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IDENTIFICATION OF 7α-,12α-DIHYDROXY-3-OXO CHOLANOIC ACID AS THE MAJOR DEGRADATION PRODUCT FROM CHOLIC BY *C. PERFRINGENS*

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SUMMARY

The major degradation product of cholic acid by *C. perfringens*, was identified as 3-dehydrocholic acid by n.m.r. and mass spectroscopic analysis of its methyl ester. 3-Dehydrocholic acid was shown to be unreactive with *P. testosteroni* 3α-hydroxysteroid dehydrogenase (forward direction) and relatively unreactive with 7α-hydroxysteroid dehydrogenase. Thus, both 3α-OH and 7α-OH bioconversion determinations in spent bacterial media have been shown to be useful in estimating the amount of 3-dehydrocholic acid formed from cholic acid in whole cell cultures of 63 strains of *C. perfringens*.

INTRODUCTION

Bile acids* can be oxidized by the normal microbial flora in man to the 3, 7 and 12-ketones [1–3]. Some species have been shown to contain stereospecific enzymes, for example, *E. coli* [4–6] and *B. fragilis* [7, 8] both contain a 7α-hydroxysteroid oxidoreductase which converts cholic acid into 7-dehydrocholic acid [1]. Cholic acid is also oxidized by *C. perfringens* but confusion still exists in the literature [9–11] as to the identity of the oxidation products. Norman and Bergman[9] concluded that the major product is 7-dehydrocholic acid on the basis of chromatographic evidence and the recrystallization of a mixture of ^{14}C-labelled product and unlabelled standard to constant S.A. Macdonald *et al.*[11], on the basis of chromatographic and enzymatic evidence disputed the claim that the major product was 7-dehydrocholic acid and tentatively identified it as 7β-,12α-dihydroxy-3-oxo-cholanoic acid.

In view of the divergent claims, it appeared that unequivocal proof of the structure of the major oxidation product was necessary. This has been obtained from nuclear magnetic resonance and mass spectroscopic data, which we now report along with chromatographic data and results of specific enzyme-catalysed conversions.

EXPERIMENTAL PROCEDURE

Materials

Chemicals. Oxo-bile acid standards were obtained from Steraloids. All other chemicals were Baker reagent grade products.

* The systematic nomenclature of bile acids referred to in this report by trivial names are as follows: cholic, 3α-,7α-,12α-trihydroxy-5β-cholanoic; chenodeoxycholic, 3α-,7α-dihydroxy-5β-cholanoic; deoxycholic, 3α-,12α-dihydroxy-5β-cholanoic; taurocholic, 3α-,7α-,12α-trihydroxy-5β-cholan-24-oyltaurine; 3-dehydrocholic, 3α-,7α-dihydroxy-3-oxo-5β-cholanoic; 7-dehydrocholic, 3α-,12α-dihydroxy-7-oxo-5β-cholanoic.

Solvent systems. Solvent systems utilized were (a) chloroform–methanol–acetic acid (40:4:2, by vol.), (b) chloroform–methanol–acetic acid (40:21:1, by vol.), and (c) benzene–dioxane–acetic acid (75:20:2, by vol.) (corresponding to solvent S_1 first described by Eneroth[12].

Detection of bile acids on t.l.c. plates. Plates were sprayed with either . Komarowsky's reagent (*p*-hydroxybenzaldehyde) [13] or Anisaldehyde reagent [14] and heated with 20 amp heat gun for 1–2 min. They were inspected under visible and long wave ultra violet light.

Oxidation of methyl cholate by C. perfringens *in a cell-free system.* A reaction mixture consisting of 0.17 M glycine–NaOH pH 9.5, 1.7×10^{-3} M NAD, 10^{-3} M methylcholate and 40 mg lyophylized cell-free preparation of *C. perfringens*, total vol. 30 ml was stirred gently overnight at room temperature. The pH was adjusted to 3–4 (pH paper) with 0.1 M HCl and extracted with two 30 ml vol. of ether. The ether was evaporated under nitrogen and the extract was dissolved in approximately 1 ml of methanol–water (5:1, v/v). The entire vol. was applied in a band on a t.l.c. plate (1.0 mm thick) and chromatographed in solvent B. End strips of the plate were sprayed with *p*-hydroxybenzaldehyde spray reagents and selectively heated with a heat gun. Unsprayed areas corresponding to the major product were removed, and eluted in a sintered glass filter with three 5 ml vol. of methanol–ether (1:1, v/v). The solvent was evaporated and the residue redissolved in a minimal vol. of the same solvent and twice recrystallized at −20°C.

