7α-Dehydroxylation of cholic acid and chenodeoxycholic acid by *Clostridium leptum*

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Abstract The rate of 7α-dehydroxylation of primary bile acids was quantitatively measured radiochromatographically in anaerobically washed whole cell suspensions of *Clostridium leptum*. The pH optimum for the 7α-dehydroxylation of both cholic and chenodeoxycholic acid was 6.5–7.0. Substrate saturation curves were observed for the 7α-dehydroxylation of cholic and chenodeoxycholic acid. However, cholic acid whole cell $K_{0.5}$ (0.37 μM) and V (0.20 μmol hr^{-1} mg protein^{-1}) values differed significantly from chenodeoxycholic acid whole cell $K_{0.5}$ (0.18 μM) and V (0.050 μmol^{-1} hr^{-1} mg protein^{-1}). 7α-Dehydroxylation activity was not detected using glycine- and taurine-conjugated primary bile acids, ursodeoxycholic acid, cholic acid methyl ester, or hyocholic acid as substrates. Substrate competition experiments showed that cholic acid 7α-dehydroxylation was reduced by increasing concentrations of chenodeoxycholic acid; however, chenodeoxycholic acid 7α-dehydroxylation activity was unaffected by increasing concentrations of cholic acid. A 10-fold increase in cholic acid 7α-dehydroxylation activity occurred during the transition from logarithmic to stationary phase growth whether cells were cultured in the presence or absence of sodium cholate. In the same culture, a similar increase in chenodeoxycholic acid 7α-dehydroxylation was detected only in cells cultured in the presence of sodium cholate. These results indicate the possible existence of two independent systems for 7α-dehydroxylation in *C. Leptum*. — **Stellwag, E. J., and P. B. Hylemon.** 7α-Dehydroxylation of cholic acid and chenodeoxycholic acid by *Clostridium leptum*. *J. Lipid Res.* 1979. **20:** 325–333.

Supplementary key words bile acids · substrate specificity · inhibitors

The final composition of bile acids in biliary bile of man is dependent upon the combined action of liver biosynthetic enzymes as well as intestinal bacterial enzymes that degrade bile acids (1). Known microbial biotransformations include deconjugation of glycine or taurine conjugated bile acids to yield free bile acids (2–6), dehydroxylation at the C_7 hydroxy group of the steroid nucleus (2, 7–11), oxidation of the hydroxy groups at C_3, C_7, and C_{12} (2, 4, 11–14) and reduction of the ketone moieties to either α or β hydroxy groups (2, 11). Quantitatively, the most important bacterial modification of the primary bile acids cholic acid and chenodeoxycholic acid is 7-α-dehydroxylation which

results in the formation of the secondary bile acids deoxycholic acid and lithocholic acid, respectively (2).

7α-Dehydroxylation of primary bile acids markedly alters the physical characteristics as well as the physiological effects of the bile acid molecule. Chemically, there is an alteration of the critical micellar concentration and a decrease in the solubility of secondary bile acids in aqueous solutions relative to their primary bile acid (15). Physiologically, it has been reported that oral administration of deoxycholic acid specifically suppresses the hepatic biosynthesis of chenodeoxycholic acid (16); however, in other studies, a decrease in the biosynthesis of both cholic and chenodeoxycholic acid has been reported (17–19). Furthermore, deoxycholic acid has been reported to be capable of inducing the secretion of water and electrolytes from the small and large intestine via an apparent effect on adenylate cyclase activity (20). Moreover, secondary bile acids have been implicated as promoters of primary chemical carcinogens in laboratory animal studies (21).

The proposed reaction mechanism for 7α-dehydroxylation of cholic acid is presented in **Fig. 1**. As elucidated by Samuelsson (15), the initial step in 7α-dehydroxylation occurs via a diaxial *trans* elimination of the 7α-hydroxy group and the 6β hydrogen atom. The proposed Δ6 intermediate thus generated is subsequently reduced by *trans* hydrogenation at the 6β and 7α positions to yield deoxycholic acid.
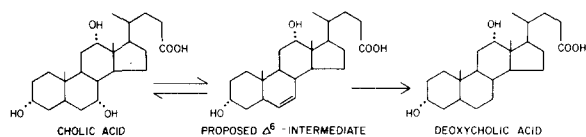
Fig. 1. Proposed reaction mechanism for 7α-dehydroxylation of cholic acid, after Samuelsson (15).

Despite the importance of 7α-dehydroxylation in bile acid metabolism, published information regarding the characteristics of this steroid biotransformation reaction is conflicting and incomplete (2). Midvedt and Norman (4) and Dickinson, Gustaffson, and Norman (22) reported a limited distribution of 7α-dehydroxylation activity in bacteria isolated from intestinal contents. In contrast, Aries and Hill (11) reported that 7α-dehydroxylase is widespread in most species of the predominant human intestinal microflora. The explanation for this discrepancy is not yet clear.

