# Effect of Chitosan Feeding on Intestinal Bile Acid Metabolism in Rats

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The effect of chitosan feeding (for 21 days) on intestinal bile acids was studied in male rats. Serum cholesterol levels in rats fed a commercial diet low in cholesterol were decreased by chitosan supplementation. Chitosan inhibited the transformation of cholesterol to coprostanol without causing a qualitative change in fecal excretion of these neutral sterols. Increased fiber consumption did not increase fecal excretion of bile acids, but caused a marked change in fecal bile acid composition. Lithocholic acid increased significantly, deoxycholic acid increased to a lesser extent, whereas hyodeoxycholic acid and the 6B-isomer and 5-epimeric 3a-hydroxy-keto-cholanoic acid(s) decreased. The pH in the cecum and colon became elevated by chitosan feeding which affected the conversion of primary bile acids to secondary bile acids in the large intestine. In the cecum, chitosan feeding increased the concentration of ***a-, B, and*** w-muricholic acids, and lithocholic acid. However, the levels of hyodeoxycholic acid and its 6B-isomer, of monohydroxy-monoketo-cholanoic acids, and of 3*a*, 6E, 7E-trihydroxy-cholanoic acid decreased. The data suggest that chitosan feeding affects the metabolism of intestinal bile acids in rats.

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Chitosan (glucosamine polymer) is known to have a marked hypocholesterolemic effect in cholesterol-fed rats (14). Sugano ***et al.*** (5) recently reported that the hypocholesterolemic effect of chitosan did not differ within the viscosity range tested (17-1620 cps), while glucosamine oligomer proved ineffective. Vahouny ***et al.*** (6) found that chitosan was effective in lowering cholesterol absorption in thoracic duct lymph.

Sugano ***et al.*** (1,4) also showed that chitosan lowered the transformation of cholesterol to coprostanol and increased the excretion of these neutral sterols. In contrast, the fecal excretion of bile acids was essentially unchanged (4). It is well known that primary bile acids are converted into secondary bile acids by intestinal bacteria (7-9). The secondary bile acids, lithocholic acid and deoxycholic acid, have been correlated with the risk of bowel cancer and gallstones (10-12). Because the ingestion of some types of dietary fibers has been shown to influence the intestinal flora (13-15), it appeared possible that chitosan may also influence bile acid metabolism. In the present study we investigated the effect of chitosan supplementation of a normal diet (0.1% cholesterol) on the metabolism of intestinal bile acids in rats.

## MATERIALS AND METHODS

*Materials.* The chitosan used in this study was donated by Professor S. Hirano, Faculty of Agriculture, Tottori University, Tottori, Japan. It was a high viscosity grade preparation with a mean molecular weight of 250,000. The degree of deacetylation was approximately 94%.

3a-Hydroxy-6-keto-5Bchoanoic acid and 3a-hydroxy-6-keto-5a-cholanoic acid were synthesized according to Thomas ***et al.*** (16) and Bergstrom ***et al.*** (17), respectively.

Reagents and organic solvents were of analytical grade. Solvents were distilled, if necessary, before use.

***Animals and diet.*** Male Wistar strain rats were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan), and were housed individually in an air-conditioned room (23 +/- 1°C) with lighting from 6 a.m. to 6 p.m. After acclimation for two weeks, the animals were divided into three groups. One group was fed a control diet consisting of a commercial powder diet (Clea CA-l, Nihon Clea Co., Tokyo, Japan), supplemented with 0.5% cellulose and 4.5% sucrose: the second group was fed a diet supplemented with 0.5% cellulose, 2.5% sucrose and 2% chitosan: the third group was fed a 5% chitosan-supplemented diet. The stock powder diet contained 25.6% protein, 4.6% fat, 4.0% fiber and all vitamins at recommended values. The diet contained 1.05 ± 0.09 ***mg/g*** of cholesterol (mean +/- S.D., n=4) and 98.0 +/- 5.1 ug (mean +/- S.D., n=4) of cholic acid as analyzed in our laboratory. The animals were maintained on these diets ***ad libitum*** for 21 days. Food intake was measured daily, and body weight was determined every second day. Feces were collected for the two days prior to killing. Animals were sacrificed by cardiac puncture under diethyl ether anesthesia. The serum was separated from blood, and the intestine was excised. The entire small intestine was divided into three segments: duodenum, jejunum and ileum, as described by Dietschy and Siperstein (18). The large intestine was divided into two parts: the cecum and colon. The intestinal contents were removed from each segment for measurement of pH and bile acid analysis. The pH of the intestinal contents was estimated directly from the samples using a Horiba Electrometer pH meter. All sampling procedures were performed between 9 and 11 a.m., and all samples were stored at -20°C prior to analysis.

