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Biotransformation of Bile Acids, Cholesterol, and Steroid Hormones

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1. Introduction

1.1. Nomenclature

Steroids are a family of organic compounds that have a five-ring perhydrocyclopentanophenanthrene nucleus. The numbering system and skeletal structures for steroids are shown in Figure 13.1. Steroids vary in the number and location of double bonds; in the type, number, and position of functional groups; and in the stereochemical configuration of substituents below (α) or above (β) the plane of the nucleus. Most steroids have 18β- and 19β-methyl groups bonded to C-10 and C-13. The hydrogen atom in steroids saturated at at C-5 can be β-oriented or α-oriented (allo). Alkyl side chains with various functional groups can be present at C-17, usually in the 17β orientation.

Estrogens (C_{18} or phenolic steroids) lack both a C-17 side chain and the 19β-methyl group and contain a 3-hydroxylated, aromatic A ring. Androgens (C_{19} steroids) lack a C-17 side chain and have a 3-oxo group and Δ^4 double bond. Corticosteroids and pregnane hormones (C_{21} steroids) contain a two-carbon side chain at C-17, a 3-oxo group, and a Δ^4 double bond. The C_{19} and C_{21} steroids are collectively termed neutral steroids. Cholesterol and other C_{27} sterols contain an eight-carbon, branched, aliphatic side chain, a 3β-hydroxy group, and a Δ^5 double bond. Bile acids possess a side chain substituted with a terminal carboxyl group. Although the side chain lengths of bile acids can vary, the most common class has a branched, five-carbon side chain with a carboxyl group at C-24 (C_{24} bile acids). The carboxyl group can be conjugated to glycine or taurine via an

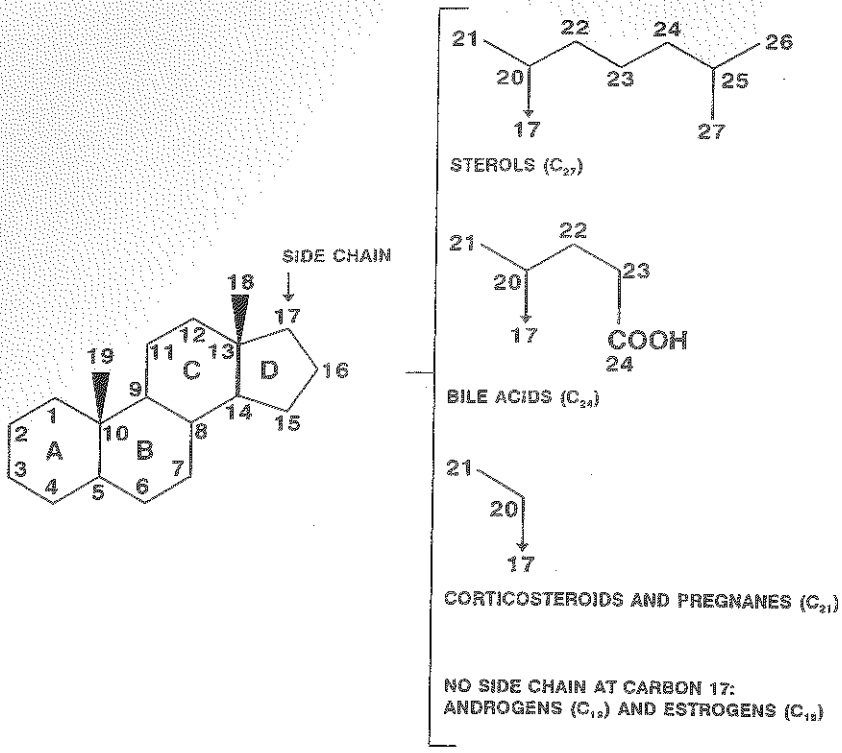

Figure 13.1. Simplified structures of steroids found in the human intestinal tract. Refer to the other figures for specific structures.

amide linkage or to other groups. Under physiological conditions, conjugated and free bile acids exist as their sodium and potassium salts. However, the term "bile acid" will be used throughout most of the text.

1.2. Systematic and Trivial Names

The systematic names of steroids mentioned in the text by their trivial names are listed in Table 13.1. We followed the guidelines proposed by Hofmann et al. (1992) for nomenclature of bile acids.

1.3. Enterohepatic Circulation

The gastrointestinal tract of humans contains about 10^{14} bacteria, represented by 300 to 400 species (Moore et al. 1988, Savage 1977). At least 99% of these

Table 13.1. Trivial and systematic name of steroids

Trivial Name	Systematic Name
Bile Acids	
5β-Cholanoic acid	5β-Cholan-24-oic acid
2-Cholenoic acid	2-Cholen-24-oic acid
3-Cholenoic acid	3-Cholen-24-oic acid
Lithocholic acid	3α-Hydroxy-5β-cholan-24-oic acid
Isolithocholic acid	3β-Hydroxy-5β-cholan-24-oic acid
Sulfolithocholic acid	3α-Sulfo-5β-cholan-24-oic acid
Deoxycholic acid	3α,12α-Dihydroxy-5β-cholan-24-oic acid
Allodeoxycholic acid	3α,12α-Dihydroxy-5α-cholan-24-oic acid
3-Dehydrocholic acid	12α-Hydroxy-3-oxo-5β-cholan-24-oic acid
3-Dehydroallodeoxycholic acid	12α-Hydroxy-3-oxo-5α-cholan-24-oic acid
3-Dehydro-4-deoxycholenoic acid	12α-Hydroxy-3-oxo-cholen-24-oic acid
3-Dehydro-4,6-deoxycholdienoic acid	12α-Hydroxy-3-oxo-6-choldien-24-oic acid
7-oxolithocholic acid	12α-Hydroxy-7-oxo-5β-cholan-24-oic acid
Ursodeoxycholic acid	3α,7β-Dihydroxy-5β-cholan-24-oic acid
Chenodeoxycholic acid	3α,7α-Dihydroxy-5β-cholan-24-oic acid
Cholic acid	3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid
Isocholic acid	3β,7α,12α-Trihydroxy-5β-cholan-24-oic acid
7-Epicholic acid	3α,7β,12α-Trihydroxy-5β-cholan-24-oic acid
12-Epicholic acid	3α,7α,12β-Trihydroxy-5β-cholan-24-oic acid
Cholyl-Coenzyme A	3α,7α,12α-Trihydroxy-5β-cholan-24-oyl-coenzyme A
Cholylglycine	3α,7α,12α-Trihydroxy-5β-cholan-24-oylglycine
Cholyltaurine	3α,7α,12α-Trihydroxy-5β-cholan-24-oyltaurine
3-Dehydrocholic acid	7α,12α-Dihydroxy-3-oxo-5β-cholan-24-oic acid
3-Dehydro-4-cholenoic acid	7α,12α-Dihydroxy-3-oxo-4-cholen-24-oic acid
7-Dehydrocholic acid	3α,12α-Dihydroxy-7-oxo-4-cholen-24-oic acid
12-Dehydrocholic acid	3α,7α-Dihydroxy-12-oxo-4-cholen-24-oic acid
β-Muricholic acid	3α,6β,7β-Trihydroxy-5β-cholan-24-oic acid
β-Hyocholic acid	3α,6α,7β-Trihydroxy-5β-cholan-24-oic acid
C_{27} Sterols	
Cholesterol	5-Cholesten-3β-ol
Coprostanone	5β-Cholestan-3-one
Coprostanol	5β-Cholestan-3β-ol
Cholestanol	5α-Cholestan-3β-ol
Campestrol	24α-Methyl-5-cholesten-3β-ol;24[R]-ergost-5-en-3β-ol
β-Sitosterol	24β-Ethyl-5-cholesten-3β-ol
Stigmasterol	3β-Hydroxy-24-ethyl-5,22-cholestadiene

(continued)

Table 13.1. *(continued).*

Trivial Name	Systematic Name
C_{19} Steroids	
11β-Hydroxy-androstenedione	11β-Hydroxy-4-androstene-3,17-dione
Dehydroepiandrosterone sulfate	3β-Hydroxy-5-androsten-17-one
Testosterone	17β-Hydroxy-4-androsten-3-one
Epitesterone	17α-Hydroxy-4-androsten-3-one
C_{21} Steroids	
Cortisol	11β,17α,21-Trihydroxy-4-pregnene-3,20-dione
3α-hydroxy-5β-tetrahydrodcortisol	3α,11β,17α,21-Tetrahydroxy-5β-pregnane-20-one
3β-hydroxy-5β-tetrahydrodcortisol	3β,11β,17α,21-Tetrahydroxy-5β-pregnane-20-one
5α-Dihydrocortisol	11β,17α,21-Trihydroxy-5β-pregnane-3,20-dione
5β-Dihydrocortisol	11β,17α,21-Trihydroxy-5α-pregnane-3,20-dione
20α-Dihydrocortisol	11β,17α,20α,21-Tetrahydroxy-4-pregnen-3-one
20β-Dihydrocortisol	11β,17α,20β,21-Tetrahydroxy-4-pregnene-3-one
21-Deoxycortisol	11β,17α-Dihydroxy-4-pregnene-3,20-dione
Cortisone	17α,21-Dihydroxy-4-pregnene-3,11,20-trione
11-Desoxycortisol	17α,21-Dihydroxy-4-pregnene-3,20-dione
16αHydroxyprogesterone	16α-Hydroxy-4-pregnene-3,20-dione
Δ^{16}-Progesterone	4,16-Pregnadience-3,20-dione
17α-Progesterone	17α-pregn-4-en-3,20-dione
17α-Hydroxyprogesterone	17α-Hydroxy-4-pregnene-3,20-dione

are obligately anaerobic bacteria; the other 1% are facultatively anaerobic bacteria. The bacterial count increases from 10^4 to 10^8/g dry weight in the jejunum to 10^{11}/g dry weight in the colon (Moore et al. 1988). During passage through the lower intestinal tract, exogenous and endogenous compounds are exposed to the indigenous microbiota, which can biotransform them to various metabolites. These metabolites can often be absorbed by the host and exert beneficial or harmful effects. The steroid components of bile are notable examples of endogenous compounds that are extensively modified by the intestinal microbiota.

