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Bile Acid Biosynthesis†

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Cholesterol homeostasis in mammals is maintained through the coordinate regulation of three major metabolic pathways in the liver (Turley & Dietschy, 1982). Two pathways supply cholesterol to cells and include an endogenous biosynthetic pathway in which acetate is converted into cholesterol (Rilling & Chayet, 1985) and an exogenous pathway in which members of the low-density lipoprotein receptor family bind and internalize cholesterol-carrying particles from the blood (Hobbs et al., 1990). A third pathway is responsible for catabolism and involves the conversion of cholesterol into hydrophilic bile acids (Danielsson, 1973).

The mass of cholesterol fluxing through the human liver per day is large and minimally encompasses a biosynthetic output of 600 mg, a receptor-mediated uptake of 300 mg, a direct secretion component of 600 mg, and a bile acid synthesis component of 100–400 mg (Turley & Dietschy, 1982). Many proteins participate in cholesterol metabolism in the liver, including over 40 enzymes in the biosynthetic pathway (Rilling & Chayet, 1985), at least two endocytic receptors, a dozen or more bile acid biosynthetic enzymes, and numerous storage, transport, and regulatory proteins. The liver employs several strategies to coordinately regulate this cadre of proteins in an effort to prevent the pathological consequences of a disruption in overall cholesterol homeostasis. These tacts include shared negative feedback regulatory mechanisms at the transcriptional level (Goldstein & Brown, 1990) and physiological intertwinings that link the activities of different key regulatory enzymes in each of the pathways.

An example of this latter form of regulation is the Jekyll and Hyde roles that bile acids play in cholesterol homeostasis. As Mr. Hyde, they are water-soluble end products of cholesterol breakdown, readily excreted via the bile and intestine. Similarly, they provide the primary stimulus for bile flow and facilitate the direct excretion of hepatic cholesterol into the bile (Turley & Dietschy, 1982). In both of these roles, bile acids serve to decrease whole body cholesterol levels and thus to increase the activities of enzymes in the supply pathways. As Dr. Jekyll, bile acids act as detergents in the small intestine, forming mixed micelles with dietary fats and cholesterol and thereby promoting their uptake (Carey, 1982). In addition, approximately 95% of the bile acids are taken up by the enterocytes and returned to the liver in an enterohepatic circulation that is very efficient at reutilizing bile acids (Carey, 1982). The combination of dietary cholesterol solubilization and decreased need for de novo bile acid synthesis due to recycling leads to an overall increase in whole body cholesterol levels and a suppression of the supply pathways.

The complex chemistry and physiology of bile acids have fascinated a wide range of experimentalists for centuries (Carey, 1982). The need to coordinately regulate supply and catabolism in order to maintain intracellular and extracellular cholesterol homeostasis, and the associated pathology of an imbalance, has served to attract more workers to these pathways. Over the last decade, the enzymes and proteins involved in cholesterol supply, including proteins involved in the biosynthesis of cholesterol (Goldstein & Brown, 1990) and endocytic receptors (Hobbs et al., 1990), have been major points of research focus. These studies have provided a wealth of insight into the components, regulation, and genetics of cholesterol supply.

Similar progress has been made recently in the cholesterol catabolic pathway. In 1989, cDNAs for two key enzymes of bile acid biosynthesis were isolated (Andersson et al., 1989a; Noshiro et al., 1989), and several more have been reported since (Table I). These advances have provided new insight and tools for examining this pathway. Here, we review the reactions, enzymes, regulation, and genetics of the main pathway of bile acid biosynthesis.

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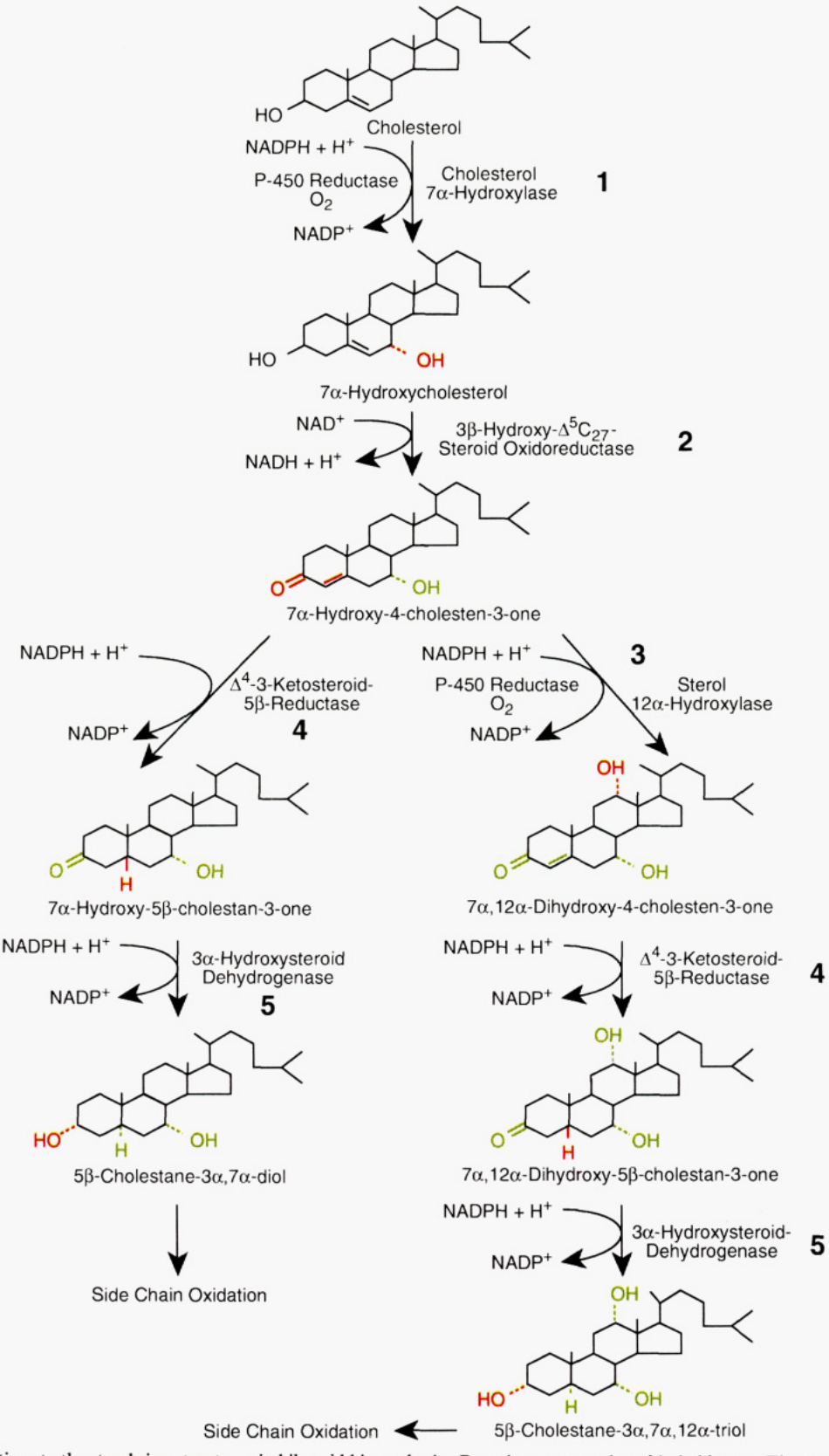


FIGURE 1: Modifications to the sterol ring structures in bile acid biosynthesis. Reactions are numbered in bold type. The modifications introduced by an individual enzyme are indicated in red on the product of the reaction. Cumulative changes to the ring structures are indicated in green. Enzymes and cofactors that catalyze a particular reaction are indicated next to the arrows. Reaction 4 is catalyzed by a Δ^4-3-oxosteroid 5β-reductase.

