EFFECTS OF SPINASTEROL AND SITOSTEROL ON PLASMA AND LIVER CHOLESTEROL LEVELS AND BILIARY AND FECAL STEROL AND BILE ACID EXCRETIONS IN MICE

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Accepted September 10, 1982

Abstract—Effects of spinasterol and sitosterol on plasma and liver cholesterol levels and biliary and fecal sterol and bile acid excretions were examined with male mice. Both phytosterols were added to the diet at a 1% concentration and fed to mice for 15 days. Spinasterol increased the fecal cholesterol excretion and decreased the plasma and liver cholesterol levels, the bile acid pool size and the fecal bile acid excretion, especially those derived from chenodeoxycholic acid. Fecal coprostanol excretion remained unchanged. These changes were similar to those produced by sitosterol. These data led to the conclusions 1) that spinasterol, as well as sitosterol, inhibits cholesterol absorption, resulting in decreases of the plasma and liver cholesterol levels and 2) that when cholesterol absorption is inhibited, the synthesis of bile acids, especially that of chenodeoxycholic acid, decreases, suggesting that the dietary cholesterol is preferentially metabolized to chenodeoxycholic acid in mice.

Sitosterol inhibits cholesterol absorption and decreases blood cholesterol level in humans (1, 2) and experimental animals (3–9). Similar effects have been reported for stigmasterol (10, 11) and diosgenin (12, 13). The mechanism of the cholesterol inhibition is not fully understood, but it is widely accepted that phytosterols form nonabsorbable complexes with cholesterol (3) or compete with cholesterol for cholesterol absorption sites (4, 5). Bile acids play an essential role in cholesterol absorption (14), but they are not thought to be involved in the action of phytosterol since sitosterol (1, 15) and diosgenin (12, 13) have been shown to inhibit cholesterol absorption without altering fecal bile acid excretion. However, we found that these phytosterols decreased fecal bile acid excretion in mice (Mizuno et al., unpublished data*).

Spinasterol is a C-29 sterol found in plants, especially Spinacea oleracea and Medicage sativa (16), and its structure resembles that of sitosterol (Fig. 1). Takeda (17) reported that spinasterol decreased the plasma cholesterol level in rabbits, but the details of its

* Presented at the 59th Kinki Regional Meeting of the Japanese Pharmacological Society (1981, Tokushima, Japan).

Fig. 1. Chemical structures of sitosterol and spinasterol.

Sitosterol

Spinasterol
effects on cholesterol and bile acid metabolism are not known. In the present experiments, we examined the effects of spinasterol on plasma and liver cholesterol levels, biliary cholesterol, phospholipid and bile acid levels, fecal cholesterol, coprostanol and bile acid levels, and the pool size and composition of bile acids in mice, and we compared them with those of sitosterol.

Materials and Methods

Animals: CD-1 (ICR) strain male mice, 6 weeks old, obtained from Charles River Japan were kept in an air-conditioned room (25±1°C, 50-60% humidity) lighted 12 hr a day (8.00 a.m. to 8.00 p.m.). Ordinary powder diet (JCL CA-1, Japan Clea, Tokyo) was provided as a basal diet, and phytosterols were supplemented at 1% to the basal diet. Since the animals consumed daily about 5 g/mouse of the diet, this resulted in a daily intake of about 50 mg/mouse of the compounds. Spinasterol was extracted from Bupleurum falcatum. Sitosterol was purchased from Merck A.G. (Lot No. 3741, Darmstadt, W. Germany).

The mice were kept individually and given the diets for 15 days utilizing a powder diet feeding apparatus (Natsume Seisakusho Co. Ltd., Tokyo, Japan), and tap water was given ad libitum. The 2-day feces were collected before and during the feeding of the phytosterols. The animals were fasted for 5 hr before autopsy, 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 ethanol with a glass rod, and biliary lipids were extracted by refluxing for 10 min at 80-90°C. After filtration, a portion was evaporated to dryness under a stream of nitrogen, and the residue was hydrolyzed in 4 ml of 1.25 N sodium hydroxide solution at 120°C for 6 hr. Cholesterol was extracted with diethyl ether, and then the bile acids were extracted with diethyl ether after the mixture had been acidified with 2 N hydrochloric acid solution (18, 19). Cholesterol was determined by GLC with a 1% SE-30 column (20). Bile acids were converted into the trifluoroacetate methyl ester derivatives and determined by gas-liquid chromatography (GLC) with a 1.5% QF-1 column (18, 20). Since cholic acid and β-muricholic acid showed almost the same retention time on the QF-1 column (20), their trifluoroacetate methyl ester derivatives were analyzed using a 1.5% AN-600 column. Phospholipids were determined by the method of Gomori (21).