Human gallbladder bile typically contains (g/g dry weight) 67% conjugated bile acids, 22% phospholipids, 4.5% protein, 4% cholesterol, 0.3% bilirubin, electrolytes, and small amounts of steroid hormones (Carey and Cahalane 1988). Cholesterol is obtained from dietary sources or synthesized de novo from acetate in the liver and other tissues. Primary bile acids are synthesized from cholesterol in the liver and conjugated to either glycine or taurine by an amide linkage at the C-24 carboxyl. The types and proportions of primary bile acids synthesized in the liver vary widely among different species of animals; the primary bile acids in humans are cholic and chenodeoxycholic acids. Neutral and phenolic steroids are synthesized from cholesterol in the adrenal cortex or gonads, carried by globulins in the bloodstream, and eventually taken up by the liver. They are

then conjugated at C-3, C-17, and C-21 hydroxy groups with glucuronic acid or sulfate before secretion in bile (Macdonald et al. 1983a). The lipid components of bile associate to form mixed micelles, which are physiologically active in fat emulsification. The gallbladder concentrates, stores, and secretes bile into the intestinal lumen during digestion.

In the terminal ileum and proximal cecum, bile acids are actively absorbed into the hepatic portal venous system and returned to the liver. This process is termed enterohepatic circulation (EHC), which has been described in detail (Carey and Cahalane 1988; Hofmann 1976, 1977, 1979; Vlahcevic et al. 1990). Greater than 95% of the bile acids secreted in bile are returned to the liver during each of the four to 12 daily cycles of the EHC. Therefore, most of the bile acid pool in humans (about 3 g) is retained within the EHC. The remaining 5% of biliary bile acids which escape active absorption, about 0.2 to 0.6 g daily, pass through the colon and are biotransformed by bacterial enzymes. The biotransformation products are largely excreted in feces, but some are passively absorbed, returned to the liver, conjugated, and secreted in bile. The bile acids lost through fecal excretion are replaced by de novo synthesis in the liver.

Biliary cholesterol is reabsorbed in the intestine and enters the mesenteric lymphatic system as chylomicrons (Cooper 1990, Stange and Dietschy 1985). These are large lipoproteins consisting of free and esterified cholesterol, apolipoproteins, dietary triglycerides, and phospholipids. The chylomicrons are then modified by lipases located on endothelial cell surfaces. Chylomicron remnants are carried in the bloodstream to the liver, where they are taken up by receptor-mediated endocytosis. About 1 g of cholesterol per day escapes intestinal absorption, passes into the colon, and is modified by bacterial enzymes. The biotransformation products of cholesterol are not absorbed by the colon but are excreted in the feces.

After secretion in bile (up to 13 mg/day), conjugated steroid hormones are deconjugated by the lower small intestinal microbiota. Most of the deconjugated steroids are passively absorbed and returned to the liver *via* EHC (Adlercreutz et al. 1979, Bokkenheuser and Winter 1983, Taylor 1971). However, some (about 2 mg/day) enter the colon, are biotransformed by the colonic microbiota, passively absorbed, and transported to the liver for conjugation. Biliary steroids may undergo several cycles of conjugation and deconjugation, but are eventually excreted in urine. Only minor amounts of steroid hormones are excreted in feces, primarily as unconjugated forms.

2. Metabolism of Bile Acids by the Intestinal Microbiota

Normal human feces contain over 20 different secondary bile acid metabolites formed by bacterial modification of the primary bile acids, cholic acids, and chenodeoxycholic acids (Ali et al. 1966, Hayakawa 1973, Midvedt 1974). Known

bacterial transformations of primary bile acids (Fig. 13.2) include (1) hydrolysis of glycine- and taurine-conjugated bile acids, yielding free bile acids (reaction 1); (2)stereospecific oxidation of hydroxy bile acids (reactions 2, 4, and 6) and reduction of the resulting oxo-bile acids to hydroxy epimers (reactions 3, 5, and 7); (3) 7-dehydroxylation—i.e., removal of 7α-hydroxy groups (reactions 8 and 9) or 7β-hydroxy groups (reaction 10); (4) monoester and polyester formation (reaction 11); and (5) removal of 3-sulfo groups (not shown).

Secondary bile acids which undergo EHC enter the bile acid pool and can thus influence the physiology of the host. Secondary bile acids are generally more hydrophobic and less effective in solubilizing lipids than primary bile acids (Carey 1985, Hofmann and Roda, 1984). Certain secondary bile acids are also cytotoxic (Vlahcevic et al. 1990), decrease de novo synthesis of cholesterol and primary bile acids (Heuman et al. 1988a,b), and possibly promote colon carcinogenesis (Cohen et al. 1980, Mower et al. 1979).

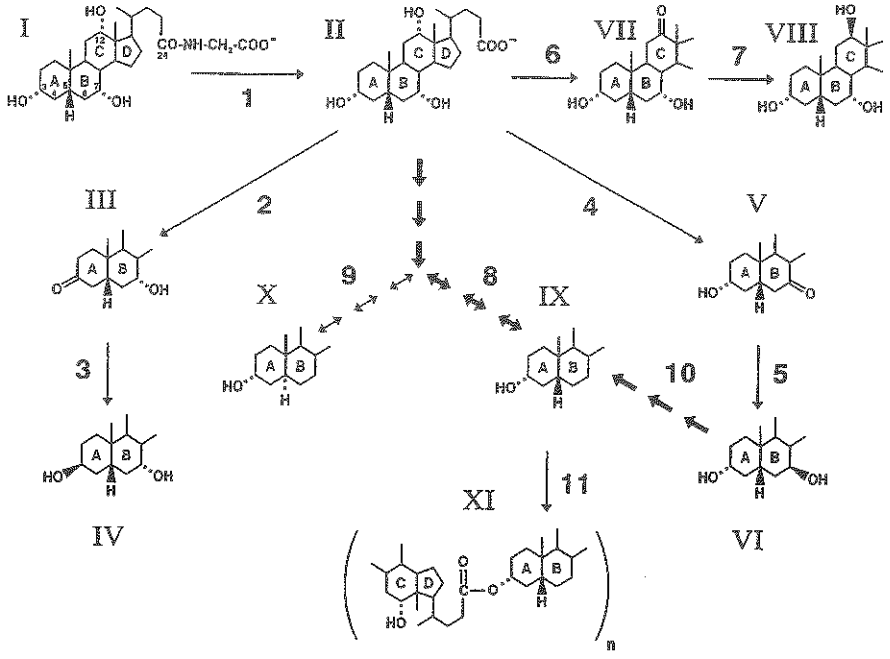

Figure 13.2. Bacterial transformations of cholic acid. Large arabic numerals refer to reactions cited in section 2. Structures: I, cholylglycine; II, cholic acid; III, 3-dehydrocholic acid; IV, isocholic acid; V, 7-dehydrocholic acid; VI, 7-epicholic acid; VII, 12-dehydrocholic acid; VIII, 12-epicholic acid; IX, deoxycholic acid; X, allodeoxycholic acid; XI, polyester of deoxycholic acid. 12-Dehydroxy analogs of intermediates I, III-VI, IX, and X can also be formed from chenodeoxycholic acid.

2.1. Deconjugation

The hydrolysis of the amide bond of glycine- and taurine-conjugated bile acids (Fig. 13.2, reaction 1) is catalyzed by conjugated bile acid hydrolase (CBH). This reaction is so rapid and complete that conjugated bile acids are usually not detected in intestinal contents (Macdonald et al. 1983a). Unconjugated bile acids are considerably less soluble than conjugated bile acids, particularly at low pH, and are less effective detergents for fat solubilization. Therefore, they must be reconjugated by the liver after EHC. CBH activity depresses growth in poultry (Feighner and Dashkevicz 1988) and contributes to the small-bowel syndrome in humans (Gorbach and Tabaqchali 1969, Northfield et al. 1973). CBH activity has been detected in intestinal strains of *Bacteroides*, *Bifidobacterium*, *Fusobacterium*, *Clostridium*, *Lactobacillus*, *Peptostreptococcus*, *and Streptococcus* (Hylemon and Glass 1983, Kobashi et al. 1978, Macdonald et al. 1983a, Masuda 1981, Midvedt and Norman 1967). The contribution of these genera to overall CBH activity depends on the host. For example, studies with germ-free and conventional mice indicate that lactobacilli contribute at least 74% of the total intestinal CBH activity (Tannock et al. 1989).

CBHs have been purified and characterized from *Bacteroides fragilis*, *Bacteroides vulgatus*, *Clostridium perfringens*, and *Lactobacillus* sp. strain 100–100 (Table 13.2). The optimum pH range for the activity of all CBHs is acidic, ranging from 4.2 to 6.4. Most of these enzymes are composed of one type of subunit.

Table 13.2. Characteristics of conjugated bile acid hydrolases purified from intestinal bacteria

Organism	M_r (10^3)		Subunit Composition	Apparent K_m (mM)[a]						pH Optimum	Reference[b]
	Subunit	Native		CG	DCG	CDCG	CT	DCT	CDCT		
Bacteroides fragilis	32.5	250	α_8?	0.35	0.20	0.26	0.45	0.17	0.29	4.2–4.5	1
Bacteroides vulgatus	36	140	α_4	—	—	—	+	+	+	5.6–6.4	2
Clostridium perfringens	ND[c]	ND	ND	3.6	1.2	14	37	3.5	3.0	5.6–5.8	3
Lactobacillus sp. strain 100–100	42 (α) 38 (β)										5,6
isozyme A	42		α_3	+	+	ND	0.76	+	+	4.2–4.5	
isozyme B	42, 38	115	$\alpha_2\beta_1$	+	+	ND	0.95	+	+	"	
isozyme C	42, 38	105	$\alpha_1\beta_2$	ND	ND	ND	0.45	ND	ND	"	
isozyme D	38	95 80	β_3	ND	ND	ND	0.37	ND	ND	"	
Lactobacillus plantarum 80	37.1[d]	ND	ND	+	+	+	TR	TR	TR	4.7–5.5	7

[a] CG, cholylglycine; DCG, deoxylcholylglycine; CDCG, chenodeoxycholylglycine; CT, cholyltaurine; DCT, deoxycholyltaurine; CDCT, chenodeoxycholyltaurine; +, activity detected; TR, trace of activity; —, no activity detected.

[b] References: 1. Stellway and Hylemon (1976); 2. Kawamoto et al. (1989); 3. Nair et al. (1967); 4. Gopal-Srivastava and Hylemon (1988); 5. Lundeen and Savage (1990); 6. Lundeen and Savage (1992b); 7. Christiaens et al. (1992).

[c] ND, not determined.

[d] Calculated from the deduced amino acid sequence of the conjugated bile acid hydrolase gene (GenBank accession number S51638).

