Fecal neutral steroids and bile acids from germfree rats

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ABSTRACT The amount and composition of fecal neutral sterols and bile acids excreted by adult male germfree and conventional rats have been determined. The amounts of neutral sterols excreted were 12.8 (germfree) and 19.5 (conventional) mg/kg of body wt per day. The germfree rats excreted cholesterol and lathosterol (methostenol was not assayed); the conventional rats excreted coprostanol and coprostanone in addition. The amounts of bile acids excreted were 11.3 (germfree) and 21.4 (conventional) mg/kg of body wt per day. The bile acids excreted by the rats were tentatively identified as tauro-β-muricholate, tauro-α-muricholate, and taurocholate, besides an unidentified component. The conventional rats excreted the corresponding unconjugated acids as well as many other unconjugated bile acids.

No significant correlation was found between the amount of coprosterols and the total amount of neutral sterols excreted by the conventional rats. This suggests that bacterial reduction of cholesterol is not an important mechanism of increasing neutral sterol excretion of conventional rats as compared to germfree rats. Evidence is presented that suggests that this difference in neutral sterol excretion is due to changes in intestinal secretion and sloughing between the two types of animal. The factors reponsible for the differences in bile acid excretion have not been identified.

SUPPLEMENTARY KEY WORDS coprosterols · steroid balance · intestinal microflora · cholesterol · lathosterol · dehydroxylation · deconjugation

The intestinal and fecal neutral sterols of germfree rats have been shown by Gustafsson, Gustafsson, and Sjövall (1) to be qualitatively different from those of conventional rats. They and others (2–4) have shown that germfree rats excrete no coprostanol or coprostanol analogues of the dietary plant sterols. Snog-Kjaer, Prange, and Dam (5) and Coleman and Baumann (6) demonstrated that cholesterol is reduced to coprostanol by intestinal bacteria; the mechanism of this reduction has been extensively studied by Rosenfeld, Fukushima, Hellman, and Gallagher (7–9). The fecal neutral sterols of conventional rats (reported by Danielsson (10) to be derived mainly from the intestinal wall) have been studied extensively by Baumann, Coleman, and Wells (6, 11–14) as well as by Wells and Makita (15).

Gustafsson, Bergström, Lindstedt, and Norman (16) reported that germfree rats given cholic acid-24-^{14}C by mouth eliminated the isotope only as taurocholate-24-^{14}C, in contrast to conventional rats, which excreted free cholic acid-24-^{14}C and radioactive metabolites of it in their feces. The time required for fecal excretion of one-half of the administered isotope was 2 days in the conventional group vs. 11.4 days in the germfree group. Norman and Shorb (17) have reported that human fecal bacteria can dehydroxylate cholic and chenodeoxycholic acids in vitro at the 7α position. More recently, Hill and Drasar (18) have shown that some species of human fecal bacteria can dehydroxylate the 12α as well as the 7α group. The 7α dehydroxylation

Names of steroids used in this paper are: cholesterol, cholest-5-en-3β-ol; coprostanol, 5β-cholestan-3β-ol; coprostanone, 5β-cholestan-3-one; lathosterol, 5α-cholest-7-en-3β-ol (Δ7-cholestenol); methostenol, 4α-methyl-5α-cholest-7-en-3β-ol; "7-ene sterols," a mixture of sterols containing double bonds at the 7 position, which are characterized by their rapid development of color with Liebermann–Burchard reagent; lithocholic acid, 3α-hydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; α-muricholic acid, 3α,6β,7α-trihydroxy-5β-cholanoic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholanoic acid.

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has been studied in detail by Gustafsson, Midtvedt, and Norman (19) and Hill and Drasar (20).

Conventional rats eliminate cholesterol faster than germfree rats (21). This difference has been ascribed to the action of the microflora, directly or indirectly, on sterol and bile acid metabolism. Although others have shown qualitative differences in the fecal neutral steroid excretion of germfree and conventional rats, no data were available on the quantitative aspects of the fecal neutral sterol excretion of germfree rats. Previous studies of the fecal excretion, pool size, etc. of bile acids suffered from a number of analytical shortcomings [see discussion by Grundy, Ahrens, and Miettinen (22)]. In the rat the excretion of cholic acid and its metabolites was estimated (23) as 5.1 and 1.9 mg/rat per day for conventional and germfree groups, respectively. No data were reported for the muricholic acids or their metabolites, although these may be included in the excretion reported (23).

