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THE BIOSYNTHESIS OF ETHYL ESTERS OF LITHOCHOLIC ACID AND ISOLITHOCHOLIC ACID BY RAT INTESTINAL MICROFLORA*

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SUMMARY

Recent reports concerning the tumor-promoting action of lithocholic acid in the colon and liver suggest that the metabolism of this major fecal bile acid may be important in carcinogenesis at various target sites. The metabolism of [^{14}COOH]-lithocholic acid by rat intestinal microflora derived from standard laboratory chow-fed animals produced slightly more non-polar metabolites than those incubations which utilized flora from animals on a high lean-beef regimen. Purification of the crude bacterial extracts by Sephadex LH-20 chromatography and analysis of the radioactive peaks by glass fiber paper chromatography resulted in the identification of two neutral metabolites. Confirmation of their identity as ethyl lithocholate and ethyl isolithocholate was achieved by gas–liquid chromatography and combined gas–liquid chromatography–chemical ionization mass spectrometry. The formation of ethyl esters of lithocholic acid and isolithocholic acid by the intestinal microflora requires the presence of ethanol and anaerobic incubation conditions. These data support results obtained previously with single human fecal microorganisms. Since the formation of these derivatives *in vitro* occurs under anaerobic conditions only, it is possible that such derivatives may form physiologically in the colon. The carcinogenic potential of these derivatives is under investigation.

INTRODUCTION

Epidemiological evidence indicates that a high beef-fat diet results in an alteration both in the composition and capacity of the intestinal microflora to metabolize endogenous fecal steroids and bile acids to derivatives that might be carcinogenic in the colon[1, 2]. Lithocholic acid (LA)† is a major bile acid found in the feces of animals and humans on such a diet and promotes tumorigenesis in the colon of rats treated with N-methyl-N′-nitro-N-nitrosoguanidine[3]. Also LA administration resulted in an increase in ethionine-induced hyperplastic nodules and hepatomas of rat liver[4].

Since no evidence is available to indicate whether LA itself or its metabolites are responsible for the promoting activities mentioned above, the metabolism of this compound becomes increasingly important.

We have previously studied the metabolism of LA by single human fecal microorganisms[5] and found little or no conversion of this substrate to oxidized, reduced or hydroxylated products. Since the colon contains a mixed population of microorganisms, studies with the intact intestinal microflora become more relevant. Norman and Palmer[6] have shown that the major fecal metabolites of LA in humans are isolithocholic acid (IL) and 3-keto-5β-cholan-24-oic acid (3-keto-cholanoic acid) as well as other unidentified compounds. In addition, human intestinal microflora are capable of the *in vitro* transformation of LA to IL *via* the 3-keto-cholanoic acid intermediary metabolite.

As a continuation of our interest in the metabolism of lithocholic acid, we have investigated the activity of the intestinal microflora derived from rats on a high-meat or lab-chow diet in an attempt to determine if diet can influence the metabolic products produced. In addition, we specifically analyzed the bacterial extracts for the presence of ethyl ester metabolites under different incubation conditions in order to compare these results with those obtained previously[5].

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† The following names and abbreviations for chemicals and methods have been used throughout the text: lithocholic acid (LA) = 3α-hydroxy-5β-cholan-24-oic acid; methyl lithocholate (ML) = methyl-3α-hydroxy-5β-cholan-24-oate; ethyl lithocholate (EL) = ethyl-3α-hydroxy-5β-cholan-24-oate; isolithocholic acid (IL) = 3β-hydroxy-5β-cholan-24-oic acid; methyl isolithocholate (MIL) = methyl-3β-hydroxy-5β-cholan-24-oate; ethyl isolithocholate (EIL) = ethyl-3β-hydroxy-5β-cholan-24-oate; 3-keto-cholanoic acid = 3-keto-5β-cholan-24-oic acid; Me-3-keto = methyl-3-keto-5β-cholan-24-oate; 5β-cholanoic acid = 5β-cholan-24-oic acid; Me-5β = methyl-5β-cholan-24-oate; deoxycholic acid = 3α,12α-dihydroxy-5β-cholan-24-oic acid; methyl deoxycholate = methyl-3α,12α-dihydroxy-5β-cholan-24-oate (methyl deoxy); g.f.p. = glass fiber paper chromatography; l.s.c. = liquid scintillation counting; BHI = brain heart infusion.