However, *Lactobacillus* sp. strain 100–100 produces four CBH isozymes composed of different trimeric combinations of immunologically distinct α and β subunits. These isozymes have similar kinetic properties, although the V_{max} of isozyme D with cholyltaurine is 10-fold lower than that of isozymes A, B, and C. Most CBHs hydrolyze both glycine- and taurine-conjugated dihydroxy and trihydroxy bile acids to some extent. However, the CBH from *B. vulgatus* hydrolyzes only taurine-conjugated bile acids. The CBH gene from the silage isolate, *Lactobacillus plantarum* 80, has been cloned and expressed in *Escherichia coli* MC1061 (Christiaens et al. 1992) using a direct plate screening technique (Dashkevicz and Feighner 1989). The CBH expressed in *E. coli* MC1061 extracts preferentially hydrolyzes glycine-conjugated bile acids. The deduced amino acid sequence of the CBH gene shares 52% similarity with that of penicillin V amidase from *Bacillus sphaericus*. CBH is synthesized constitutively in most intestinal bacteria. However, CBH activity in *B. fragilis* (Hylemon and Stellwag 1976) and *Lactobacillus* sp. strain 100–100 (Lundeen and Savage 1990) increases 300- and 70-fold, respectively, when the cells enter stationary phase. Furthermore, Lundeen and Savage (1990, 1992a) detected an extracellular factor in *Lactobacillus* sp. strain 100–100 which is induced by conjugated bile acids and stimulates intracellular CBH activity. The factor has an apparent M_r of 12,000 to 25,000; is stable to air, acid, heat, and pronase treatment; and can be partially extracted into organic solvents.

2.2. *Hydroxy Group Oxidation and Epimerization*

Bile acid hydroxysteroid dehydrogenases (HSDH) catalyze the reversible oxidation of hydroxy groups to oxo groups at various positions in the bile acid molecule. These enzymes are NAD(P)-dependent and are stereospecific for the α or β orientation of the hydroxy group. HSDHs are widely distributed among intestinal bacteria, including enzymes specific for α- or β-hydroxy groups at C-3, C-6, C-7, and C-12 (Hylemon and Glass 1983).

Through the concerted action of α- and β-HSDHs, the intestinal microbiota can epimerize hydroxy groups of bile acids by stereospecific oxidation (Fig. 13.2, reactions 2, 4, and 6), followed by stereospecific reduction of the resulting oxo group (reactions 3, 5, and 7). Epimerization can be performed by a single species containing both α- and β-HSDHs (intraspecies) or by cooperation between one species having an α-HSDH and another having a β-HSDH (interspecies). Epimerization of α-hydroxy groups to β-hydroxy groups is more common than the opposite conversion.

3α- AND 3β-HSDH

The 3α-hydroxy group of primary bile acids can be epimerized in vivo (Fig. 13.2, reactions 2 and 3), since the feces of humans and laboratory animals contain

low amounts of the 3β epimers (Hylemon and Glass 1983). Intraspecies 3-hydroxy epimerization has been demonstrated in fecal isolates of *C. perfringens* (Hirano et al. 1981b, Macdonald et al. 1983b), *Eubacterium lentum* (Hirano and Masuda 1981b), and *Peptostreptococcus productus* (Edenharder et al. 1989a), suggesting that these bacteria have both 3α- and 3β-HSDHs. However, to date both enzymes have been detected only in *P. productus* (Edenharder et al. 1989a). Anaerobic conditions favor epimerization of 3α-hydroxy bile acids in growing cultures or cell suspensions of *C. perfringens* or *E. lentum*, while aeration favors oxidation of these compounds to 3-oxo bile acids (Hirano et al. 1981b, Macdonald et al. 1983b).

3α- and 3β-HSDHs in various stages of purity have been characterized in *E. lentum*, *P. productus*, *Ruminococcus* sp. PO1–3, and three species of *Clostridium* (Table 13.3). These 3-HSDHs differ in pyridine nucleotide cofactor specificity, molecular weight, and substrate range. However, most have alkaline pH optima and exhibit lower K_ms for dihydroxy or dioxo bile acids than trisubstituted forms. The 3β-HSDHs from *Clostridium innocuum* and *Clostridium* sp. 25.11.c reduce 3-oxobile acids but do not oxidize 3β-hydroxy bile acids. The 3-HSDHs from *C. perfringens*, *E. lentum*, and *Ruminococcus* recognize certain 3-hydroxy- and 3-oxo-C_{19} steroids as well as bile acid substrates. All of the 3-HSDHs in Table 13.3 are synthesized constitutively. However, the synthesis of all but the

Table 13.3. Characteristics of bile acid 3α- and 3β-hydroxysteroid dehydrogenases (HSDH) from intestinal bacteria

HSDH	Organism	Purity[a]	Cofactor	Native M_r (10^3)	pH Optimum	Substrates Used[b]	Apparent K_m (mM)	Ref.[c]
3α	*Clostridium perfringens*	CE	NADP	ND[d]	11.3	3α-HBA	0.020–0.050	1
						3α-HAN	< 0.001	
3α	*Eubacterium lentum*	CE	NAD	ND	11.3	3α-HBA (CON)	0.008–0.020	2
						3α-HAN	ND[d]	
3α	*Peptostreptococcus productus*	CE	NAD	95	8.5	3α-HBA	ND	3
						3-OBA		
3β	*Peptostreptococcus productus*	CE	NAD	132	9.5	3-OBA	ND	3
3β	*Clostridium innocuum*	PP	NADH	56	10.0–10.2	3-OBA (F)	0.024–0.146	4
						3-OBA (CON)	0.0334	
3β	*Clostridium* sp. 25.11.c	CE	NADPH	104	7.3	3-OBA (F)	0.015–0.134	5
						3-OBA (CON)	0.115	
3β	*Ruminococcus* sp. PO1–3	HP	NADP	90	ND	3β-HBA (F)	0.003–0.015	6
						3-OBA (F)	0.030–0.050	
						3β-HAN	ND	
						3-OAN	ND	

[a] CE, cell extract; PP, partially purified; HP, highly purified.

[b] HBA, hydroxybile acids; OBA, oxobile acids; F, free bile acids; CON, glycine or taurine conjugates; HAN, hydroxyandrostanes; OAN, oxoandrostanes. Reduced NAD(P) was used with 3-oxo substrates.

[c] References: 1. Macdonald et al. (1976); 2. Macdonald et al. (1977); 3. Edenharder et al. (1989a); 4. Edenharder and Pfützner (1989); 5. Edenharder et al. (1989b); 6. Akao et al. (1986).

[d] ND, not determined.

3α-HSDH of *C. perfringens* is repressed by addition of their bile acid substrates to the growth medium.

6α- AND 6β-HSDH

The primary bile acid β-muricholic acid (3α,6β,7β-trihydroxy-5β-cholanoic acid) is found in the feces of germ-free rats, mice, and pigs (Madsen et al. 1976, Wostmann et al. 1979), but its 6α-epimer, β-hyocholic acid (3α,6α,7β-trihydroxy-5β-cholanoic acid) is present in the feces of conventional animals. This disparity suggests that the intestinal microbiota epimerize the 6β-hydroxy group of muricholic acid.

There is evidence for both intraspecies and interspecies epimerization of the 6β-hydroxy group. A *Clostridium* sp. (R6 × 76) isolated from rat feces converted β-muricholic acid to β-hyocholic acid (Sacquet et al. 1979). Eyssen et al. (1983) isolated strains of *E. lentum* and *Fusobacterium* sp. from rats. The *E. lentum* isolate oxidized the 6β-hydroxy group of β-muricholic acid to the 6-oxo derivative, while the *Fusobacterium* strains reduced the 6-oxo group to the 6α-hydroxy derivative. When cocultured, these two species epimerized β-muricholic acid to β-hyocholic acid. No 6α- or 6β-HSDHs have yet been purified or characterized.

7α- AND 7β-HSDH

The 7β-epimers of cholic and chenodeoxycholic acids (7-epicholic and ursodeoxycholic acids, respectively) are often detected in human bile and feces, indicating that epimerization of the 7α-hydroxy group occurs in vivo (Fig. 13.2, reactions 4 and 5). The intestinal microbiota can also epimerize 7β-hydroxy bile acids to 7α-hydroxy bile acids. However, the extent of 7-epimerization in vivo is difficult to quantitate, since it competes with the irreversible 7-dehydroxylation of bile acids (Hylemon and Glass 1983).

The epimerization of 7α-hydroxy bile acids has been demonstrated in vitro with mixed fecal cultures (Fedorowski et al. 1979, Higashi et al. 1979, Hirano et al. 1981a) and by coculturing 7α-HSDH-producing fecal isolates with 7β-HSDH-producing isolates (Hirano and Masuda 1981a, 1982b; Macdonald et al. 1982). Bacteria capable of intraspecies 7-epimerization include *Clostridium absonum* (Macdonald and Roach 1981), *Clostridium limosum* (Sutherland and Williams 1985), and lecithinase-lipase-negative strains of *Clostridium* (Edenharder and Knaflic 1981).

7α-HSDHs from members of the genera *Bacteroides*, *Clostridium*, *Escherichia*, and *Eubacterium* have been characterized at various levels of purity (Table

13.4). Although these enzymes are diverse in pyridine nucleotide requirement, molecular weight, and bile acid inducibility, all have particularly alkaline pH optima and use free and conjugated bile acid substrates efficiently. *B. fragilis* and *C. absonum* have distinct NAD- and NADP-dependent 7α-HSDHs. These enzymes in *B. fragilis* also differ in thermal stability, requirement for divalent metal cations, and gel filtration elution profiles (Hylemon and Sherrod 1975). To date, two 7α-HSDHs have been purified and characterized: an NAD-linked enzyme from *E. coli* HB101 (Yoshimoto et al. 1991), and an NADP-linked enzyme from *Eubacterium* sp. VPI 12708 (Franklund et al. 1990). Their associated genes have also been cloned and sequenced (Baron et al. 1991, Yoshimoto et al. 1991). The two enzymes are both homotetramers of comparable M_r, are synthesized constitutively, and share 36% amino acid sequence identity. However, the NAD-linked enzyme has 100-fold higher K_ms for its bile acid substrates than the NADP-linked enzyme. Both 7α-HSDH's share significant amino acid sequence homology with short chain, non-metal-containing alcohol and polyol dehydrogenases, which all contain a putative pyridine nucleotide binding domain. Sequence alignments of the two 7α-HSDHs with these enzymes reveals seven perfectly conserved amino acid residues (Baron et al. 1991). Two of these, a tyrosine and a lysine located in the middle of the amino acid sequence, are essential for catalytic activity as demonstrated by site-directed mutagenesis of *Drosophila* alcohol dehydrogenase (Chen et al. 1993).

