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Transformation of Bile Acids by *Clostridium perfringens*

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Thirty-five strains of *Clostridium perfringens* were examined for their ability to transform bile acids, both in growing cultures and by washed whole cells. All of the strains oxidized the 3α-hydroxy group to an oxo group, and all except three converted the same α-hydroxy group into a β-configuration. The oxidative 3α-dehydrogenation was barely detectable under anaerobic cultural conditions but was clearly demonstrated in an aerated system using washed whole cells, with a pH optimum between 7.0 and 9.0. The epimerizing reaction amounting to 10 to 20% conversion was observed in anaerobic cultures and also with resting cells, irrespective of oxygen supply. Both reactions were carried out with seven conventional 3α-hydroxy bile acids, thus producing a series of 3-oxo and 3β-hydroxy derivatives that could be examined for gas-liquid chromatographic and mass spectrometric behavior. No evidence for the occurrence of 7α- and 12α-hydroxysteroid dehydrogenase activities among the test strains was found. A highly potent deconjugating hydrolase was elaborated by all of the strains.

Several investigations on the bile acid-transforming activity of *Clostridium perfringens* have been published, as this organism is a well-known anaerobe indigenous to the intestinal tract of humans and lower animals. The estimated levels of this bacterium in human feces are $10^{3.2}$ (25), 10^5 (26), and $10^{0-8.7}$ (9) per g (wet weight) of feces.

Although it has been established beyond doubt that this species elaborates a potent bile salt hydrolase to split conjugated bile acids (1, 13, 20, 21), the published data concerning the metabolism of the bile acid moieties are equivocal. Norman and Bergman (22) reported the oxidation of cholic acid to 7-ketodeoxycholic acid (7α-dehydrogenation) by broth cultures of *C. perfringens*. Aries and Hill (2) and Macdonald et al. (13) demonstrated 7α- and 3α-dehydrogenase (hydroxysteroid dehydrogenase [HSDH]) activities against cholic acid by cell-free preparations of *C. perfringens*. Later, however, Macdonald et al. (15) noted a conspicuous finding that the cell-free extract of this organism selectively oxidized the 7α-OH group of cholic acid but not of chenodeoxycholic acid and that the activity was closely associated with the oxidation of the 3α-OH group. Recently, Macdonald et al. (14) reported an additional phenomenon that the oxidation of the 3α-OH group of cholic acid but not of chenodeoxycholic acid sterically hinders the enzymatic reactivity of its 7α-OH group and reconsidered the 3α-HSDH activity as being the sole actual dehydrogenation in this organism.

Besides such ambiguity as to the occurrence of 7α-HSDH activity in this organism, the epimerizing conversion of the 3α-OH group which Midtvedt and Normal (17) once suggested takes place in cultures of this species has not been reported since, in spite of frequent observations of the 3α-HSDH activity in this organism (2, 13, 14, 15).

In view of such divergence of opinion, we investigated the transformation of various bile acids by growing cultures of 35 strains of *C. perfringens*, with complementary studies with resting cells. No measurable 7α-HSDH activity was demonstrated by any of the strains, whereas 3α-HSDH activity was exhibited by all of the strains; also, many though not all of the strains were also capable of epimerizing the 3α-hydroxy group into a β-configuration. This communication is concerned with these experimental data.

MATERIALS AND METHODS

Bacterial strains. The toxigenic type cultures of *C. perfringens* listed in Table 1 were kindly supplied by N. Nishida of Kanazawa University, and the strains of *C. perfringens* for the experiments shown in Table 2 were isolated in our laboratory from human feces. Other clostridial strains are our stock cultures originally obtained from the Institute for Infectious Diseases (now renamed the Institute of Medical Science) of Tokyo University. These cultures were maintained

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TABLE 1. *Metabolites formed from conjugated and unconjugated bile acids by 23 laboratory strains of Clostridium*[a]

