Effect of bile acid deconjugation on the fecal excretion of steroids

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ABSTRACT The effect of microbiological deconjugation of bile acids on total bile acid and neutral sterol fecal excretion by adult male rats has been studied. A screening method utilizing mice allowed selection of a *Clostridium perfringens* type A strain, which accelerated cholesterol catabolism in mice. When this species of bacteria was associated with germfree rats, the fecal bile acids were excreted as free bile acids (deconjugated), however the quantities of bile acids excreted were not increased compared with those of germfree rats. Conventional rats excrete twice as much bile acids (all deconjugated) as do the germfree and *C. perfringens*-associated rats. It is, therefore, unlikely that the microbiological deconjugation of bile acids is responsible for the increased fecal excretion of bile acids seen in conventional rats.

The *C. perfringens*-associated rats excreted identical kinds and quantities of fecal neutral sterols as did the germfree rats.

SUPPLEMENTARY KEY WORDS: dehydroxylation · sterol balance in rats · fecal sterols

GERMFREE[1] rats catabolize cholesterol-26-^{14}C at a reduced rate (1), and respond to dietary cholesterol by accumulation of cholesterol in the liver to levels nearly three times greater than conventional rats (2). They excrete fewer kinds and smaller amounts of fecal neutral steroids and bile acids than do conventional rats (3, 4). These studies demonstrated that the intestinal microflora is a major factor in cholesterol metabolism in rats. However, it is not known which of many activities of the intestinal microflora is responsible for this marked difference between cholesterol and bile acid metabolism.

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

Gustafsson, Midtvedt, and Norman (5) investigated the microbiological dehydroxylation of bile acids in vitro. Pure cultures of 7α-dehydroxylating microorganisms were capable of further modifying the molecular structure of bile acids by oxidizing the C-3 and C-7 hydroxyls to keto groups. Hill and Drasar (6) studied organisms isolated from the human intestinal tract and showed that many species of bacteria were capable of both the dehydroxylation and deconjugation of bile acids in vitro. Gustafsson et al. (4, 7) also studied the metabolism of ^{14}C labeled taurocholate in vivo in previously germfree rats associated with various microbial species. Their earliest study of a rat associated with *Asperigillus niger* and *C. perfringens* type E showed no change in the rate of isotope excretion in the feces. They found partial but significant deconjugation of taurocholate in the gnotobiotic rat. A second study employing improved methods of quantitating fecal bile acids indicated that two rats monoassociated with *Escherichia coli* excreted only conjugated bile acids in amounts quantitatively identical to those excreted by germfree rats. Recent qualitative experiments (8) have shown that only deconjugated bile acids were dehydroxyl-

[1] *Germfree animal:* a gnotobiote that is free from all demonstrable associated forms of life, including bacteria, viruses, fungi, protozoa, and other saprophytic or parasitic forms; *Gnotobiote:* an animal stock or strain, derived by aseptic caesarean section or sterile hatching of eggs, that is reared and continuously maintained with germfree techniques under isolator conditions in which the composition of any associated fauna and flora, if present, is fully defined by accepted current methodology; *Defined flora animal:* a gnotobiote maintained under isolator conditions in intentional association with one or more known types of microorganisms. Such terms as "monoinfected," "monocontaminated," "monoassociated," and "polycontaminated" have been employed to describe this type of gnotobiote. Of these, the terms "monoassociated" and "polyassociated" are preferable for describing intentional, rather than accidental, association with microbes.

ated by the microbial species investigated. None of the gnotobiotic groups excreted over 25% of fecal bile acids as deconjugates.

In vitro studies of neutral sterol modification by fecal microorganisms have all utilized mixed cultures. Snog-Kjaer, Prange, and Dam (9) demonstrated that mixed fecal microorganisms are capable of saturating the double bond in cholesterol to form coprostanol. Coleman and Baumann (10) verified and expanded these studies, and showed that mixed fecal microorganisms are capable of reducing the Δ^5 double bond in many different sterols. This reaction has not been achieved by pure culture.

Gustafsson, Gustafsson, and Sjövall (11) reported that germfree rats excreted only cholesterol, lathosterol, and methostenol as compared with conventional rats which also excreted coprostanol. Kellogg and Wostmann (3) confirmed these findings and showed that while the germfree rats excreted 12 mg of neutral sterols per kg of body weight per day, conventional rats excreted 18 mg/kg of body weight per day. The fecal bile acid excretion of germfree rats was 12 mg/kg of body weight per day; all the bile acids were conjugated with taurine and were composed of β-muricholic acid, 60%; a mixture of cholic and α-muricholic acids, 25%; and an unidentified component with the chromatographic behavior of a monosubstituted cholanoic acid, 10%. The conventional rats excreted 21 mg/kg of body weight per day, all as free bile acids. These included, in addition to those cited above, lithocholic acid, deoxycholic acid, and many others.