EXPERIMENTAL

Material. The following bile acids were obtained from Omni Research, Mayaguez, PR: lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid), isolithocholic acid (3β-hydroxy-5β-cholan-24-oic acid), 3-keto-cholanoic acid (3-keto-5β-cholan-24-oic acid) and deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid). Another bile acid used in this work was 5β-cholanoic acid (5β-cholan-24-oic acid) which was purchased from Schwartz–Mann (Orangeburg, NY). [^{14}COOH]-LA was purchased from Mallinckrodt Chemical Co. (St. Louis, MO) and had a S.A. of 5.37 mCi/mM. This labeled substrate had a purity greater than 98% as judged by thin-layer chromatography (t.l.c.) described in the Methods section.

The preparation of authentic methyl and ethyl esters of LA and IL has been described[5], and the ethyl esters were used as reference standards for monitoring metabolic products produced in incubations with the rat intestinal microflora.

The scintillation cocktail used in all radioactive protocols was Aquasol (New England Nuclear, Boston, MA). All radioactive assays were conducted on an Isocap/300 Liquid Scintillation System (Nuclear Chicago, Des Plaines, IL), and samples were corrected for background and 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, MI).

METHODS

Preparation of rat mixed fecal cultures. Fischer 344 rats obtained from the Animal Farm at the Frederick Cancer Research Center had been fed either a standard Purina lab-chow or a high-meat diet consisting entirely of extra-lean ground beef. All animals had been maintained on their respective diets for at least six months, and the fecal material from these animals was used as a source of intestinal microflora for our metabolism studies. A single fecal pellet from each of the dietary groups was used to inoculate 30 ml of brain heart infusion (BHI) medium which was prepared according to procedures described in the V.P.I. Anaerobe Laboratory Manual[7]. The fecal suspension was incubated for 7 h at 37°C under anaerobic conditions in order to disperse the pellet completely. A 3 ml aliquot of this mixed fecal culture suspension was used for incubations with [^{14}COOH]-LA (1 μCi), and the experiment was continued for an additional 48 h at 37°C under anaerobic conditions before extracting the metabolites.

Incubations of unlabeled lithocholic acid with mixed fecal cultures. In experiments with unlabeled substrate, a scale-up of the radioactive protocol was used. One fecal pellet derived from a rat on a lab-chow diet was suspended in 180 ml BHI, and a 7 h preincubation period at 37°C anaerobically was used to disperse the pellet completely. Substrate (10.8 μmol) was then added to the mixed fecal cultures, and the incubation was continued for an additional 48 h at 37°C anaerobically. Controls of substrate and BHI as well as mixed fecal cultures without substrate were incubated simultaneously with the above.

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 1 vol. 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, NJ) gave a residue which was analysed as described below.

Thin-layer chromatography. Pre-coated plates with silica gel G (Analtech, Inc., Newark, DE) of 250 μm thickness were used for analytical purposes.

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.

Glass fiber paper chromatography (g.f.p.). Glass fiber sheets coated with silica gel G (Gelman Instrument Co., Ann Arbor, MI) were also used to assay unlabeled and labeled incubation mixtures with the solvent system: isooctane–isopropyl ether–acetic acid (75:30:0.5 by vol.). Visualization of spots obtained 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.

For radioactive experiments, zones of silica gel-coated g.f.p. corresponding to the mobilities (R_F) of reference standards were cut out, placed into vials and assayed by liquid scintillation counting (l.s.c.).

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.

The g.l.c. phase used for analysis was a 182 cm. long, 0.3 cm. i.d. glass column packed with 1% QF-1 on Gas Chrome Q (100–200 mesh) which was obtained from Supelco, Inc. (Bellefonte, PA). Operating conditions included oven and injection port temperatures of 210°C and detector oven at 280°C. Helium gas was used as a carrier at a flow of approximately 60 ml/min.

Gas–liquid chromatography–mass spectrometry (g.l.c.–m.s.). The g.l.c. inlet system used was a Varian Aerograph (Varian Associates, Palo Alto, CA) equipped with a 1% QF-1 column on Gas Chrome Q (100–200 mesh) that was maintained at 240°C with methane gas as both carrier (25 ml/min) and chemical ionization reagent. The effluent was ionized at 100°C, 150 V electron energy and 500 mA emmission current in a Finnegan Model 1015 (Finnigan Corp., Sunnyvale, CA) g.l.c.–m.s. instrument equipped with a Finnigan 6000 Data System.

