Influence of cholestyramine on synthesis of cholesterol and bile acids in germfree rats

Bengt E. Gustafsson, Bo Angelin, Kurt Einarsson, and Jan-Åke Gustafsson

Department of Germfree Research[1] and Department of Chemistry,[2] Karolinska Institutet, 104 01 Stockholm 60, and Department of Medicine,[3] Serafimerlasarettet, 112 83 Stockholm, Sweden

Abstract The present investigation describes the influence of partial, pharmacologic interruption of bile acid enterohepatic circulation on cholesterol and bile acid synthesis in germfree rats. Seven rats received a basal, semisynthetic diet and five rats received the basal diet supplemented with 5% cholestyramine. After 6 weeks, feces were collected in one 3- and one 4-day pool for analysis of bile acids and cholesterol. When the sampling period was concluded, the rats were killed and the liver microsomal fractions were isolated. The activities of HMG CoA reductase and 7α-hydroxylase were determined. The main fecal bile acids in the untreated rats were cholic acid and β-muricholic acid. During cholestyramine treatment cholic acid increased from 4.4 ± 0.7 to 39.5 ± 5.6 mg/kg body weight/day and β-muricholic acid from 4.5 ± 0.6 to 7.7 ± 0.9 mg/kg body weight/day. Chenodeoxycholic acid became a major bile acid averaging 10.4 ± 1.6 mg/kg body weight/day. The total amount of bile acids increased about 6–7 times and the percentage of cholic acid increased from 49.4 ± 2.0 to 68.6 ± 1.1%. The 7α-hydroxylase activity increased 4–5 times. During cholestyramine treatment the fecal excretion of cholesterol was increased from 12.0 ± 1.4 to 68.0 ± 5.0 mg/kg body weight/day. The endogenous formation of cholesterol was increased 6 times and the HMG CoA reductase activity was increased about 20 times. In conclusion, germfree rats, like conventional rats, have the ability to increase the endogenous formation of bile acids and cholesterol during interruption of the enterohepatic circulation of bile acids, which is also reflected in a stimulation of the activities of the rate-determining enzymes.

Supplementary key words HMG CoA reductase · 7α-hydroxylase · cholic acid · chenodeoxycholic acid · β-muricholic acid

Germfree rats have a reduced formation of cholesterol compared to conventional rats (1) and have a low fecal excretion of bile acids as well as cholesterol (2–4). Feeding a high cholesterol diet to conventional and germfree rats inhibits the activity of the hepatic HMG CoA reductase (1 $MgCl_2$, 3 mM; mercaptothanol, 20 mM; EC 1.1.1.34), the rate-determining enzyme in cholesterol synthesis (5–7). Simultaneously the catabolism of cholesterol to bile acids is stimulated several-fold (6–8) and so is the activity of the 7α-hydroxylase, a rate-determining enzyme in bile acid biosynthesis (6, 9–11). Interruption of the enterohepatic circulation of bile acids by cholestyramine treatment causes an enhanced output of bile acids in conventional rats (12). This procedure also leads to increased activities of HMG CoA reductase (13) and 7α-hydroxylase (9, 10, 14, 15) in conventional rats. Whether germfree rats have the ability to increase their synthesis of bile acids and cholesterol in response to the interruption of bile acid circulation caused by cholestyramine is not known.

To avoid interaction between cholestyramine and the intestinal microflora and to obtain further information about the mechanisms behind the type of cholesterol and bile acid metabolism that is characteristic of germfree rats, we have administered cholestyramine to germfree rats and determined the fecal excretion of bile acids and cholesterol. In addition, the activities of HMG CoA reductase and 7α-hydroxylase were determined. Special attention was paid to the ratio between the synthesis of cholic acid and chenodeoxycholic acid, since previous investigations have given evidence for a certain compartmentation of cholesterol in rat liver (16–19). Cholic acid should be formed from newly synthesized cholesterol to a greater extent than chenodeoxycholic acid (17).

