Polydeoxycholate in human and hamster feces: a major product of cholate metabolism

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Abstract Fecal bile acid excretion is one of the two major routes by which cholesterol is eliminated from the body, fecal cholesterol being the other. During their enterohepatic circulation, bile acids are secreted into the duodenum, pass down the jejunum and into the ileum where more than 95% is reabsorbed by the gut. Bile acids that escape reabsorption in the small intestine are metabolized by microorganisms in the large intestine. The major routes of metabolism are reported to be deconjugation, dehydroxylation, especially at the 7α-hydroxy position, and dehydrogenation of the hydroxyl moieties. There are also some reports that saponifiable metabolites containing mostly deoxycholic acid form a major component of the bile acids found in human feces. We have identified a novel metabolite of cholic acid, 3α-hydroxy polydeoxycholate, in both human and hamster feces that is the major constituent of these saponifiable metabolites. Furthermore, we have shown in hamsters that the animals that excreted more bile acid were excreting the additional bile acid as polydeoxycholate. As expected, there was a negative correlation between bile acid excretion in the feces and plasma cholesterol concentrations in these animals. We speculate that polydeoxycholate is formed in the lower gut of both humans and hamsters and that, by its formation, bile acid will be sequestered in an insoluble form, thus inhibiting its reabsorption by the gut. This process may help to reduce plasma cholesterol concentrations and coronary heart disease in humans.—**Benson, G. M., N. J. Haskins, C. Eckers, P. J. Moore, D. G. Reid, R. C. Mitchell, S. Waghmare, and K. E. Suckling.** Polydeoxycholate in human and hamster feces: a major product of cholate metabolism. *J. Lipid Res.* 1993. **34:** 2121–2134.

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The bile acids found in human and hamster bile are mostly cholic acid and chenodeoxycholic acid, the two primary bile acids produced by the liver from cholesterol (1–6). There are also smaller amounts of the microbial metabolites of these two bile acids, the secondary bile acids including deoxycholic acid and lithocholic acid, which are produced in the cecum and colon. Most of the biliary bile acids are conjugated to either glycine or taurine (4, 6–8). The bile acids are secreted into the duodenum, pass down the small intestine, and are efficiently reabsorbed by the ileum and returned to the liver. Only about 5% of the bile acid passes into the cecum and colon where microbial metabolism of the bile acids produces the metabolites found in the feces. These bile acid metabolites are reported to be nonconjugated and mostly 7α-dehydroxylated (3, 4, 9–11). Bile acid metabolites are usually extracted from feces, prior to analysis, using an hydrolysis or solvent extraction method which would be expected to either result in structural modification of some of the bile acids (12, 13) or their saponifiable metabolites (14–16) or which may be selective as to which bile acids are extracted. In studies designed to characterize novel hypolipidemic agents we wanted to identify a method for the quantitative extraction of the fecal bile acids, during which they would not be altered chemically, so that we could study differences in their profiles. The best method of monitoring the efficiency of such methods is to extract the bile acids from feces of animals in which the cholic acid and chenodeoxycholic acid metabolites have been radiolabeled separately with ^{14}C as in the method described by Eneroth, Hellström, and Sjövall (17). In studies in hamsters and subsequent studies in humans we discovered that a variable but sometimes large proportion of the cholic acid metabolites in the feces of most hamsters and humans are present as oligomers of 3α-hydroxy polydeoxycholic acid. These saponifiable metabolites of deoxycholic acid have not been characterized previously.

Abbreviations: NMR, nuclear magnetic resonance; IR, infrared; tBuOH–H_2O, mixture of tertiary butyl alcohol and water; HPTLC, high performance thin-layer chromatography; FAB-MS, fast atom bombardment-mass spectrometry; LC-MS-MS, liquid chromatography followed by mass spectrometry followed by mass spectrometry of the fragment ions; GPC, gel permeation chromatography; LDL, low density lipoprotein.

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MATERIALS AND METHODS

Hamster studies

Male Golden-Syrian hamsters (Consort, Hereford, U.K., 100–160 g) were housed in wire-bottom cages, fed on a standard powdered diet (PRD diet; Labsure, Buckinghamshire, U.K.) and given water ad libitum. The dry weight of feces from hamsters fed this diet was 1.8 ± 0.3 g per day (n = 28). Eighteen hamsters from three experiments were dosed orally with [carboxyl-^{14}C]cholic acid (74 to 130 kBq/ml/hamster) and a further five hamsters received [carboxyl-^{14}C]chenodeoxycholic acid (170 KBq/ml/hamster, Amersham International, Bucks, U.K.). Their feces were collected on either 1 or 2 subsequent days and stored at −20°C. The feces were homogenized in water (1:3 w/w) and aliquots of the homogenate were freeze-dried and either combusted in an oxidizer, to measure the total radioactivity, or were extracted with solvent. Tertiary butyl alcohol and water (2 ml, tBuOH–H_2O 1:1 (v/v) ref. 18) was added to a vial containing 100- to 130-mg aliquots of dry feces and the vials were sealed. The contents of the vials were mixed for 15 min in a shaking water bath (37°C). The solids were then sedimented by centrifugation (1500 g, 15 min) and aliquots of the supernatant were removed for the measurement of radioactivity. Also, fecal samples from individual hamsters and those pooled from several hamsters were freeze-dried and extracted in a Soxhlet apparatus, using 500 ml of chloroform–methanol 1:1 (v/v) 18 h (17). The Soxhlet extract was dried and redissolved/resuspended in methanol (ca 20 ml) using a sonic water bath. The insoluble material was sedimented by centrifugation (220 *g*, 15 min) and the supernatant was removed and pooled with five further methanol washes of the pellet (i.e., 5 × 5 ml). The methanol-insoluble pellet was dried in air then dissolved/resuspended in a known volume of chloroform (there was a slight precipitate which rose to the surface of the chloroform on standing). These two fractions will be referred to as the methanol- and chloroform-soluble fractions of the Soxhlet extract.

[^{14}C]deoxycholic acid (73 KBq) was added to another freeze-dried fecal sample pooled from six hamsters (13 g) and the sample was then extracted using the Soxhlet method. The methanol- and chloroform-soluble fractions from this sample were isolated.

The radioactivity in aliquots of all the extracts was measured by liquid scintillation counting and the extraction efficiencies were calculated.

Human studies

Fecal samples from three volunteers were taken under the direction of the Clinical Pharmacology unit, S.B. and stored at −20°C until use. The feces were freeze-dried (14–17 g dry weight) and extracted using chloroform–methanol in a Soxhlet apparatus. The chloroform-soluble fraction of the Soxhlet extract was isolated as described above. The study protocol from which these samples were obtained was approved by an independent ethics committee. A further sample, donated by one of the authors, was mixed and divided in two. One aliquot (60 g) was freeze-dried whilst the other was chilled (36 g). [^{14}C]deoxycholic acid (73 kBq) was then added to each sample. The two samples were then extracted using the Soxhlet method. The methanol- and chloroform-soluble fractions from these samples were isolated. The amount of radioactivity in both fractions was measured and extraction efficiencies were determined.

Purification

The metabolites in the chloroform-soluble (methanol-insoluble) fraction of the Soxhlet extracts from both human and hamster feces were purified as a group using silica column chromatography (45 g, Kieselgel 60, 70–230 mesh, Merck, Darmstadt, Germany, 2 cm diameter column). Samples, in chloroform, were mixed with approximately 15 g of the silica in a beaker. This was then added to the remaining silica, in chloroform, in the column. The column contents were washed with chloroform (200 ml) followed by 200 ml chloroform–glacial acetic acid 99:1 (v/v). The metabolites were eluted using a stepped gradient of methanol (1–6%, 1–2% steps, 100 ml each step) in chloroform–glacial acetic acid 99:1 (v/v). The column eluent fractions containing the metabolites were identified using high performance thin-layer chromatography HPTLC and the endogenous radioactivity, when it was present.