Important physiological and anatomical differences exist between germfree and conventional rats which may affect fecal steroid excretion in a manner not directly related to the action of intestinal microorganisms on steroids. Such differences, like the rate of sloughing of intestinal mucosa (24, 25), are inherent to the two types of rat, and the present experiments, therefore, measure the total difference in fecal steroid excretion and not just the differences attributable to the microbial activity directly and solely on steroids. Since we wished our studies to be comparable to experiments of others in the area of sterol metabolism, we did not attempt to prevent or modify the normal physiological activities of the animals by restraining, coprophagy, or skin grooming, which may affect fecal sterols. Indeed, others (26) have shown that prevention of coprophagy in the conventional rat causes major changes in cecal and fecal microbial populations, thereby rendering this rat unsuitable as a "conventional" control animal. The differences in general lipid metabolism between germfree and conventional animals have been reviewed by Kellogg and Wostmann (27). This paper reports the quantitative endogenous fecal steroid excretion of germfree and conventional rats and discusses possible factors responsible for the differences between them.

METHODS

Rats; Diets; Collections of Feces

Male Lobund strain rats (of Wistar origin) between 90 and 120 days of age were used for both the germfree and conventional experiments. 14 germfree rats were housed individually in plastic metabolism cages (Ancare Corp., Manhasset, N.Y.) inside germfree isolators made of flexible film plastic. The freedom of the animals and isolators from bacteria was verified both prior to and after the experimental period by conventional bacteriologic methods. 11 conventional rats were housed in metal wire-bottom cages, fitted with screens for collection of feces, in conventional animal quarters. An autoclaved cholesterol-free diet composed of extracted casein (24%), rice starch (60%), corn oil (5%), and supplemental vitamins and minerals was fed to both groups (28). After a 14 day (or longer) preliminary period in which the animals became adjusted to their diet and quarters, voided feces were collected quantitatively for a 5 day period, and held at incubator temperature until the collection was complete. At the end of the collection period, individual 5 day samples were homogenized with ethanol in a Waring Blendor and diluted to 100 ml. A 20 ml sample, equivalent to 1 day's excretion, was taken for analysis.

Analysis of Fecal Neutral Steroids

The analytical techniques of Miettinen, Ahrens, and Grundy (29) for the neutral sterol analyses were used throughout. These techniques employ an internal standard of cholesterol-^{14}C to correct for losses during extraction and TLC, and a second internal standard of 5α-cholestane for quantification by GLC of the sterol trimethylsilyl (TMS) ether derivatives on 1% SE-30 (methyl silicone polymer) columns. The purity of the cholesterol-^{14}C was assessed by TLC (chloroform on Silica Gel G) with nonradioactive carrier. All radioactivity was recovered in one spot having an R_f identical with that of the cholesterol standard. In our laboratory replicate analyses utilizing these methods yielded standard deviations of approximately 5%. Neutral sterols were identified by comparison with the TLC and GLC behavior of standard reference sterols and to the published work of others (1, 29).

Analysis of Fecal Bile Acids

For the analysis of bile acid the techniques of Grundy et al. (22) were used, with the exception that during purification by TLC the entire area between the fatty acid band and the origin was removed for recovery of the bile acids, because some iodine-staining bands were visible below the methyl cholate marker. An internal standard of cholic acid-^{14}C was used to correct for losses during extraction and TLC, and an internal standard of 5α-cholestane was employed for GLC of the TMS ethers of the bile acid methyl esters on 1% SE-30 and 0.5% Hi-Eff 8BP (cyclohexane dimethanol succinate) columns. The purity of the cholic acid-^{14}C was confirmed by TLC (isooctane–isopropanol–acetic acid 120:40:1).

We validated the bile acid method for use in experiments with germfree rats by the following procedures:

(*a*) Approximately 11 mg (three times as much as normally assayed) of pure taurochenodeoxycholate and taurodeoxycholate were subjected individually to the entire procedure of Grundy et al. TLC examination after hydrolysis showed no remaining conjugated bile salt. GLC analysis revealed only one symmetrical peak corresponding to the expected TMS bile acid methyl ester. Recovery of the bile acids was over 90%.

(*b*) Recovery of sodium taurocholate-24-^{14}C added to fecal homogenates from germfree rats was 90 ± 6% (SD, n = 4) after extraction, methylation, and TLC.

