Cholesterol gallstones in alloxan-diabetic mice

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Abstract Normal and alloxan-diabetic male mice (Crj-ICR) were fed a diet containing 0.5% cholesterol for 5 and 10 weeks, and gallbladder bile was analyzed for cholesterol, phospholipids and bile acids, feces for sterols and bile acids, and plasma and liver for cholesterol, phospholipids, and triglycerides. Normal mice developed no gallstones but the diabetic mice developed cholesterol gallstones with an incidence of 70% by 5 weeks and 80% by 10 weeks after feeding of the cholesterol diet. Diabetic mice fed the ordinary diet also developed stones (23%) by 10 weeks. In the diabetic mice, the gallbladder was enlarged about threefold, and biliary lipid concentration, diet intake, and fecal excretion of sterols and bile acids increased but body weight decreased. Cholic acid and β-muricholic acid comprised over 40% each of the total biliary bile acids in normal mice, but cholic acid increased to about 80% and β-muricholic acid decreased to a few percent in the diabetic mice. Fecal excretion of bile acids increased after cholesterol feeding in both normal and diabetic mice, but the increased bile acid in the normal animals was β-muricholic acid and that in the diabetic mice was deoxycholic acid. The mice that developed gallstones showed a marked increase in biliary cholesterol value and decreases in gallbladder bile and bile acid concentration, but no difference in biliary and fecal bile acid composition, bile acid synthesis, fecal sterols, or plasma and liver lipid levels. Cholesterol absorption was increased in the diabetic mice when examined by plasma $^{14}C/^{3}H$ ratio and fecal ^{14}C-labeled sterol excretion after a single oral administration of [^{14}C]cholesterol and a simultaneous intravenous injection of [^{3}H]cholesterol. These data led to the conclusion that cholesterol gallstones developed in alloxan-diabetic mice fed excess cholesterol, due to the hyperphagia and the enhancement of cholesterol absorption caused by increases in the synthesis and secretion of cholic acid. —**Akiyoshi, T., K. Uchida, H. Takase, Y. Nomura, and N. Takeuchi.** Cholesterol gallstones in alloxan-diabetic mice. *J. Lipid Res.* 1986. **27:** 915–924.

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Accompanying the extension of the human life span, the incidence of cholesterol gallstones has increased. The precise mechanism of cholesterol gallstone formation is not known but the decrease in bile acid pool produced by the increase in bile acid loss or the decrease in bile acid synthesis, as well as the increase in biliary cholesterol excretion, is generally accepted as a cause of this disease (1). Experimentally, gallstones can be produced in mice by feeding cholesterol and cholic acid in excess (2–4). When cholic acid is withdrawn (2) or replaced with chenodeoxycholic acid (5), no stones develop, suggesting that the role of cholic acid is essential in this animal model.

On the other hand, cholesterol dynamics are altered in diabetic rats. Accumulation of cholesterol in serum and liver (6, 7) and reduction of the activity of hydroxymethylglutaryl CoA (HMG-CoA) reductase of the liver (8), a key enzyme in cholesterol synthesis, are observed. Since these changes are caused by hyperphagia (9) and enhancement of cholesterol absorption (6), prevention of hyperphagia (10) or cholesterol absorption (11) normalizes serum cholesterol level and HMG-CoA reductase activity in these animals. In addition, bile acid metabolism is also altered in diabetic rats (6, 7) and mice (12). The pool size and synthesis and biliary secretion of cholic acid markedly increase, while those of chenodeoxycholic acid and β-muricholic acid decrease. Since cholic acid is essential to cholesterol absorption (13, 14), a change in bile acid metabolism may affect cholesterol dynamics in these animals.

The present experiments were done to examine whether cholesterol gallstones would form by feeding of cholesterol without cholic acid in alloxan-diabetic mice. The above-mentioned changes of bile acid metabolism in diabetic animals seemed to be lithogenic and a high incidence of cholesterol gallstones has been reported in diabetic patients (15).