HPTLC

The presence and purity of the chloroform-soluble metabolites was checked using silica HPTLC plates (Merck, Darmstadt, Germany) developed in ethyl acetate–cyclohexane–glacial acetic acid 9:9:2 (v/v). Samples were applied in bands onto the plates using an automatic TLC plate spotter (Camag Linomat IV, Merck Ltd, Poole, Dorset, U.K.). The plates on which the crude chloroform-soluble extract was applied were developed initially in chloroform which did not move the metabolites but made the metabolites mobile when the plates, once dry, were subsequently developed in ethyl acetate–cyclohexane–glacial acetic acid–chloroform 9:9:2:2 (v/v). Pre-developing the plates in chloroform prior to separating the metabolites in the crude extract probably removed contaminants from the origin which, when present, inhibited the mobility of the metabolites.

The bile acids were visualized as fluorescent bands using the charring solution of Schmitz, Assman, and Bowyer (19). Briefly, the plates were sprayed evenly with a mixture of methanol–water–concentrated sulfuric acid–$MnCl_2 \cdot 4H_2O$ (150 ml/150 ml/10 ml/1 g) until the surface of the plates remained momentarily wet. The plates were then heated uniformly at 110°C for 15 min in an oven.

The fluorescent bile acid products that formed were visible in ultraviolet light (366 nm) and were of a color characteristic for each bile acid. The position of the radioactivity on the plates, measured using a linear analyzer (RITA 68000, Isomess, Straubenhardt, Germany) or by autoradiography, corresponded to the position of the fluorescent bands.

Chemical synthesis of 3α-hydroxy polydeoxycholate

Deoxycholic acid (4.08 g) and *p*-toluenesulphonic acid (84 mg) were suspended in 250 ml toluene in a round-bottom flask fitted with a Dean and Stark condenser. The contents of the flask were refluxed for 5 h, then cooled and the solvent was removed. The flask contents were washed repeatedly with methanol to remove the methanol-soluble component of the mixture. The methanol-insoluble material, which contained the 3α-hydroxy polydeoxycholate, was dissolved in chloroform–methanol 1:1 (v/v) and a small quantity of insoluble residue was removed.

Partial hydrolysis

An aliquot of the purified chloroform-soluble metabolites from hamster feces was partially hydrolyzed in 8 M NaOH at room temperature overnight. The hydrolysate was neutralized using HCl and the sample was then purified. A C_{18} Bond Elut column fitted to a Vac-Elut apparatus (Jones Chromatography, Mid-Glamorgan, U.K.) was washed with 3 ml methanol followed by 3 ml water. The sample was then applied onto the column, the contents of which were then washed with 3 ml water, followed by two washes of methanol (2 × 3 ml) to elute any free bile acid and finally with chloroform (3 ml) to elute any unhydrolyzed metabolites. The metabolites eluted by the two methanol washes and the chloroform wash were separated by HPTLC as described above.

Complete hydrolysis

Samples of dry feces (100–130 mg) or of the chloroform-soluble metabolites were heated with 2 ml 1 M NaOH in 90% ethanol (110°C, 18 h) in borosilicate glass screw-cap vials (HPLC Technology, Macclesfield, U.K.). Aliquots of the hydrolysate (0.24 ml) were diluted with water (2.8 ml) and the bile acid metabolites were purified using C_{18} Bond Elut columns as described above but without neutralizing the pH. The purity of the extracts was also improved, without loss of bile acid, by washing the column contents with 15–20% methanol in water in place of the water alone. Complete recovery of the radiolabeled, hydrolyzed metabolites was achieved by elution with 3 ml methanol only. The methanol fractions from the columns were dried under nitrogen at 45°C.

Quantification of bile acid

Quantification of the bile acids was performed using the 3α-hydroxysteroid dehydrogenase assay described previously (20) but with all the sample volumes reduced so that the assay could be performed in 96-well microtitre plates. Briefly, purified bile acid extracts or standard bile acid (deoxycholate) were dissolved in 500 μl reaction buffer (0.1 M pyrophosphate buffer, pH 9.5, containing 0.7 mM βNAD and 0.7 mM hydrazine hydrate). Aliquots (50 μl) of these solutions were mixed with 200 μl reaction buffer containing 0.03 units of 3α-hydroxysteroid dehydrogenase (Cat No. H1506, Sigma, Dorset, U.K.) in duplicate in 96-well plates. Duplicate samples prepared with buffer but no enzyme were used as blanks. The samples were incubated at 23°C on a plate reader (Molecular Devices, Alpha Laboratories, Hampshire, U.K.) until no further increase in absorbance of the samples at 340 nm was observed. The concentrations of the bile acid in the extracts was determined using the final absorbance values in the samples and standards after subtraction of the blank values.

NMR

Samples were dissolved in $CDCl_3$ (99.8 atom %), and ^1H and ^{13}C spectra were obtained at 360 MHz and 90.5 MHz respectively on a Bruker AMX-360 using an inverse VSP probe, the latter with full broadband decoupling. Acquisition conditions were as follows; ^1H NMR: 45° pulses of 4.5 μs, 2 s recycle time, 4000 Hz spectral width, 0.3 Hz exponential signal-to-noise enhancement; ^{13}C NMR:45° pulses of 7 μs, 5 s recycle time, 25000 Hz spectral width, 10 Hz exponential signal-to-noise enhancement.

Infrared spectrometry

The IR absorbance spectra were recorded using a Perkin Elmer 1750 Fourier transform IR spectrometer equipped with an interleave accessory and a TGS detector. Samples were analyzed as solutions in chloroform. Each solution was placed in a solution cell fitted with KBr windows and having a path length of 1 mm. Sixty-four scans were averaged with a resolution of 4 cm^{-1} using a medium Norton-Beer function for apodization. The sample compartment was purged with dry nitrogen during the analysis. A spectrum of the solvent was generated under the same conditions and this was subtracted from that of the sample using the Perkin-Elmer interactive routine (IDIFF).

Hydrolysis of metabolites and deoxycholic acid ethyl ester in [^{18}O]water

Deoxycholic acid ethyl ester, deoxycholic acid (0.1 mg of each), and a similar amount of the bile acid metabolites were dried under vacuum for 48 h in borosilicate glass screw-cap vials. Sodium ethoxide (450 μl), prepared by adding 2.3 g of sodium to 90 ml of ethanol, was mixed with 50 μl [^{18}O]H_2O (98% ^{18}O, Cambridge Isotope Labs, MA). One hundred μl of ^{18}O-labeled 1 M NaOH in 90% ethanol so prepared was added to each vial and the vials

were sealed and heated overnight at 110°C. Hydrolysis of the metabolites was complete as determined by HPTLC of the hydrolysates.

Mass spectrometry of the hydrolyzed metabolites

FAB-MS. Ionization was carried out using a VG-Analytical 70-250 SEQ mass spectrometer. Samples were dissolved in the reaction mixture (1 M NaOH in 90% ethanol) and an aliquot (1–2 μl) was added to the matrix (glycerol–thioglycerol 1:1, v/v, 1–2 μl) on the tip of a static probe. Xenon was used as the bombarding gas at an energy of 8 KeV. Accelerating voltage was −8 KeV. Scans were acquired under data system control in a continuum scan from *m/z* 600–100, accumulating 4–8 scans.

LC-MS-MS. HPLC was performed using a Hewlett-Packard 1090L HPLC system fitted with a Waters Novapak C_{18} column (3.9 mm × 150 mm). An isocratic mobile phase was used (0.1 M ammonium acetate in H_2O–methanol 1:4.55 (v/v) 1 ml/min). Additional mobile phase (0.1 M ammonium acetate in H_2O–methanol 9:1 (v/v) 0.8 ml/min) was mixed with the effluent from the column in order to make up the flow required for the mass spectrometer (1.8 ml/min). Solutions of the bile acids were dissolved in the mobile phase (100–200 ng/μl) and 20 μl was injected onto the column.