(*c*) Recoveries of cholic acid-24-^{14}C added as internal standard were consistently above 90%.

The fecal bile acid methyl esters from germfree rats were separated from bile pigments by repeated TLC on preparative plates, according to Grundy et al. (22). Four bands were isolated and designated "unknowns 1, 2, 3, and 4." To assist in identification, each unknown was oxidized (30) and gas-chromatographed on an SE-30 column. The oxidation products were also subjected to TLC on Silica Gel G in chloroform–diethyl ether 85:15. The chromatogram was sprayed with a solution of 2,4-dinitrophenylhydrazine. In addition, the relative TLC mobilities of the unknowns were compared to the values reported by Hofmann (31) utilizing his system S-VII. Samples of authentic α, β, and ω-muricholic acids were obtained from Dr. W. W. Wells (Department of Biochemistry, Michigan State University) who had obtained them originally from Dr. S. L. Hsia (Department of Dermatology, University of Miami School of Medicine).

To determine whether the bile acids in the feces from each group of rats were conjugated or free, the homogenized fecal samples were saponified overnight at room temperature with 4.5% KOH; this treatment does not split the conjugates but does release neutral sterols from their esters. The sterols were extracted with hexane. The remaining sample was acidified with sulfuric acid to pH 2 and bile acids and fatty acids were extracted with diethyl ether. This extract was subjected to TLC in isoamyl acetate–propionic acid–*n*-propanol–water 4:3:2:1 on Silica Gel G [System S-VIII of Hofmann (32)] and compared to appropriate standards of taurine-conjugated, glycine-conjugated, and free cholic and deoxycholic acids. The plates were sprayed with 10% phosphomolybdic acid in ethanol and heated. The bile acids appear as blue spots on a yellow background. According to this test, all bile acids from conventional animals were free and all those from germfree rats were conjugated.

Gas–Liquid Chromatographic Procedures

A Hewlett–Packard model 402 gas chromatograph fitted with a 2 m glass U-tube column packed with 1% SE-30 on 100–120 mesh Gas-Chrom Q (purchased prepacked from Applied Science Laboratories, State College, Pa.) was conditioned at 325°C for 30 min without carrier gas flow. The analyses were performed at 240°C with flash heater at 260°C. All quantitative data were obtained with this system. A polar column of 0.5% Hi-Eff 8BP (32) coated on Gas-Chrom Q (Applied Science Laboratories) was utilized at the above temperature and conditions for identification of bile acids. Temperatures were verified by an independent thermocouple and potentiometer against an ice-distilled water reference. Nitrogen was used as carrier gas.

A standard mixture of equal parts of 5α-cholestane (the GLC internal standard), doubly recrystallized cholesterol (mp 150°C, uncorrected), and methyl cholate was analyzed as the trimethylsilyl ether derivative before and after each day's experimental analysis. In all cases the flame ionization detector response for individual standard samples from 0.3 to 70 μg injected was the same for each of the three components, as indicated by a disc integrator. Since all experimental analysis utilized well over 0.3 μg of sample, losses due to column absorption were negligible. Furthermore, the detector response on our gas chromatograph was nearly identical (± 2–3%), on a weight basis, for the TMS methyl esters of hyocholic (selected as an example of a 3,6,7-trihydroxy compound), cholic, deoxychloic, chenodeoxycholic, and lithocholic acids.

Statistics

Statistical significance between means was assessed by means of Student's "*t*" test; tests for correlations followed the methods of Snedecor (33).

RESULTS AND DISCUSSION

Neutral Sterol Excretion

The "endogenous fecal neutral sterols" reported here include cholesterol (and its neutral sterol conversion products) and lathosterol, but not plant sterols of dietary origin. The endogenous fecal neutral sterol excreted by the germfree and conventional rats averaged 12.8 and 19.5 mg/kg of body wt per day, respectively (Table 1; $P < 0.001$). Excretion by the conventional animals was similar to most values reported by others (13, 29, 34–37). The variation in intestinal flora patterns and their activities due to different genetics (38, 39), diets (39–42), and animal room characteristics may account for residual differences between our results and others.

