review

Mechanism of degradation of the steroid side chain in the formation of bile acids

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INTRODUCTION

The most important pathway for the metabolism and excretion of cholesterol in mammals is the formation of bile acids. The two major primary bile acids, cholic and chenodeoxycholic acids, are formed in the liver and secreted in bile to the intestine.

The conversion of cholesterol into bile acids involves almost all the conceivable mechanisms for conversion of a lipophilic compound into an excretable water-soluble product (for a general review, see ref. 1). Of the more than 15 different enzymes participating in the conversion, hydroxylases, oxidoreductases, and conjugating systems are of particular importance for increasing the polarity. The enzymes that modify the steroid nucleus are able to convert the nonpolar 3β-hydroxy-Δ^5-steroid into a considerably more polar 5β-cholestane-3α,7α-dihydroxy- or 5β-cholestane-3α,7α,12α-trihydroxy steroid. The enzymes involved in these conversions are mainly located in the endoplasmic reticulum and the cytosol. The enzymes involved in the steroid side-chain degradation convert the highly nonpolar C_{27}-steroid side-chain into a chain-shortened carboxylic acid conjugated to an amino acid. These enzymes are mainly located in the mitochondria and in the peroxisomes.

According to current concepts, the conversion of cholesterol into bile acids in mammals starts with the nuclear transformations, and most or all of these changes precede those of the steroid side-chain. Based on early in vivo and in vitro work on rats, the sequence of reactions shown in **Fig. 1** was formulated about 25 years ago for the conversion of cholesterol into cholic and chenodeoxycholic acids. It is evident, however, that alternative pathways exist where some or most of the changes in the steroid side-chain precede the changes in the nucleus. As the flux of bile acids probably does not regulate any of the pathways where 7α-hydroxylation is not the first step, it seems that the cholesterol 7α-hydroxylase is the only enzyme that is capable of regulating the overall conversion into bile acids.

The steroid side chain of plant sterols contains a methyl or an ethyl group in 24-position. This means that the normal mechanism for side-chain degradation cannot be operative. In view of the very low degree of absorption of these steroids, there is less need for a specialized degradative enzyme. In at least one mammalian species, however, a system has been evolved that is able to convert plant sterols into highly polar conjugated C_{21}-bile acids.

In the present review, the emphasis is on the most important mechanism for steroid side-chain degradation in connection with bile acid biosynthesis, the 27-

Abbreviations: THCA, trihydroxycoprostanoic acid; DHCA, dihydroxy-5β-cholestanoic acid (dihydroxycoprostanoic acid); CTX, cerebrotendinous xanthomatosis.

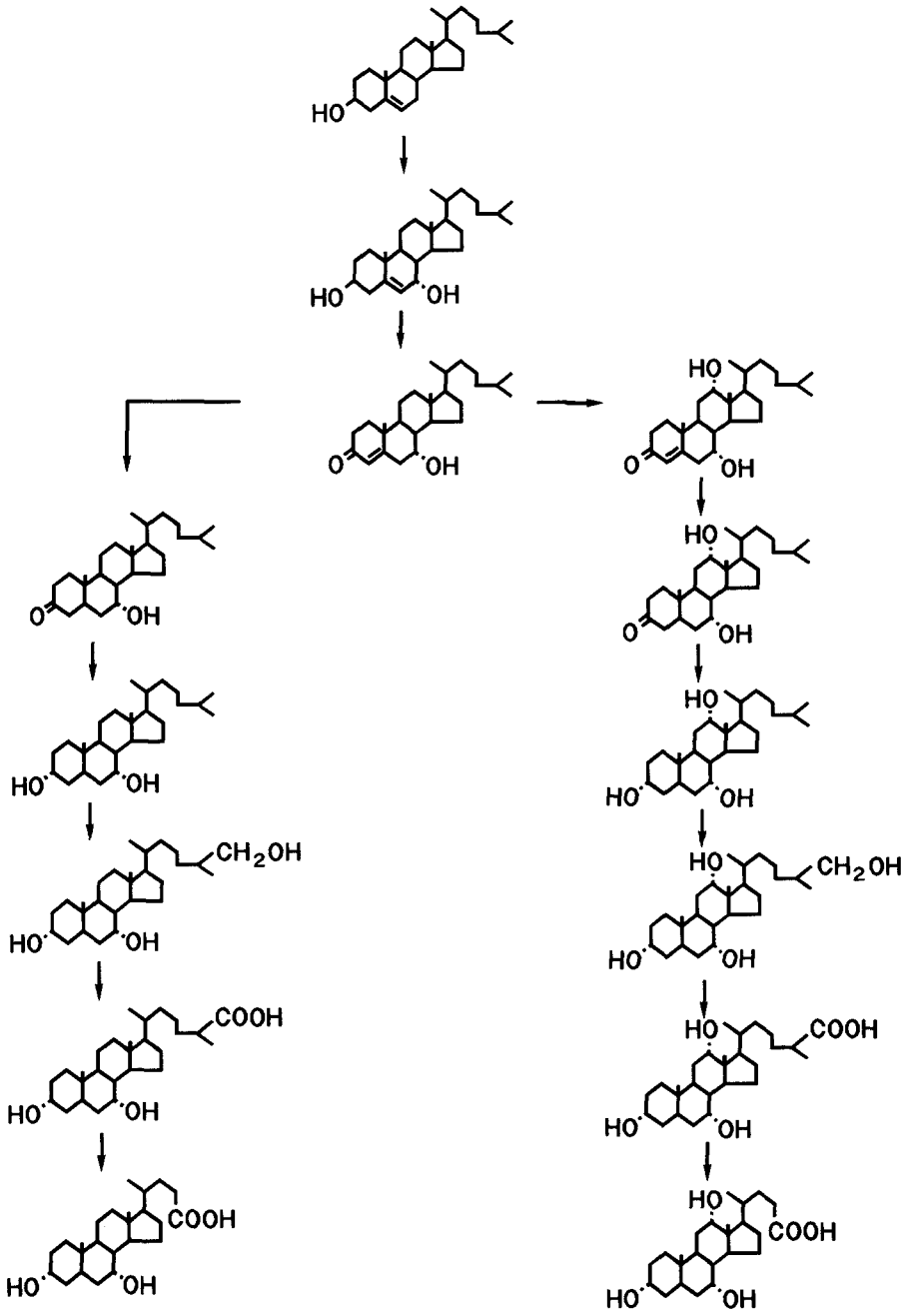

Fig. 1. Major pathway for the normal biosynthesis of bile acids in the rat.

hydroxylase pathway. An alternative pathway that operates when the major pathway is blocked will also be reviewed. Alternative substrates for side-chain degradation in different species, as well as inborn errors of metabolism affecting side-chain degradation, are also discussed. A recently discovered new pathway for degradation of the steroid side-chain of plant sterols will be mentioned.

THE 27-HYDROXYLASE PATHWAY

It is well established that the major mechanism for degradation of the cholesterol side-chain starts with oxidation of one of the two terminal methyl groups (ω-hydroxylation) followed by β-oxidation and conjugation. A simplified scheme of the reactions is shown in **Fig. 2.**