Oxidation of cholic acid in whole cell cultures. Strain 20 of *C. perfringens* was grown in a 200 ml vol. as described before [11] except in the presence of 10^{-3} M cholic acid. Four day old cultures were harvested and the spent bacterial medium adjusted to pH 3.0. Extraction, concentration of extract and t.l.c. were performed as above.

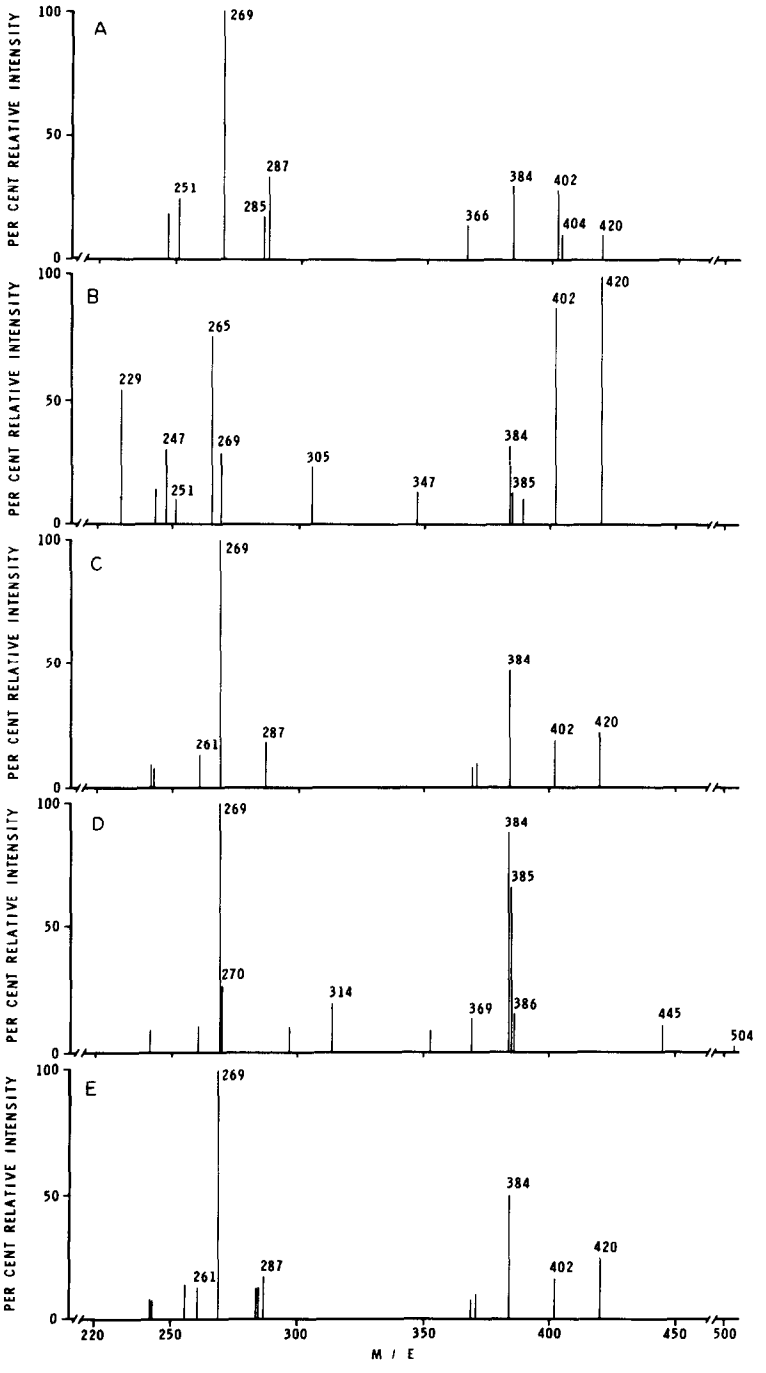

Fig. 1. Mass spectra of standards and clostridial degradation product. (A) 3α-,12α-dihydroxy-7-oxo-5β-cholanoate-Me, (B) 3α-,7α-dihydroxy-12-oxo-5β-cholanoate-Me, (C) 7α-,12α-dihydroxy-3-oxo-5β-cholanoate-Me, (D) methylated purified oxidation product from *C. perfringens*.

Preparation of the methyl ester of 3-dehydrocholic acid from P. testosteroni. Procedure was identical to that used in the oxidation of methyl cholate in a cell-free *C. perfringens* system except that 10 mg purified *P. testosteroni* 3α-hydroxysteroid dehydrogenase (Worthington) was substituted for the cell-free preparation of *C. perfringens*.