7β-HSDHs have been characterized in *C. absonum*, *Clostridium* sp. 25.11.c

Table 13.4. Characteristics of bile acid 7α-hydroxysteroid dehydrogenases from intestinal bacteria

Organism	Purity[a]	Inducibility[b]	Cofactor	M_r (10^3)		pH	Apparent K_m (mM)[c]				Ref.[d]
				Native	Subunit	Optimum	C	CG	CDC	CDCG	
Bacteroides fragilis	CE	G	NAD	ND[e]	ND	9.5–10	0.34	0.33	0.10	0.10	1,2
strains	CE	G	NADP	ND	ND	7.0–9.0	ND	ND	ND	ND	1,2
Bacteroides thetaiotaomicron	PP	G?	NAD	320	ND	8.5–9	0.22	0.32	0.048	0.083	3
Clostridium absonum	PP	I	NAD	ND	ND	9.5–11.5	0.25	ND	ND	ND	4,5
	PP	I	NADP	ND	ND	9.5–11.5	0.090	ND	0.0065	ND	4,5
Clostridium perfringens	CE	C	NADP	ND	ND	ND	ND	ND	ND	ND	6
Clostridium sp. 25.11.c	CE	S	NADP	82	ND	8.5–8.7	ND	ND	ND	ND	7
Escherichia coli	HP	C	NAD	120	28	8.5	1.2	1.25	0.43	ND	8
Eubacterium sp. VPI 12708	HP	C	NADP	124	32	8.5–10.5	0.011	0.0083	0.0056	0.0055	9

[a] CE, cell extract; PP, partially purified; HP, highly purified.

[b] G, synthesized during transition from log to stationary phse; I, bile acid inducible; S, constitutive but synthesis stimulated by bile acids; C, constitutive.

[c] C, cholic acid; CG, cholylglycine; CDC, chenodeoxycholic acid; CDCG, chenodeoxycholylglycine.

[d] References: 1. Macdonald et al. (1975); 2. Hylemon and Sherrod (1975); 3. Sherrod and Hylemon (1977); 4. Macdonald et al. (1983c); 5. Macdonald and Roach (1981); 6. Macdonald et al. (1976); 7. Edenharder et al. (1989b); 8. Yoshimoto et al. (1991); 9. Franklund et al. (1990).

[e] ND, not determined.

Table 13.5. Characteristics of bile acid 7β-hydroxysteroid dehydrogenases from intestinal bacteria

Organism	Purity[a]	Indicibility[b]	Cofactor	M_r (10^3)		pH	Apparent K_m (mM)[c]			Ref.[d]
				Native	Subunit	Optimum	UD	UDG	7OL	
Clostridium absonum	CE, PP	I	NADP	200	ND[e]	9.0–10.0	0.072	ND	ND	1,2
Clostridium sp. 25.11.c	CE	S	NADP	115	ND	8.5–8.7	ND	ND	ND	3
Eubacterium aureofaciens	CE	C	NADP	45	ND	10.5	0.108	0.909	ND	4
Peptostreptococcus productus	CE	C	NADP	53	ND	9.8	0.022	0.238	ND	4
	CE	S	NADP	82	ND	10.0	TR[f]	ND	0.0550	5
Ruminococcus sp. PO1–3	HP	ND	NADP	60	30	8.0–9.0	0.005	ND	0.0085	6

[a] CE, cell extract; PP, partially purified; HP, highly purified.

[b] I, bile acid inducible; S, constitutive but synthesis stimulated by bile acids; C, constitutive.

[c] UD, ursodeoxycholic acid; UDG, Ursodeoxycholyl glycine; 7OL, 7-oxolithocholic acid (with NADPH as electron donor).

[d] References: 1. Macdonald et al. (1983c); 2. Macdonald and Roach (1981); 3. Edenharder et al. (1989b); 4. Hirano and Masuda (1982a); 5. Edenharder et al. (1989a); 6. Akao et al. (1987).

[e] ND, not determined.

[f] TR, trace of activity.

(a lecithinase-lipase-negative strain), *Eubacterium aureofaciens*, *P. productus*, and *Ruminococcus* sp. PO1–3 (Table 13.5). All of these are NADP-dependent and have alkaline pH optima but vary widely in native M_r and bile acid inducibility. Only one, the 7β-HSDH of *Ruminococcus* sp. PO1–3, has been purified to homogeneity. It is a homodimer of a 30,000-M_r subunit. Activity of the enzyme is inhibited by sulfhydryl reagents and stimulated by dithiothreitol, suggesting the presence of a reactive sulfhydryl group in the active site.

12α- AND 12β-HSDH

Low amounts of 12-oxo and 12β-hydroxy bile acids occur in human feces (Ali et al. 1966, Eneroth et al. 1966a,b), suggesting that the intestinal microbiota carry out the 12-epimerization of primary bile acids (Fig. 13.2, reactions 6 and 7). Edenharder and Schneider (1985) demonstrated 12-epimerization of deoxycholic acid by mixed human fecal cultures and by cocultures of *Clostridium paraputrificum* and *E. lentum*, which produce 12α- and 12β-HSDH, respectively. To date, no intestinal isolate has been found to possess both 12α- and 12β-HSDH.

12α-HSDHs have been characterized in *E. lentum* and four species of *Clostridium* (Table 13.6). Most of these are NADP-dependent, have native M_rs near 100,000, and have alkaline pH optima. The 12α-HSDHs can all use cholic and deoxycholic acids as substrates but have lower K_m's for the latter. Most of these enzymes prefer unconjugated to conjugated substrates. The NADP-dependent 12α-HSDHs of *Clostridium* group P, strain C 48–50 (Braun et al. 1991) and *Clostridium leptum* (P. de Prada, Ph.D. dissertation, Virginia Commonwealth

Table 13.6. Characteristics of bile acid 12α- and 12β-hydroxysteroid dehydrogenases (HSDH) from intestinal bacteria

				M_r (10^3)			Apparent K_m (mM)[b]						
HSDH	Organism	Purity[a]	Cofactor	Native	Subunit	pH Optimum	C	DC	CG	DCG	12β-HBA	12-OBA (F,CON)	Ref.[c]
12α	*Clostridium* group P, strain C 48–50	HP	NADP	104	26	8.5–9.5	0.072	0.045	ND[d]	ND			1
12α	*Clostridium leptum*	HP	NADP	110	27	8.5	+	0.067	+	+			2
12α	*Clostridium perfringens*	CE	NAD	ND	ND	10.5	+	0.80	+	1.0			3
12α	*Eubacterium lentum*	PP	NAD	125	ND	8.0–10.5	0.059	0.028	0.25	0.17			4
12β	*Clostridium paraputrificum*	CE	NADP	126	ND	7.8, 10.0[e]					+	0.09–0.27	5

[a] HP, highly purified; PP, partially purified; CE, cell extract.

[b] C, cholic acid; CG, cholylglycine; DC, deoxycholic acid; DCG, deoxycholylglycine; 12β-HBA, 12β-hydroxybile acids; 12-oxoBA, 12-oxobile acids; F and CON, free and conjugated bile acids, respectively; +, K_m not determined, but activity detected.

[c] References: 1. Braun et al. (1991); 2. P. de Prada (1983, doctoral dissertation, Virginia Commonwealth University, Richmond; 3. Macdonald et al. (1976); 4. Macdonald et al. (1979) 5. Edenharder and Pfützner (1988).

[d] ND, not determined.

[e] for 12β-HBA oxidation with NADP and 12-OBA reduction with NADPH, respectively.

University, Richmond, Virginia, 1993) have been purified to homogeneity. Both are homotetramers of a ca. 27,000-M_r subunit. Product inhibition studies indicate that 12α-hydroxy oxidation by these enzymes proceeds by an ordered bi bi mechanism, with NADP binding first and leaving last with respect to the bile acid substrate. The N-terminal amino acid sequence of the *C. leptum* enzyme shows homology to those of short chain, non-metal-containing alcohol and polyol dehydrogenases.

NADP-dependent 12β-HSDH has been detected in *Clostridium tertium* and *Clostridium difficile* (Edenharder and Schneider 1985) and partially characterized in *C. paraputrificum* (Edenharder and Pfützner 1988) (Table 13.6). The *C. paraputrificum* enzyme oxidized 12β-hydroxy bile acids and reduced 12-oxo bile acids, although the oxidative activity was 75% lower than the reductive activity. Other properties of the enzyme are similar to those of the 12α-HSDHs. The synthesis of all 12α- and 12β-HSDHs is constitutive, although addition of 12-oxo or 12β-hydroxy bile acids to cultures of *C. paraputrificum* stimulates synthesis of its 12β-HSDH about threefold (Edenharder and Pfützner 1988).

2.3. 7-Dehydroxylation

The most physiologically significant biotransformation of bile acids in humans is the 7α-dehydroxylation of the primary bile acids: cholic and chenodeoxycholic acids, generating deoxycholic and lithocholic acids, respectively (Fig. 13.1, reac-

tion 8). Deoxycholic acid is passively absorbed from the colon and consequently comprises up to 25% of the circulating bile acid pool in humans (Sjövall 1960) and up to 95% in rabbits (Lindstedt and Sjövall 1957). The deoxycholic acid content of human bile remains relatively constant since the liver cannot 7α-hydroxylate this compound to regenerate cholic acid. However, this reaction is thought to occur in rats (Norman and Sjövall 1958). Lithocholic acid is so insoluble that most adsorbs to and is excreted in feces; only minor amounts undergo EHC.

The number of 7α-dehydroxylating bacteria in human feces is relatively low, ranging from 10^3 to 10^5/g wet weight of feces (Ferrari et al. 1977, Stellwag and Hylemon 1979). Most of the intestinal bacteria which carry out 7α-dehydroxylation are members of the genera *Clostridium* (Hayakawa and Hattori 1970, Hirano et al. 1981b,c, Stellwag and Hylemon 1979) and *Eubacterium* (Gustafsson et al. 1966, Hirano et al. 1981c, White et al. 1980). These bacteria use unconjugated but not C-24 conjugated bile acids as substrates for 7α-dehydroxylation, yet lack CBH (Batta et al. 1990, Stellwag and Hylemon 1979, White et al. 1983). Therefore, conjugated bile acids must first be deconjugated by nondehydroxylating bacteria before 7α-dehydroxylation. Moreover, bile acids conjugated at C-24 with N-methylated amino acids (Schmassmann et al. 1990a,b) or with sulfate (Kihira et al. 1991) resist both deconjugation and 7-dehydroxylation by the gut microbiota in rodents. Steric hindrance of the 7-hydroxy group also inhibits 7-dehydroxylation, as shown with 7-hydroxy bile acid analogs containing 7α- or 7β-methyl groups (Hylemon et al. 1984, Kuroki et al. 1987).

