Effects of Aminotriazole Treatment on Biosyntheses of Primary Bile Acids *in Vivo*

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The influence of aminotriazole treatment on primary bile acid biosynthesis was studied in detail. After administration of aminotriazole to rats, bile was collected for 8 h. The content of chenodeoxycholic acid in the bile was increased to 144% of the control by aminotriazole treatment, but that of cholic acid was decreased to 48.4%. In another experiment, [4-^{14}C]cholesterol was injected into rats immediately after aminotriazole treatment, and then bile was collected. The content of radioactive chenodeoxycholic acid in the bile was significantly increased to 130% of the control, but that of radioactive cholic acid was unchanged. In a similar experiment with [2-^{14}C]mevalonate, the content of radioactive chenodeoxycholic acid in the bile was hardly changed by aminotriazole treatment, but that of radioactive cholic acid was greatly decreased to 41.2% of the control. Aminotriazole treatment did not affect the ratios of tauroconjugate to glycoconjugate of the two bile acids.

Thus, aminotriazole treatment affects the syntheses of not only cholesterol (F. Hashimoto, C. Sugimoto and H. Hayashi, *Chem. Pharm. Bull.*, 38, 2532 (1990); F. Hashimoto and H. Hayashi, *Biochim. Biophys. Acta*, 1086, 115 (1991)) but also primary bile acids *in vivo*. Namely, aminotriazole treatment activated biosynthesis of chenodeoxycholic acid from exogenous cholesterol, but did not affect that of cholic acid. Aminotriazole hardly affected the synthesis of chenodeoxycholic acid through endogenous cholesterol (from mevalonate), but inhibited that of cholic acid.

Keywords aminotriazole; bile acid; cholesterol; mevalonate; peroxisome

Introduction

Peroxisomes participate in the biosyntheses of cholesterol[1-4] and bile acids.[5-8] In peroxisomes, oxidases such as urate oxidase, D-amino acid oxidase and fatty acyl-coenzyme A (CoA) oxidase are present, and produce H_2O_2 in the course of their reactions. Hydrogen peroxide thus produced is immediately removed by catalase localized in peroxisomes. However, catalase activity is irreversibly inhibited by aminotriazole. We have previously reported that the bile acid content in bile is reduced by administration of aminotriazole to rats,[7] and we recently clarified that cholesterol synthesis from mevalonate is inhibited by aminotriazole treatment *in vivo*.[9]

Bile acids are synthesized from exogenous cholesterol generated from the diet or extrahepatic organs, and endogenous cholesterol synthesized within the liver. These *de novo*-synthesized bile acids are called primary bile acids (chenodeoxycholic acid and cholic acid). The primary bile acids are converted to secondary bile acids, such as lithocholic acid and deoxycholic acid, by intestinal bacteria.

The inhibition of cholesterol synthesis from mevalonate by aminotriazole treatment[9] may at least partially contribute to the decrease of bile acid content in bile. However, the effect of aminotriazole treatment on biosyntheses of primary bile acids is still unknown. In the present work, we studied the influence of aminotriazole treatment on primary bile acid biosynthesis using ^{14}C-cholesterol as exogenous cholesterol, and ^{14}C-mevalonate as a source of endogenous cholesterol.

Materials and Methods

Materials Aminotriazole was obtained from Tokyo Chemical Industry (Japan). Bile acids were purchased from Sigma Chemicals (U.S.A.). [4-^{14}C]Cholesterol (2.22 GBq/mmol) and [2-^{14}C]mevalonolactone (1.85 GBq/mmol) were obtained from NEN Research (U.S.A.). All other reagents were of analytical grade from Wako Pure Chemicals, Ltd. (Japan).

Treatments of the Rats Male Wistar rats (250—300 g) were maintained on a 12-h light-dark cycle. Bile duct cannulation was carried out 4 h into the light cycle, and bile was collected. Aminotriazole was intraperitoneally administered to the rats at a dose of 1 mg per 1 g body weight in physiological saline at 24 h after the operation. Control rats were injected with the same volume of physiological saline instead of the aminotriazole solution.

In the radioisotope experiment, ^{14}C-cholesterol (14.8 kBq/0.2 ml of saline containing 2% Tween 80) or ^{14}C-mevalonolactone (29.6 kBq/0.2 ml of saline containing 2% Tween 80) was administered to the rats through the femoral vein immediately after the injection of aminotriazole.

