Abstract—In mice, combined addition of 1% cholesterol and 0.5% cholic acid to a diet induced cholesterol gallstones within 40 days as a result of the supersaturation of cholesterol in the bile, as has been reported. The major component of the gallstone was cholesterol, which was measured by HPLC. In this study, however, single addition of 1% cholic acid to a diet, which did not decrease cholesterol solubilizing capacity in bile, contributed to gallstone formation in mice within 50 days. The gallstones thus formed contained a large amount of palmitic acid. In the hepatic bile of this animal, palmitic acid was also detected; however, no solid material was observed by light and polarized-light microscopes. Free fatty acids such as palmitic acid seem to be dissolved in a complex micelle composed of bile acids and lecithin. This probably causes gallstone formation by reducing cholesterol solubilizing capacity in bile.

Cholesterol gallstone formation is known to be closely related to lipid composition in the bile. Cholesterol, which is hardly soluble in water, is dissolved in a complex micelle composed of bile acids and lecithin (1, 2). Cholesterol gallstone formation is frequently accompanied by a supersaturation of cholesterol in human bile (3). Increase in the proportion of cholesterol to bile acids and lecithin is particularly contributory to the stone induction in man (4, 5). In mice, addition of cholesterol and cholic acid to a diet in a final concentration of 1 and 0.5%, respectively, induces gallstones in the gallbladder within 2 months (6). In this experiment, the stones were induced only when both cholesterol and cholic acid were added to the diet, while single addition of cholic acid or cholesterol to the diet was noncontributory to the stone formation. The role of cholic acid contained in a diet in stone induction is believed to be its ability to increase cholesterol concentration in bile by enhancing absorption of cholesterol through brush-border of the small intestine.

In the preliminary report, however, we stated that single addition of cholic acid to a diet in a final concentration of 1% caused gallstone formation in the ICR strain, SPF male mice (7). This stone formation was not accompanied with a decrease in cholesterol solubilizing capacity in hepatic bile. About 90% of the bile acids contained in the bile was taurocholic acid, which is known to inhibit the cholesterol synthesis system in the rat liver (8). These data suggest that the stones thus formed may not be cholesterol gallstones and that unknown substance(s) other than cholesterol may be contained in the bile and the stones.

In the present study, we detected high contents of free fatty acids, mainly palmitic acid (hexadecanoic acid), in the gallstones and hepatic bile in mice given a diet containing 1% cholic acid. We present a more detailed report on the formation of gallstones by single addition of cholic acid to a diet and propose our hypothesis on the role of free
fatty acids in stone induction.

Materials and Methods
Handling of animals and operation procedure: Male mice (ICR strain, SPF, weighing from 18 to 22 g, 5 weeks old) were divided into groups of 6-9 animals per group. The control group was maintained with a powder of commercial chow (FM-2, Oriental, Tokyo, Japan). Other groups were fed diets containing 1% cholic acid, 1% cholesterol and 0.5% cholic acid or 1% cholesterol, respectively. The animals were maintained in 14L-10D, temperature (23±2°C), and humidity (55±5%). The bile duct was drained with a polyethylene tube under urethane (1.5 g/kg, s.c.) anesthesia, and hepatic bile was collected for 1 hr.

Determination of bile acids, lecithin and cholesterol in hepatic bile: Cholesterol, lecithin and total bile acid concentrations in hepatic bile were determined with commercial assay kits (Determiner TC555, Determiner PL, purchased from Kyowa Medex, Co. Ltd., Tokyo, Japan and Neo sterognost 3-a, from Nyegaard & Co., Oslo, Norway, respectively) according to the manufacturers' instructions.

Observation of cholesterol crystals and gallstones: The contents of a gallbladder was drained on a glass slide. Cholesterol crystals and gallstones in the contents as well as in the aliquot of hepatic bile collected in a test tube were observed as soon as possible by polarized-light and light microscopes. The presence of solid substances other than cholesterol crystals and gallstones in the contents of the gallbladder and hepatic bile was also examined carefully.

