The identification of microbial metabolites of sulfolithocholic acid

Morris I. Kelsey,[1] Jorge E. Molina,[2] Shing-Kwan S. Huang, and Kin-Kai Hwang[3]

Chemical Carcinogenesis Program, Frederick Cancer Research Center, Frederick, MD 21701

Abstract Sulfolithocholate, a major detoxified form of lithocholic acid, is biotransformed by human intestinal microflora to four major metabolites. Purification of crude extracts by Sephadex LH-20 chromatography gave three major radioactive peaks, after incubation studies, which were characterized by thin-layer and gas–liquid chromatography, as well as combined gas–liquid chromatography–mass spectrometry. Peak I has a polarity similar to that for 3β-palmitoyl-isolithocholic acid and transesterification of the metabolite showed that it was a mixture of palmitoyl, palmitoleyl, stearyl, and oleyl esters of isolithocholate. Peak II was identified as chol-3-en-24-oic acid and Peak III was a mixture of lithocholic (mainly) and isolithocholic acids. The fatty acyl derivatives of isolithocholate and Δ^3-cholenate are new microbial metabolites of sulfolithocholate and represent unique classes of bile acids that should be included in routine fecal analysis of bile acids.—**Kelsey, M. I., J. E. Molina, S -K. S. Huang, and K -K. Hwang.** The identification of microbial metabolites of sulfolithocholic acid. *J. Lipid Res.* 1980. **21:** 751–759.

Supplementary key words sulfolithocholate · gut microflora · cholenate · fatty acyl isolithocholate · lithocholate · isolithocholate

Lithocholic acid is a major fecal bile acid (1) that has been proposed as a contributing factor in human colon cancer (2). Unlike many other major bile acids, lithocholic acid has unique biological activities including cholestatic properties (3), production of bile duct hyperplasia (4), enhancement of bacterial mutagenicity (5, 6), and tumorigenicity (7, 8) of known chemical carcinogens. Recently Kelsey and Pienta (9) have shown that lithocholic acid transforms Syrian hamster embryo cells, whereas, its sulfate ester was inactive in this system.

Palmer (10) first observed sulfolithocholate in human bile and demonstrated that such derivatives might facilitate the excretion of the more hepatotoxic lithocholic acid (11). Studies of lithocholate metabolism in humans have shown that lithocholate is extensively sulfated in bile (12) and that this sulfation decreases the toxicity of increased lithocholate produced in patients receiving chenodeoxycholate therapy for gallstone dissolution (13).

Although this sulfated lithocholate is excreted into the colon via biliary transport, microbial metabolism and possible retoxification of this substrate in the large bowel produces a number of non-polar products which have not been completely identified (11, 14–16). This report presents evidence for the identification of four metabolites produced by gut microfloral metabolism of sulfolithocholate in order to extend and clarify results presented in previous studies.

MATERIALS AND METHODS

Chemicals

The following bile acids were prepared for the National Cancer Institute by Omni Research, Inc. (Mayagues, Puerto Rico): lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid; LA), isolithocholic acid (3β-hydroxy-5β-cholan-24-oic acid; ILA), and 3-keto-cholanoic acid (3-keto-5β-cholan-24-oic acid; 3-keto). The 5β-cholan-24-oic acid (5β or 5β-cholanic acid) was purchased from Schwartz/Mann (Orangeburg, NY).

All of these bile acids were found to be 95% pure, with the exception of ILA which was purified by Lipidex 5000 column chromatography (17). A column 2.5 × 54 cm was packed with Lipidex 5000 gel obtained from Packard Instrument Co. (Downers Grove,

Abbreviations: LA, lithocholic acid; ILA, isolithocholic acid; 3-keto, 3-keto-5β-cholanic acid; 5β,5β-cholanic acid; $LASO_4$, sulfolithocholate; Δ^3-cholenate, 5β-chol-3-en-24-oate; Δ^2-cholenate, 5β-chol-2-en-24-oate; ME, methyl; ME-PALM-ILA, methyl-5β-cholan-24-oyl-3β-palmitate; coprostanol, 5β-cholestan-3β-ol; cholesterol, 5-cholesten-3β-ol; cholestanol, 5α-cholestan-3β-ol; β-sitosterol, 5-cholesten-24β-ethyl-3β-ol; ME-14:1, methyl myristoleate; ME-14:0, methyl myristate; ME-15:0, methyl pentadecanoate; ME-16:1, methyl palmitoleate; ME-16:0, methyl palmitate; ME-17:0, methyl heptadecanoate; ME-18:1, methyl oleate; ME-18:0, methyl stearate; ME-20:4, methyl arachidonate; IPE, diisopropyl ether; DMP, dimethoxypropane; TLC, thin-layer chromatography; ITLC, instant thin-layer chromatography; GFP, glass fiber paper; GLC, gas–liquid chromatography; MS, mass spectrometry.

[1] To whom reprint requests should be addressed: NCI, DCCP, Landow Bldg., Rm. 8C-25, Bethesda, MD 20205.

[2] Casilla 1171, La Paz, Bolivia.

[3] Microbiological Associates, Bethesda, MD 20205.

TABLE 1. Summary of R_f values of sulfolithocholate metabolites on ITLC-SG and ITLC-SA sheets

Compounds	ITLC-SG A	ITLC-SG B
5β-Cholanate	0.91	0.97
3-Keto-5β-cholanate	0.52	0.77
ILA	0.37	0.46
LA	0.24	0.30
$LASO_4$	0.00	
	ITLC-SA A	ITLC-SA C
ME-Cholenates	0.87	0.63
ME-PALM-ILA	0.77	0.27
5β-Cholanate	0.44	
PALM-ILA	0.34	
ME-ILA	0.10	

A = isooctane–IPE–HOAC 75:30:0.3.
B = isooctane–IPE 100:25, compounds run as methyl esters.
C = isooctane–IPE 100:10.

