Apparent lack of conversion of sitosterol into C_{24}-bile acids in humans

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Abstract The metabolic fate of intravenously administered [4-^{14}C]sitosterol was studied in two healthy subjects. In marked contrast to the results of a previous investigation with [22,23-^{3}H]sitosterol, no detectable labeled C_{24}-bile acid products appeared in bile. The first and rate-limiting step in the conversion of cholesterol into bile acids is catalyzed by the liver microsomal cholesterol 7α-hydroxylase. When incubated with human liver microsomes, no detectable 7α-hydroxylation of sitosterol could be demonstrated. This was the case also when using liver microsomes from two subjects treated with cholestyramine, in which case the rate of 7α-hydroxylation of cholesterol was increased three- to sixfold. In order to bypass the rate-limiting step, the metabolic fate of ^{3}H-labeled 7α-hydroxysitosterol was studied in two volunteers. In this case there was a significant conversion into acid products in bile (18–32% excreted in bile during the first 17 h). Although part of the labeled products had chromatographic properties similar to those of cholic acid and chenodeoxycholic acid, further analysis showed that none of the products was identical to chenodeoxycholic acid and only traces at the most could be identical to cholic acid. The results suggest that healthy human subjects, in similarity with other mammalian species studied, have little or no capacity to convert sitosterol into the normal C_{24}-bile acids. —**Boberg, K. M., K. Einarsson, and I. Björkhem.** Apparent lack of conversion of sitosterol into C_{24}-bile acids in humans. *J. Lipid Res.* 1990. **31:** 1083–1088.

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The plant sterol sitosterol is a normal constituent of the human diet (1). It is absorbed to a limited extent in the intestine, circulates in plasma in low concentrations, and is excreted in bile (1). In theory, the presence of an ethyl group at C_{24} should prevent or at least obstruct conversion of sitosterol into bile acids by the same mechanism as that utilized for conversion of cholesterol into C_{24}-bile acids. In accordance with this, previous attempts to demonstrate conversion of sitosterol into normal C_{24}-bile acids in rats (2) and monkeys (3) have failed. We recently showed that there is some conversion of labeled sitosterol into unusual C_{21}-bile acids in rats (4). The mechanism of the latter conversion must, however, be different from that involved in the conversion of cholesterol into C_{24}-bile acids.

Surprisingly, an efficient formation of cholic and chenodeoxycholic acid from intravenously administered [22,23-^{3}H]sitosterol has been reported in humans (5). The rate of this conversion appeared to be about the same as that for conversion of cholesterol into bile acids.

The first and rate-limiting step in the conversion of cholesterol into bile acids is catalyzed by the cholesterol 7α-hydroxylase. If sitosterol is converted into bile acids by the same mechanism as that in the biosynthesis of bile acids from cholesterol, and the overall rate of conversion of the two steroids is about the same as suggested from the above report, sitosterol should be 7α-hydroxylated by human liver microsomes at about the same rate as cholesterol. To our knowledge, 7α-hydroxylation of sitosterol has not previously been studied in human liver microsomes. The rate of 7α-hydroxylation of sitosterol by crude rat liver microsomes is almost undetectable (6, 7).

In the present work we have reinvestigated the possibility of a conversion of sitosterol into normal bile acids in healthy human subjects. We have also studied the possibility that sitosterol can be 7α-hydroxylated in human liver microsomes.

MATERIALS AND METHODS

Labeled compounds

[4-^{14}C]Sitosterol (56 mCi/mmol) and [4-^{14}C]cholesterol (55 mCi/mmol) were obtained from Amersham International (Amersham, UK). The [4-^{14}C]sitosterol had the 20β-configuration and the ethyl group was located in the 24α-position (the naturally occurring isomer). Both the labeled cholesterol and sitosterol were pure as judged by

Abbreviations: HPLC, high pressure liquid chromatography; TLC, thin-layer chromatography; DTT, dithiothreitol.

