Changes in biliary and fecal bile acids in mice after treatments with diosgenin and β-sitosterol

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Abstract Diosgenin and β-sitosterol (1% in diet) were administered to CRJ:CD-1 male mice for 15 days, in order to examine the changes in bile acid metabolism. There were some differences between diosgenin and β-sitosterol in their effects on diet intake, liver weight, and plasma cholesterol level. However, both phytosterols caused no statistically significant changes in body weight gain, decreased cholesterol absorption to about one-third that observed in control mice, decreased liver cholesterol level, increased fecal excretion of cholesterol, and decreased fecal excretion of bile acids. Most of the increase in fecal excretion of cholesterol occurred 2 days after the start of feeding of phytosterols and gradually declined thereafter, but the levels on day 15 were nevertheless higher than those in the control mice. The fecal excretion of bile acids decreased progressively after the treatment with phytosterols. The decrease of bile acid derived from chenodeoxycholic acid was more predominant than the decrease of those derived from cholic acid, resulting in an increase of the cholic acid/chenodeoxycholic acid ratio. The biliary cholesterol, phospholipid, and bile acid mole % ratios and the lithogenic index were not changed, but the percentages of cholic acid and its related bile acids (the cholic acid group) to the total bile acids increased and those of the chenodeoxycholic acid group decreased after the treatments. The pool size of bile acids decreased in the mice given diosgenin but not in those given β-sitosterol. Distribution of bile acids between the gallbladder and intestine was not altered by either phytosterol. These data suggest that both diosgenin and β-sitosterol inhibit cholesterol absorption, decrease liver cholesterol concentration, and decrease the synthesis of bile acids, especially that of chenodeoxycholic acid.—**Uchida, K., H. Takase, Y. Nomura, K. Takeda, N. Takeuchi, and Y. Ishikawa.** Changes in biliary and fecal bile acids in mice after treatments with diosgenin and β-sitosterol. *J. Lipid Res.* 1984. **25:** 236–245.

Supplementary key words cholesterol absorption • fecal sterols • bile acid pool size • plasma and liver cholesterol

Most bile acids are formed from cholesterol. Recent studies postulate an interesting hypothesis that newly synthesized cholesterol in the liver is preferentially converted to cholic acid (1–4), but exogenously administered cholesterol is converted to chenodeoxycholic acid (5–7). Although there are some reports, contrary to this hypothesis, that cholic acid and chenodeoxycholic acid are produced in equal amounts after diets high in cholesterol are fed, our previous data with rats indicated that dietary cholesterol increased fecal excretion of bile acids, especially those derived from chenodeoxycholic acid, suggesting that exogenously administered cholesterol was preferentially converted to chenodeoxycholic acid.

In order to confirm this hypothesis, we examined fecal bile acid levels and compositions in mice fed diosgenin and β-sitosterol, because these phytosterols are known to inhibit cholesterol absorption. The precise mechanism for the inhibition is not fully understood, but it is widely accepted that the phytosterols form non-absorbable complexes with cholesterol (8, 9) or compete with cholesterol for cholesterol absorption sites (10, 11). Bile acids play an essential role in cholesterol absorption but they are not supposed to be involved in the action of phytosterols, since diosgenin has been shown to inhibit cholesterol absorption without altering fecal bile acid excretion (12, 13). As a consequence of inhibition of cholesterol absorption in rats by treatment with diosgenin or β-sitosterol, the hepatic cholesterol synthesis is enhanced (12–14), because the cholesterol synthesis is regulated by the cholesterol returning to the liver. Therefore, phytosterol treatment presents a suitable experimental model for examining the effect of dietary cholesterol on bile acid metabolism.

MATERIALS AND METHODS

CRJ:CD-1 strain male mice, 6 weeks old, obtained from Charles River Japan were kept in an air-conditioned room

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

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(25 ± 1°C, 50–60% humidity) lighted 12 hr a day (8 AM to 8 PM). Diosgenin was purchased from Wako Chemical Industries (Osaka, Japan, Lot No. SDF 1767); it was 83.5% pure as determined by TLC, GLC, and GLC–MS. The major contaminant was yamogenin (16.5%). β-Sitosterol was purchased from Merck A.G. (Darmstadt, West Germany, Lot No. 3741) and was 77% pure. The contaminants were sitostanol (16%), campesterol (6%), and campestanol (1%). Ordinary powder diet (JCL-CA-1, Japan CLEA, Tokyo, Japan) was provided as the basal diet and the phytosterols were supplemented at a level of 1% to the basal diet.

The mice were caged individually and given the diets for 15 days utilizing a powder diet-feeding apparatus (Natsume Seisakusho Co. Ltd., Tokyo, Japan). Tap water was provided ad libitum. Feces were collected for 2 days before and during the feeding of the phytosterols. Pair-fed animals were given the same amount of diet consumed by the diosgenin-fed mice. The animals were fasted for 5 hr and the gallbladder was removed under sodium methylhexabital anesthesia (125 mg/kg, i.p.). Next, blood was withdrawn from the abdominal aorta with a heparinized syringe, and the liver and small and large intestines with their contents were removed.