Bacterial strain	Designation	Amt formed (μg/ml)											
		TDCA		GDCA		CA				CDCA			
		1.00, DCA	0.89, 3β-DCA	1.00, DCA	0.89, 3β-DCA	2.02, CA	1.75, 3β-CA	1.00, DCA	2.8, 7KD	1.12, CDCA	0.96, 3β-CDCA	0.59, LCA	1.68, 7KL
C. perfringens													
Type A	WS1103	63	11	102	17	71	9			72	11		
	PB6K	68	11	102	16	90	16			76	12		
	WK47	68		112		94				90			
Type B	L5	59	10	103	18	82	13			76	11		
	NCTC 4967	64	11	95	17	77	13			71	11		
	NCTC 3110	70	11	100	16	89	15			94	15		
Type C	NCTC 3227	66	12	92	16	88	14			78	12		
	NCTC 3182	68	10	102	17	86	14			80	16		
	L7	65	12	101	18	87	14			77	12		
	C406	68	13	91	16	89	17			83	13		
Type D	D406	59	13	106	18	87	14			79	12		
Type E	L10	70	13	77	13	80	12			81	13		
	NCTC 8084	73	13	97	16	88	12			94	16		
	E406	72	12	92	16	90	16			96	16		
Type F	NCTC 3772	69	12	92	17	86	14			91	15		
C. tetani	Riku-3					92				89			
C. tetani	HA47					91				99			
C. bifermentans	317	77		117		98				90			
C. sordellii	4709	81		115		75		14	12	54		2	43
C. botulinum	Kitasato					95				94			
C. septicum	44			118		113				92			
C. histolyticum	H22					112				108			
C. sporogenes	Denken					104				94			
B. fragilis	2537	90		115		37			72	25			92
None				5		111				103			

[a] Bacterial cultures originally containing the indicated bile acid at 100 μg/ml (see the values of noncell controls) were analyzed after 6 days of anaerobic incubation. Individual bile acids were separated as methyl esters on QF-1 and noted by RRT values (methyl DCA = 1.00).

in cooked-meat medium, transfers being made every 2 or 3 months.

Transformation of bile acids by growing cultures. A buffered peptone-yeast extract broth containing the desired bile acid at 100 μg/ml was prepared as described previously (12) and used as the assay medium. The medium was dispensed in 4-ml quantities in small test tubes and seeded with a standard volume of the test strain grown in peptone-yeast extract-glucose broth for 24 h. After incubation for 6 days in an anaerobic jar with replacement of the atmosphere with a gas mixture of 90% N_2 and 10% CO_2, the spent culture medium was acidified to pH 2.0 with 6 N HCl for extraction of free bile acids.

Resting cell studies. Bacterial cells harvested by centrifugation from an early-stationary-phase culture in GAM broth (Nissui Pharmaceutical Co.) without any added bile salts were washed three times in 0.02 M sodium phosphate buffer at pH 7.0 supplemented with 0.1% sodium thioglycolate (as a reducing agent) and resuspended in the same solution at 40 mg (wet weight) per ml. The reaction mixture consisted of 1.0 ml of the cell suspension, 500 μg of bile acid, and 2.0 ml of 0.1 M sodium phosphate buffer at pH 7.0 (except for testing of pH optimum). After incubation for 4 h under anaerobic or aerobic conditions, the reaction was stopped by adjusting to pH 2.0 with HCl. Anaerobic incubation was carried out in a Thunberg tube under flushing with pure nitrogen, and aerobic incubation was in an L-shaped tube with continuous shaking in air.

Analysis of bile acids by GLC and MS. The acidified samples, spent culture media or resting cell mixtures, were extracted with ethyl acetate, and the extracted free bile acids were methylated for gas-liquid chromatography (GLC) analysis on the conventional 3% QF-1 column. Portions of the methylated samples were then converted into the complete trimethylsilyl ethers for separation on 2% OV-17 and 3% Hi Eff-8B columns. The latter phase was used since it gives a good separation of 3α- and 3β-epimers, whereas the former column was more satisfactory for the detection of oxocholanoates, the silyl ethers of which were markedly retained on Hi Eff-8B column. Individual bile acids were identified by retention time relative to that of the corresponding derivative of deoxycholic acid (relative retention time [RRT]) in reference to the previously published data for known bile acid derivatives (6, 8). The identity was further confirmed by mass spectrometry (MS) of the silyl compounds (24). Quantities of bile acids were calculated from the area of their peaks similarly related to deoxycholate. Noncell tubes were set up in every experiment and served as controls for the recovery of sample bile acids. A detailed account of these analytical procedures was given previously (12).

