Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 117–124. Pergamon Press. Printed in Great Britain

THE BIOSYNTHESIS OF ETHYL LITHOCHOLATE BY FECAL MICROORGANISMS

MORRIS I. KELSEY* and RICHARD J. THOMPSON†

*NCI Frederick Cancer Research Center,†† Frederick, Maryland 21701,
†Harleco Division of American Hospital Supply Corp., Gibbstown, New Jersey, U.S.A.

(*Received* 2 *June* 1975)

SUMMARY

The metabolism of [^{14}COOH]-lithocholic acid anaerobically by heavy cell suspensions of *Bacteroides fragilis* ss *thetaiotaomicron*, (strains 6 and 11, respectively), *Citrobacter* sp. and *Peptostreptococcus productus* I resulted in the production of a neutral metabolite (2–5%) as determined by thin-layer chromatography. An 80-fold increase in the concentrated cell suspensions of citrobacter and unlabeled lithocholic acid gave an extract which was partially purified by Sephadex LH-20 chromatography. The neutral metabolite isolated from this purification had identical thin-layer and gas–liquid chromatographic properties with those of authentic ethyl lithocholate, and combined gas–liquid chromatography–mass spectrometry data supported the structure for ethyl lithocholate and not its 3β-epimer. Further studies have shown that the formation of ethyl lithocholate requires the presence of ethanol, concentrated cell suspensions and anaerobic growth conditions. Under these circumstances *B. fragilis* ss *thetaiotaomicron* (strain No. 6) can achieve a conversion of 15% of lithocholic acid to its ethyl ester, citrobacter 10% and *P. productus* I less than 1%. The ability of select strains of fecal microorganisms to esterify a toxic bile acid such as lithocholic acid represents a novel reaction which may enhance or reduce its toxicity and/or carcinogenic potential in the colon.

INTRODUCTION

Environmental factors are generally thought to be involved in the etiology of a majority of human cancers [1]. Two integral components of our human environment include our diet and the intestinal microflora which have been postulated to be responsible for cancers of the colon, breast and stomach.

Evidence obtained from epidemiological studies [2 4] indicates that a higher incidence of colon cancer can be found in those groups whose diet is rich in beef and fat. This type of diet results in an alteration in the composition and capacity of the microflora to metabolize fecal steroids and bile acids which are derived from the diet to derivatives that might be carcinogenic to colonic tissue [1, 5].

These excretory products are more highly degraded than those sterols and bile acids found in low risk populations [2]. One of the major fecal bile acids excreted in man and animals is lithocholic acid [6–8] which is derived from the 7α-dehydroxylation of chenodeoxycholic acid by the intestinal microorganisms.

Since lithocholic acid is a toxic bile acid in humans [7] and has been shown to exert tumor-promoting activity in animal studies [9], it was of considerable interest to study its metabolism by select fecal organisms in an attempt to isolate metabolites which could then be evaluated for their carcinogenic potential.

EXPERIMENTAL

Material. The following bile acids were obtained from Omni Research, Mayaguez, Puerto Rico: lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid; LA), isolithocholic acid (3β-hydroxy-5β-cholan-24-oic acid; IL), 3-keto-5β-cholan-24-oic acid (3-keto) and deoxycholic acid (3α, 12α-dihydroxy-5β-cholan-24-oic acid). Other bile acids used in this work included 5β-cholan-24-oic acid (5β), 3β-hydroxychol-5-en-24-oic acid (3β, 5-ene) and 3β-hydroxy-5α-cholan-24-oic-acid (3β, 5α) which were purchased from Schwartz–Mann (Orangeburg, New York). The 3-keto-4-cholen-24-oic acid (3-keto, 4-ene) was prepared from 3-keto by Dr. Gary Muschik using procedures described by Kallner[10]. The sodium salt of taurolithocholic acid (TL) was obtained from Calbiochem (San Diego, California), and [^{14}COOH]-LA was purchased from Mallinckrodt Chemical Co. (St. Louis, Mo.) and had a specific activity of 5·37 mCi/mmol. This labeled substrate had a purity greater than 98% as judged by t.l.c. described in the Methods section.