In this communication we report the characterization of 7α-dehydroxylation activity in whole cells of *Clostridium leptum* using both cholic acid and chenodeoxycholic acid as substrate. We also provide preliminary evidence for the existence of two independent systems for 7α-dehydroxylation of the two primary bile acids and report levels of 7α-dehydroxylation activities in fecal samples from normal individuals.

MATERIALS AND METHODS

The organism now known as *Clostridium leptum* V.P.I. 10900 was originally isolated from a human fecal sample and was tentatively identified as a species of *Bacteroides* (2). However, after additional characterization studies by Drs. Holdeman and Moore at the Virginia Polytechnic Institute and State University, Anaerobe Laboratory, this bacterium was reclassified as a strain of *Clostridium leptum*. Stock cultures were maintained in chopped meat medium as described by Holdeman and Moore (23).

The *C. leptum* V.P.I. 10900 utilized for in vitro characterization of 7α-dehydroxylation activity was cultured anaerobically under N_2 in modified (made without salts solution) peptone–yeast extract medium containing 2 g/l glucose and 0.1 mM sodium cholate essentially as described by Holdeman and Moore (23).

Quantitative assay for 7α-dehydroxylation in *C. leptum*

Enzymatic 7α-dehydroxylation of ^{14}C-carboxyl-labeled cholic or chenodeoxycholic acid by whole cell suspensions of *C. leptum* V.P.I. 10900 was followed by measuring the rate of biotransformation to deoxycholic and lithocholic acids, respectively. Cells of *C. leptum* were harvested from a 1-liter stationary-phase culture (4 hr post-exponential) by centrifugation (13,700 g for 10 min at 25°C). The cell sediments were washed in 300 ml of 50 mM potassium phosphate buffer (pH 6.5) that had been made anaerobic by boiling for 10 min and cooling to 37°C under continuous flushing with argon gas previously passed through hot (350°C) copper filings essentially as described previously (25).

The standard reaction mixtures (1.0 ml) contained in final concentration: 2.04 μM [24-14C]cholic acid or 0.5 μM [24-14C]chenodeoxycholic acid (0.2 μCi/reaction mixture), 50 mM potassium phosphate buffer (pH 6.5), and an appropriate sample of whole cell suspension. Reaction mixtures were incubated anaerobically (37°C) in 1 × 8.5 cm test tubes equipped with rubber serum caps. The reaction mixtures were constantly flushed with argon gas via gas intake and exit needles for up to 30 min. Substrate conversion rates were linear up to 60 min. Enzymatic activity was terminated by the addition of 1.0 ml of 0.5 N HCl directly to the reaction mixtures (final pH 2.0). The acidified reaction mixtures were extracted and chromatographed as described previously (6). The regions of the chromatogram corresponding to labeled substrate and product were located by use of a radiochromatogram scanner. These regions were scraped into scintillation vials containing Triton X-100-based scintillation fluid and counted in a Beckman LS-350 liquid scintillation counter (5). A unit of enzyme activity was defined as the amount of enzyme required for the formation of 1 μmol of secondary bile acid formed per hr per mg whole cell protein under standard assay conditions. Reaction velocity was directly proportional to protein concentration over a range of 0.2–1.5 mg/ml. Protein concentration was measured by the method of Lowry et al. (24) after alkali solubilization (1 N NaOH) of whole cells.

Quantitative assays of fecal 7α-dehydroxylation activity

7α-Dehydroxylation of [24-14C]cholic acid and [24-14C]chenodeoxycholic acid by washed fecal suspensions was performed as follows. Approximately 5 g (wet weight) of freshly voided feces was suspended in 300 ml of anaerobically prepared potassium phosphate buffer (pH 6.5). The suspension was centrifuged (13,700 g for 10 min at 25°C) and the top 2–3 mm of fecal sediment was removed with a spatula, suspended in 300 ml of anaerobic buffer, and centrifuged (13,700 g for 10 min at 25°C). Again, the top 2–3 mm of fecal sedi-

ment was removed and suspended in anaerobic potassium phosphate buffer (pH 6.5) to a turbidity of 280–300 Klett units (No. 66 filter). Aliquots of this fecal suspension were assayed for 7α-dehydroxylation activity essentially as described above. Protein concentration was determined on these aliquots. To determine the levels of viable 7α-dehydroxylating intestinal bacteria, serial 10-fold dilutions were carried out on these same fecal samples as described by Holdeman and Moore (23). Aliquots (0.5 ml) of each dilution were inoculated into peptone–yeast glucose medium (4.5 ml) containing 4 nmol of labeled cholic acid (0.2 μCi/tube). After 72 hr of incubation the bile acids were extracted with ethyl acetate and bile acid products were identified by thin-layer chromatography as described below.