The incidence of gallstones in diabetic humans is reported to be about 25% (16) and is considered to be higher than that found in the general population. Although Zahor et al. (17) have been unable to confirm this, the bile in diabetes is commonly supersaturated with cholesterol (18, 19) and, in turn, there is a trend toward the development of cholesterol gallstones (20). Biliary bile

Abbreviations: GLC, gas-liquid chromatography; TLC, thin-layer chromatography.

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acid composition was also compared in insulin-treated and untreated states of diabetic patients (18) and among control, juvenile diabetic and maturity-onset diabetic subjects (19). No significant difference was found in the mean values of each biliary bile acid, although a trend toward increased cholic acid and deoxycholic acid was found in diabetic subjects.

MATERIALS AND METHODS

Animal treatment

ICR strain male mice, 6 weeks old, obtained from Charles River Japan (Crj-ICR) were kept in an air-conditioned room (25 ± 1°C, 50–60% humidity) lighted 12 hr a day (8:00 AM to 8:00 PM). Ordinary powdered diet (JCL-CA-1, Clea Japan Inc., Tokyo) was provided as a basal diet and cholesterol was added (0.5%) to the basal diet. The composition of the basal diet was as follows: 25.5% protein, 4.0% lipids, 53.5% carbohydrate, 4.0% fiber, 7.0% ash, and 6.0% water. The content of cholesterol was about 0.1%.

Ten mice each were housed in one cage and given either basal or cholesterol diet (utilizing a powdered diet feeding apparatus, Natsume Seisakusho Co. Ltd., Tokyo) and tap water ad libitum. When feces were collected, the mice were kept individually in specially designed cages (21) and 2-day feces collections were made during the last week of the experiments.

After fasting overnight, the mice were given intravenous alloxan monohydrate (50 mg/kg) and were killed 5 or 10 weeks after the treatment. Before being killed, the animals were fasted for 5 hr and the gallbladder was removed under sodium methylhexabital anesthesia (125 mg/kg, i.p.). Blood was then withdrawn from the abdominal aorta and the liver was removed.

Grades of gallstones

The presence of gallstones was examined visually by holding the gallbladder against light and gallbladders were graded from Grade 0 (no gallstones) to Grade V, which was tentatively established in normal (not diabetic) mice fed a lithogenic diet containing 0.5% cholesterol and 0.25% sodium cholate for 5 to 10 weeks according to the regimen of Tepperman, Caldwell, and Tepperman (2). The grades and the corresponding cholesterol values are given in **Table 1.** The grade was based on the cholesterol content but it corresponded well with the visual appearance.

Gallstone analyses

Components of the typical gallstones formed in the alloxan-diabetic mice given cholesterol and those in the normal mice given cholesterol and sodium cholate according to the report of Tepperman et al. (2) were analyzed by infrared spectrometry (KBr tablet method) using a Jasco A-702 spectrophotometer. Since the gallstones contained

TABLE 1. Grade of experimental gallstones in mice[a]

Grade[b]	Cholesterol Content in Gallbladder Bile		Approximate Degree of Visualized Cholelithiasis
	mg/g		
0	< 10		Gallbladder is filled with clear bile.
I	11 ~ 20		A few fine crystals are found.
II	21 ~ 30		Around ten fine crystals are found.
III	31 ~ 40		Fine crystals occupy about a half of the gallbladder.
IV	41 ~ 50		Leaflet or stratified crystals occupy over a half of the gallbladder.
V	> 50		Round gallstones are found.

[a] Crj:ICR male mice (6 weeks) were fed diet containing 0.5% cholesterol and 0.25% sodium cholate for 5 to 10 weeks.