***Analysis.*** Frozen fecal samples were lyophilized, pulverized, and weighed before analysis; 500 mg specimens of blended feces were used for crude extraction of bile acids by refluxing in 90% ethanol for 2 hr. Refluxing was repeated twice, and the pooled extracts were evaporated. The extracts were hydrolyzed by refluxing in 5% NaOH/5O% ethanol for 2 hr. And after cooling, the neutral sterols were extracted with n-hexane. The neutral sterols, analyzed by gas-liquid chromatography, were cholesterol and coprostanol. The alkaline solution was adjusted to neutrality by adding dilute HCI dropwise and then evaporated to dryness. The extracts were chromatographed on Amerlite XAD-2 (Rohm and Haas, Philadelphia, PA) to obtain the bile acid fraction. The steps used in the analysis of bile acids were absorption, elution, solvolysis, hydrolysis, extraction, and preparation of methyl ester acetate derivatives. All these steps have been previously described in detail, including the analysis of samples by GLC at 230°C using a Shimadzu Model GC-3BF unit with a coiled glass column (3mm X 1.75m), filled with 1.5% OV-1 on Gas Chrom Q 100-120 mesh (Shimadzu Co., Kyoto, Japan) (19). Quantitative results were obtained by comparing peak areas with those of known amounts of reference compounds.

In order to identify the fecal bile acids, aliquots of the bile acid fraction were fractionated on a column of silicic acid (AR, 100 mesh: Mallinckrodt. St. Louis, MO) (20), and the bile acid samples were analyzed by gas-liquid chromatography mass spectrometry (GC/MS) using a JMS-DX 303 mass spectrometer (JEOL. Tokyo, Japan) connected to a Hewlett-Packard HP 5890A gas chromatograph. A glass column (2 mm x 1.2m) packed with 1% OV-1 on Gas Chrom Q (100-120 mesh) was used. The conditions for analysis were: injection temperature, 270°C; column temperature, 250°C; ion source temperature, 250°C; ionization voltage, 70 eV; ionization current, 300 μA; carrier gas, He (27.5 ml/min.)

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**Abbreviations: GC/MS, gas-liquid chromatography mass spectrometry; MC, muricholic acid; HD, hyodeoxydiolic acid.**

The significance of differences between the means of the control and test groups was determined by one-way analysis of variance followed by Student's t-test.

## RESULTS

As shown in Table 1, there were no significant difference in growth, food intake, liver weight and dried fecal weight between the control and either of the chitosan-fed groups after 2 1 days. The serum cholesterol level was depressed by the 5% chitosan-supplemented diet group compared with the control group. There was no significant difference in fecal excretion of neutral sterols and bile acids between the control group and either of the chitosan treated groups.

The same bile acids that were found in the intestine and/or feces of control rats were also present in the respective samples from both chitosan-treated group (Table 2). Three of the compounds found in the feces-X, Y, Z-remain unidentified. The fecal bile acids were fractionated into monohydroxy (I and II), dihydroxy (III and IV), dihydroxy plus trihydroxy (V and VI), and trihydroxy (VII and VIII) fractions by chromatography on a silicic acid column. Both compounds X and Y were eluted in fraction II. The mass spectra of these compounds showed peaks at m/z 446 (M-), 386 (M+-60), 371,313, 271 and 253 [m+- (18+60+115)], indicating that they were monohydroxy-monoketo-cholanoic acid derivatives. Fragments m/z 231 and 121 from compound X are indicative of a keto group at C-12. Compound Y showed an ABC ring fragmentation ion at *m/z* 229, indicating a keto group at position 3 or 6. Fragment *m/z* 244 is indicative of a keto group at C-6. 6-Keto-5β-cholanoic acids are transformed to their 5a-forms in the presence of excess $OH^-$ or $H^+$ (2 1 ), but this allomerization is incomplete data from capillary gas chromatographic analysis, (not shown). The mass spectra of compound Z showed peaks at m/z 428, 368, 353, 313, and 253 [M-(3 x 60 + 115)], indicative of a triacetoxy derivative. The intense peak at m/z 386 [$M^+$-(60 plus an epoxide between C6 and C7)] is characteristic for the 3, 6, 7-triacetoxy compound but there were no specific fragments at *m/z* 446 and 27 1 as found for a- and B-muricholic acid. From these results, compounds X and Y appear to be 3α-hydroxy-12-keto-5B-cholanoic acid and 5-epimeric 3α-hydroxy-6 ketocholanoic acid(s), respectively. Compound Z appears to be 3α, 6E, 7E-trihydroxycholanoic acid.