The Bile Acid Synthesis Pathway. Primary bile acids are synthesized from cholesterol in the liver by the actions of at least 14 different enzymes. In the human, the hamster, and the rat, the major primary bile acids are cholic acid and chenodeoxycholic acid (Danielsson, 1973); however, the synthesis and metabolism of bile acids may differ significantly between different vertebrate species (Haslewood, 1967). The steps leading to the formation of these molecules can be divided into two broad categories: those that modify the cyclopentanoperhydrophenanthrene ring structure or nucleus (Figure 1) and those that oxidize and shorten the 8 carbon atom side chain of the sterol (Figure 2). These divisions are

Table I: Enzymes of Bile Acid Biosynthesis

reaction[a]	enzyme	mol wt[b]	subcellular localization	comments	cDNAs	reference[c]
1	cholesterol 7α-hydroxylase	56 890	endoplasmic reticulum	cytochrome P-450, liver specific	rat, human	Noshiro et al., 1989; Noshiro & Okuda, 1990
2	3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase	~46 000	endoplasmic reticulum	multiple isozymes, bile acid enzyme cDNA not yet cloned	rat, human, bovine C_{19}, C_{21} isozymes	Wikvall, 1981; The et al., 1989; Zhao et al., 1989, 1991
3	sterol 12α-hydroxylase	~56 000	endoplasmic reticulum	cytochrome P-450		Murakami et al., 1982
4	Δ^4-3-oxosteroid 5β-reductase	37 376	cytoplasm	present in many tissues	rat	Onishi et al., 1991
5	3α-hydroxysteroid dehydrogenase	37 029	cytoplasm	aldehyde/aldose reductase family	rat	Pawlowski et al., 1991
6	sterol 27-hydroxylase	57 182	mitochondria	cytochrome P-450, present in many tissues	rabbit, rat, human	Andersson et al., 1989a; Usui et al., 1990; Cali & Russell, 1991
7	alcohol dehydrogenase	~41 000	cytoplasm	multiple isozymes	human β	Deuster et al., 1986
8	aldehyde dehydrogenase	~54 000	cytoplasm	multiple isozymes, bile acid enzyme not yet identified	rat	Dunn et al., 1989; Miyauchi et al., 1991
9	bile acid coenzyme A ligase	unknown	endoplasmic reticulum	distinct from fatty acid CoA ligase		Prydz et al., 1988
10	bile acid oxidase	~52 000	peroxisome	liver specific, distinct from fatty acid oxidase		Prydz et al., 1986; Schepers et al., 1990; Gengenbacher et al., 1990
11, 12	bile acid hydratase/ dehydrogenase	~78 000	peroxisome	bifunctional enzyme, relation to fatty acid peroxisomal β-oxidation enzyme known		Prydz et al., 1986; Gengenbacher et al., 1990
13	bile acid thiolase	~40 000	peroxisome	may be the same as fatty acid peroxisomal β-oxidation enzyme		Schram et al., 1987; Gengenbacher et al., 1990

[a] Reaction numbers refer to Figures 1 and 2. [b] Exact molecular weights are derived from cDNA sequences and are for to the mature enzyme. Approximate molecular weights come from SDS–polyacrylamide gel estimates. All values are for rat enzymes. [c] References are for cDNAs when clones are reported or for seminal studies on a given enzyme in the absence of cDNAs. Only the first cloning reference is indicated; see text for others.

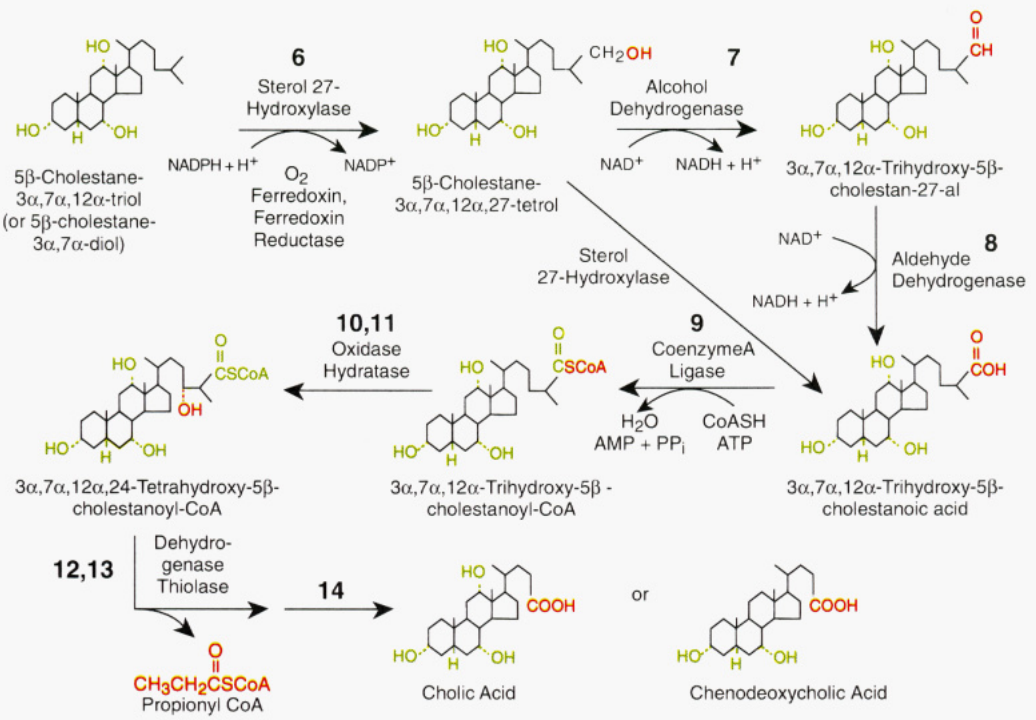


FIGURE 2: Modifications to the sterol side chain in bile acid biosynthesis. Reactions are numbered in bold type. Current changes to the side chain are indicated in red for each reaction. Cumulative changes to the side chain and ring structures of the sterol are indicated in green. Enzymes and cofactors that participate in a particular reaction are indicated next to the arrows. See text for a description of reactions taking place at step 14.

an explanatory convenience only, as many of the enzymes in the pathway exhibit substrate promiscuity, leading to uncertainty with regard to the exact order of biosynthetic steps (Björkhem, 1985).

The first step in modification of the ring structure (reaction 1, Figure 1) involves the introduction of a hydroxyl group in the axial (α) configuration at position C-7 of cholesterol. This reaction is catalyzed by a unique cytochrome P-450 enzyme, cholesterol 7α-hydroxylase (Table I), and utilizes molecular oxygen, NADPH, and a protein cofactor, cytochrome P-450 reductase. Cholesterol 7α-hydroxylase is a microsomal enzyme and is one of three cytochrome P-450s that participate in bile acid biosynthesis (Table I). The rat and human enzymes are 503 and 504 amino acids in length, respectively, and share many sequence features with other members of this large family of mixed-function monooxygenases (Noshiro et al.,

1989; Jelinek et al., 1990; Li et al., 1990; Noshiro & Okuda, 1990). Cholesterol 7α-hydroxylase catalyzes the rate-limiting step in bile acid synthesis, and the expression and activity of this enzyme are subject to multiple regulatory inputs (see below).

In the second step of the bile acid pathway, 7α-hydroxycholesterol is acted upon by a microsomal 3β-hydroxy-C_{27}-steroid oxidoreductase (reaction 2, Figure 1). This reaction utilizes NAD^+ as a cofactor. The 3β-hydroxy-C_{27}-steroid oxidoreductase isozyme that participates in bile acid biosynthesis shows an absolute preference for C_{27} sterol substrates (Wikvall, 1981) and is thus different from recently described isozymes that act on neutral C_{19} and C_{21} steroids (see below). This difference is further evident by the fact that an inborn error of bile acid synthesis involving the 3β-hydroxy-C_{27}-steroid oxidoreductase does not affect endocrine function (Clayton et al., 1987). Historically, the C_{27}-specific isozyme was the first bile acid synthetic enzyme purified to homogeneity, a feat accomplished by Wikvall in 1981. Complementary DNAs encoding several different 3β-hydroxysteroid oxidoreductase isozymes that participate in steroid hormone metabolism have been isolated from multiple species (The et al., 1989; Zhao et al., 1989, 1991; Lorence et al., 1990); however, a cDNA encoding an enzyme with a preference for the C_{27} sterols has not yet been reported.

The 7α-hydroxy-4-cholesten-3-one product of 3β-hydroxy-C_{27}-steroid oxidoreductase can take one of two paths in bile acid biosynthesis (Figure 1). If this intermediate is acted upon by the second microsomal cytochrome P-450 enzyme of the pathway, sterol 12α-hydroxylase (reaction 3, Figure 1), then the resulting product is ultimately converted into cholic acid. The sterol 12α-hydroxylase enzyme has been substantially purified from rat liver and shown to be a microsomal cytochrome P-450 with a molecular weight of 56 000 (Murakami et al., 1982). A cDNA encoding sterol 12α-hydroxylase has not yet been reported; however, the ability to purify the protein suggests that the cloning will be accomplished shortly. 7α-Hydroxy-4-cholesten-3-one can alternatively serve as a substrate for a soluble Δ^4-3-oxosteroid 5β-reductase (reaction 4, Figure 1) to yield a sterol intermediate that is ultimately converted into chenodeoxycholic acid. This enzyme also catalyzes the identical reaction on 7α,12α-dihydroxy-4-cholesten-3-one, the product of 12α-hydroxylation. Cholic acid and chenodeoxycholic acid, as well as their secondary and tertiary metabolites, have different physicochemical and physiological properties (Carey, 1982). This branch point is therefore of potential regulatory significance. However, a correlation between sterol 12α-hydroxylase activity in the liver and the ratio of these two bile acids has not been found (Björkhem et al., 1983), suggesting that other steps in the bile acid pathway influence the cholic acid to chenodeoxycholic acid ratio.