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HPLC and gas-liquid chromatography as trimethylsilyl ether. Under the chromatographic conditions used, the 20α- and 20β-isomers of sitosterol were separated. [7β-^{3}H]7α-Hydroxysitosterol (20 mCi/mmol) was synthesized by oxidation of sitosterol acetate with CrO_3 to yield 7-oxo-sitosterol acetate (8), followed by reduction with tritium-labeled sodium borohydride. A mixture of the 7α- and 7β-hydroxy isomers was obtained. The 7α-isomer was isolated by preparative thin-layer chromatography (TLC) using toluene–ethyl acetate 3:7 (v/v) as mobile phase. The material obtained was pure as judged by TLC and gas-liquid chromatography of the trimethylsilyl (TMS) ether derivative. The labeled sterols were purified by high pressure liquid chromatography (HPLC), cf. below, immediately before use.

Administration of sterols to human subjects and collection of bile

[4-^{14}C]Sitosterol was dissolved in 1 ml of ethanol. The solution was passed through a Millex 0.22-μm filter (Millipore Co., Bedford, MA), mixed with 20 ml of 0.9% (w/v) NaCl, and slowly administered intravenously with a simultaneous infusion of 0.9% NaCl to two healthy persons. The injected doses were 16.6×10^6 cpm (60 μg) (subject I) and 8.9×10^6 cpm (31 μg) (subject II). Five months later the same persons received [7β-^{3}H]7α-hydroxysitosterol, prepared and injected as described above. The injected doses were 21.3×10^6 cpm (210 μg) (subject I) and 17.4×10^6 cpm (170 μg) (subject II). After an additional 8 months, subject II received [4-^{14}C]cholesterol, prepared and injected as above. The dose was 23×10^6 cpm (80 μg) in this case.

Bile was collected through a duodenal tube 17, 24, and 68 h after injection of [4-^{14}C]sitosterol and 17 and 42 h after injection of [7β-^{3}H]7α-hydroxysitosterol and [4-^{14}C]cholesterol. The subjects had been fasting for a minimum of 4 h, and Ceruletid (Takus, Farmitalia) (0.3 μg/kg body weight) was given as an intramuscular injection to stimulate contraction of the gallbladder.

The recovery of radioactivity in bile was calculated by measuring the amount of cholic acid in the different bile samples and multiplying the radioactivity in the sample by the ratio between the cholic acid pool size and the amount of cholic acid in the sample.

The pool of cholic acid in subject II (male) was 1373 mg, as measured by the Lindstedt technique (9). It was assumed that subject I (female) had a normal pool size (760 mg) (10).

The studies were approved by the ethical committee as well as by the isotope committee at Huddinge Hospital.

Extraction procedures and chromatography

Aliquots of the bile were hydrolyzed in 4 M KOH at 110°C for 18 h. Neutral sterols were extracted with n-hexane. After acidification of the water phase to pH 1.0 with hydrochloric acid, bile acids were extracted with ethyl ether. The ether extract was washed to neutrality with water, blown to dryness under the stream of N_2, and redissolved in chloroform–methanol 2:1 (v/v). Aliquots of the hexane and ether extracts were analyzed by HPLC, as described previously (11). A Supelcosil LC-18 column (250 × 4.6 mm, 5 μm particle size, from Supelco Inc., Bellefonte, PA) was used at a flow rate of 1 ml/min. The chromatograph was equipped with a constant-flow pump (ConstaMetric III, Laboratory Data Control, Milton Roy Co., St. Petersburg, FL) and a differential refractometer (R-401, Waters Assoc., Milford, MA). Bile acids were separated with methanol–water 70:30 (v/v); the water was adjusted to pH 3.0 (with phosphoric acid) as the mobile phase. Typical retention times (min) were: cholic acid, 24; chenodeoxycholic acid, 50; deoxycholic acid, 56; and lithocholic acid, 120. Sterols were eluted with methanol–water 95:5 (v/v) as the mobile phase. Retention times (min) were: cholesterol, 41, and sitosterol, 52. Fractions of 1 ml were collected, 10 ml of Packard Insta Gel II scintillation liquid (Packard Instrument Co., Downers Grove, IL) was added, and radioactivity was assayed in a Packard Tri-Carb liquid scintillation analyzer, model 1500. Methylated aliquots of the bile acid fraction were analyzed by TLC on plates coated with Silica Gel H (Merck, Darmstadt, FRG), using trimethylpentane–ethyl acetate–acetic acid 10:10:2 (by volume) as solvent. The plates were assayed for radioactivity by autoradiography and by scraping off suitable zones which were eluted with methanol and counted as described above.