The human intestinal isolate, *Eubacterium* sp. VPI 12708, has a bile acid-inducible enzyme system which dehydroxylates 7α-hydroxy bile acids (White et al. 1980, 1981, 1983). A mechanism for bile acid 7α-dehydroxylation in this organism has been proposed, based on the isolation of bile acid intermediates and conversion of these intermediates to dehydroxylated products (Björkhem et al. 1989, Coleman et al. 1987a, Hylemon et al. 1991, Mallonee et al. 1992). This mechanism is illustrated in Figure 13.3, with cholic acid as the dehydroxylation substrate; however, this pathway might also function in the 7α-dehydroxylation of chenodeoxycholic acid to lithocholic acid. Cholic acid (I) is first actively transported into the bacterial cell by a specific carrier protein. Inside the cell, cholic acid is conjugated at the C-24 carboxyl either to ADP-3′-phosphate (Coleman et al. 1987a) or to coenzyme A (CoA) by an ATP-dependent bile acid CoA ligase (Mallonee et al. 1992). The cholic acid conjugate (II) is oxidized twice and dehydrated, yielding 3-dehydro-4,6-deoxycholdienoic acid (V). This intermediate is reduced to 3-dehydro-4-deoxycholenoic acid (VI), which is reduced twice to yield deoxycholic acid (VIII). Deoxycholic acid is transported out of the cell by a specific carrier. Most of the steps in the pathway appear to be pyridine nucleotide-dependent. However, reduction of 3-dehydro-6-deoxycholenoic acid, an analog of the 3-dehydro-4,6-deoxycholdienoic acid intermediate (V), is stimu-

gest that reduction of the 3-dehydro-4-deoxycholdienoic intermediate (VI) generates both the 5β and 5α configurations of the C-5 hydrogen. Allobile acids are the major bile acid class in lower animals and certain fishes (Elliott 1971), and small amounts occur in humans and other mammals (Eneroth et al. 1966b, Tammar 1966).

Bile acid 7α-dehydroxylation activity in *Eubacterium* sp. VPI 12708 is induced by culturing the organism in the presence of unconjugated C_{24} bile acids which have a 7α-hydroxy group (White et al. 1980). This treatment also induces the biosynthesis of several new polypeptides presumably involved in 7α-dehydroxylation (Paone and Hylemon 1984, White et al. 1981). Most of the genes encoding these polypeptides are clustered on a large, cholic acid-inducible operon (>10 kb) containing nine or more open reading frames (*baiA2-baiI*) (Franklund et al. 1993, Mallonee et al. 1990, White et al. 1988a,b) (Fig. 13.4). This operon encodes gene products with M_rs of 9,000, 19,500, 27,000, 47,500, 50,000, 58,000, 59,000, and 72,000. Two additional cholic acid-inducible genes, *baiA1* and *baiA3*, are located on separate monocistronic transcripts about 1 kb long (Coleman et al. 1987b, 1988; Gopal-Srivastava et al. 1990; White 1988b) (Fig. 13.4). These genes encode identical 27,000-M_r polypeptides which share 92% amino acid sequence identity with the *baiA2* gene product. Enzymatic functions have tentatively been assigned to several of the *bai* gene products based on biochemical activities of the gene products expressed in *E. coli* or on amino acid sequence homology to

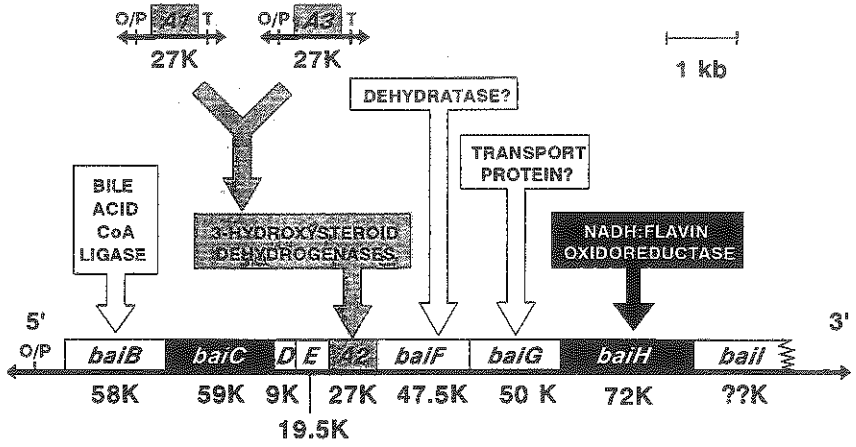

Figure 13.4. Bile acid inducible (*bai*) genes in *Eubacterium* sp. VPI 12708. The predicted M_rs in thousands (K) of the gene products are shown below each gene, and their proposed functions in bile acid 7α-dehydroxylation above the genes. Genes that share homology are shaded or blackened. Symbols: O/P, operator/promoter region; T, stem-loop terminator structure; *A1*, *A2*, *A3*, *D*, and *E*–genes *baiA1*, *baiA2*, *baiA3*, *baiD*, and *baiE*, respectively.

proteins with known functions. The 58,000-M_r protein has ATP-dependent bile acid-CoA ligase activity (Mallonee et al. 1992). The three 27,000-M_r proteins share homology with short-chain NAD(P)-dependent alcohol/polyol dehydrogenases and are thus probably 3α-HSDHs which catalyze 3α-hydroxy oxidation or 3-oxo reduction of intermediates II, VIII, and X (Fig. 13.3) (Coleman et al. 1988). The 50,000-M_r protein has homology to bacterial tetracycline export proteins and may thus function in bile acid transport (D.H. Mallonee and P.B. Hylemon, unpublished data). The 47,500-M_r protein has homology to carnitine dehydratase of *E. coli* (D.H. Mallonee and P.B. Hylemon, unpublished data). The 72,000-M_r protein has NADH:flavin oxidoreductase activity and has been purified from cell extracts of *Eubacterium* sp. VPI 12708 (Franklund et al. 1993, Lipsky and Hylemon 1980). It has amino acid sequence homology with certain soluble NAD(P)H dehydrogenases, trimethylamine dehydrogenase, and the 59,000-M_r protein encoded by the above operon, but its physiological role in 7α-dehydroxylation is unclear (Franklund et al. 1993).

The 7β-hydroxy bile acid ursodeoxycholic acid is used clinically to dissolve cholesterol gallstones and to treat certain cholestatic liver diseases. Washed fecal suspensions convert this compound to lithocholic acid (Fedorowski et al. 1979). *Eubacterium* sp. strains VPI 12708 (White et al. 1982) and C-25 (Takamine and Imamura 1985) can 7β-dehydroxylate ursodeoxycholic acid, forming lithocholic acid. This reaction proceeds directly in these strains, and not by 7α-dehydroxylation of chenodeoxycholic acid formed by 7β-epimerization of ursodeoxycholic acid. The 7β-dehydroxylation activity in *Eubacterium* sp. strain C-25 is greatly stimulated by coincubation with *Bacteroides distasonis* strain K-5 whole cells or cell extracts; the latter strain had no 7-HSDH or 7-dehydroxylation activity alone. The 7α-dehydroxylation and 7β-dehydroxylation activities in *Eubacterium* sp. VPI 12708 have similar bile acid inducibility, cofactor requirements, reaction kinetics, and chromatographic behavior, suggesting that they are catalyzed by the same enzyme system (White et al. 1982, 1983).

2.4. Esterification

Saponifiable derivatives (esters) of bile acids have been detected in human and rodent feces from untreated subjects or from subjects given oral doses of 24-^{14}C-labeled bile acids (Benson et al. 1993, Korpela et al. 1986, 1988, Norman 1964, Norman and Palmer 1964). The proportion of saponifiable bile acids in human feces is estimated to be 10% to 37% of the total bile acid content and consists mainly of deoxycholic acid esters (Korpela et al. 1986, 1988). Recently, Benson et al. (1993) demonstrated that human and hamster feces contain considerable amounts of deoxycholic acid oligomers, formed by esterification of the C-24 carboxyl group of one molecule with the 3α-hydroxy group of the next (Fig. 13.2, reaction 11). The chain lengths of these polyesters range from two to 22

deoxycholic acid subunits. Since these compounds are not present in bile, they are probably produced by intestinal bacteria.

Kelsey and Sexton (1976) showed that mixed cultures from human feces incubated anaerobically with 24-^{14}C-lithocholic acid and ethanol produced the C-24 ethyl esters of lithocholic acid and isolithocholic acid. However, under similar conditions, fecal isolates of *B. thetaiotaomicron*, *Citrobacter* sp., and *P. productus* produced only the lithocholic acid ester (Kelsey and Thompson 1976). Edenharder and Hammann (1985) showed that strains of *Bacteroides*, *Eubacterium*, and *Lactobacillus* isolated from human feces converted cholic acid to deoxycholic acid and the C-24 methyl ester of deoxycholic acid. However, this activity was only rarely detected in mixed fecal cultures. The esterification activity in the pure cultures did not require addition of exogenous methanol and was lost after serial transfers of the cultures. To date, enzymes catalyzing bile acid esterification by intestinal bacteria have not been purified.

2.5. Desulfation

Bacterially produced lithocholic acid which escapes fecal excretion is returned to the liver via EHC. There, it is conjugated to glycine or taurine at the C-24 carboxyl group and esterified with sulfate at the 3α-hydroxy group before secretion in bile. After deconjugation of the glycine or taurine moiety by bacterial CBH, the sulfate group can also be removed by bacterial sulfatases. Sulfobile acids are poorly absorbed from the gastrointestinal tract (De Witt and Lack 1980) but are readily excreted in feces. Studies with germ-free and conventional rats indicate that bacterial desulfation of sulfobile acids decreases their excretion and promotes EHC of the desulfated products (Eyssen et al. 1985, Robben et al. 1988).