DNA sequence analysis of a recently isolated rat cDNA predicts the Δ^4-3-oxosteroid 5β-reductase to be a soluble enzyme of 327 amino acids with no overt hydrophobic features (Onishi et al., 1991). Interestingly, the sequence of the Δ^4-3-oxosteroid 5β-reductase is not related to that of the corresponding steroid 5α-reductase (Andersson et al., 1989b), an enzyme that catalyzes a reaction with opposite stereoselectivity on many of the same sterol substrates and is responsible for the formation of allo (5α-H) bile acids (Elliott, 1971).

The products of the Δ^4-3-oxosteroid 5β-reductase reactions are the substrates for a soluble 3α-hydroxysteroid dehydrogenase enzyme (reaction 5, Figure 1). This enzyme appears to be ambivalent with respect to pyridine nucleotide cofactor, utilizing NAD or NADP, and catalyzes both the oxidation and reduction of a variety of substrates (Penning et al., 1991, and references therein). Three groups have recently reported the isolation of cDNA clones encoding rat liver 3α-hydroxysteroid dehydrogenases (Cheng et al., 1991; Pawlowski et al., 1991; Stolz et al., 1991). The cDNA encodes a soluble protein of 322 amino acids that shares sequence identity with several other aldo/keto reductase proteins. Expression of one of the cDNAs in *Escherichia coli* yielded an enzyme that catalyzed the oxidation of androsterone to 5α-androstanedione, utilizing NAD as a cofactor (Cheng et al., 1991). A second cDNA encoding an enzyme with 70% sequence identity to 3α-hydroxysteroid dehydrogenase was isolated by Cheng et al. (1991), raising the possibility of multiple isozymes.

The products of the 3α-hydroxysteroid dehydrogenase reaction next undergo oxidation and shortening of the side chain (Figure 2). The first step in this portion of the pathway is catalyzed by a mitochondrial cytochrome P-450 sterol 27-hydroxylase (reaction 6, Figure 2). The sterol 27-hydroxylase resides in the inner membrane of the mitochondria (Taniguchi et al., 1973) and utilizes molecular oxygen, NADPH, and two protein cofactors, ferredoxin and ferredoxin reductase, to introduce a hydroxyl group at the C-27 position of the bile alcohol intermediates (Wikvall, 1984). The enzyme is often referred to as a sterol 26-hydroxylase in the literature; however, on the basis of the stereochemistry of the reaction catalyzed by this mitochondrial enzyme (Danielsson, 1960), it is now more correctly termed a sterol 27-hydroxylase (Björkhem, 1985).

Complementary cDNAs encoding the rabbit, rat, and human sterol 27-hydroxylases have been isolated and shown to encode cytochrome P-450 enzymes of approximately 530 amino acids (Andersson et al., 1989a; Usui et al., 1990; Su et al., 1990; Cali & Russell, 1991). Each cDNA encodes a protein with a cleavable mitochondrial-type signal sequence and appears to be transcribed from a single-copy gene. The enzyme has been purified to homogeneity from rabbit liver and, in the presence of ferredoxin and ferredoxin reductase, will oxidize several different sterol substrates, including cholesterol, and vitamin D_3 (Wikvall, 1984; Su et al., 1990; Akiyoshi-Shibata et al., 1991). Two unique characteristics of sterol 27-hydroxylase have been described. First, unlike most other cytochrome P-450 enzymes that show a restricted tissue expression pattern (Gonzalez, 1989), sterol 27-hydroxylase mRNA and enzyme activity are present in many different tissues (Skrede et al., 1986; Andersson et al., 1989a; Su et al., 1990). This finding suggests that the actions of the enzyme are not limited to hepatic bile acid synthesis. Second, the protein can catalyze multiple oxidation reactions at the C-27 position of bile acid intermediates (Andersson et al., 1989a; Dahlbäck & Holmberg, 1990; Cali & Russell, 1991). Thus, not only will the enzyme form an alcohol product as shown in reaction 6 of Figure 2, but it will go on to form the carboxylic acid derivative of the sterol (3α,7α,12α-trihydroxy-5β-cholestanoic acid) as shown by the long arrow in Figure 2.

The formation of this latter intermediate can also be accomplished by the concerted actions of a cytoplasmic NAD-linked alcohol dehydrogenase (reaction 7, Figure 2) and an NAD-requiring aldehyde dehydrogenase (reaction 8, Figure 2). Highly purified preparations of these two enzymes catalyze the formation of the aldehyde and carboxylic acid intermediates, respectively (Okuda et al., 1969; Taniguchi et al., 1973).

An alcohol dehydrogenase from human liver that participates in bile acid biosynthesis has been identified as a $\beta_2\beta_2$ isozyme (Okuda & Okuda, 1983), while the exact aldehyde dehydrogenase isozyme has not yet been identified. At present, the relative proportion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid formed in vivo by sterol 27-hydroxylase versus that formed by alcohol and aldehyde dehydrogenase is not known.

In reaction 9 of the pathway, a microsomal coenzyme A ligase utilizes ATP and a molecule of coenzyme A to form a thioesterified 5β-cholestanoic acid derivative. Pedersen, Björkhem, and colleagues have shown that this enzyme resides almost exclusively in the microsomal fraction of rat liver cells and that the distribution of the bile acid ligase is different from that of fatty acid ligases such as palmitoyl-CoA ligase (Prydz et al., 1988). It is not known whether distinct bile acid ligases exist for the cholic acid and chenodeoxycholic acid intermediates.

The terminal steps of side-chain oxidation take place mainly in the peroxisome (Pedersen & Gustafsson, 1980) and are thought to follow a pathway similar to that of the β-oxidation of fatty acids in this organelle (Lazarow & Fujiki, 1985). Thus, the CoA ester of 5β-cholestanoic acid is initially acted upon by an oxidase enzyme and a bifunctional dehydrogenase/hydratase enzyme to yield a 24-hydroxylated species (reactions 10 and 11, Figure 2). The formation of this product (3α,7α,12α,24-tetrahydroxy-5β-cholestanoyl-CoA) goes through a Δ^{24} intermediate (Farrants et al., 1989), followed by action of the hydratase activity of the bifunctional enzyme to form the C-24 hydroxylated species. The oxidase enzyme involved in bile acid synthesis has recently been shown to be chromatographically distinct from the fatty acid oxidase in both the human and the rat (Casteels et al., 1990; Schepers et al., 1990).

The dehydrogenase activity of the bifunctional enzyme next forms a 24-oxo intermediate, and a subsequent thiolytic cleavage results in the loss of a propionyl-CoA and the formation of a C_{24} bile acid–CoA product (reactions 12 and 13, Figure 2). Genetic evidence suggests that a single thiolase enzyme may act on both fatty acids and bile acid intermediates (Schram et al., 1987). Overall, there is a paucity of information regarding the biochemistry of the bile acid biosynthetic enzymes in the peroxisome. This situation may soon be remedied by the use of cloned cDNA probes encoding their fatty acid oxidizing counterparts (Osumi et al., 1987, and references therein) in low-stringency hybridization or polymerase chain reactions.