Identification of 7α-dehydroxylation products

Products generated using cholic acid or chenodeoxycholic acid as substrates were isolated from *C. leptum* whole cell reaction mixtures and identified by thin-layer chromatography (Baker-flex Silica gel 1B-2, J. T. Baker Chemical C., Phillipsburg, NJ). Steroids were chromatographed in solvent systems S_1, S_6, and S_{12} as described by Eneroth (26). Reaction products were chromatographed separately and as mixtures with authentic bile acid standards. Bile acid 7α-dehydroxylation products and known standards were treated individually with specific 3α-, 7α-, and 12α-hydroxysteroid dehydrogenases. The enzymatic conversion product generated by steroid dehydrogenase treatment was then chromatographed separately and as a mixture with known bile acid standards in solvent systems S_1, S_6, and S_{12} to confirm identification.

Chemicals and enzymes

[24-^{14}C]Cholic (50 mCi/mmol) and [24-^{14}C]chenodeoxycholic acids (50 mCi/mmol) were purchased from New England Nuclear, Boston, MA. [24-^{14}C]-Lithocholic acid (50 mCi/mmol) was obtained from Amersham Searle Corp., Arlington Heights, IL. The labeled lithocholic acid was purified by thin-layer chromatography prior to use. 3α-Hydroxysteroid dehydrogenase was obtained from Worthington Biochemicals, Freehold, NJ. 7α-Hydroxysteroid dehydrogenase and 12α-hydroxysteroid dehydrogenase were isolated as described previously (13, 25). Ursodeoxycholic acid, hyocholic acid, and cholic acid methyl ester were purchased from Steraloids, Wilton, NH. All other bile acids and bile salts were obtained from Calbiochem, San Diego, CA. Dicyclo-

TABLE 1. Relative mobilities of bile acid standards and identification of secondary bile acids generated by *Clostridium leptum*

Solvent Systems[a]	S1	S6	S12
Relative Mobility[b]	R_D	R_C	R_D
Mobility of standard (cm)	7.0	3.0	6.8
Bile acid standards			
3α, 7α, 12α	0.21	1.00	0.17
3α, 12α	1.00	3.16	1.00
3α, 7α	0.94	2.83	0.91
3α	1.73	4.66	2.00
7α, 12α, 3-keto[c]	0.72	2.20	1.00
12α, 3-keto[c]	1.52	4.40	2.00
3-keto[c]	1.97	5.33	2.38
3α, 12α, 7-keto[d]	0.90	1.50	0.66
3α, 7-keto[d]	1.35	3.50	1.17
3α, 7α, 12-keto[e]	0.54	1.50	0.63
3α, 12-keto[e]	1.47	4.06	1.77
Product identification			
Product of cholic acid 7α-dehydroxylation			
Untreated	0.97	3.12	1.00
Derivative of treatment[c]	1.50	4.40	1.97
Derivative of treatment[d]	0.98	3.14	0.98
Derivative of treatment[e]	1.48	4.06	1.79
Product of chenodeoxycholic acid 7α-dehydroxylation			
Untreated	1.74	4.66	1.98
Derivative of treatment[c]	2.00	5.32	2.36
Derivative of treatment[d]	1.71	4.70	1.98
Derivative of treatment[e]	1.72	4.66	1.98

[a] Described by Eneroth (26).
[b] Mobility of deoxycholic acid (R_D) and cholic acids (R_C).
[c] Derived by treatment with 3α-hydroxysteroid dehydrogenase.
[d] Derived by treatment with 7α-hydroxysteroid dehydrogenase.
[e] Derived by treatment with 12α-hydroxysteroid dehydrogenase.

hexylcarbodiimide (DCCD) and acriflavin were purchased from Sigma Chemical Co., St. Louis, MO.

RESULTS

Identification of 7α-dehydroxylation reaction products

The R_f values of cholic, chenodeoxycholic, deoxycholic, and lithocholic acid standards as well as the 3α-, 7α-, and 12α-hydroxysteroid dehydrogenase-derived keto-bile acid derivatives of these standards in solvent systems S_1, S_6, and S_{12} are presented in **Table 1.** R_f values obtained from chromatography of the 7α-dehydroxylation reaction product and 3α-, 7α-, and 12α-hydroxysteroid dehydrogenase-derived product show that cholic acid and chenodeoxycholic acid are enzymatically transformed by *C. leptum* to deoxycholic and lithocholic acid, respectively. These results confirm and extend the conclusion obtained by Hayakawa (2) using infrared
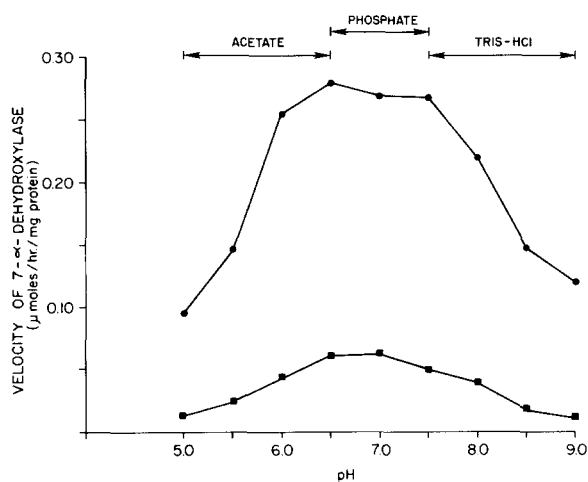
Fig. 2. Effect of pH on the 7α-dehydroxylation by washed whole cell suspensions of *C. leptum* of cholic acid (●) or chenodeoxycholic acid (■). Values presented for pH are the values of the buffer. All buffers were used at a concentration of 50 mM.