Excretion of neutral sterols by germfree animals was two-thirds of that of the conventional control rats. As reported by other workers (1–4), the germfree rats excreted no coprostanol or copro analogues of the plant

TABLE 1 ENDOGENOUS FECAL NEUTRAL STEROID EXCRETION OF GERMFREE AND CONVENTIONAL MALE RATS (3–4 months old)

	Germfree 14 Rats	Conventional 11 Rats
	mg/kg body wt/day	
Steroids excreted	12.8 ± 3.0	19.5 ± 5.2*
Composition	%	
5-ene sterols	89 ± 3	34 ± 16
Lathosterol	11 ± 3	9 ± 5
5β,3-OH-sterol	0	55 ± 12
5β-3-one	0	1.2 ± 0.6

Values are means ±SD.
* Difference between germfree and conventional means is significant ($P < 0.001$).

sterols. In addition, we could not detect coprostanone in germfree feces. The conventional rats excreted coprostanol, coprostanone, and the corresponding plant sterol analogues, as reported by others (1).

Gustafsson et al. (1) report finding methostenol in the feces of both germfree and conventional rats, and Neiderhiser and Wells (43) report that 5.9% of the total neutral fecal sterols of endogenous origin in conventional rats consisted of methostenol. However, the methods used in our study do not permit methostenol to be identified separately from dietary plant sterols.

The diet consumption was not measured for the conventional group, so that any loss of neutral sterol due to bacterial degradation could not be estimated by determining the recovery of dietary plant sterols, as recently described by Grundy, Ahrens, and Salen (44). However, diet consumption was accurately measured for 9 of 14 rats in the germfree group. The fecal recovery of β-sitosterol in these animals averaged 92.4 ± 14.0% over the 5-day collection period. Only one value (118%) exceeded 100%. These recoveries suggest that on a group average basis a 5-day collection period is adequate to average out variations in amounts of feces voided daily. If bacterial degradation of neutral sterol had occurred in the feces of the conventional group either before or after defecation the real differences in neutral steroid excretions between the two groups would be even greater than that indicated in Table 1.

Since the diet employed in our study was free from both cholesterol and lathosterol, the amounts of these substances, as well as of coprostanol and coprostanone, excreted in the feces can be considered to be endogenous in origin. Coprostanol can be synthesized by intestinal microflora from cholesterol (5, 6) and no other endogenous source of this compound has been reported. Conversion to coprostanol, a virtually unabsorbable sterol (45), would lower the amount of cholesterol reabsorbed and thereby increase the total fecal neutral sterol excretion. This would hold true, however, only if significant coprostanol formation occurred at or proximal to the intestinal site of cholesterol absorption. Wells, Anderson, and Quan Ma (46) and Wilson (47) could detect little or no coprostanol in the first half of the small intestine of rats, where Swell et al. (48) have shown that the main part of cholesterol absorption occurs. Although Wilson's animals were free to practive coprophagy, the recycling of coprostanol appeared to be negligible (47).

No significant ($P < 0.05$) correlation existed (Table 2) between the percentage of any of three forms of endogenous sterol (cholesterol, coprostanol, coprostanone) and the amount of neutral sterols excreted by the conventional rats. Therefore, the increased excretion of neutral sterols by the conventional animals appeared not to be due primarily to coprostanol formation, although these data must be interpreted with caution, since some coprostanol may have been formed in voided feces after excretion.

A correlation coefficient of +0.61 ($P < 0.05$) was found between the percentage of lathosterol in neutral sterols excreted by conventional rats and the amount of neutral sterols excreted. A correlation coefficient of +0.21 was found for similar data from germfree rats. This correlation was not statistically significant because of a large variability contributed by the data obtained from three of the rats. When these were excluded, the correlation coefficient for the germfree group became +0.74 ($P < 0.01$; n = 11). The above correlations suggested that a source of sterol containing lathosterol was the probable source of the increase in excreted fecal neutral sterol. Wells, Coleman, and Baumann (49) have shown that 7-ene sterols constitute a natural component of intestinal mucosa, but are not present in bile.

Miller and Baumann (50) have reported that 7-ene sterols make up approximately one-third of the sterols of rat skin. Some of these would be ingested by grooming, but under the experimental conditions it was impossible to assess their possible contribution to the fecal sterols. The small amounts of cholesterol and its precursors that may be ingested in this way would probably be almost

TABLE 2 CORRELATION COEFFICIENTS BETWEEN PERCENTAGES OF INDIVIDUAL STEROLS AND THE TOTAL AMOUNT OF FECAL NEUTRAL STEROLS EXCRETED

Sterol	Rat Type and No.	Correlation	Significance
Cholesterol	Conventional (11)	−0.10	none
Lathosterol	Conventional (11)	+0.61	$P < 0.05$
Lathosterol	Germfree (14)	+0.21*	none*
Coprostanol	Conventional (11)	+0.08	none
Coprostanone	Conventional (11)	+0.10	none
Coprostanone vs. coprostanol	Conventional (11)	+0.34	none

* See discussion.

totally absorbed, as has been suggested (10) for biliary cholesterol. Therefore, it appears improbable that the pronounced differences in neutral sterol excretion would be related to amounts of skin sterol ingested in the two experimental groups.