TABLE 2. *Metabolites formed from conjugated and unconjugated bile acids by 20 local isolates of C. perfringens*[a]

Strain	Amt formed (μg/ml)								
	GDCA			CA			CDCA		
	1.00, DCA	0.89, 3β-DCA	1.78, 3k-DCA	2.02, CA	1.75, 3β-CA	3.8, 3k-CA	1.12, CDCA	0.96, 3β-CDCA	2.14 3k-CDCA
I3	107	19	3	50	7	NC[b]	84	19	12
I35	100	17	2	51	8	NC	92	20	3
IVB101	109	20	1	51	7	NC	85	14	6
S1	90	13	3	48	6	3	86	9	6
S2	89	13	3	45	7	3	87	9	5
S3	105		2	66		3	75		3
SN1	123		1	67		NC	95		2
SN2	107	18	2	53	6	NC	79	11	3
SN3	104	18	2	59	6	NC	86	12	2
SN4	93	14	2	51	7	3	82	9	4
SN5	103	13	3	59	9	3	90	13	4
SN6	109	17	2	56	7	NC	88	12	2
HU1	105	14	1	60	9	3	88	14	5
F1	103	12	NC	57	8	3	91	13	4
F2	101	13	1	56	8	3	91	14	5
F3	94	13	2	52	8	2	90	13	4
FG1	99	12	1	51	9	3	90	13	1
FG3	100	12	1	58	8	4	85	13	6
FG4	110	16	1	58	7	NC	87	13	2
HC1	102	14	2	57	8	4	87	13	5

[a] See Table 1.
[b] Small peak on chromatogram not counted by integrator.

Bile acids. Bile acids are referred to by abbreviations of trivial names, to which the following systematic names are given: CA, cholic acid (3α,7α,12α-triol-5β-cholanoic acid); CDCA, chenodeoxycholic acid (3α,7α-diol-5β-cholanoic acid); DCA, deoxycholic acid (3α,12α-diol-5β-cholanoic acid); 7KD, 7-ketodeoxycholic acid (3α,12α-diol-7-one-5β-cholanoic acid); 7KL, 7-ketolithocholic acid (3α-ol-7-one-5β-cholanoic acid); LCA, lithocholic acid (3α-ol-5β-cholanoic acid); and UDCA, ursodeoxycholic acid (3α,7β-diol-5β-cholanoic acid). The 3β-hydroxy and 3-oxo derivatives of these 3α-hydroxy acids are indicated by the addition of the prefix "3β" or "3k" to the trivial name (e.g., 3β-CDCA stands for the 3β-hydroxy epimer of CDCA). CA was obtained from Sigma Chemical Co., CDCA was obtained from Tokyo Tanabe Pharmaceutical Co., and purified UDCA was kindly donated by T. Setoguchi, Miyazaki Medical College. Other free bile acids were purchased from Gaschro-Kogyo Co. Sodium salts of taurine and glycine conjugates of DCA (TDCA and GDCA, respectively) were from Calbiochem. Except for GDCA, which contained 5% free bile acid as an impurity, all of the preparations were pure when tested by GLC.

RESULTS

Transformation of bile acids by 23 laboratory strains of *Clostridium*. Table 1 summarizes the metabolites formed from conjugates of DCA and unconjugated CA and CDCA by 6-day cultures of these strains. All 15 strains of *C. perfringens* completely hydrolyzed the conjugates of DCA (comparable amounts of DCA were freed after hydrolysis of sterile assay medium in 3 N NaOH at 121°C for 4 h), and all except one (strain WK47) modified the freed DCA to a compound eluting at an RRT of 0.89 (as methyl ester on QF-1), just before the DCA peak. Similar compounds with RRTs of 1.75 and 0.96 were produced as the sole product from CA and CDCA, respectively. In reference to the reported RRT values for known bile acid derivatives (6, 8) and taking into consideration the contention that an axial β-OH group gives a shorter retention time than an equatorial α-OH group at the same carbon atom (23), these minor peaks may be ascribed to the 3β-hydroxy epimers from the respective 3α-hydroxylated major bile acids. The extent of the epimerizing conversion was 10 to 20%, irrespective of the kind of bile acid and the strain of bacterium.

None of the clostridial species other than *C. perfringens* was able to perform the epimerizing reaction described above. Deconjugation was effected by three strains; *C. bifermentans* and *C. sordellii* split both conjugates, but *C. septicum* hydrolyzed only the glycine conjugate with strict specificity. *C. sordellii* strain 4709 deserves attention because of its ability to remove or oxidize the 7α-OH group in CA and CDCA, the typical two primary bile acids of human bile. *Bacte-*

roides fragilis strain 2536, included for comparison, oxidized but did not dehydroxylate the 7α-OH group.

Transformation of bile acids by 20 isolates of *C. perfringens*. Twenty newly isolated strains of *C. perfringens* were tested in the manner described above (Table 2). All of the strains completely hydrolyzed GDCA to release DCA, and all except two strains (S3 and SN1) converted about 10 to 20% of the DCA into its 3β-hydroxy epimer, by analogy with the previous finding. CA and CDCA underwent a similar change.