The final steps of bile acid biosynthesis (reaction 14, Figure 2) are not well understood. How and in what form the product of the thiolase enzyme exits the peroxisome is not known. The molecule could leave either as a CoA derivative or as an unesterified cholic or chenodeoxycholic acid. A microsomal bile acid CoA synthetase has been reported (Lim & Jordan, 1981), suggesting that the bile acid may leave the peroxisome as the free acid prior to reconjugation with CoA. The CoA-conjugated bile acid is a substrate for the last enzyme in the pathway, a bile acid–CoA:amino acid *N*-acyltransferase that utilizes either glycine or taurine to form the corresponding *N*-acyl bile acid. For clarity, this reaction is not shown in Figure 2, although it represents an essential final step in the biosynthesis of the primary acids prior to secretion into bile. Quantitatively, 98% of secreted bile acids are in the conjugated form. The human transferase enzyme has recently been purified to homogeneity and shown to be a soluble protein of molecular weight 50 000 that is active as a monomer and utilizes both glycine and taurine as substrates (Johnson et al., 1991). This enzyme is enriched in rat peroxisomes (Kase & Björkhem, 1989), raising the possibility that the soluble enzyme isolated by Johnson et al. (1991) may have been released from this organelle and that both the terminal steps of bile acid side-chain oxidation and conjugation occur in the peroxisome. The ratio of glycine to taurine conjugated bile acids is normally 3:1 in adult man (Sjövall, 1959), which probably reflects the relative hepatic abundance of the two amino acids given the enzyme's near-identical affinity for these two substrates. For a similar reason, this ratio can differ during development (Setchell et al., 1988b) and between species.

The aminoacyl bile acids are water soluble and are actively secreted from the parenchymal cells of the liver into the common bile duct (Erlinger, 1982; Nathanson & Boyer, 1991). In most mammals, the majority of bile is stored in the gall bladder and released upon hormonal stimulation into the duodenum (Carey, 1982). It is here that bile acids act to solubilize dietary fats and cholesterol, thereby facilitating the uptake of these nutrients by the enterocytes. During passage through the gastrointestinal tract, primary bile acids undergo modifications by numerous bacterial enzymes, resulting in the formation of secondary and tertiary bile acids (Hylemon, 1985). As a result of these extensive bacterial modifications, the diversity of bile acids in various biological fluids including bile is large and is aptly illustrated by the detailed mass spectrometric analyses published in recent years [e.g., Almé et al. (1977), Setchell et al. (1983), Shoda et al. (1988), and Nakagawa et al. (1990)].

Conservation of the bile acid pool is facilitated by the efficient reabsorption of bile acids from the gastrointestinal tract. Absorption can take place by several mechanisms which mainly include passive nonionic diffusion and active transport and is determined largely by the chemical form of the bile acid and its pK_a (Carey, 1982). It is beyond the scope of this review to discuss bile acid transport save to indicate that the major site for reabsorption for bile acids is the terminal ileum.

One Pathway, Multiple Organelles. A consideration of the subcellular localizations of the many enzymes in the bile acid pathway (Table I) demonstrates that sterol intermediates travel a complex itinerary en route to becoming primary bile acids. In the pathway described in Figures 1 and 2, synthesis of bile acids begins in the endoplasmic reticulum with the action of cholesterol 7α-hydroxylase. Figure 3 shows an indirect immunofluorescence study in which antibodies raised against the rat cholesterol 7α-hydroxylase were used to identify the subcellular locale of the enzyme expressed from a transfected cDNA in COS cells. The reticular staining pattern is readily evident. The first two or three steps of the pathway (Figure 1, Table I) take place in this organelle and from here intermediates follow a cytoplasm → mitochondria → cytoplasm → endoplasmic reticulum → peroxisome → endoplasmic reticulum → bile route. We again emphasize the fact that the exact order of metabolic steps in the pathway may not be that outlined in Figures 1 and 2, and in fact a different order has been demonstrated in cultured human hepatoblastoma cells (Axelson et al., 1991). Nevertheless, all intermediates must at some point enter and exit these various organelles. How the bile acid intermediates and conjugated bile acids are successfully trafficked through cells is not known; however, several sterol binding proteins in the liver and intestine have been postulated to have a role in this shuttling (LeBlanc & Waxman, 1990; Lin et al., 1990).

Regulation of Bile Acid Biosynthesis. Most of our knowledge regarding the regulation of bile acid production has been gathered in the rat. The absence of a gall bladder in this

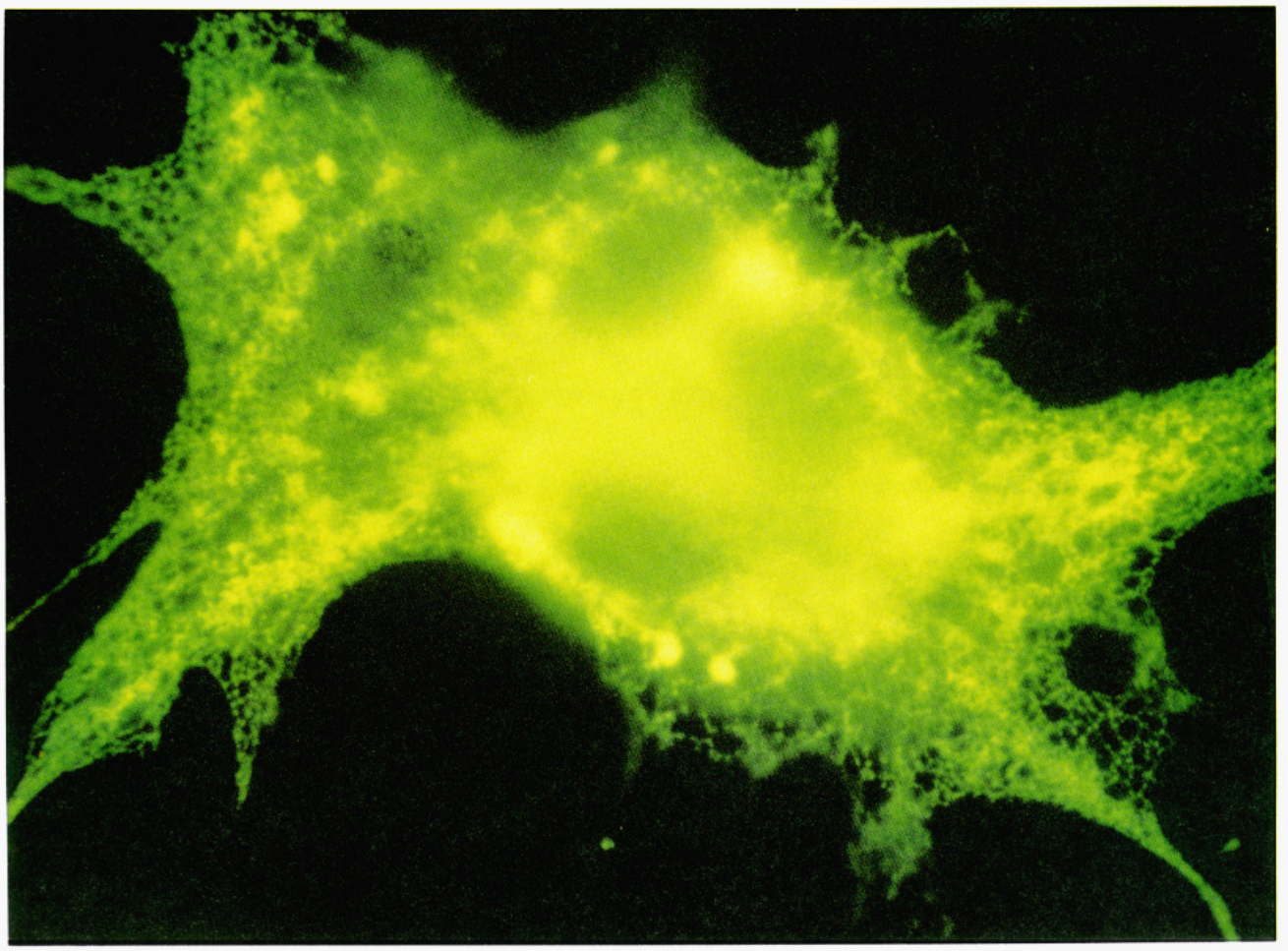


FIGURE 3: Localization of cholesterol 7α-hydroxylase in the endoplasmic reticulum. A cDNA encoding the rat cholesterol 7α-hydroxylase was expressed in Simian COS cells as described previously (Jelinek et al., 1990). Rabbit polyclonal antibodies raised against the purified protein were used to visualize the expressed cholesterol 7α-hydroxylase by indirect immunofluorescence using a modification of protocols described by Yamamoto et al. (1984). A fluorescein-labeled goat anti-rabbit second antibody was used. Staining of transfected cells with preimmune serum did not reveal any fluorescence (data not shown).

species, coupled with an unusually long small intestine, results in high levels of bile acid biosynthesis (Turley & Dietschy, 1982). These anatomical and biochemical differences, and the quite different manner by which the rat metabolizes endogenous and exogenous cholesterol (Turley & Dietschy, 1982), suggest that not all of the results obtained in the rat may be extrapolated to other species. Nevertheless, most mammals share the same rate-limiting step in bile acid biosynthesis, and in this regard the rat is an exemplary species. In the late 1960s, this step was shown to be catalyzed by cholesterol 7α-hydroxylase (Danielsson et al., 1967; Shefer et al., 1968, 1970). These and subsequent investigations have shown that once 7α-hydroxycholesterol is formed in the liver, primary bile acid synthesis soon results. Despite the difficulty posed by measuring an enzyme that both acts on and is regulated by sterols, early studies demonstrated that cholesterol 7α-hydroxylase activity is induced by blocking the return of bile acids to the liver, either by surgical interruption of the enterohepatic circulation (Danielsson et al., 1967) or by dietary administration of anion-exchange copolymers that bind bile acids in the small intestine and thereby prevent their uptake and feedback regulation (Huff et al., 1963). Conversely, infusion or dietary administration of bile acids leads to a decrease in enzyme activity (Shefer et al., 1970). The picture with respect to cholesterol regulation of the enzyme was less clear, with some studies finding an induction of activity and others a suppression. Resolution of this discrepancy had to await the isolation of cDNA and antibody probes for cholesterol 7α-hydroxylase.