MS–MS was carried out on a Finnigan MAT TSQ 46 equipped with a Nova 4X Superincos data system and fitted with a Finnigan MAT thermospray interface. The vaporizer temperature was 127°C and the jet temperature was 250°C. MS–MS was carried out using argon as the collision gas in the collision cell at pressures from 1.8 to 5 mTorr and collision energies of 5 to 30 eV.

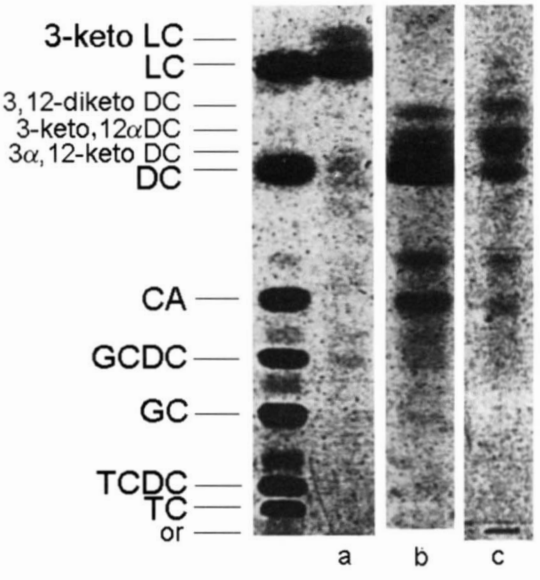


Fig. 1. Typical autoradiographs of HPTLC plates on which endogenously radiolabeled metabolites of (a) chenodeoxycholic acid or (b) cholic acid, extracted from hamster feces using tBuOH–H_2O, have been separated. Also, the endogenously radiolabeled metabolites of cholic acid found in the methanol-soluble fraction of the chloroform–methanol extract from hamster feces (c). The biliary bile acid pools of hamsters were radiolabeled with either [^{14}C]chenodeoxycholic acid or [^{14}C]cholic acid as described in Materials and Methods. The feces from the hamsters were collected on either 1 or 2 subsequent days and the bile acids were extracted using either tBuOH–H_2O or chloroform–methanol in a Soxhlet apparatus. The methanol-soluble fraction of the Soxhlet extract was prepared as described in Materials and Methods. The tBuOH–H_2O and methanol-soluble extracts were then purified using C_{18} Bond Elut columns before being applied to the HPTLC plates. The plates were developed in ethyl acetate–methanol–glacial acetic acid 7:2:1 (v/v) (ref 41) to 3 cm from the origin followed, after drying, by ethyl acetate–cyclohexane–glacial acetic acid 9:9:2 (v/v) to 8.5 cm from the origin. The plates were then exposed to autoradiography film (^{3}H Hyperfilm, Amersham International, Bucks, U.K.) for between 4 and 6 weeks. The bile acids were then visualized by charring. The 12-keto bile acids could only be charred to fluorescent products after the plates had been dried to remove residual acetic acid and then sprayed with sodium borohydride in ethanol (1 mg/ml) to reduce the ketone. (or, origin; TC, taurocholic acid; TCDC, taurochenodeoxycholic acid; GC, glycocholic acid; GCDC, glycochenodeoxycholic acid; CA, cholic acid; DC, deoxycholic acid; LC, lithocholic acid.) Tentative identification based on R_f and color of known standards were also given to 3α, 12-keto DC = 3α-hydroxy, 12-keto-5β-cholanoic acid, 3-keto, 12α DC = 3-keto, 12α-hydroxy-5β-cholanoic acid, 3,12-diketo DC = 3,12-diketo-5β-cholanoic acid, 3-keto LC = 3-keto-5β-cholanoic acid)

Gel permeation chromatography (GPC)

GPC was performed using three 30-cm columns in series packed with μ-Styragel (10 μm bead size) of porosity 500 Å, 10^3 Å and 10^4 Å (Waters Associates) and fitted with a 100-μl injection loop. Tetrahydrofuran was used as the elution solvent (0.5 to 1 ml/min). A differential refractive index detector (Waters Associates, Model 401) was used to detect the bile acid and, when radioactive bile acids were being separated, timed fractions of the column eluent were collected and the radioactivity was measured by liquid scintillation counting.

Hamster plasma cholesterol determination

Blood was taken by cardiac puncture whilst the hamsters were under halothane anesthesia. Plasma was prepared and cholesterol concentrations were determined using the Merck Chod-Iodide kit (BDH, Merck Ltd, Poole, U.K., Merck No. 14106, 14107 and 14108).

RESULTS

Hamster studies

Tertiary-butyl alcohol and water (tBuOH–H_2O 1:1, v/v, (18)) extracted all of endogenous chenodeoxycholic acid metabolites (99% ± 4%) as well as the simple/non-polymeric cholic acid metabolites from hamster feces. The major bile acids in these extracts, characterized by HPTLC, were lithocholic acid and deoxycholic acid (**Fig. 1**). The proportion of simple cholic acid metabolites varied considerably in the feces from different hamsters (8–96%). However, the intra-animal variability in extraction efficiency of feces taken on 2 consecutive days was small (9 ± 7%, mean ± SD of the difference between the 2 days, n = 9). The cholic acid metabolites which were in-

TABLE 1. Percentage extraction efficiency of [^{14}C]cholic acid metabolites from hamster feces

Sample	Percentage Extraction Efficiencies			
	tBuOH–H_2O Soluble	Chloroform–Methanol-Soluble	Methanol-Soluble	Chloroform-Soluble
1	12	67	17	
2	27	64	22	42
3	19	60	20	37
4	90	87	80	5
5 (pooled)	34	66	31	35
6 (pooled)	33	66	35	31

Percentage extraction efficiency of [^{14}C]cholic acid metabolites from hamster feces using tBuOH–H_2O 1:1 (v/v) in glass vials, or chloroform–methanol 1:1 (v/v) in a Soxhlet apparatus. Feces from individual hamsters or those pooled from several hamsters were extracted using both procedures as described in Methods. The solvent from the Soxhlet extract was removed and the residue was washed repeatedly with methanol to remove the methanol-soluble metabolites. The methanol-insoluble residue was dissolved in chloroform. The amount of radioactivity in the extracts was measured and the results were compared with the total radioactivity in the feces which was measured using an oxidizer.

soluble in tBuOH–H_2O were also insoluble in methanol, ethyl acetate, hexane, diethyl ether, and acetone. These metabolites will be referred to as the saponifiable metabolites because bile acids could only be solubilized after hydrolysis in ethanolic sodium hydroxide.

A larger proportion of the radiolabeled cholic acid metabolites could be extracted from hamster feces using chloroform and methanol in a Soxhlet apparatus (60–87%) than using tBuOH–H_2O (12–90%, **Table 1**). The chloroform–methanol extract was dried and washed exhaustively with methanol. The methanol fraction contained approximately the same proportion of the total radioactivity in the feces (17–80%) as did the tBuOH–H_2O extracts. All of the previously characterized bile acid metabolites are soluble in methanol. The bile acids in the methanol fractions were shown, using HPTLC, to be the same as those found in the tBuOH–H_2O extracts (see Fig. 1). The cholic acid metabolites in the methanol-insoluble fraction were found to be soluble in chloroform and tetrahydrofuran only. This chloroform-soluble fraction contained saponifiable cholic acid metabolites that were characterized as polymers of deoxycholic acid.

None of the methods we tested could extract all of the cholic acid metabolites from all of the feces. In those feces that were poorly extracted using tBuOH–H_2O (<34% of the total radioactivity) there remained about 30% of the metabolites (see Table 1) that could not be extracted using any of the solvents tested even when extracted under reflux conditions. These insoluble cholic acid metabolites were partially characterized as discussed below.

