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Bile Acids in Bile of Germfree and Conventional Dogs[1] (40059)

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Germfree dogs have been reared for a number of years, mostly to be used for the study of various surgical problems (1). However, they are also of interest to the gnotobiologist because they are mammals not subject to the usual cecal enlargement found in the germfree rodent. The gastrointestinal function of the dog may possibly be more similar to man's than that of the rodent, and with this in mind, a collaborative study of the gastrointestinal function of the germfree dog was begun.[2] Gallbladder bile from germfree and conventional dogs was available for a preliminary study of the dog as a model for the investigation of sterol metabolism. This is the first report on the biliary bile acids of the germfree dog.

In addition to being the main catabolic end product of endogenously and exogenously derived cholesterol,[3] the actual presence of bile acids in the gut affects a number of intestinal parameters. They influence the absorption of lipids (2), but may also affect Ca and Mg absorption (3) since, in the gut a substantial part of these metals may be bound to fatty acids (4). They appear to affect the renewal rate of the intestinal mucosa (5, 6) and aspects of intestinal metabolism (7, 8), including transport related ATP-ases (9).

The absence of an intestinal microflora leads to extensive qualitative and quantitative changes in bile acids of rats and mice (10, 11). In conventional rabbits the major biliary bile acid is deoxycholic acid, which is absent in the bile of the germfree rabbit (12). Preliminary data comparing germfree and conventional gerbils indicate chenodeoxycholic acid as a major bile acid in germfree, but not conventional gerbils maintained at the Lobund Laboratory.[4] In bile of germfree piglets hyocholic acid predominates, while in conventional piglets hyocholic and hyodeoxycholic occur in comparable amounts (13). The data in this report show much less extensive differences between germfree and conventional dog bile, with cholic acid being the dominant bile acid in both cases.

Materials and methods. Pure-bred germfree and conventional Beagle dogs of mixed sexes and 5–6 months old were obtained from Louisiana State University Medical Center, Department of Surgery in New Orleans, Louisiana. After caesarian birth the germfree puppies had been housed in isolators described by Heneghan, Longoria and Cohn (14) and fed autoclaved Borden Esbilac (The Borden Co., New York, NY) with 1 ml filter sterilized Vi-Syneral Multiple Vitamins (B vitamins plus vitamins A, D, E, and C) (U.S. Vitamin and Pharmaceutical Corp., New York, NY) per liter of milk diet. A filter-sterilized 2.5% methionine solution had been added to the milk so each puppy received approximately 30 mg of methionine per day until they were weaned to autoclaved Purina

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[2] This project involved Drs. J. B. Heneghan, Louisiana State University Medical Center, New Orleans, LA; Helmut A. Gordon, University of Kentucky Medical Center, Lexington, KY; Silvio Baez, Albert Einstein College of Midicine, New York, N.Y.; Esther Kokas, University of North Carolina, Chapel Hill, N.C.; and Bernard S. Wostmann, University of Notre Dame, Notre Dame, IN. Preliminary results were presented at the 11th Annual Meeting of the Association for Gnotobiotics, Guelph, Ontario, Canada, June 9–13, 1974.

[3] The following systematic names are given to bile acids and sterols referred to by trivial names: cholesterol—cholest-5-en-3β-ol; cholic acid—3α,7α,12α-Trihydroxy-5β-cholanic acid; α-muricholic acid—3α,6β,7α-Trihydroxy-5β-cholanic acid; β-muricholic acid—3α,6β,-7β-Trihydroxy-5β-cholanic acid; Chenodeoxycholic acid—3α,7α-Dihydroxy-5β-cholanic acid; Deoxycholanic acid—3α,12α-Dihydroxy-5β-cholanic acid; Hyodeoxycholanic acid—3α,6α-Dihydroxy-5β-cholanic acid; Lithocholanic acid—3α,Hydroxycholanic acid; 7-ketolithocholic acid—3α-Hydroxy-7-ketocholanic acid; 7-ketodeoxycholanic acid—3α,12α-Dihydroxy-7-ketocholanic acid.

[4] Wostmann, B. S. Unpublished data, 1975.