Sephadex LH-20 chromatography. Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ) was swollen in isooctane–chloroform–methanol (2:1:1 by vol.) overnight and was packed by gravity into a column (1.9 × 66 cm.) to give a bed vol. of approx. 152 ml (51 cm.).

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 g.f.p.–l.s.c. in radiolabeled extracts.

Using these columns bile acid reference standards were eluted in the following average bed volumes: 5β-cholanoic acid (50%), ethyl lithocholate (EL) and ethyl isolithocholate (EIL) (53%), 3-keto-cholanoic acid (66%) and LA and IL (82%).

RESULTS

In contrast to work with individual strains of intestinal anaerobes, a mixed-fecal population of organisms produces a much more complicated metabolic profile. Studies[6, 8, 9] have shown that LA can be converted to its 3β-epimer via oxidation at the C-3 hydroxyl group using mixed fecal cultures. Since we reported the formation of EL by pure strains of organisms[5], it was reasonable to expect that both EL and EIL might be produced in incubations with LA added as an ethanolic solution to mixed fecal cultures.

Figure 1 shows the separation of LA, IL and their respective ethyl esters by g.f.p. indicating good resolution of the epimeric acids and esters. It should be noted, however, that the ethyl and methyl esters of LA and IL do not separate from each other by g.f.p. but do separate by g.l.c.[5]. Using the g.f.p. system in conjunction with l.s.c. analyses of the individual zones gave results shown in Table 1. It is apparent from these data that the lab-chow profile demonstrated more conversion of LA to EIL and more nonpolar metabolites, whereas the high-meat data

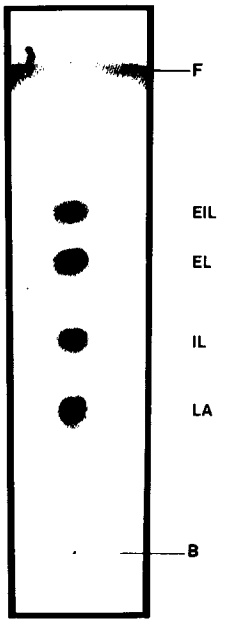
Table 1. Radioactive distribution of metabolites produced by the incubation of [^{14}COOH]-LA with rat intestinal microflora. Silica gel-coated g.f.p. sheets were used to separate the metabolites

		ORIGIN OF THE MICROFLORA					
ZONE		HM_{30}[1]		BHI-CONTROL		LC_{30}[1]	
		cpm	%	cpm	%	cpm	%
1		21.6	(0.12)	4.1	(0.03)	49.8	(0.42)
2		107.5	(0.63)	19.5	(0.14)	142.9	(1.21)
3		135.6	(0.79)	20.8	(0.15)	116.2	(0.98)
4	EIL	676.2	(3.98)	17.1	(0.12)	573.4	(4.87) EIL
5	EL	1625.7	(9.57)	66.2	(0.47)	796.4	(6.77) EL
6	IL	2360.8	(13.9)	55.4	(0.39)	1468.8	(12.48) IL
7	LA	10768.2	(63.4)	13262.2	(95.7)	6965.2	(59.22) LA
8		179.1	(1.05)	157.4	(1.13)	226.0	(1.92)
9	Base[2]	1107.5	(6.5)	258.5	(1.86)	1422.5	(12.09)
Total cpm		16982.2		13861.2		11761.2	

g.f.p. Solvent: isooctane: isopropyl ether: HOAC (75:30:0.5)

[1] HM_{30} or LC_{30} represents the incubation of [^{14}COOH]-LA with mixed fecal cultures which were prepared by dispersing a single fecal pellet from an animal on a high-meat or laboratory-chow diet, respectively, in 30 ml of BHI medium.

[2] The percent total activity recovered in the respective organic extracts was 100% (HM_{30}), 89% (BHI) and 75% (LC_{30}). In general, recoveries range from 80–90% depending upon the amount of emulsions present.