Determination of Bile Acid Content Bile acid content in bile was colorimetrically determined according to the method of Eastwood *et al.*,[10] or assayed by gas-liquid chromatography. For gas-liquid chromatography, a bile sample was diluted with 9 volumes of 0.5 M phosphate buffer (pH 7.0), and passed through a Sep-pak C_{18} cartridge (Waters, U.S.A.). After successive washing of the cartridge with water and 1.5% ethanol, bile acid was eluted with 90% ethanol,[11] and dried under nitrogen. Bile acids were hydrolyzed in 10% KOH–50% ethanol solution at 121 °C for 4 h. The hydrolyzate was acidified and extracted with ethyl acetate, followed by addition of estriol (100 μg) as an internal standard. The mixture was allowed to react with hexafluoroisopropyl alcohol and trifluoroacetic anhydride at 60 °C for 45 min. After evaporation of the reagent, the derivatives were dissolved in *n*-hexane. An aliquot was subjected to gas-liquid chromatography (Silicone DC QF-1 2%, Chromosorb WHP 80/100 glass column, 3 mm i.d. × 2 m, injection temperature 240 °C, column temperature 220 °C, flow rate of N_2 45 ml/min).

Incorporation of Radioactivity into Bile Acids in Bile A bile sample was diluted with 9 volumes of 0.5 M phosphate buffer (pH 7.0), and then bile acid was eluted with 90% ethanol through a Sep-pak C_{18} cartridge as stated above. Bile acids in bile are mainly present as tauro- or glyco-conjugates. Taurocholate, glycocholate, taurochenodeoxycholate and glycochenodeoxycholate (200 μg each) were added to the concentrated eluate as markers. These samples were separated into 2 fractions and subjected to thin-layer chromatography using LK 6F plates (20 × 20 cm, thickness 0.25 mm, Whatman, U.S.A.). For good separation of tauro- and glyco-conjugates, one fraction was developed with the solvent system of *n*-butyl alcohol–acetic acid–water (17 : 2 : 1, v/v/v), and the other fraction was developed with the solvent system of chloroform–ethyl alcohol–28% ammonium (25 : 35 : 1, v/v/v). After development, the plates were sprayed with 20% H_2SO_4 and heated. With the former solvent system, taurochenodeoxycholate (*Rf* value 0.353) was developed to nearly the same position as glycocholate (*Rf* 0.406) on thin-layer chromatography, and so the areas corresponding to taurocholate (*Rf* 0.244) and glycochenodeoxycholate (*Rf* 0.567) were scraped into centrifuge tubes. With the latter solvent system, glycochenodeoxycholate (*Rf* 0.170) was developed to nearly the same position as taurocholate (*Rf* 0.217), and therefore the areas corresponding to glycocholate (*Rf* 0.085) and taurochenodeoxycholate (*Rf* 0.366) were scraped off. Bile acid was extracted with Folch's solution and the solvent was evaporated off. Radioactivities of the samples were determined with an Aloka scintillation counter (Model LSC-700, Japan) in scintillation fluid consisting of 2,5-diphenyloxazole (PPO) (4 g) and

1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) (50 mg) in 1 l of toluene and Triton X-100 (2 : 1).

Results and Discussion

Changes in Bile Acid Composition in Bile In order to study the effect of aminotriazole treatment on biosyntheses of primary bile acids, we tried to identify the time when secondary bile acids disappear and primary bile acids account for nearly all the bile acids in bile. When bile acid was colorimetrically assayed,[10] its excretion rate into bile was found to be 12.1 ± 2.7 mg/h (mean ± S.D. of 5 animals) during the first 2 h after the operation, and then decreased rapidly. The excretion rate from 8 to 24 h was almost constant (0.92 ± 0.26 mg/h). Figure 1(A) shows the excretion rate of bile acids into bile collected at 2-h intervals from 14 h after implantation of a bile duct fistula. Bile acids were determined by gas-liquid chromatography. Secondary bile acids (deoxycholic acid, ursodeoxycholic acid and lithocholic acid) gradually disappeared, and primary bile acids (cholic acid and chenodeoxycholic acid) began to increase at about 24 h after the operation. Figure 1(B) shows the composition of bile acids in the bile. Primary bile acids accounted for about 75% of the total up to 18 h, and more than 95% after 24 h. From these results, we decided to use the bile at 24 h after the operation in order to study the effect of aminotriazole treatment on syntheses of primary bile acids.