Liquid scintillation cocktails used in all radioactive protocols included Instagel (Packard Instruments, Warrenville, Illinois) and Aquasol (New England Nuclear, Boston, Massachusetts). All radioactive assays were conducted on an Isocap/300 Liquid Scintillation System (Nuclear Chicago, Des Plaines, Illinois), and samples were corrected for background and

* M. I. Kelsey: To whom correspondence should be addressed.

†† Research sponsored by the National Cancer Institute under Contract No. NO1-CO-25423 with Litton Bionetics, Inc.

Table 1. Metabolism of sodium taurolithocholate by intestinal microorganisms

Source	Strain	FCRC(#)[1]	Amount[2] LA
VPI	Bacteroides fragilis ss distasonis	3	-
	Acidaminococcus fermentans	4	(+)
	Fusobacterium praunsnitzii	5	-
	Bacteroides fragilis ss thetaiota-omicron	6	++
	Bifidobacterium infantis	7	++
	Peptostreptococcus sp A	8	-
	Peptostreptococcus productus II	9	+
	Bifidobacterium adolescentis	10	++
	Streptococcus intermedius	20	(+)
UCLA	Fusobacterium sp 2	21	(+)
	Fusobacterium sp 2	22	-
	Fusobacterium sp 3	23	+
	Fusobacterium sp 4	24	+

1. Numbers refer to the Frederick Cancer Research Center (FCRC) collection of microorganisms.
2. (+) = faint spot on t.l.c.; + = moderate spot; ++ = strong spot.

quenching which was determined by the external standard ratio method.

All other reagent-grade solvents used were purchased from either Fisher Scientific Co. (Pittsburgh, Pa.) or Burdick–Jackson (Muskegon, Michigan).

METHODS

Growth of microorganisms. Microorganisms used in these studies had either been isolated from the feces of a polyposis patient by the staff of the Anaerobe Laboratory (Virginia Polytechnic Institute and State University, Blacksburg, Virginia) or were provided by Dr. Sydney Finegold of U.C.L.A. as indicated in Table 1. Brain heart infusion (BHI) medium was prepared according to procedures described in the V.P.I. Anaerobe Laboratory Manual [11].

Cultures used in these studies were maintained either on pre-reduced BHI agar slants, BHI or chopped meat media [11]. The latter cultures were used as seed inocula for preparation of cell suspensions described in the Results section. All operations were conducted with the V.P.I. Anaerobic Culture System (Bellco Glass, Inc., Vineland, N.J.) which employed oxygen-free CO_2 for maintaining anaerobic growth conditions.

Seed cultures were incubated from 18–24 h at 37°C and incubations with added substrates were continued for 18–72 h as indicated.

Extraction techniques. After the designated incubation periods, cell suspensions of organisms were allowed to cool to room temperature before acidifying to pH 1 with 12 N HCl. Five vol. of chloroform–methanol (2:1 v/v) were used to extract the bile acid metabolites, and the organic layer was washed once with one volume of water corresponding to the original vol. of cell suspensions. Removal of the organic solvent at 30–35°C using a rotary evaporator equipped with a Dewar condenser (Buchler Co., Fort Lee, N.J.) gave a residue which was analyzed as described below.

Thin-layer chromatography. Pre-coated plates with silica gel G (Analtech, Inc., Newark, Delaware) of either 250 or 500 mμ thickness were used for analytical or preparative purposes, respectively. The S-VIII system of Eneroth [12] consisting of isoamyl acetate–propionic acid–n-propanol–water (4:3:2:1 by vol.) was used to detect the deconjugation of TL to LA by certain fecal microorganisms listed in Table 1.

The t.l.c. system of benzene–dioxane–acetic acid (100:10:1 by vol.) was used to assay the purity of [^{14}COOH]-LA and the production of metabolites as well as monofunctional bile acid reference standards used in these studies.

Purification of methyl lithocholate (ML) by preparative t.l.c. was achieved on a 500 mμ-thick plate which was pre-washed and developed in an acetone–benzene (10:90 v/v) system. After drying at room temperature the plate was sprayed with water, and the gel corresponding to the ML was scraped and eluted with 15 ml of methanol; the solvent was evaporated to dryness on a rotary evaporator, and the residue was further characterized by appropriate analytical methods described below.

For radioactive experiments, zones of silica gel corresponding to the mobilities (R_F) of reference standards were scraped into vials and assayed by liquid scintillation counting (LSC).