ISOOCTANE: ISOPROPYL ETHER: ACETIC ACID (75:30:0.5)

Fig. 1. Separation of LA, IL and their ethyl esters by silica gel-coated glass fiber paper chromatography. The metabolites are designated as follows: LA (lithocholic acid), IL (isolithocholic acid), EL (ethyl lithocholate) and EIL (ethyl isolithocholate).

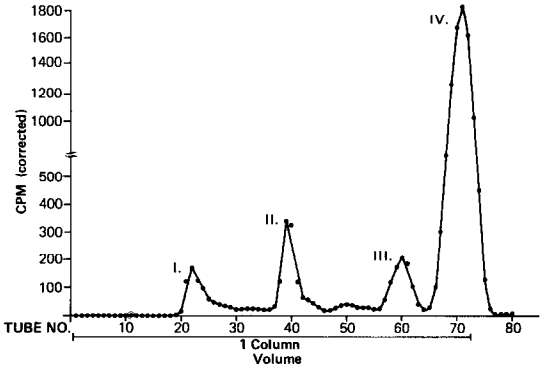


Fig. 2. Sephadex LH-20 chromatography purification of bacterial extract prepared from the incubation of [^{14}COOH]-LA and rat intestinal microflora. A solvent mixture of isooctane–chloroform–methanol (2:1:1 by vol.) was used to elute the metabolites from the column. The size of the fractions was 2 ml, and 20 μl aliquots were used for l.s.c. analyses. The column volume was 152 ml.

Table 2. Analysis of peaks from Sephadex LH-20 purification (see Fig. 2) of an extract derived from the incubation of LA with lab-chow microflora. The numbers represent the percent distribution of the total c.p.m. of the extract. Reference standards used included LA (lithocholic acid), EL (ethyl lithocholate), EIL (ethyl isolithocholate), Me-3-keto (methyl-3-keto-5β-cholan-24-oate) and Me-5β (methyl-5β-cholan-24-oate)

	% DISTRIBUTION OF RADIOACTIVITY				
COMPOUND	ACID I	METHYLATED I	ACID II	METHYLATED II	COMPOUND
Me-5β	4.9	5.2	0	1.1	Me-5β
	2.9	1.2	1.6	1.2	
Me-3-Keto	2.8	1.9	0.7	0.6	Me-3-Keto
	4.7	3.5	3.2	1.3	
EIL	0.8	1.0	25.7	25.2	EIL
	0.5	1.1	15.0	6.8	
EL	0.9	0.8	28.1	36.2	EL
	0.3	0.5	0.4	6.9	
IL	1.0	1.2	0.4	5.1	IL
	1.3	1.5	0.7	0.6	
LA	2.6	4.6	12.6	2.3	LA
	22.6	29.2	3.9	4.8	
Baseline	55.1	48.2	7.5	7.7	Baseline
Total cpm	641.9	515.7	926.6	888.3	

Table 3. Determination of the presence of LA and its metabolites in extracts from rat intestinal microflora (see Fig. 2). The numbers represent percent distribution of the total c.p.m. of the extract. Reference standards used included LA (lithocholic acid), IL (isolithocholic acid), ML (methyl lithocholate), MIL (methyl isolithocholate), 3-keto (3-keto-cholanoic acid), Me-3-keto (methyl-3-keto-5β-cholan-24-oate), 5β (5β-cholanoic acid) and Me-5β (methyl-5β-cholan-24-oate)

	% DISTRIBUTION OF RADIOACTIVITY				
	ACID III	METHYLATED III	ACID IV	METHYLATED IV	
Me-5β +5β	0.3	0.6	0	0.1	Me-5β +5β
	0.4	3.2	0	0	
Me-3-Keto	0.5	67.6	0	0.1	Me-3-Keto
	0.8	0.6	0	2.4	
MIL + 3-Keto	75.3	4.2	0.2	18.7	MIL + 3-Keto
ML	1.1	4.8	12.8	66.0	ML
IL	1.3	1.0	18.8	1.5	IL
LA	4.9	1.8	66.4	9.0	LA
	4.0	3.9	0.8	1.0	
Baseline	11.5	12.2	1.0	1.0	Baseline
Total cpm =	994.4	1000.9	7737.9	8555.4	