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Dog Chow (Ralston Purina Co., St. Louis, MO) supplemented with 1.25 ml Vi-Syneral Vitamins per liter of drinking water. Conventional control animals had been housed in standard stainless steel cages, two to a cage in the open animal facility, and were fed autoclaved Purina Dog Chow and water *ad lib.*

Samples of gallbladder bile were obtained at autopsy, and diluted 1:20 with 50% (v/v) ethanol for storage. Bile acids were prepared by our methodology (15) which is summarized here. For analysis, ethanol was added to an aliquot of diluted bile sample to a final ethanol concentration of approximately 75% (v/v), and NaOH was added to 0.50 *N*. Carrier-free cholic acid-[^{14}C] was added to determine recovery after sample preparation (see hereafter). The mixture was refluxed 1 hr over steam, cooled, and the neutral sterols removed by four extractions with hexane. The water phase, containing the bile acids, was made more basic (0.8 *N*), and the mixture autoclaved to hydrolyze conjugated bile acids. After cooling, the mixture was acidified with HCl and bile acids were extracted once with chloroform:methanol (2:1) and thrice with chloroform. Bile acids were methylated overnight by dissolving in 10 ml of 5% acetylchloride in methanol.

The material was streaked onto 20 × 20 cm thin layer chromatographic (TLC) plates (250 nm thick), and developed once in chloroform:benzene (1:1) to remove fatty acids. The plate was then developed in isooctane:isopropanol:HAc (100:60:1) to carry the bile acids away from the origin, the latter containing most of the pigmented material. After elution of bile acids from the gel with acetone, they were streaked on a second TLC plate and developed in chloroform:acetone:methanol (70:25:5); this separates the bile acid mixture into three fractions suitable for gas–liquid chromatography (GLC) analysis: a lower zone of trihydroxy bile acids, a middle zone containing hyodeoxycholic acid (when present) and the upper zone carrying all dihydroxy and keto-BAs (15).

As an internal standard for GLC quantitation, 5α-cholestane was added to the eluted bile acid fractions. An aliquot representing one-fourth of the sample was used to determine cholic acid-[^{14}C] recovery to correct for losses during the foregoing analytical procedure. One milligram methyl cholate carrier was added and the mixture incorporated in 10 ml 2,5-diphenyloxazole (PPO) (5 g/l) in toluene, with 0.5 ml of methanol added to assure complete solution of all material. Radioactivity was determined with a Beckman Instruments LS-250 Liquid Scintillation System using the automatic quench correction (AQC).

The remaining three-fourths of the sample was dried and the bile acids converted to trimethylsilyl (TMS) ethers for flame ionization GLC on 1% SE-30 and on 3% QF-1, both supported on 100/200 mesh Gas Chrom Q (Applied Science Laboratories, Inc., State College, PA), for quantitation and identification (15). Quantitation was done by integration of GLC peaks with an Autolab System IV Computing Integrator (Autolab Division of Spectra-Physics, Santa Clara, CA).

Quantitative corrections were made based on the recovery of the cholic acid-[^{14}C]. Recoveries were always better than 85%.

Results. With the limited number of dogs available no obviously significant difference between total bile acids in bile of germfree and conventional dogs could be found (Table I). The bile acids of all dog bile samples, from both germfree and conventional animals ranged from 23 to 44 mg/ml bile, with the germfree dogs tending to show higher values. In both germfree and conventional dogs, cholic acid was the dominating component, with approximately 4% chenodeoxycholic being present. In the conventional animals 10–20% deoxycholic acid was present, accounting for the decrease in cholic acid. Only traces of keto-bile acids were found in the bile of either group, usually with a GLC

TABLE I. BILE ACIDS IN GALLBLADDER BILE OF ADULT GERMFREE (GF) AND CONVENTIONAL (CV) BEAGLE DOGS.

	GF (8)[a] %	CV (4) %
Cholic acid	94.9 ± 0.7[b]	81.6 ± 2.2
Chenodeoxycholic acid	4.6 ± 0.6	3.7 ± 0.6
Deoxycholic acid	—	12.1 ± 3.1
Ketones	trace	trace
Total bile acids mg/ml	38.8 ± 2.6	28.5 ± 2.9

[a] Number of animals in parenthesis.
[b] Mean ± SE.

retention time that suggested 12-keto-3α cholanic acid.

Discussion. Gans and Butz (18) found gallbladder bile acids in conventional dogs to consist largely of taurocholate and taurodeoxycholate. Earlier, Gans (19) had reported 30–40% taurodeoxycholate determined by a chemical method. The present data on conventional dog material are more in line with the findings of Quarfordt and coworkers (20). Using conventionally reared mongrel dogs, they found the biliary bile acids to be cholic (69%), chenodeoxycholic (9%) and deoxycholic (21%) acids with total bile acid concentration of 20.2 mg/ml bile. Differences between these and the present data may be due to dog breed, diet or different methods of quantitation.