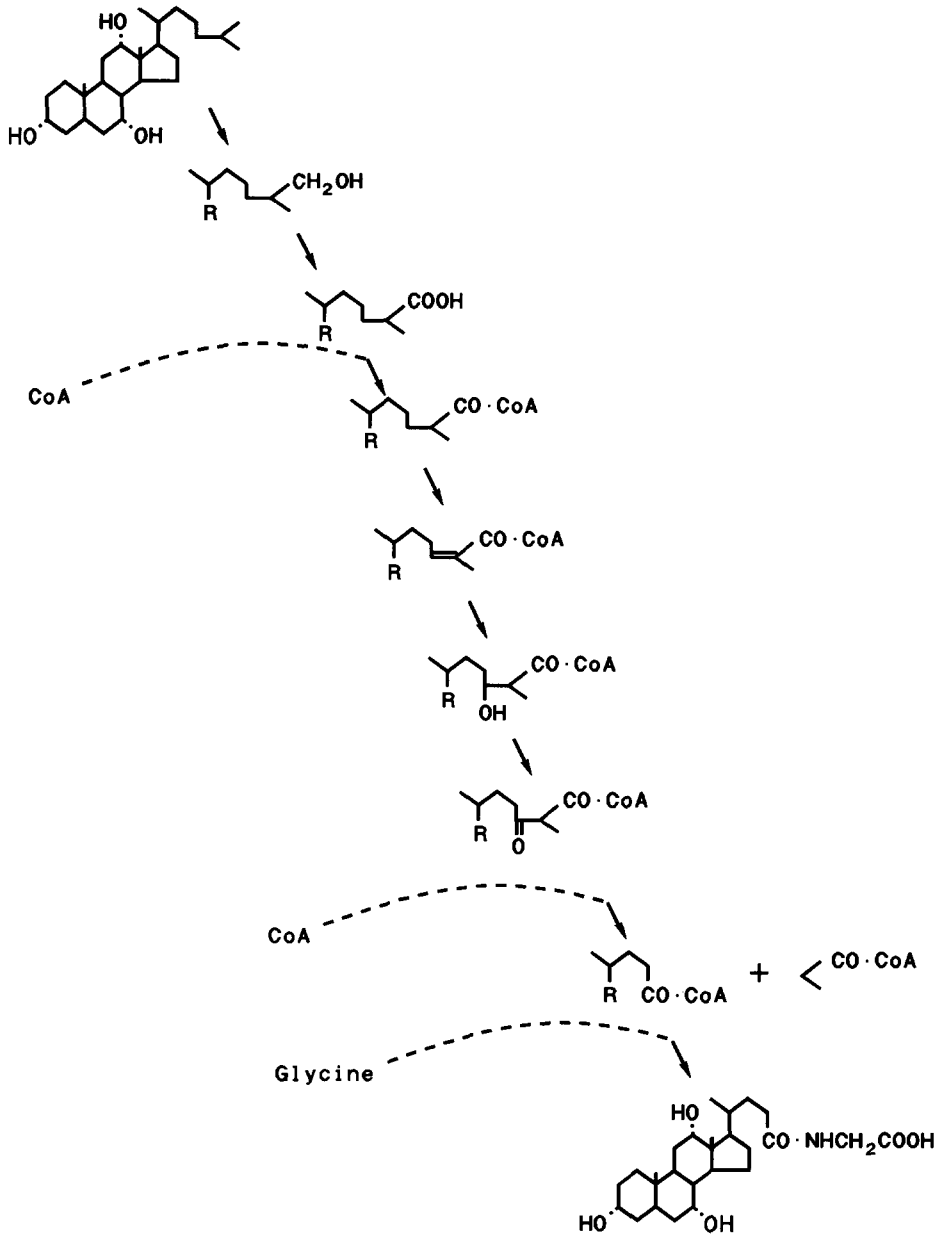

Fig. 2. Side-chain oxidation and conjugation in connection with bile acid biosynthesis from cholesterol. The 27-hydroxylase pathway for the conversion of 5β-cholestane-3α,7α,12α-triol into cholic acid is shown.

The above pathway will be referred to as the "ω-hydroxylase pathway" or the "27-hydroxylase pathway."

27-Hydroxylation

Inasmuch as hydroxylation of one of the two terminal methyl groups in the steroid side-chain creates an asymetric carbon atom at C-25, ω-hydroxylation may be stereospecific. Popjak et al. (2) have suggested that the 25-pro-R methyl group should be denoted C-26 and the 25-pro-S methyl group C-27. There is a mitochondrial ω-hydroxylase in mammalian liver that almost exclusively attacks the C-27 methyl group (for a general review, see ref. 1). This hydroxylase should thus be denoted "27-hydroxylase," although it has been referred to as "26-hydroxylase" in most previous work. There is also a microsomal ω-hydroxylase in mammalian liver, which has a specificity towards the C-26 methyl group (3). In humans, this microsomal 26-hydroxylase has little activity compared to the mitochondrial 27-hydroxylase (4). In accordance with this, trihydroxycoprostanoic acid isolated from human bile has the 25-R-configuration (5), indicating that it is

formed by an initial attack by the mitochondrial 27-hydroxylase. In addition to 26-hydroxylase activity, both rat and human liver microsomes contain 23-, 24-, and 25-hydroxylases active towards the C_{27}-steroid side-chain (cf below). In the rat, the microsomal 26-hydroxylase may be of importance and there is some evidence that in this species the enzyme may be involved in the regulation of the ratio between cholic acid and chenodeoxycholic acid formed in the liver (6).

The microsomal 26-hydroxylase has a high substrate specificity and the mitochondrial 27-hydroxylase has a low substrate specificity (see below). The mitochondrial 27-hydroxylase is located not only in the liver but also in fibroblasts (7), brain (8), kidney (9), and several other organs and tissues (10).

On the basis of experiments with rat liver peroxisomes, it has been suggested that there is an ω-hydroxylase in peroxisomes active toward C_{27}-steroids (11). Attempts to confirm this in our laboratory and in the laboratory of Pedersen have failed, however.

In view of the lesser importance of the 26-hydroxylase in mammals, only the 27-hydroxylase will be discussed in this review. Early work by Björkhem and Gustafsson (12, 13) and by Taniguchi, Hoshita, and Okuda (14) and Okuda, Weber, and Ullrich (15) established that this enzyme is a mixed function oxidase containing cytochrome P-450. The enzyme is bound to the inner mitochondrial membrane. Thus the activity was low with intact mitochondria and NADPH as a co-factor (13, 14). Under such conditions citric acid and isocitric acid, which are able to penetrate the inner mitochondrial membranes, stimulate 27-hydroxylation much more efficiently than NADPH (13, 14). It is evident that citric acid and isocitric acid generate NADPH inside the mitochondrial membranes. With leaking mitochondria, NADPH stimulates the reaction as efficiently as isocitrate. There is some evidence for the presence of different transport mechanisms for different substrates through the mitochondrial membranes (16). This may be of some importance for the different rates of hydroxylation of different substrates by intact liver mitochondria.

Pedersen, Oftebro, and Vänngård (17) and Sato et al. (18) reported simultaneously that small amounts of cytochrome P-450 could be solubilized from the inner membranes of rat liver mitochondria. The cytochrome P-450 fraction obtained was active toward cholesterol as well as toward 5β-cholestane-3α,7α,12α-triol in the presence of ferredoxin, ferredoxin reductase, and NADPH. Ferredoxin and ferredoxin reductase were active regardless of whether they were isolated from rat liver mitochondria or bovine adrenal mitochondria (19). Pedersen, Björkhem, and Gustafsson (19) showed that the substrate specificity of a reconstituted system from rat liver mitochondria was similar to that in intact mitochondria. The rate of 27-hydroxylation was, however, about 10-fold higher in the reconstituted system than in intact mitochondria, indicating that the transport of the steroid through the mitochondria membranes may be a limiting factor (19).

Wikvall (20) and Dahlbäck and Wikvall (21) were the first to purify the mitochondrial 27-hydroxylase to homogeneity. They used rabbit liver as enzyme source. Later, other groups characterized the rat (22), human (23), and pig (24) 27-hydroxylases. Andersson et al. (10) recently made an extensive characterization of the rabbit liver 27-hydroxylase. The protein sequence of the 499 amino acid enzyme shares many of the hallmarks of a cytochrome P-450 structure, including an overall hydrophobic nature (36% of the amino acids in this protein have either aromatic or hydrophobic side-chains) and a conserved cystein residue (located at position 444 in the 27-hydroxylase) that is thought to be a ligand for the heme iron. The structure of the enzyme displays a similarity with the mitochondrial cholesterol side-chain cleavage enzyme (34%) and to the adrenal mitochondrial 11β-hydroxylase (33%). A signal sequence of 36 residues was found to precede the coding region and this signal sequence is common to sequences that direct proteins into the mitochondrion. The 27-hydroxylase was regarded as a member of a novel cytochrome P-450 gene family. Several research groups in the bile acid field have now decided to call the gene CYP 27.

Andersson et al. (10) were also able to express the 27-hydroxylase activity in cultured monkey COS cells. 27-Hydroxylase enzyme activity in COS-M6 cells transfected with the cDNA was enhanced by co-transfection of a plasmid encoding bovine adrenodoxin but not by a plasmid encoding NADPH cytochrome P-450 reductase. As could have been expected from previous work, mRNA measurements showed that the enzyme was expressed in several different organs and tissues (10). Most interesting, the abundance of the mRNA for the enzyme paralleled the cholesterol biosynthesis capacity of the tissues that were assayed. Thus the mRNA levels were high in the liver, duodenum, adrenal gland, and lung and less abundant in the kidney and spleen.

