

## THE INFLUENCE OF DIETARY CHOLESTEROL ON THE LITHOGENESITY OF BILE IN RATS TREATED WITH CLOFIBRATE (II)\*<sup>1)</sup>

By

Toshio KAWAMOTO, Goro KAJIYAMA, Akira MARUHASHI,  
Toshiyuki MIZUNO, Katsushi YAMADA, Masamichi FUJIYAMA  
and Akima MIYOSHI

*1st Department of Internal Medicine, Hiroshima University School of Medicine, Hiroshima 734, Japan*

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### ABSTRACT

The HMG CoA reductase and cholesterol 7 $\alpha$ -hydroxylase activities were determined and fecal bile acids were analyzed in rats fed with a low (0.16%) or a high (1%) cholesterol diet in order to investigate the influence of dietary cholesterol on the lithogenesis of bile in rats treated with clofibrate ( $\alpha$ -p-chlorophenoxy isobutyl ethyl ester).

The HMG CoA reductase activity displayed no change five days after administration of 200 mg/kg/day clofibrate to both the rats treated with a low or high cholesterol diet, although the activity fell considerably by administration of the cholesterol diet. But clofibrate produced a slight elevation of cholesterol 7 $\alpha$ -hydroxylase activity in both diet groups.

The fecal output of deoxycholic acid significantly increased in the rats fed with a low cholesterol diet but not in those fed with a high cholesterol diet after clofibrate administration.

The foregoing results indicate the possibility that the enlarged poolsize of trihydroxy bile acids after clofibrate administration in the rats fed with a low cholesterol diet as seen in the previous experiment<sup>1)</sup> will be due to the increased conversion of cholesterol into bile acids but not due to inhibition of bile acid output into feces.

On the contrary, the absence of increase in the bile acid pool size in the rats fed with a high cholesterol diet after clofibrate administration<sup>1)</sup> appeared to be due to deficiency of substrate in the liver. The substrate is meant to be the newly synthesized cholesterol in the liver, which was reduced by the feed back inhibition of exogenous cholesterol, and led to production of lithogenic bile with the aid of excess biliary cholesterol accelerated by another action of clofibrate.

Clofibrate produced an increase in the liver weight of these animals, but failed to cause not only any light microscopic changes in the liver tissue but also any changes in the serum liver function tests.

\*<sup>1)</sup> 川本敏雄, 梶山悟朗, 丸橋 暉, 水野敏之, 山田勝士, 藤山正道, 三好秋馬: クロフィブレート投与ラット胆汁の胆石形成度に対する食餌性コレステロールの影響(II)

## INTRODUCTION

Clofibrate has been very well known to affect the lipid and bile acid metabolism in the liver with influence not only upon the serum lipids but also upon biliary lipids and bile acids; viz., the effect of clofibrate reducing serum cholesterol leads to an application of clofibrate for the treatment of hypercholesterolemia, and clofibrate at the same time induces formation of lithogenic bile because it promotes excretion of cholesterol into bile from serum.

However, the bile of patients administered with clofibrate is not usually supersaturated with cholesterol and rarely produces gallstones.

In the previous experiment<sup>1)</sup>, the rats treated with clofibrate, when fed with a low cholesterol (0.16%) diet, were found to have increased pool size of trihydroxy bile acids as determined by the bile acid washout technique, as compared with the control rats. But on the contrary, when they were fed with a high cholesterol (1%) diet, clofibrate did not induce an adequate increase in the trihydroxy bile acid pool size and accelerated the excretion of biliary cholesterol, resulting in an elevation of lithogenesis of bile.

These results indicate that the conversion of cholesterol into trihydroxy bile acid was promoted in the liver of rats treated with clofibrate when fed with a low cholesterol diet but not when fed with a high cholesterol diet.

On the other hand, there is a possibility of the increase in trihydroxy bile acid pool size in rats when clofibrate inhibits the conversion of the primary bile acids into the secondary bile acids and, therefore, prevents their excretion into feces.