spectrometry and mixed melting point analysis of the 7α-dehydroxylation reaction product isolated from growing cultures of *C. leptum* in medium containing cholic acid.

pH optimum for 7α-dehydroxylation

The initial rates of primary bile acid 7α-dehydroxylation were measured over 30 min under standard assay conditions and a pH range of 5.0–9.0. Since the reaction was found to be sensitive to molecular oxygen, the pH values of each reaction mixture could not be measured directly and the values presented are those of the buffer used. 7α-Dehydroxylation activity in washed whole cell suspensions of *C. leptum* had an optimum pH of 6.5–7.0 using either cholic or chenodeoxycholic acid as substrate (**Fig. 2**).

Substrate specificity

7α-Dehydroxylation of cholic acid, chenodeoxycholic acid, and glycocholic acid was followed by radiochromatographic assay as outlined above. The 7α-dehydroxylation of the other bile acids was followed by incubating the bile acids in question with whole cell suspensions, extraction of the substrate and product, and chromatography on silica gel thin-layer plates. The plates were then sprayed with sulfuric acid–methanol and charred by heating to 150°C for 10 min. This method allows detection of conversion rates as low as 0.005 μmol hr^{-1} mg whole cell protein^{-1}.

Several bile acids bearing the 7α-hydroxy group were examined for activity as substrates of 7α-dehydroxylation using washed whole cell suspensions

of *C. leptum*. Anaerobically incubated whole cells 7α-dehydroxylated both cholic and chenodeoxycholic acids. A 4-fold higher maximal activity was demonstrated using the trihydroxy as compared to the dihydroxy primary bile acid (**Table 2**). 7α-Dehydroxylation activity was not detectable for glycine- or taurine-conjugated primary bile acids, cholic acid methyl ester, hyocholic acid, or ursodeoxycholic acid (Table 2). $3\alpha,7\alpha$-Dihydroxy-12-keto-5β-cholanoic acid was tested as a substrate for 7α-dehydroxylation in anaerobically incubated whole cell suspensions of *C. leptum*. The end product of the reaction, as assayed by thin-layer chromatography, was deoxycholic acid with cholic acid accumulating as a transient intermediate.

Kinetics of 7α-dehydroxylation by whole cells

Substrate saturation kinetics were carried out using cholic and chenodeoxycholic acid as substrates (**Figs. 3** and **4**). Saturation curves were used to estimate $K_{0.5}$ and V values for cholic and chenodeoxycholic acids. $K_{0.5}$ is defined as the substrate concentration yielding the half-maximal rate of 7α-dehydroxylation using whole cell suspensions. $K_{0.5}$ (0.37 μM) and V (0.20 μmol hr^{-1} mg protein^{-1}) values for 7α-dehydroxylation activity differed significantly from those for chenodeoxycholic acid $K_{0.5}$ (0.18 μM) and V (0.050 μmol hr^{-1} mg protein^{-1}).

Substrate competition

The data illustrated in **Fig. 5** show the results of mixed primary bile acid competition experiments. A saturating level of radiolabeled primary bile acid substrate and increasing concentrations of a non-labeled competing primary bile acid were simultaneously incubated in the presence of whole cell sus-

TABLE 2. Apparent $K_{0.5}$ and V values for 7α-dehydroxylation in whole cells of *Clostridium leptum*

Bile Acid	$K_{0.5}$ $(\mu M)^a$	V^b
$3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholanoic acid^c	0.37	0.200
$3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholanoyl glycine^c	—^{e}	<0.001
$3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholanoyl taurine^d	—^{e}	<0.005
$3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholanoyl methyl ester^d	—^{e}	<0.005
$3\alpha,6\alpha,7\alpha$-trihydroxy-5β-cholanoic acid^d	—^{e}	<0.005
$3\alpha,7\alpha$-dihydroxy-5β-cholanoic acid^c	0.18	0.050
$3\alpha,7\alpha$-dihydroxy-5β-cholanoyl glycine^d	—^{e}	<0.005
$3\alpha,7\alpha$-dihydroxy-5β-cholanoyl taurine^d	—^{e}	<0.005
$3\alpha,7\beta$-dihydroxy-5β-cholanoic acid^d	—^{e}	<0.005