TABLE 1

**Growth, Food Intake. Liver Weight. Fecal Weight. and Fecal Neutral Sterols and Bile Acids in rats[a]**

| Observations | Control (n=5) | 2% Chitosan (n=4) | 5% Chitosan (n=6) |
|---|---|---|---|
| Growth[b] (g) | 70.0 +/- 6.5 | 65.0 +/- 11.0 | 78.0 +/- 9.4 |
| Food intake (g/day) | 23.5 +/- 0.9 | 24.2 +/- 1.1 | 24.8 +/- 0.6 |
| Liver weight (g. 100g body weight) | 5.1 +/- 0.3 | 4.6 +/- 0.3 | 4.6 +/- 0.2 |
| Dried fecal weight (g/day) | 3.0 +/- 0.3 | 3.2 +/- 0.3 | 2.9 +/- 0.1 |
| Serum cholesterol (g/dL) | 45.2 +/- 2.9 | 41.8 +/- 3.3 | 36.1 +/- 2.3' |
| Fecal neutral sterol excretion (mg. day) | 15.87 +/- 1.97 | 14.87 +/- 2.21 | 17.68 +/- 0.97 |
| Fecal bile acid excretion (mg. day) | 12.16 +/- 0.90 | 9.94 +/- 1.14 | 12.64 +/- 0.80 |

[a] Data are expressed as mean +/- SEM.
[b] Weight gain.
[c] Significantly different from control, $p<0.05$.

TABLE 2

Profiles of Bile Acids by GLC and GC/MS Analysis

| Bile acid | Retention time relative to deoxycholic acid (18.2 min.) OV-1 | Characteristic ions (*m/z*) in GC/MS analysis[a] |
|---|---|---|
| Lithocholic | 0.77 | |
| Deoxycholic | 1 .oo | |
| Chenodeoxycholic | 1.18 | |
| Compound X | 1.23 | 446 (61.8), 428 (3.7), 386 (82.3), 371 (10.3), 313 (17.6), 271 (25.0), 253 (20.6), 213 (100), 121 (69.9) |
| Cholic | 1.31 | |
| Compound Y | 1.33 | 446, 386, 371, 313, 271, 253, 244, 229 |
| Hyodeoxycholic and its 6B-isomer | 1.50 | |
| a-Muricholic | 1.99 | |
| Compound Z | 2.12 | 428 (17.0), 386 (100), 368 (18.8), 353 (8.9), 313 (10.7), 253 (17.0) 211 (8.9), 159 (42.9) |
| w-Muricholic | 2.35 | |
| B-Muricholic | 2.48 | |

"Number in parentheses shows the relative intensity.

**TABLE 3**

Composition of Fecal Bile Acids and Neutral Sterols in Rat Feces'

| Composition | Dietary group: Control (n=5) | 2% Chitosan (n=4) | 5% Chitosan (n=6) |
|---|---|---|---|
| Bile acid (%) | | | |
| Lithocholic | 3.5 +/-t 0.8 | 5.9 +/- 1.6 | 15.3 +/- 2.7[b] |
| Deoxycholic | 12.0 +/- 2.6 | 13.4 +/- 4.1 | 20.0 +/- 4.1 |
| Hyodeoxycholic and its 6BP-isomer (HD) | 22.4 +/- 6.8 | 20.9 +/- 6.4 | 1.3 +/- 0.2[b] |
| a-Muricholic | 0.3 +/- 0.1 | 2.2 +/- 0.1 | 1.8+/- 0.1 |
| B-Muricholic | 5.5 +/- 1.2 | 5.2 +/- 0.2 | 5.6 +/- 0.1 |
| w-muricholic (w-MC) | 21.0 +/- 5.6 | 18.7 +/- 4.6 | 29.2 +/- 3.6 |
| 3a-Hydroxy-12-keto-5Bcholanoic | 16.7 +/- 3.5 | 16.5 +/- 4.1 | 20.0 +/- 3.1 |
| 5-Epimeric 3α-hydroxy-6-keto-cholanoic | 14.3 +/- 2.7 | 13.4 +/- 4.4 | 1.1 +/- 0.1[b] |
| 3α, 6ξ, 7ξ-Trihydroxycholanoic | 4.3 +/- 0.7 | 3.8 +/- 1.2 | 5.7 +/- 0.4 |
| w-MCHD ratio | 0.94 | 0.89 | 22.5 |
| Neutral sterol (%) | | | |
| Coprostanol | 57.5 +/- 13.0 | 54.2 +/- 13.1 | 21.6 +/- 3.6[b] |
| Cholesterol | 42.5 +/-f 8.1 | 45.8 +/- 12.7 | 78.4 +/- 6.1[b] |