Included in the table is an additional compound which eluted more slowly than the parent bile acid. It was produced in far less quantity than were the 3β-epimers and varied in quantity from one culture to another. In the light of the published RRT values (6, 8), these additional peaks were attributable to the 3-oxo derivatives of the respective major acid, a possible intermediate in the conversion of the hydroxy group from the α- to the β-position. It should be stressed in this connection that, in contrast with the constant formation of the 3-oxo acids by every strain, not quite all of the strains produced the 3β-hydroxy epimers.

Resting cell studies of transformation of bile acids. To increase the yield of the 3-oxo products, the conversion of 3α-hydroxy bile acids was tested by the use of resting cells which can be incubated under both aerobic and anaerobic conditions and with variation in medium pH (Fig. 1). As against a few percent under anaerobic conditions, 30 to 40% of the 3α-hydroxy acids was converted into 3-oxo products under aerobic conditions, with a broad pH optimum from 7.0 to 9.0. The activity was significantly reduced at pH 6.0 and barely detectable at pH 5.0, suggesting the inhibition of the oxo production in medium containing a fermentable carbohydrate. The epimerizing conversion which probably involves a reductive process (from 3-oxo to 3β-OH) was somewhat suppressed under aerobic conditions but retained no less than 10% conversion aerobically over a broad pH range between 5.0 and 9.0.

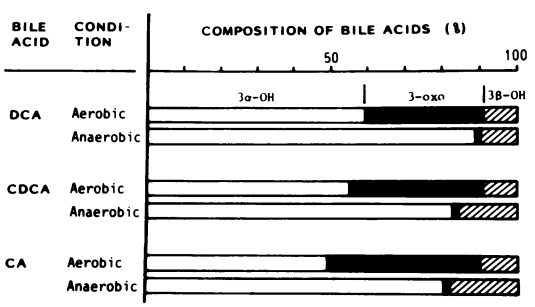

FIG. 1. *Transformation of three representative bile acids by resting cells of C. perfringens under aerobic and anaerobic conditions. Washed whole cells of strain PB6K (see Table 1) were incubated with each of the bile acids at pH 7.0 for 4 h. Metabolites were quantified as methyl esters on QF-1 and expressed in percentage composition.*

Transformation of seven 3α-hydroxy bile acids by resting cells and identification of 3-oxo and 3β-hydroxy products by GLC and MS. From the above data, seven conventional bile acids, all of which had an α-OH group at C-3, were incubated with resting cells of *C. perfringens* at pH 7.0 under aerobic conditions to provide the respective 3β-hydroxy and 3-oxo products. The metabolites were separated on QF-1 after methylation of carboxyl group and rechromatographed on OV-17 and Hi Eff-8B after trimethylsilylation of all hydroxy substituents; all of the GLC effluents were submitted to MS analysis. (The systematic GLC and MS data of these novel bile acids will be published elsewhere [S. Hirano, N. Masuda, M. Tamaki, and H. Akimori, Acta Med. Univ. Kagoshimaensis, in press].) Figure 2 presents examples of chromatograms in which the silyl derivatives of the metabolites were chromatographed on OV-17 and partly on Hi Eff-8B. As the figure shows, the respective 3β-hydroxy and 3-oxo substituents were invariably derived from all seven of the 3α-hydroxy bile acids.

DISCUSSION

In contrast with the results of the studies of Norman and Bergman (22), Aries and Hill (2), and Macdonald et al. (13, 15), the present work did not provide any evidence for the occurrence of 7α-HSDH activity among the 35 strains of *C. perfringens*. By analysis of metabolites by means of GLC which is sufficiently sensitive to detect a few micrograms of bile acids, no 7-oxo products were detected from either CA or CDCA under any experimental conditions, by growing or resting cells or under anaerobic or aerobic incubation. 12α-HSDH activity suggested by Macdonald et al. (15) also was not demonstrable by any of the strains. The sole HSDH activity effected by these strains was 3α-dehydrogenation. The reason for the discrepancy is not clear at present, but the survey of the successive data of Macdonald et al. (13–15), quoted previously, seems to offer some suggestions. They reached 3α-dehydrogenation as the sole reaction starting from the idea of multi-dehydrogenases.