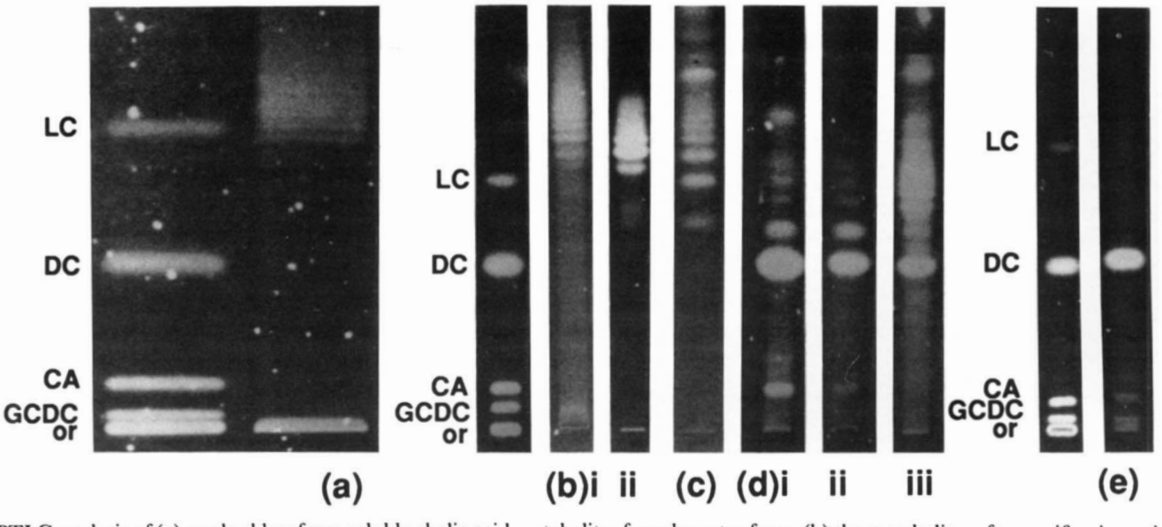


Fig. 2. HPTLC analysis of (a) crude chloroform-soluble cholic acid metabolites from hamster feces, (b) the metabolites after purification using silica column chromatography from (i) hamster and (ii) human feces, (c) synthetic 3α-hydroxy poly-deoxycholate, (d) (i) and (ii) methanol-soluble, and (iii) chloroform-soluble fractions of partially hydrolyzed metabolites from hamster feces, and (e) completely hydrolyzed metabolites from hamster feces (or, origin; GCDC, glycochenodeoxycholic acid; CA, cholic acid; DC, deoxycholic acid; LC, lithocholic acid).

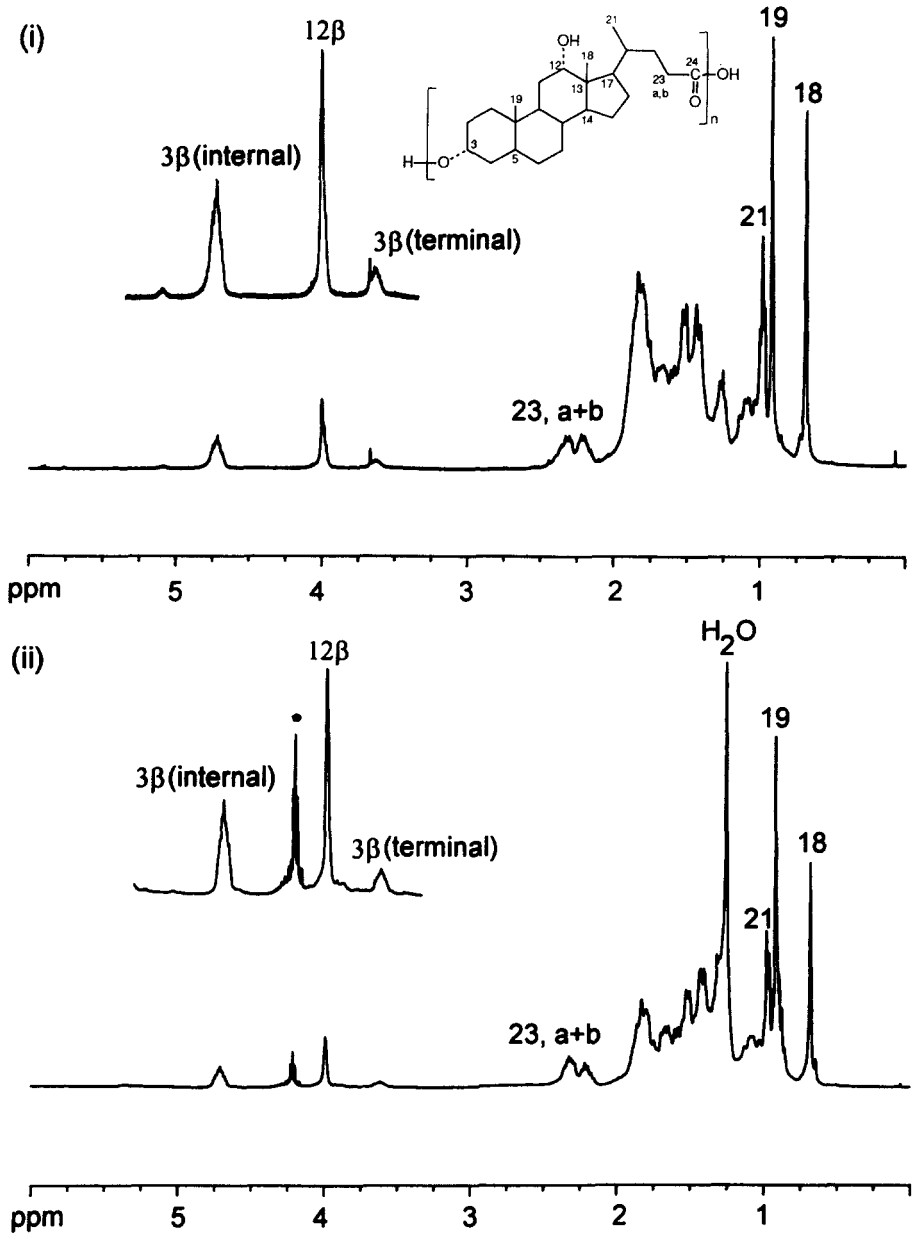
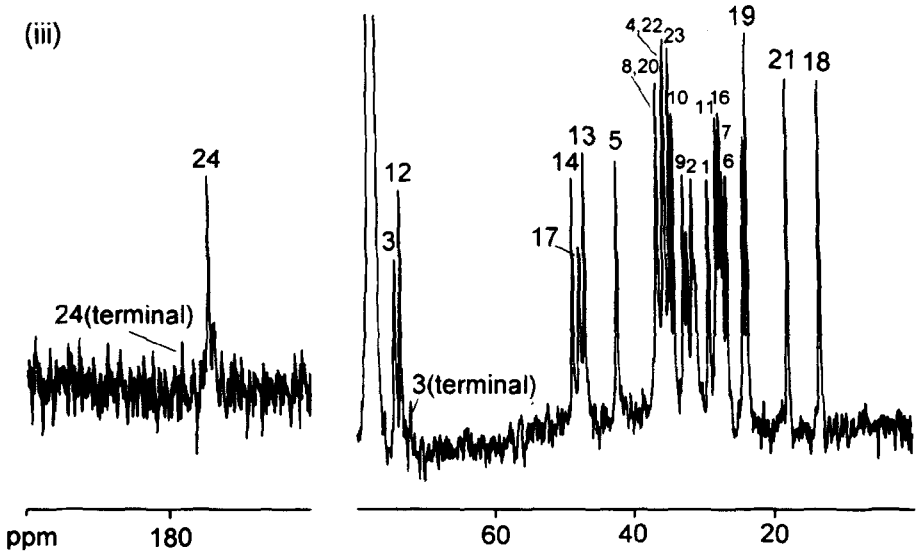