[b] The grade was based on the cholesterol content in gallbladder bile but corresponded well with the visualized cholelithiasis.

protein, which was evident by the absorption peaks at 1650 cm^{-1} and 1530 cm^{-1} (**Fig. 1**), the relative protein content was calculated from a linear relationship between the albumin content against cholesterol (a%), and the ratio (d) of the absorption at 1530 cm^{-1} (of albumin) and 1053 cm^{-1} (of cholesterol). The equation was $a = 56.5 \cdot d - 0.6$ ($r = 0.999$), where d = D1530/D1053. The absorption peaks of lecithin ($V_{C=O}$ 1750–1730 cm^{-1}, $V_{P=O}$ 1200–1100 cm^{-1} and 1100–1000 cm^{-1}) and bilirubin (1692, 1612, 1249 cm^{-1}) were not found (Fig. 1). The content of calcium was not determined.

Lipid determination

The gallbladder was crushed in 20 ml of ethanol with a glass rod and biliary lipids were extracted by refluxing for 10 min at 80–90°C (12). After filtration, a portion was evaporated to dryness under a stream of nitrogen and the residue was hydrolyzed in 2 ml of 1.25 N NaOH at 120°C for 6 hr. Cholesterol was extracted with diethyl ether, then the bile acids were extracted with diethyl ether after the mixture had been acidified with 2 N HCl (21, 22). Cholesterol was determined by GLC using a 1.5% SE-30 column (23). Bile acids were converted into trifluoroacetate methyl ester derivatives and determined by GLC using a 1.5% QF-1 column (22, 23). Cholesterol in serum and liver and sterols and bile acids in feces were determined by procedures described previously (22, 24). Phospholipids in bile, serum, and liver were quantified by the method of Gomori (25). Triglyclerides in plasma and liver were determined by the method of Fletcher (26).

In the present experiments, the gallbladder bile was analyzed together with gallbladder tissue, but the weight of one sample of gallbladder tissue was about 1 mg or less when examined after the bile had been washed out with saline. The mean cholesterol content in one sample of gallbladder tissue was 2.62 μg and phospholipid content was 39.4 μg; these values did not seem to affect the values of biliary lipids. The biliary lipid concentrations were expressed on a weight basis since the bile volume of one mouse was so small. The specific gravity of normal and diabetic mouse bile was 1.035–1.043.

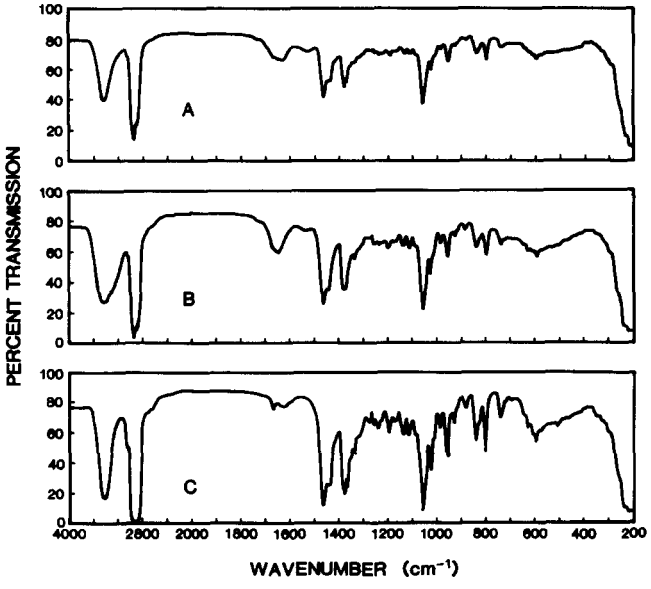

Fig. 1. Infrared spectra of gallstones found in an alloxan-diabetic mouse given cholesterol (A) and in a normal mouse given cholesterol and sodium cholate (B), and of authentic cholesterol (C).