The 3α-HSDH was synthesized by all of the strains, constitutively in bacterial cultures with no added bile salt, and acted on a whole range

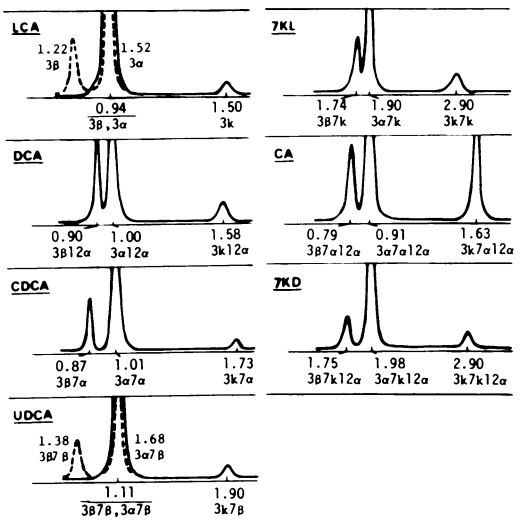

FIG. 2. *Chromatographic separation of 3β-hydroxy and 3-oxo products from seven 3α-hydroxy bile acids by C. perfringens resting cells (strain PB6K). Washed whole cells were incubated for 4 h with each of the bile acids at pH 7.0 under aerobic conditions. Bile acid extracts were separated as methylcholanyl trimethylsilyl ethers on OV-17. For LCA and UDCA, the same silylated samples were rechromatographed on Hi Eff-8B to differentiate the 3α- and 3β-hydroxy epimers (broken lines). Individual bile acids are noted by RRT values (the silyl ether of methyl DCA = 1.00), and identity is given by substituents in 5β-cholanoic acid (α, α-OH; β, β-OH; k, oxo group).*

of 3α-hydroxy bile acids showing broad substrate specificity. The 3-oxo production, however, was barely detectable under anaerobic cultural conditions, but highly enhanced (to 30 to 40% conversion) by aeration of resting cells (no enhancement by the same cell preparation incubated under anaerobic conditions). It seems likely that atmospheric oxygen may act as an efficient electron acceptor of the dehydrogenation or that a highly elevated redox potential of the medium is needed for the oxidative process to proceed.

Besides the oxidative 3α-HSDH activity, epimerizing conversion of the 3α-hydroxy group into a β-position was effected by most but not all of the strains. The yield of 3β-epimers amounted to 10 to 20%, irrespective of the kind of bile acid and of the bacterial strain and independently of the state of cells, growing or resting. It is worthy of note that, although the appearance of these 3β-hydroxy epimers was accompanied in each case by the formation of a 3-oxo acid, the reverse was not necessarily the case, as exemplified by the three strains which were capable of oxidizing but failed to epimerize the 3α-OH group. If the epimerizing conversion is carried out by way of an oxo intermediate, i.e., by oxidation of the 3α-OH group to a 3-oxo group followed by the stereospecific reduction of the oxo group to a 3β-OH group, these three strains lack the latter step of the reaction sequence. This cannot be explained by one-enzyme concept as suggested by Hayaishi et al. (10), but favors the involvement of two separate enzyme entities in the conversion as evidenced in *Pseudomonas testosteroni* (4, 16). It seems highly probably that the three strains are devoid of 3β-HSDH though provided with 3α-HSDH, whereas most of the strains of *C. perfringens* elaborate both α- and β-enzymes to epimerize the 3α-OH group. It should be noted that the epimerizing conversion by resting cells took place to a considerable extent even under continuous shaking in the air. The reductive process of 3β-HSDH, if it were involved, seems to have been considerably oxygen tolerant.

Similar microbial 3-epimerization attendant upon oxo formation in several strains of *C. perfringens*, *Bacillus cereus*, and *Eubacterium* species has been reported by Midtvedt and Norman (17) and in two strains of anaerobic streptococci by Dickinson et al. (5). A comprehensive evaluation of *C. perfringens* in this respect, however, was attempted for the first time in this study. The prevalence of this property among the individual strains of this species contributes to the understanding of the constant appearance of various 3β-hydroxy bile acids in in vitro cultures of human feces (S. Hirano, N. Masuda, and H. Oda, J. Lipid Res., in press) and rat intestinal contents (18, 19), and also of the occurrence of these epimeric bile acids as normal components of fecal bile acids of humans (3, 7, 11).

In this study, 3-oxo and 3β-hydroxy derivatives were obtained from conventional 3α-hydroxy bile acids by the action of *C. perfringens*. In other words, a whole range of these novel bile acids can be provided with ease by bacteriological measures. It is convenient for this purpose that the organism affects only the 3α-OH substituent with these products and exerts no effect on other parts of the steroid nucleus.

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