Bile acid desulfation has been studied in mixed fecal cultures (Kelsey et al. 1980, 1981; Pacini et al. 1987; Palmer 1972) and fecal isolates. The bacterial genera capable of bile acid desulfation include *Clostridium* (Borriello and Owen 1982, Huijghebaert and Eyssen 1982, Robben et al. 1986), *Peptococcus* (Van Eldere et al. 1988), and *Fusobacterium* (Robben et al. 1989). The desulfation products of 3-sulfolithocholic acid generated by mixed and pure cultures include lithocholic acid, isolithocholic acid and certain of its 3β-fatty acyl esters, 2-cholenoic acid, 3-cholenoic acid, and 5β-cholanoic acid.

Bile acid sulfatase activity in pure cultures of fecal bacteria requires a 3α- or 3β-sulfo group and a free C-24 or C-26 carboxyl group. Bile acids sulfated at positions other than C-3 are not desulfated. Desulfation by these bacteria is also stereospecific, depending on the configuration of both the 3-sulfo group (3α or 3β) and the C-5 hydrogen atom (5α or 5β). These configurations also determine the desulfated products formed. For example, *Fusobacterium* sp. strains H35 and H83 convert 3α- or 3β-sulfo-5β-bile acids to 5β-cholen-3-oic acids but convert

3β-sulfo-5α-bile acids to 5α-cholen-2-oic acids and 3α-sulfo-5α-bile acids; no products are formed from 3α-sulfo-5α-bile acids (Robben et al. 1989). Bile acid desulfation could hypothetically occur by elimination or hydrolysis. However, the mechanism has not been determined, and enzymes catalyzing the reaction have not been purified.

2.6. Physiological Function of Bile Acid Metabolism in Intestinal Bacteria

Bile acid deconjugation and desulfation may provide growth substrates for the bacteria which perform these reactions, or perhaps inhibit the growth of competing bacteria due to the toxicity of the free bile acids produced. Dehydroxylating bacteria may gain a similar competitive advantage, since dehydroxylated bile acids are considerably more hydrophobic and toxic than their parent compounds (Binder et al. 1975).

Esterification and epimerization of hydroxy bile acids may reduce their toxicity and thus protect bile acid-sensitive bacteria. For example, ursodeoxycholic acid (7β-hydroxy) is less hydrophobic than chenodeoxycholic acid (7α-hydroxy) and presumably less deleterious to cell membranes (Armstrong and Carey 1982). Similarly, bile acid esters are sparingly soluble and may form harmless aggregates.

Oxidation of hydroxy bile acids by HSDHs may generate reducing equivalents for electron transport phosphorylation or biosynthetic purposes. In fact, addition of exogenous electron acceptors such as menadione or fumarate to cultures of *B. thetaiotaomicron* greatly increases the oxidation of cholic acid to 7-dehydrocholic acid (Sherrod and Hylemon 1977). Oxo- and ring unsaturated intermediates formed during epimerization and 7-dehydroxylation reactions may serve as electron sinks or as terminal electron acceptors for electron transport chains. Because 7α-dehydroxylation is a net reductive process, it may serve as a key electron accepting reaction in the energy metabolism of dehydroxylating bacteria. These bacteria may occupy a unique niche in the colon ecosystem since their numbers are relatively low (Sect. 2.3) while the amounts of their bile acid substrates are considerable (up to 600 mg).

3. Metabolism of Cholesterol by the Intestinal Microbiota

3.1. Reduction to Coprostanone and Coprostanol

The colon receives up to 1 g of cholesterol per day originating from bile, the diet, and sloughing of intestinal mucosa. Cholesterol is reduced to coprostanol and

minor amounts of coprostanone by the colonic microbiota (Fig. 13.5). Together, cholesterol and its transformation products comprise 95% of the total neutral steroid detected in rat and human feces (McNamara et al. 1981). The role of the intestinal microbiota in the formation of coprostanol and coprostanone was confirmed by comparing the fecal sterols of germ-free and conventional rats (Kellogg and Wostmann 1969); the former excrete unmodified cholesterol, but the latter also excrete coprostanol and coprostanone in amounts up to 55% of the total fecal sterols. The cecum is the site where cholesterol is most extensively transformed in rats (Eyssen et al. 1972, Kellogg 1973). The proportion of cholesterol converted in vivo varies within populations. Wilkins and Hackman (1974) found that 23 out of 31 in a group of North American Caucasians excreted high amounts of coprostanol and coprostanone in their feces (88% of the total sterols), while the other eight excreted only low amounts (10%).

Both mixed fecal biota (Snog-Kjaer et al. 1956) and fecal isolates have been shown to convert cholesterol to its transformation products. Crowther et al. (1977) reported that strains of *Bifidobacterium*, *Clostridium*, and *Bacteroides* reduce cholesterol to coprostanol, although this has not been confirmed. Most cholesterol-reducing bacteria that have been isolated and characterized are members of the genus *Eubacterium*.

A cholesterol-reducing bacterium, *Eubacterium* ATCC 21408, was isolated

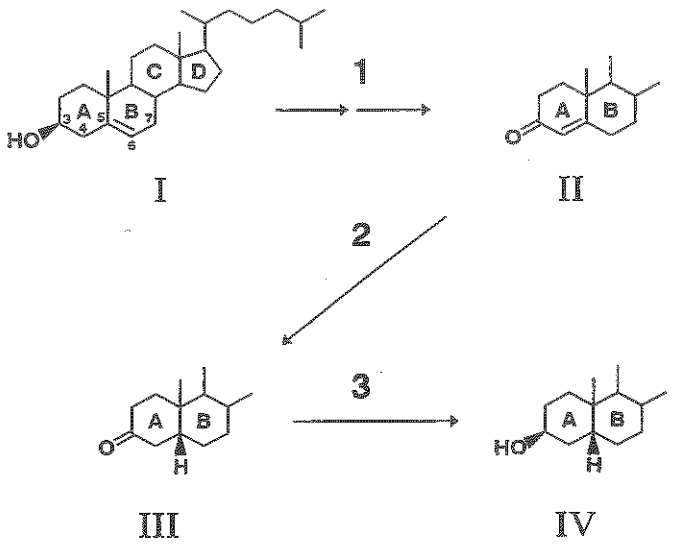

Figure 13.5. Bacterial transformations of cholesterol. Large Arabic numerals refer to reactions cited in section 3. Structures: I, cholesterol; II, 4-cholesten-3-one; III, coprostanone; IV, coprostanol.

from rat cecal contents, using a cholesterol-rich calf brain powder medium (Eyssen et al. 1973). This organism required 3β-hydroxy-Δ^5 animal or plant sterols for growth, including cholesterol, stigmasterol, campesterol, and β-sitosterol, and reduced them to 3β-hydroxy-5β-saturated derivatives. In addition, the organism reduced 4-cholesten-3-one, coprostanone, and its 5α-epimer (cholestanone) to coprostanol (see Fig. 13.5 for structures). Reduction of the Δ^5 double bond required a 3β-hydroxy group, since sterols with a 3α-hydroxy group, with other C-3 substituents, or without a 3-hydroxy group were not reduced. Double bonds at positions other than Δ^4 or Δ^5 were not reduced. Eyssen et al. (1973) suggested that this bacterium used cholesterol as an external electron acceptor, since it required up to 2.0 mg/mL cholesterol for optimal growth. Subsequently, using brain-supplemented media, Sadzikowski et al. (1977) isolated cholesterol-reducing gram-positive diplobacilli from rat cecal contents and human feces, and Mott and Brinkley (1979) isolated the cholesterol-reducing *Eubacterium* strain 403 from baboon feces. In all of these studies (Eyssen et al. 1973, Mott and Brinkley 1979, Sadzikowski et al. 1977), the bacteria would not form viable colonies on solid media, and were purified in broth culture by serial dilution, filtration, and addition of selective inhibitors.

Mott and Brinkley (1979) discovered that the cholesterol-reducing *Eubacterium* strains ATCC 21408 and 403 required both cholesterol and a plasmalogen component of calf brain lipids, plasmenylethanolamine, for growth. Plasmalogens are phosphoglyceride analogs in which the C-1 substituent of glycerol is a long chain 1-alkenyl ether. These membrane phospholipids are particularly abundant in muscle cells, nerve cells, and certain anaerobic bacteria. Although the role of the plasmalogen was unclear, its alkenyl ether group was metabolized during growth. Moreover, plasmalogens with a 1-alkyl ether rather than a 1-alkenyl ether would not support growth of these *Eubacterium* strains (Mott et al. 1980). Ultimately, Brinkley et al. (1980) developed a solid medium containing lecithin, brain powder, and 5% added cholesterol, which adequately supported colony formation by cholesterol-reducing *Eubacterium* strains. With this medium, nine new strains of cholesterol-reducing bacteria were isolated from baboon feces and intestinal contents (Brinkley et al. 1982). Unlike previous cholesterol reducers, none of these strains required cholesterol plus plasmalogen for growth, although seven required plasmalogen for cholesterol reduction. The above studies suggest that cholesterol-reducing intestinal bacteria can be classified into three categories: (1) those that require plasmalogen (and cholesterol) for growth and cholesterol reduction; (2) those that require plasmalogen for cholesterol reduction but not growth; and (3) those that require plasmalogen for neither cholesterol reduction nor growth.

The mechanism of cholesterol reduction was studied in cultures of *Eubacterium* ATCC 21408 fed [4β-^{3}H,4-^{14}C]-cholesterol (Eyssen and Parmentier 1974, Parmentier and Eyssen 1974) (Fig. 13.5). The reaction most likely proceeds by a

sequential oxidation of the 3β-hydroxy group and isomerization of the Δ^5 to a Δ^4 double bond, forming 4-cholesten-3-one (II), reduction of the Δ^4 double bond, yielding coprostanone (III), and reduction of the 3-oxo group, yielding coprostanol (IV). This hypothesis is further supported by the detection of coprostanone in feces and by the reduction of 4-cholesten-3-one and coprostanone to coprostanol by fecal bacteria (Eyssen et al. 1973, Mott et al. 1980). To date, the enzymology of cholesterol reduction has not been studied.

4. Metabolism of Steroid Hormones by the Intestinal Microbiota

Steroid hormones that undergo EHC are conjugated to either glucuronide or sulfate at C-3, C-17, and C-21 by the liver and secreted in biliary bile (Laatikainen 1970b). Total concentrations of steroids in human bile show gender differences, with adult men and women secreting approximately 13 mg/day and 6 mg/day, respectively (Laatikaninen 1970a). Pregnant women are reported to have an increased biliary secretion of steroid hormones (Eriksson et al. 1970). Four 17α-hydroxylated C_{21} steroids have been identified in biliary bile, including 5β- (and 5α-)pregnan-3α,17α,20α-triol and 5-pregnen-3α (and 3β),17α,20α-triol (Laatinkanien 1970c).