Estimation of cholesterol and free fatty acids in gallstones: After observation with microscopes, gallbladder bile (content of the gallbladder) was suspended in 1 ml of distilled water, and the suspension was passed through a glass filter (Whatman GF-A, 25 mm in diameter, Whatman Ltd., England). The filter was washed with 10 ml of distilled water and dried at room temperature. Lipids in the substances trapped on the filter were extracted three times with 1 ml of a mixture of chloroform and methanol (2:1) at room temperature. The extracts obtained by these washings were mixed together, and the solvent was evaporated by vacuum. The residue thus obtained was redissolved with 50 μl of chloroform-methanol, and 10 μl of the aliquot was used for determination of cholesterol amount by high performance liquid chromatography (HPLC). The solvent of the remaining extract (40 μl) was evaporated again by vacuum for free fatty acid analysis.

Cholesterol was estimated by HPLC. A Shimadzu HPLC model LC-5A equipped with a sample injector, model Rheodyne 7125 (Rheodyne, CA, U.S.A.), and a variable UV detector, model SPD-2AM (Shimadzu Co., Kyoto, Japan), was used with a reverse phase column of 8x100 mm (internal diameter) packed with Radialpak Bondapak C18 (Waters Assoc., MA, U.S.A.). The flow rate was 2.0 ml/min at room temperature. UV detection was performed at 210 nm. Detector response was detected with a Shimadzu model JEOL Recorder JR-251A (Nihondenshi, Tokyo, Japan). The mobile phase was methanol and acetonitril (65:35).

Free fatty acids in hepatic bile and in the extract of the substances trapped on glass filter were also analyzed by HPLC according to the method of Passi et al. (9), using the same equipment described above. The amount of cholesterol and free fatty acids in the gallstones was expressed as mg per gallbladder content.

Results
Formation of cholesterol crystals and gallstones in the gallbladder was detected not only by observations using polarized-light and light microscopes but also by direct estimation of cholesterol amount in the solid substances trapped on the glass filter obtained by filtering a suspension of the gallbladder contents. Cholesterol crystals and gallstones were detected by observation using a polarized-light microscope as the major components of these solid materials. No solid materials and liquid crystals were detected by observations using polarized-light and light microscopes in the eluent obtained by washing the glass filter. Therefore, almost all of the cholesterol crystals and gallstones were assumed to be trapped...
on the glass filter. When a pre-weighed powder of cholesterol in place of gallbladder contents was applied on a glass filter, and the cholesterol trapped on the filter was then washed, extracted and analyzed by HPLC according to the same procedure mentioned above, the recovery of the cholesterol was about 95%. Therefore, the values obtained by HPLC indicate the contents of cholesterol in the gallstones and the crystals formed in the gallbladder.

Figure 1 shows the amounts of cholesterol in the solid substances trapped on the glass filter obtained from the contents of the gallbladder in mice fed a diet containing 1% cholesterol and 0.5% cholic acid (Fig. 1a), 1% cholic acid (Fig. 1b), and 1% cholesterol (Fig. 1c). Solid cholesterol amount in the gallbladder of mice, maintained with a diet containing both cholesterol and cholic acid, increased within 30 days and was markedly enhanced on the 40th day of the experiments (P<0.01 vs. control group). The extent gradually increased up to the 100th day of the experiments (P<0.005). Cholesterol crystals were observed with a polarized-light microscope on the 20th day of the feeding, and gallstones (large aggregates of cholesterol crystals) appeared on the 40th day. On the other hand, by single addition of 1% cholic acid to a diet, the amount of cholesterol was increased on the 50th day of the experiments (P<0.005 vs. control group) and gallstones occurred on the 60th day. These values were increased markedly on the 80th and 100th day of the experiments. These gallstones were somewhat globular and white, and substances other than cholesterol crystals were attached to the large aggregates of cholesterol crystals. Therefore, these do not seem to be pigment gallstones and to be somewhat different from those induced in mice fed a diet containing 1% cholesterol and 0.5% cholic acid.