Preparation of human liver microsomes and assay of cholesterol 7α-hydroxylase activity

Liver biopsies were obtained from one female with adenomyoma of the gallbladder (age 39 years), two untreated females with gallstones in connection with cholecystectomy (age 58 and 52 years), one female and one male who were operated on due to Crohn's disease (age 60 and 40 years), and two females with gallstones (age 33 and 41 years) who were treated with cholestyramine (Questran^R, Bristol) in a dose of 8 g twice daily for 2–3 weeks preoperatively. The patients were hospitalized in the surgical ward 1–2 days before operation. They were given the regular diet. The cholecystectomy was performed between 8 and 9 AM. After opening of the abdomen, a 2–3 g liver biopsy was cut out from the left liver lobe. A small specimen was sent for histological examination. The rest of the biopsy was immediately placed in ice-cold homogenizing medium and transported to the laboratory within 10 min.

The liver homogenate (10% w/v) was prepared in nine volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaCl, 0.3 M sucrose, 10 mM EDTA, and 10 mM DTT. The homogenate was then centrifuged at 20,000 *g* for 15 min at 4°C. The supernatant was centrifuged at

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100,000 *g* for 60 min. The microsomal fraction obtained was suspended in the homogenizing medium lacking DTT and recentrifuged at 100,000 *g* for 60 min. The resulting microsomal fraction was suspended in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, to give a final concentration of 10% (w/v). The microsomal content was determined by the method of Lowry et al. (12). The concentration of free cholesterol was determined by isotope dilution–mass spectrometry as described previously (13) with the modification described in ref. 14.

The standard assay system consisted of 0.5 ml of the microsomal preparation corresponding to about 0.5–1 mg of protein, 0.5 ml 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM NADPH to a total volume of 1 ml (15). In addition, 100 μg of Tween 80 with or without 50 μg of unlabeled sitosterol was added. It has been shown that this amount of Tween 80 in itself has no effect on the cholesterol 7α-hydroxylase activity in human liver microsomes (16). The enzyme assay was carried out in duplicate for 15 min at 37°C in a waterbath. The reaction was stopped by the addition of 10 ml of chloroform–methanol 2:1 (v/v). Deuterium-labeled 7α-hydroxycholesterol, 150 pmol, dissolved in 25 μl of benezene, was added as internal standard. The chloroform phase was removed and evaporated to dryness under N_2 at about 40°C. The sterol fraction was dissolved in chloroform–methanol 2:1 (v/v) and subjected to thin-layer chromatography using toluene–ethyl acetate 30:70 (v/v) as developing solvent. The bands corresponding to 7α-hydroxycholesterol and 7α-hydroxysitosterol were eluted with methanol, evaporated to dryness under N_2, and converted into trimethylsilyl ether before analysis by gas–liquid chromatography–mass spectrometry as described previously (15). Each set of experiments also included two assays in which the mass of 7α-hydroxycholesterol was determined in the incubation mixture extracted at zero-time.

In the mass spectrometric analysis the ions at *m/z* 456 (M–90 ion in the mass spectrum of derivative of unlabeled 7α-hydroxycholesterol), *m/z* 458 (M–90 ion in the mass spectrum of derivative of $^2H_2$-labeled 7α-hydroxycholesterol) and *m/z* 484 (M–90 in the mass spectrum of derivative of unlabeled 7α-hydroxysitosterol) were followed. The derivative of 7α-hydroxysitosterol should thus be detected in the tracing of the ion at *m/z* 484 with a retention time slightly longer than that of the derivative of 7α-hydroxycholesterol.

RESULTS

In vivo experiments with [4-^{14}C]sitosterol and [4-^{14}C]cholesterol

Analysis of the bile sampled 16–17 h after the injection of [4-^{14}C]sitosterol showed that 5.3% and 8.9%, respectively, of the injected dose was recovered in the bile of the two subjects. Smaller amounts of radioactivity were found in the bile after 43 and 68 h (**Table 1**).