IL); it had previously been expanded in ethylene dichloride–methanol–water 10:70:30 (v/v/v) to give a column volume of 229 ml. Approximately 45 mg of ILA was dissolved in the mobile phase, and purified ILA was collected in an elution volume of 600–700% of the column volume as judged by thin-layer chromatography (18). The LA contaminant was found in fractions corresponding to 534–560% of the column volume, and approximately 16 mg of pure ILA was recovered using this procedure.

Applied Science Laboratories (State College, PA) was the source of cholestane and neutral sterol derivatives including: 5β-cholestane, coprostanol (5β-cholestan-3β-ol), cholesterol (5-cholesten-3β-ol), cholestanol (5α-cholestan-3β-ol) and β-sitosterol (5-cholesten-24β-ethyl-3β-ol). Methyl esters of fatty acids were also obtained from Applied Science Laboratories: methyl myristoleate (14:1), methyl myristate (14:0), methyl pentadecanoate (15:0), methyl palmitoleate (16:1), methyl palmitate (16:0), methyl heptadecanoate (17:0), methyl oleate (18:1), methyl stearate (18:0), and methyl arachidonate (20:4).

Most reagent-grade solvents were purchased from Burdick-Jackson (Muskegon, MI) and certain solvents for TLC from Fisher Scientific Co. (Pittsburgh, PA). All solvent mixtures are given as proportions by volume. PCS cocktail (Amersham Searle, Arlington Heights, IL) was routinely used for radioactive measurements using an ISOCAP/300 Liquid Scintillation System (Nuclear-Chicago, Des Plaines, IL).

Synthesis of substrates and reference standards

Methylation. Preparation of methyl esters utilized either diazomethane generated from Diazald (Aldrich Chemical Co., WI) according to the manufacturer's instructions or dimethoxypropane (DMP) using a slight modification of the method of Shaw and Elliott (19). Methylation consisted of dissolving the sample (usually 1 mg or less) in 110 μl of a solution of DMP–methanol–HCl 5:5:1 and allowing the vial to remain at room temperature for 1 hr in the dark. The solution was then evaporated to dryness under a stream of nitrogen, reconstituted in solvent, and analyzed.

Preparation of sulfolithocholate (3α-sulfooxy-5β-cholan-24-oic acid, $LASO_4$). The synthesis of labeled and unlabeled $LASO_4$ consisted of reacting LA with a sulfur trioxide pyridine complex (Upjohn Co., Kalamazoo, MI) according to detailed procedures already described (20). Preparation of [^{14}COOH]-$LASO_4$ utilized [^{14}COOH]LA (sp act 59 mCi/mmol) that was obtained from Amersham-Searle; the labeled lithocholic acid was 98% pure as shown by TLC (18). The purified [^{14}COOH]-$LASO_4$ has a final specific activity of 0.37 μCi/μmol (0.38 μCi/μmol calculated) and a radiochemical purity of 98% (20).

Synthesis of cholenates. Preparation of a mixture of cholenic acids, 5β-chol-2-en-24-oic (Δ^2-cholenate) and 5β-chol-3-en-24-oic (Δ^3-cholenate), consisted of methylation of 600 mg of $LASO_4$ (21) in 20 ml of methanol with 200 ml of an ether solution of freshly distilled diazomethane. After 1.5 hr at 25°C with stirring, the solvents were removed under a stream of nitrogen, and the product was analyzed by TLC using ITLC-SA sheets (see section on glass fiber paper chromatography) developed in isooctane–diisopropyl ether 100:20. Results indicated that the methylation was essentially complete with some formation of the methyl cholenates since a major spot was noted having an R_f similar to ME-5β (**Table 1**).

Conversion of the remaining dimethyl-$LASO_4$ to ME-cholenates required 37 hr of reflux in 300 ml of toluene previously stored over potassium carbonate. Following removal of the toluene by rotary evaporation, isolation of the crude ME-cholenates included a liquid-liquid extraction of the residue with ethyl acetate–water 300:50. Upon removal of the ethyl acetate, the residue was dried in vacuo at 55°C for several hours, yielding 363 mg of crude cholenates.

Purification of the ME-cholenates by column chromatography (1.8 × 29 cm) required 40 g of silica gel (Hi-Flosil, 60/200 mesh; Applied Science Laboratories, Inc.) that was eluted sequentially with 400 ml each of 0–2% diisopropylether in isooctane collected in 10-ml fractions. The majority of the ME-cholenates was found in the 1% IPE in isooctane fraction as monitored by ITLC described above, and 175 mg of purified product was recovered.

Gas–liquid chromatographic analysis on a column of 1%-SE-30/QF-1 maintained at 210°C [see Gas–liquid

chromatography (GLC) section] indicated a mixture of ME-Δ^3-cholenate (58%) and Δ^2-cholenate (42%) with relative retention times (with respect to 5β-cholestane) equivalent to ME-cholenate standards analyzed by mass spectrometry (MS) (see conditions in the MS section). Major ions for the Δ^3-isomer (see also Fig. 6) included m/z = 372 (M^+), 357 (M-15), 257, 230, 215, and 201, whereas the Δ^2-cholenate also contained ions at m/z = 318, which indicated the presence of a symmetrical olefin and loss of ring A (11), and 203. No further attempts were made to separate and purify the individual cholenates since the gas–liquid chromatographic separation and analysis by mass spectrometry were sufficient to facilitate identification of the unknown cholenate metabolite isolated from microfloral incubations.