Biliary lipid determination

The gallbladder was crushed in 20 ml of ethanol with a glass rod and biliary lipids were extracted by refluxing for 10 min at 80–90°C. After filtration, a portion of the extract was evaporated to dryness under a stream of nitrogen and the residue was hydrolyzed in 4 ml of 1.25 N NaOH at 120°C for 6 hr. Cholesterol was extracted with diethyl ether and after acidification with 2 N HCl the bile acids were extracted with diethyl ether (5, 15). Cholesterol was determined by GLC on a 1% SE-30 column (16). Bile acids were converted to methyl ester trifluoroacetate derivatives and determined by GLC using a 1.5% QF-1 column (15, 16). Since cholic and β-muricholic acids had similar retention times on the QF-1 column, their methyl ester trifluoroacetate derivatives were analyzed using a 1.5% AN-600 column. The column was originally used for the analysis of bile acid methyl ester acetate derivatives (17, 18), but we applied bile acids as methyl ester trifluoroacetate derivatives since trifluoroacetylation was much easier than acetylation. This procedure gave a good separation for cholic acid and β-muricholic acid as shown in **Table 1.** Cholesterol and taurocholic acid added to the bile were quantitatively (almost 100%) recovered by the present procedures. Phospholipids were determined by the method of Gomori (19). The recovery of phospholipids was not examined. In the present experiments, the gallbladder bile was analyzed together with gallbladder tissue, but the weight of one sample of gallbladder tissue was about 1 mg or

TABLE 1. Relative retention times of bile acid methyl ester trifluoroacetate derivatives on 1.5% QF-1, 1.5% AN-600, and 1% SE-30 columns

5β-Cholanoic Acid[a]	1.5% QF-1	1.5% AN-600	1% SE-30
3α	0.68	0.79	1.16
3α,12α	1.00	1.00	1.00
3α,6β,7α	1.16	1.15	0.77
3α,7α	1.26	1.46	1.16
3α,6α	1.37	1.54	1.25
3α,7β	1.41	1.67	1.32
3α,7β,12α	1.80	1.80	0.97
3α,6β,7β	1.92	2.50	1.26
3α,7α,12α	1.93	2.23	1.03
3α,6α,7β	2.09	2.71	1.27
3α, 12=0	2.14	3.21	1.90
3α, 7=0	2.25	3.49	1.86
3α, 6=0	2.43		1.81
3α,12α, 7=0	2.86	3.89	1.48
3α,7α, 12=0	3.52	5.50	1.77
3α, 7=0, 12=0	5.59	11.97	2.82
3=0, 12α		2.67	1.32
3=0, 7α,12α		4.40	1.31

[a] 5β-Cholanoic acid possessing hydroxyl and oxo groups at positions indicated.

less when examined after the bile had been washed out with saline. The mean cholesterol content in one sample of gallbladder tissue was 2.62 μg and phospholipid content was 39.4 μg; these values did not seem to affect the values of biliary lipids. Lithogenic indexes were calculated by the formula of Thomas and Hofmann (20).

Fecal sterol and bile acid determination

Fecal sterols and bile acids were determined as reported previously (5, 16, 21). Briefly, dried and powdered feces were extracted with absolute ethanol and petroleum ether and hydrolyzed in 1.25 N NaOH at 120°C for 6 hr. After extraction of the sterols with 2.5 vol of diethyl ether three times, the hydrolyzate was acidified to pH 2 or below with 2 N HCl, and bile acids were extracted with 2.5 vol of diethyl ether three times. The sterols and bile acids were quantified by GLC on a 1% SE-30 column and a 1.5% QF-1 column, respectively.

In order to determine the extraction efficiency of sterols and bile acids from feces, a dose of [4-^{14}C]cholesterol (1 μCi/68 μg) (Amersham Japan Co., Tokyo, Japan) was administered orally to male mice and the feces were collected daily for 1 week. The feces were extracted by procedures described previously (5, 16, 21). Portions of the feces before ethanol extraction and the residues after ethanol extraction were combusted utilizing a Packard Tri-Carb Sample Oxidizer Model B 0306 and the radioactivity was determined. The ethanol extract was evaporated to dryness, hydrolyzed in 1.25 N NaOH at 120°C for 6 hr, and sterols and bile acids were extracted with diethyl ether. The radioactivity in the ether extracts was also determined by the combustion method. The extraction efficiency was 95 ± 0.9% with ethanol, 92 ± 1.4%

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with ether, and the efficiency after the entire procedure was 87 ± 1.1% (mean ± SE, 12 determinations).

Tissue bile acid determination

The small and large intestines with their contents were homogenized with distilled water and lyophilized. The lyophilized preparations were extracted three times with 100 ml of absolute ethanol by refluxing for 1 hr at 85–90°C. The filtrates were combined and evaporated to dryness under reduced pressure. The residue for the large intestine was dissolved in 10–15 ml of 70% methanol and neutral lipids were removed by extraction with an equal volume of n-hexane. The 70% methanol layer was evaporated to dryness. The residues were dissolved in 1.25 N NaOH, hydrolyzed at 120°C for 6 hr, and acidified with 2 N HCl. The bile acids were then extracted with diethyl ether and quantified by GLC on 1.5% QF-1 and 1.5% AN-600 columns. The extraction efficiency of the intestinal bile acids was not examined; inasmuch as the efficiency for the fecal bile acids was over 90% (Table 1, ref. 21), similar values would be expected for the intestinal bile acids.

Plasma and liver lipid determination

Blood was centrifuged at 3000 rpm for 15 min to separate plasma. About 1 g of the largest lobe of the liver (*lobus sinistra externa*) was excised and homogenized with 9 vol of ice-chilled water using an Ultra-Turrax TP 18-10 (IKE-WERK, Janke & Kunkel KG, West Germany). The plasma and the liver homogenate were extracted with 10 vol of ethanol by refluxing for 20 min at 90–95°C. Portions of the extracts were hydrolyzed in 15% KOH–50% ethanol solution at 75–80°C for 20 min and cholesterol was extracted three times with 2.5 vol of petroleum ether. The cholesterol was quantified by GLC on a 1% SE-30 column. Phospholipids were determined on the ethanol extracts by the method of Gomori (19). The recovery of plasma and liver cholesterol was almost 100%; phospholipid recovery was not examined.