MATERIALS

Glutaryl-[3-^{14}C]HMG CoA (sp act 20 μCi/mg), [mevalonic-5-^{3}H(N)]DL-mevalonic acid (dibenzylethylene-diamine salt, sp act 25 μCi/mg), [4-^{14}C]-cholesterol (sp act 145 μCi/mg), [1,2-^{3}H]cholesterol

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; HMG, 3-hydroxy-3-methylglutaryl; cholic acid; 3α,7α,12α-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid; 3α,7α-dihydroxy-5β-cholanoic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholanoic acid.

[1] B. Gustafsson

[2] J-Å. Gustafsson.

[3] B. Angelin and K. Einarsson.

(sp act, 110 μCi/mg), and [24-^{14}C]cholic acid (sp act 138 μCi/mg) were purchased from New England Nuclear Corp., Boston, MA. The radioactive compounds were analyzed by thin-layer chromatography and were found to be more than 98% pure. The radioactively labeled HMG CoA was diluted with unlabeled material, obtained from P-L Biochemicals, Inc., Milwaukee, WI, to yield a specific radioactivity of 1.45 μCi/mg.

Unlabeled DL-mevalonic acid lactone, NADP, NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co., St. Louis, MO.

METHODS

Animals and preparations of microsomes

Germfree male rats of the Long-Evans strain weighing about 200–300 g were used. They were reared according to the technique of Gustafsson (20, 21) and were fed a standardized diet ad libitum (21). This diet is semisynthetic with 10% (w/w) arachis oil as source of fat and contains 0.004% (w/w) cholesterol. Seven animals were given the basal diet and five animals received the basal diet supplemented with 5% (w/w) of cholestyramine. After the animals had been maintained on the experimental diets for 6 weeks, feces were collected in one 3-day and one 4-day pool which were stored at −20°C until analyzed. A long feeding time was chosen because germfree rats have a very long transit time of intestinal contents and, after a change of diet, it takes several weeks until a new steady state in cholesterol and bile acid metabolism is reached (22).

When the sampling period was concluded, the rats were killed by a blow on the head immediately after they had been removed from the germfree isolators at 9 AM. The livers were taken out and chilled on ice. One part of the liver was homogenized in 9 volumes of a medium containing 0.3 M sucrose, 0.075 M nicotinamide, 0.002 M EDTA, and 0.02 M mercaptoethanol using a Potter-Elvehjem Teflon–glass homogenizer equipped with a loosely fitting pestle. The microsomes were prepared as described recently (1) and were suspended in the homogenizing medium in a volume corresponding to that of the 20,000 *g* supernatant fluid. The protein concentration was determined by the method of Lowry et al. (23). This microsomal fraction was used for the assay of HMG CoA reductase activity.

Another part of the liver was homogenized in 4 volumes of 0.25 M sucrose solution containing 0.001 M EDTA, and the isolated microsomal fraction was suspended in a 0.1 M phosphate buffer, pH 7.0, containing 0.028 M nicotinamide as described by Björkhem and Danielsson (19). The protein concentration (23) and cholesterol content (24) of the microsomes were determined. This microsomal fraction was used for the assay of 7α-hydroxylase activity.

Assay of HMG CoA reductase activity

The assay system has recently been described (1) and was similar to that developed by Shefer et al. (13). Each incubation flask contained, in a volume of 0.85 ml, 0.2 ml of microsomal fraction; phosphate buffer, pH 7.2, 100 mM; NADP, 3 mM; glucose-6-phosphate, 10 mM; glucose-6-phosphate dehydrogenase, 5 enzyme units; mevalonate: NADP oxidoreductase, [3-^{14}C]HMG CoA, 0.2 mM. The incubation was conducted for 15 min at 37°C and was stopped by the addition of 0.1 ml of 5 M HCl. Tritium-labeled and unlabeled mevalonic acid were added as internal standards and the incubation mixture was further shaken for 30 min at 37°C. The mixture was extracted and subjected to TLC, and the radioactivity of mevalonic acid lactone was determined. The recovery of tritium-labeled internal standard was about 60%.