The liver mitochondrial 27-hydroxylase seems to be of little importance for the regulation of bile acid biosynthesis and the composition of bile acids formed. The activity is high compared to that of the enzyme that catalyzes the rate-limiting step, cholesterol 7α-hydroxylase. It has been reported that the rat enzyme is slightly inhibited by biliary drainage and that it is not affected by, for example, starvation and phenobarbital treatment (12). Biliary obstruction seems to have different effects on the rat mitochondrial 27-hydroxyl-

ase, depending on the substrate (25). Whether these effects are due to effects on the 27-hydroxylase per se or to effects on the transfer of steroids to the site of the enzyme could not be established. Saarem and Pedersen (26) was well as Dahlbäck (27) reported that the level of the enzyme is higher in female than in male animals. Cholic acid and starvation was found to reduce the amount of mitochondrial cytochrome P-450-27 and the catalytic activity by 30–60%. There were no parallel changes in the mRNA encoding the enzyme however (27).

The rat, human, and pig 27-hydroxylases have catalytical properties similar to those of the rabbit enzyme (22–24). The deduced amino acid sequences of the rat and the human enzymes indicated that it is 73% and 81%, respectively, identical to the rabbit enzyme (23, 28). It may be mentioned in this connection that Raza and Avadhani (29) isolated two β-naphthoflavone-inducible species of cytochrome P-450 from rat liver that possessed 27-hydroxylase activity in addition to hydroxylase activity toward various xenobiotics. In contrast to the cytochrome P-450 fractions described above, the preparation studied by Raza and Avadhani (29) could be reconstituted with both microsomal NADPH-cytochrome P-450 reductase and mitochondrial ferredoxin + ferredoxin reductase.

The possibility has been discussed that the 27-hydroxylase may be of importance for the overall regulation of cholesterol biosynthesis (for a review, see ref. 30). One of the products, 27-hydroxycholesterol, is thus known to be a potent inhibitor of the rate-limiting enzyme in cholesterol synthesis, the HMG-CoA reductase (31). The finding by Andersson et al. (10) that the abundance of the mRNA for the enzyme paralleled the cholesterol biosynthesis capacity of the tissues is also in agreement with this possibility. Since it is possible to survive a complete or almost complete lack of the enzyme activity for a life-time (cf below), it is evident that the enzyme activity cannot be obligatory for the down-regulation of cholesterol synthesis in various tissues. In recent work from this laboratory, the possibility was excluded that a mitochondrial 27-hydroxylation is of importance in the down-regulation of HMG-CoA reductase by dietary cholesterol in mice (E. Lund and I. Björkhem, unpublished results). It was shown that 27-hydroxylation of 26,26,26,27,27,27-$^2H_6$-cholesterol in mouse liver mitochondria was associated with a marked isotope effect. When such deuterated cholesterol was added to the diet, it was able to suppress HMG-CoA reductase as efficiently as unlabeled cholesterol.

It may be speculated that the 27-hydroxylase may be involved in the transport of cholesterol out from the cell. Thus 27-hydroxycholesterol and, in particular, its oxidation product 3β-hydroxy-5-cholestenoic acid are considerably more polar than cholesterol. Direct evidence for this hypothesis is still lacking, however.

Pedersen, Holmberg, and Björkhem (32) and Björkhem et al. (33) showed that a crude preparation of cytochrome P-450 from rat liver mitochondria was able to catalyze not only 27-hydroxylation of various C_{27}-steroids but also 25-hydroxylation of cholesterol and vitamin D_3. A kinetic study suggested that different enzymes, or at least different binding sites, may be involved in the 25- and 27-hydroxylations (33). In accordance with this contention, Dahlbäck (34) showed that monoclonar antibodies against a purified 27-hydroxylase inhibited 27-hydroxylation but not 25-hydroxylation by a reconstituted system containing highly purified, apparently homogeneous mitochondrial cytochrome P-450, ferredoxin, and ferredoxin reductase. In contrast to these results, however, Ohyama et al. (35) reported that partial denaturation by heating and treatment of the enzyme by N-bromosuccinimide inactivated the two enzyme activities in a similar manner. It has also been shown that COS-cells transfected with the 27-hydroxylase plasmid expressed both 27- and 25-hydroxylase activity (27). At least in these cells, the same gene must therefore be responsible for both activities.

Post-translational changes in the primary gene product or presence of a specific 27-hydroxylase in addition to the more nonspecific 27-hydroxylase (possessing both 27- and 25-hydroxylase activity) may explain the apparently conflicting results from the different groups. Another possibility is that the binding of nonpolar substrates like cholesterol and vitamin D_3 to the active site of the enzyme may differ from the corresponding binding of more polar substrates like 5β-cholestane-3α,7α,12α-triol. In accordance with this latter hypothesis we have found that cyclosporin inhibits 27-hydroxylation (and 25-hydroxylation) of cholesterol and 25-hydroxylation of vitamin D but not 27-hydroxylation of more polar C_{27}-steroids catalyzed by an apparently homogeneous cytochrome P-450 preparation from rabbit liver (H. Dahlbäck, I. Björkhem, and H. Princen, unpublished observation).

Conversion of 27-hydroxysteroid to 27-carboxysteroid

The liver has a very high capacity to catalyze oxidation of 27-hydroxysteroids into the corresponding 27-carboxysteroids. Three different systems have been described.

In 1965 Okuda and Danielsson (36) synthesized the 27-aldehyde, believed to be the intermediate in the above conversion. This aldehyde was efficiently oxidized into 3α,7α,12α-trihydroxy-5β-cholestanoic acid (trihydroxycoprostanoic acid, (THCA) in the cytosolic, microsomal, and mitochondrial fractions of a rat liver homogenate. The soluble and the mitochondrial frac-

tions were most efficient. The soluble 5β-cholestane-3α,7α,12α,27-tetrol-NAD-dehydrogenase and 3α,7α,12α-trihydroxy-5β-cholestan-27-al-NAD-dehydrogenase were later purified by Okuda and Takigawa (37–39). It was suggested that the enzymes could be identical to alcohol and aldehyde dehydrogenase, respectively. In accordance with this, Waller, Theorell, and Sjövall (40) and Björkhem, Jörnwall, and Åkesson (41) reported that recrystallized alcohol dehydrogenase from horse liver was able to catalyze oxidation of the tetrol into the carboxylic acid. The SS isoenzyme was found to be most efficient (41).

In accordance with the investigation by Okuda and Danielsson (36), Dahlbäck et al. (42) reported that rabbit liver mitochondria contain an NAD-dependent enzyme system capable of catalyzing oxidation of the tetrol into the carboxylic acid.

Andersson et al. (10) and Dahlbäck and Holmberg (43) showed that the purified mitochondrial cytochrome P-450-27 was able to catalyze formation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid from 5β-cholestane-3α,7α,12α,27-tetrol when reconstituted with ferredoxin, ferredoxin reductase, and NADPH. The possibility was clearly excluded that the enzymatic activity was derived from contaminating mitochondrial and cytosolic dehydrogenases. The intermediate aldehyde could not be isolated. It is possible, however, that an intermediate aldehyde may be dismutated to give equal amounts of the tetrol and the carboxylic acid.

The relative importance of the three systems is not known. Sjövall, Andersson, and Lieber (44) showed that deer mice, which genetically lack soluble alcohol dehydrogenase, have the same bile acid pool size and composition as animals with normal levels of the enzyme. It was therefore concluded that the soluble alcohol dehydrogenase cannot be obligatory for bile acid biosynthesis.

Inasmuch as the 27-hydroxylation step occurs inside the mitochondria, it is attractive to suggest that one or both of the two mitochondrial systems are most important for the conversion. Because the carboxylic acid is considerably more polar than its precursor, it is possible that the conversion is of importance for the transport of the bile acid intermediate from the mitochondria.