^{a} Estimated as substrate concentration yielding ½ maximal rate.
^{b} Estimated from substrate saturation curves and reported as μmol hr^{-1} mg protein^{-1}.
^{c} Carboxyl-^{14}C-labeled bile acids.
^{d} Unlabeled bile acids.
^{e} — indicates insufficient activity to permit calculation.
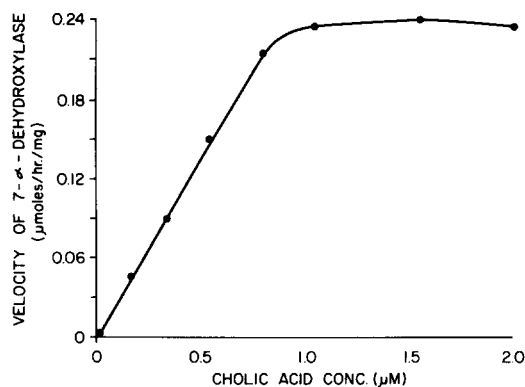
Fig. 3. Effect of cholic acid concentration on 7α-dehydroxylation activity. Cholic acid was added to initiate the reaction. Each reaction mixture contained approximately 0.70 mg of whole cell protein. Initial rates of 7α-dehydroxylation were measured over a time course of up to 30 min.

pensions of *C. leptum*. Increasing concentrations of chenodeoxycholic acid caused a decrease in the apparent conversion rate of cholic acid to deoxycholic acid. In contrast, increasing concentrations of cholic acid caused only a slight decrease in the initial rate of chenodeoxycholic acid conversion to lithocholic acid (**Fig. 5**).

Regulation of 7α-dehydroxylation activities in whole cells

Bile acid conversion rates using cholic acid and chenodeoxycholic acid as substrates were measured at various points during the different growth phases of *C. leptum* cultured on modified peptone-yeast glucose medium. The conversion rate of cholic acid to deoxycholic acid was 10- to 12-fold higher in washed stationary phase cells compared to conversion rates obtained with washed early logarithmic phase cells. The increased conversion rate of stationary
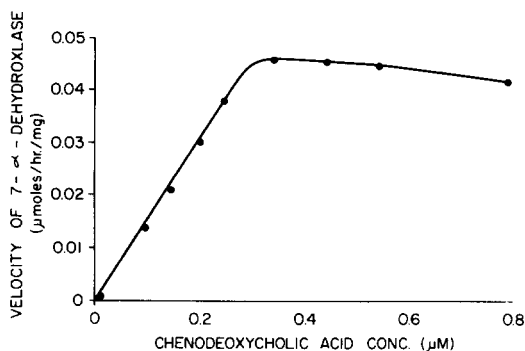

Fig. 4. Effect of chenodeoxycholic acid concentration on 7α-dehydroxylation rate. Chenodeoxycholic acid was added to initiate the reaction. Each reaction mixture contained approximately 0.70 mg of whole cell protein. Initial rates of 7α-dehydroxylation were measured over a time course of up to 30 min.
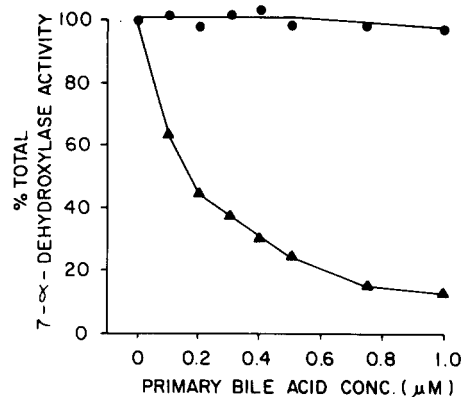

Fig. 5. Effect of unlabeled chenodeoxycholic acid concentration on 7α-dehydroxylation rate measured at a saturating level (2.0 μM) of radiolabeled cholic acid (▲); and effect of unlabeled cholic acid concentration on 7α-dehydroxylation rate measured at a saturating level (0.50 μM) of radiolabeled chenodeoxycholic acid (●). Bile acid mixtures were added simultaneously to initiate the reaction. Each reaction mixture contained approximately 0.70 mg of whole cell protein. Initial rates of 7α-dehydroxylation were measured by taking multiple samples over a time course of 30 min.

