney microsomes
↓
Sodium cholate solubilization
↓
Polyethylene glycol precipitation
↓
Octylamine-Sepharose chromatography
↓
Hydroxylapatite chromatography
↓
High performance Q-Sepharose chromatography

Purification of 27-hydroxylating cytochrome P450 from pig liver mitochondria

Cytochrome P450 active in 27-hydroxylation was partially purified from liver mitochondria from newborn and six months old pigs as described in Paper V. The procedure was similar to that described by Axén et al [98], except that aminohexyl-Sepharose was used instead of octylamine-Sepharose. The mitochondrial preparations used were mixtures of liver tissue from three individuals of each age.

Purification of 1α- and 27-hydroxylating cytochrome P450 from pig kidney mitochondria

Kidney cortex mitochondria were prepared from 20-25 kg of pig kidneys and 1α- and 27-hydroxylating cytochrome P450 was partially purified as described in Paper I. The methodology was similar to the one described by Postlind [99]. The procedure is summarized below:

Pig kidney mitochondria
↓
Osmotic disruption in 10 mM phosphate buffer
↓
Sonication
↓
Sodium cholate solubilization
↓
DEAE-Sepharose chromatography
↓
Aminohexyl-Sepharose chromatography
↓
Hydroxylapatite chromatography
↓
High performance Q-Sepharose chromatography 1
↓
High performance Q-Sepharose chromatography 2

Expression of recombinant human CYP7A and CYP27A in *E. coli*

Expression of human CYP7A in *E. coli* and purification of the *E. coli*-expressed protein were performed in essentially the same way as described by Karam and Chiang [97], with slight modifications. The expression plasmid pJL/H7α1.5 containing cDNA encoding for human CYP7A was transformed into *E. coli* strain DH5α instead of TOPP3 cells. Two litres of "terrific broth" (2.4% yeast extract, 1.2% tryptone, 0.4% glycerol, 17 mM KH_2PO_4 and 72 mM KH_2PO_4) containing 100 mg ampicillin/liter were inoculated with a 6 h culture of transformed DH5α-cells in Luria-Bertani (LB) broth containing 100 mg of ampicillin/liter. The culture was grown at 37°C with shaking at 200 rpm until the OD_{600} reached 0.5-0.6. Protein synthesis was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) and δ-aminolevulinic acid (δ-ALA) to a final concentration of 1 mM and 0.2 mM respectively and growing the *E. coli* culture under good aeration at 30°C for 16 h with shaking at 150 rpm. The *E. coli* cells were harvested and lysed and the recombinantly expressed protein was purified by octylamine-Sepharose and hydroxylapatite chromatography according to the methods described by Karam and Chiang [97] with modifications as described in Paper III.

Expression of human CYP27A in *E. coli* was performed as described by Axén et al [38].

Mammalian cell cultures

Mammalian cell lines were grown in Dulbecco's Modified Eagle Medium, supplemented with 10% fetal calf serum and antibiotics, on 60 mm or 100 mm culture dishes in a humidified incubator at 37°C in an atmosphere of 95% air and 5% CO_2.

Human embryonic kidney cells (293 cells) were assayed for endogenous 7α-hydroxylase activity towards cholesterol, oxysterols, dehydroepiandrosterone and pregnenolone by addition of the substrates, dissolved in either dimethylsulfoxide or 45% 2-hydroxypropyl-β-cyclodextrin, to the medium and incubation for 24 or 72 h. Following incubation with substrate, the medium was collected and extracted and the organic phase was analyzed for 7α-hydroxylated metabolites as described below.

COS-M6 cells were transfected by electroporation with pSVL vector containing cDNA encoding for human CYP7A or rat CYP7A as described in Papers III and IV. COS cells transfected with pSVL vector without the CYP7A cDNA-insert or pSVL vector with the cDNA inserted in reversed position were used as negative controls. Following transfection, the cells were cultured for 48 h in medium containing oxysterols or dehydroepiandrosterone,

dissolved in dimethylsulfoxide. Catalytic activity towards cholesterol was assayed with endogenous substrate. Following incubation, medium and cells were harvested separately. The cells were suspended in 50 mM Tris-acetate buffer and homogenized. Cells and medium were extracted and the organic phase was analyzed for 7α-hydroxylated metabolites as described below.

Incubation procedures

Incubations were carried out at 37°C for 5-60 min with microsomes or purified microsomal or mitochondrial cytochrome P450 enzyme fractions. All substrates were dissolved in 25 μl acetone prior to addition to the incubation mixture.

The substrates [4-^{14}C]cholesterol, 20(*S*)-hydroxycholesterol (unlabeled), 22(*R*)-hydroxycholesterol (unlabeled), 24-[3α-^{3}H]hydroxycholesterol, 24-hydroxycholesterol (unlabeled), 25-[26,27-^{3}H]hydroxycholesterol, 25-hydroxycholesterol (unlabeled), 27-hydroxycholesterol (unlabeled), [4-^{14}C]dehydroepiandrosterone, [7-^{3}H]pregnenolone, [4-^{14}C]testosterone or [1-^{14}C]lauric acid were incubated with varying amounts of microsomes or purified microsomal cytochrome P450 fractions and NADPH in a total volume of 1 ml of 50 mM Tris-acetate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. NADPH-cytochrome P450 reductase [100] was added in incubations with purified microsomal enzyme fractions. In incubations with testosterone, dilauroylglycero-3-phosphorylcholine was added. Triton X-100 and dithiothreitol were added in incubations with cholesterol and in all incubations with CYP7A purified from *E. coli*. Triton X-100 was not added in incubations with rat liver microsomes. 7α-Hydroxycholesterol (unlabeled) was incubated with microsomes and NAD^+ in a volume of 1 ml of 100 mM phosphate buffer pH 7.4, containing 20% glycerol and 0.1 mM EDTA.

The substrates 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol, 1α-hydroxyvitamin D_3 (unlabeled) or 25-hydroxyvitamin D_3 (unlabeled) were incubated with purified mitochondrial cytochrome P450 fractions, NADPH, ferredoxin and ferredoxin reductase [40] in a volume of 1 ml of 50 mM Tris-acetate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA.

Incubations with oxysterols, cholesterol, 1α-hydroxyvitamin D_3 and 25-hydroxyvitamin D_3 were terminated with 5 ml of trichloroethane/methanol (2:1) and incubations with dehydroepiandrosterone, pregnenolone and testosterone with 5 ml of ethyl acetate. The incubations with lauric acid and 5β-cholestane-3α,7α,12α-triol were terminated with 5 ml of ethanol.

Analysis of incubation mixtures

Incubations with 27-hydroxycholesterol and unlabeled 25-hydroxycholesterol were analyzed as described in Papers II and IV. The analysis procedure was similar to that described by Toll et al [76]. The 7α-hydroxylated products were converted to 7α,27-dihydroxy-4-cholesten-3-one and 7α,25-dihydroxy-4-cholesten-3-one by incubation with cholesterol oxidase prior to HPLC analysis. The samples were subjected to straight phase HPLC with hexane/isopropanol as the mobile phase and steroids with a 3-oxo-Δ^4-structure were monitored at 240 nm. Incubations containing radiolabeled 25-hydroxycholesterol were analyzed by thin layer chromatography. The chromatoplates were developed once in a solvent system consisting of toluene/ethyl acetate 40:60 (v/v) and scanned for radioactivity using a Berthold Tracemaster 20 TLC scanner [19].