Peroxisomal β-oxidation of coprostanoic acids

Early experiments by Masui and Staple (45) showed that 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid is a probable intermediate in the conversion of THCA into cholic acid and that the 24-hydroxylation step was catalyzed by the mitochondrial fraction of a rat liver homogenate. It was subsequently shown that this hydroxylation was also catalyzed by the microsomal fraction together with the cytosol and ATP (46). The final oxidation and thiolytic cleavage were reported to be catalyzed by the mitochondrial fraction (45, 47, 48) but also by the soluble fraction (45, 47) and by the microsomal fraction combined with the 100,000 *g* supernatant (48). In these early experiments no attempts were made to separate out other subcellular fractions and marker enzymes were not used to check for cross-contamination of the fractions.

In view of the controversial results, the subcellular localization of the enzyme system responsible for the overall conversion of THCA into cholic acid and of DHCA (3α,7α-dihydroxy-5β-cholestanoic acid, dihydroxycoprostanoic acid) into chenodeoxycholic acid was reinvestigated by Pedersen and Gustafsson (49) and by Pedersen's group in collaboration with the author (50, 51). The initial experiments showed that a peroxisome-enriched fraction from rat liver had the highest capacity to catalyze conversion of THCA into cholic acid. When THCA and DHCA were incubated with different subcellular fractions of rat liver, the highest specific activities for conversion into cholic acid and chenodeoxycholic acid were observed in a light mitochondrial fraction that also contained the highest activity of the peroxisomal marker enzymes (50, 51). After separation of the light mitochondrial fraction on sucrose or Nycodenz gradients, it was shown that the fractions containing the highest peroxisomal marker activities also catalyzed formation of cholic acid from THCA and chenodeoxycholic acid from DHCA. The conversion required the presence of NAD, CoA, ATP and Mg^{2+}. With peroxisomes prepared on sucrose gradient, FAD stimulated the reaction. The reaction was stimulated by KCN and unaffected by inhibitors of the mitochondrial respiratory chain. Small amounts of 24-OH-THCA could be isolated from incubations with THCA.

The findings were consistent with the view that the reaction sequence for oxidative cleavage of the C_{27}-steroid side-chain is similar to that of the peroxisomal β-oxidation of fatty acids, involving a THCA-CoA synthetase, an FAD-dependent oxidase, a hydratase, an NAD-dependent dehydrogenase, and a thiolase (Fig. 2). A Δ^{24}-unsaturated steroid should be an intermediate in this reaction. Indirect evidence for the intermediate formation of such a compound was obtained from experiments performed in the presence of $^{2}H_2O$ or in an atmosphere of $^{18}O_2$ (52). In the former case there was an incorporation of one atom of deuterium into the 25-position, whereas in the latter case there was no incorporation of isotope. Using specific incubation conditions without NAD, it was later possible to show the direct conversion of THCA into Δ^{24}-THCA (53). It was also shown that the Δ^{24}-double bond had the ZZ-configuration.

The first step in the overall conversion of THCA into cholic acid is the activation to yield a CoA derivative. The microsomal fraction of a rat liver homogenate was found to have the highest catalytic activity, whereas the peroxisomal fraction had little or no capacity to catalyze this conversion (54). The THCA-CoA oxidase was found to be a specific system, not identical to that involved in peroxisomal fatty acid oxidation. In contrast to the fatty acid oxidation system, clofibrate did not stimulate the THCA oxidase (55) and treatment of rats with partially hydrogenated marine oil was found to have different effects on the two systems (56). Schepers et al. (57) described a partial purification of the THCA-CoA oxidase from rat liver peroxisomes and showed that it was different from palmitoyl-CoA oxidase. The THCA-CoA oxidase had an apparent molecular mass of 139 kDa and consisted mainly, if not exclusively, of one polypeptide component of 69 kDa.

The next two steps in the conversion are catalyzed by a bifunctional enzyme, enoyl-CoA hydratase/ β-hydroxyacyl-CoA dehydrogenase. This enzyme seems to be the same as that involved in peroxisomal fatty acid oxidation. Using photoaffinity labeling of isolated intact rat liver peroxisomes with tritium-labeled 7,7-azo-3α,12α-dihydroxy-5β-cholestan-27-oyl-CoA, Gengenbacher et al. (58) showed an incorporation of radioactivity into polypeptides that immunoprecipitated with antibodies toward both the bifunctional enzyme and the thiolase involved in peroxisomal β-oxidation of fatty acids.

The final step in the sequence of reactions leading to a conjugated bile acid is catalyzed by a bile acid-CoA:amino acid N-acyltransferase. Kase et al. (59) and Kase and Björkhem (60) showed that the highest specific amidation activity of both choloyl-CoA and chenodeoxycholoyl-CoA was always found in the most peroxisome-rich fractions of a rat liver homogenate. The microsomal fraction contained less activity, while the cytosol was inactive. In previous work substantial amounts of enzyme activity were found in the cytosol. This may be due to leakage of peroxisomal enzymes into the cytosol during the preparation of the different subcellular fractions. Striking differences were observed in the K_m values and the saturation concentrations for glycine and taurine in the peroxisomal system. It was suggested that most of the de novo synthesized bile acids conjugated to taurine by the peroxisomal systems, whereas the bile acids deconjugated in the gut and recirculation to the liver might be activated and amidated by the microsomal enzyme system prior to biliary secretion.

Very recently Johnson et al. (61) isolated a bile acid-CoA:amino acid N-acyltransferase from the soluble fraction of homogenized purified frozen human liver. After purification, the reduced denatured enzyme migrated as a single 50 kDa protein band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A similar molecular mass was obtained for the native enzyme by HPLC gel filtration. The purified enzyme was found to use glycine, taurine, and 2-fluoro-β-alanine but not alanine as substrates. It was suggested that a single monomeric enzyme is responsible for conjugation of bile acids with both glycine and taurine in human liver.

THE 25-HYDROXYLASE PATHWAY

In addition to the 27-hydroxylase pathway, there is an alternative mechanism for degradation of the steroid side-chain in 5β-cholestane-3α,7α,12α-triol. It was shown early on in both rat (64) and human (4) that 5β-cholestane-3α,7α,12α-triol is efficiently 25-hydroxylated in the microsomal fraction. Shefer et al. (63) and Salen et al. (64) further demonstrated that the product, 5β-cholestane-3α,7α,12α,25-tetrol, is converted into 5β-cholestane-3α,7α,12α,24α,25-pentol, 5β-cholestane-3α,7α,12α24β,25-pentol, 5β-cholestane-3α,7α,12α,23,25-pentol, and 5β-cholestane-3α,7α,12α, 25,26-pentol in the presence of microsomes fortified with NADPH. In the presence of NAD, 5β-cholestane-3α,7α,12α,24β,25-pentol, but not the other 5β-cholestanepentols formed, is converted into cholic acid by soluble enzymes (**Fig. 3**). The latter conversion must be assumed to involve acetone. These experiments demonstrated the existence of a new pathway for side-chain degradation in cholic acid biosynthesis that does not involve hydroxylation at C-26 or the participation of mitochondria. The relative importance of this pathway has been a matter of controversy (cf below). Since

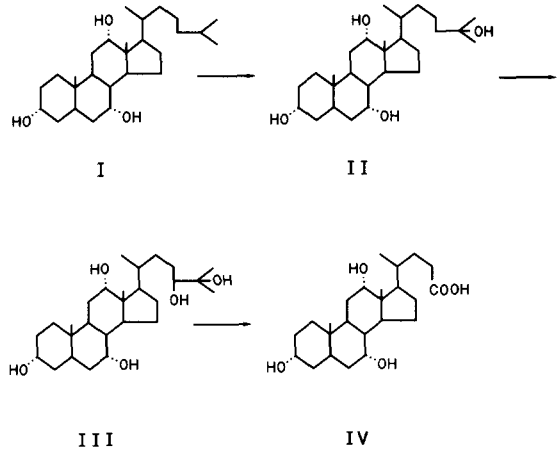

Fig. 3. Formation of cholic acid from 5β-cholestane-3α,7α,12α-triol by the 25-hydroxylase pathway described by Salen et al. (64) I = 5β-cholestane-3α,7α,12α-triol; II = 5β-cholestane-3α,7α,12α,25-tetrol; III = 5β-cholestane-3α,7α,12α,24(S),25-pentol; IV = cholic acid.

there is little or no 25-hydroxylation of 5β-cholestane-3α,7α-diol in liver microsomes, the 25-hydroxylase pathway cannot be of importance in the formation of chenodeoxycholic acid.