Determination of cholic acid and β-muricholic acid

Since cholic acid and β-muricholic acid showed almost the same retention time on a QF-1 column GLC (22, 23), they were first separated by TLC and then quantified by GLC. The methylated bile acid fraction was applied to a silica gel TLC plate (precoated silica gel GF, 250 nm, Uniplate, Analtech, Newark, DE) and developed in a solvent system of benzene–acetone 7:3. Spots were visualized with iodine vapor and those corresponding to methylcholate and methyl-β-muricholate were scraped off. They were extracted with methanol, trifluoroacetylated, and then quantified by GLC. The recovery rates were about 80% for both bile acids. In practice, the composition ratio of cholic acid and β-muricholic acid was determined by the present procedures and the amounts of both bile acids were calculated from the data obtained by the GLC procedures described previously (22, 23).

Other determinations

Plasma glucose level was determined using the "Blood Sugar-GOD-Perid Test" (Boehringer Mannheim, West Germany). Plasma creatinine, urea-N, and bilirubin levels were determined by the use of Technicon SMAC (C 9100) system (Technicon Instruments Corp., Tarrytown, NY).

Cholesterol absorption

Cholesterol absorption was determined by the dual isotope ratio method reported by Zilversmit (27) and Zilversmit and Hughes (28) with a slight modification (29), and by the determination of fecal excretion of ^{14}C-labeled sterol after a single oral administration of [^{14}C]cholesterol (29). Cholesterol absorption was calculated by the plasma $^{14}C/^{3}H$ ratio (27, 28), but since the diabetic mice showed marked hyperphagia, the specific activity of [^{14}C]cholesterol given orally was decreased by dietary cholesterol, and therefore we corrected the values using the specific activities of ^{14}C-labeled sterols in the feces. The equation for correction was: corrected $^{14}C/^{3}H$ ratio = $^{14}C/^{3}H$ × sp act of ^{14}C-labeled sterols in normal mouse feces/sp act of ^{14}C-labeled sterols in diabetic mouse feces.

Five weeks after the injection of alloxan monohydrate, each mouse received a single oral administration of 2.0 μCi of [4-^{14}C]cholesterol (5.0 mg) suspended in 0.2 ml of 5% gum arabic solution containing 20% ethanol. At the

same time, they received an intravenous injection of 0.5 μCi of [1,2-^{3}H]cholesterol (5 μg) suspended in 0.1 ml of saline solution containing 5% ethanol. [4-^{14}C]Cholesterol and [1,2-^{3}H]cholesterol were obtained from Amersham Japan Co. (Tokyo, Japan). The tracers were administered at 9 AM. Feces were collected daily for 3 days at 24-hr intervals. The gallbladder, blood, and liver were removed under anesthesia 72 hr after administration of the tracers. The feces were homogenized in a minimum volume of water and extracted with absolute ethanol by procedures described previously (21, 23, 29). Portions of the feces before extraction and the residue after extraction were combusted utilizing a sample oxidizer (Packard Tri-Carb Model B 0306) and the radioactivity was determined. The extraction efficiency was over 98%.

The plasma and sterol fractions of the gallbladder bile and feces were combusted and the radioactivities for ^{14}C and ^{3}H were determined, respectively. The procedures for extraction of sterols from gallbladder bile and feces were described above.

In other experiments, 1 μCi of [4-^{14}C]cholesterol (1.0 mg) suspended in the same medium described above was administered orally to both normal and diabetic mice (5 weeks after alloxan injection), and changes in plasma radioactivities and fecal ^{14}C-labeled sterol excretion were examined. The data on plasma radioactivities were obtained from different animals since they were killed at the times scheduled (1–72 hr); data on fecal ^{14}C-labeled sterols were obtained from the same animals up to 7 days.

Statistical analysis

Statistical significance was estimated by Student's *t*-test. A P value of less than 0.05 was considered to be significant.

RESULTS

Body weight, liver weight, diet intake, and plasma biochemical parameters

General and plasma biochemical parameters of alloxan-diabetic mice are given in **Table 2.** The diabetic mice ate more than twice the normal amount of diet, but they showed almost no gain in body weight. The liver weight increased in the diabetic mice, and the increase was remarkable when calculated on body weight basis (g/10 g body weight). The plasma glucose level increased markedly (ca. 800 mg/dl) and was about four times that of the normal mice. Plasma creatinine and urea-N levels increased in the alloxan-diabetic mice, suggesting kidney failure in these animals. Plasma bilirubin levels, however, were not changed by 10 weeks.