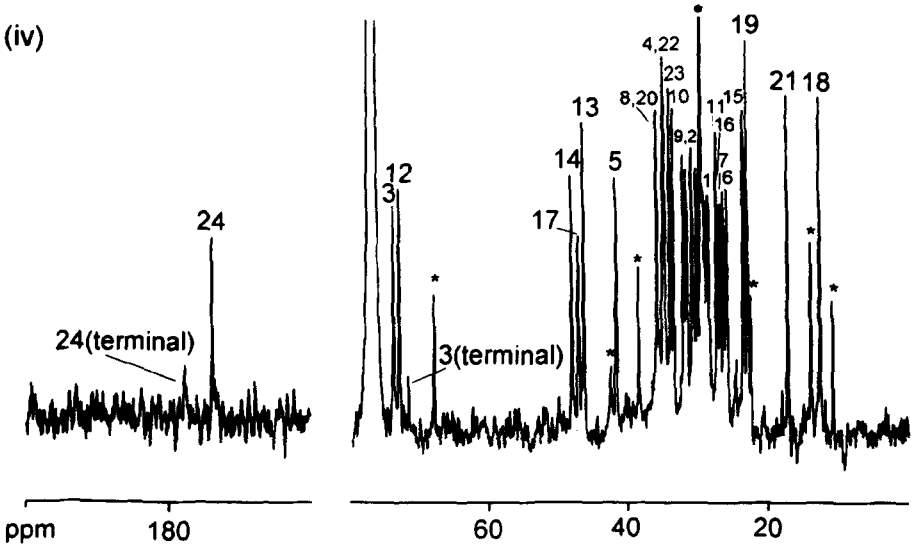
Fig. 3. Proton (i and ii) and ^{13}C (iii and iv) NMR spectra of synthetic (i and iii) and human (ii and iv) polydeoxycholate. Selected assignments are shown; the spectra are fully consistent with the literature (42, 43) and with the down-field shifts induced at the 3-position by esterification of the 3-hydroxyl group in the benzyl ester of the 3α-hydroxy hexanoyl ester of deoxycholic acid (v). An approximate value of the mean polymer length can be obtained from the ratio of the integrals of the ^1H signals of the esterified and nonesterified 3β-hydrogen; ca 4 to 7 in the case of both the human and hamster polydeoxycholate. (* = impurities).

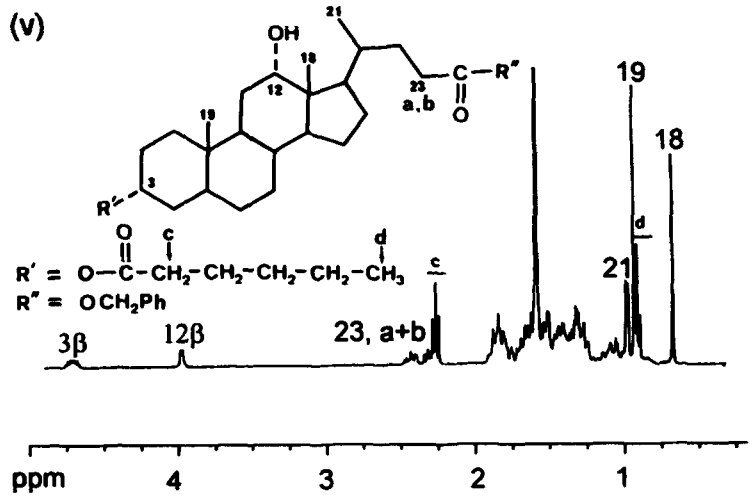
Human studies

The bile acids in the feces from three volunteers were extracted using the Soxhlet method. The chloroform-soluble fractions from the Soxhlet extracts were prepared and, after hydrolysis, the bile acids were quantified. These chloroform-soluble fractions contained between 2% and 14% of the total bile acid in the feces. The Soxhlet method extracted all of the bile acid from the feces, as has been reported previously (17), because there was no bile acid detected in the fecal residue after extraction.

Radiolabeled deoxycholic acid was added to either fresh or freeze-dried human feces or freeze-dried hamster feces in order to determine whether methanol-insoluble metabolites could be formed during extraction using the Soxhlet method. All of the radioactivity was recovered in the methanol-soluble fractions indicating that the extrac-







tion method was not capable of forming the methanol-insoluble metabolites. The chloroform-soluble fractions from these samples were shown to contain polymers of deoxycholic acid.

Purification of the chloroform-soluble cholic acid metabolites

The chloroform-soluble fractions from human and hamster feces were partially purified using silica column chromatography, and aliquots of the purified samples were characterized using HPTLC (**Fig. 2b**). The radioactive metabolites purified from hamster feces had the same chromatographic properties as the metabolites in the crude chloroform-soluble extract from which they were purified (Fig. 2a), which showed that the purification process did not selectively remove metabolites. The cholic acid metabolites in the extracts did not consist of a single compound but chromatographed on the HPTLC plates as a broad peak in which distinct bands were visible at the lower edge. The even spacing and the large number of bands provided the first indication that the compounds might belong to a series of related structures. We initially considered that the metabolites might belong to series of simple carboxylic acid or hydroxy esters of deoxycholic acid with short chain alcohols or long chain fatty acids. However, these esters, when synthesized, were found to be soluble in methanol and were also excluded by comparison of their R_f values on HPTLC and by NMR analysis. Further analysis of the metabolites led us to believe they might be polymers. Polydeoxycholic acid was, therefore, synthesized and, using HPTLC, was shown to contain the same bile acid metabolites as were present in the chloroform-soluble cholic acid metabolites from human and hamster feces (Fig. 2c). Further analysis of the synthetic material by proton-NMR confirmed its structure as 3α-hydroxy polydeoxycholate in which approximately 75% of the 3α-hydroxy groups as well as a trace of the 12α-hydroxy groups (4%) were esterified (**Fig. 3**).

Characterization of the chloroform-soluble cholic acid metabolites

Partially hydrolyzed chloroform-soluble metabolites from hamster feces contained deoxycholic acid, and a trace of cholic acid, and another fluorescent product with an R_f on HPTLC between that of deoxycholic acid and the unhydrolyzed metabolites (Fig. 2d). This band is probably the deoxycholic acid dimer as deoxycholic acid and a trace of cholic acid were the only bile acids present after complete hydrolysis of the metabolite from both human and hamster feces (Fig. 2e). After complete hydrolysis of the metabolites, no compounds other than these two bile acids were detected in the extract by HPTLC and charring.

Proton- and ^{13}C-NMR spectra of the metabolites were compared with those of synthetic polydeoxycholic acid (Fig. 3). The proton-NMR results confirmed that deoxycholic acid was present in the metabolites and that it was not esterified to a fatty acid, a potential metabolic route that has been identified in vitro (21). Integration of the proton-NMR spectra showed that approximately 75–86% of the 3α-hydroxy groups were esterified in the metabolites from both humans and hamster feces. These data imply that between three out of four and six out of seven of the hydroxy groups in polydeoxycholate are involved in internal ester bonds whilst the remaining groups is an unesterified terminal group. The average chain length of the polydeoxycholate was, therefore, between 4 and 7 units. The average chain length for the synthetic polydeoxycholic acid was about 4 units. The ^{13}C-NMR results also showed that most of the deoxycholic acid in the metabolites was esterified at the carboxyl as well as the 3α-hydroxy groups. There was no evidence from the NMR spectra for any other significant component to the samples other than polydeoxycholic acid.