Cholesterol 7α-hydroxylase activity in microsomes and purified fractions was analyzed using [4-^{14}C]cholesterol and thin layer chromatography as described [96]. Cholesterol 7α-hydroxylase activity in COS cells was assayed with the endogenous cholesterol as substrate. Following extraction of cell homogenate and medium, the samples were incubated with cholesterol oxidase and subjected to HPLC in a way similar to the analysis of incubations with 27-hydroxycholesterol, with modification of the mobile phase as described in Paper III.

Assays for analyses of incubations with unlabeled 20(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol and 24-hydroxycholesterol were developed by modification of the assays for incubations with 27-hydroxycholesterol and unlabeled cholesterol. Since these oxysterols are less polar than 27-hydroxycholesterol but more polar than cholesterol, it was assumed that 7α-hydroxylated products formed from 20(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol and 24-hydroxycholesterol would have retention times intermediate to 7α,27-dihydroxy-4-cholesten-3-one and 7α-hydroxy-4-cholesten-3-one. Experiments with different HPLC mobile phases were performed to screen for peaks corresponding to enzymatically derived products. Incubations were performed with and without NADPH-cytochrome P450 reductase or NADPH in order to detect if the HPLC peaks found would correspond to cytochrome P450-mediated enzyme activity. The mobile phases and retention times for the analyses of these products were those described in Papers III and IV. Incubations with ^{3}H-labeled 24-hydroxycholesterol were analyzed by radio HPLC as described in Paper III [101].

Incubations with dehydroepiandrosterone and pregnenolone were analyzed by reversed phase HPLC as described in Paper II using methanol/water as the mobile phase. Elution of

labeled steroids was monitored by a Radiomatic™ 150TR Flow Scintillation Analyzer (Packard). The assay conditions were based on the procedure described by Akwa et al [86].

The incubations with 5β-cholestane-3α,7α,12α-triol were extracted with acidified ether and analyzed by thin layer chromatography. The chromatoplates were developed once in a solvent system consisting of ethyl acetate/isooctane/acetic acid, 50:50:17 (v/v/v) and scanned for radioactivity using a Berthold Tracemaster 20 TLC scanner [40].

Incubations with 25-hydroxyvitamin D_3 and 1α-hydroxyvitamin D_3 were analyzed by straight phase and reversed phase HPLC as described [42,102]. Incubations with lauric acid were extracted and assayed by thin layer chromatography as described by Dahlbäck and Wikvall [102]. Formation of 7α-hydroxy-4-cholesten-3-one from 7α-hydroxycholesterol was analyzed by HPLC as described by Furster [103].

The identities of 7α-hydroxylated metabolites formed from cholesterol, 20(*S*)-hydroxycholesterol, 24-hydroxycholesterol, 27-hydroxycholesterol, dehydroepiandrosterone and pregnenolone were verified by gas chromatography-mass spectrometry (GC/MS) as described [75,78,101,104]. Previous to GC/MS the metabolites were converted to trimethylsilyl ethers.

Immunological methods

The methodology used for Western blot experiments was similar to that described by Andersson and Jörnvall [105]. For details on the procedure see Papers I and V. The immunoreactive bands were visualized either by alkaline phosphatase detection or by enhanced chemiluminescence (ECL). The monoclonal antibody against porcine CYP27A, used in immunoblotting, was the one produced and described in a previous report from this laboratory [42]. The polyclonal antibody against an unknown 55 kDa pig kidney mitochondrial protein was produced as described in Paper I.

Incubations with antibody-coupled Sepharose were performed as previously described [41].

Induction of rat liver CYP7A by cholestyramine treatment

Rats (Sprague-Dawley strain) were maintained either on a regular chow diet or a diet supplemented with 3% (w/w) cholestyramine. The animals were sacrificed after six days and the 7α-hydroxylation of cholesterol, 20(*S*)-hydroxycholesterol, 24-hydroxycholesterol, 25-

hydroxycholesterol, 27-hydroxycholesterol and dehydroepiandrosterone was examined in liver microsomes prepared from untreated and cholestyramine-treated rats as described under Incubation procedures.

Isolation of RNA and Northern blot analysis

Total RNA was isolated from pig liver tissue with the RNeasy total RNA Midi isolation kit (Qiagen). Poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose chromatography according to Sambrook et al [106].

Northern blot analysis was carried out as previously described [49]. For details see Paper V. The cDNA probes used were a 1.7 kB CYP27A pig kidney cDNA fragment [49], a 1.5 kB *Nco*I-*Nco*I fragment of human CYP7A cDNA excised from a pJL vector containing human CYP7A [97] and a 1.6 kB *Eco*RI-*Not*I fragment of human CYP7B cDNA excised from a pCMV6 vector containing human CYP7B. After Northern blotting with CYP27A, CYP7A or CYP7B cDNA probes the filters were stripped and probed with a 1.7 kB *Bam*HI-*Sal*I fragment of human β-actin cDNA.

Other methods

NADPH-cytochrome P450 reductase was prepared from pig liver microsomes as described by Yasukochi and Masters [100]). Ferredoxin and ferredoxin reductase were prepared from bovine adrenal mitochondria as described by Wikvall [40].

Protein concentrations in microsomal fractions and cell homogenates were determined by the method of Lowry [107]. The concentration in purified protein fractions was estimated by measuring the absorbance at 280 nm (concentration in mg/ml = absorbance of protein at 280 nm). Cytochrome P450 concentration in purified fractions was estimated by measuring the absorbance at 416 nm as an indication of total heme content [108].

SDS-PAGE was performed according to Laemmli [109] with modifications as described in Paper II and the gels were silverstained as described by Wray et al [110].

Sequence analysis of peptides from porcine oxysterol 7α-hydroxylase was performed by mass spectrometry (Q-TOF) [111].

RESULTS AND DISCUSSION

Purification and biochemical characterization of microsomal oxysterol 7α-hydroxylase in pig liver (Papers II, III, IV, and V)

Paper II describes the purification of cytochrome P450 active in 7α-hydroxylation of 27-hydroxycholesterol from pig liver microsomes. Sodium cholate solubilization, precipitation with polyethylene glycol and chromatography on DEAE-Sepharose, hydroxylapatite, S-Sepharose, Q-Sepharose and S-Sepharose resulted in a cytochrome P450 enzyme fraction with 7α-hydroxylase activity towards 25-hydroxycholesterol, 27-hydroxycholesterol, pregnenolone, and dehydroepiandrosterone (Table 1). This "oxysterol 7α-hydroxylase fraction" did not 7α-hydroxylate cholesterol or testosterone. The apparent K_m values for 7α-hydroxylation by the oxysterol 7α-hydroxylase fraction were 4 μM for 27-hydroxycholesterol, 4 μM for 25-hydroxycholesterol, 13 μM for dehydroepiandrosterone and 12 μM for pregnenolone. SDS-PAGE of the purified cytochrome P450 fraction showed one major and two to three minor protein bands.