This paper reports studies carried out with pure cultures of organisms in the gnotobiotic mice and rats with the purpose of identifying the microbiological species involved in the qualitative and quantitative modification of neutral sterol and bile acid excretion by the intestinal microflora.

SCREENING STUDIES WITH MICE

Methods

Initial screening of species of microorganisms was conducted in monoassociated mice. Adult germfree Lobund mice of Swiss Webster origin, were injected introcardially with cholesterol-26-^{14}C and were subsequently transferred in groups of five to glass jars with fiber glass filter tops. They were monoassociated via inoculation with organisms listed in Table 1.

After a period of 5 days, the monoassociated animals were killed. One mouse was used to verify the microbiological status of the group. All the animals were briefly autoclaved to soften the skin, and the entire animal was homogenized in a Waring Blendor. An aliquot of the homogenate was counted on planchettes for residual ^{14}C, and the data were expressed as the percentage of the dose of ^{14}C originally injected. Two control groups were included in each experimental series. One control group was maintained germfree, and the other was conventionalized by exposing germfree animals to a slurry of the intestinal and cecal contents of conventional mice.

TABLE 1 PERCENTAGE OF RADIOACTIVITY RETAINED IN THE CARCASS OF MICE 5 DAYS AFTER INJECTION OF CHOLESTEROL-26-^{14}C

Experiment	Germfree	*Clostridium perfringens* Gnotobiote	Conventionalized Germfree
		%	
1	65.8 ± 8.0*	59.2 ± 6.9	53.6 ± 10.7
2	69.1 ± 5.3	45.8 ± 6.5	45.0 ± 12.0
3	Contaminated	36.2 ± 2.9; 30.7 ± 3.7	35.0 ± 6.5

Other species tested which were not significantly different from germfree controls included: *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Streptococcus liquefaciens*, and *Clostridium difficile*.

* Each value is the average of five mice ± SD.

Results

Table 1 shows the bacterial species investigated and the percentage recovery (± SD) of the ^{14}C, which was initially injected into the animals as cholesterol-26-^{14}C. The conventionalized control mice consistently retained a lower level of the injected ^{14}C than did their germfree counterparts. Table 1 shows that *C. perfringens*-associated mice had a ^{14}C retention similar to that of the conventional control animals. The microorganisms were reisolated from these animals and identified as *C. perfringens*, type A (ND757). Since none of the other microbial species studied resulted in a lower than germfree retention of ^{14}C, this species was selected for further studies in the rat.

STEROID BALANCE STUDIES WITH RATS

Methods

2-month-old male germfree Lobund rats of Wistar origin, were maintained on cholesterol-free, semisynthetic diet L-474-E12 (12) for at least 4 weeks for adjustment to diet and quarters, and subsequently were monoassociated by oral inoculation with *C. perfringens* type A obtained from the screening tests in mice. After an additional 4 week period, the feces were collected and analyzed as described elsewhere (3, 13, 14). Bacterial examination of the animals at the conclusion of the experimental period verified the presence of the miroorganism in the gastrointestinal tract. Additional tests were conducted to determine if other contaminating species were present.

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 and butanol. This extract was analyzed by TLC on Silica Gel G plates developed in isoamyl acetate–propionic acid–*n*-propanol–water 4:3:2:1 (System S-VIII of Hofmann [15]), and compared with appropriate standards of taurine-conjugated, glycine-conjugated, and free cholic and deoxycholic acid. 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 and gnotobiotic animals were free, and all those from germfree rats were conjugated.

Results

12 rats were monoassociated with *C. perfringens* type A (ND757) as described above. Six of the rats were accidentally contaminated during the experiment with a streptococcal species in addition to the *Clostridium*. Examination of the quantitative and qualitative bile acid and neutral sterol excretion of the monoassociated and the diassociated groups showed no apparent or statistical differences in any of the parameters measured. Therefore, the data were pooled and analyzed statistically as one population. Table 2 shows the bile acid and neutral sterol excretion of these gnotobiotic rats as compared with data collected from rats in the germfree or conventional state.