Several authors (10, 51, 52) have concluded that the fecal neutral sterols are predominantly of intestinal origin and arise by secretion from or sloughing of cells by the intestine. The comparative rate of sloughing of cells from the intestinal villi can be calculated from data of Abrams, Bauer, and Sprinz (24) and Matsuzawa and Wilson (25). The villi of germfree mice slough only an average of 62% as many cells per unit time as their conventional counterparts. Unpublished studies in our laboratory have shown the small intestinal mucosal cholesterol of rats to be 14.75 (germfree) and 17.25 mg/kg of body wt (conventional). Utilizing the above data on mucosal renewal rates in germfree and conventional mice (24, 25), we calculate the small intestinal cell sloughing to contribute 3.8 and 8.9 mg/kg of body wt per day, respectively, the difference of 5.1 mg of cholesterol per kg of body wt per day accounting for nearly 80% of the difference (Table 1) between the two groups in excretion of neutral sterols.

Wilson and Reinke (53) have shown that sterol synthesized in the intestinal tract can be excreted to the lumen of the intestine, from which it is not reabsorbed to any appreciable extent. Gordon and Bruckner-Kardoss (54) demonstrated that, in the germfree rat, the lower small intestine [the site of maximal intestinal sterol synthesis (55)] had only 63% the surface area and 66% the dry weight of the same part of the intestine in conventional rats. Thus the lower excretion of neutral sterols by germfree rats (66% of conventional) could also be explained either by a lowered secretion (which should be related to mass of tissue and (or) surface area), or by a combination of lowered secretion and rate of sloughing.

Dietschy has recently reported that the concentration of luminal bile salts controls the rate of cholesterogenesis in the gastrointestinal tract (56). Since the amount and types of bile acids excreted by germfree and conventional rats differed markedly (see below), it is possible that the differences in neutral sterol excretion might be related, at least partially, to the difference in intestinal bile acids between the germfree and conventional groups. However, correlation coefficients between the neutral sterol and bile acid fecal excretion of rats in the two groups were only + 0.34 (germfree) and +0.04 (conventional) showing little, if any, quantitative relationship between the two excretory forms of steroid.

Thus the following observations support the view that most of the increase in fecal neutral sterol excretion in conventional rats as compared to germfree rats is due to an increase in sloughing and (or) secretion of sterols from the intestinal walls, rather than to the formation of unabsorbable neutral sterols by bacterial action: (*a*) the major site of sterol absorption is prior to the major site of coprostanol formation (46–48); (*b*) there was no correlation between the amounts of total sterol excreted and the amount of coprostanol or coprostanone excreted; (*c*) others have concluded that most fecal neutral sterols arise from materials sloughed by or excreted from the intestinal tract and our data indicating the correlation between lathosterol and the total neutral sterol excretion support this; and (*d*) the differences in cell sloughing, surface area, and weight of the germfree as compared to the conventional intestine are similar to the differences seen in the fecal excretion of neutral sterols between the two groups.

Bile Acid Excretion

Fecal excretion of bile acids by germfree rats averaged 11.3 ± 2.4 and by conventional rats 21.4 ± 9.9 mg/kg of body wt per day ($P < 0.005$). The coefficients of variation (SD ÷ mean × 100), an adjusted reflection of the variability in the data, were 21 and 46%, respectively. This suggested that at least half of the quantitative variation seen in fecal excretion of bile acids in the conventional animal was derived from effects of the intestinal microflora.