It may be mentioned that the microsomal 25-hydroxylase is not active towards cholesterol (4). As mentioned above, the mitochondrial fraction of a liver homogenate is able to catalyze 25-hydroxylation of cholesterol (13). The rate of this hydroxylation is, however, very low and it seems unlikely that a pathway involving 25-hydroxycholesterol is of importance in the biosynthesis of bile acids.

RELATIVE IMPORTANCE OF THE 25- AND 27-HYDROXYLASE PATHWAYS

On the basis of the efficient 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol in human liver microsomes and the accumulation of 5β-cholestane-3α,7α,12α,25-tetrol in bile and feces of patients with cerebrotendinous xanthomatosis (CTX, see below), Salen et al. suggested that the 25-hydroxylase pathway may be the major mechanism for biosynthesis of cholic acid in humans (64). There are, however, a number of experimental findings that suggest that the 27-hydroxylase pathway is most important in the biosynthesis of both cholic acid and chenodeoxycholic acid.

First, there are two known inborn errors of metabolism in the 27-hydroxylase pathway in humans (cf below). In both cases the metabolic block leads to accumulation of intermediates (or their metabolites) in the 27-hydroxylase pathway. In one of the diseases (CTX), one of the accumulated products is also an intermediate in the 25-hydroxylase pathway. Taken together, however, the metabolic consequences of the metabolic blocks clearly support the contention that the 27-hydroxylase pathway is the more important.

Second, the 25-hydroxylase pathway generates acetone as a cleavage product, whereas the 27-hydroxylase pathway generates propionic acid. In contrast to acetone, propionic acid is rapidly oxidized to carbon dioxide. By studying the relative formation of radioactive carbon dioxide and acetone from cholesterol labeled with ^{14}C in the terminal methyl groups, Duane et al. (65, 66) showed in both rat and human that the 25-hydroxylase pathway is a minor one under normal conditions (**Fig. 4**).

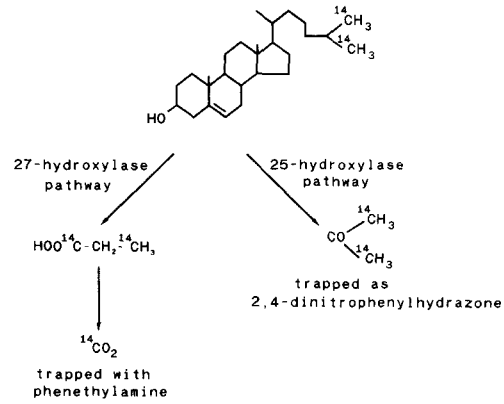

Fig. 4. Experimental approach used by Duane et al. (65, 66) to evaluate the relative importance of the 25- and the 27-hydroxylase pathways in the biosynthesis of bile acids in rat and humans.

SPECIES DIFFERENCES AND ALTERNATIVE SUBSTRATES FOR THE 27-HYDROXYLASE PATHWAY

In the sequence of reactions shown in Fig. 1, all the nuclear changes precede the changes in the steroid side-chain. Thus 5β-cholestane-3α,7α-diol is the substrate for the 27-hydroxylase in the formation of chenodeoxycholic acid and 5β-cholestane-3α,7α,12α-triol is the corresponding substrate in the formation of cholic acid. This pathway is most probably predominant in rats but the situation may be different in humans.

Since the mitochondrial 27-hydroxylase has a broad substrate specificity (4, 12, 13), it is evident that pathways may exist where the 27-hydroxyl group is introduced at a stage where only part of the nuclear changes have occurred. In the extreme case cholesterol may be 27-hydroxylated prior to the introduction of the 7α-hydroxyl group. Mitropoulos et al. (67) and Mitropoulos and Myant (68) showed that there was a small conversion of cholesterol into 27-hydroxycholesterol, 3β-hydroxy-5-cholenoic acid, lithocholic acid, and chenodeoxycholic acid in rat liver mitochondria fortified with cytosol (**Fig. 5**). Lithocholic acid is, however, a less efficient precursor to chenodeoxycholic acid and all available evidence suggests that a pathway involving lithocholic acid as intermediate is of little or no importance under normal conditions. Such a pathway may, however, be of some importance under cholestatic conditions, for example. Thus it has been shown that urine from infants with biliary atresia and patients with liver disease contains elevated concentrations of 3β-hydroxy-5-cholenoic acid (for a review, see ref. 1).

Among the many possible alternative pathways in which the 27-hydroxyl group is introduced at a stage prior to completion of the nuclear changes (for a review see ref. 1), there is at present experimental support for the presence of three specific such pathways in human liver.

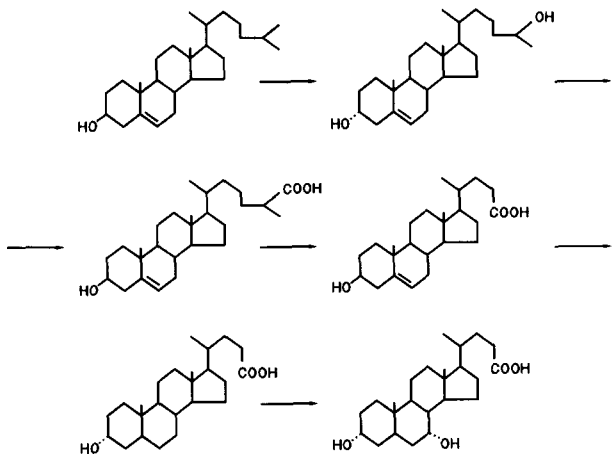

Fig. 5. Mitochondrial conversion of cholesterol into chenodeoxycholic acid (67, 68).

Pathway involving 7α,27-dihydroxy-4-cholesten-3-one as intermediate

7α-Hydroxy-4-cholesten-3-one is an efficient substrate for the mitochondrial 27-hydroxylase in both rat and human liver (4, 11). The product of the mitochondrial 27-hydroxylase, 7α,27-dihydroxy-4-cholesten-3-one, is rapidly converted into both cholic acid and chenodeoxycholic acid in vivo in humans (69, 70). Good evidence that this pathway is of importance in humans is our finding that the lack of the mitochondrial 27-hydroxylase in patients with CTX (see below) leads to a substantial accumulation of 7α-hydroxy-4-cholesten-3-one and its metabolite, 7α,12α-dihydroxy-4-cholesten-3-one (71). It is not known with certainty how 7α,27-dihydroxy-4-cholesten-3-one is further metabolized. One alternative would be completion of the side-chain cleavage prior to further nuclear changes. Axelsson, Mörk, and Everson (72) recently described such a pathway in cultured HepG2 cells. They isolated and identified all intermediates from 7α-hydroxy-4-cholesten-3-one to chenodeoxycholic acid from the incubation medium. In this pathway side-chain degradation was completed prior to 5β-reduction of the A-ring.

Pathway involving 7α,12α,27-trihydroxy-4-cholesten-3-one as intermediate

The accumulation of 7α,12α-dihydroxy-4-cholesten-3-one in patients with a lack of the mitochondrial 27-hydroxylase indicates that this compound may also be an important substrate for the 27-hydroxylase in vivo (71). In accordance with this, a pathway starting with side-chain oxidation of 7α,12α-dihydroxy-4-cholesten-3-one was very recently described in HepG2 cells (72). Most of the intermediates from this compound to cholic acid were identified in the incubation medium of these cells and the amounts of the products were shown to change in a characteristic manner by an alteration of conditions. According to these authors (72), cholic acid would be formed from 7α,12α-dihydroxy-4-cholesten-3-one by the sequence of 27-hydroxylation, oxidation, and degradation of the side-chain and A-ring reduction. An alternative pathway to cholic acid was also described including reduction of the intermediate 7α,12α-dihydroxy-3-oxo-4-cholestenoic acid to form 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) prior to side chain cleavage (72).

Whether or not bile acids are formed by the same mechanism in normal hepatocytes as in the above hepatoblastoma cells is not known with certainty. The fact that two of the described intermediates, 7α,12α,27-trihydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-3-oxo-4-cholestenoic acid, are present in human blood supports the contention that the pathway is significant (73).