Analysis by HPLC of the hexane and ether extracts showed that the major part (>90%) of the radioactivity in all the samples was present as unmetabolized sitosterol. No radioactivity was present in the cholesterol fraction. Only small amounts (less than 10%) were more polar than sitosterol. The majority of these polar products eluted around 10 min with methanol–water 95:5 (v/v) as the mobile phase. The amount of these polar products was very low on a weight basis, and the specific radioactivity

TABLE 1. Recovery of radioactivity in bile after administration of [4-^{14}C]sitosterol and [4-^{14}C]cholesterol[a]

Time after Administration	Recovery of ^{14}C from	
	[4-^{14}C]Sitosterol	[4-^{14}C]Cholesterol
Subject I 17 h	5.3% (>90% sitosterol <3% C_{24}-bile acids)	
Subject I 24 h	3.5% (>90% sitosterol <3% C_{24}-bile acids)	
Subject I 68 h	<3%[b] (>90% sitosterol <3% C_{24}-bile acids	
Subject II 16 h	8.9% (>90% sitosterol <3% C_{24}-bile acids)	3.2% (34% cholesterol; 66% C_{24}-bile acids
Subject II 43 h	4.6% (>90% sitosterol <3% bile acids)	7.5% (4% cholesterol; 96% C_{24}-bile acids)

[a]The total recovery in bile was calculated with use of the radioactivity and cholic acid content in each bile sample and the pool size of cholic acid. In subject II, the pool size of cholic acid had been measured to be 1370 mg. In subject I, normal pool size (760 mg) was assumed.

[b]Analysis of the cholic acid failed in this case and only a maximum value can be given.

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could not be determined. Due to lack of sufficient amounts, no attempts were made to identify this material. Most important, no radioactivity at all could be detected in the regions of the normal bile acids (cholic, chenodeoxycholic, and deoxycholic acids) in a system using methanol–water (pH 3.0) 70:30 (v/v) as eluent. No other radioactivity peaks appeared within the first 120 min (approximate retention time of lithocholic acid) of the chromatography. Analysis of a methylated ether extract of bile from subject I by TLC followed by autoradiography of the plate showed trace amounts of radioactivity with a retention time between that of chenodeoxycholic acid and lithocholic acid. This finding was not reproducible in bile from subject II, and could possibly represent a C_{29}-bile acid from sitosterol. Under the conditions used, it was thus not possible to detect any metabolites from sitosterol with properties of the normal bile acids. Considering the detection limits and the amount of radioactivity recovered, it was calculated that less than 3% of the excreted radioactivity could be present as normal bile acids.

This would mean that less than 0.3% of the administered [4-^{14}C]sitosterol could have been converted into normal bile acids in any of the two subjects.

For reasons of comparison, [4-^{14}C]cholesterol was administered to subject II (whose cholic acid pool size was exactly known). After 16 h, 3.2% of the administered radioactivity was present in bile, 66% of which was C_{24}-bile acids. After 43 h, 7.5% of the radioactivity was present in bile, 96% of which was C_{24}-bile acids.

In vitro experiments with sitosterol

Hydroxylation in 7α-position is the first and rate-limiting step in the conversion of cholesterol into bile acids, and the possibility was therefore studied that sitosterol could be 7α-hydroxylated in human liver microsomes. The results of these experiments are summarized in **Table 2.** Addition of sitosterol to the microsomes was found to depress the 7α-hydroxylase activity towards cholesterol by 9–46% in the incubations with microsomes from untreated patients and patients with Crohn's disease. In the incubations with the stimulated microsomes from the two gallstone patients treated with cholestyramine, the depression caused by the added sitosterol was 60% and 56%, respectively. The depressive effect of the added sitosterol is in accord with results of previous work with rat liver microsomes (17, 18). The reason for the higher depression of sitosterol in the incubations with liver microsomes from cholestyramine-treated patients is most likely the fact that the cholesterol 7α-hydroxylating system is less saturated with substrate cholesterol under such conditions (16).