Pool size and synthesis of bile acids

The pool size of bile acids was obtained by totaling the amounts in the bile and small and large intestines (22). In a steady state, the amount of bile acids excreted into the feces is presumed to correspond to that synthesized in the liver.

Cholesterol absorption

Cholesterol absorption was determined by the dual isotope ratio method reported by Zilversmit (23) and Zilversmit and Hughes (24) with a slight modification. After feeding the phytosterols for 2 weeks, each mouse received by oral administration 2 μCi of [4-^{14}C]cholesterol (2 mg) suspended in 0.2 ml of 5% gum arabic solution containing 20% ethanol. At the same time, they received by intravenous injection 0.5 μCi of [1,2-^{3}H]cholesterol (5 μg) suspended in 0.1 ml of saline solution containing 5% ethanol. [4-^{14}C]Cholesterol and [1,2-^{3}H]cholesterol were obtained from Amersham Japan Co. (Tokyo, Japan). The administration of the tracers was performed at 9 AM, 1 hr after the time for darkness during our experiments. Feces were collected daily for 3 days at 24-hr intervals (12 hr for the first day). The gallbladder and blood were obtained 60 hr after the administration of the tracers. The feces were homogenized in ethanol and extracted with absolute ethanol by procedures described previously (5, 16, 21). Portions of the feces before extraction and the residues after extraction were combusted utilizing a Sample Oxidizer (Packard Tri-Carb Model B 0306) and the radioactivity was determined. The extraction efficiency was over 98%.

The plasma and both fractions of sterols and bile acids of the gallbladder bile and feces were combusted and the radioactivities for ^{14}C and ^{3}H were determined separately. The procedures for extraction of sterols and bile acids from gallbladder bile and feces are described above.

Statistical analysis

The results are expressed as mean values and standard errors of the mean. Student's *t* test was used to determine

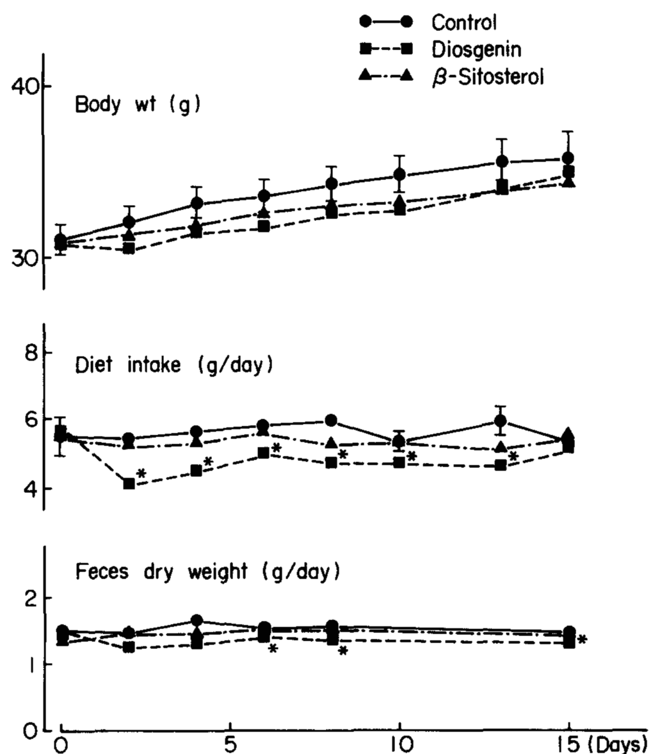

Fig. 1. Changes in body weight, diet intake, and feces dry weight in diosgenin- and β-sitosterol-fed mice. Each point represents the mean ± SE of five mice. An asterisk (*) means significant difference from control ($P < 0.05$).

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TABLE 2. Effects of diosgenin and β-sitosterol on fecal excretion of sterols and bile acids in mice

	Total Sterols				Total Bile Acids			
	Control	Pair-fed	Diosgenin	β-Sitosterol	Control	Pair-fed	Diosgenin	β-Sitosterol
	mg/day							
No. of mice	5	5	5	5	5	5	5	5
Day 0	4.63 ± 0.47[a]		4.74 ± 0.32	4.63 ± 0.19	2.33 ± 0.24[a]		2.15 ± 0.11	2.04 ± 0.22
2	4.67 ± 0.56	3.87 ± 0.29	9.45 ± 0.69*	7.30 ± 0.27*				
4	4.74 ± 0.39	3.85 ± 0.11	7.75 ± 0.44*	5.95 ± 0.25*	1.98 ± 0.15	2.17 ± 0.15	1.49 ± 0.10*	1.50 ± 0.18
6	4.41 ± 0.40		7.20 ± 0.59*	5.61 ± 0.50				
8	4.13 ± 0.39		6.31 ± 0.31*	5.36 ± 0.54	2.07 ± 0.11		1.39 ± 0.16*	1.20 ± 0.07*
15	3.76 ± 0.29	3.29 ± 0.15	5.80 ± 0.42*	5.64 ± 0.33*	2.19 ± 0.28	2.40 ± 0.19	1.27 ± 0.11*	1.24 ± 0.04*

[a] Mean ± SE.
* Statistically significant compared to the control values ($P < 0.05$).

statistical significance. Since diosgenin decreased diet intake, pair-fed experiments were performed. However, the restriction of diet caused only minor effects on cholesterol and bile acid levels. Therefore, the treated animals were generally compared with the controls (not restricted). Comparisons with pair-fed controls were also carried out as indicated.