Pathway involving 27-hydroxycholesterol as intermediate

Human liver mitochondria are able to 27-hydroxylate cholesterol (4). Krisans et al. (74) have shown that 27-hydroxycholesterol can be further metabolized into 3β-hydroxy-5-cholenic acid in rat liver peroxisomes. The cofactor requirement was the same as that in the peroxisomal conversion of THCA into cholic acid (74). Anderson, Kok, and Javitt (75) reported that 27-hydroxycholesterol is rapidly converted into both cholic and chenodeoxycholic acid in vivo in humans. 27-Hydroxycholesterol and 3β-hydroxy-5-cholenoic acid intermediates in the above pathway are present in relatively high concentrations in human blood (76–78). We have found that the concentration of 27-hydroxycholesterol is considerably lower in the circulation of rats and rabbits than in humans (I. Björkhem, unpublished observation) possibly indicating that pathways involving 27-hydroxycholesterol are less important in the former species.

Axelsson and Sjövall (73) and Axelsson, Mörk, and Sjövall (76) have denoted the pathway to chenodeoxycholic acid starting with 27-hydroxylation of cholesterol and proceeding via C_{27}-acids the "acid" pathway. The one starting with 7α-hydroxylation of cholesterol was called the "neutral" pathway. Since they found that the levels of the circulating intermediates in the neutral pathway were increased after cholestyramine treatment, whereas the intermediates in the acid pathway were unaffected, they predicted that the two pathways were regulated separately. They also studied the

behavior of 7α-hydroxy-3-oxo-4-cholestenoic acid in the human circulation under different conditions (73). This compound may be formed in both the "neutral" and the "acid" pathway. In accordance with this, the level of this acid was increased in the circulation after cholestyramine treatment but not to the same degree as 7α-hydroxy-4-cholesten-3-one (73).

Further support for the presence of a pathway starting with 27-hydroxylation comes from work by Princen et al (79) and from collaborative work of H. Dahlbäck, I. Björkhem, and H. Princen (unpublished observation). It was found that cyclosporin selectively inhibits 27-hydroxylation of cholesterol but not 27-hydroxylation of other 7α-hydroxylated bile acid intermediates in liver mitochondria and in a reconstituted purified cytochrome P-450 system. Cholesterol 7α-hydroxylase was not affected by the inhibitor. Hepatocytes from rat and human hepatoblastoma cells (HepG2 cells) responded with decreased bile acid formation when exposed to cyclosporin. Such a decrease would be expected if part of the bile acids is formed in a pathway involving 27-hydroxylation as a first and rate-limiting step. It may be mentioned that cyclosporin has a hypercholesterolemic effect and it may be speculated that this may be due in part to a blocking of the "acid" pathway for bile acid biosynthesis.

If 27-hydroxycholesterol is an intermediate in a pathway to cholic and chenodeoxycholic acid, the 7α-hydroxyl group may be introduced at different stages. It was recently reported (80) that 27-hydroxycholesterol is efficiently 7α-hydroxylated in pig liver mitochondria. In preliminary experiments we found little or no 7α-hydroxylase activity toward 27-hydroxycholesterol in human liver mitochondria (I. Björkhem, E. Reihnér, and K. Einarsson, unpublished observations). Significant such activity was found in the microsomal fraction of a homogenate from human liver. Another group (J. Shoda, A. Toll, M. Axelsson, F. Pieper, K. Wikvall, and J. Sjövall, unpublished observations) found significant 7α-hydroxylase activity in both the microsomal and mitochondrial fractions. Whether or not the microsomal 7α-hydroxylase active towards 27-hydroxycholesterol is the same as that active towards cholesterol is not known.

The relative importance of the pathway including 27-hydroxycholesterol as an intermediate is not known and one can only speculate about this. According to the work by Axelson and Sjövall (73) and Princen et al. (79), this pathway may be responsible for up to 50% of all bile acids formed in human liver under basal conditions. In a situation where bile acid biosynthesis is up-regulated, e.g., by a bile fistula or by cholestyramine treatment, the contribution of the "acid" pathway may be considerably less (73).

INBORN ERRORS IN SIDE-CHAIN CLEAVAGE

There are two known types of inborn metabolic errors that affect cholesterol side-chain cleavage, cerebrotendinous xanthomatosis (CTX) and various peroxisomal disorders. In CTX there is a specific defect in one of the enzymes. In the peroxisomal disorders there is a lack of intact peroxisomes and thus also a lack of the peroxisomal enzymes necessary for β-oxidation of cholestanoic acids.

Cerebrotendinous xanthomatosis

This rare, inherited lipid storage disease is characterized by xanthomas, progressive neurological dysfunction, cataracts, and the development of xanthomatous lesions in the brain and lung (for a general review, see ref. 81). In contrast to other diseases with tendon xanthomatosis, plasma cholesterol levels are remarkably low. Large deposits of cholesterol and cholestanol are present in most tissues. In 1974, Setoguchi et al. (82) made the key discovery that CTX patients have a defect in bile acid biosynthesis, with incomplete oxidation of the C_{27}-steroid side-chain, leading to excretion of large amounts of C_{27}-bile alcohols in bile, feces, and urine. The formation of bile acids, in particular chenodeoxycholic acid, was reduced.

There has been a controversy in the past between our laboratory and that of Salen et al. concerning the location of this defect (for a general review, see ref. 81). It is however, now established that the basic metabolic defect is located to the mitochondrial 27-hydroxylase. We have shown, for example, that fibroblasts from CTX patients do not express this enzyme activity and that fibroblasts from heterozygotes have an activity about 50% of normal (7). Very recently Cali et al. (83) showed that the 27-hydroxylase gene in patients with CTX contained two cys→arg mutations. When genes with these mutations were expressed in COS-cells, the expressed enzyme was found to be inactive.

The lack of the mitochondrial 27-hydroxylase leads to extensive accumulation of a number of substrates for the enzyme, such as 7α-hydroxy-4-cholesten-3-one and 5β-cholestane-3α,7α,12α-triol. The accumulation of 7α-hydroxy-4-cholesten-3-one may be due in part to the up-regulated cholesterol 7α-hydroxylase (see below) which leads to increased levels of both 7α-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one in the liver and the circulation (81, 84, 85). The up-regulation of the cholesterol 7α-hydroxylase is due to the low formation of primary bile acids, in particular chenodeoxycholic acid. Among the primary bile acids, chenodeoxycholic acid seems to be the most efficient suppressor of the human cholesterol 7α-hydroxylase

(86). Since bile acids are involved also in the regulation of the HMG-CoA reductase (1, 87, 88), cholesterol synthesis is also increased in CTX. As a consequence, LDL turnover is increased (89). The possibility has been discussed that the increased cholesterol synthesis and LDL-turnover in CTX may be in part a direct effect of the lack of 27-hydroxycholesterol or its metabolites (30, 73). Treatment of patients with chenodeoxycholic acid seems to normalize cholesterol synthesis, however (90).

Because 7α-hydroxy-4-cholesten-3-one cannot be metabolized by the usual pathway to bile acids, more unusual pathways are used (**Fig. 6**). We have shown that one product of 7α-hydroxy-4-cholesten-3-one is cholestanol (81). This compound is formed by dehydration of 7α-hydroxy-4-cholesten-3-one to 4,6-cholestadien-3-one by a specific enzyme in liver microsomes (91). The latter steroid is rapidly converted into cholestanol (92). It appears likely that at least a substantial part of the cholestanol accumulating over the decades in the brain and tendons of patients with CTX is formed from accumulated bile acid intermediates.

Because the accumulated 5β-cholestane-3α,7α,12α-triol cannot be metabolized by the usual pathway to bile acids, the 25-hydroxylase pathway may be used (**Fig. 7**). Since the enzymes involved in this pathway appear to have a limited capacity, some of the intermediates in this pathway accumulate and are excreted in bile and feces. Thus patients with CTX excrete high amounts of 5β-cholestane-3α,7α,12α,25-tetrol (81, 82). Also other metabolites of 5β-cholestane-3α,7α,12α-triol, such as 5β-cholestane-3α,12α,12α,24α-25-pentol and 5β-cholestane-3α,7α,12α,23-25-pentol, are excreted. In total, gram amounts of all the above bile alcohols are excreted daily in bile and feces. Treatment with chenodeoxycholic acid suppresses the up-regulated cholesterol 7α-hydroxylase and results in a drastically reduced formation of bile alcohols and cholestanol (90).