In the present study, the activities of HMG CoA reductase and cholesterol 7 $\alpha$ -hydroxylase were determined in the liver of rats treated with clofibrate, to clarify whether or not the enlarged bile acid pool size is accompanied with and accordingly caused by the intensified activity of the enzymes that regulate the bile acid synthesis either directly or indirectly.

The daily fecal output of bile acid was at the same time measured to evaluate the effect of clofibrate on the conversion of the primary bile acids to the secondary in the animal intestine.

The influence of cholesterol diet and clofibrate, and the mechanism of clofibrate action

on biliary cholesterol were discussed on the basis of the results of the present study in relation to the previous experiment.

In addition to the above effect on the biliary lipids, clofibrate seems to have some other effects on the liver metabolism and function. Therefore, researches have been made into such effects of this agent on the liver weight, morphology and various liver function tests.

## MATERIALS AND METHODS

Female Wistar rats weighing 200–250 g were obtained from Miyamoto Animal Experimental Laboratory, Japan.

These animals were divided into two principal groups, i. e. one group which was fed with a stock diet contaminated with approximately 0.16% cholesterol (low cholesterol diet) and the other group with 1% cholesterol (high cholesterol diet) for three weeks respectively. 0.5% taurocholic acid was added to the latter diet to enhance cholesterol absorption in the animal intestine. 200 mg/kg per day of clofibrate was given to halves of both groups orally by means of a fine catheter at 6 P.M. for a period of 5 days in succession prior to an experimental operation. The other halves without clofibrate treatment were used as the control group. The rats were maintained with diets and water ad libitum for a period of three weeks. The animals fed with a high cholesterol diet did not develop diarrhea and gained weight at the same time rate as the control. The animals were studied in the fasting state on the final day of the dietary experimentation.

### ENZYME ASSAYS

The specific activities of hepatic microsomal HMG CoA reductase and cholesterol 7 $\alpha$ -hydroxylase were determined by the following procedures.

#### *Preparation of microsomes:*

Microsomes were prepared basically according to the method described by Shefer et al.<sup>2)</sup> The rats were killed by decapitation between 9 : 00 A.M. and 10 : 00 A.M. The livers were excised, immediately weighed and chilled on ice. All subsequent operations were carried out at 0–5°C. The blood was extruded from the liver through saline. The liver (1.0 gram) was then homogenized in a loose-fitting Potter-Elvehjem homogenizer (0.5 mm radial clear-

ance) with a 4-fold volume of ice-cold homogenizing medium containing 0.3 M sucrose, 75 mM nicotinamide, 2.5 mM neutralized EDTA and 25 mM neutralized reduced glutathione. The homogenate was centrifuged at 800 g for 10 minutes to get rid of nuclei and cell debris. The supernatant solution was centrifuged at 6,700 g for 10 minutes to precipitate and eliminate the mitochondrial fractions. The supernatant solution thus obtained was further centrifuged at 9,500 g for 10 minutes and the precipitate was removed. The microsomal fraction was then precipitated at 105,000 g for 60 minutes. This microsomal pellet was suspended in the 4 ml homogenizing medium and prepared so as to contain approximately 1 mg protein per milliliter. The protein concentration of the fraction was determined with Folin phenol reagent. This suspension was used in appropriate quantity for the determination of both HMG CoA reductase and cholesterol 7 $\alpha$ -hydroxylase activities.

#### *Hepatic HMG CoA reductase:*

The hepatic HMG CoA reductase activity was determined by a procedure fundamentally corresponding to that described by Schoenfield et al.<sup>3,4)</sup> The standard assay system comprised in a volume of 0.75 ml, 100mM phosphate buffer at pH 7.4, 3 mM MgCl<sub>2</sub>, 3 mM NADP, 10 mM glucose-6-phosphate, 3 enzyme units of glucose-6-phosphate dehydrogenase, 20 mM 2-mercaptoethanol, 0.35 mM HMG CoA-3-<sup>14</sup>C-CoA and 0.2 to 0.4 mg microsomal protein.