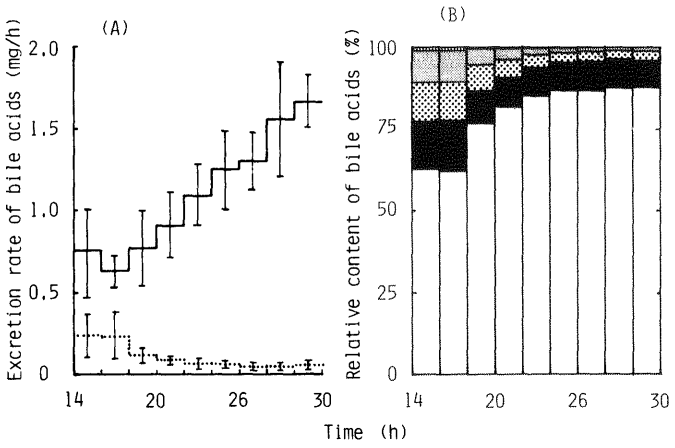

Fig. 1. Changes in Excretion Rate and Composition of Bile Acids in Bile after Bile-Duct Fistulization

Bile was collected at 2-h intervals from 14 h after implantation of a bile duct fistula. Bile acids were extracted from the bile, and analyzed by gas-liquid chromatography after hydrolysis as described in the text. Data are means ± S.D. of 3 animals. (A), excretion rates of primary bile acids (——) and secondary bile acids (– – –). The former group contains chenodeoxycholic acid and cholic acid. The latter contains lithocholic acid, deoxycholic acid and ursodeoxycholic acid; (B), relative content of bile acids in bile. ▥, lithocholic acid; ▨, deoxycholic acid; ▩, ursodeoxycholic acid; ■, chenodeoxycholic acid; □, cholic acid.

Effects of Aminotriazole Treatment on Content of Primary Bile Acids in Bile Figure 2 shows the effect of aminotriazole treatment on cumulative excretion of chenodeoxycholic acid and cholic acid into the bile. The time of 24 h after the operation is displayed as zero time. Chenodeoxycholic acid rapidly increased after aminotriazole treatment, reaching 144% of the control at 8 h. In contrast, cholic acid was clearly decreased to 48.4% of the control. The cholic acid: chenodeoxycholic acid ratio was changed from 11.6 to 3.92 by aminotriazole treatment. The excretion rate of bile of control rats was almost constant (0.794 ± 0.133 ml/h, mean ± S.D. of 4 animals), and aminotriazole treatment had essentially no effect on this (data not shown).

Influence of Aminotriazole Treatment on Biosyntheses of Primary Bile Acids from ^{14}C-Cholesterol Figure 3 shows the effect of aminotriazole treatment on the cumulative radioactivities of primary bile acids synthesized from ^{14}C-cholesterol. Bile acid is synthesized in the liver and immediately excreted into bile, and so the radioactivity of bile acid in the bile represents *de novo* synthesis of bile acid.

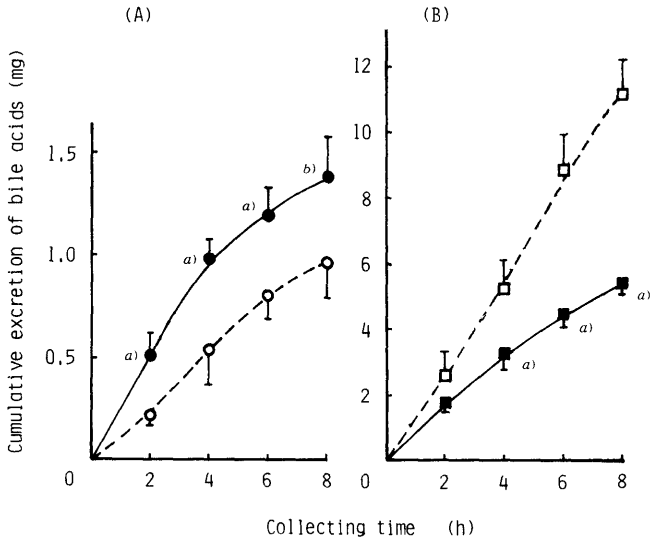

Fig. 2. Effects of Aminotriazole Treatment on Cumulative Excretion of Bile Acids into Bile