Synthesis of methyl-5β-cholan-24-oyl-3β-palmitate (ME-PALM-ILA). The starting materials for this synthesis included ME-ILA and palmitoyl chloride. The ME-ILA was prepared by dissolving 1000 mg of ILA in 100 ml of 2% methanolic HCl (w/v) and heating the reaction mixture at 55–60°C for 3 hr. Following removal of the solvent by rotary evaporation, a portion of the residue (500 mg) was dissolved in 2 ml of 0.5% acetone in benzene and applied to a column (2.5 × 35.5 cm) containing 50 g of silica gel washed with 500 ml of benzene. Stepwise elution with 0–2% acetone in benzene (collecting 10-ml fractions) gave pure ME-ILA mainly in the 2% acetone–benzene fractions. Detection of the ME-ILA by ITLC-SG sheets (see GFP chromatography) developed in isooctane–IPE 100:40 (v/v) indicated that the purified ME-ILA (240 mg) was not contaminated with ME-LA (the main contaminant, 90 mg).

Preparation of palmitoyl chloride consisted of dissolving 1 g of palmitic acid (Matheson Co., Inc., East Rutherford, NJ) in 3 ml of thionyl chloride (Fisher Scientific Co., Pittsburgh, PA) and stirring the mixture in a closed test tube overnight at 25°C. Removal of the reagent with a stream of nitrogen gave 1.03 g of product having an infrared band at 1800 cm^{-1} (–COCl) and no absorption at 1720 cm^{-1} (COOH).

Two hundred mg of ME-ILA were dissolved in 5 ml of dry pyridine and a 1-ml solution of palmitoyl chloride (~1 g) in pyridine was added to the reaction mixture with stirring. The reaction was continued overnight at 25°C and was evaporated to dryness under nitrogen. Extraction of the residue with methanol–hexane 20:80 partially purified the product which was found in the hexane fraction. Removal of the solvent by rotary evaporation in vacuo followed by vacuum dessication at 40°C overnight gave 400 mg of crude product.

Purification of the ME-PALM-ILA by silica gel column chromatography (40 g of silica gel slurried in benzene gave a column 2.5 × 28.5 cm) using benzene elution (10-ml fractions) gave a pure product (single spot) in fractions 22–36 as determined on ITLC-SA sheets developed in either isooctane–diethyl ether 100:6 (v/v) or isooctane–IPE–acetic acid 75:30:0.3. Mass spectral analysis (see below) using a solid probe/EI ionization technique gave a very small M^+ at m/z = 628 with major fragments at m/z = 372, (expected ME-cholenate fragment from loss of fatty acid) 357, 257, 230, 215, and 201.

Additional proof of structure of the ME-PALM-ILA consisted of alkaline hydrolysis of a portion of the product (4.5 mg) with 2 ml of 4% potassium hydroxide in methanol (w/v) for 1 hr at 50°C in a closed vial with stirring. Addition of 2 ml of 1.2 N HCl to the reaction mixture followed by extraction with chloroform–water 10:2 (v/v) gave a product that contained two major spots having R_f values corresponding to palmitic acid and ILA, respectively, on an ITLC-SA sheet developed in solvent *A* (Table 1). The fatty acid would have an R_f equal to that for 5β. In addition, remethylation of a similar preparation with diazomethane gave ME-ILA and ME-palmitate on 1% QF-1 and 1% SE-30 gas–liquid chromatographic columns, respectively (see GLC conditions and Tables 2A and 2B).

Synthesis of 5β-cholan-24-oyl-3β-palmitate (PALM-ILA). To prepare the acid form of the PALM-ILA, a simple procedure described by Kemp and Mercer (22) was adapted. A mixture of 10 mg of purified ILA, 3 mg of p-toluenesulfonic acid, and 42 mg of palmitic acid was refluxed in 5 ml of dry benzene for 6 hr. After removal of the solvent by rotary evaporation, 48 mg of crude product was obtained and was assayed on an ITLC-SA sheet developed in solvent *A* (Table 1). Partial purification of the desired compound was accomplished using a Sephadex LH-20 column (column vol = 160 ml) eluted with isooctane–chloroform–methanol 2:1:1. Fractions of 5 ml were collected and 6 mg of the desired product was found in fractions 13–14. Analysis of the fractions with ITLC-SA sheets developed in system *A* (R_f of PALM-ILA was 0.38) indicated traces of a very nonpolar contaminant. Hydrolysis of the product gave two spots (by ITLC-SA chromatography described above) corresponding to ILA and palmitic acid. Methylation of the hydrolyzed mixture followed by gas–liquid chromatographic analysis on 1% SE-30 and 1% QF-1 columns (see GLC methods) gave ME palmitate and ILA peaks, respectively. An infrared spectrum (dried film) of the reference standard palmitoyl-isolithocholic acid (PALM-ILA) revealed bands at 1730 cm^{-1} (ester of carboxylic acid) and 1700 cm^{-1} (carbonyl of free carboxylic acid). Bands were also present at 1100–1200 cm^{-1} due to an ester (C-O stretch) and

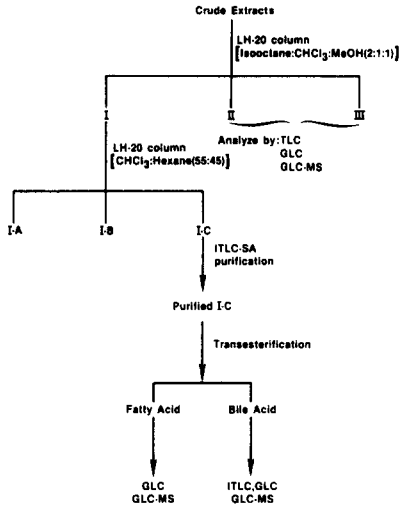


Fig. 1. Protocol for purification and analysis of microbial metabolites of $LASO_4$. Note that I-C was methylated prior to ITLC-SA purification.

double bands between 1200–1250 cm^{-1} were due to an ester of a secondary axial alcohol (23).