RESULTS

Body weight and diet intake

Changes in body weight and diet intake are shown in **Fig. 1.** Diosgenin-fed mice consumed less diet, especially at the beginning of the feeding period, but β-sitosterol-fed mice ate almost the same amount of diet as control mice. The dietary phytosterols caused no statistically significant changes in body weight gain. The pair-fed mice, however, showed a slight decrease in the final body weight (ca. 13%, $P < 0.05$), while the diosgenin-fed mice showed about 8% decrease ($P < 0.05$) compared with the control mice. This was probably due to the fact that the pair-fed mice could not eat all the diet provided in the powder diet feeding apparatus and left up to 1 g (0.2 to 1 g) of the diet daily.

Fecal sterols and bile acids

Changes in the fecal excretion of sterols and bile acids are shown in **Table 2.** The total sterol (cholesterol plus coprostanol) excretion increased markedly soon after the start of the diosgenin feeding and decreased thereafter. β-Sitosterol also increased the fecal sterol excretion but to a lesser extent than diosgenin. **Fig. 2** shows the changes in the individual excretion of cholesterol and coprostanol. The cholesterol excretion increased markedly on day 2 and was followed by a gradual decrease, whereas no significant difference was found between the effects of diosgenin and β-sitosterol. On the other hand, the coprostanol excretion increased in diosgenin-fed mice but not in mice fed β-sitosterol. The fecal excretion of sterols in the pair-fed mice was slightly lower than that in the control mice but remained almost constant throughout the experimental period (Table 2). However, the relative amount of coprostanol was slightly higher in the pair-fed mice than in the controls.

The total bile acid excretion in the control and pair-fed mice remained almost constant, but excretion in the diosgenin and β-sitosterol-fed mice decreased with time, and the degree of decrease was similar (Table 2).

Deoxycholic acid and β- and ω-muricholic acids were the major constituents of fecal bile acids. Considerable

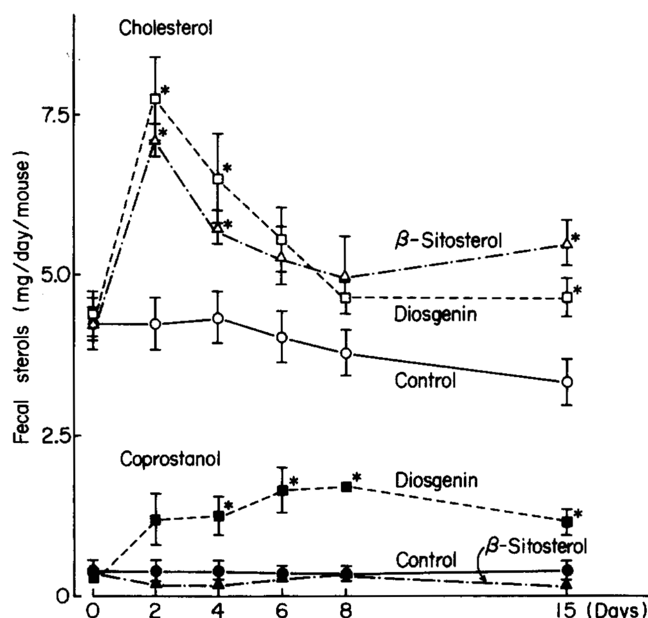

Fig. 2. Changes in fecal excretion of cholesterol and coprostanol in diosgenin- and β-sitosterol-fed mice. Each point represents the mean ± SE of five mice. An asterisk (*) means significant difference from control ($P < 0.05$).

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TABLE 3. Effects of diosgenin and β-sitosterol on fecal bile acid excretion (on day 15) in mice

	Control	Pair-fed	Diosgenin	β-Sitosterol
		mg/day per mouse		
No. of mice	5	5	5	5
Cholic acid group	0.75 ± 0.110[a]	0.80 ± 0.118	0.58 ± 0.059	0.47 ± 0.044*
Deoxycholic acid	0.51 ± 0.10 (22.6)	0.65 ± 0.10 (26.4)	0.36 ± 0.05 (27.8)	0.29 ± 0.03* (23.6)
Cholic acid	0.04 ± 0.02 (1.9)		0.03 ± 0.00 (2.3)	0.02 ± 0.00 (1.9)
3α,12α-Dihydroxy-7-oxo-	0.17 ± 0.08 (7.9)	0.10 ± 0.01 (4.2)	0.19 ± 0.03 (14.9)	0.14 ± 0.02 (11.2)
7β-Cholic acid	0.04 ± 0.01 (1.6)	0.05 ± 0.02 (2.1)	0.01 ± 0.00 (0.5)	0.01 ± 0.00 (1.0)
Chenodeoxycholic acid group	1.21 ± 0.164	1.32 ± 0.136	0.59 ± 0.037*	0.56 ± 0.027*
Lithocholic acid	0.16 ± 0.03 (7.2)	0.17 ± 0.02 (6.9)	0.16 ± 0.04 (12.2)	0.14 ± 0.03 (10.9)
Ursodeoxycholic acid	0.01 ± 0.00 (0.4)	0.06 ± 0.01 (2.3)	<0.01 (0.3)	<0.01 (0.3)
α-Muricholic acid	0.07 ± 0.02 (3.0)	0.15 ± 0.02 (6.2)	0.05 ± 0.01 (3.8)	0.02 ± 0.01* (1.7)
β-Muricholic acid	0.50 ± 0.09 (22.5)	0.42 ± 0.06 (17.3)	0.15 ± 0.04* (11.7)	0.13 ± 0.02* (10.2)
ω-Muricholic acid	0.45 ± 0.08 (21.4)	0.44 ± 0.03 (18.5)	0.19 ± 0.04* (15.5)	0.27 ± 0.04 (21.9)
3α-Hydroxy-6-oxo-	0.01 ± 0.01 (0.5)	0.04 ± 0.01 (1.6)	0.06 ± 0.01 (4.9)	0.01 ± 0.00 (1.2)
Others[b]	0.24 ± 0.06 (10.9)	0.27 ± 0.04 (12.0)	0.08 ± 0.01* (6.3)	0.20 ± 0.04 (16.2)
CA/CDCA ratio	0.63 ± 0.057	0.61 ± 0.058	0.99 ± 0.072*	0.84 ± 0.074*