Assay of 7α-hydroxylase activity

The assay system has recently been described by Björkhem and Danielsson (19). To a mixture of 3 ml of microsomal fraction, 2 ml of buffer, and 5 μmol of NADPH, 10 μg of [4-^{14}C]cholesterol was added as a suspension in buffer containing 3 mg of Tween 80. The incubation was carried out for 15 min at 37°C and was terminated by the addition of 20 volumes of $CHCl_3$–CH_3OH 2:1 (v/v). The incubation extract was subjected to thin-layer chromatography and the products were analyzed as described previously (25). The activity of the 7α-hydroxylase was calculated by multiplying the percentage conversion of cholesterol to 5-cholestene-3β,7α-diol and the microsomal content of cholesterol. The enzyme activity was then expressed as pmol/mg protein per min. The radioactive substrate is assumed to be in equilibrium with the endogenous substrate pool to the same extent in microsomes from untreated rats as in microsomes from cholestyramine-treated rats. This assumption is considered to be valid since the cholesterol content of the liver microsomes from the treated rats was not different from that of the untreated rats (19).

Analysis of fecal bile acids and neutral steroids

Feces were disintegrated in water and refluxed in 70% (v/v) aqueous ethanol for 2 hr. After filtration, the residue was refluxed in $CHCl_3$–CH_3OH 1:1 (v/v) for 1 hr and the extracts were combined.

An aliquot (1/10 or 1/5) of the combined extracts

TABLE 1. Influence of cholestyramine on activities of HMG CoA reductase and 7α-hydroxylase in liver microsomes from germfree rats

Rats	HMG CoA Reductase	7α-Hydroxylase
	pmol/mg protein/min	
Untreated rats[a]	34.0 ± 4.4	12.4 ± 2.6
Cholestyramine-treated rats[a]	712 ± 124	54.6 ± 7.3
Significance[b] of differences	$P < 0.001$	$P < 0.001$

[a] Values are the means ± SEM from seven untreated and five cholestyramine-treated rats.
[b] Students *t* test.

was hydrolyzed with 1 M KOH in 50% aqueous ethanol for 10 hr at 110°C. The saponification mixture was first extracted with petroleum ether; the petroleum ether phase was discarded. The saponification mixture was then acidified and extracted with ethyl acetate. The ethyl acetate extract was washed with water until neutral and the solvent was evaporated. [24-^{14}C]Cholic acid was used as an internal standard for the correction of losses. The residue of the ethyl acetate extract was methylated with diazomethane. It was then trimethyl silylated and analyzed by gas–liquid chromatography using 1% HiEff 8 BP as the stationary phase. The column temperature was 225°C. External bile acid standards were used for the quantitation.

Another aliquot of the combined extracts (1/10 to 1/5) was hydrolyzed with 1 M KOH in 50% aqueous ethanol for 1 hr. The saponification mixture was extracted with petroleum ether. The petroleum ether phase was evaporated. [1,2-^{3}H]Cholesterol was used as an internal standard in the extraction procedure to correct for losses. The residue of the extract was silylated and analyzed by GLC using 1% HiEff 8 BP as the stationary phase.

RESULTS

HMG CoA reductase activity

As seen in **Table 1,** the amount of mevalonic acid lactone formed in untreated rats was 34.0 ± 4.4 pmol/mg protein per min. In the cholestyramine-treated rats, the formation of mevalonic acid lactone was 712 ± 124 pmol/mg protein per min, i.e., about 20 times the reaction rate found in untreated rats.

7α-Hydroxylase activity

In the untreated rats, the 7α-hydroxylase activity averaged 12.4 ± 2.6 pmol/mg protein per min (Table 1). In the cholestyramine-treated animals, the hydroxylase activity was increased 4–5 times compared with the untreated rats.