Previous studies suggested the possibility of one common enzyme responsible for the 7α-hydroxylation of 27-hydroxycholesterol and dehydroepiandrosterone in extrahepatic tissues [77,78,87,89]. As described in Paper II, a series of experiments was performed to study whether there are one or several enzymes 7α-hydroxylating 27-hydroxycholesterol and dehydroepiandrosterone in pig liver. The activities towards 27-hydroxycholesterol and dehydroepiandrosterone copurified, but the ratio between 27-hydroxycholesterol and dehydroepiandrosterone 7α-hydroxylation varied considerably in different purification steps and between different preparations. The 7α-hydroxylase activity towards dehydroepiandrosterone varied more than 10-fold in different preparations. The effects of the enzyme inhibitors disulfiram, N-bromosuccinimide, ketoconazole, metyrapone and α-naphthoflavone on the 7α-hydroxylation of 27-hydroxycholesterol and dehydroepiandrosterone in the purified 7α-hydroxylase fraction were examined. All five inhibitors were found to affect the activities towards the two substrates in a similar way. Substrate inhibition experiments showed that dehydroepiandrosterone inhibited 27-hydroxycholesterol 7α-hydroxylation whereas 27-hydroxycholesterol had almost no inhibitory effect on dehydroepiandrosterone 7α-hydroxylation. Experiments to examine the nature of inhibition by dehydroepiandrosterone on 27-hydroxycholesterol 7α-hydroxylation indicated a noncompetitive

Table 1. 7α-Hydroxylation by purified cytochrome P450 fractions from pig liver and by recombinant human and rat CYP7A

	Pig liver microsomes	Oxysterol 7α-hydroxylase fraction	Cholesterol 7α-hydroxylase fraction	Human CYP7A expressed in E. coli	Human CYP7A expressed in COS cells	Rat CYP7A expressed in COS cells
	nmol/nmol P450/min				nmol/mg cell protein/48 h	
Cholesterol	0.002-0.005	≤ 0.005	1.1	16.2	0.9-6.0[3]	0.8-2.5[3]
20(S)-Hydroxycholesterol	≤ 0.005	≤ 0.005	1.4	6.6	1.0	3.1
22(R)-Hydroxycholesterol	≤ 0.005	≤ 0.005	0.2	≤ 0.03	≤ 0.2	ND
24-Hydroxycholesterol	0.008	≤ 0.005	0.3	6.3	0.5	0.5
25-Hydroxycholesterol	0.35	3.8	0.5	3.1	1.6	0.8
27-Hydroxycholesterol	0.5	4.8	0.4	7.3	4.5	2.6
Dehydroepiandrosterone	5.9	43.2	0.4	≤ 0.2[2]	≤ 0.2	ND
Pregnenolone	3.2	11.7	ND[1]	≤ 0.2[2]	ND	ND

[1]Not determined

[2]The detection limit for assay of 7α-hydroxylated metabolites from dehydroepiandrosterone and pregnenolone was slightly higher than for assay of 7α-hydroxylated metabolites from oxysterols. The present data cannot exclude 7α-hydroxylase activity towards dehydroepiandrosterone or pregnenolone less than 0.2 nmol/nmol P450/min.

[3]The cholesterol 7α-hydroxylase activity varied depending on which oxysterol that was added to the cell medium.

inhibitory mechanism. The finding that 27-hydroxycholesterol and dehydroepiandrosterone were not able to competitively inhibit the 7α-hydroxylation of each other indicates that the 7α-hydroxylations of dehydroepiandrosterone and 27-hydroxycholesterol are not located to the same active site. If so, this could be explained either by the existence of one enzyme with several active sites or by the existence of several enzymes catalyzing the reactions. Taken together, the results suggest the existence of at least two closely related enzymes involved in the 7α-hydroxylation of 27-hydroxycholesterol and dehydroepiandrosterone in pig liver. The possibility of overlapping substrate specificity of the 7α-hydroxylating enzymes cannot be excluded.

A cDNA isolated from mouse hippocampus, encoding an oxysterol 7α-hydroxylase (CYP7B) catalyzing 7α-hydroxylation of 25-hydroxycholesterol, 27-hydroxycholesterol, dehydroepiandrosterone and pregnenolone, was reported by Stapleton et al [89] and Rose et al [87]. This enzyme was later shown to be expressed also in several other tissues in mice and humans including liver and kidney [90]. In order to compare the porcine oxysterol 7α-hydroxylase fraction with recombinant CYP7B, the highly purified porcine oxysterol 7α-hydroxylase preparation was used for sequence analysis as described in Paper V. Three peptide fragments, 16-18 amino acids in length, generated by proteolytic cleavage, were subjected to sequence analysis using mass spectrometry (Q-TOF). The sequences of these peptides showed high homology with the deduced sequences of human (65-94% identity) and murine (41-89% identity) CYP7B. It may therefore be concluded that the purified porcine oxysterol 7α-hydroxylase fraction contains CYP7B or an enzyme belonging to the CYP7B subfamily.

7α-Hydroxylation of 24-hydroxycholesterol, 20(*S*)-hydroxycholesterol and 22(*R*)-hydroxycholesterol by purified cytochrome P450 fractions from pig liver (Papers III and IV)

The net flux over the brain-blood barrier of 24(*S*)-hydroxycholesterol, a steroid important for brain cholesterol homeostasis, was reported to be similar to the uptake of this steroid in the liver [14,101,112]. Since there was no renal elimination, 24(*S*)-hydroxycholesterol is apparently eliminated by the liver. Data obtained with human subjects suggested a probable metabolism of this steroid rather than excretion in unmetabolized form. If 24(*S*)-hydroxycholesterol is converted by the liver into bile acids, it must undergo 7α-hydroxylation. The possibility of 7α-hydroxylase activity towards 24-hydroxycholesterol was

the refore investigated with purified 7α-hydroxylating cytochrome P450 fractions from pig liver. The results of Paper III show that 24-hydroxycholesterol is 7α-hydroxylated in the liver. In view of the structural similarity between 24(*S*)-hydroxycholesterol and other side-chain hydroxylated cholesterol derivatives, it seemed likely that 24(*S*)-hydroxycholesterol could be a substrate for oxysterol 7α-hydroxylase. However, the highly purified porcine oxysterol 7α-hydroxylase fraction, active towards 25-hydroxycholesterol and 27-hydroxycholesterol, did not 7α-hydroxylate 24-hydroxycholesterol. Formation of 7α,24-dihydroxycholesterol was found in another partially purified pig liver microsomal cytochrome P450 enzyme fraction, showing high 7α-hydroxylase activity towards cholesterol, the "cholesterol 7α-hydroxylase fraction" (Table 1). Consequently, it appeared that 24-hydroxycholesterol was metabolized in the liver by an enzyme different from oxysterol 7α-hydroxylase. To obtain further information concerning the 7α-hydroxylase activity towards 24-hydroxycholesterol and to investigate its possible relation to the cholesterol 7α-hydroxylase enzyme, the cholesterol 7α-hydroxylase fraction was incubated in the presence of 7-oxocholesterol, a strong inhibitor of cholesterol 7α-hydroxylase [113]. 7-Oxocholesterol was found to be an effective inhibitor also of the 7α-hydroxylase activity towards 24-hydroxycholesterol. A concentration of 3 μM inhibited the formation of 7α,24-dihydroxycholesterol by 70%.