After incubation at 37°C for 30 minutes in an open tube which was kept shaking, the reaction was stopped by addition of 0.1 mg of 10 N NaOH. A small amount of <sup>3</sup>H-mevalonic acid (approximately 25,000 d. p. m.) and 3 mg of unlabelled mevalonolactone were added to each tube, which was left overnight. On the next day the tubes were supplied with 0.3 ml of 5 N H<sub>2</sub>SO<sub>4</sub> and shaken at 37°C for 45 minutes to lactonize the mevalonate. The tubes after cooling to room temperature were then supplied with 0.5 ml of 100 per cent ethanol and 1.0 g of anhydrous Na<sub>2</sub>SO<sub>4</sub>. The mixture was extracted four times with 7 ml of ethyl ether. The combined extracts were evaporated over Na<sub>2</sub>SO<sub>4</sub> and re-dissolved in 0.2 ml acetone.

The extracts in acetone were plotted and developed on a silica gel G plate in a benzene acetone solution (1 : 1 v/v). The layers in Rf

of 0.5 and 0.75 were scraped off the plate and placed into a counting vial.

A liquid scintillation fluid was added and the radioactivity was counted. A Packard Automatic Tri-carb 3330 liquid scintillation counter was used for simultaneous <sup>14</sup>C and <sup>3</sup>H mixed counting. Quenching and crossover were corrected with the external standard.

The results were obtained as the nanomoles of enzyme activity per milligram of microsomal protein from the equation:

$$\text{moles of mevalonate } ^{14}\text{C formed} = \frac{\text{Mevalonate } ^3\text{H added (d. p. m.)}}{\text{Mevalonate } ^3\text{H recovered (d. p. m.)}} \times \frac{\text{Mevalonate } ^{14}\text{C recovered (d. p. m.)}}{\text{Specific activity HMG } ^{14}\text{C CoA d. p. m./mole}}$$

#### *Cholesterol 7 $\alpha$ -hydroxylase*

The hepatic cholesterol 7 $\alpha$ -hydroxylase enzyme that catalyzes oxidation of 4-<sup>14</sup>C-cholesterol to 7 $\alpha$ -hydroxy-4-<sup>14</sup>C cholesterol was assayed essentially as described previously by Schoenfield et al.<sup>3,4)</sup> The enzyme activity, however, was measured in the microsomal fraction of the liver with the same homogenizing medium as used for HMG CoA reductase. 0.5 mg to 1.0 mg of microsomal protein was used.

The incubation mixture (1.0 ml) comprised 70 mM potassium phosphate buffer at pH 7.5, 5 mM MgCl<sub>2</sub>, 4 mM NADP, 8 mM glucose-6-phosphate, 5 units of glucose-6-phosphate dehydrogenase, 3 mM EDTA, 2 mM 2-mercaptoethanol, 0.75  $\times$  10<sup>5</sup> d. p. m. 4-<sup>14</sup>C cholesterol, 0.5 micromoles of unlabelled cholesterol as a carrier and 0.4 to 1.0 mg microsomal protein. Incubation was conducted in air for 30 minutes in a shaking water bath at 37°C and terminated by adding 20 ml of chloroform: methanol (2 : 1). The precipitate was filtered off and 4 ml of 0.1 M sodium chloride solution was added to the filtrate which was then centrifuged at 2,000 r. p. m. for 10 minutes. The upper phase was aspirated off and the organic solvent was evaporated to dryness under N<sub>2</sub> in a 50°C water bath. The sterol fraction was dissolved in 0.2 ml of acetone and applied together with the reference compounds to the plates precoated with silica gel G. The plates developed with benzen-ethyl acetate (2 : 3 v/v) as a solvent.