Single addition of 1% cholesterol to the diet had no affect on the induction of cholesterol crystals and gallstones. However, the amount of solid cholesterol increased on the 20th day of the experiments (P<0.005 vs. control group) and gallstones occurred on the 60th day. These gallstones were somewhat globular and white, and substances other than cholesterol crystals were attached to the large aggregates of cholesterol crystals. Therefore, these do not seem to be pigment gallstones and to be somewhat different from those induced in mice fed a diet containing 1% cholesterol and 0.5% cholic acid. Single addition of 1% cholesterol to the diet had no affect on the induction of cholesterol crystals and gallstones. However, the amount of solid cholesterol increased on the 20th day of the feeding (P<0.01 vs. control group). The reasons why this temporary increase in cholesterol amount occurred can not be explained at present. In this group, neither cholesterol crystals nor gallstones were observed with a polarized-light microscope and a solid substance containing cholesterol was not trapped on the glass filter during the period between the 30th and 100th day of the experiments.

As shown in Fig. 2a, single addition of 1% cholic acid to the diet increased total bile acid concentration in hepatic bile during the experiments, while bile acid concentration did not change significantly in the other groups. Figure 2b illustrates changes in lecithin concentration in hepatic bile. In both the groups that showed gallstone formation, lecithin concentration was increased during a course of the experiment. On the contrary, the group given 1% cholesterol kept a slightly sub-normal level to the 80th day of the experiments. In all groups, cholesterol concentration was high during the first 40 days of the feeding. These high cholesterol concentrations were maintained up to 100 days in both the groups that showed gallstone formation.
formation (Fig. 2c). On the other hand, cholesterol concentration in the hepatic bile from mice given a diet containing 1% cholesterol were reduced to the normal level on the 50th day of the feeding.

Figure 3 indicates molar percent of cholesterol among bile acid, lecithin and cholesterol in hepatic bile calculated from the data presented in Fig. 2. The molar percent of cholesterol in hepatic bile was elevated by additions of cholesterol and cholic acid to the diet. On the contrary, single addition of cholic acid to a diet had no effect. These results indicate that gallstone formation in mice fed both cholesterol and cholic acid corresponds to a lower cholesterol solubilizing capacity in the bile. In the present study, major biliary lipids (i.e., bile acid, lecithin and cholesterol) in gallbladder bile were not estimated because all gallbladder contents were used for estimating cholesterol and free fatty acid amount in the solid substances in the gallbladder. In the previous report, these lipid concentrations in gallbladder bile were somewhat higher than those in the hepatic bile, but the relative amounts of these lipids were almost the same as those of the hepatic bile. Thus, the molar percent of cholesterol in hepatic bile is assumed to be related to that in gallbladder bile.

The appearance of gallstones in mice given 1% cholic acid, however, seems not to be accompanied with a supersaturation of cholesterol in hepatic and gallbladder bile, because the value of the molar percent of cholesterol is the same as that obtained from the control animals. Therefore, it is possible that unknown substances other than cholesterol which contribute to gallstone induction and/or constitute gallstones may exist in bile. We expected that one of these factors was a saturated free fatty acid, such as palmitic acid (hexadecanoic acid), which is practically insoluble in water, but may be dissolved in a complex micelle composed of bile acids and lecithin in bile. So we tentatively analyzed for free fatty acids in the hepatic bile.
Free Fatty Acids in the Gallstones

As shown in Fig. 4, free fatty acids (myristic acid, palmitic acid and stearic acid) were detected by HPLC from the hepatic bile and gallstones in mice maintained on a diet containing 1% cholic acid for 50 days. The main component of the free fatty acids in the hepatic bile and gallstones was palmitic acid.

Figure 5 illustrates the relationship between the amount of cholesterol and the amount of free fatty acids in the extract from the solid substances (mainly, cholesterol crystals and gallstones) trapped on the glass filter by filtering the contents of a gallbladder. In the present study, weight of the solid substances on the glass filter could not be estimated because at the 10th and 20th day of the feeding, the contents were too small to measure these weights exactly.