[a] Mean ± SE (values in parentheses represent % of the total bile acids).
[b] Others comprise unidentified peaks with relative retention times of 0.74 and 0.85.
* Statistically significant compared to the control values ($P < 0.05$).

amounts of 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid and lithocholic acid were also found. **Table 3** shows the amounts of individual bile acids in the feces on day 15. In the phytosterol-fed mice, deoxycholic acid and muricholic acids decreased but 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid or lithocholic acid did not change. The decrease in bile acids derived from chenodeoxycholic acid was larger than that in the cholic acid group, and the ratio CA/CDCA increased. The changes in both bile acid groups are shown in **Fig. 3.** The chenodeoxycholic acid group decreased more markedly than the cholic acid group after the feeding of diosgenin or β-sitosterol.

Biliary cholesterol, phospholipids, and bile acids

Gallbladder bile weight and biliary lipid concentration are shown in **Table 4.** Diosgenin decreased the phospholipid concentration and β-sitosterol decreased the cholesterol concentration, but neither phytosterol caused a statistically significant decrease in the bile acid concentration or the lithogenic index. The pair-fed mice showed a decrease in phospholipid concentration.

Biliary bile acid compositions are compared in **Table 5.** The biliary bile acids in the control mice consisted of cholic acid (about 50%), β-muricholic acid (35%), and minor amounts of deoxycholic acid, 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid, chenodeoxycholic acid, ursodeoxycholic acid, α-muricholic acid, ω-muricholic acid, and 3α-hydroxy-6-oxo-5β-cholanoic acid. When diosgenin was fed for 15 days, cholic acid markedly increased but β-muricholic, chenodeoxycholic, ursodeoxycholic, ω-muricholic, and 3α-hydroxy-6-oxo-5β-cholanoic acids decreased. Mice fed β-sitosterol showed similar changes in the bile acid composition.

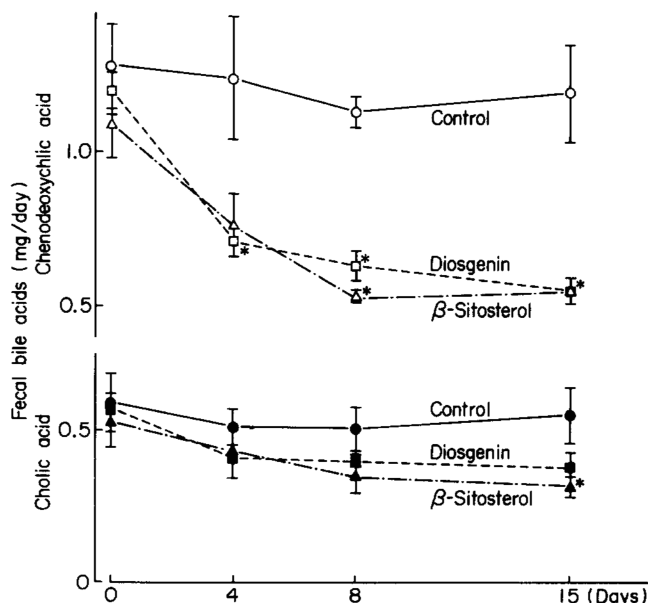

Fig. 3. Changes in fecal excretion of bile acids in diosgenin- and β-sitosterol-fed mice. Each point represents the mean ± SE of five mice. The cholic acid group is comprised of deoxycholic acid, cholic acid, 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid, and 7β-cholic acid, and the chenodeoxycholic acid group includes lithocholic acid, ursodeoxycholic acid, hyodeoxycholic acid, muricholic acids, and 3α-hydroxy-6-oxo-5β-cholanoic acid. An asterisk (*) means significant difference from control ($P < 0.05$).