Fecal bile acids

The major bile acids in feces from untreated rats were cholic acid and β-muricholic acid (**Table 2**). Only trace amounts of chenodeoxycholic acid were found. Table 2 summarizes the results of treatment with cholestyramine. The amount of cholic acid increased about 9 times, and the excretion of β-muricholic acid was almost doubled. Chenodeoxycholic acid became a major bile acid. The total amount of bile acids excreted increased 6–7 times and the ratio between cholic acid and β-muricholic acid plus chenodeoxycholic acid increased from about 1.0 to 2.2.

Fecal excretion of cholesterol

The amount of cholesterol excreted per day in the untreated germfree rats averaged 12.0 ± 1.4 mg/kg body weight (**Table 3**). In the cholestyramine-treated rats, the excretion of cholesterol was increased 5–6 times to 68.0 ± 5.0 mg/kg body weight.

Endogenous formation of cholesterol

The daily synthesis of cholesterol could be calculated as the sum of fecal excretion of bile acids and cholesterol minus dietary intake of cholesterol. From the daily food consumption and from the diet composition, the intake of cholesterol could be calculated to about 1 mg per day. Table 3 summarizes the results. The synthesis of cholesterol has been expressed as mg/kg body weight per day. The formation of cholesterol was stimulated about 6 times during cholestyramine treatment.

DISCUSSION

The fecal bile acids of the germfree rat largely consist of end products of bile acid metabolism. The major bile acids are cholic acid and β-muricholic acid (2–4, 7). Cholic acid is primarily formed in the liver and is not further metabolized. β-Muricholic acid is an end product of chenodeoxycholic acid. The latter bile acid is formed from cholesterol in the liver and is 6β-hydroxylated to α-muricholic acid which is converted to β-muricholic acid (for a review, see ref. 26). With an efficient reabsorption and frequent recyclings of chenodeoxycholic acid, it is quantitatively transformed to β-muricholic acid. During cholestyramine treatment, the enterohepatic circulation of bile acids is partly broken. As a consequence of this, chenodeoxycholic acid now becomes a major bile acid in feces.

No attempt was made in the present investigation to quantitate sulfated bile acids in feces from male germfree rats. Even though bile acid sulfates con-

TABLE 2. Influence of cholestyramine on fecal excretion of bile acids in germfree rats

Rats	Cholic Acid	β-Muricholic Acid	Chenodeoxy-cholic Acid	β-Muricholic Acid + Chenodeoxy-cholic Acid	Total Bile Acids	% Cholic Acid of Total Bile Acids
			mg/kg b.w./d			
Untreated rats[a]	4.4 ± 0.7	4.5 ± 0.6	Trace	4.5 ± 0.6	8.9 ± 1.3	49.4 ± 2.0
Cholestyramine-treated rats[a]	39.5 ± 5.6	7.7 ± 0.9	10.4 ± 1.6	18.1 ± 2.4	57.6 ± 7.9	68.6 ± 1.1
Significance[b] of difference	$P < 0.001$	$P < 0.01$		$P < 0.001$	$P < 0.001$	$P < 0.001$

[a] Values are the means ± SEM of fecal pools collected from seven untreated and five cholestyramine-treated rats.
[b] Student's *t* test.
b.w. = body weight.

stitute a quantitatively important part of large intestinal bile acids in germfree female rats, they seem to be of negligible quantitative significance in large intestine from germfree male rats (27). It is therefore reasonable to assume that bile acid sulfates should form a very minor part of the total bile acids excreted in feces from germfree male rats.

Cholestyramine treatment led to a several-fold increase of the fecal excretion of bile acids. This is in accordance with an earlier report on conventional rats (12) and stresses the fact that the influence of cholestyramine on bile acid metabolism is not mediated via an effect on the intestinal microflora. Cholic acid synthesis was stimulated to a larger extent than that of chenodeoxycholic acid. In contrast, Huff, Gilfillan, and Hunt (12) found that the excretion of cholic acid in conventional rats was almost unchanged, whereas the excretion of dihydroxy bile acids was stimulated several times after cholestyramine feeding. Conventional rats with a bile fistula, however, excrete more cholic acid than chenodeoxycholic acid (28). In accordance with previous results from conventional rats (9, 10, 14, 15), the 7α-hydroxylase activity was stimulated by cholestyramine treatment. The increase of the enzyme activity was of the same order as the increase of the bile acid synthesis.