Oxysterols, including 24-hydroxycholesterol, have been reported to influence cholesterol homeostasis in several ways. 20(*S*)-Hydroxycholesterol, 22(*R*)-hydroxycholesterol, 24-hydroxycholesterol, 25-hydroxycholesterol and 27-hydroxycholesterol are ligands of the LXR, a ligand-dependent transcription factor that stimulates bile acid biosynthesis [22,68,69]. Furthermore, 20(*S*)-, 25- and 27-hydroxycholesterol are considered to suppress the transcription of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis [1,8]. Whereas the metabolism of 25-hydroxycholesterol and 27-hydroxycholesterol has been extensively studied [19,24,72-83,90] little is known about a possible hepatic metabolism of 20(*S*)-hydroxycholesterol and 22(*R*)-hydroxycholesterol. The results of paper IV show that 20(*S*)-hydroxycholesterol and 22(*R*)-hydroxycholesterol are 7α-hydroxylated in pig liver (Figs. 6 and 7, respectively). The enzyme fraction found to 7α-hydroxylate these two oxysterols was the one that also 7α-hydroxylated 24-hydroxycholesterol, *i. e.* the cholesterol 7α-hydroxylase fraction (Table 1).

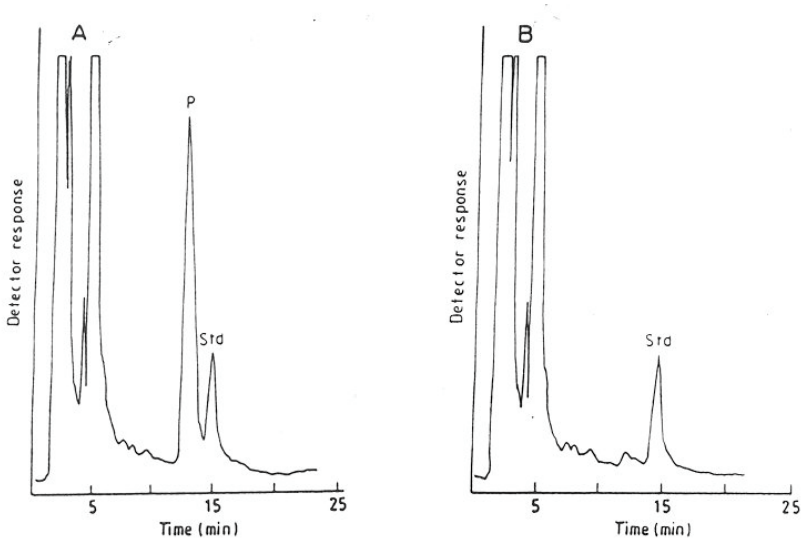

Fig. 6. HPLC analysis of incubations with 20(*S*)-hydroxycholesterol. HPLC of an incubation with 20(*S*)-hydroxycholesterol and the purified reconstituted pig liver cholesterol 7α-hydroxylase fraction (A) and the corresponding control (an incubation without NADPH-cytochrome P450 reductase) (B). An enzymatically derived product was eluted after 12-13 min.

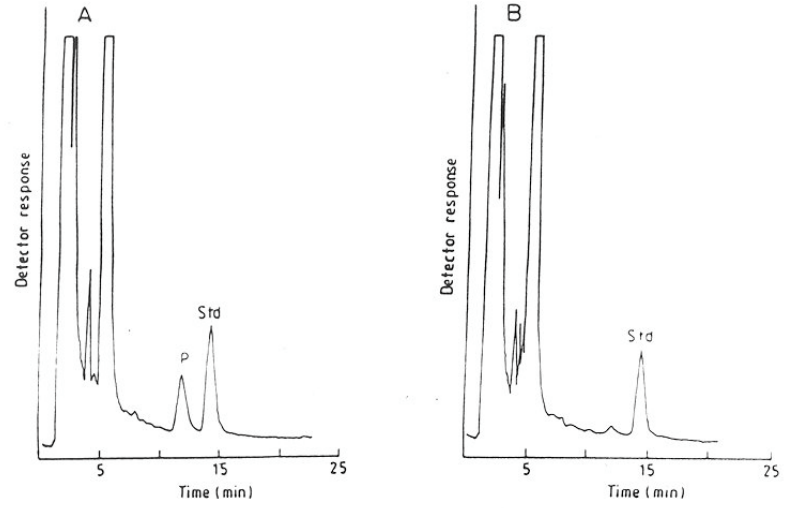

Fig. 7. HPLC analysis of incubations with 22(*R*)-hydroxycholesterol. HPLC analysis of an incubation with 22(*R*)-hydroxycholesterol and the purified reconstituted pig liver cholesterol 7α-hydroxylase fraction (A) and the corresponding control (an incubation without NADPH-cytochrome P450 reductase) (B). An enzymatically derived product was eluted after 12 min.

7α-Hydroxylation of oxysterols by recombinantly expressed human and rat cholesterol 7α-hydroxylase (CYP7A) (Papers III and IV)

Because the studies with purified enzyme fractions from pig liver indicated that the enzyme responsible for 7α-hydroxylation of 20(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol and 24-hydroxycholesterol might be the cholesterol 7α-hydroxylase, experiments were carried out with human CYP7A expressed in *E. coli*. Recombinant *E. coli*-expressed human CYP7A was found to 7α-hydroxylate both cholesterol and 24-hydroxycholesterol (Table 1). The enzyme showed a preference for the 24(*S*)-isomer over the 24(*R*)-isomer. Experiments with human CYP7A also showed 7α-hydroxylase activity by this enzyme towards 20(*S*)-hydroxycholesterol. Surprisingly, human *E. coli*-expressed CYP7A 7α-hydroxylated not only 20(*S*)-hydroxycholesterol and 24-hydroxycholesterol but also 25-hydroxycholesterol and 27-hydroxycholesterol. No 7α-hydroxylase activity was detected towards 22(*R*)-hydroxycholesterol, dehydroepiandrosterone or pregnenolone (Table 1). The apparent K_m values for the 7α-hydroxylation of 20(*S*)-, 24-, 25-, and 27-hydroxycholesterol by *E. coli*-expressed human CYP7A were found to be 8, 6, 4, and 3 μM, respectively. A K_m of 3 μM was obtained for cholesterol 7α-hydroxylation.