The value for bile acid excretion in conventional rats of 21.4 mg/kg of body wt per day was in reasonable agreement with the values reported by others (22, 23, 57–62). Gustafsson et al. (23) have reported that the amount of cholic acid and its metabolites excreted by conventional animals was about double that of their germfree counterparts. Their method employed titration of the chromatographically purified bile acids, and their values therefore probably included the amount of the muricholic acids as well as of cholic acid and its metabolites. Their values (recalculated on a mg/kg of body wt per day basis) were 8.2 and 18.9 for germfree and conventional groups, respectively. All of the bile acids excreted by our conventional rats had a TLC mobility similar to those of the free cholanoic acid markers, whereas those extracted from the germfree rats had a mobility (Table 3) similar to that of taurocholate. A trace of material from the germfree group was seen in an area corresponding to glycine-conjugated bile acids. This is in agreement with the report of Gustafsson et al. (16).

After deconjugation, the four bile acids isolated from germfree feces were tentatively identified as cholic, β-muricholic, and α-muricholic acids (plus one unidentified) by GLC, oxidation, and TLC (Table 3). Unknown *1* yielded an oxidation product that was

TABLE 3 TLC AND GLC BEHAVIOR OF METHYL ESTERS OF BILE ACIDS ISOLATED FROM GERMFREE RAT FECES, COMPARED TO REFERENCE STANDARDS

Material Analyzed	TLC in Hofmann's System		GLC of TMS Ethers on SE-30	GLC of TMS Ethers on Hi-Eff-8BP	TLC of Oxidation Product		GLC of Oxidation Product on SE-30	Probable Identification of Unknowns
	S-VIII R_f	S-VII Rm*	RRT†	RRT‡	R_f	2,4-DNP Color	RRT†	
3α,7α,12α free acid	0.70							
Ditto, taurine conjugate	0.10							
Ditto, glycine conjugate	0.35							
Chloroform–methanol fecal extract	0.07–0.17							Mixed taurine conjugates
Methyl esters of:								
Unknown *1*		1.36	2.87	0.74	0.42	Peach	—	α-Muricholic acid
3α,6β,7α, reference§		(1.34)‖	2.86	0.77				
3α,6α,7α, reference		1.78 (1.73)	—	1.00	0.42	Peach	—	
Unknown *2*		2.14	3.84	1.20	0.42	Peach	—	β-Muricholic acid
3α,6β,7β, reference		(2.08)	3.81	1.14				
3α,6α,7α, reference		1.78 (1.73)	—	1.00	0.42	Peach	—	
Unknown *3*		—	2.87	0.79	0.26	Yellow	4.50	Cholic acid
3α,7α,12α, reference		1.00	2.86	0.80	0.26	Yellow	4.23	
Unknown *4*			2.20	none	0.83	Yellow	No major peak	Unidentified
3α, reference			2.17	1.72	0.85	Pale yellow	2.04	

Unknowns *1–4* were from TLC bands after deconjugation.
* Relative to methyl 3α,7α,12α-trihydroxy-5β-cholanoate.
† Relative to 5α-cholestane.
‡ Relative to methyl 3α,6α,7α-trihydroxy-5β-cholanoate.
§ Reference compounds are hydroxy-substituted 5β-cholanoate methyl esters unless specified otherwise.
‖ Values in parentheses calculated from Hofmann (32).

identical in TLC and GLC behavior and 2,4-DNP color reaction to that of an oxidized 3,6,7-trihydroxy bile acid methyl ester. The TMS ether derivative had GLC retention times identical to those of the TMS ether of methyl 3α,6β,7α-trihydroxy-5β-cholanoate both on SE-30 and on Hi-Eff 8BP. The TLC relative mobility of the methyl ester was that expected for α-muricholic acid (32).

The methyl ester of unknown *2* yielded an oxidation product identical with that of unknown *1* methyl ester, again identifying it as a 3,6,7-trihydroxycholanoic acid. The retention times of its TMS ether–methyl ester derivative on the polar and nonpolar GLC columns agreed with those of the corresponding derivative of 3α,6β,7β-trihydroxy-5β-cholanoate. TLC relative mobility of the methyl ester agreed with published values (32) for methyl β-muricholate.

Unknown *3* corresponded in all the above respects to cholic acid. Unknown *4* yielded an oxidation product whose identity could not be definitely established, although its mobility was typical of a monoketone. Small amounts of other bile acids may have been present but remained unidentified because they cochromatographed with major components.