Studies of neutral steroid hormone patterns in feces of germ-free and conventional animals indicate that extensive biotransformations of steroid hormones are carried out by the intestinal microbiota (Eriksson and Gustafsson 1970, Eriksson et al. 1969a, Gustafsson 1968). Known microbial biotransformations include (Table 13.7, Fig. 13.6) (1) hydrolysis of glucuronide and sulfate conjugates by microbial glucuronidases and sulfatases (not shown); (2) 21-dehydroxylation (reaction 10); (3) side chain cleavage (reaction 5); (4) saturation of the Δ^4 double bond (reactions 1 and 4); (5) oxidation/reduction of hydroxy/oxo groups at C-3, C-17, and C-20 (reactions 2, 3, 6–8, and 9); and (6) 16α-dehydroxylation, yielding 17β-side chains (Fig. 13.7). Steroid hormone metabolites generated by the intestinal microbiota can be absorbed and secreted in the urine (Wade et al. 1959). However, the significance of the intestinal biotransformations of steroid hormones to the host is not clear.

4.1. Hydrolysis of Glucuronides and Sulfates

Steroid hormones that are secreted into bile are deconjugated in the intestine by bacterial sulfatases and glucuronidases (Eriksson and Gustafsson 1970). Glucuronidase activity may be derived from intestinal bacteria or mucosal cells. However, the sulfatases are strictly bacterial, and the intestinal microbiota can hydrolyze sulfate conjugates in the 3α-, 3β-, 17β-, and 21-hydroxy positions (Macdonald et al. 1983a). Bacterial desulfation has been demonstrated to increase

Table 13.7. Steroid hormone modifications by intestinal microbiota

Biotransformation(s)	Organism(s)	Enzyme(s)	Cofactor(s)	M_r (10^3)	Comments	Ref[a]
Hydrolysis of sulfate and glucuronide conjugates	*Clostridium* sp. *Peptococcus* sp. *Eubacterium* sp. *Lactobacillus* sp. *Bacteroides* sp. *Escherichia coli*	sulfatase(s) and β-glucuronidase(s)	— —	? ?	β-glucuronidase activity found both in bacteria and intestinal mucosa	1–3
21-CH_3OH → 21-CH_3	*Eubacterium lentum*	21-dehydroxylase	$FMNH_2$	582[b]	Requires α-ketol steroids as substrates	4
C_{17} side chain → 17-oxo	*C. scindens*, *Eubacterium desmolans*	desmolase	AD, B_{12}, divalent cations	?	Steroid inducible	5,6
4-ene → 5α-H	*Clostridium* sp. J-1, *Eubacterium* sp. strain 144	5α-reductase	?	?	Soluble and membrane bound forms; H_2 or pyruvate stimulatory	7,8
4-ene → 5β-H	*C. paraputrificum* *C. innocuum*	5β-reductase	NADH	?		9–11
16α-OH → Δ^{16}	*Eubacterium* sp. strain 144	16α-dehydratase	—	42.4[c]	Δ^{16} steroid chemically reactive	12,13
Δ^{16} → 17α-Pregnanes	*Eubacterium* sp. strain 144	Δ^{16}-steroid reductase	?	?	H_2 or pyruvate stimulatory	14
3-oxo → 3β-OH	*C. innocuum*	3β-hydroxysteroid dehyrogenase	NADH	80.0[b]		11
3-oxo → 3α-OH	*E. lentum* *C. paraputrificum*	3α-hydroxysteroid dehydrogenase	NAD(P)H	?		10,15
17-oxo → 17β-OH	*E. lentum* *C. innocuum* *B. fragilis*	17β-hydroxysteroid dehydrogenase	?	?		16
17-oxo → 17α-OH	*Eubacterium* sp. VPI 12708	17α-hydroxysteroid dehydrogenase	NADPH	42.0[c]	O_2-sensitive	17
20-oxo → 20β-OH	*B. fragilis* *Bifidobacterium adolescentis*	20β-hydroxysteroid dehydrogenase	?	?		18
20-oxo → 20α-OH	*C. scindens*	20α-hydroxysteroid dehydrogenase	NADH	40.0[c]	Uses only steroids with 17α, 21-dihydroxy group	19

[a] Selected references: 1. Graves et al. (1977); 2. Van Eldere et al. (1988); 3. Van Eldere et al. (1991); 4. Feighner and Hylemon (1980); 5. Winter et al. (1984a); 6. Krafft et al. (1987); 7. Bokkenheuser et al. (1983); 8. Glass et al. (1991); 9. Schubert et al. (1967); 10. Glass et al. (1979); 11. Stokes and Hylemon (1985); 12. Glass and Lamppa (1985); 13. Glass et al. (1982); 14. Watkins and Glass (1991); 15. Bokkenheuser et al. (1979); 16. Winter et al. (1984b); 17. dePrada et al. (1994); 18. Winter et al. (1982a); 19. Krafft and Hylemon (1989)

[b] Native M_r.

[c] Subunit M_r.

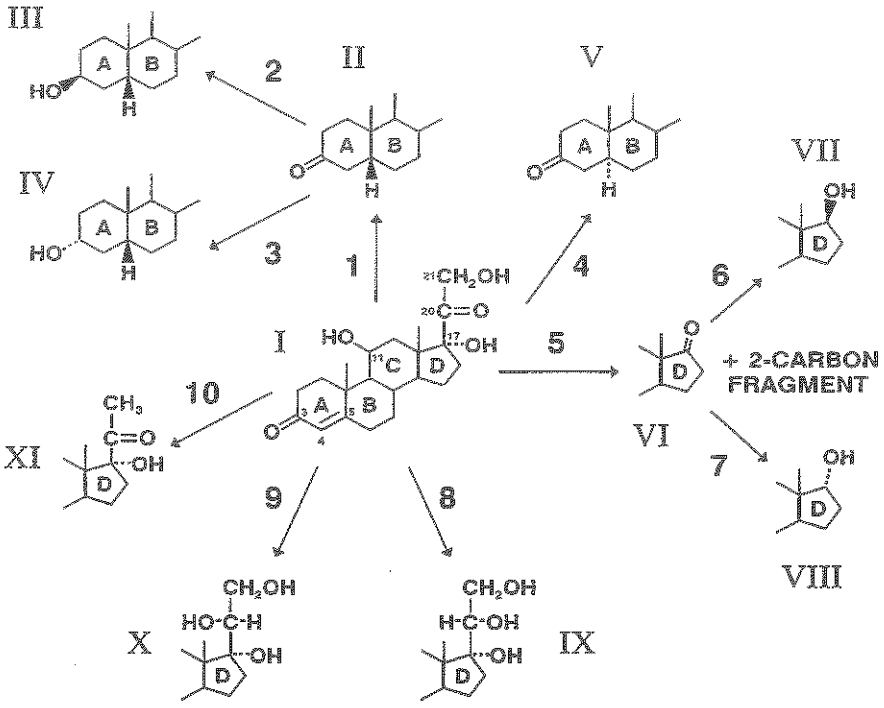

Figure 13.6. Bacterial transformations of cortisol. Large arabic numerals refer to reactions cited in section 4. Structures: I, cortisol; II, 5β-dihydrocortisol; III, 3α-hydroxy-5β-tetrahydrocortisol; IV, 3β-hydroxy-5β-tetrahydrocortisol; V, 5α-dihydrocortisol; VI, 11β-hydroxy-androstenedione; VII, 11β,17β-Dihydroxy-4-androsten-3-one; VIII, 11β,17α-Dihydroxy-4-androsten-3-one; IX, 20α-dihydrocortisol; X, 20β-dihydrocortisol; XI, 21-deoxycortisol. Intermediates VII and VIII have not been detected in vivo. However, their 11-dehydroxy analogs, testosterone and epitestosterone, are formed by the action of 17α- and 17β-hydroxysteroid dehydrogenases on androstenedione (Section 4).

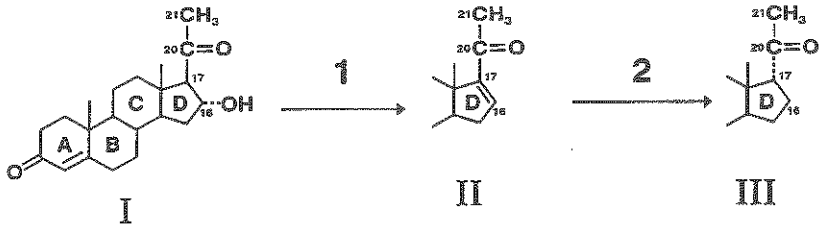

Figure 13.7. Bacterial metabolism of 16α-hydroxyprogesterone. Large arabic numerals refer to reactions cited in Section 4. Structures: I, 16α-hydroxyprogesterone; II, Δ^{16}-progesterone; III, 17α-progesterone.

the elimination of dehydroepiandrosteone sulfate from rats by increasing its EHC and urinary excretion (Van Eldere et al. 1990). Steroid sulfatase activity has been detected in certain species of the genera *Clostridium*, *Lactobacillus*, *Eubacterium*, *Peptococcus*, and *Bacteroides* (Van Eldere et al. 1988). However, the substrate specificity for steroid sulfates varies considerably among these species, suggesting that there are many different steroid sulfatases among the intestinal microbiota. Indeed, *Peptococcus niger* synthesizes at least three different steroid sulfatases (Van Eldere et al. 1991).

4.2. 21-Dehydroxylation of Corticosteroids

21-Dehydroxylation of corticosteroids is restricted to those that undergo EHC (Fig. 13.6, reaction 10). The role of intestinal bacteria in 21-dehydroxylation was first shown in the late 1960s by comparing the steroid hormone patterns in germfree and conventional animals (Eriksson et al. 1969b, Gustafsson 1968, Gustafsson and Sjövall 1968). Studies by Eriksson et al. (1969) showed that anaerobic incubation of fecal suspensions with 3β,21-dihydroxy-5α-pregnane-20-one resulted in the formation of 3,20-dihydroxypregnane metabolites. Moreover, it has also been demonstrated that the removal of the 21-hydroxy group of corticosteroids by intestinal bacterial shifts the secretion from biliary to renal.