After thin-layer chromatography, the internal standards including 7 $\alpha$ -hydroxycholesterol and the auto-(oxidation) products were visualized

by iodine vapor. The iodine was sublimated at room temperature. The appropriate zones were then scraped off into test tubes and extracted with 5 ml of methanol by vigorous stirring. An optimal amount of these extracts was transferred into a liquid scintillation vial containing 10 ml of "aquasol".

The radioactivity was measured by a Packard Automatic Tri-carb 3330 liquid scintillation counter with necessary corrections of background and quenching. The enzyme activity was expressed as the radioactivity incorporated into 7 $\alpha$ -hydroxycholesterol per milligram of microsomal protein (the original 3-<sup>14</sup>C-cholesterol specific activity is already known). Along with the samples, the blanks were run wherein the enzyme reaction was stopped at 0 time. The results with correction of losses during the extraction procedures were calculated from the initial radioactivity of cholesterol and the total counts recovered after extraction.

#### DETERMINATION OF FECAL BILE ACID

The total amount of feces of a whole day both before and after administration of clofibrate were collected and weighed. The whole samples, after having been heated at 80°C for 30 minutes, were crushed into fine powder. 1.0 g of sample was filtrated into a 50 ml teflon-coated crucible after extraction with 10 ml of chloroform-methanol (2 : 1 v/v). The solvent was evaporated to dryness under nitrogen stream, and the residue was dissolved in 10 ml of 1N NaOH. Bile acid was determined by gas-liquid chromatography after having been hydrolyzed, acidified and methylated in the same way<sup>1)</sup> as was employed for determination of bile acid in bile.

#### EXAMINATION OF LIVER WEIGHT, MORPHOLOGY AND FUNCTION TESTS

The livers having been weighed, the liver specimens were prepared for light microscopic observation. Blood obtained by heart-puncture was centrifuged and serum was used for the standard liver function tests, e. g., total protein, GPT, total bilirubin, alkaline phosphatase and LAP.

### RESULTS

#### I) HMG CoA reductase and cholesterol 7-hydroxylase activities

##### 1) HMG CoA reductase activity

The HMG CoA reductase activity in the rats fed with a low cholesterol diet averaged  $1.220 \pm 0.268$  in the control rats and  $1.231 \pm 0.447$  n mol/mg protein/min. in the rats treated with clofibrate, respectively. The rats fed with a high cholesterol diet showed a reduction in the enzyme activity to approximately one fifth of that in the rats fed with a low cholesterol diet. However, clofibrate failed to bring about any change in the enzyme activity which was  $0.210 \pm 0.035$  in the control and  $0.232 \pm 0.031$  n mol/mg protein/min. in the rats treated with clofibrate, respectively. (Ref: Table 1)

##### 2) Cholesterol 7 $\alpha$ -hydroxylase activity

The cholesterol 7 $\alpha$ -hydroxylase activity in the rats fed with a low cholesterol diet averaged  $0.393 \pm 0.048$  in the control and  $0.450 \pm 0.168$  in the rats treated with clofibrate, respectively. Clofibrate administration to the rats fed with a high cholesterol diet also produced a slight increase in the enzyme activities, i. e. from  $0.409 \pm 0.079$  in the control rats without clofibrate to  $0.483 \pm 0.051$  nmol/mg protein/min. in the rats treated with clofibrate. There was, however, no statistical significance in the difference in the enzyme activity levels between them. (Ref: Table 1)

##### II) Fecal bile acid output

Administration of clofibrate to the rats fed with a low cholesterol diet produced an increase, in the output of fecal deoxycholic acid (mg/dl) (from  $113.6 \pm 24.1$  to  $219.3 \pm 34.8$ ;  $p < 0.005$ ) and in the output of fecal lithocolic acid (mg/dl) (from  $79.0 \pm 18.4$  to  $153.7 \pm 35.5$ ). The fecal output of deoxycholic acid as well as lithocolic acid did not increase in the rats fed with a high cholesterol diet (from  $3946.7 \pm 650.4$  to  $3130.8 \pm 761.5$ , and from  $2348.0 \pm 506.5$  to  $2442.8 \pm 501.2$ , respectively) after administration of clofibrate. (Ref: Table 2)