Cholestyramine treatment led to a 20-fold increase of the HMG CoA reductase activity. However, part of the endogenous formation of cholesterol occurs extrahepatically, predominantly in the intestinal mucosa (29), and the present study gives no information on whether the synthesis of intestinal cholesterol was stimulated to the same extent as that of hepatic cholesterol.

It was considered unnecessary to monitor fecal excretion of cholesterol by β-sitosterol since the absence of the intestinal microflora in germfree rats results in the excretion of unmetabolized cholesterol in the feces (30). The neutral steroid excretion was higher in the cholestyramine-treated rats compared with the untreated rats. This increased neutral steroid excretion could depend on several factors: *1*) an increased biliary secretion of cholesterol, *2*) an increased secretion of newly synthesized cholesterol from the intestinal wall to the intestinal lumen, *3*) a less efficient absorption of cholesterol from the intestine, or *4*) a combination of two or three of these possibilities. Germfree rats have a more efficient absorption of dietary cholesterol compared with conventional rats (31), which could probably be ascribed to the higher concentration of bile acids in the small intestine (32). During cholestyramine treatment, bile acids are removed from the intestine at a higher rate and the concentration of bile acids in the intestine probably diminishes, which might influence cholesterol absorption. However, a more extensive malabsorption of cholesterol in the cholestyramine-treated rats seems less likely since these rats did not lose weight when compared with the untreated rats.

As mentioned in the introduction, several lines of evidence indicate that a certain compartmentation of cholesterol exists in the rat liver (16–19). Cholic acid has been reported to be formed from newly synthesized cholesterol to a larger extent than chenodeoxycholic acid (17). In a previous investigation, we showed that an increased dietary input of cholesterol to the liver of germfree rats, which results in an inhibition of cholesterol synthesis and an increased bile

TABLE 3. Influence of cholestyramine on fecal excretion and endogenous formation of cholesterol

Rats	Fecal Excretion of Cholesterol	Endogenous Formation of Cholesterol
	mg/kg b.w./d	
Untreated rats[a]	12.0 ± 1.4	20.5 ± 2.3
Cholestyramine-treated rats[a]	68.0 ± 5.0	123.4 ± 7.9
Significance[b] of differences	$P < 0.001$	$P < 0.001$

[a] Endogenous formation of cholesterol was calculated as fecal excretion of cholesterol plus bile acids minus dietary intake of cholesterol. The values are the means ± SEM of seven untreated and five cholestyramine-treated rats.
[b] Student's *t* test.

acid formation, stimulated the synthesis of chenodeoxycholic acid to a larger extent than that of cholic acid (7). The present study shows that the synthesis of cholic acid is stimulated more than that of chenodeoxycholic acid during cholestyramine treatment when both cholesterol and bile acid formation are increased.

In conclusion, germfree rats, like conventional rats, have the ability to increase the endogenous formation of bile acids and cholesterol during interruption of the enterohepatic circulation of bile acids which is also reflected in a stimulation of the activities of the rate-determining enzymes. In a previous investigation, we showed that germfree rats have compensatory mechanisms that are activated when the animals are given increased amounts of cholesterol, i.e., a reduced HMG CoA reductase activity and an increased formation of bile acids (7). Taken together, these facts indicate that the higher cholesterol concentration in liver from germfree rats than in liver from conventional rats (11, 33) may not be explained solely on the basis of an increased intestinal absorption of cholesterol in germfree animals. It is probable that the large pool of bile acids that is present in the enterohepatic circulation of germfree rats (11, 32) leads to a decreased 7α-hydroxylation of cholesterol (34) and consequently to a less efficient conversion of cholesterol to bile acids. This would tend to counteract the compensatory mechanism protecting the rat from cholesterol accumulation and may be a contributing factor to the higher hepatic cholesterol levels in germfree than in conventional rats.

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