To date, all bacteria capable of 21-dehydroxylation are members of the genus *Eubacterium*. Bokkenheuser et al. (1977) successfully isolated a bacterium from human fecal suspension, which was capable of the 21-dehydroxylation of corticosteroids with an α-ketol side chain. The bacterium was later identified as a strain of *Eubacterium lentum* (Bokkenheuser et al. 1979). Feighner et al. (1979) demonstrated 21-dehydroxylase activity in cell extracts of *E. lentum* and showed that the enzyme required reduced free flavins for activity. The M_r of 21-dehydroxylase was estimated to be 582,000 by gel filtration chromatography. 21-Dehydroxylation increased sevenfold when whole cells of *E. lentum* were sparged with hydrogen gas (Feighner and Hylemon 1980).

21-Dehydroxylation of corticosteroids requires a 20-oxo substrate and reduced flavins. Holland and Riemland (1984) studied the mechanism of 21-dehydroxylation using substrates labelled with deuterium at C-17 and C-21. Their proposed mechanism is consistent with an initial formation of an enediol followed by reduction, dehydration, and enol-oxo tautomerism, yielding the 21-dehydroxylated steroid product. Such a mechanism suggests that 21-dehydroxylation requires more than one enzymatic activity. However, 21-dehydroxylase has not yet been purified to homogeneity or characterized.

4.3. Side-Chain Cleavage

Evidence for the side-chain cleavage of glucocorticoids by members of the intestinal microbiota was first reported by Nabarro et al. (1957) (Fig. 13.6, reac-

tion 5). Studies by Eriksson and Gustafsson (1971) demonstrated that mixed fecal biota from healthy human subjects removed the side-chain of cortisol. Bokkenheuser et al. (1984) isolated from human feces a species of *Clostridium* that had desmolase (C17-C20 lyase) activity. The bacterium was classified as a new species, *Clostridium scindens*. It was demonstrated using whole cells of this bacterium that side-chain cleavage required a hydroxy group at C-17 and preferred an α-ketol group at C-20 or C-21 for optimal activity (Winter et al. 1984a). It was shown later that desmolase activity in *C. scindens* was induced by cortisol, cortisone, 11-desoxycortisol, and 17α-hydroxyprogesterone. Desmolase activity in cell extracts of *C. scindens* prepared from induced cultures required NAD^+ and a divalent metal cation—i.e., Mg^{2+}, Mn^{2+}, or Fe^{2+} (Krafft et al. 1987). Additional studies demonstrated that desmolase activity was further stimulated by the addition of vitamin B_{12} to cell extracts of *C. scindens* (A.E. Krafft and P.B. Hylemon, unpublished data). Finally, Morris et al. (1986) isolated from cat feces a new species, *Eubacterium desmolans*, which had desmolase activity.

4.4. Reduction of Ring A

Reduction of ring A in steroid hormones markedly decreases their hormonal activity. The reduction of ring A in steroid hormones is normally thought to be carried out in the liver. However, the intestinal microbiota rapidly reduce ring A of steroid hormones containing the Δ^4-3-oxo-steroid structure. Bacterial reduction of such hormones occurs by a sequential two step reaction (Glass et al. 1979). The Δ^4 double bond is initially reduced to either to the 5α- or 5β configuration (Fig. 13.6, reactions 1 and 4) by stereospecific 5α- or 5β-steroid reductases. The 3-oxo group is then reduced to either a 3α- or 3β-hydroxy group by stereospecific 3α- or 3β-HSDHs (Fig. 13.6, reactions 2 and 3). Species of intestinal clostridia have been shown to carry out the reduction of Δ^4-3-oxo-steroids. *Clostridium innocuum* and *Clostridium paraputrificum* reduce Δ^4-3-oxosteroids to 3β-5β and 3α-5β derivatives, respectively (Bokkenheuser et al. 1979, Glass et al. 1979, Stokes and Hylemon 1985), while *Clostridium* sp. J-1 produces a 3β-5α derivative (Bokkenheuser et al. 1983). Ring A in synthetic progestins, which are used in oral contraceptives, is reduced three to 10 times more slowly than in naturally occurring progestins (Bokkenheuser et al. 1983). This observation may explain the pharmacological superiority of synthetic progestins over natural progestins. In cell extracts prepared from *C. innocuum* or *C. paraputrificium*, NADH markedly stimulated the 5β-reductase and 3α- and 3β-HSDH activities. The relative molecular weight of 5β-reductase and 3β-HSDH from *C. innocuum* was estimated to be 80,000 by gel filtration chromatography. *Eubacterium lentum* has also been reported to have both 3α- and 3β-HSDH activities and can epimerize the 3α-hydroxy group to the 3β-orientation (Bokkenheuser et al. 1979).

4.5. *Oxidation/Reduction of 17- and 20-Hydroxy/Oxo Groups*

The reduction of the 17-oxo group to a 17β-hydroxy derivative can be carried out by a number of species of intestinal bacteria including: *Eubacterium lentum, Clostridium paraputrificum, Clostridium* J-1, *Clostridium innocuum*, and *Bacteroides fragilis*. The 17-oxo function of phenolic steroids is reduced specifically by certain species of the genera *Eubacterium* and *Clostridium*, while the 17-oxo group of androstenedione is reduced solely by *B. fragilis* (Winter et al. 1984b). The reduction of the 17-oxo group of androstenedione to the 17α-hydroxy derivative (yielding epitestosterone) was recently discovered in the human intestinal isolate, *Eubacterium* sp. VPI 12708 (de Prada et al. 1994). A steroid inducible 17α-HSDH was purified and characterized from this organism. The enzyme was oxygen-sensitive and was highly specific for NADPH and the 17-oxo group of C_{19} steroids. The N-terminal amino acid sequence suggested that this enzyme belongs to a disulfide reductase gene family (de Prada et al. 1994). The presence of both 17α- and 17β-HSDHs suggests that the intestinal microbiota can epimerize the 17β-hydroxy group of testosterone to the 17α-hydroxy group of epitestosterone. The pathways of epitestosterone biosynthesis in the body are controversial. However, there has been renewed interest in the biosynthesis of this compound because of its potential as an antiandrogen (Bicikova et al. 1992). In addition, the International Olympic Committee uses the urinary ratio of testosterone to epitestosterone as a marker for testosterone doping in athletes (Kicman et al. 1990).

Bacteroides fragilis and *Bifidobacterium adolescentis* have been reported to have 20β-HSDH activity (Bokkenheuser et al. 1975, Winter et al. 1982a). Reduction of the 20-oxo group makes the steroid resistant to further modification of the steroid side chain. Steroid substrate specificity studies using whole cells of *B. fragilis* and *B. adolescentis* suggest that there may be more than one form of 20β-HSDH among the intestinal microbiota. However, 20β-HSDH has not been purified or characterized from members of the intestinal biota.

20α-HSDH has been detected in a variety of mammalian tissues (Nancarrow et al. 1981) and, in microorganisms, isolated from soil and water. Studies by Winter et al. (1984a) showed that the intestinal microbiota contain 20α-HSDH activity. Bokkenheuser et al. (1984) isolated from human feces a species of *Clostridium* that had 20α-HSDH activity. This bacterium was later determined to be a new species, *Clostridium scindens* (Morris et al. 1985). The 20α-HSDH from *C. scindens* has been purified and characterized (Krafft and Hylemon 1989). The enzyme was NAD^+/NADH dependent and was highly specific for adrenocorticosteroids having 17α- and 21-hydroxy groups. The subunit M_r of the enzyme was approximately 40,000 Da. The N-terminal amino acid sequence (first 11 residues) suggested that this enzyme may belong to the glyceraldehyde-3-phosphate dehydrogenase gene family (Krafft and Hylemon 1989).

4.6. 16α-Dehydroxylation

Steroid hormones with a 17α side chain have been detected in feces and urine of rats and humans (Calvin and Lieberman 1962, Eriksson et al. 1969a). These steroid hormones have been shown to originate from the 16α-dehydroxylation of steroid hormones by the intestinal microbiota. 16α-Dehydroxylation activity has been detected in human feces and rat intestinal contents (Eriksson and Gustafsson 1971). Two intestinal strains of *Eubacterium* with 16α-dehydroxylation activity were isolated by Bokkenheuser et al. (1980). Winter et al. (1982b) showed that the 16α-dehydroxylation of corticoids by isolates of intestinal bacteria was a two-step biotransformation (Fig. 13.7). The 16α-hydroxysteroid substrate was dehydrated to a Δ^{16}-steroid intermediate (reaction 1), which accumulated in culture media and was subsequently reduced to a 17α side chain steroid product (reaction 2). Glass et al. (1982) showed that the Δ^{16} steroid intermediate would react nonenzymatically with L-cysteine in the culture medium, forming a 16-thioether bond. The high reactivity of Δ^{16} steroids is believe to be due to the formation of a highly reactive α,β-unsaturated ketone associated with the D-ring of the steroid. It is not known if cysteine conjugates of steroids are formed in vivo; however, Δ^{16} steroids have been detected in the urine of female subjects (Calvin and Lieberman 1962). A 16α-dehydratase has been purified from *Eubacterium* sp. strain 144 and was found to have a subunit M_r of 42,400 and native M_rs of 181,000 and 326,000 by gel filtration chromatography (Glass and Lamppa 1985). The reduction of Δ^{16} steroids by whole cell suspensions of *Eubacterium* sp. strain 144 required growth in the presence of hemin and an inducing steroid. In addition, it was necessary to incubate the cell suspensions in the presence of either pyruvate or molecular hydrogen. It was proposed that hemin was used for the synthesis of a cytochrome-containing electron transport chain which then uses Δ^{16} steroids as an electron acceptor (Glass et al. 1991, Watkins and Glass 1991). It was discovered that these same growth conditions induced a membrane-associated steroid 5α-reductase which also required either pyruvate or molecular hydrogen as an electron donors. These results suggest that these steroid biotransformations might be linked to energy metabolism in this bacterium.

5. Summary

In the 1960's and early 1970's, the various intestinal biotransformations of bile acids, cholesterol, and steroid hormones were shown to occur in intact animals and humans. In the late 1970's and 1980's, intestinal bacteria catalyzing these steroid biotransformations were isolated and identified. Enzymes catalyzing some of the steroid modifications have been purified and characterized, and the corresponding genes cloned and sequenced. Some steroid modifying enzymes have

been assigned to gene families. In the late 1980's, a multi-step biochemical pathway for bile acid 7-dehydroxylation was discovered. However, the physiological significance of the steroid biotransformations to intestinal bacteria has not been determined. Finally, a thorough understanding of the importance of these biotransformations to the host in health and disease awaits further research.

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