The enzyme is present in trace quantities in the liver and resisted purification until 1985 when Andersson et al. (1985) described a procedure for obtaining a highly purified preparation of rat cholesterol 7α-hydroxylase. Subsequently, several groups adapted this procedure to obtain sufficient quantities of the enzyme for protein sequencing, antibody production, and cloning (Ogishima et al., 1987; Noshiro et al., 1989; Chiang et al., 1990; Nguyen et al., 1990; Jelinek et al., 1990; Li et al., 1990; Sundseth & Waxman, 1990; Jelinek & Russell, 1990; Nishimoto et al., 1991). Figure 4 summarizes the findings of many studies on the structure of the rat cholesterol 7α-hydroxylase gene, mRNA, and protein.

The use of cDNA and antibody probes has demonstrated that cholesterol 7α-hydroxylase mRNA and protein are found only in the liver and that a variety of dietary and hormonal stimuli impact on the expression of the gene (Table II). Bile acids such as cholic acid and chenodeoxycholic acid mediate negative feedback regulation and decrease steady-state levels of mRNA and immunodetectable protein (Jelinek et al., 1990; Shefer et al., 1991).

Interestingly, not all bile acids share this ability to suppress cholesterol 7α-hydroxylase. The 7β-hydroxy epimers of cholic acid and chenodeoxycholic acid (ursocholic acid and ursodeoxycholic acid, respectively) have little or no ability to regulate expression of the enzyme (Shefer et al., 1991, and

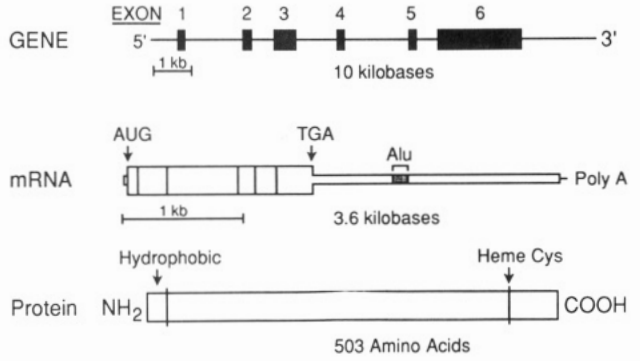


FIGURE 4: Structural features of the rat cholesterol 7α-hydroxylase gene, mRNA, and protein. A schematic of the 10-kilobase gene is shown at the top of the figure with exons indicated by black boxes and 5′-flanking, intron, and 3′-flanking sequences by thin connecting lines. The major 3.6-kilobase mRNA transcribed from the unique rat gene is shown in the center. The coding region is represented by the thick portion of the diagram, while 5′-flanking and 3′-flanking regions are shown by thin portions. The limits of individual exons are indicated by vertical lines within the coding region. The location of a middle repetitive *Alu* sequence in the 3′-untranslated region of the mRNA is indicated by a shaded block. The 503 amino acid protein is shown at the bottom with two landmark features common to microsomal cytochrome P-450 enzymes.

Table II: Regulation of Rat Cholesterol 7α-Hydroxylase[a]

	cholesterol 7α-hydroxylase			
stimulus	activity	protein	mRNA	transcription
bile acids	↓	↓	↓	↓
bile acid binding resins	↑	↑	↑	↑
cholesterol	↑	↑	↑	↑
diurnal rhythm				
dark	↑	↑	↑	?
light	↓	↓	↓	?
starvation	↓	↓	↓	?
thyroid hormone	↑	?	↑	?
glucocorticoids[b]	↓	↓	↓	?

[a] See text for individual references. [b] Data from rats treated with dexamethasone (100 mg/kg body weight; Li et al., 1990). This glucocorticoid agonist induces cholesterol 7α-hydroxylase in some liver cell lines (Leighton et al., 1991).

references therein). This difference may be linked to a decreased ability of the 7β-hydroxy bile acids to promote cholesterol uptake in the small intestine (see below).

Both dietary cholesterol (Jelinek et al., 1990; Nguyen et al., 1990) and thyroid hormone (Ness et al., 1990) induce the amount of cholesterol 7α-hydroxylase mRNA and protein in the liver. Expression is also governed by a presumably hormone-mediated diurnal rhythm, reaching a zenith in the middle of the dark period of the 12-h light/dark cycle and a nadir in the middle of the light portion of the cycle (Chiang et al., 1990; Noshiro et al., 1990; Sundseth & Waxman, 1990).

Transcriptional regulation of the cholesterol 7α-hydroxylase gene is responsible for changes in steady-state mRNA levels (Pandak et al., 1991). The nuclear run-on data of Figure 5 illustrate the ability of different diets to regulate transcription of this gene. The observed changes can be dramatic, as shown by the near absence of transcription when primary bile acids are fed versus the robust transcription seen in the presence of either the bile acid binding resin cholestid or cholesterol (Figure 5). Diets that induce cholesterol 7α-hydroxylase in the liver do not induce transcription of the gene in the kidney or lung (Figure 5), tissues that express multiple cytochrome P-450 genes (Gonzalez, 1989). Similarly, these diets do not affect transcription of other liver cytochrome P-450 genes such as cytochrome P-450II C12 (Zaphiropoulos et al., 1988) or of other steroid- and sterol-metabolizing enzymes such as

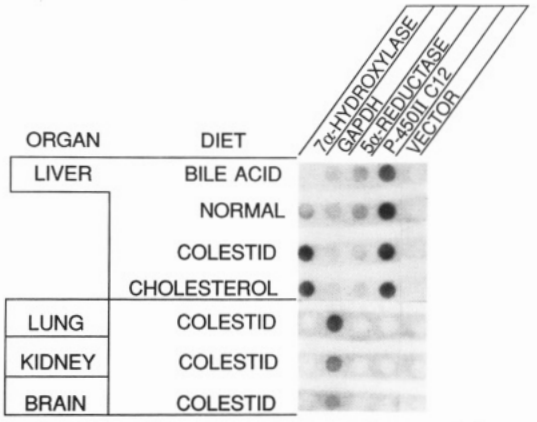


FIGURE 5: Transcriptional regulation of the rat cholesterol 7α-hydroxylase gene. An autoradiogram from a nuclear run-on experiment is shown. Nuclei were isolated from the indicated organ from animals maintained for 14 days on rat chow with the following supplements: bile acid = 0.2% (w/w) chenodeoxycholic acid, normal = no supplement; colestid = 2.0% (w/w) colestid (Upjohn Corp., Kalamazoo, MI); cholesterol = 1.0% (w/w) cholesterol in 10% (v/w) corn oil. Plasmid DNAs (10 μg) corresponding to the indicated cDNA (GAPDH = glyceraldehyde-3-phosphate dehydrogenase; 5α-reductase = steroid 5α-reductase; P-450II C12 = cytochrome P-450 II C12) or the pBluescript vector alone were fixed to nitrocellulose and hybridized to [^{32}P]-labeled run-on RNAs as described by Yuan and Tucker (1984).

steroid 5α-reductase (Figure 5).