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TABLE 4. Effects of diosgenin and β-sitosterol on biliary cholesterol, phospholipids and bile acids, and lithogenic index in mice

	Control	Pair-fed	Diosgenin	β-Sitosterol
No. of mice	5	5	5	5
Gallbladder bile (mg/mouse)	22 ± 1.8[a]	24 ± 1.6	21 ± 1.9	20 ± 4.2
Cholesterol (mg/g bile)	1.22 ± 0.07	1.37 ± 0.21	1.02 ± 0.09	0.69 ± 0.03*
Phospholipids (mg/g bile)	16.1 ± 1.44	11.2 ± 0.78*	10.7 ± 0.44*	13.3 ± 1.20
Bile acids (mg/g bile)	51.1 ± 3.52	45.2 ± 4.16	43.9 ± 1.57	47.7 ± 4.37
Cholesterol (mol %)	2.0 ± 0.26	2.4 ± 0.09	1.8 ± 0.10	1.4 ± 0.10
Phospholipids (mol %)	12.3 ± 0.52	10.8 ± 0.63	13.3 ± 1.10	12.0 ± 0.46
Bile acids (mol %)	85.7 ± 0.68	86.6 ± 0.70	84.9 ± 1.19	86.6 ± 0.54
Lithogenic index	0.23 ± 0.029	0.29 ± 0.017	0.25 ± 0.019	0.17 ± 0.010

[a] Mean ± SE.
* Statistically significant compared to the control value ($P < 0.05$).

Pool size and distribution of bile acids

The pool size of bile acids decreased markedly in the diosgenin-fed mice but not in the β-sitosterol-fed animals (**Table 6**). Under our experimental conditions of 12 hr darkness from 8 AM to 8 PM and 5 hr fasting, about 25% of the bile acids were localized in the gallbladder, 60% in the small intestine, and 13% in the large intestine. Diosgenin and β-sitosterol caused no significant change in the distribution of bile acids between the gallbladder and intestine.

Plasma and liver lipid levels and liver weight

Diosgenin markedly decreased plasma and liver cholesterol levels but caused no significant change in the phospholipid levels (**Table 7**). β-Sitosterol did not significantly alter plasma cholesterol level, but markedly decreased liver cholesterol levels. Diosgenin caused an almost twofold increase in liver weight but β-sitosterol did not. The pair-fed mice showed no significant change.

Cholesterol absorption

Radioactivity values of ^{14}C and ^{3}H (derived from oral [^{14}C]cholesterol and intravenous [^{3}H]cholesterol) in the plasma, liver, and bile and in the feces are shown in **Table 8** and **Table 9,** respectively. ^{14}C in the plasma, liver, and bile (Table 8) decreased in the mice treated with diosgenin and β-sitosterol, although the decrease in the liver ^{14}C levels in the diosgenin-treated mice was statistically insignificant ($P > 0.05$) on account of large fluctuation of the values. On the other hand, the ^{3}H radioactivity in the plasma, liver, and bile did not change. The cholesterol absorption calculated from the plasma $^{14}C/^{3}H$ ratio was 26.7% in the normal mice but decreased in the phytosterol-fed mice. Time-course changes in the $^{14}C/^{3}H$ ratio

TABLE 5. Effects of diosgenin and β-sitosterol on biliary bile acid composition on day 15

	Control	Pair-fed	Diosgenin	β-Sitosterol
No. of mice	5	5	5	5
Cholic acid group (%)	58.4 ± 1.9[a]	55.7 ± 4.1	79.5 ± 2.7*	67.6 ± 2.4*
Deoxycholic acid	0.9 ± 0.1	1.9 ± 0.3*	0.5 ± 0.1*	2.4 ± 0.5*
Cholic acid	55.5 ± 1.9	52.5 ± 4.0	77.5 ± 2.7*	63.1 ± 2.6*
3α,12α-Dihydroxy-7-oxo-	2.0 ± 0.2	1.3 ± 0.1*	1.5 ± 0.1	2.1 ± 0.4
Chenodeoxycholic acid group (%)	40.1 ± 1.8	42.3 ± 4.1	18.9 ± 2.3*	30.1 ± 1.3*
Chenodeoxycholic acid	1.4 ± 0.1	1.0 ± 0.11	0.4 ± 0.1*	0.7 ± 0.0*
Ursodeoxycholic acid	4.3 ± 0.2	5.4 ± 0.8	1.8 ± 0.1*	2.7 ± 0.2*
α-Muricholic acid	0.7 ± 0.2	1.3 ± 0.3	0.6 ± 0.2	0.5 ± 0.1
β-Muricholic acid	28.2 ± 1.7	28.1 ± 3.0	14.0 ± 2.4*	20.8 ± 2.1*
ω-Muricholic acid	3.8 ± 0.7	5.8 ± 0.9	1.5 ± 0.2*	3.9 ± 0.7
3α-Hydroxy-6-oxo-	1.7 ± 0.4	0.7 ± 0.1	0.5 ± 0.2*	1.4 ± 0.4
Others[b]	1.5 ± 0.2	2.0 ± 0.3	1.6 ± 0.6	2.4 ± 1.3
CA/CDCA ratio	1.48 ± 0.12	1.41 ± 0.25	4.53 ± 0.69*	2.27 ± 0.18*

[a] Mean ± SE.
[b] Others comprise unidentified peaks with relative retention times of 0.74 and 0.85.
* Statistically significant compared to the control value ($P < 0.05$).

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TABLE 6. Effects of diosgenin and β-sitosterol on distribution and pool size of bile acids in mice

	Control	Diosgenin	β-Sitosterol
No. of mice	5	5	5
Pool size (mg/mouse)	6.57 ± 0.48[a]	3.31 ± 0.28*	5.39 ± 0.28
Bile	1.61 ± 0.38 (24.7%)	0.87 ± 0.12 (26.3%)	0.97 ± 0.25 (18.0%)
Small intestine	4.05 ± 0.67 (62.2)	2.03 ± 0.19* (61.3)	3.76 ± 0.31 (69.8)
Large intestine	0.85 ± 0.16 (13.0)	0.40 ± 0.11* (12.1)	0.66 ± 0.06 (12.2)

[a] Mean ± SE (values in parentheses represent % of the total bile acids).
* Statistically significant compared to the control value ($P < 0.05$).

were examined in another experiment (**Fig. 4**). The ratio for the control mice decreased on day 2, but remained at the same level on day 3. The ratios for the diosgenin- and β-sitosterol-treated mice were fairly constant. These data suggested that the specific activities of [^{14}C]- and [^{3}H]cholesterol were parallel in plasma.