[a] Data are expressed as mean +/- SEM.
[b] Significantly different from control, $p<0.05$.

**TABLE 4**

pH of Intestinal Contents[a]

| Intestine | Control (n=5) | 2% chitosan (n=4) | 5%Chitosan (n=6) |
|---|---|---|---|
| Duodenum | 6.32+-0.10 | 6.28 +- 0.11 | 6.45 +- 1.10 |
| Jejunum | 6.25 +- 0.13 | 6.32 +- 0.13 | 6.45 +- 0.05 |
| Heum | 7.13 +- 0.26 | 7.05 +- 0.13 | 7.01 +- 0.06 |
| Cecum | 5.88 +-f 0.06 | 6.18 +-f 0.20 | 6.52 +-_ 0.09' |
| Colon | 5.79 +- 0.04 | 6.06 +- 0.20 | 6.52 +- O.O9b |

"Data are expressed as mean +/- SEM.
[b]Significantly different from control,$p<0.05$.

**TABLE 5**

Comnosition of Bile Acids in the Duodenum[a]

| Bile acid | Composition (%): Control (n=5) | 5% Chitosan (n=6) |
|---|---|---|
| Deoxycholic | 7.0 +- 1.4 | 4.4 +- 0.7 |
| Chenodeoxycholic | 4.0 +- 0.0 | 2.7 +- 0.4[b] |
| Hyodeoxycholic | 11.1 +- 1.5 | 4.8 +- 0.8[b] |
| Cholic | 53.1 +- 3.4 | 59.7 +- 6.2 |
| a-Muricholic | 3.5 +- 0.5 | 3.3 +- 0.5 |
| B-Muricholic | 21.5 +- 2.5 | 25.1 +- 4.6 |

[a]Data are expressed as mean +/- SEM.
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The composition of fecal bile acids and neutral sterol is shown in Table 3. In control rats, 3α-hydroxy-12-keto-5β-cholanoic acid,ω-muricholic acid and hyodeoxycholic acid and its 6β-isomer were the predominant bile acids (>15%); small amounts (<5%) of a-muricholic acid, lithocholic acid, and 3α, 6ξ, 7ξ-trihydroxycholanoic acid were also present. Feeding 5% chitosan to rats resulted in an increase in lithocholic acid, a smaller increase in deoxycholic acid ($p<0.2$), and a decrease in hyodeoxycholic acid and its 6β-isomer, and in 5-epimeric 3α-hydroxy-6-ketocholanoic acid(s). There was a decrease in coprostanol and an increase in cholesterol in the 5% chitosan-treated group compared with controls.

Table 4 shows the pH of the contents of various intestinal segments. The pH of the contents of the cecum and of the colon in the 5% chitosan-treated group was higher than for the controls.

Table 5 shows the bile acid compositioni in the duodenum. In the chitosan-treated group, hyodeoxycholic acid and its 6β-isomer, as well as chenodeoxycholic acid were decreased as compared to controls.

As shown in Table 6, in the cecum of the chitosan-fed group, lithocholica cid, and a-, β- and o-muricholic acids increases. By contrast, hyodeoxycholic acid and its 6β-isomer, monohydroxy-monoketo-cholanoic acids, and 3α, 6ξ, 7ξ-trihydroxy-cholanoic acid decreased. Chitosan feeding expanded the neutral sterol pool and concomitantly caused a decrease in coprostanol and an increase in cholesterol.