##### III) Liver weight and morphology

There was a slight increase in the liver weight in the rats fed with a low cholesterol diet after clofibrate administration, though the difference was not statistically significant between the control and the rats treated with clofibrate. However, the increase in the liver weight was significant in the rats fed with a high cholesterol diet by clofibrate administration. (Ref: Table 3)

There were no pathological deteriorations of the liver cells by clofibrate administration for five days in both the rats fed with low and

**Table 1.** HMG CoA reductase and cholesterol 7 $\alpha$ -hydroxylase activities in rats fed with low or high cholesterol diet (n mol/mg protein/min.)

Group	Treatment	n	HMG CoA reductase	cholesterol 7 $\alpha$ -hydroxylase
Stock diet (low cholesterol)	Control	3	1.220 $\pm$ 0.268	0.393 $\pm$ 0.048
	Clofibrate-treated	3	1.231 $\pm$ 0.447	0.450 $\pm$ 0.168
Cholesterol diet (high cholesterol)	Control	3	0.210 $\pm$ 0.035	0.409 $\pm$ 0.079
	Clofibrate-treated	3	0.232 $\pm$ 0.031	0.483 $\pm$ 0.051

M $\pm$ SE n: the number of rat**Table 2.** Fecal bile acids in rats fed with low or high cholesterol diet ( $\mu$ g/100 g rat/day)

Group	Treatment	n	Deoxycholic acid	Lithocholic acid
Stock diet (low cholesterol)	Before Clofibrate	5	113.6 $\pm$ 24.1	79.0 $\pm$ 18.4
	After Clofibrate	5	219.3 $\pm$ 34.8	153.7 $\pm$ 35.5
Cholesterol diet (high cholesterol)	Before Clofibrate	5	3946.7 $\pm$ 650.4	2348.0 $\pm$ 506.5
	After Clofibrate	5	3130.8 $\pm$ 761.5	2442.8 $\pm$ 501.2

M $\pm$ SE n: the number of rat \* p<0.005**Table 3.** Liver weight of rats fed with of rats fed with low or high cholesterol diet

Group	Treatment	n	Liver weight (g/100 g rat)
Stock diet (low cholesterol)	Control	26	2.87 $\pm$ 0.13
	Clofibrate-treated	9	3.23 $\pm$ 0.10
Cholesterol diet (high cholesterol)	Control	10	3.29 $\pm$ 0.06
	Clofibrate-treated	10	4.00 $\pm$ 0.08

M $\pm$ SE n: the number of rat \* p<0.001

high cholesterol diets. (Ref: Fig.1)

## IV) Liver function tests

Serum cholesterol, GPT, total bilirubin, alkaline phosphatase and LAP were examined in both the rats fed with low and high cholesterol

diets. No remarkable abnormality was observed as a result of these tests after clofibrate administration as shown in Table 4. (Ref: Table 4)