Cholesterol and taurocholic acid added to the bile were quantitatively (almost 100%) recovered by the present procedures. The recovery of phospholipids was not examined.

Fecal sterol and bile acid determination: Fecal sterols and bile acids were determined as reported previously (19, 20, 22). Briefly, dried and powdered feces were extracted with absolute ethanol and petroleum ether and hydrolyzed in 1.25 N sodium hydroxide solution at 120°C for 6 hr. After extraction of the sterols with 2.5 volumes of diethyl ether three times, the hydrolysate was acidified to pH 2 or below with 2 N hydrochloric acid, and bile acids were extracted with 2.5 volumes of diethyl ether three times. The sterols and bile acids were quantified by GLC with a 1% SE-30 column and a 1.5% QF-1 column, respectively. The recovery of the fecal sterols was almost 100% by the procedures, but that of the bile acids was about 90% (22).

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 to 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 sodium hydroxide solution, hydrolyzed at 120°C for 6 hr, and acidified with 2 N hydrochloric acid. Next, the bile acids were extracted with diethyl ether and quantified by GLC with 1.5% QF-1 and 1.5% AN-600 columns. The recovery rate for the intestinal bile acid extraction was not examined, but since that for the fecal bile acids was about 90% (22), similar values would be expected for these procedures.

**Plasma and liver lipid determination:**

Plasma was separated by centrifugation at 3000 rpm for 15 min. About 1 g of the largest lobe of the liver (lobus sinistra externa) was excised and homogenized with nine volumes of ice-chilled water using an ULTRATURRAX TP 18-10 (IKW-WERK, Jänke & Kunkel KG, West Germany). The plasma and the liver homogenate were extracted with 10 volumes of ethanol by refluxing for 20 min at 90-95°C. Portions of the extracts were hydrolyzed in 15% potassium hydroxide-50% ethanol solution at 75-80°C for 20-min, and cholesterol was extracted three times with 2.5 volumes of petroleum ether. The cholesterol was quantified by GLC with a 1% SE-30 column. Phospholipids were determined with the ethanol extracts by the method of Gomori (21). The recovery of the plasma and liver cholesterol was almost 100%, but that for phospholipids was not examined.

**Pool size and synthesis of bile acids:** The pool size of bile acids was obtained by summing the amounts in the bile and small and large intestines (23). In a steady state, the amount of bile acids excreted into the feces was presumed to correspond to that synthesized in the liver.

**Statistical analysis:** The results are expressed as mean values and standard errors of the means. The Student's t-test was used to determine statistical significance.

**Results**

1) **Body weight and diet intake:** Changes in body weight and diet intake are shown in Fig. 2. Spinasterol- and sitosterol-fed mice consumed almost the same amounts of diet as the control, but their body weights were slightly lower than those of the control mice.

2) **Plasma and liver cholesterol and phospholipids:** Spinasterol markedly decreased plasma and liver cholesterol levels, but caused no significant change in the phospholipid levels (Table 1). Sitosterol did not significantly decrease the plasma cholesterol level, but markedly decreased the liver cholesterol level. Liver weights were not changed in both groups.

3) **Biliary cholesterol, phospholipids and bile acids:** Table 2 shows the gallbladder bile
weight and biliary lipid concentrations after the treatment with spinasterol and sitosterol for 15 days. The cholesterol concentrations were reduced, but the phospholipid and bile acid concentrations were not changed.

Biliary bile acid compositions are compared in Table 3. The biliary bile acids in the control mice mainly consisted of cholic acid (about 56%), \( \beta \)-muricholic acid (30%) and minor amounts of deoxycholic acid, 3\( \alpha \), 12\( \alpha \)-dihydroxy-7-oxo-5\( \beta \)-cholanoic acid, chenodeoxycholic acid, ursodeoxycholic acid, hyodeoxycholic acid, \( \alpha \)- and \( \omega \)-muricholic acids and 3\( \alpha \)-hydroxy-6-oxo-5\( \beta \)-cholanoic acid.