The total bile acid excretion of the gnotobiotic rats was 7.1 ± 3.5 mg/kg of body weight per day. Germfree rats excreted 11.3 ± 2.4 mg/kg of body weight per day ($P < 0.005$). All fecal bile acids of the gnotobiotic rats were unconjugated, while the fecal bile acids of the germfree rats were conjugated with taurine. The percentage of the fecal bile acids represented by cholic plus α-muricholic acid was essentially the same in the two groups. The percentage consisting of β-muricholic acid was significantly lower in the gnotobiotic group, 23.6%, than in germfree rats, 60.5% (Table 2 and Fig. 1). No difference in cecal size was observed between the gnotobiotic and germfree rats.

An estimate of the amount of bacterial activity toward bile acids can be obtained as outlined in a previous paper (3). The percentage of bile acids excreted, which were bacterially modified, was 34.3 ± 14.1% for the gnotobiotic group compared with 57.7 ± 9.3% for the conventional rats. The neutral sterol excretion of the gnotobiotic rats was similar in both character and amount to that seen in germfree rats. The total neutral sterol excretion of the gnotobiotic group was 12.7 ± 4.2 mg/kg of body weight per day; germfree rats excreted 12.8 ± 3.0 mg/kg of body weight per day. No 5-β-neutral sterols were detected in fecal neutral sterols from either the germfree or the gnotobiotic rats.

DISCUSSION

Previous studies had shown that the release of ^{14}C from the 26-position of cholesterol was faster in conventional than in germfree rats (1). The excretion of the radioactivity in feces, presumably in the form of cholesterol and its metabolites, was significantly greater in the conventional than in the germfree rats. This indicated a faster rate of cholesterol breakdown to bile acids and a faster neutral sterol excretion in the conventional than in the germfree rats. These findings were upheld by the direct measurement of the fecal bile acid and neutral sterol excretion of germfree and conventional rats (3). The greater cholesterol-26-^{14}C retention in the germfree than in the conventional mice reported here, appeared to confirm the data obtained with germfree and conventional rats.

Screening for microorganisms possibly involved in the cholesterol catabolism-enhancing effect of the conventional intestinal microflora was done in mice since this allowed a much simpler technical approach than did the use of rats. Utilizing the isotopic clearance test, the results suggested that association with *C. perfringens* type A (ND757) caused an increase in cholesterol catabolism comparable with the effect evoked by the presence of

TABLE 2 FECAL STEROID EXCRETION OF GERMFREE, *C. perfringens*-ASSOCIATED (GNOTOBIOTIC), AND CONVENTIONAL RATS

Parameter Examined	Animal Status		
	Germfree	Gnotobiotic	Conventional*
Number of rats	12	12	14
Endogenous fecal neutral sterol excretion *mg/kg of body wt per day*	12.8 ± 3.0[a]	12.7 ± 4.2[e]	19.5 ± 5.2[a,e]
Fecal bile acid excretion *mg/kg of body wt per day*	11.3 ± 2.4[b]	7.14 ± 3.53[b]	21.4 ± 9.9[b]
% of fecal bile acids conjugated with taurine	100%	None	None
% of fecal bile acids which are bacterially modified	None	34.3 ± 14.1[c]	57.7 ± 9.3[c]
% of fecal bile acids present as cholate and μ-muricholate	29.5 ± 9.6	27.3 ± 10.1	—
% of fecal bile acids which is β-muricholate	60.5 ± 9.1[d]	23.6 ± 8.6[d]	—

[a,b,c,d,e] Any values with the same superscript are significantly different at $P < 0.05$.
* See reference 3.

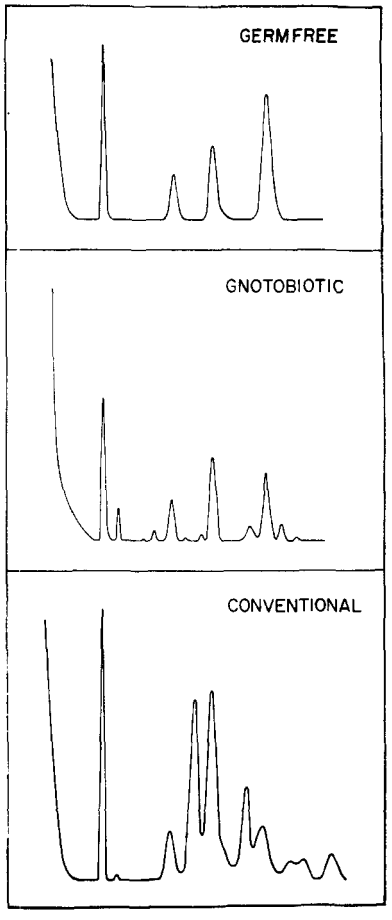

FIG. 1. GLC of fecal bile acids from germfree, gnotobiotic, and conventional rats. The first peak in each chromatogram is the internal standard 5α-cholestane. Instrument; Hewlett-Packard 402 gas chromatograph. Column; 2 m glass U-tube packed with 1% SE-30 on 80–120 mesh Gas Chrom Q. Isothermal 220°C. Nitrogen carrier gas 75 ml/min. Flame ionization detector. Bile acids analyzed as the trimethylsilyl ethers of the bile acid methyl esters (3).