A comparison of the infrared (IR) absorbance spectra (**Fig. 4**) obtained for the synthetic polydeoxycholate and the metabolites, in chloroform, confirmed the presence of alcohol (νOH = 3617 cm^{-1}), carboxyl (νOH = 3517 cm^{-1}, νC=O = 1719 cm^{-1}) and ester (νC=O = 1719 cm^{-1}) functional groups in the metabolites. A second derivative of the 1719 cm^{-1} carbonyl band showed that it consisted of two closely overlapping components at 1721 cm^{-1} and 1709 cm^{-1} which could be assigned to νC=O (ester) and

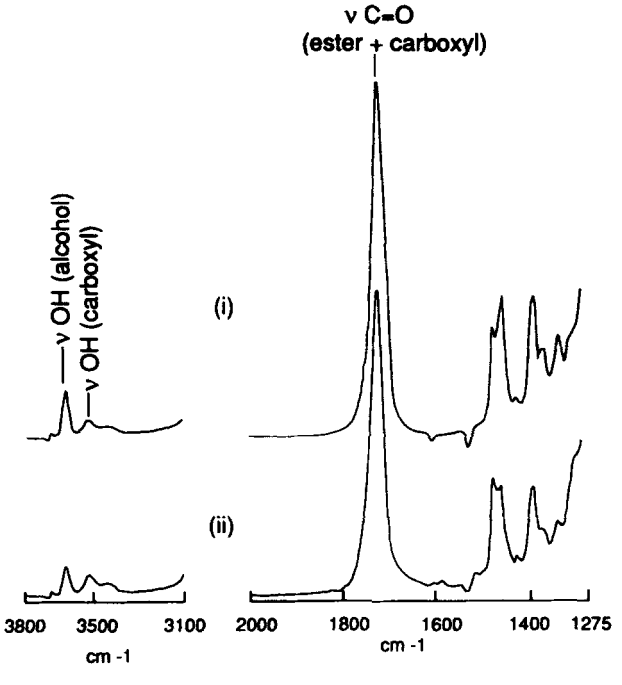


Fig. 4. Infrared absorbance spectra of i) synthetic polydeoxycholic acid, and ii) the same chloroform-soluble cholic acid metabolites that were analyzed by NMR.

vC=O (carboxyl) respectively, the latter band having about half the intensity of the former.

We were unable to obtain mass spectra of the metabolites themselves. However, mass spectroscopic evidence for the ester function was obtained by hydrolysis of the esters in [^{18}O]water. During hydrolysis the oxygen from water will be incorporated into the carboxyl group of an esterified carboxylic acid but will not be incorporated into a free carboxylic acid (22). If the deoxycholic acid in the chloroform-soluble metabolites is esterified at the carboxyl group, then hydrolysis of the ester in [^{18}O]water would result in a deoxycholic acid molecule that is 2 mass units heavier (*m/z* 394) than normal deoxycholic acid (*m/z* 392). Chloroform-soluble cholic acid metabolites from human and hamster feces were hydrolyzed in the presence of [^{18}O]water. Deoxycholic acid was also treated under the same hydrolysis conditions to confirm that no exchange of ^{18}O takes place in the free acid. Ethyl deoxycholate was also hydrolyzed to prove the incorporation of ^{18}O into the bile acid on hydrolysis of an ester. Negative ion fast atom bombardment mass spectrometry (FAB-MS) showed the deprotonated molecules from deoxycholic acid and [^{18}O]deoxycholic acid at *m/z* 391 and *m/z* 393, respectively (**Fig. 5A**). There was no enrichment with ^{18}O after treating deoxycholic acid under ^{18}O-hydrolytic conditions. Ethyl deoxycholate was enriched after hydrolysis in [^{18}O]H_2O with an enrichment factor of 82%. The metabolite had an enrichment factor of 71%. These enrichments are, therefore, solely due to hydrolysis of the ester function and not to any exchange with free deoxycholic acid. The lower enrichment seen with the metabolite is due to the presence of a free carboxy terminal deoxycholic acid in the polymer chain and implies that there are, on average, six esterified carboxyl groups for every free carboxyl in the chloroform-soluble metabolites.

As much as 30% of the radiolabeled cholic acid metabolites in hamster feces could not be extracted by any solvent tested. These insoluble metabolites were partially

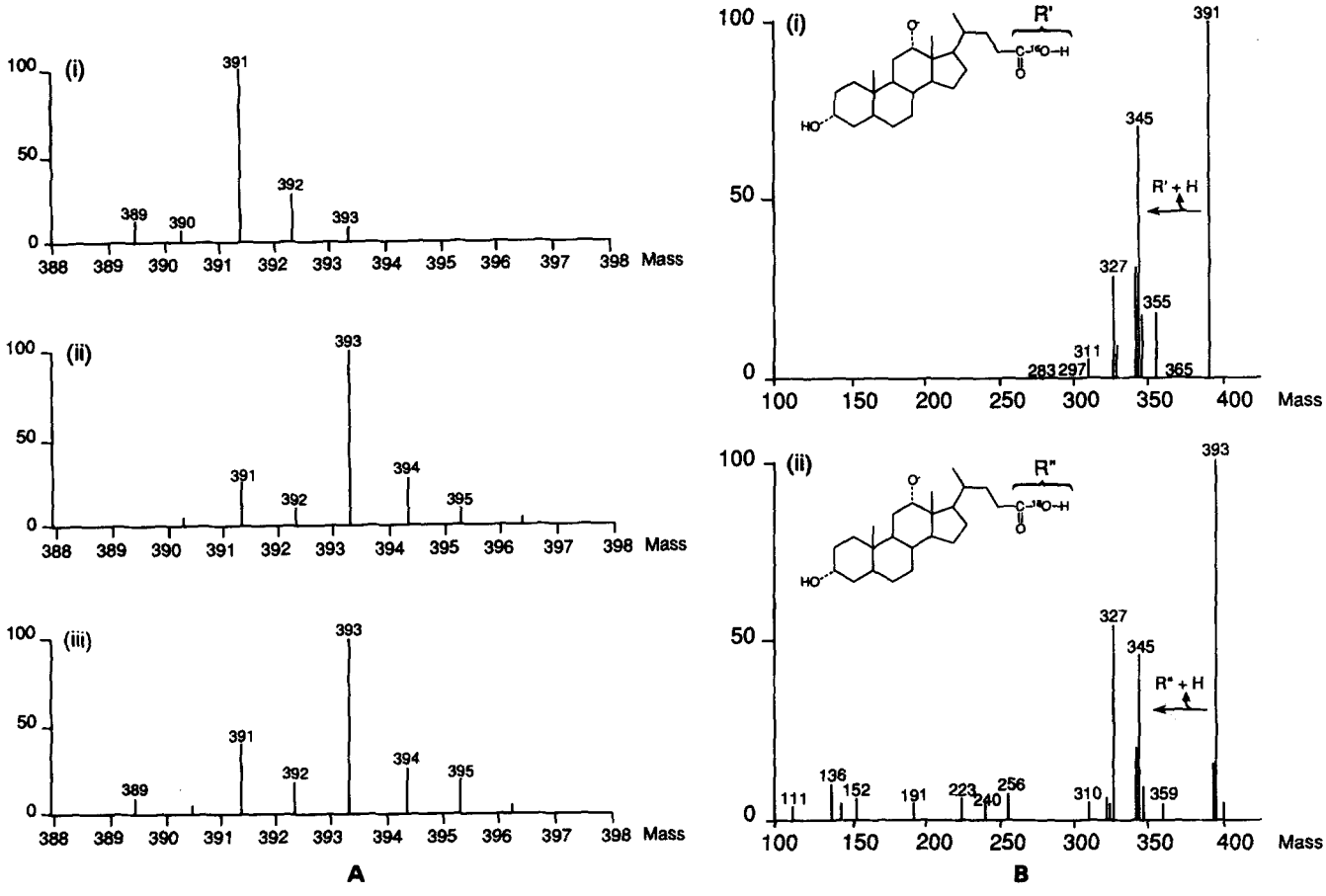


Fig. 5. A) Negative ion FAB-MS spectra of the products of hydrolysis of i) deoxycholic acid, ii) ethyl deoxycholic acid, and iii) the chloroform-soluble cholic acid metabolites from hamster feces in [^{18}O]H_2O. B) LC-MS-MS analysis of [^{16}O]- or [^{18}O]deoxycholic acid produced during the hydrolysis of the chloroform-soluble cholic acid metabolites in ^{18}O enriched water. B) The mass spectra show the fragment ions of the negative ions of i) deoxycholic acid (391) and ii) [^{18}O]deoxycholic acid (393). The spectra of the fragments for both deoxycholic acid and [^{18}O]deoxycholic acid contain an ion at *m/z* 345 which must result from the loss of the ^{18}O-containing part of the molecule. The ion at *m/z* 345 corresponds to the loss of formic acid and thus the ^{18}O must be incorporated in the carboxyl group of the deoxycholic acid.

characterized by hydrolysis in [^{18}O]water. Small samples of feces, which had previously been extracted with chloroform–methanol, were hydrolyzed in [^{18}O]water. The hydrolysate was shown, using HPTLC, to contain only deoxycholic acid and a trace of cholic acid. The deoxycholic acid in the hydrolysate of feces and of a standard ester, ethyl deoxycholate, were shown by liquid chromatography followed by mass spectrometry (LC–MS) to be enriched with ^{18}O by 73.7% and 76.6%, respectively. This implies that most of the insoluble cholic acid metabolite in hamster feces was composed of deoxycholic acid of which about 96% was esterified at the carboxyl group.