60.5 ± 9.1% of the germfree fecal bile acids excreted consisted of β-muricholic acid, 29.5 ± 9.6% consisted of a mixture of cholic and α-muricholic acids (these eluted together from SE-30 columns under our conditions) and 10.0 ± 9.4% was the unidentified material with the chromatographic behavior of a monosubstituted bile acid. Since Grundy et al. (22) reported that in studies of human fecal bile acids all peaks eluting after 5α-cholestane were bile acids, we assumed this material to be a bile acid and included it in the calculations. The mobilities of the bile acids from conventional animals were (on the SE-30 column) those of cholic acid, α- and β-muricholic acids, chenodeoxycholic acid, deoxycholic acid, and lithocholic acid. Other peaks seen were not identified.

We have assumed that materials chromatographing on the SE-30 column with the same relative mobilities as the bile acids from germfree animals were primary bile acids and all materials not chromatographing with those mobilities were bacterially modified steroids (secondary bile acids). Although we have not checked all possible secondary bile acids, none of the known major ones cochromatographed with the primary bile acids as TMS ethers. On these assumptions we calculated the percentage of bile acids excreted from the conventional rat that were bacterially modified (not including deconjugation) as 57.7 ± 9.3% (Table 4). This would include chenodeoxycholic acid, which was found to occur in germfree bile and the feces of the conventional rat but not in germfree feces.

TABLE 4 FECAL BILE ACIDS EXCRETED BY GERMFREE AND CONVENTIONAL MALE RATS

	Germfree (14)	Conventional (11)
Fecal bile acid excretion mg/kg body wt/day ± SD	11.3 ± 2.4	21.4 ± 9.9*
Approximate % of bacterial modification	None	57.7%
Types of bile acids found	Taurocholic Tauro-β-muricholic Tauro-α-muricholic Unidentified compound	Cholic β-Muricholic α-Muricholic Chenodeoxycholic Deoxycholic Lithocholic Many others

* Difference between conventional and germfree means is significant ($P < 0.005$).

Preliminary studies in our laboratory of the bile acid content of the bile of germfree rats have indicated that a dihydroxy bile acid similar in behavior to chenodeoxycholic acid was present. Chenodeoxycholic acid is a primary bile acid, synthesized in rat liver from cholesterol (62). The absence of this material from the feces of the germfree rats was an indication that biliary chenodeoxycholic acid was completely reabsorbed in the intestinal tract. It has been shown that chenodeoxycholic acid can be converted in the liver to α-muricholic acid (62–66), which can be further metabolized to β-muricholic acid (67). Thus, β-muricholic acid and cholic acid are end-products of hepatic bile acid metabolism in the rat. Our data show that β-muricholic acid comprised 60% of the excreted bile acids in germfree rats and, although a complete separation could not be made by GLC between α-muricholic and cholic acids, the amount of α-muricholic acid was considerably less than that of cholic acid. These last results must be regarded as preliminary because insufficient data are available about the losses of these two materials on the Hi-Eff 8BP columns under our conditions.

Thus, the fecal bile acids of the germfree rat largely consisted of materials that are end-products of bile acid metabolism. The predominance of the muricholic acids in the fecal excretion of these animals was probably a reflection of the fact that the reabsorbed chenodeoxycholic acid could be converted only into muricholic acids in the liver of the rat and not into cholic acid (62–66). What appeared in the feces of conventional animals as chenodeoxycholic acid because of incomplete reabsorption was seen in the feces of germfree animals as the muricholic acids. Similarly α-muricholic acid, if reabsorbed and further metabolized by the liver, would be converted to β-muricholic acid (67). There was strong evidence, therefore, that in the germfree rat, enhanced reabsorption increased the number of cycles of the bile acids through the enterohepatic system and that, consequently, the degree of metabolism of bile acids was greater than it was in the conventional animal. This view is supported by the data of Gustafsson et al. (16), who have shown that the half-life of cholic acid-^{14}C in the germfree rat is from three to five times as long as in the conventional rat.

Hill and Drasar have shown (20) that some intestinal microorganisms are capable of dehydroxylating bile acids in the 12α-position, with consequent formation of chenodeoxycholic acid from cholic acid. This would place chenodeoxycholic acid in the position of being both a primary bile acid, capable of being synthesized from cholesterol in the liver, and a secondary bile acid, capable of being produced by intestinal bacteria from cholic acid. The production of chenodeoxycholic acid by the intestinal bacteria would tend to shift metabolic pools of cholic acid towards metabolic pools of muricholic acids in conventional rats, since it is generally accepted that the 12α-hydroxyl group could not be restored in bile acids by the liver. Therefore, the only further metabolic fates of chenodeoxycholic acid converted from cholic acid by the intestinal bacteria would be (*a*) excretion in the feces, (*b*) further metabolism to α-muricholic and β-muricholic acids (and their possible bacterial conversion products) as discussed above, or (*c*) further dehydroxylation to lithocholic acid and its metabolities. Danielsson et al. (68) have reported the presence of small amounts of tritium-labeled lithocholic acid in the feces of patients given tritiated cholic acid. However, there was a possibility that this was due to trace amounts of tritiated impurities.