phase cells occurred irrespective of the presence or absence of 0.1 mM sodium cholate in the growth medium (**Fig. 6**). However, the conversion rate of chenodeoxycholic acid to lithocholic acid increased 8- to 10-fold in washed stationary phase cells compared to that obtained for early logarithmic phase cells only when *C. leptum* was cultured in the presence of 0.1 mM sodium cholate. The conversion
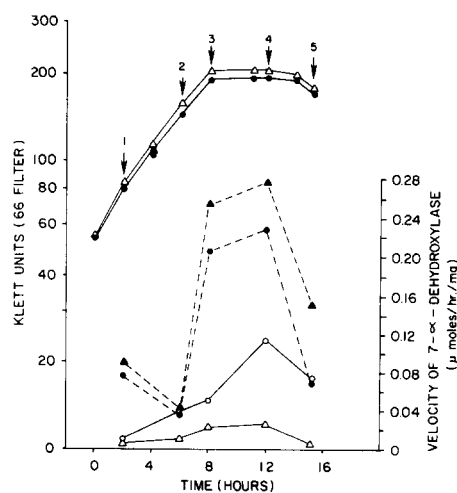

Fig. 6. Regulation of 7α-dehydroxylase activities in *C. leptum*. A 1-liter starter culture grown on peptone-yeast glucose medium without sodium cholate was harvested during the exponential phase of growth and was used to inoculate (0 hr) two 3-liter flasks of medium with (●) or without (△) 0.1 mM sodium cholate. Symbols indicate 7α-dehydroxylation activities in washed whole cells at time points (numbered arrows) indicated on growth curve in the presence (circles) or absence (triangles) of sodium cholate. 7α-Dehydroxylase activities are shown using either cholic (dashed lines) or chenodeoxycholic (solid lines) acid as substrates.
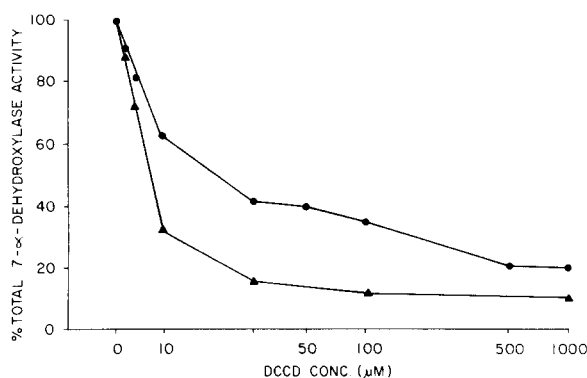
Fig. 7. Effect of DCCD concentration on 7α-dehydroxylase activity using cholic (▲) or chenodeoxycholic acid (●) as substrate. Cell suspensions of *C. leptum* were incubated with DCCD solubilized in dimethyl sulfoxide (DMSO) for 15 min prior to initiation of primary bile acid addition. A saturating level of primary bile acid was added to initiate the reaction. Each reaction mixture contained approximately 0.60 mg of whole cell protein. Initial rates of 7α-dehydroxylation were measured over a time course of up to 30 min under standard assay conditions. Control experiments using DMSO alone did not inhibit the 7α-dehydroxylation of either cholic or chenodeoxycholic acid.

rate of chenodeoxycholic acid to lithocholic acid showed less than a 2-fold increase during the various growth phases when *C. leptum* was cultured in the absence of 0.1 mM sodium cholate (Fig. 6).

Inhibitors of 7α-dehydroxylation rates in whole cells

The data presented in **Fig. 7** show the conversion rates of cholic and chenodeoxycholic acids at increasing concentrations of the bacterial ATPase inhibitor DCCD. Incubation in the presence of DCCD resulted in stronger inhibition of the cholic acid conversion rates than chenodeoxycholic acid conversion rates. Inhibition of cholic acid 7α-dehydroxylation saturated at approximately 25 μM DCCD, whereas inhibition of chenodeoxycholic acid 7α-dehydroxylation saturated between 100 and 500 μM DCCD (Fig. 7). Although cholic acid and chenodeoxycholic acid 7α-dehydroxylations were differentially sensitive to DCCD inhibition, decreased 7α-dehydroxylation of cholic acid was closely paralleled by decreased chenodeoxycholic acid 7α-dehydroxylation at increasing acriflavin concentrations (**Table 3**).

7α-Dehydroxylation in cell extracts of *C. leptum*

Breakage of cells of *C. leptum* by passage through a French pressure cell at 15,000 lb in^{-2} under a stream of argon gas resulted in a complete loss of cholic and chenodeoxycholic acid 7α-dehydroxylation activity. The loss of activity occurred regardless of precautions taken with respect to oxygen exposure of the cell extracts.

TABLE 3.   Acriflavin inhibition of 7α-dehydroxylation activity in whole cells of *Clostridium leptum* V.P.I. 10900

Acriflavin conc.[b]	7α-Dehydroxylation Activity[a]			
	Cholic Acid[c]		Chenodeoxycholic acid[c]	
μM	%		%	
0	0.210	100	0.056	100
25	0.187	89	0.051	91
50	0.161	77	0.048	86
100	0.130	62	0.037	66
200	0.069	33	0.021	37

[a] Rate of 7α-dehydroxylation is measured as μmoles of substrate converted to product hr^{-1} mg protein^{-1} under standard assay conditions.