Analytical methods

Glass fiber paper (GFP) chromatography. GFP sheets coated with silica gel (ITLC-SG) or silicic acid (ITLC-SA) were obtained from Gelman Instrument Co. (Ann Arbor, MI). Routine analysis of metabolites on ITLC-SG or ITLC-SA sheets developed in various solvents are summarized in Table 1, which gives the R_f values of all pertinent reference standards. All solvent ratios described are expressed as proportions by volume. Visualization of spots consisted of charring the ITLC sheets after spraying with a 50% solution of sulfuric acid. Radioactive measurements of ITLC-SG or ITLC-SA sheets consisted of cutting out zones corresponding to the R_f of reference standards which were spotted on the sides and assaying the activity by liquid scintillation spectrometry.

Sephadex LH-20 column chromatography. Extracts of incubations were purified on a column of Sephadex LH-20 (15, 18) having a bed volume of 160 ml, and 2-ml fractions collected were assayed by liquid scintillation counting (see Fig. 2). Peaks containing radioactivity were pooled and analyzed by GFP chromatography.

For additional purification of Peak I (**Figs. 1** and 2), a second Sephadex LH-20 column (vol = 147 ml) was used with a neutral solvent system consisting of chloroform–hexane 55:45 as the mobile phase (24).

GLC methods. Bile acid and neutral sterols were analyzed on a 1.8 m × 6.35 mm (O.D.) column of 1% QF-1 coated on Gas Chrom Q (100–120 mesh; Lot UP-001) obtained from Applied Science Laboratories. The column was housed in a Hewlett-Packard (Avondale, PA) 5730A gas chromatograph which maintained an oven temperature of 210°C, injection port at 250°C, and a flame ionization detector at 300°C. Helium gas was used as the carrier with a flow of approximately 60 ml/min. A sample of 5β-cholestane was coinjected with all samples to obtain relative retention times.

Eventually the above phase deteriorated, and a column packed with 3% OV-210 coated on Chromosorb WHP (Supelco, Inc., Bellefonte, PA) gave similar separations as summarized in **Table 2.** Operating conditions were similar to those above except that the oven temperature was maintained at 220°C.

A mixture of 1% SE-30/1% QF-1 coated on Gas Chrom Q (100–120 mesh) was obtained from Supelco, Inc. and was used occasionally to assay synthetic preparations of methyl-Δ^3/Δ^2-cholenates. A column (1.5 m × 3 mm (I.D.)) was maintained at 210°C with detector/injection port temperatures at 280°C using a Shimadzu 4 BM gas chromatograph. Helium was also used as a carrier (~60 ml/min), and 5β-cholestane was coinjected with all samples to determine relative retention times. The separation of these isomers was identical to that obtained on the QF-1 and OV-210 columns.

Analysis of fatty acids (**Table 3**) was done routinely on a 1% SE-30 column (2 m × 3 mm (I.D.)) coated on Gas Chrom Q (100–120 mesh; Supelco, Inc.). The oven temperature was 140°C, and the injection port and flame ionization detector temperatures were 280°C. Helium gas was used as a carrier (60 ml/min), and methyl arachidonate was coinjected as an external standard for relative retention time determinations.

Alternative analysis (see Table 3) of fatty acids was performed on a 1.8 m × 6.4 mm (O.D.) column containing 10% Silar 10C coated on Gas Chrom Q (Applied Science Laboratories). Oven temperature was 165°C with injection port and detector temperatures maintained at 250°C and 300°C, respectively.

TABLE 2. Summary of retention times for neutral sterols and bile acid methyl esters

Compound	3% OV-210		1% QF-1	
	ART	RRT	ART	RRT
#5β-Cholestane	3.12	1.00	1.43	1.00
ME-Chol-3-enoate	6.14	1.97	2.84	1.98
ME-Chol-2-enoate	6.53	2.10	3.01	2.10
ME-5β-Cholanate	6.53	2.10	3.01	2.10
Coprostanol	8.34	2.68	3.92	2.74
Cholesterol	9.63	3.09	4.55	3.18
Cholestanol	10.34	3.32	4.88	3.41
β-Sitosterol	15.05	4.83	7.32	5.11
ILA	18.21	5.85	8.77	6.12
LA	20.42	6.56	9.86	6.89
3-Keto	39.06	12.54	18.99	13.26

ART, actual retention time.
RRT, relative retention time.

Areas and retention times were determined on both gas chromatographs using a Hewlett-Packard 3354 laboratory data system linked through a Hewlett-Packard 1865 A/D converter.

Mass spectrometry (MS). Low resolution mass spectra were obtained by combined GLC–MS using a Varian Aerograph (Varian Associated, Palo Alto, CA) GLC equipped with a 2 m × 2 mm (I.D.) column of either 3% SE-30 at 200°C or a 3% OV-210 coated on Chromosorb WHP (100–120 mesh) (Supelco, Inc.) that was maintained at 240°C. The gas chromatograph was interfaced with a Finnigan Model 3300 (Finnigan Corp., Sunnyvale, CA) mass spectrometer. Electron impact ionization of both GLC effluents and direct probe samples occurred at 70 ev; filament current was 0.5 m amps, and a scan was made every 5 seconds. Data acquisition utilized a Finnigan 6000 Data System which corrected the spectra for background.