The transcription factors that mediate regulation of the cholesterol 7α-hydroxylase gene have not yet been identified; however, the large changes in expression that occur and the recent isolation and characterization of the rat gene (Jelinek & Russell, 1990; Nishimoto et al., 1991) suggest that insight into these proteins will be rapidly forthcoming. The 5′-flanking sequence of the gene contains several transcriptional landmarks, including a consensus TATAAA sequence and a liver-preferential LF-B1 transcription factor binding site. The ability to dissect the roles of these sequences in transcriptional regulation of this gene may be limited by the degree to which dietary changes can be reproduced in vitro. Along these lines, a rat hepatoma cell line that mimics the cholesterol-induced transcription and some aspects of hormonal regulation of the gene has recently been described (Leighton et al., 1991). The use of these cells in transfection studies may eliminate traditional problems encountered in mimicking dietary responses in tissue culture.

Many questions remain with regard to the regulation of cholesterol 7α-hydroxylase. It is not known if a bile acid or a sterol metabolite exerts the suppressive effect observed in diets containing bile acids. The contrasting regulatory properties of bile acids and cholesterol suggest that their relative intracellular ratios or flux across the liver may act to titrate expression of the gene in an as yet unknown manner (see below). The activity and specificity of cell surface bile acid transport proteins in the small intestine and the liver may also contribute to the regulation of cholesterol 7α-hydroxylase. Another parameter to be considered is the rate of cholesterol secretion and uptake under various dietary regimens and how this secretion correlates with cholesterol 7α-hydroxylase activity and transcription. Finally, it has been shown in some studies that posttranscriptional regulation of the enzyme may occur (Shefer et al., 1991; Jones et al., 1991), adding a possible additional level of control.

Coordinate Regulation of Cholesterol Catabolism and Supply. Regulatory cross-talk between the pathways of cholesterol supply and catabolism occurs in the liver and can

Table III: Genetics of Bile Acid Biosynthesis[a]

disease	gene	reaction[b]	inheritance pattern	frequency
cerebrotendinous xanthomatosis	sterol 27-hydroxylase	6	autosomal recessive	rare; 150–200 cases worldwide
3β-Δ^5-C_{27}-hydroxysteroid oxidoreductase deficiency	3β-hydroxy-C_{27}-steroid oxidoreductase	2	autosomal recessive	rare (?),[c] 7 cases
Δ^4-3-oxosteroid 5β-reductase deficiency	Δ^4-3-oxosteroid 5β-reductase	4	autosomal recessive	rare (?),[c] 14 cases

[a] See text for references. [b] From Figures 1 and 2. [c] Detection methods only recently available; true incidence not yet measured.

be readily demonstrated in the rat by dietary manipulation. For example, feeding animals bile acid binding resins leads to a decrease in the return of bile acids to the liver and a consequent increase in cholesterol 7α-hydroxylase (Table II) and of de novo bile acid synthesis. This increase, in turn, depletes intracellular cholesterol pools, causing an increase in the synthesis of enzymes in the cholesterol biosynthetic pathway and of the low-density lipoprotein receptor (Goldstein & Brown, 1990). Conversely, when cholesterol is included in the diet, enzymes in the supply pathway are suppressed and cholesterol 7α-hydroxylase is induced (Jelinek et al., 1990; Li et al., 1990; Duckworth et al., 1991; Pandak et al., 1991). This induction in turn leads to an increase in bile acid production and to an increase in excretion in this species (Björkhem et al., 1991). When primary bile acids are fed, both cholesterol 7α-hydroxylase and enzymes in the supply pathways are suppressed (Spady et al., 1986; Jelinek et al., 1990; Duckworth et al., 1991). The response of the supply pathways can be explained by an increased absorption of dietary cholesterol mediated by cholic or chenodeoxycholic acid and a subsequent increased return of the sterol to the liver in the form of chylomicron particles (Spady et al., 1986). In addition, down-regulation of cholesterol 7α-hydroxylase decreases de novo synthesis of bile acids and further increases the hepatic pool of cholesterol, and hence the suppression of cholesterol synthesis and uptake.

Genes in the cholesterol supply pathways coordinately respond to sterols through conserved sterol regulatory elements (SREs) in their 5′-flanking regions (Goldstein & Brown, 1990). The presence of one or more SREs leads to a suppression of transcription in the presence of excess sterols and an induction in the absence of sterols. Can the regulation of cholesterol 7α-hydroxylase by different diets be adapted to this paradigm? On the one hand, transcription must increase in response to dietary cholesterol but decrease in response to bile acids (Figure 5). This response, as well as others, could be mediated by interactions between a positive SRE (i.e., one that *induces* transcription in response to sterols) and a negative bile acid response element (BARE) in the rat cholesterol 7α-hydroxylase gene, with the ratio of bile acids to cholesterol titrating the ultimate output of the gene. Thus, in cholesterol feeding, the ratio of intrahepatic bile acids to cholesterol decreases, leading to increased transcription of cholesterol 7α-hydroxylase. Conversely, when bile acids are fed, this ratio increases (despite the relative increase in solubilization of dietary cholesterol), leading to decreased transcription of the gene. This seesaw regulation predicts that neither the SRE nor the BARE is absolutely dominant over the other but that both elements contribute to expression of the gene.

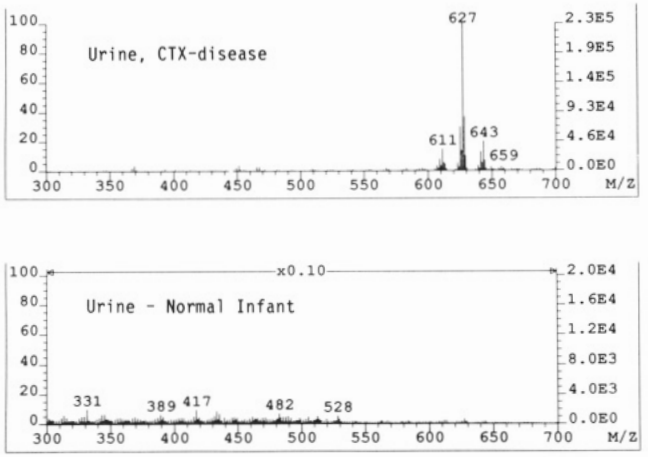
Two recent studies have addressed this question directly. Björkhem et al. (1991) showed that blocking the return of cholesterol to the liver in rats fed a high cholesterol diet does not prevent the increase in cholesterol 7α-hydroxylase activity and mRNA. They further showed that high cholesterol diets increase the fecal excretion of bile acids. These results indicate that the stimulation of the enzyme is due to a decreased return of bile acids to the liver and not to substrate stimulation. In a second study, Spady and Cuthbert (1992) showed that a dietary combination of bile acid and cholesterol led to an increase in the levels of cholesterol 7α-hydroxylase enzyme activity and mRNA. These results suggest that the contributions of the putative positive SRE may outweigh those of the BARE.

The importance of cholesterol in regulating the expression of cholesterol 7α-hydroxylase can be shown in another way. Drug-induced inhibition of cholesterol biosynthesis in the rat at one of two different steps leads to an acute decrease in both cholesterol 7α-hydroxylase activity and bile acid biosynthesis (Pandak et al., 1990a,b). Similar results have recently been reported in a small human study (Mitchell et al., 1991). The fact that the enzyme is saturated with respect to cholesterol substrate in the endoplasmic reticulum under conditions of both cholesterol starvation and repletion (Björkhem & Åkerlund, 1988) suggests that the decrease observed may represent transcriptional regulation (via the putative positive SRE) and not substrate availability.

Is it cholesterol or a metabolite that is responsible for the observed regulation of cholesterol supply and catabolism? An attractive, but as yet untested, hypothesis to explain interpathway regulation postulates the formation of a corepressor (in the classic genetic sense) by the bile acid biosynthetic enzyme sterol 27-hydroxylase (Andersson et al., 1989a; Javitt, 1990). The addition of oxysterols such as 25-hydroxycholesterol and 27-hydroxycholesterol to the medium of cultured cells leads to a rapid decrease in the transcription of genes containing SREs (Goldstein & Brown, 1990). Sterol 27-hydroxylase has a low, but demonstrable, ability to utilize cholesterol as a substrate, producing 27-hydroxycholesterol (Wikvall, 1984). Coupling these two observations leads to the teleologically pleasing hypothesis that accumulation of intracellular cholesterol results in transfer of the molecule to the mitochondria and subsequent oxidation by the sterol 27-hydroxylase enzyme. The resulting oxysterol could then diffuse to the nucleus or perhaps be actively transported by oxysterol binding proteins (Dawson et al., 1989) to this organelle, leading to a subsequent suppression of cholesterol supply pathway genes. The response of cholesterol 7α-hydroxylase to oxysterols is not yet known; however, these compounds are substrates for the enzyme (Leighton et al., 1991), and like cholesterol, they may induce transcription of the gene. A regulatory role for sterol 27-hydroxylase is further supported by the observation that the enzyme is expressed in multiple tissues and that the level of mRNA correlates well with a given tissue's cholesterol biosynthetic capacity (Andersson et al., 1989a). In addition, individuals with inherited mutations in the sterol 27-hydroxylase gene abnormally accumulate sterols in multiple tissues (see below).