Methylation of microbial oxidation product. Purified oxidation product isolated from *C. perfringens* spent bacterial medium was stirred with excess diazomethane in ether–methanol (9:1, v/v) for 30 min at room temperature. Diazomethane was prepared according to van Berge Henegouwen *et al.*[15].

Mass spectroscopy studies. Mass spectra were obtained on a 21-104 mass spectrometer operating under the following conditions: direct probe temperature, 120°C; source temperature, 150°C; ionization potential, 70 ev; accelerating voltage, 800 V.

Nuclear magnetic resonance studies. n.m.r. Spectra were obtained from 0.5% solutions of compound in deuterochloroform on a Varian CFT 20 operating in proton mode.

Use of 3α-, 7α- and 12α-hydroxysteroid dehydrogenases in the characterization of the major degradation product of C. perfringens. About 40 μg (0.10 μmol) of microbial oxidation product or standard was introduced into a cuvette (vol. 3.0 ml) containing 0.17 M glycine–NaOH pH 9.5, 1.7×10^{-3} M NAD, and 50 μl of purified *P. testosteroni* 3α-hydroxysteroid hydrogenase [16, 17] (Worthington). Alternately, *E. coli* 7α-hydroxysteroid dehydrogenase (4 mg/ml) [16] or NADP dependent 12α-hydroxysteroid dehydrogenase from *Clostridium sp* gr P (40 mg/ml) [18] were used. Increase in absorbance at 340 nm was monitored with a Beckman DBGT spectrophotometer and 10 inch recorder. The reverse reactions were achieved similarly, except that 0.10 sodium phosphate buffer, pH 6.0 and 10^{-4} M NAD(P)H were used.

Tandem oxidation studies of cholic acid and chenodeoxycholic acid with 3α- and 7α-hydroxysteroid dehydrogenases. Conditions identical to those described above were used with substrate cholic or chenodeoxycholic acids; 3α- and then 7α-hydroxysteroid dehydrogenase were introduced into the cuvette sequentially (allowing the first phase of the reaction to complete before introducing the second enzyme). The enzyme addition sequence was varied.

Estimation of the loss of measureable 3α- and 7α-OH groups from taurocholic acid and deconjugation in spent bacterial media in 63 strains of C. perfringens. Strains of *C. perfringens* [11, 19] were grown in 10 ml vol. of freshly autoclaved brain heart infusion broth containing 10^{-3} M taurocholic as previously described [10]. Total measureable 3α- and 7α-OH group determinations and deconjugation in the spent bacterial medium were done as before [10].

RESULTS AND DISCUSSION

A direct comparison of the mass spectra of the methyl esters of the 3-, 7- and 12-dehydrocholic acids shows that the three isomers are readily distinguished from one another (Fig. 1A, B, C). The mass spectra of the *C. perfringens* oxidation product of methylcholate was essentially superimposable upon that of 3-dehydrocholic acid (Fig. 1D). Proton nuclear magnetic resonance spectral comparisons confirmed that the oxidation had taken place at the 3-position. Table 1 gives the chemical shifts of the predominant peaks of the standards and oxidation product.

Table 1. Chemical shift data for methyl cholanoates*

	C_{18}—CH_3		C_{19}—CH_3		OCH_3	CHOR
	obs	calc†	obs	calc†	obs	obs
3α-, 12α-Dihydroxy-7-oxo-5β-	0.67	0.70	1.16	1.19	3.64	—, 3.85
3α-,7α-Dihydroxy-12-oxo-5β-	1.01	1.03	1.00	1.02	3.64	—, 3.83
7α-,12α-Dihydroxy-3-oxo-5β-	0.73	0.73	0.99	1.02	3.64	3.87, 3.81
Methylated oxidation product from *C. perfringens*	0.73	—	0.99	—	3.64	3.87, 3.81

* Chemical shifts are δ values in ppm relative to tetramethylsilane.
† Calculated according to Bhacca and Williams[20].

Table 2. Thin layer chromatography studies of commercial standards and purified degradation product from *C. perfringens*

Cholanoic acid or methyl ester	R_F value*				Color with Anisaldehyde or *p*-hydroxybenzaldehyde
	A	B	C	C†	
3α-,12α-Dihydroxy-7-oxo-5β-‡	0.74	0.62	0.50	0.45	khaki–green
3α-,7α-Dihydroxy-12-oxo-5β-‡	0.74	0.62	0.50	0.45	gray–green
7α-,12α-Dihydroxy-3-oxo-5β-§	0.79	0.66	0.52	1.31	wine–rose
Oxidation product by *C. perfringens*	0.79	0.66	0.52	—	wine–rose
3α-,12α-Dihydroxy-7-oxo-5β-Me‡	1.2	1.5	0.90	—	khaki–green
3α-,7α-Dihydroxy-12-oxo-5β-Me‡	1.2	1.5	0.90	—	gray–green
7α-,12α-Dihydroxy-3-oxo-5β-Me‡	1.3	1.7	1.0	—	wine–rose
Methylated oxidation product from *C. perfringens*	1.3	1.7	1.0	—	wine–rose
Methylated oxidation product by *P. testosteroni* 3α-hydroxysteroid dehydrogenase	1.3	1.7	1.0	—	wine–rose