Glass fibre paper chromatography (g.f.p.). Glass fibre sheets coated with silica gel G (Gelman Instrument Co., Ann Arbor, Michigan) were also used to assay unlabeled and labeled incubation mixtures with the

solvent system: isooctane–isopropyl ether–acetic acid (75:30:1 by vol.). Visualization of spots by either t.l.c. or g.f.p. was achieved by charring plates or sheets which had been sprayed with an ethanol–sulfuric acid–water (2:2:1 by vol.) solution.

Preparation of bile acid derivatives. Methyl esters of bile acids were prepared using diazomethane generated from Diazald (Aldrich Chemical Company, Inc., Milwaukee, Wisconsin) according to procedures based on the work of deBoer and Backer[13]. Preparation of ethyl lithocholate (EL) and ethyl isolithocholate (EIL) was carried out by dissolving the respective acids (28 mg) in 2·0–5·0 ml of ethanol-hydrochloric acid (1·0 ml of 12 N HCl and 40 ml of ethanol) and heating the solution for 1 h at 55°C with occasional shaking. After standing at room temperature for 12–15 h the solutions were extracted with 5 vol. of chloroform which was washed with one volume of water. The organic layer was evaporated to dryness at 30–35°C using a rotary evaporator, and the residues obtained were used as aids in the identification of metabolites.

Silylation of methyl deoxycholate was carried out with Tri-Sil in dimethylformamide, whereas N-trimethylsilylimidazole was used for the mono-functional bile acids. Both reagents were obtained from Pierce Chemical Co. (Rockford, Illinois).

Gas–liquid chromatography (g.l.c.). Analyses by g.l.c. were performed on a Shimadzu, model 4 BM, Gas Chromatograph (American Instrument Co., Silver Spring, Md.) using silanized coiled glass columns. A 91 cm. long, 0·3 cm. i.d. column of 3% OV-17 on 80–100 mesh SW was packed by the manufacturer and was operated at an oven and injection port temperature of 260°C and detector at 280°C using helium as a carrier with a flow of approximately 60 ml/min.

The second g.l.c. phase used for analysis was a 182 cm. long, 0·3 cm. i.d. glass column packed with either 1% or 3% QF-1 on Gas Chrome Q (100–120 mesh) which was obtained from Supelco (Bellefonte, Pa.). Operating conditions included oven and injection port temperatures of 235°C or 210°C and detector oven at 280°C. Helium gas was again used as a carrier at a flow of approximately 60 ml/min.

Gas–liquid chromatography–mass spectrometry (g.l.c.–MS). The g.l.c. inlet system used was a Varian Aerograph (Varian Associates, Palo Alto, California) equipped with a 1% QF-1 column on Gas Chrome Q (100–120 mesh) that was maintained at 240°C with methane gas as carrier (25 ml/min). The effluent was ionized with methane at 100°C, 150 V electron energy and 500 mA emission current in a Finnegan Model 1015 (Finnigan Corp., Sunnyvale California) g.l.c.–MS instrument equipped with a System 250 Data System (Systems Industries, Sunnyvale, California).

Sephadex LH-20 chromatography. Approximately 7 g of Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, N.J.) were swollen in isooctane–chloroform–methanol (2:1:1 by vol.) overnight and packed into a column (1 × 36 cm.) that contained a bed vol. of 28 ml. Extracts were dissolved in a minimal vol. of the above solvent, and the column was eluted with the same solvent collecting 1–2 ml fractions. These fractions were assayed either by t.l.c. or g.f.p. in the case of unlabeled extracts or by combined t.l.c. (g.f.p.)-LSC in radiolabeled extracts.

RESULTS

Deconjugation of TL. Since the hydrolysis of conjugated bile acids by intestinal bacteria has been reported previously [14–16], we examined a number of purified strains in our laboratory for their capacity to split the amide bond of TL. The substrate was added as an aqueous solution (final concentration of 0·1 mg/ml) to cell suspensions of each organism which had been prepared from an inoculum grown in BHI medium anaerobically at 37°C for 15–18 h prior to addition of the bile salt.

Extraction of the acidified suspension in the usual manner gave an extract which was analyzed by t.l.c. using the S-VIII solvent system of Eneroth [12]. Table 1 indicates that 9 of the 13 strains tested were capable of producing LA, and deconjugation was more prevalent among the bacteroides and bifidobacterium genera as was found by Drasar, Hill and coworkers [14, 15].