It was not possible to detect any conversion of the added sitosterol into 7α-hydroxysitosterol under the conditions used. The detection limit of the assay should be about 0.2–0.3 pmol/min per mg protein. This means that if there was a 7α-hydroxylation of sitosterol in the stimulated microsomes, the rate of this conversion must be less than 2% of that of cholesterol.

In vivo experiment with [7β-^{3}H]7α-hydroxysitosterol

In view of the finding that there was no significant conversion of [4-^{14}C]sitosterol into normal bile acids in vivo and no significant 7α-hydroxylation of sitosterol in vitro, it was of interest to study the metabolic fate of the expected product in the in vitro experiment, 7α-hydroxysitosterol. By using this compound, the rate-limiting enzyme is thus bypassed. After administration of [7β-^{3}H]7α-hydroxysitosterol to the two healthy volunteers, 18 and 32%, respectively, of the dose was excreted in the bile after 17 h. At 42 h somewhat less radioactivity was recovered in one of the subjects and about the same amount in the other. In contrast to the experiments with [4-^{14}C]sitosterol, almost the entire amount (99%) of the radioactivity from [7β-^{3}H]7α-hydroxysitosterol appeared as acidic products.

TABLE 2. 7α-Hydroxylation of endogenous cholesterol and exogenous sitosterol in human liver microsomes

Source of Liver Microsomes	7α-Hydroxylation of Cholesterol		7α-Hydroxylation of Added Sitosterol
	Tween Added	Tween and Sitosterol Added	
	pmol/min/mg		
Patient with adenomyoma of the gallbladder	16.0	10.5	<0.3
Gallstone patient I	3.4	1.9	<0.3
Gallstone patient II		3.6	<0.3
Crohn patient I	3.1	1.7	<0.3
Crohn patient II	15.3	14.0	<0.3
Cholestyramine-treated patient I	57.7	23.3	<0.3
Cholestyramine-treated patient II	34	15	<0.3

For experimental details, see Materials and Methods.

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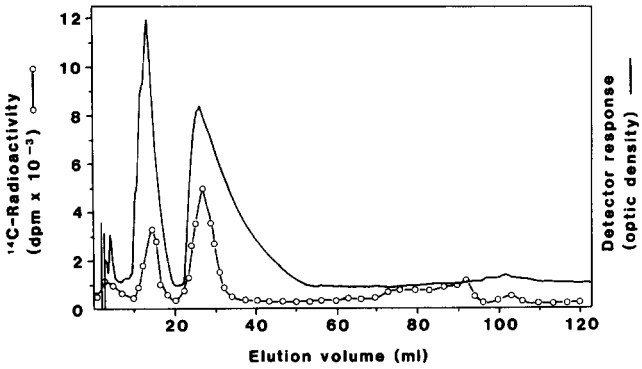

Fig. 1. Reversed phase HPLC of hydrolyzed bile sample from a subject to whom ^{3}H-labeled 7α-hydroxysitosterol had been administered 17 h previously; (○—○) radioactivity; (—) absorbance.

Fig. 1 shows the distribution of radioactivity after HPLC analysis of an aliquot of the ether extract of bile from one of the subjects (I) injected with [7β-^{3}H]7α-hydroxysitosterol. Two major radioactivity peaks were present, appearing with about the same retention times as those of cholic acid and chenodeoxycholic acid, respectively. Minor peaks were eluted at about 75 and 90 min. The pattern was essentially identical in bile from subject II. When methylated aliquots of the ether extracts were analyzed by TLC, no radioactivity had retention time as chenodeoxycholic acid, while about 15% co-chromatographed with cholic acid. Attempts to crystallize the cholic acid fraction to constant specific activity failed. After four crystallizations, the specific activity had decreased to less than 25% of the original level. This means that less than 4% of the total radioactivity could be identical with cholic acid.