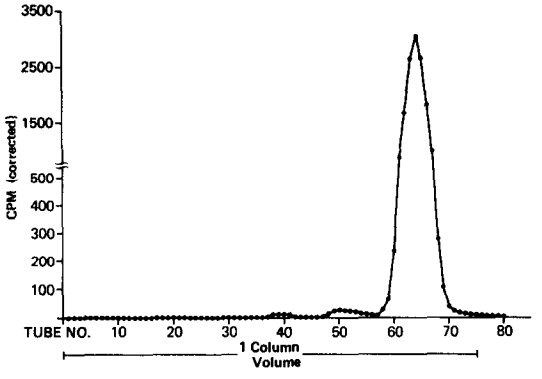


Fig. 3. Sephadex LH-20 purification of extract LA + BHI incubation performed anaerobically. Conditions for purification and assay were identical with those given in Fig. 2.

resulted in increased levels of IL and EL. Although the differences between the profiles may be small, the lab-chow data in other experiments consistently demonstrated the presence of more non-polar metabolites.

Figure 2 contains data obtained from LH-20 fractionation of the crude extract using the solvent system described in the Methods section. Each peak was analysed as its acid and methyl ester by g.f.p., and the results shown in Tables 2 and 3 represent the actual separation of the zones analysed by l.s.c. Peak 1 contained nonpolar metabolites in the 5β-cholanoic acid region (solvent front) that did not shift upon methylation; peak II corresponded to the EL and EIL regions which also did not shift upon methylation. Other unidentified metabolites in the peak II region had R_F values different from the epimeric ethyl esters of LA and IL.

The analyses of peaks III and IV by combined g.f.p.–l.s.c. are summarized in Table 3 and show that peak III contained a major metabolite whose polarity was identical with that of 3-keto-cholanoic acid and shifted upon methylation to the methyl ester region of 3-keto-cholanoic acid; peak IV corresponded to LA and IL which migrated to their respective methyl ester regions upon treatment with diazomethane.

The control (LA + BHI) shown in Fig. 3 contains no activity in the region of peaks I–III but has a single labeled peak corresponding to unmetabolized LA according to g.f.p. techniques.

Additional confirmation of the presence of IL, EL and EIL was obtained in experiments with unlabeled LA incubated with rat mixed fecal cultures (see Methods).

Purification of the crude bacterial extract by LH-20 chromatography resulted in a number of metabolites in the region of interest. Since the column used for this separation had been previously calibrated from the radioactive experiments, the location of EIL and EL in fractions 37–39 (49–52% of the column elution vol.) was easily confirmed by g.l.c. analysis as shown in Fig. 4. Retention times of 0.53 (EIL) and 0.59 (EL) relative to methyl deoxycholate (methyl deoxy), which was used as an external reference standard, agreed well with previously published data[5].

Similarly, IL and LA were eluted in fractions 55–63 corresponding to a column vol. of 73–84%, and their g.l.c. retention times were identical to those values expected[5].

The controls representing LA + BHI or cells without substrate did not exhibit metabolites on g.l.c. with retention times of metabolites of interest.

Combined g.l.c.–m.s. analyses of the pooled peaks confirmed the presence of EIL with peaks at

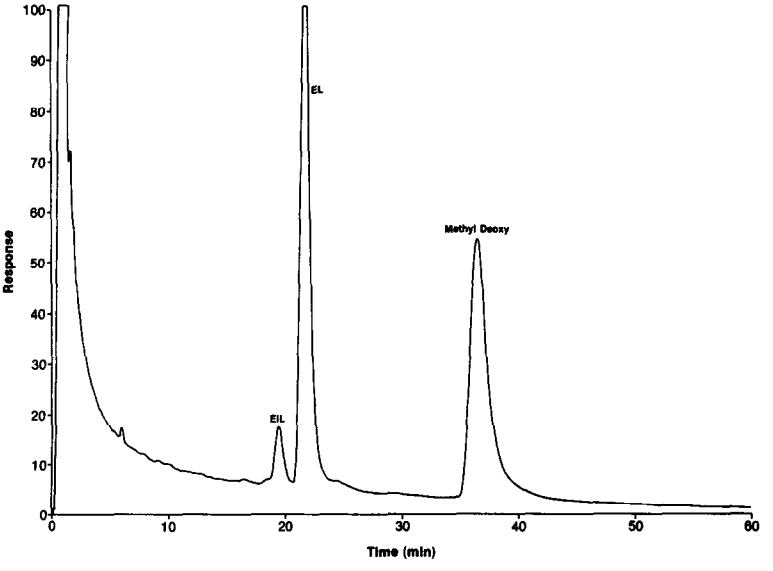


Fig. 4. G.l.c. profile of EIL and EL produced anaerobically from the incubation of LA with rat mixed fecal cultures. Methyl deoxycholate (Methyl Deoxy) was used as an external reference standard. Conditions are given in the Methods section.