One of the extracts derived from the incubation of TL with *B. fragilis* ss *thetaiotaomicron* (strain No. 6) was methylated and purified by preparative t.l.c. in benzene–acetone (90:10 v/v). The gas–liquid chromatographic profile on 3% QF-1 was identical with that of ML both as the free hydroxyl and trimethylsilyl (TMSi) derivative. Combined g.l.c.–MS gave a spectrum which was identical with that of authentic ML with no molecular ion (M^+) at *m/e* 390 and a base peak of *m/e* of 372 (loss of water).

[^{14}*COOH*]-*LA.* [^{14}COOH]-LA (1 mμ Ci) was added as an ethanolic solution to heavy cell suspensions (2·5–3·0-fold concentrated) of *B. fragilis* ss *thetaiotaomicron* (strain No. 11) citrobacter (strain No. 17) and *P. productus* I (strain No. 18) since preliminary experiments had shown these strains to be the most active. The concentrated cell suspensions were prepared by inoculating 30 ml of BHI medium from stock BHI stab cultures anaerobically and incubating the cell suspension for 18 h at 37°C in 50-ml, screw-capped, Teflon-lined test tubes. The organisms were then transferred in a CO_2 atmosphere into stainless steel centrifuge tubes fitted with rubber gasket-lined caps that maintained an anaerobic environment and were spun at 2500 ***g*** for 20 min. The cells were resuspended in 10 ml of BHI and were spun as described above two additional times. The pellet obtained from this procedure was suspended in a final volume of 10 ml BHI from which 5 ml were used for incubation purposes.

A control of [^{14}COOH]-LA in BHI without bacteria was included with all incubations which were maintained as stationary cultures for 24 h at 37°C.

Table 2. Summary of radioactive distribution[1] of extracts from incubation of [^{14}COOH]-LA with FCRC strains No. 11, 17 and 18[2]

COMPD.	ZONE	CONTROL dpm	%ACTIV.	STRAIN #11 dpm	%ACTIV.	STRAIN #17 dpm	%ACTIV.	STRAIN #18 dpm	%ACTIV.
	1	0	0.00	14	0.08	2	0.01	34	0.16
5β	2	7	0.05	37	0.20	8	0.05	31	0.15
3-keto	3	54	0.41	424	2.28	769	5.18	943	4.52
3-keto-Δ^{4}	4	56	0.43	46	0.25	37	0.25	50	0.24
LA	5	12,913	98.36	17,602	94.56	13,790	92.80	19,410	92.07
Baseline	6	102	0.78	492	2.64	254	1.71	603	2.86
Total dpm		13,128		18,615		14,860		21,081	

Solvent: Benzene: Dioxane: Acetic Acid (100:10:1 by vol.)

1. All d.p.m. are corrected for background and quenching.
2. Numbers refer to the following strains: No. 11 = *B. fragilis* ss *thetaiotaomicron* No. 17 = citrobacter No. 18 = *P. productus* I.

The extracts derived from these incubations contained from 74–100% of the total activity administered to the cultures and 94% in the case of the control. In Table 2 a summary of the radioactive profiles of these extracts indicates a metabolite which is less polar than LA in the 3-keto region. This region of the plate contained 2–5% of the total activity, whereas the control (LA + BHI) contained only 0·4% of the activity of the extract. Although not indicated here, the extract from *B. fragilis* ss *thetaiotaomicron* (strain No. 6 assayed prior to the extracts in Table 2) contained 3% of its total activity in this area.*

Purification of the latter extract by Sephadex LH-20 chromatography using an isooctane–chloroform–methanol (2:1:1 by vol.) system gave the profile indicated in Fig. 1. The small peak preceding the main area of activity which contained unmetabolized LA was a region in which previous studies had shown to contain 3-keto and/or esters of other monofunctional bile acids [17].

Fractions 9–13 from a purified extract of strain No. 17 were each divided into two equal portions, and one of each of these halves was methylated with diazomethane. The results of the analyses of the acidic and methylated portions of each of the above fractions by g.f.p. chromatography are summarized in Table 3. There was a shift in activity upon methylation for fractions 12–13 corresponding to a change from LA to ML. However, the activity for fractions 9 and 10 remained in the 3-keto and/or ML region thus indicating that the metabolite had possibly been esterified prior to the methylation step.