Incubation protocol

Fresh feces from a single individual were collected in a stainless steel container which was built at the Frederick Cancer Research Center according to specifications described by Pryke and White (25). A sample of 4.5 g (wet weight) of fecal material was dispersed in 180 ml of prereduced Vogel-Bonner E (VBE) medium and maintained under anaerobic conditions prior to incubation with labeled substrate. An aliquot of 30 ml of the suspension of microorganisms was incubated with [^{14}COOH]-$LASO_4$ (2.04 μmol; sp act = 0.37 μCi/μmol) in 1 ml of water. Controls for this experiment included 48-hr anaerobic incubations of intestinal microflora without added substrate (endogenous lipid control) as well as labeled $LASO_4$ in VBE medium (chemical stability control). These incubations were continued as static cultures for 48 hr at 37°C under anaerobic conditions and then extracted as described below.

Extraction techniques. All incubations were cooled to room temperature, acidified with 12 N HCl to pH 1–2 and extracted with five volumes of chloroform–methanol 2:1 (v/v) as described previously (26).

TABLE 3. Separation of fatty acid methyl esters

Compound	10% Silar 10C		1% SE-30	
	ART	RRT	ART	RRT
14:0	3.46	1.00	4.10	0.10
14:1	4.53	1.31	3.82	0.09
15:0	4.53	1.31	6.44	0.15
16:0	6.00	1.73	10.23	0.25
16:1	7.38	2.13	8.98	0.22
17:0	7.93	2.29	16.31	0.39
18:0	10.55	3.05	26.12	0.63
18:1	12.57	3.63	22.01	0.53
20:4			41.76	1.00

ART, actual retention time.
RRT, relative retention time.

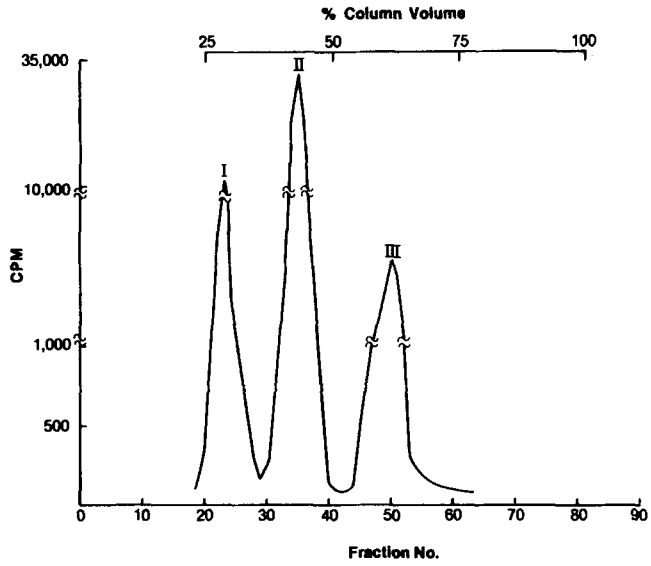


Fig. 2. Purification of an extract from the incubation of [^{14}COOH]-$LASO_4$ with human intestinal microflora using Sephadex LH-20 chromatography. The mobile phase of isooctane–chloroform–methanol 2:1:1 eluted the metabolites from a column having a bed volume of 160 ml.

RESULTS

Isolation of metabolites

Results presented here and in previous reports (15, 16, 20) show that $LASO_4$ is not converted to metabolites such as the Δ^3-cholenate or PALM-ILA in the absence of bacteria or as a result of our isolation procedure.

Each extraction was chromatographed on a column of Sephadex LH-20 using isooctane–chloroform–methanol 2:1:1 as the mobile phase. **Fig. 2** indicates three major radioactive peaks having column volumes of 29, 44, and 63%, respectively. Previous studies in this laboratory (15, 16) have shown that Peaks I and II are fairly non-polar products having a polarity similar to 5β-cholanic acid, whereas, Peak III eluted in a column volume similar to LA and ILA.

To characterize Peak I further, the above incubations were performed in triplicate. Following LH-20 chromatography, all of the Peaks I were combined and rechromatographed on a second LH-20 column using a mobile phase of chloroform–hexane 55:45 which has been used by Krause and Hartman (24) to isolate cholesteryl esters from adipocytes.

Identification of Peak I-C

Analysis of Peak I-C (see Fig. 1) by ITLC-SA chromatography using solvent *A* (**Fig. 3**) showed that

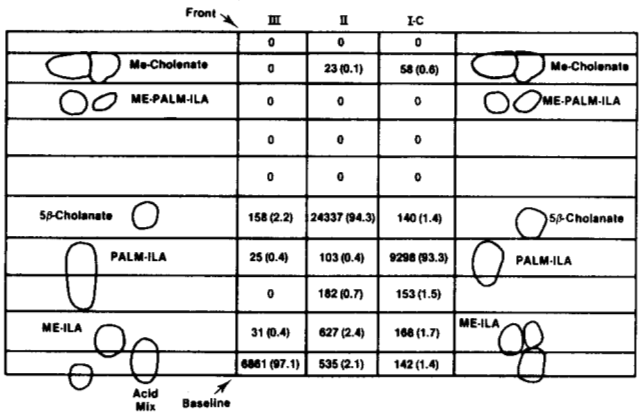


Fig. 3. ITLC-SA analysis of Peaks I-C, II, and III which are major metabolites of $LASO_4$. The developing solvent was isooctane–IPE–acetic acid 75:30:0.3. Numbers in the grid refer to DPM, and the numbers in parentheses refer to the percentage of total activity associated with each zone. Approximately 0.5–1% of the total extract (pooled extract of three incubations) was analyzed as indicated. Note the R_f of cholenic and cholanic acids are identical as their acid or methyl ester derivatives.