After extraction of the lipids from the glass filter, hardly any solid substance could be observed on the filter by polarized-light and light microscopes. In the mice fed 1% cholesterol and 0.5% cholic acid, the main component of the solid materials was cholesterol. On the other hand, significant amounts of free fatty acids were detected from the solid materials in the mice maintained with 1% cholic acid. The gallstones induced by combined administration of cholesterol and cholic acid are assumed hereafter to be cholesterol gallstones from its lipid composition. However, the gallstones, which are abundant in free fatty acids, appearing in the mice maintained on a diet containing 1% cholic acid seem to be somewhat different from the cholesterol gallstone.

Discussion

In the present study, combined administration of cholesterol and cholic acid to a diet induced cholesterol crystals and gallstones in the gallbladder of mice in accordance with the results of Tepperman et al.
The cholesterol crystals and gallstones were detected not only by observation using polarized-light and light microscopes but also by direct measurement of cholesterol amount in the extract from solid substances trapped on the glass filter obtained by filtering the contents of a gallbladder. These solid substances were solubilized almost completely by extraction with chloroform and methanol. Therefore, the main components of these substances are assumed to be lipids.

Estimation of the weight of these solid substances in an individual mouse was difficult because the value at the early periods of gallstone formation was too small to be determined exactly. However, in some cases in which large gallstones were induced in mice fed a diet containing 1% cholesterol and 0.5% cholic acid for long periods, the weights of the solid substances could be measured, and the values were almost the same as the amount of cholesterol extracted from the solid substances in the gallbladder. Thus, the gallstones induced by combined administration of cholesterol and cholic acid probably are cholesterol gallstones.

Gallstones could also be induced by a 1% cholic acid-containing diet. Tepperman et al. (6) failed to induce gallstones in mice given 1% cholic acid. The reasons for this discrepancy are not known at present. This may result from differences in the diets used in the studies. In the future additional experiments must be done 1% cholic acid-containing diets not used in the present study. In the preliminary study, addition of 0.5% cholic acid to the diet was not so effective for stone induction. Few animals formed gallstones when they were maintained on this diet for 100 days. On the other hand, addition of 1.5% cholic acid to the diet inhibited body weight gain. Thus 1.5% cholic acid seems to be an "over dose". Therefore, addition of 1.0% cholic acid is most effective for gallstone formation. The gallstones contained significant amounts of free fatty acids, mainly palmitic acid. Therefore, these gallstones seem to be somewhat different from those induced by combined administration of cholesterol and cholic acid.

The hepatic bile in mice fed a diet containing 1% cholic acid also contained high concentrations of free fatty acids. No solid substance was observed in the hepatic bile by observations using polarized-light and light microscopes. These results indicate that free fatty acids, such as palmitic acid which is hardly soluble in water, exist in the solubilized form in hepatic bile. It seems likely that free fatty acids in the hepatic bile, as well as cholesterol, are dissolved in complex micelles composed of bile acids and lecithin. If the micelles actually dissolve free fatty acids, the cholesterol solubilizing capacities of the micelles will be diminished by solubilizing free fatty acids in place of cholesterol. Therefore, free fatty acids as well as cholesterol in the hepatic bile are probably contributory to stone induction, and the stone thus formed probably contains significant amounts of free fatty acids.

Although the "triangle theory" of Admirand and Small (4) has been used for evaluating cholesterol solubilizing capacity in bile, free fatty acids will be also important in determining the capacity. The source of free fatty acids in hepatic bile and the changes of the concentrations in hepatic and gallbladder biles by administration of cholic acid are now under investigation.

In the present study, the extent of the formation of cholesterol crystals and gallstones is expressed as the cholesterol amount extracted from solid materials, mainly gallstones and cholesterol crystals, in the contents of a gallbladder. Although this is a very useful and sensitive method to evaluate the degree of gallstone formation, it is difficult to distinguish between the cholesterol crystals and the gallstones. Therefore, it is necessary to observe the contents of the gallbladder by polarized-light and light microscopes prior to the extraction of the lipids.

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