Incubations of unlabeled LA with citrobacter. A heavy cell suspension of citrobacter was prepared by inoculating 24 tubes containing 30 ml each of BHI medium with an inoculum from seed cultures of the organisms. The cultures were grown for 18 h anaerobically at 37°C and then spun down at 2500 *g* for 20 min. The pellets were combined and resuspended twice with fresh medium under anaerobic conditions, and the washed pellet was suspended in 120 ml of fresh medium giving a 6-fold concentrated suspension of cells. LA (1·86 μmol) was added as an ethanolic

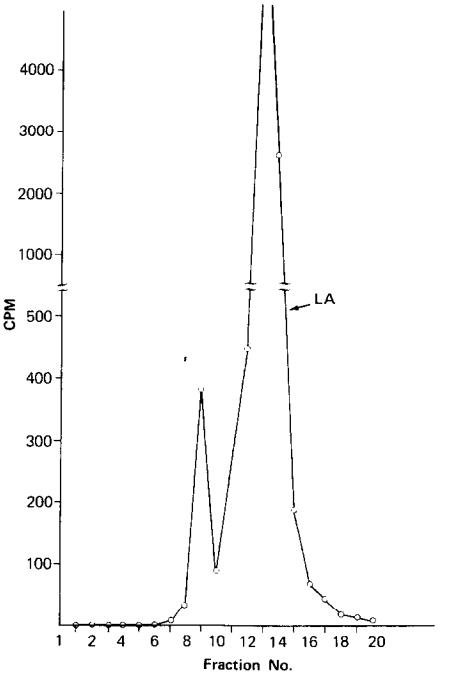

Fig. 1. Fractionation of an extract from *B. fragilis* ss *thetaiotaomicron* I by Sephadex LH-20 chromatography. The size of the fractions collected was 2 ml.

* It should be noted that 3 ml of a 4-fold concentrated cell suspension were used in incubations with strain No. 6.

Table 3. Glass fibre paper chromatographic profile of ^{14}C-labeled acid and ester fractions from Sephadex LH-20 purified extract of citrobacter (strain No. 17)

ZONE	COMPOUNDS	FRACTION #9 ACID	FRACTION #9 ESTER	FRACTION #10 ACID	FRACTION #10 ESTER	FRACTION #12 ACID	FRACTION #12 ESTER	FRACTION #13 ACID	FRACTION #13 ESTER
1	5β-and Me-5β	0	2	6	7	0	0	0	0
2	Me-3-keto	0	0	1	8	0	0	0	0
3		1	0	1	2	0	0	0	0
4	3-keto or ML	95	96	75	70	0	95	0	95
5		1	1	1	1	0	0	0	0
6	LA	1	0	4	3	98	3	98	3
7	Baseline	2	1	10	10	1	1	1	1
Total dpm		570	440	126	103	2818	2286	7338	5987

Solvent: isooctane: isopropyl ether: acetic acid (75:30:1.0 by vol.)

solution (1·0 ml to 30 ml of this heavy cell suspension), and the incubation was continued anaerobically for 24 h at 37°C. For purposes of discussion this sample will be known as LA + citrobacter. Controls included in this study were LA (1·86 μmol) in BHI medium (LA + BHI) and 30 ml of cells (citrobacter) without substrate.

The g.l.c. chromatograms of the unmethylated extracts on 3% OV-17 are shown in Figs. 2A–2C and indicate a peak with a relative retention time (RRT) of 0·63, using methyl deoxycholate as a reference standard, for the LA + citrobacter, whereas the LA + BHI and citrobacter samples gave flat responses in this region.

Following purification by Sephadex LH-20 chromatography, the partially purified metabolite gave a single peak with RRT = 0·63 both before and after methylation with diazomethane.

Gas–liquid chromatographic retention times of some monofunctional bile acids. A number of monofunctional bile acid reference standards were assayed by g.l.c. on 3% OV-17 as their hydroxyl and TMSi derivatives. Table 4 indicates that the metabolite isolated in fraction No. 9 from the LH-20 column had an RRT which was similar to a number of the standards listed in its hydroxyl form but as the TMSi derivative its RRT was unique (1·09).