Fecal excretion of ^{14}C-labeled sterols increased but those of ^{14}C-labeled bile acids decreased in the diosgenin- and β-sitosterol-treated mice (Table 9). Cholesterol absorption rate calculated from the fecal ^{14}C-labeled sterols was 25.1% in the normal mice and 7.8% and 9.7% in the diosgenin-treated and β-sitosterol-treated mice, respectively. These values almost coincide with those obtained by the plasma isotope ratio method (Table 8).

DISCUSSION

Diosgenin and β-sitosterol decreased cholesterol absorption and markedly increased fecal excretion of cholesterol in mice, in agreement with previous reports on rats (12–14, 25, 26). The increase of fecal cholesterol excretion, however, was most predominant on day 2 and gradually declined thereafter, although the levels on days 8 to 15 were 30–40% higher than the control level. The reason for this decline is not known, but a cholesterol pool should have been present in the intestinal lumen which was excreted upon the first administration of phytosterols. As is obvious in Fig. 2, diosgenin increased coprostanol excretion but β-sitosterol did not. Therefore, the total sterol (cholesterol plus coprostanol) secretion was higher in the mice given diosgenin than in those given β-sitosterol in the present experiments.

In contrast to the increase of fecal sterol excretion, fecal bile acid excretion was decreased by treatment with the phytosterols (Fig. 3 and Table 3). At that time, most of the bile acids decreased, but the bile acids derived from chenodeoxycholic acid decreased more than those derived from cholic acid, resulting in an increase in the CA/CDCA ratio. The fecal bile acid excretion increases after a cholesterol-rich diet feeding (5, 27) but decreases after a cholesterol-free diet (5). A similar decrease in fecal bile acid excretion was found in mice fed a fat-free diet.[5] Therefore, the dietary cholesterol content seems to be a major determinant for the amount of fecal bile acid, and the decrease of fecal bile acids after phytosterol treatment will be a reflection of the decrease in cholesterol absorption.

The primary bile acids synthesized in the liver are cholic and chenodeoxycholic acids. Both are derived from cholesterol, but the type of bile acid formed seems to be determined by the source of cholesterol. Ogura et al. (1, 2) and Mitropoulos et al. (3) demonstrated that newly synthesized cholesterol in the liver is mainly converted to cholic acid. This was further confirmed by the experiments of Gustafsson et al. (4) who showed that cholestyramine, which prevents bile acid absorption from the intestine, causes increases in cholesterol and cholic acid synthesis. On the other hand, when excess cholesterol

[5] Uchida, K., et al. Unpublished data.

TABLE 7. Effects of diosgenin and β-sitosterol on plasma and liver cholesterol and phospholipid concentrations in mice

	Control	Pair-fed	Diosgenin	β-Sitosterol
No. of mice	5	5	5	5
Plasma cholesterol (mg/100 ml)	69 ± 5.7[a]	68 ± 4.5	30 ± 1.8*	58 ± 5.3
Plasma phospholipids (mg/100 ml)	228 ± 18.9	202 ± 10.3	191 ± 27.1	237 ± 16.8
Liver cholesterol (mg/g)	2.7 ± 0.19	3.0 ± 0.30	1.8 ± 0.08*	2.0 ± 0.05*
Liver phospholipids (mg/g)	49.2 ± 1.21	47.2 ± 1.90	49.6 ± 1.21	47.6 ± 1.15
Liver wt (g)	1.69 ± 0.08	1.55 ± 0.07	3.08 ± 0.05*	1.65 ± 0.08

[a] Mean ± SE.
* Statistically significant compared to the control value ($P < 0.05$).

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TABLE 8. Effects of diosgenin and β-sitosterol on distribution of radioactivities of orally administered [^{14}C]cholesterol and intravenous [^{3}H]cholesterol in plasma, liver and bile, and cholesterol absorption in male mice

	^{14}C			^{3}H		
	Control	Diosgenin	β-Sitosterol	Control	Diosgenin	β-Sitosterol
No. of mice	5	4	5	5	4	5
Body weight (g)	37.4 ± 1.36[a]	36.8 ± 0.93	37.4 ± 1.54			
Liver weight (g)	2.15 ± 0.18	2.78 ± 0.18*	1.83 ± 0.15			
Gallbladder bile (mg/mouse)	21.1 ± 2.78	21.7 ± 6.75	19.4 ± 2.39			
Plasma cholesterol (10^3 dpm/ml)	29.8 ± 4.19	9.6 ± 2.25*	12.8 ± 1.14*	20.5 ± 1.30	16.5 ± 1.27	24.8 ± 2.24
Liver cholesterol (10^3 dpm/g)	57.1 ± 10.87	25.1 ± 8.89	22.7 ± 1.27*	56.6 ± 2.16	49.0 ± 4.14	55.2 ± 5.01
Bile cholesterol (10^3 dpm/g)	41.6 ± 8.14	18.4 ± 2.44*	10.6 ± 2.12*	33.4 ± 2.54	31.0 ± 4.71	23.2 ± 3.53
Bile bile acids (10^3 dpm/g)	1150 ± 125.6	362 ± 73.9*	565 ± 76.7*	945 ± 57.7	607 ± 119.0	1146 ± 125.0
Cholesterol absorption[b] (%)	26.7 ± 3.18	11.2 ± 3.20*	11.4 ± 1.36*			