### Table 1. Effects of spinasterol and sitosterol on plasma and liver cholesterol and phospholipid levels in mice

|                      | Control       | Spinasterol  | Sitosterol   |
|----------------------|---------------|--------------|--------------|
| Plasma cholesterol (mg/100 ml) | 69±5.7*       | 48±1.3*      | 58±5.3*      |
| Plasma phospholipids (mg/100 ml) | 228±18.9      | 188±8.0      | 237±18.8     |
| Liver cholesterol (mg/g) | 2.7±0.19      | 2.1±0.11*    | 2.0±0.05*    |
| Liver phospholipids (mg/g) | 49.2±1.21     | 47.3±0.18    | 47.6±1.15    |
| Liver wt. (g)         | 1.69±0.08     | 1.57±0.04    | 1.65±0.08    |

* Mean±S.E. in 5 mice. *Statistically significant (P<0.05)

### Table 2. Effects of spinasterol and sitosterol on gallbladder bile, cholesterol, phospholipid and bile acid concentrations in mice

|                      | Control       | Spinasterol  | Sitosterol   |
|----------------------|---------------|--------------|--------------|
| Gallbladder bile (mg/mouse) | 22±1.8*       | 22±1.0       | 20±4.2       |
| Cholesterol (mg/g bile)  | 1.22±0.07     | 0.95±0.12    | 0.69±0.03*   |
| Phospholipids (mg/g bile) | 16.1±1.44     | 14.2±1.52    | 13.3±1.20    |
| Bile acids (mg/g bile)   | 51.1±3.52     | 46.8±7.27    | 47.7±4.37    |

* Mean±S.E. in 5 mice. *Statistically significant (P<0.05)

### Table 3. Effects of spinasterol and sitosterol on biliary bile acid composition (on day 15) in mice

|                      | Control       | Spinasterol  | Sitosterol   |
|----------------------|---------------|--------------|--------------|
| Cholic acid group (%) | 58.4±1.9*     | 52.6±1.8     | 67.6±2.4*    |
| Deoxycholic acid      | 0.9±0.1       | 1.1±0.3      | 2.4±0.5*     |
| Cholic acid           | 55.5±1.9      | 49.7±2.0     | 63.1±2.6*    |
| 3\( \alpha \), 12\( \alpha \)-Dihydroxy-7-oxo- | 2.0±0.2       | 1.9±0.4      | 2.1±0.4      |
| Chenodeoxycholic acid group (%) | 40.1±1.8    | 45.5±1.8     | 30.1±1.3*    |
| Chenodeoxycholic acid | 1.4±0.1       | 0.9±0.2      | 0.7±0.0*     |
| Ursodeoxycholic acid  | 4.3±0.2       | 2.9±0.5*     | 2.7±0.2*     |
| Hyodeoxycholic acid   | 4.3±0.2       | 2.9±0.5*     | 2.7±0.2*     |
| \( \alpha \)-Muricholic acid | 0.7±0.2   | 1.4±0.3      | 0.5±0.1      |
| \( \beta \)-Muricholic acid | 28.2±1.7    | 34.7±1.8*   | 20.8±2.1*    |
| \( \omega \)-Muricholic acid | 3.8±0.7     | 5.1±1.4     | 3.9±0.7      |
| 3\( \alpha \)-Hydroxy-6-oxo- | 1.7±0.4      | 1.4±0.3      | 1.4±0.4      |
| Others                | 1.5±0.2       | 1.8±0.3      | 2.4±1.3      |
| CA/CDCA ratio         | 1.4±0.1       | 1.17±0.09    | 2.27±0.18*   |

* Mean±S.E. in 5 mice. *Statistically significant (P<0.05)
In sitosterol-fed mice, deoxycholic and cholic acids markedly increased; but chenodeoxycholic, ursodeoxycholic (including hyodeoxycholic) and \( \beta \)-muricholic acids decreased. Spinasterol, however, showed no significant change in the total values of both cholic acid and chenodeoxycholic acid groups, but it decreased ursodeoxycholic-hyodeoxycholic acid and increased \( \beta \)-muricholic acid.