Large-scale incubation of LA with strain No. 17. In order to characterize this unknown metabolite further, an 83-fold scale-up of our incubation conditions above that used in the radioactive experiments was carried out by adding 5·8 mg LA in 8·3 ml ethanol to 6-fold concentrated cells of strain No. 17 in a final vol. of 240 ml of BHI medium. After the usual incubation and extraction procedures, the extract was purified by LH-20 chromatography as described previously.

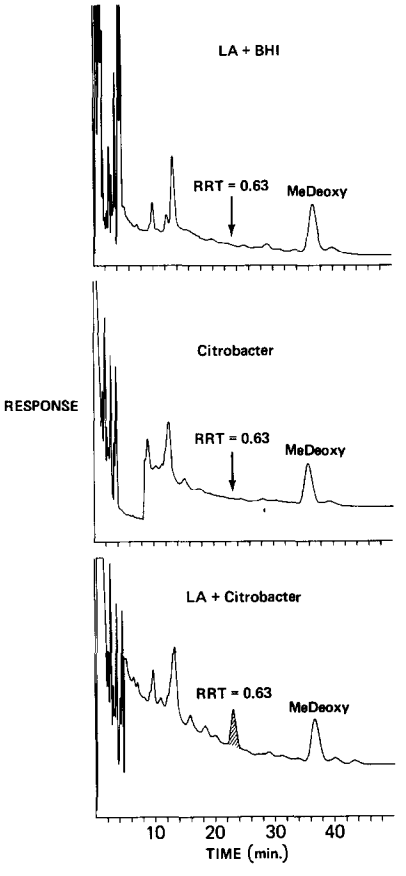

Fig. 2A: The g.l.c. profile on 3% OV-17 of an extract derived from the incubation of LA and BHI medium using methyl deoxycholate (Me Deoxy) as an external reference standard.

Fig. 2B: Profile of an extract derived from the incubation of heavy cell suspensions of citrobacter.

Fig. 2C: Chromatogram of an extract derived from the incubation of LA with a heavy cell suspension of citrobacter showing the appearance of an unidentified metabolite (shaded area) at a relative retention time (RRT) of 0·63.

Table 4. Retention times of monofunctional bile acids as their methyl (Me) and ethyl (Et) esters compared with fraction No. 9 from a Sephadex LH-20 purified extract of citrobacter

GLC RETENTION TIMES ON OV-17 (260°)

	OH			OTMSi		
Substituent	ART(min)	RRT	RRT*	ART(min)	RRT	RRT*
Me - 5β	9.1	0.23	0.20	9.3	0.60	0.62
Me - 3β, 5ε	20.4	0.54	0.51	13.8	0.89	0.86
Me - 3α, 5β	21.7	0.54	0.52	14.5	0.94	0.93
Me - 3β, 5δ	24.6	0.62	-	18.5	1.21	-
Me - 3β, 5α	25.3	0.63	0.60	18.8	1.22	1.20
Me - 3=O, 5β	25.3	0.63	0.61	24.0	1.55	-
Me - 3α, 12α, 5β	40.2	1.00	-	15.3	1.00	-
Et - 3β, 5β	19.7	0.54	-	15.4	1.04	-
Et - 3α, 5β	22.9	0.63	-	16.2	1.09	-
Fraction #9	-	0.63	-	-	1.09	

GLC RETENTION TIMES - 1% QF-1

	OH (235°)			OTMSi (210°)		
Substituent	ART(min)	RRT	RRT*	ART(min)	RRT	RRT*
Me - 5β	2.4	0.20	0.15	-	-	-
Me - 3β, 5β	5.6	0.48	0.44	10.5	0.85	0.83
Me - 3α, 5ε	6.2	0.53	0.49	11.1	0.90	0.91
Me - 3β, 5α	-	-	0.53	-	-	1.20
Et 3β, 5β	6.7	0.58	-	12.5	1.01	-
Et 3α, 5β	7.3	0.63	-	13.5	1.09	-
Fraction #9	-	0.63	-	-	1.09	-
Me - 3=O, 5β	11.9	0.97	1.00	-	-	-
Me - 3α, 12α, 5β	12.3	1.00	1.00	12.4	1.00	-

* Elliott W. H., Walsh L. B., Mui M. M., Thorne M. A. and Siegfried C. M.: *J. Chromatog.* **44** (1969) 452–464.