Peroxisomal disorders

The most serious of the peroxisomal diseases affecting bile acid synthesis is Zellweger's disease (for a general review, see ref. 93). This rare congenital disorder is characterized by multiple craniofacial abnormalities, generalized hypotonia, central nervous system abnormalities, hepatomegaly, and renal cortical cysts. Most often the afflicted infants die within 6 months. Other peroxisomal disorders also affecting bile acid biosynthesis are infantile Refsum's disease, and neonatal adrenoleukodystrophia (93). Patients with Zellweger's disease lack intact peroxisomes and most of the peroxisomal enzymes are reduced or absent. Consequently these patients have several severe metabolic disturbances (reduced capacity to oxidize fatty acids, prostaglandins, phytanic acid, reduced synthesis of plasmalogens) (93).

In accordance with our finding that peroxisomes contain the enzymes involved in β-oxidation of the CoA derivative of trihydroxycoprostanoic acid, patients with Zellweger's disease, infantile Refsum's disease,

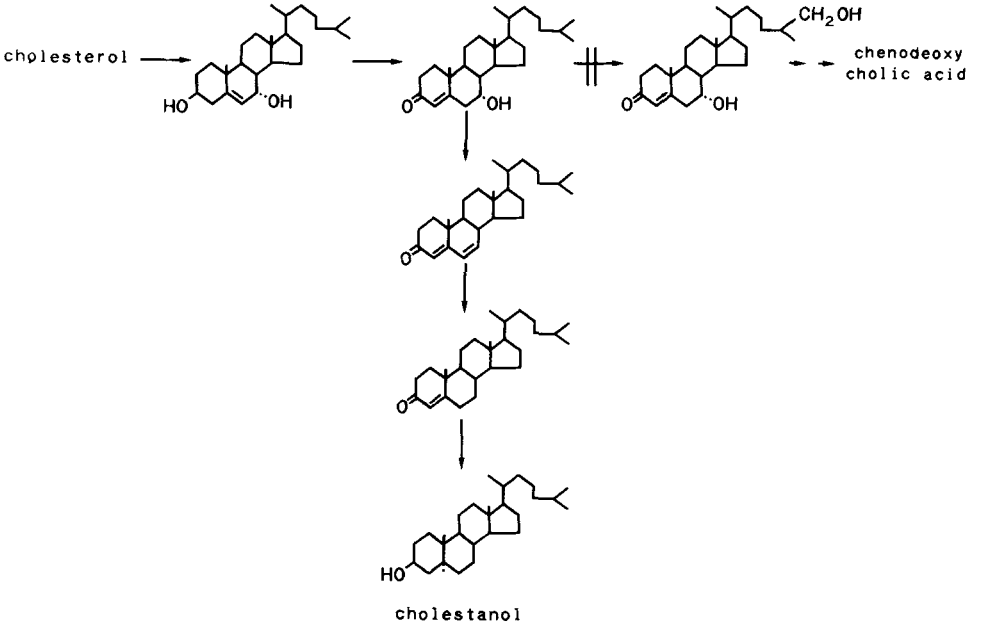

Fig. 6. Metabolic consequence of the accumulation of 7α-hydroxy-4-cholesten-3-one in patients with CTX. Mechanism of formation of excess cholestanol (81).

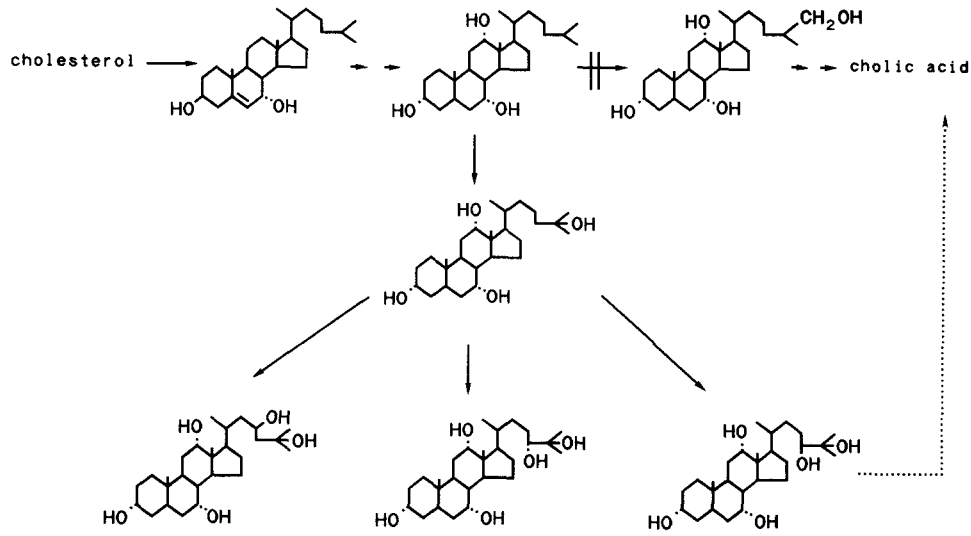

Fig. 7. Metabolic consequence of the accumulation of 5β-cholestane-3α,7α,12α-triol in patients with CTX. Mechanism of formation of C_{27}-bile alcohols and utilization of the 25-hydroxylase pathway for formation of cholic acid.

and neonatal adrenoleukodystrophia accumulate THCA and a number of metabolites of this compound (for general reviews, see refs. 93 and 94). The most important metabolites that accumulate in the above diseases are summarized in **Fig. 8.** One interesting and quantitatively important metabolite of THCA acid is a dicarboxylic C_{29}-acid (94). The mechanism behind the formation of this chain-elongated derivative is not known. It should be emphasized that the metabolic block is not complete since patients with above peroxisomal disorders also form some cholic acid. In collaborative studies together with Pedersen and Kase we have shown, however, that the cholic acid and chenodeoxycholic acid pool sizes are drastically reduced (95, 96). After administration to Zellweger patients, different labeled intermediates in bile acid biosynthesis, carrying an intact C_{27}-steroid side-chain, were found to be rapidly converted into THCA but then only very slowly to cholic acid (95, 96). A liver biopsy from a patient with Zellweger's disease had no significant ability to convert THCA into cholic acid (96). There was, however, some conversion of 3α,7α-dihydroxy-5β-cholestanoic acid into chenodeoxycholic acid. The latter finding may explain why there is little or no accumulation of 3α,7α-dihydroxy-5β-cholestanoic acid in this disease. Another explanation is that 3α,7α-dihydroxy-5β-cholestanoic acid is efficiently 12α-hydroxylated by the human liver (97). Yet, another explanation was presented by Axelson et al. (72) who found an accumulation of 7α-hydroxy-3-oxo-4-choles-

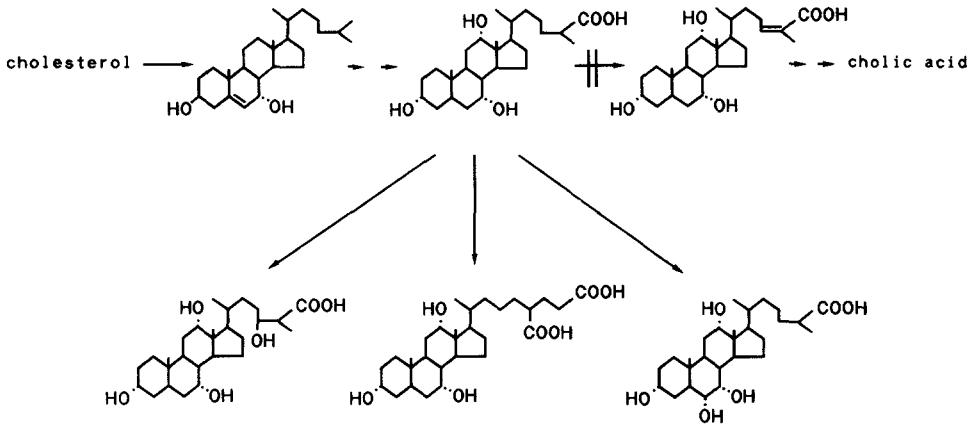

Fig. 8. Metabolic consequence of the accumulation of THCA in patients with the Zellweger disease. Only the quantitatively most dominating metabolites of THCA are shown.