Aminotriazole was intraperitoneally injected into rats 24 h after implantation of a bile duct fistula. Control rats were injected with saline instead of aminotriazole. Bile was collected at 2-h intervals after the treatment. Bile acids were extracted from the bile and analyzed by gas-liquid chromatography after hydrolysis. (A), chenodeoxycholic acid; (B), cholic acid. Closed symbols, aminotriazole; open symbols, control. Data are means ± S.D. of 4 animals. *a*) and *b*) represent significant changes (*a*), $p<0.005$; *b*), $p<0.01$).

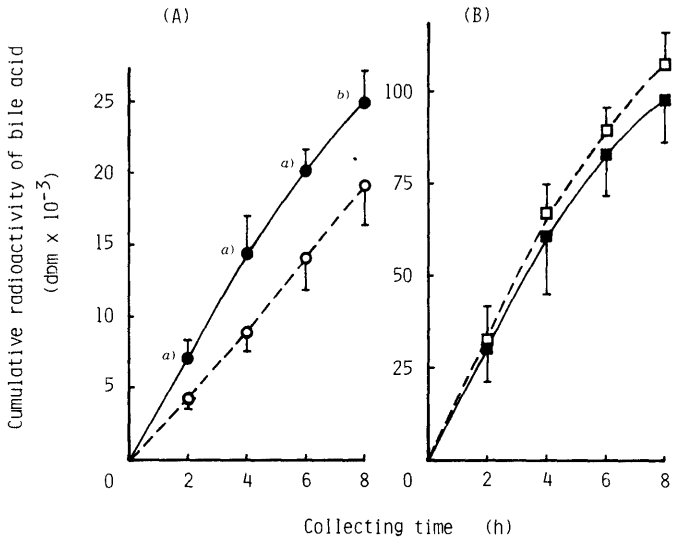

Fig. 3. Effects of Aminotriazole Treatment on Biosyntheses of Bile Acids from ^{14}C-Cholesterol

Aminotriazole was intraperitoneally injected into rats 24 h after implantation of a bile duct fistula. ^{14}C-Cholesterol was also administered almost simultaneously to the same rats through the femoral vein. Control rats were injected with saline instead of aminotriazole. Bile was collected at 2-h intervals. Bile acids were extracted and subjected to thin-layer chromatography. The radioactivities of bile acids were determined as described in the text. (A), chenodeoxycholic acid (taurochenodeoxycholate and glycochenodeoxycholate); (B), cholic acid (taurocholate and glycocholate). Closed symbols, aminotriazole; open symbols, control. Data are means ± S.D. of 5 animals. *a*) and *b*) represent significant changes (*a*), $p<0.005$; *b*), $p<0.01$).

In control rats, the radioactivities of chenodeoxycholic acid and cholic acid synthesized from ^{14}C-cholesterol for 8 h correspond to 2.15% and 12.1%, respectively, of the injected total radioactivity. The radioactivity of chenodeoxycholic acid was increased to 1.30 times the control by the aminotriazole treatment (Fig. 3(A)), but that of cholic acid was hardly changed (Fig. 3(B)). The cholic acid: chenodeoxycholic acid ratio of the radioactivity in the bile was changed from 5.62 to 3.92 by aminotriazole treatment.

When we studied the synthesis of total bile acids from exogenous cholesterol, as described in a previous paper, we could not find any effect of aminotriazole treatment.[9] However, in the present study, synthesis of chenodeoxycholic acid from exogenous cholesterol was increased by aminotriazole treatment (Fig. 3(A)). The content of chenodeoxycholic acid in total bile acids is small (Fig. 1(B)), and this may be the reason no significant effect of aminotriazole could be found in the earlier work, even if synthesis of chenodeoxycholic acid had been increased by the treatment. On the other hand, synthesis of cholic acid from exogenous cholesterol was not affected by aminotriazole treatment (Fig. 3(B)). These results indicate that synthesis of chenodeoxycholic acid from exogenous cholesterol was specifically activated by aminotriazole treatment. Namely, the pathway of synthesis of chenodeoxycholic acid may be at least partly different from that of cholic acid.