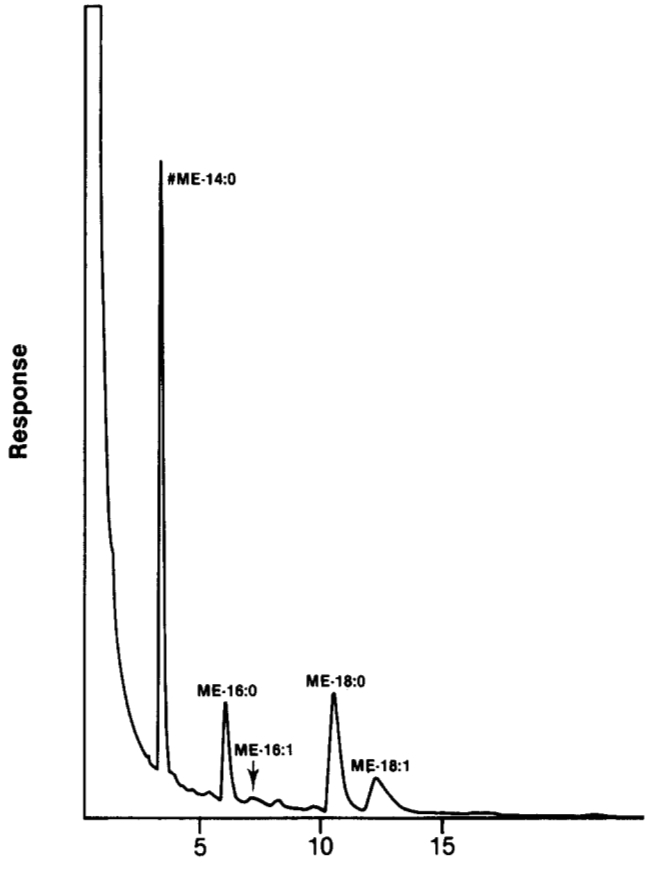
the majority of the activity centered in the PALM-ILA region. Methylation of this compound with DMP (see Experimental Procedures) resulted in a shift in the R_f to the ME-PALM-ILA (75; 261 nmol) zone using the same system.

Previous studies (15, 16) of the microfloral metabolism of $LASO_4$ showed considerable contamination of Peaks I and II (Fig. 1) with endogenous fatty acids and neutral sterols. Principal fatty acids found in these extracts have included 14:0, 15:0, 16:0, 17:0, and 18:0, whereas, coprostanol, cholesterol, and β-sitosterol were the major fecal sterols. Since Peak I-C (Fig. 1) is believed to be a fatty acyl derivative of ILA (from previous experiments like those described here), it was imperative to remove all traces of endogenous fatty acids. Using an ITLC-SA sheet developed in solvent *C*, all ME-fatty acids migrate to the ME-5β-cholenate region, whereas, all the sterols are more polar than the metabolite (ME-PALM-ILA type structure).

The methylated extract was applied to two ITLC-SA sheets containing appropriate standard markers (ME-cholentates, ME-PALM-ILA). After development, the edges of the chromatogram were sprayed with sulfuric acid, charred, and the appropriate regions marked. Approximately 6% of the radioactive band was removed and six metabolite regions counted. The activity associated with the ME-PALM-ILA region accounted for 77% (116 nmol) of the sample, whereas, 22% of the rest of the mass was concentrated on the baseline. This latter material contained 68% of its activity in the ME-ILA region of an ITLC-SG sheet developed in solvent *B*.

An injection of 2% of the purified metabolite (eluted from the sheet with chloroform–methanol 2:1) on a Silar 10C column (see Experimental Procedures) gave no fatty acid peaks, indicating no contamination with fatty acids. Using a one-pot, one-step transesterification procedure to conserve sample, the metabolite was hydrolyzed in 3% methanolic-hydrochloric acid (27) for 1 hr at 70°C. After cooling to room temperature, the reagent was removed under a stream of nitrogen, reconstituted in 200 μl of benzene and a 4-μl aliquot was analyzed by GLC on the same Silar 10C column. **Fig. 4** shows clearly that the predominant fatty acids associated with the metabolite were palmitic, stearic, and oleic acids which accounted for the majority of the endogenous fatty acids present in the fecal extracts.

Combined GLC–MS analysis of the fatty acids on a 3% SE-30 column, which resulted in the unsaturated



#	RT	AREA	AREA %	RRT	R AREA %	NAME
1	3.52	56206.	47.8	1	0	#14:0
2	6.17	14604	12.4	1.752	23.8	16:0
3	7.27	1652	1.4	2.066	2.7	16.1
4	8.36	1405	1.2	2.374	2.3	
5	10.81	28905	24.6	3.071	47	18:0
6	12.55	14907	12.7	3.566	24.2	18:1

Fig. 4. GLC analysis of Peak I-C on Silar 10C (conditions in the text) following ITLC-purification and transesterification. Methyl 14:0 was coinjected with the extract to determine relative retention times (RRT). Note that no Me-14:0 was found in the extract itself.