The g.l.c. profiles on OV-17 were identical with those described previously (i.e. RRT = 0·63 and 1·09 for the hydroxyl and TMSi derivatives, respectively), and the sample was analyzed by g.l.c.–MS. The mass spectra indicated a small molecular ion at $m/e = 404$, loss of water at $m/e = 386$ and a combined loss of ethanol and water at $m/e = 340$.

Since the mass spectrum fits the expected fragmentation pattern of an ethyl ester of LA and the metabolite had an RRT similar to that for ML (see Table 4), both EL and the epimeric EIL were synthesized as described in the Methods section.

Table 4 summarizes the g.l.c. RRT data on QF-1 and OV-17 for the methyl and ethyl esters of LA and IL and clearly points out that the metabolite had identical retention times with those for EL only.

Figures 3A–C compare the mass spectra for EL, EIL and the metabolite (RO43-59-1) isolated from incubation of LA with strain No. 17. It is clear that the fragmentation of the metabolite agrees more closely with that found for EL since only a weak molecular ion is observed as opposed to the more pronounced M^+ for the EIL. This same effect of the M^+ differences has also been observed for ML and its epimer, methyl-3β-hydroxy-5β-cholanate. The peak at $m/e = 433\ (M + 29)^+$ is due to the addition of C_2H_5 from the ionizing gas.

Requirements for EL biosynthesis

Ethanol. TL (1·86 μmol) was added as an aqueous or ethanolic solution to a heavy cell suspension (6-fold concentrated) of strain No. 17, and the incubation was carried out for 24 h anaerobically at 37°C. The extracts obtained by the usual procedure were assayed by g.l.c. on 3% OV-17 and indicated that EL was produced from the TL added as an ethanolic solution only. From these results it is apparent that ethanol is an obligatory component of the incubation system.

Aeration. The usual BHI medium (180 ml) was prepared in 250 ml Nalgene centrifuge tubes under aerobic conditions and was inoculated with a seed culture of strain No. 17 which is a facultative organism. After shaking the tubes which were fitted with cotton plugs for 24 h at 37°C, good growth was observed, and 6-fold concentrated cell suspensions were prepared as described previously with the exception that all operations were conducted aerobically. The LA (1·86 μmol) was added as an ethanolic solution, and the incubation was continued aerobically with shak-

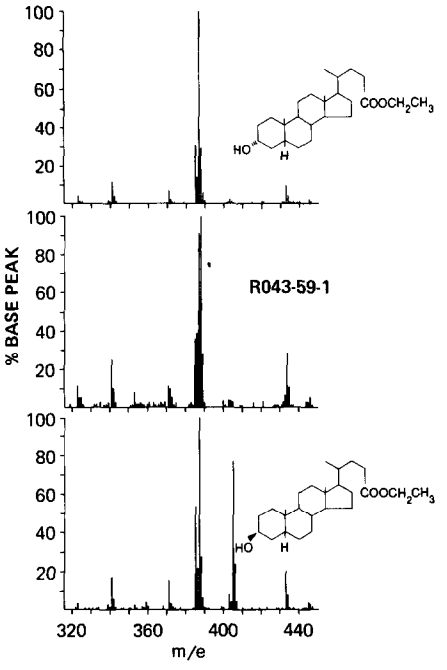

Fig. 3A is the fragmentation pattern of authentic EL. Figure 3B represents the mass spectrum of the unknown metabolite (R043-59-1). Figure 3C is the fragmentation pattern of EIL.

ing for 24 h. Controls run simultaneously included LA and ethanol incubated with BHI and a heavy cell suspension without substrate. After the usual extraction procedures, the extracts were analyzed by g.l.c. on 3% OV-17, and no EL was observed. Therefore, anaerobic conditions appear to be necessary for ester formation.

Heavy cell suspensions. In experiments in which LA was added as an ethanolic solution to an unconcentrated cell suspension of strain No. 17 the g.l.c. profiles on 3% OV-17 indicated no presence of EL. Although some EL might be formed with unconcentrated cell suspension, the amount present would be too small to be detected by our methods described here. Despite this possibility these results suggest that heavy cell suspensions are required for optimal production of EL.