Effect of Aminotriazole Treatment on Biosyntheses of Primary Bile Acids from ^{14}C-Mevalonate Mevalonate is commonly used in studies of biosyntheses of cholesterol and bile acids, since it is a readily available, effective precursor which enters the mevalonate pathway after the rate limiting enzyme of cholesterol synthesis, 3-hydroxy-3-methylglutaryl-CoA reductase. Figure 4 shows the cumulative radioactivities of primary bile acids derived from ^{14}C-mevalonate. In control rats, the radioactivities of chenodeoxycholic acid and cholic acid synthesized during 8 h correspond to 0.62% and 4.55%, respectively, of the injected total radioactivity. The radioactivity of chenodeoxycholic acid was unaffected by aminotriazole treatment (Fig. 4(A)), but that of cholic acid was clearly decreased to 41.2% of the control at 8 h (Fig. 4(B)). The cholic acid: chenodeoxycholic acid ratio of the radioactivity in the bile was changed from 7.33 to 3.49 by aminotriazole treatment. In control and treated rats, synthetic rate of chenodeoxycholic acid from ^{14}C-mevalonate was the highest during the first 2 h, differing from the result with ^{14}C-cholesterol.

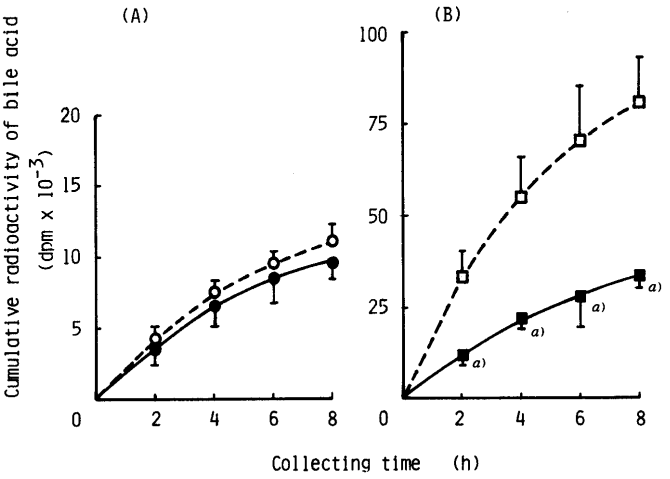

Fig. 4. Effects of Aminotriazole Treatment on Biosyntheses of Bile Acids from ^{14}C-Mevalonate

^{14}C-Mevalonate was administered instead of ^{14}C-cholesterol as in Fig. 3. (A), chenodeoxycholic acid (taurochenodeoxycholate and glycochenodeoxycholate); (B), cholic acid (taurocholate and glycocholate). Closed symbols, aminotriazole; open symbols, control. Data are means±S.D. of 5 animals. *a)* represents significant changes ($p<0.005$).

In the previous paper, synthesis of endogenous cholesterol from mevalonate was shown to be inhibited by aminotriazole treatment[9]; therefore, we expected that syntheses of both chenodeoxycholic acid and cholic acid from mevalonate would be inhibited by such treatment. However, although the synthesis of cholic acid was inhibited, that of chenodeoxycholic acid was not decreased (Fig. 4). We considered that synthesis of chenodeoxycholic acid from endogenous cholesterol as well as that from exogenous cholesterol (Fig. 3(A)) may be activated by aminotriazole treatment. Consequently, synthesis of chenodeoxycholic acid from mevalonate may be apparently unchanged, even though the synthesis of endogenous cholesterol from mevalonate was inhibited by aminotriazole.

It is not yet fully understood whether hepatic cholesterol that originates from different sources is distributed in a similar way to cholic acid and chenodeoxycholic acid. In the present study, we found that the cholic acid: chenodeoxycholic acid ratio of bile acids derived from ^{14}C-mevalonate in control rats was higher than that of bile acids derived from ^{14}C-cholesterol (Figs. 3 and 4). These results indicate the preferential utilization of endogenous cholesterol for cholic acid synthesis. Furthermore, as can be seen in Fig. 2, the content of chenodeoxycholic acid increased rapidly after the aminotriazole treatment, but that of cholic acid was clearly decreased. In view of this result, together with the radioisotope data (Figs. 3 and 4), the increase in content of chenodeoxycholic acid by aminotriazole treatment may be due to the enhancement of the synthesis of chenodeoxycholic acid from exogenous cholesterol (Fig. 3(A)), and the clear decrease in cholic acid may be due to the reduction of the synthesis of cholic acid through endogenous cholesterol (Fig. 4(B)). Namely, the decrease of cholic acid content by aminotriazole treatment seems to be independent of the increase of chenodeoxycholic acid content. These results strongly suggest that chenodeoxycholic acid is mainly synthesized from exogenous cholesterol in the liver, and cholic acid, from endogenous cholesterol. These results indicate that pools of exogenous and endogenous cholesterol are independently present in the liver.[12-14]