Experiments were also performed with human CYP7A, transiently expressed in simian COS cells. Transfection of COS cells with pSVL vector containing human CYP7A cDNA and incubation with oxysterols added to the medium resulted in formation of 7α,20(*S*)-dihydroxycholesterol, 7α,24-dihydroxycholesterol, 7α,25-dihydroxycholesterol, and 7α,27-dihydroxycholesterol by transfected cells. The CYP7A-mediated 7α-hydroxylase activity towards 20(*S*)-, 24-, 25-, and 27-hydroxycholesterol was 1.0, 0.5, 1.6, and 4.5 nmol/mg cell protein/48 h, respectively. As expected, cells transfected with CYP7A cDNA also showed a high rate of formation of 7α-hydroxycholesterol from endogenous cholesterol (0.9-6.0 nmol/mg in different experiments) (Table 1). No 7α-hydroxylase activity was found towards 22(*R*)-hydroxycholesterol or dehydroepiandrosterone. Thus, the results of these experiments supported the results obtained with *E. coli*-expressed enzyme and with porcine microsomal cytochrome P450 fractions. The finding that 22(*R*)-hydroxycholesterol was 7α-hydroxylated in experiments with porcine but not human enzyme may reflect species differences with respect to the 7α-hydroxylation of oxysterols.

It was noted that 7α-hydroxycholesterol was present both within the cells and in the cell medium of transfected cells, whereas 7α-hydroxylated metabolites formed from oxysterols were found almost exclusively in the cell medium. This is in agreement with reported

findings that side chain-hydroxylated cholesterol derivatives are more easily secreted from some cells [13,114]. The CYP7A-transfected cells 7α-hydroxylated 27-hydroxycholesterol in concentrations as low as 0.5 μM, which corresponds to the reported physiological concentration of this oxysterol [114,115]. Interestingly, an increase of the concentration of added 27-hydroxycholesterol markedly decreased the 7α-hydroxylase activity by transfected COS cells towards cholesterol. An increase of added 27-hydroxycholesterol from 0.5 to 5 μM decreased cholesterol 7α-hydroxylation about fivefold.

Experiments were also performed with rat CYP7A cDNA expressed in COS cells. Recombinantly expressed rat CYP7A was able to 7α-hydroxylate the same substrates as human CYP7A. The 7α-hydroxylase activity mediated by recombinant rat CYP7A towards 20(*S*)-, 24-, 25- and 27-hydroxycholesterol was 3.1, 0.5, 0.8 and 2.6 nmol/mg cell protein/48 h, respectively. The 7α-hydroxylase activity towards cholesterol by rat CYP7A varied between 0.8 and 2.5 nmol/mg cell protein/48 h in different experiments (Table 1).

Effects of 7-oxocholesterol on the 7α-hydroxylation of 27-hydroxycholesterol by pig liver enzyme fractions (Paper IV)

To further study the hepatic 27-hydroxycholesterol 7α-hydroxylase activity, the pig liver cholesterol 7α-hydroxylase and oxysterol 7α-hydroxylase fractions (see Table 1) were incubated with 7-oxocholesterol, an inhibitor of CYP7A [113]. A marked difference was found in the effects of 7-oxocholesterol on the activity in the two fractions. This compound had very little effect on the 27-hydroxycholesterol 7α-hydroxylase activity in the oxysterol 7α-hydroxylase fraction. In contrast, the 27-hydroxycholesterol 7α-hydroxylase activity in the cholesterol 7α-hydroxylase fraction was inhibited by about 80% at a concentration of 3 μM 7-oxocholesterol. These results are in agreement with a role for CYP7A in hepatic 7α-hydroxylation of 27-hydroxycholesterol.

Effect of cholestyramine treatment on the 7α-hydroxylation of oxysterols in rat liver (Paper IV)

The 7α-hydroxylase activities towards oxysterols and dehydroepiandrosterone were measured in microsomes prepared from rats treated with cholestyramine, an inducer of CYP7A [59,67] (Fig. 8). Dehydroepiandrosterone was included in this experiment as a negative control and as a marker substrate for CYP7B. Treatment with cholestyramine

increased 7α-hydroxylation of both cholesterol and 20(*S*)-hydroxycholesterol about threefold. The 7α-hydroxylation of 25-hydroxycholesterol and 27-hydroxycholesterol was also stimulated, although not to the same extent as the 7α-hydroxylation of cholesterol and 20(*S*)-hydroxycholesterol. The increase of 7α-hydroxylase activity in cholestyramine-treated rats as compared with untreated rats was about 70% for 25-hydroxycholesterol 7α-hydroxylation and 50% for 27-hydroxycholesterol 7α-hydroxylation (Fig. 8). These differences were statistically significant ($P < 0.01$). The 7α-hydroxylase activities towards dehydroepiandrosterone or 24-hydroxycholesterol in rat liver, however, were not significantly increased by cholestyramine treatment.

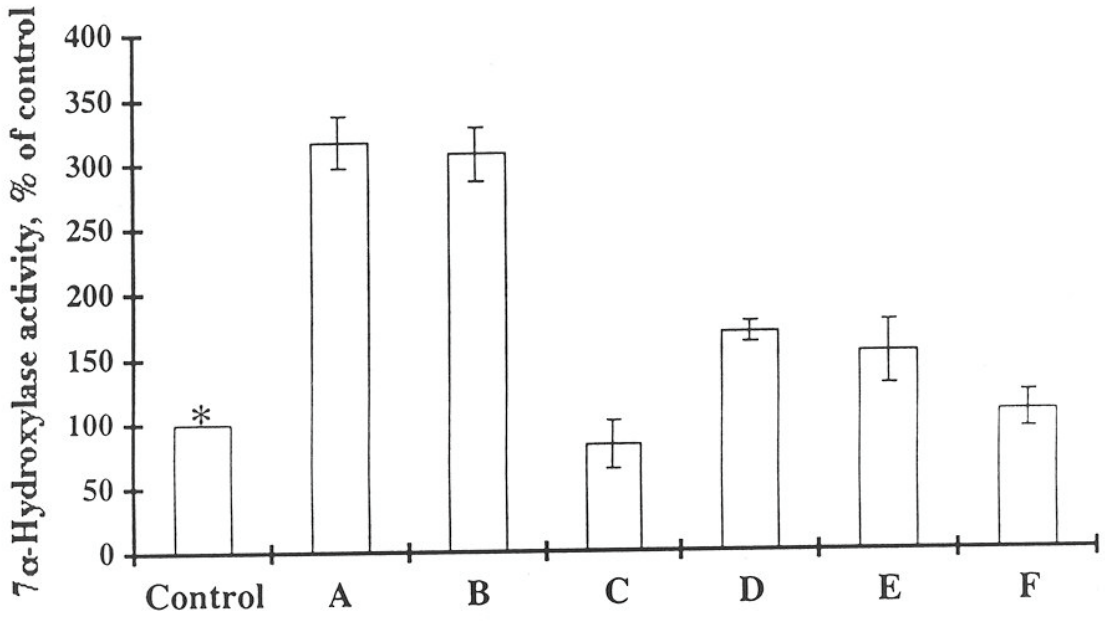

Fig. 8. Effects of cholestyramine treatment on the 7α-hydroxylase activities towards A) cholesterol, B) 20(*S*)-hydroxycholesterol, C) dehydroepiandrosterone, D) 25-hydroxycholesterol, E) 27-hydroxycholesterol and F) 24-hydroxycholesterol by rat liver microsomes. The results are given as percent of the activity in microsomes from untreated rats (*control).