The predominant bile acid of the germfree Beagle dog is cholic acid; this, together with the other primary bile acid, chenodeoxycholic acid, represents at least 99.5% of the total gallbladder bile acids. In conventional dogs, deoxycholic acid is presumably formed by bacterial degradation of cholic acid in the gut and recirculates via the liver without being completely rehydroxylated. Chenodeoxycholic acid occurs in approximately the same quantity as found in the germfree animal. Cholic acid, deoxy- and chenodeoxycholic acids represent 99.3% of the material found in the bile acid fraction of the conventional dog bile.

Biliary bile acid composition reflects, to a large extent, the qualitative composition of bile acids that are functionally active in the small intestine. In the most frequently used animal models, the rat and the mouse, bile acid composition is considerably different from that in man (Table II). Although the bile acids in dog and human bile are not quantitatively comparable, they are much more similar than are those of rodents and man. Human gallbladder bile consists mainly of cholic acid (44%), chenodeoxycholic acid (33%), and deoxycholic acid (19%) representing 96% of the total bile acids with the remainder lithocholic acid (4%) (21). Total bile acid concentration falls in the same range as found for the dog (Table II), but 75% of human biliary acids are conjugated with glycine and only 25% with taurine (23), whereas almost all the bile acids in both dog (18) and rat (24) bile are taurine conjugated. β-Muricholic acid, very much in evidence as a primary bile acid in rat (10) and mouse (11) bile, is present neither in dog nor in human bile.

Moreover, the bile acid patterns of the germfree rat and mouse differ very substantially, both quantitatively and qualitatively, from those of their conventional counterparts (10, 11). In the dog, germfree and conventional animals differ only in the presence of a moderate amount of deoxycholic acid in conventional dog bile. Total amounts of bile acids appear to fall in the same range, although there seems to be a trend towards higher values in the germfree animal reminiscent of the more pronounced differences found in murine rodents (10, 25). Evidently

TABLE II. PERCENTAGE OCCURRENCE OF MAJOR BILE ACIDS IN BILE OF VARIOUS MAMMALIAN SPECIES.

	Dog		Rat (10)		Mouse		Human	
Bile Acid	GF	CV	GF[a]	CV[a]	GF (11.25)	CV[b]	GB (23) feces[c]	CV (21)
Chenodeoxycholic acid	4.6[d]	3.7	0.6	4.2	1.5	—	++	33.4
Cholic acid	94.9	83.6	49.4	74.8	24.5[e]	52.9	++	44.2
Deoxycholic acid	—	12.1	—	1.0	—	3.5	—	18.6
β-muricholic acid[f]	—	—	49.2	15.3	68.0	37.5	—	—
Hyodeoxycholic acid	—	—	—	3.0	—	trace	—	—
Bile acids total (mg/ml)	36.8	28.5	10.12	4.16	not given		?	37.9

[a] Lobund rats of Wistar origin.

[b] Wostmann, B. S. Unpublished data, 1975.

[c] 2 gnotobiotic (GB) children, one GF 3 months then contaminated with a bacterium of the *Klebsiella-aerobacter* group and *Candida albicans* and the other with a spore-former.

[d] Percent of total bile acids.

[e] Includes 1.7% of cholic acid-7-SO_4 derived from cholic acid, according to personal communication from H. Eyssen, University of Leuven, Belgium, 1974.

[f] This fraction includes 5–10% α-muricholic acid (10).

the intestinal microflora exerts far less influence on the bile acid pattern of the dog than on that of any of the other species studied thus far.

The present data indicate similarities that point to the possible usefulness of the dog as a model for the study of the microbial factor in bile acid and cholesterol metabolism as it relates to cardiovascular disease in man. The unexpectedly small differences, both qualitative and quantitative, between bile acids of germfree and conventional dogs suggest that in general, germfree dogs may be used advantageously in studies of intestinal function and metabolism that require absence or control of the intestinal microflora. The similarity in bile acid composition would warrant the assumption that bile acid-dependent function in the small intestine of gnotobiotic dogs would be comparable to that of conventional animals.

Summary. The amount and composition of gallbladder bile acids of eight germfree and four conventional pure-bred Beagle dogs were determined. Unlike in other mammalian species, no major differences in bile acid composition were found between germfree and conventional dogs. Germfree dog bile contained on the average 94.9% cholic acid and 4.6% chenodeoxycholic acid. Conventional dog bile contained 83.6% cholic acid, 3.7% chenodeoxycholic acid and in addition, 12.1% deoxycholic acid. Bile from both germfree and conventional dogs contained traces of keto–bile acids. The average amounts of total bile acids in mg/ml bile were 36.8 ± 2.6 (germfree) and 28.5 ± 2.9 (conventional). Except for differences in conjugation, dog and human biliary bile acid patterns are qualitatively comparable, and differ from those in rats and mice in that muricholic and related bile acids are found only in the latter group.

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