$m/e = 433$ (addition of reagent gas to the parent molecule, mol. wt. = 404) and $m/e = 405$ $(M + H)^+$. In addition peaks at $m/e = 387$ and 341 were also identical with those found for authentic EIL and are possibly due to the loss of water and water + ethanol, respectively, from the $(M + H)^+$ ion. The exact mechanisms for the fragmentation of bile acids by chemical ionization m.s. are currently under investigation[10]. EL had a similar profile with the exception that no $(M + 1)^+$ peak was observed[5].

IL and LA analysed as their methyl esters had fragmentation patterns similar to those for their respective ethyl esters. Fragments at $m/e = 419$, and 373 were presumably due to the addition of ionizing gas to the parent molecule and loss of water from the $(M + 1)^+$ peaks, respectively. Again, an $(M + 1)^+$ peak at $m/e = 391$ was seen for methyl isolithocholate (MIL), but none was present in the fragmentation of methyl lithocholate (ML)[5]. Additional fragments at $m/e = 357$ and 341 were identical with those observed for authentic samples, and mechanisms for these fragmentations will require further studies. Fragmentations which were common to both methyl and ethyl ester derivatives differed by 14 atomic mass units.

The influence of oxygen on the nature of metabolic products formed by the intestinal microflora could be demonstrated clearly from the Sephadex LH-20 fractionation of the crude bacterial extracts. Figure 5 shows that four major peaks of activity are obtained under normal anaerobic conditions; these peaks correspond to unknown nonpolar metabolites (peak No. 1), EL and EIL (peak No. 2), 3-keto-cholanoic acid

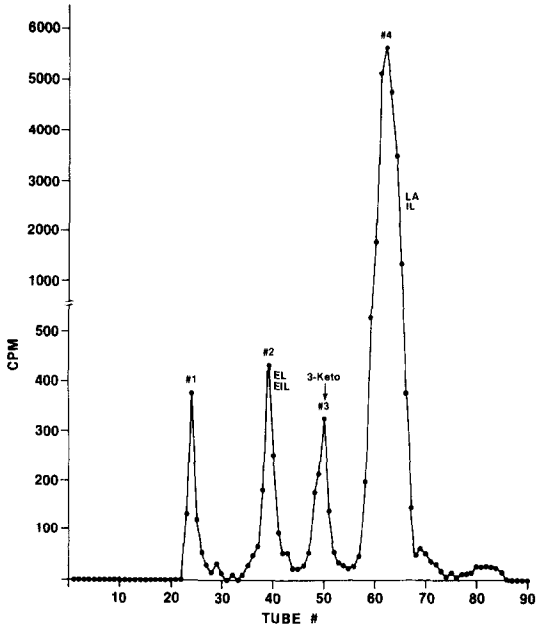


Fig. 5. The metabolism of [^{14}COOH]-LA anaerobically by rat intestinal microflora. Conditions for Sephadex LH-20 purification and assay of metabolites are identical to those described for Fig. 2.

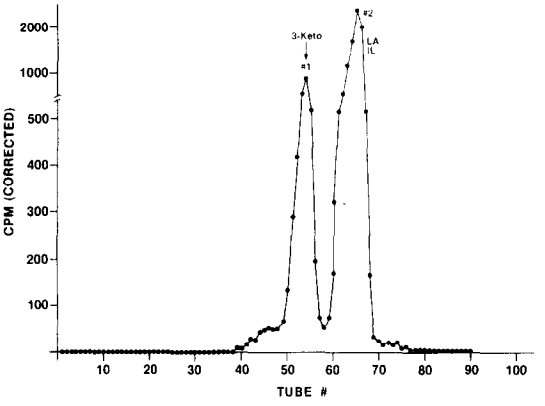


Fig. 6. Aerobic metabolism of [^{14}COOH]-LA by rat intestinal microflora. Conditions for Sephadex LH-20 purification and assay of metabolites are identical to those described for Fig. 2.