* Relative to the mobility of 3α-,7α-dihydroxy-5β-cholanoic acid (chenodeoxycholic acid).
† Values from Eneroth[12].
‡ Obtained from Steraloids.
§ From *P. testosteroni* 3α-hydroxysteroid dehydrogenase oxidation of cholic acid

Table 3. Reactivity of microbial oxidation products and standard with group specific oxidoreductases*

Compound	3α-Hydroxysteroid dehydrogenase		7α-Hydroxysteroid dehydrogenase		12α-Hydroxysteroid dehydrogenase	
	forward	reverse	forward	reverse	forward	reverse
C. perfringens oxidation product	−	+	−	−	+	−
P. testosteroni 3α-hydroxysteroid dehydrogenase oxidation product (3-dehydrocholic acid)	−	+	−	−	+	−

* See text for reaction conditions.

Thin layer chromatography using three solvent systems including one reported [12] to clearly separate the 3-dehydrocholic acid from the 7- and 12-dehydrocholic acids proved ineffective in separating the acids or esters (Table 2). The mobility of the 3-dehydrocholic acid was very similar to that of the 7- or 12-isomers, and not markedly different as reported by Eneroth[12]. However, the color obtained with either anisaldehyde [14] or *p*-hydroxybenzaldehyde spray reagent [13] clearly distinguishes these compounds.

In characterizing the *C. perfringens* cholic acid oxidation product with 3α-, 7α- and 12α-oxidoreductases, the results (Table 3) indicated the presence of a 3-carbonyl group and a 12α-hydroxyl group, but were surprisingly negative for the 7α-hydroxyl group. The relative lack of reactivity of this compound with *E. coli*, 7α-hydroxysteroid dehydrogenase was verified by testing the 3-dehydrocholic acid obtained from oxidation of cholic acid by *P. testosteroni*, which behaved identically (Table 3).

When cholic acid was subjected to sequential tandem oxidation studies, with *P. testosteroni* 3α-hydroxysteroid dehydrogenase and *E. coli* 7α-hydroxysteroid dehydrogenase, the relative unreactivity of the 7α-hydroxysteroid dehydrogenase, against the 3-dehydrocholic acid-intermediate was demonstrated (Fig. 2). However, in contrast to cholic acid, oxidation of the 3 position of chenodeoxycholic acid did not tend to abolish the reactivity of this substrate with 7α-hydroxysteroid dehydrogenase (Fig. 2).

It has already been shown that cholic acid displays a Michaelis constant (K_M value) more than a factor of ten greater than that of 3α-,7α-dihydroxy-5β-cholanoic acid [4]. Haslewood[6] has shown that sulfation of the 3 position of either of these molecules abolishes their reactivity with the 7α-hydroxysteroid dehydrogenase. Thus, it is evident that substituents not adjacent but sterically close to the 7 position can have a significant effect on the reactivity of the substrate with this enzyme.

Consistent with these results, estimation of the 3α-OH and 7α-OH bioconversion of cholic acid [10] by 63 different strains of *C. perfringens* indicated a direct correlation ($r = 0.90$) between the disappear-