Renal 7α-hydroxylation of oxysterols (Paper IV)

A cytochrome P450 fraction active in 7α-hydroxylation was isolated from microsomes of pig kidney as described in Paper IV. This enzyme fraction showed 7α-hydroxylase activity

towards the same steroids as the liver oxysterol 7α-hydroxylase fraction, *i. e.* 25-hydroxycholesterol, 27-hydroxycholesterol, pregnenolone, and dehydroepiandrosterone. No catalytic activity was detected towards 20(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol, 24-hydroxycholesterol or cholesterol (Table 2).

The endogenous 7α-hydroxylase activity towards cholesterol, oxysterols, dehydroepiandrosterone and pregnenolone was also studied in human embryonic kidney cells (293 cells). These cells displayed high 7α-hydroxylase activity towards 25-hydroxycholesterol, 27-hydroxycholesterol, dehydroepiandrosterone and pregnenolone. In contrast, no 7α-hydroxylase activity was detected towards 20(S)-, 22(R)-, 24-hydroxycholesterol or cholesterol (Table 2).

Whereas CYP7A is considered to be expressed only in the liver, CYP7B mRNA has been detected in extrahepatic tissues, including kidney [90]. The findings that pig kidney cytochrome P450 and human kidney cells do not 7α-hydroxylate 20(S)-, 22(R)- or 24-hydroxycholesterol support the conclusion that CYP7B is not responsible for the 7α-hydroxylation of these sterols.

Table 2. 7α-Hydroxylation by partially purified pig kidney cytochrome P450 and by human embryonic kidney cells

	Pig kidney cytochrome P450	**Human embryonic kidney cells**
	7α-Hydroxylation (nmol/nmol P450/min)	**7α-Hydroxylation (nmol/mg protein/24 h)**
Cholesterol	**≤ 0.005**	**≤ 0.05**
20(S)-Hydroxycholesterol	**≤ 0.005**	**≤ 0.05**
22(R)-Hydroxycholesterol	**≤ 0.005**	**≤ 0.05**
24-Hydroxycholesterol	**≤ 0.005**	**≤ 0.05**
25-Hydroxycholesterol	**0.18**	**1.7**
27-Hydroxycholesterol	**0.21**	**2.0**
Dehydroepiandrosterone	**0.29**	**1.3**
Pregnenolone	**0.25**	**1.4**

Physiological role(s) of CYP7A-mediated 7α-hydroxylation of oxysterols

The finding that CYP7A is able to 7α-hydroxylate oxysterols is surprising since this enzyme has been believed to be specific for cholesterol and cholestanol and not active towards side-chain modified steroids. The results of the experiments with cholestyramine-treated rats indicate a physiological role for CYP7A in the 7α-hydroxylation of 20(S)-hydroxycholesterol, 25-hydroxycholesterol and 27-hydroxycholesterol. As expected, the data on 25-hydroxycholesterol and 27-hydroxycholesterol suggest that CYP7A is not the sole enzyme responsible for the 7α-hydroxylation of these oxysterols, which is consistent with the concept that CYP7B is important in these reactions. The finding that 7α-hydroxylation of 24-hydroxycholesterol is not significantly stimulated by cholestyramine treatment suggests that yet another enzyme may play an important role for this reaction in rat liver. In fact, very recently Li-Hawkins et al [116] reported the cloning of a novel 7α-hydroxylase in mouse and human, CYP39A, selective for 24-hydroxycholesterol. At this stage it is not possible to conclude which of these 7α-hydroxylases that plays the major role for 24-hydroxycholesterol 7α-hydroxylation in human liver.

The results of the present investigation may have implications for our understanding of the pathways of bile acid biosynthesis as well as for oxysterol-mediated regulation of gene expression. The present data show that CYP7A has the ability to participate in both the neutral and the acidic pathways of bile acid biosynthesis. A physiologically significant role for CYP7A in 27-hydroxycholesterol 7α-hydroxylation is supported also by the results obtained with COS cells. In these experiments, 27-hydroxycholesterol was efficiently 7α-hydroxylated by living, transfected cells at physiological concentrations of substrate [114,115] in an environment containing large amounts of endogenous cholesterol, competing for the active site of the enzyme [115]. It is notable that presence of 27-hydroxycholesterol substantially decreased the 7α-hydroxylase activity of these cells towards cholesterol.

The oxysterols 7_-hydroxylated by CYP7A, 20(S)-, 24-, 25- and 27-hydroxycholesterol, are reported to be ligands for the LXR receptor [22,68,69]. Thus, some of the ligands of the liver X receptor, a nuclear receptor which induces expression of CYP7A in rodents, are metabolized by the very enzyme they induce. No information is available on what effects the 7α-hydroxylated derivatives of 20(S)-, 24- or 27-hydroxycholesterol may have on the LXR. However, according to a report by Janowski et al [22] 25-hydroxycholesterol is a severalfold more potent ligand for LXRα than 7α,25-dihydroxycholesterol. It may be speculated that 7α-

hydroxylation of LXR ligands could reflect a way of controlling the level of expression of CYP7A by means of a feed-back mechanism.

CYP27 as a renal 25-hydroxyvitamin D3 1α-hydroxylase (Paper I)

Previous studies reported that purified hepatic mitochondrial 27-hydroxylase and recombinant expressed human CYP27A are able to catalyze 25-hydroxyvitamin D_3 1α-hydroxylation [38]. It was also reported that 1α,25-hydroxyvitamin D_3 suppresses both the renal mitochondrial 1α-hydroxylase activity and the CYP27A mRNA levels [117]. These findings suggest a possible role for CYP27A as a renal 1α-hydroxylase. In Paper I the role for CYP27A in renal 1α-hydroxylation of 25-hydroxyvitamin D_3 is studied. Cytochrome P450 catalyzing 1α- and 27-hydroxylation but not 24-hydroxylation of 25-hydroxyvitamin D_3 was partially purified from pig kidney mitochondria by several chromatographic steps. The highest activities found were 516 pmol/mg/min for 27-hydroxylation and 118 pmol/mg/min for 1α-hydroxylation. The ratio between the 1α- and 27-hydroxylase activities was the same in all purification steps. Attempts to separate the 1α- and 27-hydroxylase activities by various chromatographic methods were unsuccessful. A monoclonal antibody directed against pig liver CYP27A [42] immunoprecipitated both the 1α- and 27-hydroxylase activities towards 25-hydroxyvitamin D_3 in the purified renal cytochrome P450 fraction as well as in a solubilized, crude cytochrome P450 extract considered to represent the major part of renal mitochondrial 25-hydroxyvitamin D_3 hydroxylase activity. Since the purification and antibody experiments indicated that most of the renal mitochondrial 1α-hydroxylase activity is associated with CYP27A, further experiments were carried out to study this enzymatic activity. Addition of increasing amounts of vitamin D_3, a known substrate for CYP27A, to the solubilized crude mitochondrial cytochrome P450 extract decreased the 1α- and 27-hydroxylase activities towards 25-hydroxyvitamin D_3 in a parallel fashion. Addition of equimolar amounts of vitamin D_3 to the incubation mixture decreased the 1α- and 27-hydroxylase activities by 50%, as would be expected if a single enzyme catalyzed both reactions. In contrast, the 24-hydroxylation of 25-hydroxyvitamin D_3 was not inhibited by the addition of vitamin D_3. Experiments were also performed with human liver CYP27A recombinantly expressed in *E. coli* [38]. These experiments confirmed that CYP27A is able to catalyze 1α- and 27-hydroxylations but not 24-hydroxylation of 25-hydroxyvitamin D_3. To summarize, the results show that the 1α-hydroxylase activity but not the 24-hydroxylase activity is associated with CYP27A in pig kidney mitochondria. The findings suggest a role

for CYP27A as a renal mitochondrial 25-hydroxyvitamin D_3 1α-hydroxylase. The obtained data do not exclude the existence of additional mitochondrial 1α-hydroxylases in kidney.