Confirmation that the ^{18}O in the [^{18}O]deoxycholic acid, produced by hydrolysis of the metabolites in [^{18}O]water, was in the carboxyl group was supplied by LC–MS–MS analysis (Fig. 5B).

Gel permeation chromatography (GPC) was used to measure the molecular weights of the metabolites. The radiolabeled, chloroform-soluble cholic acid metabolites from hamster feces were separated on a GPC column with a size exclusion, for polystyrene standards, greater than 670,000 daltons (**Fig. 6**). Potential binding of the bile acids to the column was investigated by measuring the elution volumes of cholic acid, deoxycholic acid, and lithocholic acid which might be expected to bind differently if they bind at all. The elution volumes for all three bile acids were the same and were what would be expected for molecules of their molecular weights. These results indicate that the bile acids do not bind to the column. Deoxycholic acid eluted from this column with 27.7 ml of tetrahydrofuran and the chloroform-soluble cholic acid metabolites with between 21 ml and 26 ml of tetrahydrofuran. The elution volume for deoxycholic acid was just greater than that of the lowest molecular weight polystyrene standard. However, by extrapolating the curve, the calculated molecular weight for deoxycholic acid was 560 (true mol wt 392). The cholic acid metabolites had a range of molecular weights between 840 and 12470 with 3000 being the most abundant molecular weight.

We investigated the relationship between plasma cholesterol concentrations and total bile acid and polydeoxycholic acid excretion in the feces (**Fig. 7**). Fecal samples were taken from two groups of untreated male hamsters for 1 or 3 days prior to a blood sampling. Samples of dry fecal homogenate were extracted repeatedly using tBuOH–H_2O so that only the saponifiable bile acids remained. The total fecal bile acids and the saponifiable bile acids were then measured using the complete hydrolysis and enzyme methods described above. Significant negative correlations were found between plasma cholesterol and fecal bile acid excretion for each group and for the combined data. The linear correlation coefficients for the combined data were -0.63 ($P = 0.001$) and -0.59 ($P = 0.003$) for the total and saponifiable bile acid (polydeoxycholate), respectively.

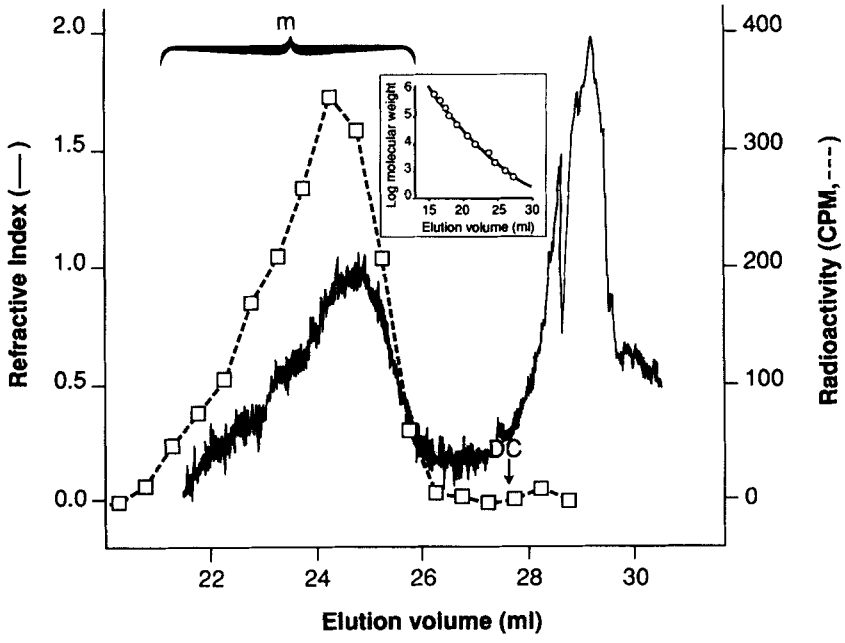


Fig. 6. Gel permeation chromatography (GPC) of the chloroform-soluble cholic acid metabolites (m) isolated from hamster feces. (DC, elution position of deoxycholic acid). Inset: The calibration curve for linear polystyrene using the same GPC columns.

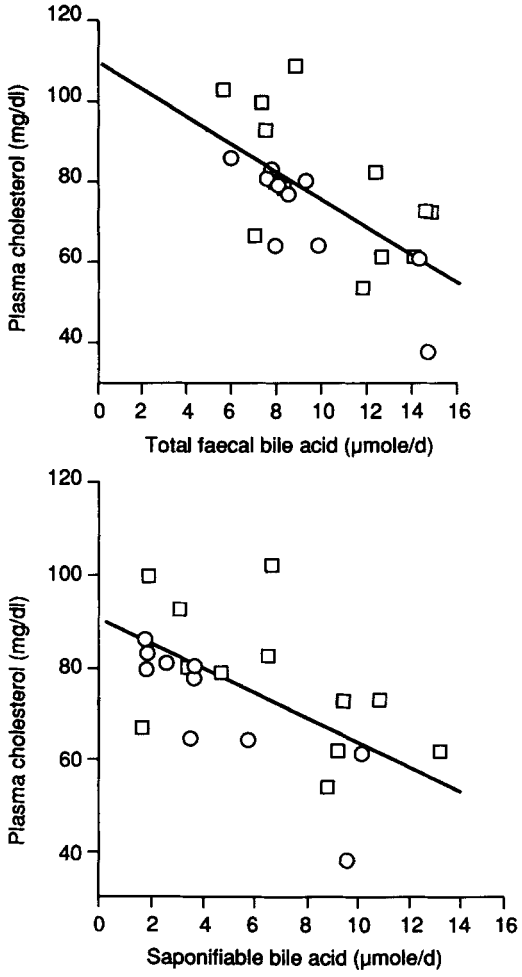


Fig. 7. The relationship between plasma cholesterol concentrations and (top) total and (bottom) saponifiable bile acid (polydeoxycholate) in the feces from two groups of hamsters. Fecal samples were taken from untreated male hamsters for 3 days (○) or one day (□) prior to a sample of blood being taken by cardiac puncture. Samples of dry fecal homogenate were extracted repeatedly using tBuOH-H_2O so that only the saponifiable bile acids remained. The total fecal bile acids and the saponifiable bile acids were then measured using the complete hydrolysis and enzyme assays described in Methods.

DISCUSSION

The bile acids found in feces are an extremely complex mixture produced by bacterial metabolism of the two primary bile acids cholic acid and chenodeoxycholic acid. Our initial objective in these studies was to develop a method for extracting all of the bile acids from hamster feces without modifying them chemically so that we could investigate the effect of different hypolipidemic agents on the fecal bile acid profile. We used feces from hamsters that had been dosed with either [carboxyl-^{14}C]cholic acid or [carboxyl-^{14}C]chenodeoxycholic acid so that all of the bile acid metabolites were labeled with a nonlabile isotope. In addition to using the endogenously radiolabeled bile acids to measure extraction efficiencies, we also used them to validate the methods for purification and analysis of the bile acids.