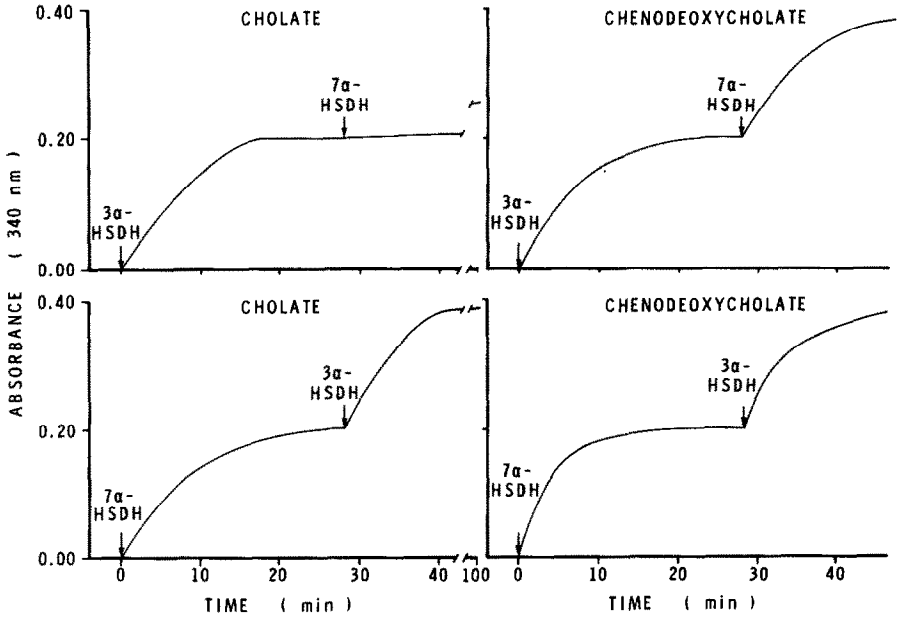

Fig. 2. Tandem oxidation of 0.10 μmol of (A) cholic acid with 3α- and then 7α-hydroxysteroid dehydrogenase, (B) cholic acid with 7α- and then 3α-hydroxysteroid dehydrogenase, (C) chenodeoxycholic acid with 3α- and then 7α-hydroxysteroid dehydrogenase, (D) chenodeoxycholic acid with 7α- and then 3α-hydroxysteroid dehydrogenase.

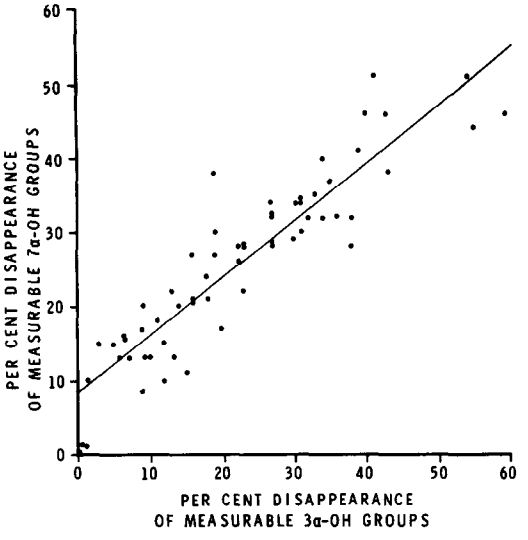

Fig. 3. Percent disappearance of 3α-OH groups (abscissa) plotted against percent disappearance of 7α-OH groups for spent bacterial media of 63 strains of *C. perfringens* grown in the presence of 3α-,7α-,12α-trihydroxy-5β-cholanoic acid.

ance of measureable 3α-OH groups and the disappearance of measureable 7α-OH groups (Fig. 3). t.l.c. of extracts of these strains revealed one major product identified as 7α-,12α-dihydroxy-3-oxo-5β-cholanoic (6 strains tested). The amount of compound visible on t.l.c. correlated directly with the amounts of 3α- and 7α-OH bioconversion. Those strains which did not produce 3-dehydrocholic acid (t.l.c. results) did not bioconvert at the 3α- and 7α-OH positions (Table 4).

The reason for the somewhat greater values for 7α-OH bioconversion than for 3α-OH bioconversion (Fig. 3 and Table 4) is not clear. A number of barely detectable minor products observable on t.l.c. of *C. perfringens* spent bacterial media may collectively represent an increase in reaction products devoid of measureable 7α-OH groups; one minor product has been previously identified as 12α-hydroxy-3,7-dioxo-5β-cholanoic acid [11]. Differences between the various strains of *C. perfringens* in their ability to produce this 3-dehydrocholic acid were quite reproducible in repeated experiments and appear as genuine strain differences. The relationship between the ability of a given strain to produce this compound and the oxidoreductase content of the organism is being investigated. The identity of minor products by *C. perfringens* is also being further pursued.

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Table 4. Reproducibility of 3α- and 7α-OH bioconversion and deconjugation data and formation of 7α-,12α-dihydroxy-3-oxo-5β-cholanoic acid by whole cell cultures of *C. perfringens*

Strain*	Estimated % 3α-OH bioconversion		Estimated % 7α-OH bioconversion		% Deconjugation		Presence of 7α-,12α-dihydroxy-3-oxo-5β-cholanoic acid on t.l.c. of spent bacterial medium
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	
4	6	3	5	6	100	100	—
10	43	55	36	44	101	97	+ + +
20	44	54	43	51	98	100	+ + +
23	41	41	42	40	103	100	+ + +
33	6	2	8	1	100	106	—
43	16	12	16	10	100	100	+

* Numbering system as described earlier [11, 19].

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