Several explanations could be offered for the highly significant differences between the fecal bile acid excretion of the germfree and the conventional animals. It is possible that the bacterially modified bile acids were less readily absorbed than are the primary and (or) conjugated bile acids. Gustafsson and Norman (69) have shown that 35–40% of cholic acid-^{14}C and its metabolites in conventional rat cecum and colon contents were found in a water-insoluble fraction that sediments when centrifuged at 25,000 *g* for 60 min. In contrast, in germfree rats less than 5% was recovered in this fraction. There was relatively more bile acid which had undergone 7-dehydroxylation present in the water-insoluble fraction than in the supernatant fraction from the conventional rats. These observations have been confirmed by Norman (70). Similar data by Norman and Shorb (17) have shown lithocholic acid to be adsorbed to the sediment while its chenodeoxycholic precursor was in the supernantant fraction. Thus, deoxycholic acid would be less available for reabsorption because of its presence in the ceca in a predominantly water-insoluble

form. These differences were not present, however, in the small intestine where active bile salt absorption occurs (70). Comparisons of the concentration ratios of various types of di- and trihydroxy bile acids in the portal blood vs. the small intestine of rats indicated that most of these bile acids were absorbed to about the same extent (71). Danielsson et al. (68) reported, however, that lithocholic acid was practically absent from bile because it was absorbed from the intestinal tract to only a limited extent. Thus the microbial formation of lithocholic acid would increase the fecal excretion of bile acids.

The intestinal tract of the germfree rat might absorb bile acids more *efficiently* than the intestinal tract of its conventional counterpart, although no data on this point are yet available. Against this hypothesis are the lower surface area and dry weight (54) of germfree compared to conventional gastrointestinal tracts, discussed above. On the other hand, the gastrointestinal transit time has been shown to be 2–3 times shorter in conventional than germfree mice (72), but unpublished studies in our laboratory have indicated that the corresponding difference in rats is much smaller (only 20%).

Total Fecal Steroid Excretion

The combined fecal neutral sterols and bile acids of the germfree and the conventional groups (Table 5) were 24.1 and 40.9 mg/kg of body wt per day, respectively. Since the diet contained no cholesterol, these data also represent the negative sterol balance of the two experimental groups and, if we assume a steady state in these adult animals, indicate that steroid production in the conventional groups was 170% of that in the germfree group. The bile acids composed 46.8% (germfree) and 52.3% (conventional) of the total excreted steroids. As pointed out above, the correlation coefficients between neutral sterols and bile acids indicate no strong interrelationship between the two forms of steroid excretion, since neither coefficient is significantly different from zero at the 5% probability level (33).

TABLE 5 TOTAL FECAL STEROID EXCRETION OF GERMFREE AND CONVENTIONAL MALE RATS

	Germfree (14)	Conventional (11)
Total excretion mg/kg body wt/day ± SD	24.1 ± 4.2	40.9* ± 11.3
Coefficient of variation	17%	28%
Percent bile acids	46.8 ± 5.5	52.3 ± 10.0
Correlation coefficient between neutral and acidic steroids	+0.34	+0.04

* Difference between conventional and germfree means is significant ($P < 0.001$).

Wostmann, Wiech, and Kung (21) have shown that cholesterol-26-^{14}C is metabolized to $^{14}CO_2$ faster in the presence of a conventional microflora. Data on bile acid elimination obtained by Gustafsson et al. (23) and further detailed in this paper suggest that the greater bile acid excretion in conventional rats as compared to germfree counterparts is a major cause of the enhanced cholesterol catabolism found in those animals. The increased neutral sterol excretion, on the other hand, appears to be caused, at least in part, by the higher rate of desquamation of the intestinal mucosa in the conventional rat. This may be of minor importance for the turnover of cholesterol from the blood–liver pool of the rat, since cholesterol is synthesized in the intestine from acetate and when secreted into the lumen is not reabsorbed to any great extent (53).

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