Recently, cDNA encoding another mitochondrial 25-hydroxyvitamin D_3 1α-hydroxylase has been isolated from mouse [118], rat [119,120] and human [121,122]. This enzyme was designated CYP27B since its amino acid sequence showed 41-44% identity to that of CYP27A. Thus, it composes a new subfamily of the CYP27 family.

Developmental variation of enzymes in bile acid biosynthesis (Paper V)

Except for a limited amount of information on developmental changes of CYP7A [123,124] the variation of the bile acid forming enzymes during different periods of life remains unclear. A recent report describing a newborn child with a mutation in the CYP7B gene suggested that this enzyme may be essential for bile acid formation in early human life [20]. This is in contrast to the findings in mice [18,19] where murine CYP7B is not present in the newborn but is upregulated later in life. Instead, CYP7A appears to be critical for normal lipid absorption in the newborn mouse. Studies of the development of these enzymes in humans are hampered by the difficulty in obtaining tissue material from humans of different ages. The pig, which is probably biochemically more related to humans than rodents are, may be more useful for studies of bile acid forming enzymes than rats and mice. Paper V describes the investigation of the developmental variation of 7α-hydroxylating and 27-hydroxylating cytochromes P450 (CYP7A, CYP7B and CYP27A) and 3β-hydroxy-Δ^5-C_{27} steroid dehydrogenase, key enzymes in bile acid biosynthesis, in pigs of different ages. The results of this study are summarized in Table 3.

CYP7A levels in livers from newborn and six months old pigs

Cholesterol 7α-hydroxylase activity was measured in partially purified cytochrome P450 extracts prepared from liver microsomes of newborn (five days old) and six months old pigs. The 7α-hydroxylase activity towards cholesterol was found to be about twice as high in livers from six months old pigs (3.1 ± 0.2 pmol/mg/min) as compared with livers from newborns (1.4 ± 0.4 pmol/mg/min). Since the domestic pig reaches sexual maturity at about six months, the data would reflect the variation between infancy and adolescence. CYP7A mRNA levels also increased with age. The developmental pattern found for CYP7A is in agreement with previously reported findings [123,124].

Table 3. Changes in levels of CYP27A,CYP7A,CYP7B and 3β-hydroxy-Δ^5-C_{27} steroid dehydrogenase with age

The table shows a comparison of the levels of protein and mRNA in pigs aged from newborn to six months old. For detailed information see Paper V.

	CYP27A	3β-hydroxy-Δ^5-C_{27} steroid dehydrogenase	CYP7A	CYP7B	
				liver	*kidney*
mRNA levels	unchanged	ND[1]	increased	increased	ND
Catalytic activity	unchanged	increased	increased	increased[2]	decreased[2]
Immunodetectable protein	decreased	ND	ND	ND	ND

[1]Not determined [2] As measured with microsomes

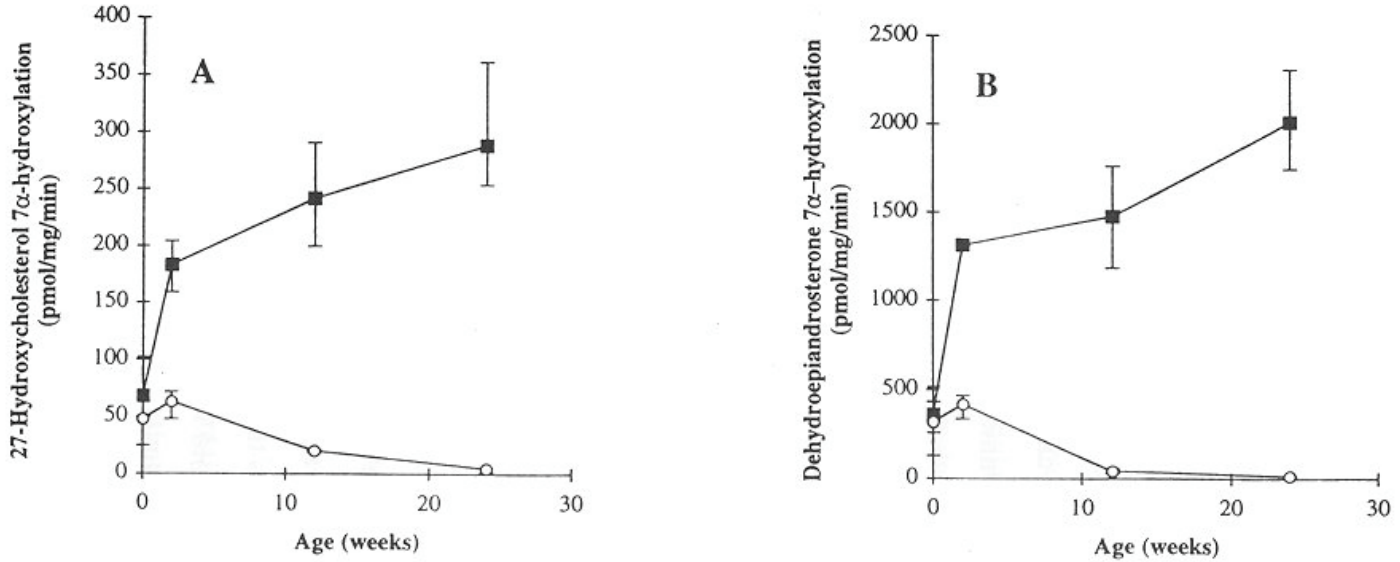

Fig. 9. Age-dependent variation of the 7α-hydroxylase activity towards 27-hydroxycholesterol (A) and dehydroepiandrosterone (B) in microsomes from liver (■)and kidney (O). The data represent the means and range.

Hepatic microsomal 3β-hydroxy-Δ^5-C_{27} steroid dehydrogenase activity in pigs of different ages

The dehydrogenase/isomerase activity towards 7α-hydroxycholesterol was measured in liver microsomes from pigs varying in age from newborn (≤ five days) to six months. The rate of formation of 7α-hydroxy-4-cholesten-3-one increased about fivefold (from 255 to 1221 pmol/mg/min) during this age period.