**4) Fecal sterols and bile acids:** Changes in the fecal excretion of sterols and bile acids are given in Table 4. The total sterol (cholesterol plus coprostanol) excretion markedly increased soon after the start of the phytosterol feeding, but the increases gradually diminished thereafter. Figure 3 shows the changes in the individual excretion of cholesterol and coprostanol. The cholesterol excretion markedly increased on day 2 and was followed by a gradual decrease. The value on day 15 was still high in the sitosterol-fed mice (\( P<0.05 \)), but not in the spinasterol-fed mice. The coprostanol excretion was not affected throughout the period.

The total bile acid excretion in the control mice remained almost constant, but those in the treated mice decreased with time, and no significant difference was found between the two groups (Table 4).

Table 5 shows the amounts of individual bile acids in the feces on day 15. Deoxycholic acid and \( \alpha \)-, and \( \beta \)-muricholic acids decreased after the treatments. \( \alpha \)-Muricholic acid also decreased, but this was statistically insignificant (\( P>0.05 \)). When the fecal bile acids were divided into two groups according to their metabolic fate, the cholic acid group and the chenodeoxycholic acid group, the decrease in the chenodeoxycholic acid group was larger than that in the cholic acid group, and their ratio (CA/CDCA) increased. The changes in both bile acid groups are shown in Fig. 4. The chenodeoxycholic acid group decreased more markedly than the cholic acid group.

### Table 4. Effects of spinasterol and sitosterol on fecal excretion of sterols and bile acids in mice

| Total sterols (mg/day) | Total bile acids (mg/day) |
|-----------------------|--------------------------|
|                       | Control | Spinasterol | Sitosterol | Control | Spinasterol | Sitosterol |
| Day 0                | 4.63±0.47\(^{a2}\) | 5.35±0.41 | 4.63±0.19 | 2.33±0.24\(^{a3}\) | 2.00±0.13 | 2.04±0.22 |
| 2                    | 4.67±0.56 | 6.75±0.37\(^*\) | 7.30±0.27 | 1.98±0.15 | 1.44±0.10\(^*\) | 1.50±0.18 |
| 4                    | 4.74±0.39 | 6.02±0.21\(^*\) | 5.95±0.25\(^*\) | 2.07±0.11 | 1.23±0.04\(^*\) | 1.20±0.07\(^*\) |
| 6                    | 4.41±0.40 | 5.51±0.39 | 5.61±0.50 | 2.19±0.28 | 1.20±0.07\(^*\) | 1.24±0.04\(^*\) |
| 8                    | 4.13±0.39 | 4.66±0.40 | 5.36±0.54 |               |            |            |
| 15                   | 3.76±0.29 | 4.12±0.20 | 5.64±0.33\(^*\) |               |            |            |

\(^{a2}\) Mean±S.E. in 5 mice. \(^*\)Statistically significant against the control (\( P<0.05 \)).
Table 5. Effects of spinasterol and sitosterol on fecal bile acid excretion<sup>a</sup>) (on day 15) in mice