[b] Cell suspensions of *C. leptum* were preincubated with acriflavin for 15 min prior to initiation of the reaction.

[c] A saturating level of primary bile acid was added to initiate the reaction.

Reduced forms of benzyl viologen, methyl viologen, phenosafranin, Janus green B, indigocarmine, phenazine methosulfate, and methylene blue failed to restore 7α-dehydroxylation activity in *C. leptum* cell extracts. Furthermore, incubation of cell extracts under H_2 or in the presence of 10 mM pyruvate-reduced or oxidized pyridine nucleotides (1 mM), reduced or oxidized flavin nucleotides (1 mM) and reducing agents such as sodium dithionite (5 mM), cysteine (5 mM), or 2-mercaptoethanol (5 mM) were unsuccessful in reconstituting 7α-dehydroxylating activity.

7α-Dehydroxylation activity in fecal samples

The bile acid conversion rates in anaerobically washed fecal samples ranged from 0.005 to 0.008 μmol hr^{-1} mg protein^{-1} for chenodeoxycholic acid and 0.006 to 0.012 μmol hr^{-1} mg protein^{-1} for cholic acid from four individuals. The levels of 7α-dehydroxylating bacteria in these same fecal samples ranged from approximately 10^4 to 10^6 viable cells per gram wet weight feces (**Table 4**).

DISCUSSION

Intestinal anaerobic bacteria are responsible for a wide variety of bile acid biotransformations in man (2). Recently, however, it has become clear that the extent of these biotransformations is limited by the constraints inherent to the strictly reducing anaerobic environments of the human colon (28). Reductive reaction sequences are strongly favored in the anaerobic environment of the gut. For example, cholesterol, neutral steroid hormones, bile pigments, unsaturated fatty acids, and bile acids (9, 28–30) are

all ultimately reduced in the gastrointestinal tract by the action of enzymes associated with intestinal bacteria. Although bile acids may be enzymatically oxidized under certain conditions by intestinal bacteria, virtually all bile acids isolated from feces of normal individuals are in a reduced form (1). Consequently, it is apparent that the reductive 7α-dehydroxylation of free primary bile acids is quantitatively the most important enzymatic alteration of bile acids in the human intestinal tract.

Our studies of primary bile acid 7α-dehydroxylation by the human intestinal anaerobe *Clostridium leptum* have demonstrated that 7α-dehydroxylation is exceedingly sensitive to inhibition by molecular oxygen. Further, we have been unable to detect 7α-dehydroxylation activity in cell extracts prepared under anaerobic conditions. Aries and Hill (11) have previously reported 7α-dehydroxylase to be widespread among most intestinal anaerobic bacteria and these investigators also reported that cell-free extracts prepared from *Bacteroides fragilis* NCTC 9343 were capable of catalyzing the 7α-dehydroxylation of cholic acid. However, we have been unable to detect 7α-dehydroxylase activity in whole cells or cell extracts of this same strain of *B. fragilis* using a variety of incubation conditions. The explanation for this discrepancy is unclear at the present time. We have been unable to detect 7α-dehydroxylase activity in 70 different strains of intestinal *Bacteroides* species.[3] However, Erdenharder and Stemrova (31) reported that they were able to detect 7α-dehydroxylase in 3 of 39 strains of intestinal *Bacteroides* species.

The 7α-dehydroxylation of cholic acid and chenodeoxycholic acid in anaerobically incubated whole cells of *C. leptum* was detectable over a pH range of 5.0–9.0. The optimum pH for cholic acid and chenodeoxycholic acid 7α-dehydroxylation was 6.5 to 7.0. These results support the findings of Samuel et al. (32) who detected 7α-dehydroxylation in human fecal samples over a pH range of 5.0–8.5.

A high degree of substrate specificity was exhibited by the 7α-dehydroxylase(s) in washed whole cells of *C. leptum* (Table 2). In this regard, bile acids with moieties covalently bound to the 24-carboxyl group, such as the conjugated bile acids and cholic acid methyl ester, were unable to serve as substrates for 7α-dehydroxylation. Furthermore, 7α-dehydroxylation of hyocholic acid and ursodeoxycholic acid was undetectable under our assay conditions. A recent report by Federowski et al. (33) indicates that the intestinal microflora of the rhesus monkey cannot degrade the

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[3] Stellwag, E. J., and P. B. Hylemon. Unpublished data.