CYP27A levels in livers from newborn and six months old pigs

In contrast to 3β-hydroxy-Δ^5-C_{27} steroid dehydrogenase and CYP7A, liver mitochondrial CYP27A did not increase with age. 27-Hydroxylation of 5β-cholestane-3α,7α,12α-triol and 25-hydroxylation of 1α-hydroxyvitamin D_3, reactions considered to be mediated by CYP27A, were measured in a partially purified cytochrome P450 extract, isolated from liver mitochondria of newborn (five days old) and six months old pigs. The catalytic activity towards 5β-cholestane-3α,7α,12α-triol in livers from newborn and six months old pigs was 2.5 and 1.9 nmol/mg/min respectively. The activity towards 1α-hydroxyvitamin D_3 was 0.16 nmol/mg/min for both ages. Western blotting with an antibody directed against CYP27A indicated somewhat higher levels of CYP27A protein in the newborn pigs. The CYP27A mRNA levels were found to be about the same in newborn and six months old pigs. The data taken together suggest that CYP27A is expressed to a similar degree in livers of newborn and six months old pigs.

CYP7B levels in livers and kidneys from pigs of different ages

The 7α-hydroxylation of 27-hydroxycholesterol and dehydroepiandrosterone, substrates typical for CYP7B, was measured in liver and kidney microsomes from pigs varying in age from newborn (≤ five days) to six months as well as in partially purified liver microsomal cytochrome P450 extracts. Interestingly, age-dependent changes in the enzymatic activity towards these substrates showed different patterns depending on the tissue studied. In liver microsomes the activities were lowest in the newborns and increased in parallel to become about fivefold higher in livers from six months old pigs than in livers from newborns. The 7α-hydroxylation of 27-hydroxycholesterol and dehydroepiandrosterone by kidney microsomes, however, decreased markedly with age to become about 10-20 times lower in kidneys from six months old pigs than in kidneys from newborns (Fig. 9).

The contrasting patterns in liver and kidney may have different reasons. One possibility is that 7α-hydroxylation of 27-hydroxycholesterol and dehydroepiandrosterone in the liver is

performed also by other enzymes than CYP7B, some of which are not present in the kidney. Another possible explanation could be that the results are due to tissue-specific developmental regulation of CYP7B. Hepatic CYP7B mRNA levels was found to be severalfold higher in six months old pigs than in newborns which supports the latter explanation. The present results do not exclude, however, the possibility of additional hepatic 7α-hydroxylating enzymes active towards these substrates. As shown in Paper IV 27-hydroxycholesterol 7α-hydroxylation is catalyzed also by CYP7A, but CYP7A does not 7α-hydroxylate dehydroepiandrosterone. In addition to CYP7B and CYP7A, there may be another hitherto unrecognized enzyme in the liver, able to catalyze 7α-hydroxylation of dehydroepiandrosterone and/or 27-hydroxycholesterol. The 7α-hydroxylase activity towards 27-hydroxycholesterol and dehydroepiandrosterone measured in partially purified liver cytochrome P450 extracts did not show the same pattern as in liver microsomes. The activity in purified fractions from liver microsomes of newborn and six months old pigs appeared to decrease with age (from 242 to 99 pmol/mg/min for 27-hydroxycholesterol 7α-hydroxylation and from 1673 to 751 pmol/mg/min for dehydroepiandrosterone 7α-hydroxylation). It is unlikely that the enzyme fraction from six months old pigs lost some of its catalytic activity during purification as particular care was taken to handle the preparations from the different ages in exactly the same way. Furthermore the 7α-hydroxylation of cholesterol (catalyzed by CYP7A) in these purified fractions was higher in the six months old pigs than in the newborns (see under ”CYP7A levels in livers from newborn and six months old pigs”). If there are additional 7α-hydroxylases, the enzymes may not coelute or may have different sensitivity to destabilizing factors during purification.

The function of CYP7B in the kidney remains unknown. The results of the present study indicate that the renal enzyme is important for the newborn. An important role for CYP7B in newborns is consistent with the results obtained by Setchell et al [20] on a human infant with a mutation in the CYP7B gene. In addition to the severe damage to the liver, the autopsy of this patient also showed enlarged and histologically abnormal kidneys [20].

Very little information is available on possible regulatory mechanisms for CYP7B [16,24]. The results of the present study indicate a tissue-specific developmental regulation of this enzyme. The age-dependent variation in liver and kidney suggest that hormonal factors are involved in the regulation of CYP7B.

SUMMARY AND CONCLUSIONS

A cytochrome P450 enzyme fraction active in 7α-hydroxylation was purified from pig liver microsomes. This enzyme fraction 7α-hydroxylated 25- and 27-hydroxycholesterol, dehydroepiandrosterone and pregnenolone but not cholesterol or testosterone. Peptide sequence analysis indicated that the purified porcine oxysterol 7α-hydroxylase fraction contains CYP7B or an enzyme belonging to the CYP7B subfamily. Purification and inhibition experiments indicated that at least two closely related enzymes are involved in the 7α-hydroxylation of 27-hydroxycholesterol and dehydroepiandrosterone in pig liver.

Human cholesterol 7α-hydroxylase, CYP7A, expressed in *E. coli* and in COS cells was found to catalyze 7α-hydroxylation of 20(*S*)-, 24-, 25- and 27-hydroxycholesterol. This enzyme was previously considered specific for cholesterol and cholestanol. A partially purified cholesterol 7α-hydroxylase enzyme fraction from pig liver showed 7α-hydroxylase activity towards the same oxysterols as metabolized by recombinant human and rat CYP7A. The 7α-hydroxylase activity towards 20(*S*)-, 25-, and 27-hydroxycholesterol in rat liver was significantly increased by treatment with cholestyramine, an inducer of CYP7A. Cytochrome P450 of renal origin showed 7α-hydroxylase activity towards 25- and 27-hydroxycholesterol, dehydroepiandrosterone and pregnenolone but not towards 20(*S*)-, 22(*R*)-, 24-hydroxycholesterol or cholesterol. The results indicate a physiological role for CYP7A as an oxysterol 7α-hydroxylase, in addition to the previously known human oxysterol 7α-hydroxylase, CYP7B.

Cytochrome P450, catalyzing 1α- and 27-hydroxylation but not 24-hydroxylation of 25-hydroxyvitamin D_3, was partially purified from pig kidney mitochondria. Purification and inhibition experiments as well as experiments with a monoclonal antibody against CYP27A indicated that one single enzyme catalyzes both 1α- and 27-hydroxylation. The findings suggest a role for CYP27A in renal 25-hydroxyvitamin D_3 1α-hydroxylation.

The expression of CYP7A, CYP7B and CYP27A during development was investigated. The levels of CYP27A were similar in livers of newborn and six months old pigs whereas the levels of CYP7A increased. Expression of CYP7B showed different developmental patterns depending on the tissue studied. The activity towards 27-hydroxycholesterol and dehydroepiandrosterone in liver microsomes as well as hepatic CYP7B mRNA levels increased with age whereas the activity in kidney microsomes showed a marked age-dependent decrease. The results indicate a tissue-specific developmental regulation of this enzyme.

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