DISCUSSION

The present finding that there was no significant conversion of intravenously administered [4-^{14}C]sitosterol into C_{24}-bile acids in humans is in marked contrast to the results of the previous early study on the metabolic fate of [22,23-^{3}H]sitosterol (5). No simple explanation can be found for the different results. In principle a 4-^{14}C-label should be more stable than a 22,23-^{3}H-label both from a metabolic and a chemical point of view. It was reported in the previous study with [22,23-^{3}H]sitosterol that the labeled bile acids obtained had a specific radioactivity approximately the same as that of the reisolated sitosterol. This is surprising in view of the fact that a C_{24}-bile acid formed from [22,23-^{3}H]sitosterol should lose all the ^{3}H in 23-position during the alkaline saponification step. The specific radioactivity of the bile acid formed should thus be expected to be about half or less of that of the precursor. If the loss of ^{3}H from the bile acids is taken into account, the rate of formation of bile acids from [22,23-^{3}H]sitosterol would, in fact, be twice that calculated by the authors, which would mean that sitosterol would be converted into bile acids even more efficiently than cholesterol.

Our finding that there is no significant conversion of [4-^{14}C]sitosterol into C_{24}-bile acids is in agreement with previous in vivo studies on monkeys (3) and rats (2). In a recent study we found that rats, in particular female Wistar rats, can convert sitosterol into highly polar trihydroxylated C_{21}-bile acids (4). No such significant conversion could be detected in the present study. Small amounts of radioactivity were recovered in a chromatographic fraction more polar than sitosterol but less polar than lithocholic acid. This fraction weighed very little, reflecting the very small pool of sitosterol and sitosterol metabolites. The pool of sitosterol in humans is normally less than 1% of that of cholesterol (1) and this must be taken into account when comparing the percentage conversion of the two compounds into bile acids.

Since the conversion into product with chromatographic properties as bile acids was very low in spite of the vanishingly small pool of sitosterol, it is evident that the enzymatic capacity for the conversion is extremely low.

If sitosterol is converted into bile acids as effectively as cholesterol, and by the same mechanism, the rate of 7α-hydroxylation of sitosterol could be expected to be similar to that of cholesterol. In accordance with the in vivo results, we found no significant conversion of sitosterol into 7α-hydroxysitosterol by human liver microsomes. This result was also obtained when the cholesterol 7α-hydroxylating system was stimulated by preoperative treatment of the patient with cholestyramine. In accordance with previous results (17, 18), the addition of sitosterol to the crude microsomal fraction had an inhibitory effect on the 7α-hydroxylation of cholesterol. In a recent study by Shefer et al. (18) it was shown that 7α-hydroxylation of sitosterol was barely detectable in crude rat liver microsomes, but could be detected after removal of endogenous cholesterol by acetone. We have found the rate of 7α-hydroxylation of sitosterol to be about 2% of that of cholesterol both in crude and in acetone-treated rat liver microsomes (17).

We cannot exclude the possibility that acetone treatment of the human liver microsomes used here would have resulted in a detectable 7α-hydroxylation of sitosterol. Lack of sufficient material prevented such experiments. In any case the results presented here show that even if a 7α-hydroxylation of sitosterol exists, it must be of very little importance from a quantitative point of view.

It was considered to be of interest to see whether the human liver could convert sitosterol into the normal C_{24}-bile acids after the critical 7α-hydroxylating step had been bypassed. Administration of labeled 7α-hydroxysitosterol

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resulted in a significant conversion into acid products in bile. The major part of these acid products were, however, not identical to the normal C_{24}-bile acids. Whether or not these products have a completely or incompletely oxidized side-chain remains to be established. Due to the relatively small degree of conversion no extensive attempts to identify the product could be made.

In theory, a conversion of sitosterol and other plant sterols into C_{24}-bile acids could occur to yield cholesterol which might then be converted into bile acids by the usual mechanism. A dealkylation of sitosterol to yield desmosterol has been reported in tobacco hornworms (19) and Florida land crabs (20), but never in mammals. No significant dealkylation of sitosterol could be demonstrated in the present study since there was no recovery of ^{14}C from [4-^{14}C]sitosterol either in cholesterol or C_{24}-bile acids. It should be pointed out that the subjects studied here were healthy with assumed normal pools of sitosterol. In patients with sitosterolemia, the pool of plant steroids might be at least one order of magnitude higher than normal (21) and the possibility cannot be completely excluded that such conditions may lead to induction of enzyme(s) not normally utilized.

To summarize, the present study shows that healthy human subjects, in similarity with the other mammalian species hitherto studied, have little or no capacity to convert sitosterol into the normal C_{24}-bile acids.

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