The ratio of cholic acid to chenodeoxycholic acid was reduced by aminotriazole treatment (Figs. 2, 3 and 4). The mechanism regulating the ratio between cholic acid and chenodeoxycholic acid is not well understood. Microsomal 12α-hydroxylation is the unique step in the formation of cholic acid and is likely to be of regulatory importance for the ratio between newly synthesized cholic acid and chenodeoxycholic acid. Introduction of a 26-hydroxyl group seems to prevent subsequent 12α-hydroxylation in rat liver and the 26-hydroxylase could thus also have a regulatory role.[15] In the preset study, biosynthesis of chenodeoxycholic acid from at least exogenous cholesterol was activated by aminotriazole treatment (Fig. 3(A)). It is not yet known whether 12α- and/or 26-hydroxylase are affected by this treatment. If the mechanism of action of amino-

triazole is clarified, investigation of the regulation of the ratio between cholic acid and chenodeoxycholic acid may be able to progress.

In the conjugation process of bile acids, bile acid acyl-CoA ester is formed by microsomes, and cytosolic enzymes catalyze the coupling to taurine or glycine.[16] Kase *et al.* found that peroxisomes of rat liver also conjugate choloyl-CoA with either taurine or glycine.[17] The ratio of tauroconjugate to glycoconjugate is reported to be influenced by cholestyramine, hydrocortisone and other factors.[16,18,19] In the present study, ratios were calculated from the radioactivities of tauroconjugate and glycoconjugate of primary bile acids in the bile, which was accumulated for 8 h, of the same rats as shown in Figs. 3 and 4. In control rats, the ratios of tauroconjugate to glycoconjugate of chenodeoxycholic acid synthesized from ^{14}C-cholesterol and ^{14}C-mevalonate were 3.35 ± 0.59 (mean $\pm$ S.D. of 5 animals) and 2.97 ± 0.65, respectively. The ratios of cholic acid synthesized from ^{14}C-cholesterol and ^{14}C-mevalonate were 20.7 ± 3.6 and 21.9 ± 4.8, respectively. Thus cholic acid synthesized from ^{14}C-cholesterol and ^{14}C-mevalonate was mostly present as tauroconjugate in the bile of the rats. Aminotriazole treatment had virtually no effect on the ratios (data not shown).

Recently we found that 4,4-dimethyl-5α-cholest-8-en-3β-ol and 4α-methyl-5α-cholest-7-en-3β-ol were accumulated in the liver after inhibition of cholesterol synthesis by aminotriazole treatment *in vivo*. Therefore, we considered that the 4α-methyl sterol oxidase reaction is inhibited by aminotriazole treatment.[20] 4α-Methyl sterol oxidase had been reported to be localized in microsomes; however, recent reports[2-4,21,22] suggest that this enzyme is also localized in peroxisomes. It is not yet clear whether the 4α-methyl sterol oxidase reaction of peroxisomes and/or microsomes is directly inhibited by aminotriazole, or whether the elimination of the 4α-methyl group is suppressed by H_2O_2 accumulated owing to the inhibition of catalase acivity of peroxisomes by aminotriazole treatment. In any event, it was elucidated that aminotriazole treatment affects not only cholesterol synthesis but also synthesis of primary bile acids *in vivo*.

In conclusion, we have clarified that aminotriazole treatment differently affects the biosyntheses of chenodeoxycholic acid and cholic acid. Namely, aminotriazole does not affect synthesis of cholic acid from exogenous cholesterol, but activates that of chenodeoxycholic acid. Further, aminotriazole treatment does not affect biosynthesis of chenodeoxycholic acid through endogenous cholesterol, but does inhibit that of cholic acid. Therefore, aminotriazole may be useful as a tool to elucidate the regulatory mechanism of the ratio between cholic acid and chenodeoxycholic acid.

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