|                          | Control            | Spinasterol       | Sitosterol        |
|--------------------------|--------------------|-------------------|-------------------|
| Cholic acid group<sup>b</sup>) | 0.75±0.110<sup>a</sup>) | 0.45±0.030<sup>*</sup>) | 0.47±0.044<sup>*</sup>) |
| Deoxycholic acid         | 0.51±0.10 (22.6)   | 0.29±0.03<sup>*</sup> (24.3) | 0.29±0.03<sup>*</sup> (23.6) |
| Cholic acid              | 0.04±0.02 (1.9)    | 0.03±0.00 (2.4)   | 0.02±0.00 (1.9)   |
| 3α, 12β-Dihydroxy-7-oxo  | 0.17±0.08 (7.9)    | 0.13±0.02 (10.5)  | 0.14±0.02 (11.2)  |
| 7β-Cholic acid           | 0.04±0.01 (1.6)    | 0.01±0.00 (0.8)   | 0.01±0.00 (1.0)   |
| Chenodeoxycholic acid    | 1.21±0.164         | 0.53±0.041<sup>*</sup>) | 0.56±0.027<sup>*</sup>) |
| Lithocholic acid         | 0.16±0.03 (7.2)    | 0.11±0.02 (9.2)   | 0.14±0.03 (10.9)  |
| Ursodeoxycholic acid     | 0.01±0.00 (0.4)    | <0.01 (0.0)       | <0.01 (0.3)       |
| Hyodeoxycholic acid      |                    |                   |                   |
| α-Muricholic acid        | 0.07±0.02 (3.0)    | 0.02±0.01<sup>*</sup> (2.0) | 0.02±0.01<sup>*</sup> (1.7) |
| β-Muricholic acid        | 0.50±0.09 (22.5)   | 0.12±0.01<sup>*</sup> (10.3) | 0.13±0.02<sup>*</sup> (10.2) |
| ω-Muricholic acid        | 0.45±0.08 (21.4)   | 0.27±0.04 (23.0)  | 0.27±0.04 (21.9)  |
| 3α-Hydroxy-6-oxo-         | 0.01±0.01 (0.5)    | 0.00±0.00 (0.4)   | 0.01±0.00 (1.2)   |
| Others                   | 0.24±0.06 (10.9)   | 0.21±0.032 (17.2) | 0.20±0.04 (16.2)  |
| CA/CDCA ratio            | 0.63±0.057         | 0.86±0.059<sup>*</sup>) | 0.84±0.074<sup>*</sup>) |

<sup>a</sup>) mg/mouse per day.  <sup>b</sup>) Mean±S.E. in 5 mice.  (% of the total.  *Statistically significant against the control (P<0.05)
5) Pool size and distribution of bile acids:
As shown in Table 6, the pool size of bile acids decreased about 20% after the treatments with spinasterol and sitosterol, but the latter decrease was statistically insignificant (P<0.05). Under our experimental conditions of 12 hr darkness from 8.00 p.m. to 8.00 a.m. and 5 hr fasting, about 25% of the bile acids became localized in the gallbladder, about 60% in the small intestine and about 13% in the large intestine. Both phytosterols caused no significant change in the distribution of bile acids, but showed a tendency to decrease the bile acid in the bile.

Discussion
Spinasterol and sitosterol markedly increased the fecal excretion of cholesterol and decreased the liver cholesterol levels in mice. These data suggest that spinasterol, as well as sitosterol, inhibited cholesterol absorption, in agreement with the previous reports on sitosterol (1-9) and diosgenin (12, 13).

The increase in the fecal cholesterol excretion, in the spinasterol and sitosterol-fed mice, however, was highest on day 2 and gradually declined thereafter. Grundy and Mok (15) observed a similar trend of change in humans after treatment with sitosterol. Changes in the diet intake would alter the amounts of fecal sterols since the diet is a source of exogenous cholesterol, but the diet intakes for the spinasterol- or sitosterol-fed mice were almost comparable to that for the control mice. Another source of fecal sterols is biliary cholesterol, but the amount was very small, being at maximum 5% of that in the diet. In addition, fecal excretion of coprostanol in the spinasterol and sitosterol treated mice remained unchanged, and fecal bile acid excretion decreased. Cohen et al. (24) observed that sitosterol produced no increase in the fecal coprostanol excretion in rats. Therefore, we cannot explain the decline of the fecal cholesterol excretion, but a cholesterol pool, which would be excreted upon the first administration of phytosterols, should have been present in the intestinal lumen.

Table 6. Effects of spinasterol and sitosterol on the pool size and distribution of bile acids in mice

|                  | Control          | Spinasterol     | Sitosterol      |
|------------------|------------------|-----------------|-----------------|
| Pool size (mg/mouse) | 6.51±0.48*       | 5.27±0.17*      | 5.39±0.28       |
| Bile             | 1.61±0.38 (24.7%)| 0.94±0.24 (17.8%)| 0.97±0.25 (18.0%)|
| Small intestine  | 4.05±0.67 (62.2%)| 3.52±0.15 (66.8%)| 3.76±0.31 (69.8%)|
| Large intestine  | 0.85±0.16 (13.0%)| 0.81±0.10 (15.4%)| 0.66±0.06 (12.2%)|

*a) Mean±S.E. in 5 mice. ( ) % of the total
As mentioned above, fecal bile acid excretion, especially of those derived from chenodeoxycholic acid, was decreased by the spinasterol and sitosterol treatments (Table 4 and Fig. 3). This was not consistent with previous reports on humans after sitosterol treatment (1, 15) or on rats after diosgenin treatment (12, 13). This discrepancy may be due to the species difference between humans, mice and rats.