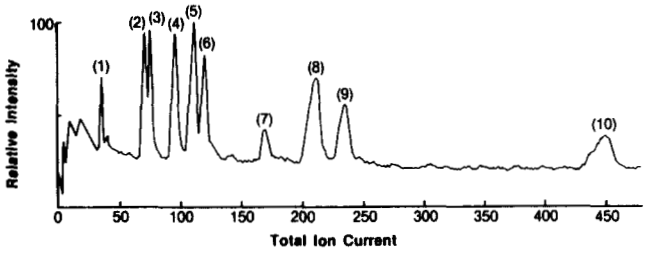


Fig. 5. Reconstructed GLC chromatogram used to analyze "STERBILE," which is a mixture of #5 β-cholestane (1), ME-Δ^3-cholenate (2), ME-Δ^2-cholenate + ME-5β (3), coprostanol (4), cholesterol (5), cholestanol (6), β-sitosterol (7), ME-ILA (8), ME-LA (9), and ME-3-keto (10). Note that there is a good separation of the isomeric cholenates and baseline resolution of the epimeric lithocholates. Mass spectra of these standards were compared with those found for Peaks I-C, II, and III.

fatty acids eluting ahead of their respective saturated derivatives (as opposed to the Silar 10C column), gave identical results with characteristic fragments for methyl palmitate at: (m/z = 270 (M^+), 239, 227, 213, 199, 185, 171, 157, 149, 143, 129, 115, 101, 87, and 74 (base peak)), oleate (m/z = 296 (M^+), 264, 222, 180, 166, 123, 101, 87, 74, 69, and 55 (base peak)), and stearate (m/z = 298 (M^+), 267, 255, 213, 199, 185, 171, 157, 143, 129, 111, 101, 87, and 74 (base peak)). A few minor GLC peaks present were too small to be identified.

ITLC-SG analysis of the transesterified metabolite using solvent *B* found the activity in the ME-ILA (88%, 162 nmoles) region. The remainder of the activity (8%) was concentrated at the solvent front presumably due to some ME-cholenate formed during the transesterification.

GLC–MS conditions were standardized by using the synthetic mixture of "STERBILE" as shown in the reconstructed chromatogram in **Fig. 5.** There was good resolution between the isomeric cholenates and baseline resolution of the epimeric lithocholates facilitated the identification of the bile acid portion of Peak I-C.

Analysis of the transesterified metabolite gave major fragments at m/z = 390 (M^+), 375, 372, 357, 290, 264, 257, 248, 233, 230, 215, and 201, that are identical with ME-ILA and confirm the identity of Peak I as a mixture of 3β-fatty acyl-ILA derivatives.

Identification of Peak II

Fig. 3 shows that Peak II activity is associated with the 5β-cholanic/cholenic acid region. Similarly, methylation of this metabolite results in a shift of the total activity (93%, 1839 nmol) to the methyl-5β-cholanate/cholenate region of the chromatogram. GLC of this metabolite (similar to Fig. 5) indicated clearly that this compound was specifically the Δ^3-cholenate. Other endogenous lipids present included coprostanol, cholesterol, cholestanol, and β-sitosterol. Major fatty acids associated with this extract (Silar 10C data not shown) included ME-16:0, 16:1, 18:0, and 18:1. GLC–MS data (**Figs. 6A–6C**) also support the identification of the metabolite (Fig. 6B) as the Δ^3-cholenate as opposed to the Δ^2-isomer. These data agree with Palmer's (11) suggestion as to the identity of this metabolite.

Identification of Peak III

Chromatographic analysis using an ITLC-SG sheet developed in solvent mixture *B* gives excellent separation of ME-LA and ME-ILA. Using this system, methylated Peak III contained 82% (329 nmoles) of the activity which was associated with ME-LA and 9% (37 nmol) having an R_f identical with that of ME-ILA.

In support of the ITLC-analysis of this extract, the GLC profile on a 3% OV-210 column showed the presence of ILA and LA as the major bile acid metabolites. GLC–MS analysis gave fragments at m/z = 390 (M^+), 372, 357, 341, 318, 257, 248, 230, 215, and 201 for ME-ILA and identical fragments (no M^+) for ME-LA.

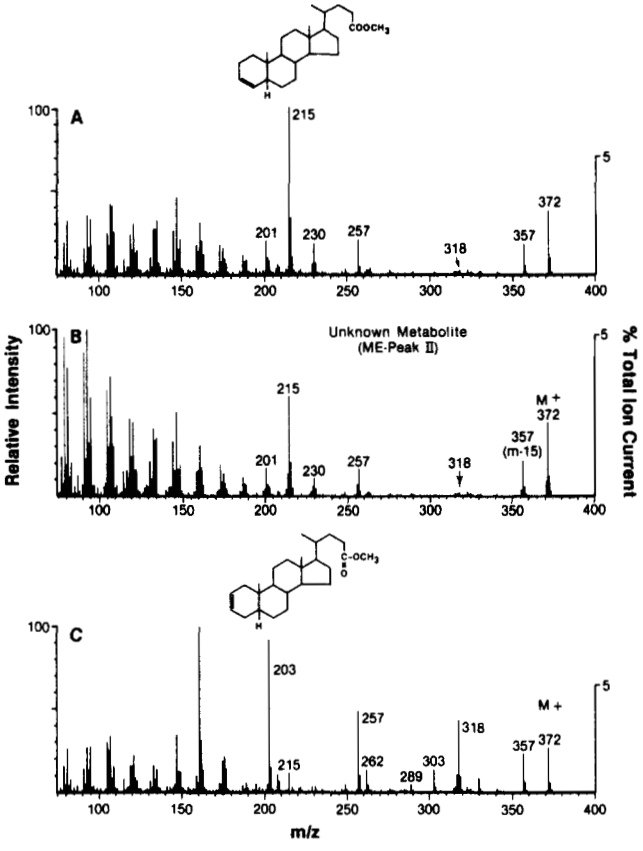


Fig. 6. Mass spectrum of methyl-5β-chol-3-enoate (Δ^3-cholenate). Conditions are given in text. B. Mass spectrum of methylated Peak II. C. Mass spectrum of methyl-5β-chol-2-enoate. Note that the mass spectrum of the metabolite agrees with that for the Δ^3-cholenate.