Formation of EL by select fecal microorganisms. Although studies conducted previously with [^{14}COOH]-LA indicated that four strains converted LA to EL in 2–5% yields, these experiments were conducted with 2·5–3·0-fold cell suspensions of the respective organisms (see Table 2).

Therefore, the formation of EL was studied under more optimal incubation conditions using 6-fold concentrated cell suspensions of strains No. 6, 11, 17 and 18, and LA (1·86 μmol) which was added as an ethanolic solution. After the usual incubation and extraction procedures, the extracts were purified by LH-20 chromatography and then assayed by g.l.c. on 1% QF-1. Only strains No. 6 and No. 17 indicated significant conversions of 15 and 10% respectively, whereas the incubations with strains No. 11 and No. 18 resulted in the formation of trace levels of EL.

DISCUSSION

Bile acids have been suggested to enhance the rate of colonic tumor formations in animals treated with known carcinogens [9, 18, 19], and LA specifically has been shown to enhance tumor incidence in rats intrarectally instilled with a single dose of N-methyl-N′-nitro-N-nitrosoguanidine [9]. Since LA is a major fecal bile acid its metabolism by individual intestinal microorganisms becomes important in assessing its possible role in the etiology of colon cancer. In this report we have described the esterification of LA by several organisms which represent some major genera present in the large intestine.

Conditions which were found to be optimal for the esterification process were established with the faculatative organism, citrobacter. The formation of EL requires the presence of ethanol, heavy cell suspensions and anaerobic incubation conditions.

In a comparison of select fecal organisms using 6-fold concentrated cell suspensions, LA was converted to EL in yields of 10–15% by *B. fragilis* ss *thetaiotaomicron* (strain No. 6) and citrobacter. Trace levels of this derivative were found with *B. fragilis* ss *thetaiotaomicron* (strain No. 11) and *P. productus* I. Although earlier data (Table 2) indicated a similar conversion of 2–5% for these organisms using 3-fold concentrated cell suspensions, the above results may be attributed to an increased enzyme concentration and/or change in metabolic activity of these organisms which were maintained in culture some months after the initial experiments.

The esterification of LA with ethanol by select fecal microorganisms represents a novel biosynthetic process whose physiological significance is unknown at the present time. Furthermore, the intestinal bacteria are also capable of deconjugating TL by hydrolysis of the amide bond and esterifying the free carboxyl group of the LA which is produced from the above catabolic process to form EL. The esterification of a bile acid is not an artifact of either the incubation or extraction procedures since both radioactive and unlabeled controls which contained ethanolic solutions of the substrates did not produce EL under these conditions.

The observation that EL is formed from LA in cultures of citrobacter under anaerobic conditions only is significant since the colon is essentially anaerobic and thus provides the conditions necessary for the growth of the organisms studied here as well as other significant genera. It is possible that these organisms may produce small amounts of ethanol as part of their general metabolism and thus theoretically provide the conditions necessary for esterification of LA and other fecal bile acids as well. However, such metabolites produced *in vivo* will have to be confirmed in analyses of bile acid metabolites in fecal material.

The fact that such metabolites have not been identified previously in fecal samples is probably due to the fact that such derivatives have not been observed in biological systems and/or the analytical methods employed were not sufficiently sensitive or specific.

Although carboxylic acid esters of bile acids have not been reported previously, sulfate esters of the 3α-hydroxyl group of monofunctional bile acids have been described by Norman and Palmer[7] and Palmer and Bolt[20]. Sulfation of the hydroxyl group probably occurs in the liver [21], but mixed populations of fecal microorganisms and not isolated strains [22] are capable of desulfating the ester to LA and possibly other metabolites [7]. Although it is unlikely that much lithocholic acid sulfate would be present in the colon due to the action of the mixed fecal flora, the sulfate esterification may be important in abolishing the toxicity of LA [23] in other parts of the mammalian system.

Similarly the esterification of LA with ethanol may be involved in either enhancing or reducing the toxicity and/or carcinogenic potential of this compound in the large intestine.

Acknowledgements—The authors wish to thank Dr. Gary Muschik and Mr. Jim Schroer for their cooperation and assistance with the g.l.c. MS analyses. We also would like to express our appreciation to Dr. Milton W. Slein and Ms. Mary Wilson for their comments and suggestions during the course of this work and to Mrs. Sharyn Sexton for her fine technical assistance in the completion of these studies.

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