TABLE 4. 7α-Dehydroxylation activities in mixed fecal populations of intestinal bacteria[a]

Donor No.	Rate of 7α-Dehydroxylation		Ratio[b] Rate	Levels of 7α-Dehydroxylating Bacteria in Feces[c]
	Cholic Acid	Chenodeoxy-cholic Acid		
1	6.35	5.56	1.14	$\approx 5 \times 10^5$
2	10.35	6.89	1.51	ND[d]
3	7.72	6.60	1.17	$\approx 10^4 - 10^5$
4	11.98	8.30	1.44	$\approx 10^5$

[a] 7α-Dehydroxylation activity is expressed as μmol $\times 10^3$ primary bile acid converted to secondary bile acid hr^{-1} mg protein^{-1}.
[b] Cholic acid 7α-dehydroxylation activity/chenodeoxycholic acid 7α-dehydroxylation activity.
[c] Per gram wet weight of freshly voided feces.
[d] Not determined.

7β-hydroxy group of ursodeoxycholic acid. It is not clear whether the intestinal microflora of man can carry out 7β-dehydroxylation of ursodeoxycholic acid. Clearly, a free carboxyl group at carbon 24 and a 7α-hydroxy group in the B ring of the steroid nucleus are required for 7α-dehydroxylation activity in whole cells of *C. leptum*.

Substrate saturation kinetics were performed for the 7α-dehydroxylation of both cholic and chenodeoxycholic acid. The maximum velocity of 7α-dehydroxylation of cholic acid was 4-fold higher than that for chenodeoxycholic acid. Interestingly, the whole cell $K_{0.5}$ values for cholic acid (0.37 μM) 7α-dehydroxylation are quite low when compared to K_m values generally obtained for soluble enzymes of intestinal anaerobes, e.g., bile salt hydrolase and 7α-hydroxysteroid dehydrogenase (5, 13). However, 7α-dehydroxylation was measured in whole cells and the possibility exists that the rate is limited by these conditions. We have been unsuccessful in demonstrating the uptake of radiolabeled bile acids by whole cell suspensions of *C. leptum* under a variety of incubation conditions. For this reason, we favor the notion that steroid modification reactions occur in or on the cytoplasmic membrane of intestinal anaerobic bacteria (5, 13).

The inhibition of 7α-dehydroxylation in whole cells of *C. leptum* by metabolic inhibitors such as 2,4-dinitrophenol, carbonyl-cyanide-M-chlorophenyl hydrazone (9), and N,N^1-dicyclohexylcarbodiimide (Fig. 7) might suggest that 7α-dehydroxylation is coupled to an energy generating system. Marked inhibition of 7α-dehydroxylation rates by the flavin analog acriflavin indicated that a flavin-linked reaction may be required for 7α-dehydroxylation (34).

Substrate competition experiments (Fig. 5) showed that increasing concentrations of unlabeled chenodeoxycholic acid decreased the rate of cholic acid 7α-dehydroxylation. Such a result indicates that the 7α-

dehydroxylation of cholic acid and chenodeoxycholic acid may be mediated by the same enzyme. In contrast, increasing concentrations of cholic acid caused only a slight decrease in the initial rate of chenodeoxycholic acid 7α-dehydroxylation (Fig. 5). This result suggests that two independent systems may mediate the 7α-dehydroxylation of cholic and chenodeoxycholic acids.

The indication that two independent systems may mediate the 7α-dehydroxylation of the primary bile acids in *C. leptum* is supported by the differential regulation of cholic acid and chenodeoxycholic acid 7α-dehydroxylation activities. The cholic acid 7α-dehydroxylation rate increased 10- to 12-fold during the stationary phase of cell growth in *C. leptum* independent of the presence or absence of sodium cholate (0.1 mM) in the culture medium (Fig. 6). In contrast, chenodeoxycholic acid 7α-dehydroxylation rate measured in the same culture increases 6- to 8-fold during the stationary phase of growth only when sodium cholate was included in the culture medium. The increase in chenodeoxycholic acid 7α-dehydroxylation rate in the absence of sodium cholate in the culture medium was less than 2-fold.

Further evidence for the existence of independent systems for primary bile acid 7α-dehydroxylation derives from the observation (Fig. 7) that the rate of 7α-dehydroxylation of cholic acid is more sensitive to DCCD inhibition than that of chenodeoxycholic acid. The 7α-dehydroxylation of cholic acid is approximately 10-fold more sensitive to inhibition than that of chenodeoxycholic acid.

The results reported in this paper also support the notion that intestinal bacteria capable of carrying-out 7α-dehydroxylation of primary bile acids represent only a small fraction of the human intestinal microflora (Table 4) and that 7α-dehydroxylase does not appear to be widely distributed among members of the predominant bacterial microflora. However, additional studies are required to determine the distribution of 7α-dehydroxylase in different intestinal bacteria and to determine the physiological significance of this unique steroid biotransformation reaction to the bacterium and host.▮▮

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