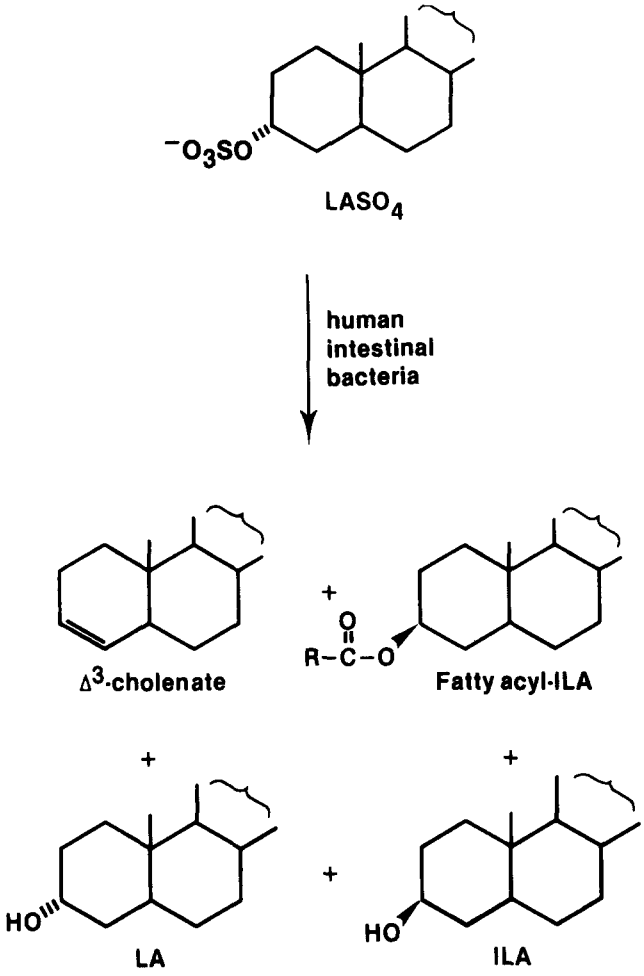


Fig. 7. Summary of microfloral metabolism of $LASO_4$ to non-polar derivatives. The fatty acyl-ILA represents a mixture of fatty acyl derivatives including 16:0, 16:1, 18:0, and 18:1 which reflect the composition of endogenous fatty acids present in these extracts.

DISCUSSION

Results presented in this report have shown that human gut microflora are capable of converting $LASO_4$ into four major metabolites as summarized in **Fig. 7.** The identification of a 3β-fatty acyl-derivative of ILA (Peak I-C, Fig. 2) now clarifies observations by Norman and Palmer (14) who observed saponifiable derivatives of ILA in the feces of humans given an oral dose of [24-^{14}C]-LA. Using in vitro incubations, we have now been able to identify this major metabolite as a mixture of fatty acyl derivatives including palmitoyl, palmitoleyl, stearyl, and oleyl esters of ILA. These results are further confirmed by the fact that these fatty acids are major constituents in our extracts and are common major metabolites in human feces (28, 29). Since the bile acid portion of the molecule was labeled, it was easily monitored by ITLC-chromatography both before and after transesterification. Data presented in Fig. 3 indicate that the metabolite contains a free carboxyl group which, after methylation, has an R_f similar to ME-PALM-ILA, which was used as a model compound.

Additional data (not presented here) regarding the identity of Peak I included low resolution, direct probe mass spectra of the intact metabolite which gave a base peak at m/z = 372; this represents the methyl cholenate fragment of the molecule similar to that observed with ME-PALM-ILA. This fragmentation of acyl esters of bile acids is also characteristic of cholesteryl esters such as cholesteryl palmitate[4] and cholesteryl ethers (30).

The formation of the Δ^3-cholenate (Peak II, Fig. 2) and not the Δ^2-isomer clarifies earlier observations by Palmer (11) and Kelsey (15) who were not certain of the exact isomer formed. According to Palmer (11), a structure of this type might have interesting pharmacological and toxicological properties perhaps more potent than LA, and data presented here show that the intestinal microorganisms are capable of forming this metabolite from sulfolithocholate.

Peak III contains mostly LA and ILA, thus indicating the presence of sulfatase enzymes in the fecal incubations. In examining the structures of these metabolites with respect to Peaks I and II, it would be interesting to know if the fatty acyl derivative is formed via esterification with ILA similar to cholesteryl esters (31), or if some form of displacement of the sulfate ester with inversion of configuration occurs. Similarly, the formation of the cholenate might occur via elimination of the fatty acyl or sulfate group (21). Additional studies with labeled substrates are planned to elucidate these new biosynthetic pathways.

The identification of the 3β-fatty acyl-ILA and Δ^3-cholenic acid as new microfloral metabolites of sulfolithocholic acid represent new types of bile acids which have not been examined routinely in the feces of individuals at high- and low-risk for colon cancer. Preliminary in vitro studies done at this institution (16), and in collaboration with Dr. Martin Lipkin of Memorial Sloan-Kettering Institute with low- and high-risk subjects, show the presence of significant amounts of both types of metabolites. These data suggest that further screening studies are necessary to obtain statistical evaluation of such biochemical markers as possible indicators of neoplasia.

It would be important to know if the fatty acyl-ILA and cholenate derivatives represent activation or detoxification of LA-type metabolites. With sufficient quantities of such metabolites, in vitro studies (5, 6, 9)

[4] Kelsey, M. I., J. E. Molina, S -K. S. Huang, and K -K. Hwang. Unpublished results.

of these new metabolites should provide important clues regarding their possible roles as modifiers of carcinogenesis.

The authors wish to thank Drs. R. Hallmark, J. Kawalek, and W. Lijinsky for their comments and helpful suggestions. This research was supported by Contract No. N01-CO-75380, with the National Cancer Institute, NIH, Bethesda, MD 20205.

Manuscript received 10 September 1979 and in revised form 14 April 1980.

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