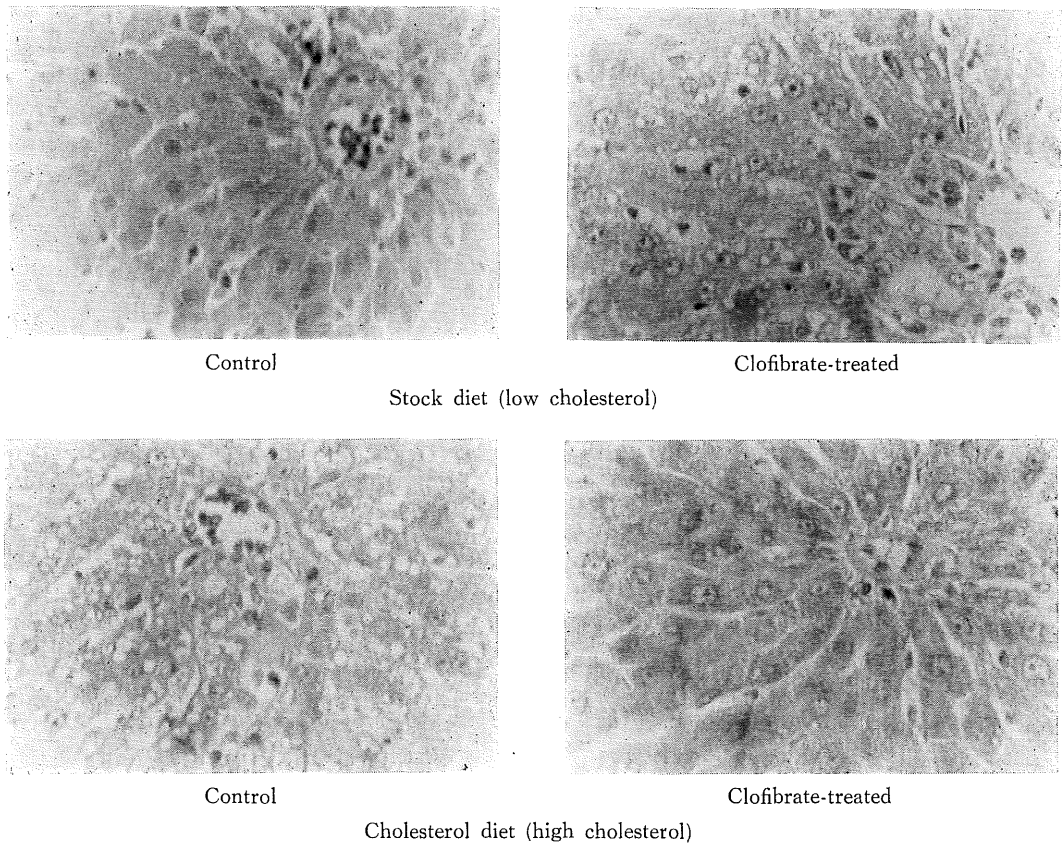


Fig. 1. Microscopic findings of liver

Table 4. Serum liver function tests in rats fed with low or high cholesterol diet

Group	Treatment	n	Total Protein (g/dl)	GPT(U)	Total Bilirubin (mg/dl)	Alkaline Phosphatase (U)	LAP (U)
Stock diet (low cholesterol)	Control	5	6.5±0.1	24.8±3.3	0.4±0.2	10.2±1.4	69.6±4.1
	Clofibrate-treated	7	6.8±0.1	24.7±2.4	0.5±0.1	12.7±1.2	73.7±3.5
Cholesterol diet (high cholesterol)	Control	8	6.7±0.2	21.3±1.8	0.3±0.1	14.2±1.3	97.8±8.5
	Clofibrate-treated	10	7.0±0.2	20.1±2.9	0.3±0.1	12.0±1.3	87.5±7.4

M±SE n: the number of rat

## DISCUSSION

Mosbach et al.<sup>5)</sup> stated in their earlier study that clofibrate mixed in a standard chow inhibited the HMG CoA reductase activity and decreased the serum cholesterol level in rats after 2 weeks from feeding.

In the present experiment, although clofibrate administration for a period of 5 days failed to inhibit the enzyme activity in the rats fed with a low cholesterol diet, the serum cholesterol level was significantly lowered in the rats treated with clofibrate as compared with the control fed with a low cholesterol diet. This result

can be interpreted to indicate that administration of clofibrate for a period of only 5 days was insufficient for inhibiting HMG CoA reductase activity.