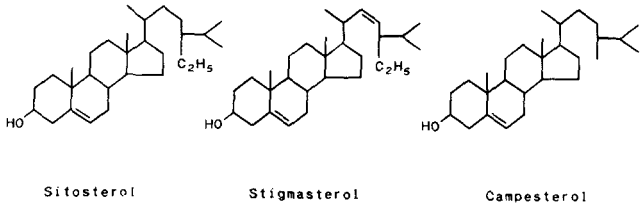

Fig. 9. Structures of the most important phytosterols.

tenoic acid and its hydrolyzed product, 3-oxo-4,6-cholestadienoic acid, in blood from a patient with this disease. This finding suggests that 7α-hydroxy-3-oxo-4-cholestenoic acid could be the substrate for the peroxisomal side-chain degradation instead of 3α,7α-dihydroxy-5β-cholestanoic acid.

In any case, it is evident that the accumulation of THCA acid and its metabolites in the peroxisomal diseases reflects the importance of the peroxisomes for the last step in the biosynthesis of cholic acid from cholesterol.

MECHANISM OF DEGRADATION OF THE STEROID SIDE CHAIN OF PLANT STEROIDS

The structure of the three most common plant sterols is shown in **Fig. 9.** These plant sterols are normal constituents of the human diet. They are absorbed to a very limited extent from the intestine, circulate in plasma in low concentrations, and are excreted in bile (for a review, see ref. 81).

In theory, the presence of an ethyl group at C-24 should prevent or at least obstruct conversion of the plant sterols to bile acids. The possibility must be considered that sitosterol is first dealkylated to cholesterol and then converted into bile acids. Such a dealkylation occurs in some worms and crabs (98, 99) but attempts to demonstrate such a pathway in mammals have failed hitherto. In accordance with this, attempts to demonstrate conversion of sitosterol into normal C_{24}-bile acids in rats (100) and monkeys (101) have failed. In an early study, Salen, Ahrens, and Grundy (102) reported an efficient formation of cholic and chenodeoxycholic acid from intravenously administered [22,23-^{3}H]sitosterol in humans. In a reinvestigation with [4-^{14}C]sitosterol we could not find any significant conversion into labeled C_{24}-bile acids in two healthy subjects (103). In order to bypass the rate-limiting step, the metabolic fate of ^{3}H-labeled 7α-hydroxysitosterol was also studied. In this case there was a significant conversion into acid products in bile. Although part of the labeled products had the chromatographic properties of cholic and chenodeoxycholic acid, further analysis showed that none of the products was identical to chenodeoxycholic acid and only traces at the most could be identical to cholic acid. The results suggested that healthy human subjects, like other mammalian species studied, have little or no capacity to convert sitosterol into the normal C_{24}-bile acids.

Several previous studies have shown that sitosterol is converted into polar compounds in the bile acid fraction of rat bile (100, 104, 105). Very recently we were able to identify most of these products (106, 107). The major part of the ^{14}C radioactivity recovered as bile acids in bile after intravenous administration of [4-^{14}C]sitosterol to female Wistar rats was found to be considerably more polar than cholic acid and only trace amounts had chromatographic properties similar to those of cholic acid and chenodeoxycholic acid. It was shown that the polar products were di- and trihydroxylated C_{21}-bile acids.

Using mass spectrometry, NMR, stereospecific dehydrogenases and reagents, the major trihydroxylated C_{21}-bile acids were identified as 5β-pregnan-3α,11β,15β-triol-21-oic acid, 5β-pregnan-3α,11β,15α-21-oic acid, and 5β-pregnan-3α,11β,16-triol-21-oic acid. The major dihydroxylated C_{21}-bile acid was identified by the same means as 5α-pregnan-3α,12α-diol-21-oic acid (**Fig. 10**) (see refs. 97, 98).

Considerably less C_{21}-bile acids were formed from labeled sitosterol in male than in female Wistar rats. The C_{21}-bile acids formed in male rats did not contain a 15-hydroxyl group. Conversion of labeled sitosterol into C_{21}-bile acids also occurred in adrenalectomized and ovariectomized rats, indicating that endocrine tissues were not involved. Experiments with isolated perfused liver gave direct evidence that the overall conversion of sitosterol into C_{21}-bile acids occurs in this organ. Attempts to demonstrate production of C_{21}-bile acids in isolated hepatocytes have failed hitherto.

After intravenous injection of deuterium-labeled sitosterol in bile fistula female Wistar rats, the isolated C_{21}-bile acids were found to contain very little isotope,

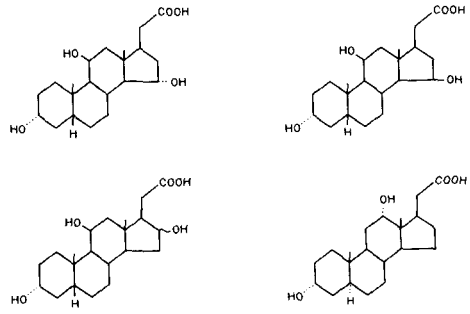

Fig. 10. Structures of the major C_{21}-bile acids formed from cholesterol and plant sterols in female Wistar rats (97, 98).

indicating a high dilution with endogenous material. In view of the small amounts of sitosterol in the diet, it appeared likely that C_{21}-bile acids may also be formed from a more abundant precursor, such as cholesterol. It was subsequently shown, using a mixture of [4-^{14}C]cholesterol and [22-^{3}H]cholesterol, that there was an efficient conversion of the administered material into the above C_{21}-bile acids. The conversion was surprisingly high, up to about 25%. It is thus evident that a blocking group at C_{24} is not obligatory for degradation of the steroid side-chain beyond the C_{24} stage. It was further shown that campesterol, carrying a methyl group in 24-position, was also converted into the above C_{21}-bile acid in female Wistar rats (106).

The mechanism for formation of the C_{21}-bile acids can only be speculated about at the present stage of knowledge. The most likely mechanism seems to be a primary attack by a mixed function oxidase at C_{21}, followed by further oxidation to give a C_{21}-carboxylic group. The latter compound may then be further oxidized to give a C_{21}-bile acid as final product (**Fig. 11**). At present, however, there is no direct evidence of this hypothetical "21-hydroxylase pathway." In preliminary experiments we synthesized labeled 21-hydroxycholesterol and administered it to female Wistar rats. Most of the polar products obtained were not identical to the above C_{21}-bile acids. It is thus likely that some metabolic step(s) in the steroid nucleus precedes the 21-hydroxylation. It is tempting to suggest that the sex-specific 15-hydroxylation may facilitate the formation of C_{21}-bile acids.

The high capacity of the female rat liver to convert cholesterol into compounds considerably more polar than the usual bile acids may be of some regulatory importance in the overall cholesterol balance. The role of C_{21}-bile acids in other mammalian species is uncertain, however. In humans there seems to be little or no formation of such bile acids (E. Lund, K. Muri-Boberg, and I. Björkhem, unpublished observation).

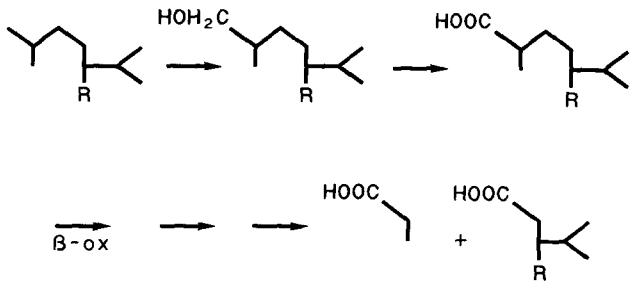

Fig. 11. Suggested mechanism for formation of C_{21}-bile acids from phytosterols and cholesterol in female Wistar rats (97, 98).

CONCLUDING REMARKS

Three different mechanisms are known for the degradation of the cholesterol side chain.

The major mechanism, the "27-hydroxylase pathway," involves a primary attack by a mitochondrial cytochrome P-450 system followed by peroxisomal β-oxidation. Metabolic blocks in this pathway lead to accumulation of intermediates, with severe metabolic consequences.

The "25-hydroxylase pathway" involves a primary attack by a microsomal 25-hydroxylase. This pathway has a low capacity and seems to be of importance only when the 27-hydroxylase pathway is blocked.

In addition to the above systems, a complementary system has been evolved in at least one mammalian species that is able to degrade both cholesterol and plant sterols into highly polar C_{21}-bile acids. It is probable that this degradation is initiated by attack of a 21-hydroxylase. The relative importance of the hypothetical "21-hydroxylase pathway" in different mammalian species is not known.

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