Clearly, there are many exciting hypotheses to be tested regarding coordinate regulation of cholesterol catabolism and supply. The availability of cloned genes for key players in the pathways should allow a rapid molecular dissection of the complex regulatory networks that permeate cholesterol homeostasis.

Genetics of Bile Acid Biosynthesis. Three inherited defects in bile acid biosynthesis have been described to date (Table III). The first was identified in 1974 by Salen and co-workers,



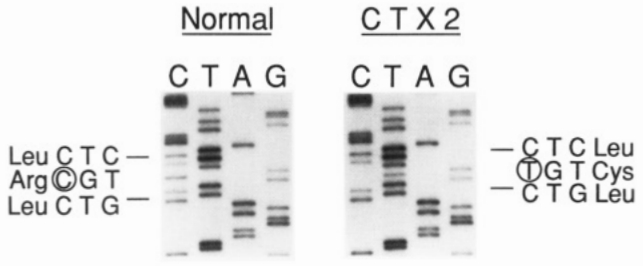


FIGURE 6: (A, top) Negative-ion fast atom bombardment ionization–mass spectra of urinary bile acid metabolites from a typical individual with cerebrotendinous xanthomatosis (CTX), compared with that obtained from a normal subject. The spectrum of the patient is characterized by the presence of intense signals corresponding in mass to a series of polyhydroxylated bile alcohols not normally found in the urine of healthy individuals (bottom spectrum). (B, bottom) DNA sequences of sterol 27-hydroxylase cDNAs from a normal subject and from a CTX homozygote [CTX 2 of Cali et al. (1991)] in the region surrounding arginine 362. Messenger RNA encoding sterol 27-hydroxylase was amplified and subjected to DNA sequence analysis as described by Cali et al. (1991).

who noted that subjects with the rare autosomal recessive disease cerebrotendinous xanthomatosis (CTX) excreted abnormal C_{27} bile alcohols and significantly reduced levels of C_{24} bile acids, particularly chenodeoxycholic acid (Setoguchi et al., 1974). The failure to synthesize normal levels of bile acids and the resulting disruption in cholesterol metabolism leads to a buildup of this sterol and its 5α-reduced derivative, cholestanol, in the tissues of affected individuals. Accumulation in the central nervous system causes progressive neurological dysfunction and eventual death (Menkes et al., 1968).

The metabolic defect in CTX was initially thought to be due to a mutation in a sterol 24-hydroxylase enzyme involved in a minor pathway of cholic acid biosynthesis (Salen et al., 1979). However, subsequent experiments by Björkhem, Pedersen, and colleagues (Oftebro et al., 1980) indicated that the defect resides in the sterol 27-hydroxylase enzyme (reaction 6) of the major bile acid biosynthetic pathway outlined in Figures 1 and 2. Multiple lines of evidence have since confirmed the defect in sterol 27-hydroxylase (Skrede et al., 1986; Miki et al., 1986; Björkhem & Skrede, 1989). Most recently, the molecular basis of CTX has been established through the cloning of the normal sterol 27-hydroxylase cDNA (Cali & Russell, 1991) and the use of this probe to analyze the mRNA in two unrelated CTX subjects (Cali et al., 1991). Point mutations that converted arginine codons to cysteine codons at positions 362 and 446 were identified, and the recreation of these mutations in a cDNA by site-directed mutagenesis followed by expression analysis indicated that both changes

Table IV: 3β-Hydroxy-Δ^5-C_{27}-steroid Oxidoreductase Activity in Cultured Human Fibroblasts[a]

	nmol (mg of protein)$^{-1}$ h^{-1}
control values	6.22–21.57
mother	6.8
father	2.50
patient U	<0.10

[a] Fibroblasts were cultured from 18 different healthy subjects. Enzyme activity was assayed with 1 mg of protein and incubated with 0.1 mM 7α-hydroxycholesterol and 1.5 mM NAD in 0.1 M phosphate buffer (pH 7.5) for 20 min. Data taken from Buchmann et al. (1990).

inactivated the enzyme. In addition, the newer studies localized the gene to the distal long arm of chromosome 2 (bands q33–qter), in agreement with the autosomal recessive inheritance pattern of the disease (Cali et al., 1991).

CTX is readily and specifically diagnosed by analysis of urinary sterol metabolites using negative-ion fast atom bombardment ionization–mass spectrometry (FAB–MS) (Egestad et al., 1985; Setchell & Street, 1987). Figure 6A compares typical spectra generated from the analysis of the urine from a normal individual with that obtained from a CTX subject. The presence of polyhydroxylated bile alcohol glucuronides is characteristic of the disease, and analysis of these by GC–MS indicates that they represent the 5β-cholestane-3α,7α,12α-triol nucleus with additional hydroxylations in the side chain at positions C-22, C-23, C-24, and C-25 but not at position C-27 (Setchell & Street, 1987). The availability of cDNA clones and sequence has provided additional molecular methods for diagnosis. Figure 6B shows a DNA sequence analysis around the region of the cDNA encoding arginine 362 and the substitution mutation present in the CTX 2 subject (Cali et al., 1991). The ability to diagnose CTX by chemical and/or molecular biological methods is a significant therapeutic advance. The disease is readily treated by oral primary bile acid therapy if recognized in the first decades of life prior to significant accumulation of sterols in the central nervous system (Salen et al., 1975; Berginer et al., 1984). Primary bile acids, but not ursodeoxycholic acid, act by feedback inhibition, thereby preventing further mobilization and accumulation of cholesterol and bile alcohols.

The technique of fast atom bombardment mass spectrometry was used to discover two additional genetic disorders in bile acid biosynthesis (Table III). In 1987, Clayton et al. described a defect in 3β-hydroxy-C_{27}-steroid oxidoreductase (reaction 2, Figure 1) in an individual with giant-cell hepatitis. This subject and a subsequently identified older child with the disorder (Setchell, 1990; Setchell et al., 1990a), excreted in their urine multiple C_{24} bile acids having a 3β-hydroxy-Δ^5 structure.

Confirmation of the primary enzyme defect was accomplished by assaying the activity of the 3β-hydroxy-C_{27}-steroid oxidoreductase in cultured fibroblasts from the original family (Table IV). Negligible activity was found in the patient, while the enzyme activity in fibroblasts from the parents was lower than normal, consistent with a heterozygous carrier phenotype (Buchmann et al., 1990).

A third disorder in bile acid biosynthesis was initially described by Setchell et al. (1988a) in monochorionic twin boys and has since been identified in more than a dozen infants with neonatal hepatitis and cholestasis. The defect was shown to be due to a deficiency in activity of the cytosolic Δ^4-3-oxosteroid 5β-reductase enzyme (reaction 4, Figure 1). Infants with the deficiency synthesize abnormally high levels of C_{24} bile acids that retain the Δ^4-3-oxo configuration of the sterol substrates (i.e., 7α-hydroxy-4-cholesten-3-one and 7α,12α-

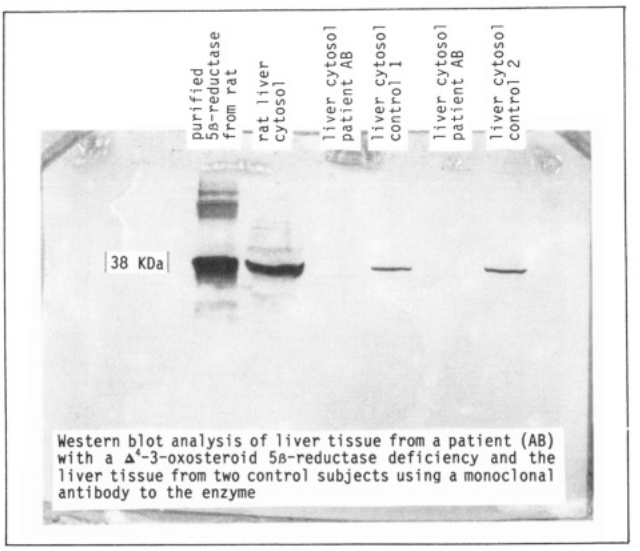


FIGURE 7: Immunoblotting of Δ^4-3-oxosteroid 5β-reductase in normal and deficient liver cells. The immunoblot shows duplicate lanes of the cytosolic fraction from the liver of a patient diagnosed with Δ^4-3-oxosteroid 5β-reductase deficiency and controls representing two different patients with liver disease but who synthesized primary bile acids.