[a] Mean ± SE.
[b] Cholesterol absorption was calculated from the ^{14}C/^{3}H ratio in plasma according to the method reported by Zilversmit and Hughes (24).
* Statistically significant compared to the control value ($P < 0.05$).

was fed to rats, bile acid synthesis increased, especially that of chenodeoxycholic acid (5–7). This tendency has been observed in rats, and also seems to be the case in mice as judged from the present data. On the other hand, Schwartz et al. (28, 29) showed that cholic acid and chenodeoxycholic acid were formed from HDL-cholesterol at the same ratio in man. Therefore, the relationship between bile acid formed and precursor cholesterol sources differ with species.

The bile acids were located mainly in the small intestine under our experimental conditions (Table 6), and their composition was very similar to that in the bile. The bile acids in the large intestine amounted to about 13% of the total but the composition was very different from that in the small intestine and bile, as evidenced by a decrease in the CA/CDCA ratio. This suggested that cholic acid was more efficiently absorbed from the small intestine than β-muricholic acid which was derived from chenodeoxycholic acid and is a major bile acid in mice.

The total bile acids in the bile and small and large intestines were considered to correspond roughly to the pool size. The bile acids were also distributed in the liver and blood, but since the amounts in these tissues were less than 2% they were excluded from the calculation. Diosgenin markedly decreased the pool size without affecting the distribution ratio of bile acids (Table 6). β-Sitosterol slightly decreased the pool size but the change was statistically insignificant.

Diosgenin decreased the plasma and liver cholesterol concentrations but not those of phospholipids. β-Sitosterol

TABLE 9. Effects of diosgenin and β-sitosterol on fecal excretion of radioactivities of orally administered [^{14}C]cholesterol and intravenous [^{3}H]cholesterol in male mice

	^{14}C			^{3}H		
	Control	Diosgenin	β-Sitosterol	Control	Diosgenin	β-Sitosterol
No. of mice	5	4	5	5	4	5
Feces sterols (10^3 dpm/mouse)						
Day 1	2963 ± 260.6[a]	3467 ± 293.1	3765 ± 46.9	25 ± 3.6	60 ± 9.0*	24 ± 4.9
Day 2	290 ± 76.4	334 ± 148.0	173 ± 44.6	84 ± 10.3	194 ± 4.5*	63 ± 2.4
Day 3	77 ± 7.4	96 ± 35.5	35 ± 6.2*	57 ± 6.2	100 ± 10.1*	41 ± 2.3
Days 1–3	3330 ± 215.2	4058 ± 80.4*	3972 ± 48.5*	166 ± 18.8	354 ± 23.4*	124 ± 5.9
Feces bile acids (10^3 dpm/mouse)						
Day 1	149 ± 15.4	121 ± 16.8	105 ± 11.0	21 ± 1.3	17 ± 1.2	21 ± 2.3
Day 2	98 ± 14.5	56 ± 26.7	44 ± 13.6	74 ± 5.2	49 ± 4.1	61 ± 11.9
Day 3	94 ± 11.2	26 ± 9.2*	35 ± 6.0*	78 ± 9.0	40 ± 5.5	54 ± 7.0
Days 1–3	341 ± 22.9	203 ± 18.1*	184 ± 20.9*	172 ± 12.5	106 ± 8.7*	133 ± 20.0
Cholesterol absorption[b] (%)						
Days 1–3	25.1 ± 2.56	7.8 ± 1.85*	9.7 ± 1.10*			

[a] Mean ± SE.
[b] Cholesterol absorption was calculated from the total amount of sterols excreted into feces.
* Statistically significant compared to the control value ($P < 0.05$).

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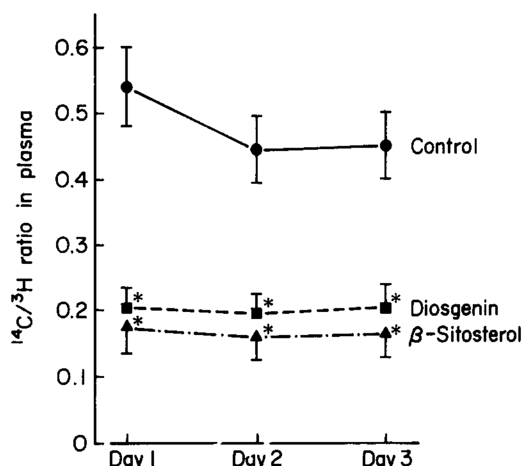

Fig. 4. Time-course changes of the $^{14}C/^{3}H$ ratio in plasma after oral administration of [^{14}C]cholesterol and intravenous injection of [^{3}H]cholesterol in diosgenin- and β-sitosterol-fed and control male mice. Each point represents the mean ± SE of five mice. An asterisk (*) means significant difference from control ($P < 0.05$).

showed no hypocholesterolemic effect but decreased the liver cholesterol level. These data obtained in the present experiments on mice were substantially consistent with previous observations in man and other animals (8). The liver cholesterol concentration in the diosgenin-fed mice decreased, but since the liver weight increased in this group, the total amount of cholesterol in the liver increased about 20% (ca. 1 mg/mouse). Therefore, diosgenin appears to cause a redistribution of cholesterol from plasma into liver in mice, contrary to what is found in rats (13). β-Sitosterol, on the other hand, decreased both the concentration and total amount of liver cholesterol.

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