However, as mentioned above, a significantly larger pool size of trihydroxy bile acid (cholic acid:  $p < 0.05$ ) was observed in the rats treated with clofibrate than in the control rats when fed with a low cholesterol diet. Therefore, a slight decline in the serum cholesterol level in the rats fed with a low cholesterol diet seen in the previous experiment<sup>11</sup> was considered to have been caused by the selective conversion of cholesterol into trihydroxy bile acids but not by inhibition of HMG CoA reductase activity.

The activity of cholesterol 7 $\alpha$ -hydroxylase, the rate-limiting enzyme of bile acid synthesis, slightly increased in the rats fed with a low cholesterol diet by clofibrate administration, which fact supports that the accelerated conversion of cholesterol into bile acid, especially into trihydroxy bile acid, resulted in the enlargement of bile acid pool size. But there was not brought forth by clofibrate administration any significant difference in the cholesterol 7 $\alpha$ -hydroxylase activity between the rats with and without clofibrate, although the enzyme activity was seen increased by 14.5% in the rats treated with clofibrate.

In contrast to these results obtained by the authors, Einarsson et al.<sup>6</sup> reported that while clofibrate stimulated all the hydroxylation reactions of androstene-3, 17-dione, it did not affect 7 $\alpha$ -hydroxylation of cholesterol in the Sprague-Dawley strain fed with a crushed rat diet supplemented with 0.3% clofibrate for 3 week ad libitum.

On the other hand, however, the enlargement of pool size of trihydroxy bile acid can also be assumed as a result of the lowered intestinal conversion of the primary bile acid into the secondary bile acid (from cholic acid to deoxycholic acid) due to the inhibited bacterial 7 $\alpha$ -dehydroxylation by the non-specific or specific action of excess bowel clofibrate.

However, the gas-chromatographic analysis of fecal bile acid revealed a significant increase in the output of deoxycholic acid in the rats fed with a low cholesterol diet, proving the absence of inhibition of intestinal bacterial 7 $\alpha$ -hydroxylation by clofibrate.

These results, therefore, seem to suggest after

all that although there was no significant difference between the control and the clofibrate treated rats fed with a low cholesterol diet, the slightly elevated cholesterol 7 $\alpha$ -hydroxylase activity enlarged the trihydroxy bile acid pool.

This interpretation is also in conformity with the research of Kudchodkar<sup>7</sup>) proposing that the conversion of cholesterol into cholic acid was promoted in human beings by clofibrate. But this problem would require further re-confirmation to arrive at a solution.

On the other hand, a high cholesterol diet reduced the HMG CoA reductase activity, though clofibrate failed to produce any effect on the enzyme activity of the rats fed with a high cholesterol diet as well. The reduced enzyme activity would be accompanied with feed back inhibition of cholesterol synthesis, and would inhibit bile acid synthesis resulting in a reduced bile acid pool size because newly synthesized cholesterol is preferentially converted into bile acid in the liver of rats as proven by many investigators<sup>8-10</sup>).

In spite of clofibrate having slightly elevated the cholesterol 7 $\alpha$ -hydroxylase activity as seen in the rats fed with a high cholesterol diet in the present experiment, the rats fed with a high cholesterol diet had very small pool size of bile acid which was not re-expanded by clofibrate in the previous experiment<sup>11</sup>).

The reduced cholesterol as the substrate caused by a high cholesterol diet is considered inadequate to parallel the elevated cholesterol 7 $\alpha$ -hydroxylase activity and to expand the bile acid pool size in the animals under these conditions. Furthermore, the excess excretion of dietary cholesterol which was brought about by clofibrate administration, resulted in producing lithogenic bile which may cause precipitation of cholesterol crystals and develop into gallstones.

The influence of clofibrate on the liver and its action of inducing abnormalities in the liver function tests in association with the side effect have been frequently reported.

The present experiment demonstrated that administration of clofibrate for only 5 days induced an increase in the liver weight but neither caused any morphological change nor influenced the various standard serum liver function tests.

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