dihydroxy-4-cholesten-3-one) and negligible amounts of primary bile acids. This enzyme is not expressed in fibroblasts, but evidence in support of a primary enzyme defect was recently established by immunoblot analysis of liver samples using a monoclonal antibody directed against the cytosolic rat liver Δ^4-3-oxosteroid 5β-reductase (Figure 7). This monoclonal antibody was capable of recognizing the human Δ^4-3-oxosteroid 5β-reductase in livers from control subjects and from individuals with liver disease not due to disruption of bile acid biosynthesis but does not detect the enzyme in the liver from two patients biochemically diagnosed with this inborn error (K. D. R. Setchell, K. Okuda, and I. Björkhem, 1991, unpublished data). Many patients with liver disease synthesize Δ^4-3-oxo bile acids (Clayton et al., 1988); however, the distinguishing feature of the genetic deficiency is the very high proportion of Δ^4-3-oxo bile acids relative to primary bile acids and the supporting finding of significant amounts of allo (5α-H) bile acids. These allo bile acids are of hepatic origin [many allo bile acids are found in feces and are the result of intestinal bacterial synthesis (Hylemon, 1985)] and arise because the intracellular concentrations of the sterol precursors exceed the K_m for the hepatic steroid 5α-reductase which is much higher than that of the Δ^4-3-oxosteroid 5β-reductase.

The cause of the liver disease in these two bile acid defects is thought to be due to the accumulation of potentially cholestatic and hepatotoxic atypical bile acids, exacerbated by the lack of primary bile acids that are essential for generating bile acid-dependent bile flow. Furthermore, the unique hepatic histology characterized by immature bile canaliculi suggests that synthesis of primary bile acids may be a requirement in early life for normal morphologic development of the liver. This hypothesis is supported by the near normalization in morphology that was seen in these patients following primary bile acid therapy (Setchell et al., 1990b). Primary bile acid therapy in these latter two defects has proven to be beneficial in normalizing liver dysfunction. The rationale for this approach to treatment is that downregulation of endogenous bile acid synthesis by feedback inhibition of cholesterol 7α-hydroxylase limits futher synthesis of the atypical bile acids that are potentially hepatotoxic. The therapy also stimulates bile flow in these patients, thereby facilitating the biliary secretion of toxic substances such as bilirubin.

The molecular basis of these diseases is not yet known; however, the recent cloning of 3β-hydroxysteroid dehydrogenase cDNAs (Table I) and of a Δ^4-3-oxosteroid 5β-reductase cDNA (Onishi et al., 1991) may provide DNA probes that will facilitate obtaining this information.

Defects in bile acid biosynthesis are also present in individuals suffering from one of several peroxisomal disorders (Lazarow & Moser, 1989); however, these, metabolic conditions do not represent primary bile acid defects but instead should be considered to be secondary to defects in peroxisomal function. In subjects with a generalized failure to assemble viable peroxisomes, pleiotropic effects are detected in the multiple biosynthetic pathways that take place wholly or in part in this subcellular organelle. The bile acid intermediates that are found in serum, urine, and bile in affected individuals reflect a deficiency in the last steps of the path, i.e., side-chain oxidation (reactions 10–13, Figure 2), and include a variety of polyhydroxylated cholestanoic acid derivatives (Lawson et al., 1986; Setchell et al., 1991). These include a predominance of C-1, C-6, and C-24 hydroxylated metabolites of 3α,7α,12α-trihydroxy-5β-cholestenoic acid which arise in part because these hydroxylation pathways are quantitatively important in bile acid biosynthesis during normal development (Setchell et al., 1988a). These pathways may reflect a means by which the liver protects itself against the accumulation of hydrophobic and more cytotoxic bile acids because of an immature enterohepatic circulation in early life. Overall, the biochemical derangements with respect to bile acid synthesis in these disorders serve to highlight the importance of the peroxisome in primary bile acid biosynthesis.

It is highly likely that additional diseases in bile acid biosynthesis will be discovered through the new facile screening of urinary metabolites by fast atom bombardment mass spectrometry. Newborns with cholestasis or liver dysfunction represent an informative population for screening. In addition, numerous reports in the literature have described increases or decreases in aspects of bile acid metabolism that may contribute to hypertriglyceridemic states (Angelin et al., 1987) or hypocholesterolemic states (Kern, 1991). The development of cholesterol gallstones may also have a genetic basis in an as yet undefined aspect of bile acid biosynthesis (Turley & Dietschy, 1982).

Summary. To conclude, the last several years have seen a resurgence of interest in the biosynthesis of bile acids. This focus has come about due to the central roles that these molecules play in cholesterol and fat metabolism and due to recent advances in their chemistry, biochemistry, and molecular biology. The application of probes generated by these methodologies has begun to generate novel insight into bile acid metabolism, regulation, and genetics. The next several years should be equally exciting.

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Accelerated Publications

Detection and Characterization of an Early Folding Intermediate of T4 Lysozyme Using Pulsed Hydrogen Exchange and Two-Dimensional NMR†

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ABSTRACT: Two-dimensional ^{1}H–^{15}N NMR techniques combined with pulsed hydrogen–deuterium exchange have been used to characterize the folding pathway of T4 lysozyme. In the unfolded state, there is little differential protection of the various amides from hydrogen exchange. In the native folded structure, 84 amides of the 164 residues are sufficiently spectrally resolved and protected from solvent exchange to serve as probes of the folding pathway. These probes are located in both the N-terminal and C-terminal domains of the native folded structure of the protein. The studies described here show that at least one intermediate is formed early during refolding at low denaturant concentrations. This intermediate (or intermediates) forms very rapidly (within the 10-ms temporal resolution of our mixing device) under the conditions used and is completed at least 10 times faster than the overall folding event. The intermediate(s) protect(s) from exchange a subset of amides in the N-terminal and C-terminal regions of the protein. In the final folded states these protected regions correspond to two α-helices and a β-sheet region. These amides are protected from exchange by factors between 20 and 200 as compared to the fully unfolded protein. Protection of this magnitude is consistent with the formation of somewhat exposed secondary structure in these regions and could represent a "molten globule"-like or a "framework"-like structure for the intermediate(s) in which specific parts of the sequence form isolated secondary structures that are not stabilized by extensive tertiary interactions.

The lysozyme produced by T4 bacteriophage is a monomeric protein with no disulfide bonds. Figure 1 shows the amino acid sequence of T4 lysozyme with secondary structure segments denoted as helix A, B, C, D, E, F, G, and H and β-sheets determined by both crystallographic and solution methods (Matthews & Remington, 1974; McIntosh et al., 1990). The N-terminal domain (13–71) has more β-sheet-like structure, and the C-terminal domain (1–12, 72–164) is largely α-helical. The two domains are linked in part by a long helical segment (helix C).

The folding and stability of this 164-residue protein have been the subject of extensive study using X-ray crystallographic and solution methods (Alber et al., 1987; Becktel & Baase, 1987; Hudson et al., 1987; Matthews, 1987; Weaver & Matthews, 1987). A large collection of point mutants has been generated to investigate the structural and thermodynamic consequences of modification of particular residues in the protein [see Bell et al. (1990)]. Under most conditions, the equilibrium between the folded and unfolded states of most of the lysozyme variants is two-state, showing essentially no significant equilibrium population of any intermediate species. Similarly, the recent kinetic studies of Schellman and coworkers show no evidence for the accumulation of kinetic intermediates (Chen et al., 1991) in the folding pathway in the presence of high concentrations of denaturants. These elegant studies were designed to investigate the folding pathway in a regime where the relaxation times are accessible using manual mixing techniques. The denaturant concentrations needed to slow the relaxation rates to this extent reveal very interesting interactions of the protein with solvent but mask events that occur under conditions where the native form is most stable.

We were particularly interested in the possibility that we might detect and begin a structural analysis of early kinetic intermediates in the folding pathway. In this event, the observed intermediate(s) would reflect a population of protein molecules in which there are no obvious slow kinetic barriers such as hindered rotation of a peptide bond separating members of the majority population. The kinetic properties of such a population are likely to provide interesting insights into the early events in folding. Structural information about the

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