(peak No. 3) and IL and LA (peak No. 4) as determined by combined g.f.p.–l.s.c. of the acid and ester derivatives. Using aerobic conditions in a parallel experiment run simultaneously with the above, only two peaks (Fig. 6) are obtained corresponding to 3-keto-cholanoic acid (peak No. 1) and the mixture of IL and LA (peak No. 2). Therefore the formation of ethyl ester derivatives requires anaerobic conditions, whereas aerobic conditions favor the expected oxidized derivative primarily.

DISCUSSION

The metabolism of LA by a mixed population of organisms from animals on different dietary regimens resulted in slight differences which appear to be more apparent in the production of unidentified nonpolar, or more chemically modified derivatives by the lab-chow diet. Hill has suggested that populations whose diet is rich in beef and fat excrete more highly degraded bile acids which may be carcinogenic to colonic tissue[11]. Although the results presented here appear to contradict this hypothesis, it should be noted that the data were obtained from rodents and not humans and, furthermore, the high-meat animals were fed lean meat which was not supplemented with fat. Studies are now in progress to monitor the metabolism of LA by human intestinal microflora which are derived from subjects on different diets in order to ascertain if dietary differences will result in more non-polar or chemically modified derivatives.

Previously, we have observed that EL was produced by certain select fecal microorganisms including two strains of Bacteroides, *Citrobacter* sp. and *Peptostreptococcus productus* I. However, when incubating LA as an ethanolic solution[5] neither IL nor 3-keto-cholanoic acid was produced and detected in these bacterial extracts.

With mixed populations of organisms from rats, IL and 3-keto-cholanoic as well as other unidentified nonpolar metabolites are produced, and esterification

of all of these derivatives with ethanol is theoretically possible. Data presented here show that both EL and EIL are formed by the intestinal microflora and can be separated from their respective acid precursors by Sephadex LH-20 chromatography. Additional evidence in support of their structure included g.f.p.-l.s.c. data as well as g.l.c.–m.s. separation and fragmentation. Ethyl-3-keto-5β-cholan-24-oate was not identified in the bacterial extracts since no authentic reference material was prepared, but future studies will include screening for this metabolite as well.

The requirement of anaerobic conditions for the esterification of LA and IL by the intestinal microflora was clearly shown in the Sephadex LH-20 profiles (Figs. 5 and 6). Although some 3-keto-cholanoic acid is produced under anaerobic conditions, the aerobic incubations performed simultaneously with the anaerobic experiment resulted in optimal 3-keto-cholanoic acid formation. The presence of LA and IL in the aerobic incubation did not result in the formation of their respective ethyl esters. These results agree with our previous data which demonstrated a requirement for anaerobic conditions for the biosynthesis of EL using the facultative organism, Citrobacter[5].

Evidence that LA may be involved in mammalian carcinogenesis at various target sites includes its role as a tumor-promoting agent in the colon[3] and liver[4]. Unpublished observations from our laboratory[12] have suggested that LA and its metabolites (including EL) only produced a comutagenic response in the Ames mutagen assay[13] with suboptimal levels of a carcinogen in the presence of the mammalian microsomal activation system. Therefore, further studies regarding the metabolism of this major fecal bile acid are justified and should extend to the physiological derivatives such as the taurine conjugate and sulfate ester using human rather than rodent intestinal microflora. These experiments are currently in progress.

The formation of ethyl esters of both LA and IL anaerobically by the intestinal microflora *in vitro* represents a novel metabolic pathway of LA, but the significance of such derivatives is unknown at present. The comutagenic activity of EL suggests that such compounds might also act as tumor-promoting agents but further work would be required to demonstrate this *in vivo*.

In addition, the presence of unknown non-polar derivatives formed only under anaerobic conditions is interesting since the colon is essentially anaerobic. These derivatives should be identified and tested in the bacterial mutagenesis system for their mutagenic or comutagenic activities since such highly degraded compounds may be carcinogenic in the large intestine and/or other target sites.

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