conventional microflora. The rat study, however, in which the excretion was directly measured by chemical means did not support this contention. While the associated mice cleared cholesterol-26-^{14}C approximately 50% faster than did the germfree mice, the associated rats did not demonstrate increased fecal steroid excretion which accompanied the higher cholesterol catabolism in conventional rats. Since these two methods have been shown to agree in germfree and conventional rats, it seems likely that the differences seen in the present study are due to differences in response between the rat and mouse to the microorganisms under study. Association of rats with *C. perfringens* did not cause a measurable hydrogenation of the Δ^5 double bond of the cholesterol molecule, and the associated rats showed no increase in fecal neutral sterol excretion. The fecal bile acid excretion of germfree rats has been shown (3) to consist of α-muricholic, β-muricholic, and cholic acid taurine conjugates as well as an unidentified component with the chromatographic mobility of a monosubstituted cholanoic acid. In the clostridial-associated rats, the GLC pattern of bile acids obtained after purification via TLC indicated several additional bile acids in the feces (Fig. 1). The highly significant reduction in β-muricholic acid excretion (Table 2) suggests that in the clostridial-associated rat, the bacterial activity toward the substituents of the steroid nucleus is chiefly directed at β-muricholic acid. The nature of the changes wrought by *C. perfringens* on this molecule have not been investigated. A small amount of material (2–3% of the total bile acids) with a retention time of authentic deoxycholic acid (Fig. 1) was found in the excretions of these gnotobiotic rats, indicating some possible 7-dehydroxylation of cholic acid.

The most striking difference noted between the germfree and gnotobiotic groups was the absence of any taurine-conjugated bile acids in the rats associated with *C. perfringens*. This 100% deconjugation is much more extensive than reported by others (4, 7, 8) using *C. perfringens* and other microbial species as pure or mixed cultures in gnotobiotic rats.

Gustafsson, Bergström, Lindstedt, and Norman (7) reported that a rat diassociated with a mold and *C. perfringens* type E excreted bile acids which were partially deconjugated, but that the rate of fecal excretion of ^{14}C after oral administration of ^{14}C-cholic acid, was unchanged. The present data support the latter observation since the amount of cholic acid (and α-muricholic acid) excreted was not significantly different for rats in the two states. However, cholic acid (and α-muricholic acid) represents only a small (< 30%) and relatively constant contribution to the fecal bile acid excretion of our germfree and gnotobiotic groups (Table 2).

The gnotobiotic rats excreted significantly less total fecal bile acids than did the germfree rats. Although the mechanism of bile acid absorption has been extensively studied, which of the several mechanisms known to exist is quantitatively more important in vivo and what importance absorption in the lower gut plays in bile acid absorption, are not yet understood (16). These questions must be answered before the lowered excretion can be attributed to any specific microbial activity on the bile acids.

Conventional rats catabolize cholesterol at a much faster rate than do germfree rats (1) and excrete 90% more fecal bile acids (21 mg/kg per day vs. 11, Table 2) (3, 4). The conventional rat's fecal bile acids were totally deconjugated (no deconjugation in germfree bile acids), and 60% were additionally bacterially modified. The gnotobiotic rat's fecal acids were also totally deconjugated and 34% bacterially modified. However, this group's total deconjugation of the fecal bile acids was

accompanied by no increase in fecal bile acid excretion (7 mg by the gnotobiotic vs. 21 mg by the conventional). It thus seems improbable that the bacterial deconjugation of bile acids in the conventional rats was an important factor in increasing fecal excretion over the comparable germfree values.

Some difference(s), as yet unknown, existed between the gnotobiotic and conventional groups which caused an increase in fecal bile acid excretion of the conventional rats. Further studies of cholesterol and bile acid metabolism in vivo with pure and mixed cultures of bacteria are needed to lend insight into this difference and to clarify the general role of the intestinal microflora in the cholesterol–bile acid metabolism of the host.

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