All of the endogenously radiolabeled chenodeoxycholate metabolites could be extracted using tertiary butyl alcohol and water (tBuOH-H_2O 1:1, v/v, ref. 18). However, this method extracted an extremely variable proportion (8–96%) of the endogenously radiolabeled cholic acid metabolites. Complete extraction of the cholate metabolites could only be achieved after hydrolysis of the feces. The method using tBuOH-H_2O is reported to extract all of the bile acid added to human, rabbit, and rat feces and we also demonstrated that it could extract all of the [^{14}C]cholate, [^{14}C]chenodeoxycholate, [^{14}C]lithocholate, and [^{14}C]deoxycholate added to hamster feces. These results lead us to investigate the reason for the variable extraction efficiencies of the cholate metabolites by attempting to identify the unextracted metabolites. Using chloroform and methanol in a Soxhlet apparatus, a higher proportion of the cholic acid metabolites was extracted (60–87%, Table 1). The extraction efficiency of this method was also less than had been anticipated because this method has previously been shown to extract all of the endogenously radiolabeled bile acid from human feces (17). The Soxhlet extract was dried and the residue was separated into methanol-soluble and methanol-insoluble (chloroform-soluble) material. The methanol-soluble fraction was shown to contain an equivalent proportion of the total cholic acid metabolites and the same profile of bile acids as the tBuOH-H_2O extracts (Fig. 1). This result allowed us to develop a rapid method for separating the simple bile acid metabolites in the feces from the saponifiable metabolites by washing the feces with tBuOH-H_2O. In the chloroform-soluble fraction of the Soxhlet extracts from hamster feces and subsequently from human feces, we found metabolites that appeared to belong to a series of related structures that were only soluble in chloroform and tetrahydrofuran. The physical data obtained on these metabolites were consistent with them having the structures of a series of oligomers of deoxycholic acid. The average chain length of these oligomers was determined using NMR and mass spectrometry after hydrolysis in [^{18}O]H_2O to be approximately 4–7 units. The range of chain lengths was determined using GPC to be approximately 2–22 units in the hamster. The deoxycholic acid in polydeoxycholic acid is joined together by ester bonds from the 3α-hydroxy group of one molecule to the carboxyl group of the next (**Fig. 8**). The oligomers of different lengths presumably chromatograph on HPTLC according to their chain length and, when partially hydrolysed, could be reduced to an oligomer (probably the dimer) with a shorter chain length that accumulated temporarily in the hydrolysis mixture.

The bile acid in human feces was composed of either simple monomeric bile acids or a variable proportion of

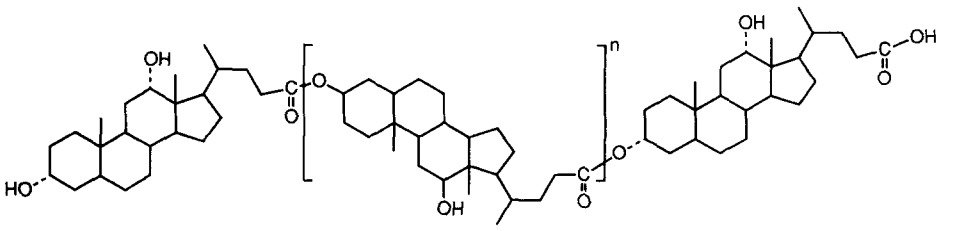


Fig. 8. Schematic diagram of the proposed structure of 3α-hydroxy polydeoxycholate found in human and hamster feces.

short chain-length polymers of deoxycholic acid all of which could be extracted using chloroform and methanol. In addition to these metabolites, hamster feces also contained an insoluble metabolite of deoxycholic acid, 96% of which was esterified at the carboxyl group. Significant quantities of the insoluble metabolites were only found in those hamster feces with >66% of the cholic acid metabolites in the saponifiable fraction (see Table 1), thus accounting perhaps for their absence from human feces with its relatively small saponifiable fraction. These data suggest to us that these insoluble metabolites may also be polyesters but with an average chain length of about 26 deoxycholic acid units. Such metabolites may well be insoluble.

Saponifiable bile acid metabolites have been found previously in human feces (10, 23–25) and rat feces (26, 27) and are produced by the microflora derived from the feces of either rats (28) or humans (21, 29). Korpela, Fotsis, and Aldercreutz (24) found that saponifiable bile acid metabolites containing mostly deoxycholic acid constituted 24% ± 6% of the total bile acid in human feces. Normal (10) also showed that human feces contain at least 25% of radiolabeled cholic acid metabolites as saponifiable derivatives of 3,12-dihydroxy-cholanic acid, curiously, mainly the 3β isomer. The saponifiable metabolites produced in vivo have not been characterized previously, probably because of their unusual structure. They may have been extracted along with the simple bile acids (17, 30) but will then have been hydrolyzed during or after the extraction procedure or may even have been lost during purification procedures in which their recoveries were not monitored.

The amount of polydeoxycholate in hamster feces is difficult to quantify because we have not found a solvent system that will extract all of the radioactive cholic acid metabolites out of all of the hamsters' feces. We could extract all of the bile acid metabolites except for the saponifiable fraction from both human and hamster feces by using $tBuOH-H_2O$. If all of the saponifiable metabolites were polyesters then these comprise an often large but very variable proportion of the total bile acids in feces (15–27% of the total fecal bile acid in humans and 4–92% of the cholic acid metabolites in hamsters).

It is reported that microorganisms, principally bacteria, can produce polyesters of β-hydroxy fatty acids (31). These polyesters, which can be extracted using chloroform, are only produced under adverse conditions for growth such as limited nitrogen supply and they are believed to act as an energy reserve. As the polyesters of deoxycholic acid are not present in human or hamster bile, it is probable that those found in the feces are produced by microorganisms in the cecum and colon. The production of the polyester may involve the synthesis of deoxycholic acid coenzyme A by a thiokinase followed by polymerization by a polymerase as has been proposed for the polyesters of the hydroxy fatty acids (31). The incorporation of only deoxycholic acid and a trace of cholic acid into the polymer implies that the enzymes are highly specific.

It has been shown that bile acids, and deoxycholic acid in particular, are detrimental to certain bacteria (32–35). The microorganisms that make the polyester probably do so in order to reduce the toxicity of the deoxycholic acid with which they would otherwise be in contact.

There may also be consequences of the bacterial formation of polydeoxycholate in the gut for bile acid and cholesterol metabolism in both hamsters and humans. The effect of gut flora on increasing bile acid excretion in the feces has been known for over 20 years (4). The discovery of polydeoxycholic acid in feces may help to explain the mechanism behind this increase. Bile acids are synthesized from cholesterol in the liver and some of this cholesterol is derived from low density lipoprotein (LDL) in the plasma. High LDL cholesterol concentrations are associated with an increased risk of developing coronary heart disease (36). Chemical bile acid sequestrants such as cholestyramine and colestipol are effective hypocholesterolemic agents in both humans and hamsters because they cause an increase in bile acid excretion in the feces (20, 37). Polydeoxycholate will probably be insoluble in the intestinal contents and will, therefore, not be reabsorbed by the gut. We have shown that the plasma cholesterol concentrations in untreated hamsters were inversely related to fecal bile acid excretion and more specifically to the excretion of saponifiable bile acid (Fig. 7). It is possible, therefore, that the synthesis of polyde-

oxycholic acid will have a similar hypocholesterolemic effect in humans as it has in the hamster.

The formation of polydeoxycholate may also be beneficial in preventing other diseases such as colorectal cancer (25), cholesterol gallstones (38), and certain diarrheal conditions (39, 40) in which bile acid metabolism in the colon is believed to play a part.

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