## Discussion

Chitosan feeding induced a significant reduction of plasma cholesterol in rats fed a hypercholesterolemic diet consistent with previous reports (l-5). The present study also shows that chitosan feeding (5%) reduces serum cholesterol levels in rats fed a low cholesterol diet (0.1%). Dietary fiber may alter the excretion or metabolism of cholesterol and bile acids in the gut and thus influence plasma cholesterol levels. Cholesterol is converted to coprostanol by intestinal microflora (22). In our experiments, chitosan feeding suppressed the formation of coprostanol, as was observed earlier by Sugano *et al.* (1,4). While chitosan increased the excretion of neutral sterols in rats fed a high cholesterol diet, there was no increase in fecal neutral sterols in our experiments with rats fed a low cholesterol diet. The cholesterol pool in the cecum was expanded by chitosan feeding; however, the amount of cholesterol that accumulated in the cecum may have been insufficient to increase the excretion of cholesterol. It is, therefore, likely that cholesterol that was not absorbed by the proximal region of the small intestine, the site of cholesterol absorption (23), accumulated in the cecum which may explain decreased plasma cholesterol levels.

Dietary fiber affected the composition of fecal bile acids but did not alter their excretion. Primary bile acids are converted to secondary bile acids by intestinal bacteria in the cecum and colon (24). Bacterial 7α-dehydroxylation converts cholic acid to deoxycholic acid (7), and chenodeoxycholic acid to lithocholic acid (8). Lithocholic acid and deoxycholic acid are carcinogenic and toxigenic (10, 11). Although lithocholic acid and deoxycholic acid are detoxified by hydroxylation in liver in the course of enterohepatic circulation (25-28), chitosan feeding caused an increase in lithocholic acid and a smaller increase in deoxycholic

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| Composition | Dietary group: Control (n=5) | 2% Chitosan (n=4) | 5% Chitosan (n=6) |
|---|---|---|---|
| Bile acid (%) | | | |
| Lithocholic | 3.5 ± 0.8 | 5.9 ± 1.6 | 15.3 ± 2.7 |
| Deoxycholic | 12.0 ± 2.6 | 13.4 ± 4.1 | 20.0 ± 4.1 |
| Hyodeoxycholic and its 6β-isomer (HD) | 22.4 ± 6.8 | 20.9 ± 6.4 | 1.3 ± 0.2[b] |
| a-Muricholic | 0.3 ± 0.1 | 2.2 ± 0.1 | 1.8 ± 0.1 |
| β-Muricholic | 5.5 ± 1.2 | 5.2 ± 0.2 | 5.6 ± 0.1 |
| ω-Muricholic (ω-MC) | 21 .O ± 5.6 | 18.7 ± 4.6 | 29.2 ± 3.6 |
| 3α-Hydroxy-12-keto-5β-cholanoic | 16.7 ± 3.5 | 16.5 ± 4.1 | 20.0 ± 3.1 |
| 5-Epimeric 3α-hydroxy-6-keto-cholanoic | 14.3 ± 2.7 | 13.4 ± 4.4 | 1.1 ± 0.1[b] |
| 3α,6ξ,7ξ-Trihydroxycholanoic | 4.3 ± 0.7 | 3.8 ± 1.2 | 5.7 ± 0.4 |
| ω-MC/HD ratio | 0.94 | 0.89 | 22.5 |
| Neutral sterol (%) | | | |
| Coprostanol | 57.5 ± 13.0 | 54.2 ± 13.1 | 21.6 ± 3.6[b] |
| Cholesterol | 42.5 ± 8.1 | 45.8 ± 12.7 | 78.4 ± 6.1[b] |

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| Heum | 7.13 ± 0.26 | 7.05 ± 0.13 | 7.01 ± 0.06 |
| Cecum | 5.88 ± 0.06 | 6.18 ± 0.20 | 6.52 ± 0.09[b] |
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TABLE 5

Composition of Bile Acids in the Duodenum[a]

| Bile acid | Composition (%): Control (n=5) | 5% Chitosan (n=6) |
|---|---|---|
| Deoxycholic | 7.0 ± 1.4 | 4.4 ± 0.7 |
| Chenodeoxycholic | 4.0 ± 0.0 | 2.7 ± 0.4[b] |
| Hyodeoxycholic | 11.1 ± 1.5 | 4.8 ± 0.8[b] |
| Cholic | 53.1 ± 3.4 | 59.7 ± 6.2 |
| a-Muricholic | 3.5 ± 0.5 | 3.3 ± 0.5 |
| β-Muricholic | 21.5 ± 2.5 | 25.1 ± 4.6 |

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