In a steady state, the amount of fecal bile acids is assumed to represent the amount of bile acids synthesized daily. Under our experimental conditions, bile acid synthesis decreased, and chenodeoxycholic acid formation was depressed much more than cholic acid formation after the treatment with phytosterols (Fig. 3), resulting in an increase in the CA/CDCA ratio (Table 5). The decrease in bile acid synthesis would be due to the decrease in cholesterol absorption, and the decrease in cholesterol absorption in turn would increase hepatic cholesterol synthesis since it is regulated through the negative feedback mechanism and mainly by the amount of exogenous cholesterol (25). We did not determine the hepatic activities for synthesizing cholesterol in the present experiments with mice but others have reported an increase in cholesterol synthesis in rats given sitosterol (6) and diosgenin (13).

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. (26, 27) and Mitropoulos et al. (28) demonstrated that newly synthesized cholesterol in the liver is mainly converted to cholic acid. This was confirmed by the experiments of Gustafsson et al. (29) who showed that prevention of bile acid absorption by the feeding of cholestyramine resulted in increases of cholesterol and cholic acid syntheses. On the other hand, excessive feeding of cholesterol increased the synthesis of bile acids, especially that of chenodeoxycholic acid (14, 18, 30). In other words, newly synthesized cholesterol in the liver is mainly metabolized to cholic acid, but cholesterol from the diet to chenodeoxycholic acid. This biological regulation for bile acid synthesis was observed in rats, but if a similar regulation mechanism took place in mice, this would explain the marked decrease in chenodeoxycholic acid synthesis after the treatment with phytosterols. The biliary CA/CDCA ratio increased after the treatment with sitosterol reflecting the decrease of chenodeoxycholic acid synthesis, but the ratio in the mice given spinasterol did not increase (Table 3). This discrepancy should be examined in further experiments. The fecal CA/CDCA ratios on days 4 and 8 in the control mice were 0.64 and 0.69, while those in the spinasterol-fed mice were 0.84* and 1.02*, respectively (*statistically significant against the control, P<0.05).

The mechanism by which phytosterols inhibit cholesterol absorption is not fully understood, but the following explanations have been suggested: a) formation of a nonabsorbable complex with cholesterol (3), b) inhibition of cholesterol esterification (31) which is a usual, but not an obligatory reaction for sterol absorption, and c) competition with cholesterol for the absorption sites in the intestinal mucosa (4, 5). In addition to these, a decrease of bile acids in the bile and small intestine may be a cause for the decrease of cholesterol absorption since bile acids play an essential role in cholesterol absorption.

In regard to the hypocholesterolemic effect of phytosterols, an "extra absorptive" action of the phytosterols is proposed, since sitosterol decreased the serum cholesterol level in people maintained on a cholesterol-free diet (32). Tabata et al. (33) suggested that the phytosterols enhance cholesterol conversion to bile acids. If the phytosterols
act directly on the cholesterol metabolism, they should be absorbed at least to some extent. In the present study, sitosterol was found to be absorbed to almost double its concentration in the plasma, liver and bile on day 15, while spinasterol was absorbed to a far less extent since its amount of spinasterol in the plasma, liver and bile was almost negligible. Ikeda and Sugano (34) showed that the absorption rate was smaller for sitostanol than for sitosterol, probably due to saturation of the $\Delta^6$ double bond. The low absorption rate for spinasterol may be related to its chemical structure (Fig. 1) since it possesses no $\Delta^5$ double bond but a $\Delta^7$ double bond.

Acknowledgements: We sincerely thank Dr. S. Seo of Shionogi Research Laboratories for the preparation of spinasterol and also Messrs. H. Takase and Y. Nomura and Dr. S. Hashimoto of the same laboratories for their help and discussions. We are also indebted to Miss F. Koyama for preparing the manuscript.

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