Gallstones in alloxan-diabetic mice

The occurrence of gallstones in normal and alloxan-diabetic mice is shown in **Table 3.** Normal animals did not develop gallstones when sodium cholate was not added to the cholesterol diet, but the diabetic mice developed gallstones without dietary cholate within 5 weeks. Three out of 13 diabetic mice developed gallstones even by feeding of the ordinary diet for 10 weeks. The cholesterol content in the gallbladder bile including gallstones increased in the animals fed the cholesterol diet for a longer period.

Appearances of typical gallstones formed in an alloxan-diabetic mouse given cholesterol and those in a normal mouse given cholesterol and sodium cholate are shown in **Fig. 2, A and B,** respectively. These stones were found in one animal each in both cases.

TABLE 2. Body weight, liver weight, diet intake, and plasma biochemical parameters

	Normal Mice				Diabetic Mice[a]			
	Ordinary Diet		Cholesterol Diet		Ordinary Diet		Cholesterol Diet	
	5 Weeks	10 Weeks	5 Weeks	10 Weeks	5 Weeks	10 Weeks	5 Weeks	10 Weeks
Experiment I								
No. of mice	10	10	10	10	10	13	10	7[b]
Body weight (g)	37[c]	40	38	41	26[d]	29[d]	27[d]	32[d]
Liver weight (g)	1.8	1.8	1.9	1.9	2.0	2.2[d]	2.3[d]	2.7[d]
Diet intake (g/day)	5.0	5.2	5.7	4.7	11.2[d]	12.4[d]	11.8[d]	12.4[d]
Water intake (ml/day)		6.4		6.6		71.3[d]		73.7[d]
Plasma glucose (mg/dl)		174		217		845[d]		892[d]
Experiment II								
No. of mice		8				7		11
Plasma glucose (mg/dl)		253				777[d]		793
Plasma creatinine (mg/dl)		0.44				0.58[d]		0.62
Plasma urea-N (mg/dl)		27				42[d]		40
Plasma bilirubin (mg/dl)		0.10				0.09		0.08

[a] A dose of 50 mg/kg of alloxan was injected intravenously 5 to 10 weeks before.
[b] Three mice out of 10 died at 28, 41, and 42 days after the start of the experiment.
[c] Mean value.
[d] Statistically significant compared to the corresponding normal mice ($P < 0.05$).

TABLE 3. Occurrence of gallstones in normal and alloxan diabetic mice

Duration Experiment	Animal	Diet	Occurrence of Gallstones		Grade[a]					
					0	I	II	III	IV	V
5 Weeks	Normal	Ordinary diet	0/10	0%	10					
5 Weeks	Normal	Cholesterol diet	0/10	0%	10					
5 Weeks	Normal	Cholesterol diet + cholic acid[b]	7/10	70%	3	1	5	1		
5 Weeks	Alloxan[c]	Ordinary diet	0/10	0%	10					
5 Weeks	Alloxan[c]	Cholesterol diet	7/10	70%	3	2	2	2		1
10 Weeks	Normal	Ordinary diet	0/10	0%	10					
10 Weeks	Normal	Cholesterol diet	0/10	0%	10					
10 Weeks	Normal	Cholesterol diet + cholic acid[b]	7/10	70%	3		1	2	2	2
10 Weeks	Alloxan[c]	Ordinary diet	3/13	23%	10		1	1		1
10 Weeks	Alloxan[c]	Cholesterol diet	8/10	80%	2		1	3	4	

[a]Grade is shown in Table 1.
[b]Cholic acid (0.25%) was added to the 0.5% cholesterol diet.
[c]A dose of 50 mg/kg of alloxan was injected intravenously.

Fig. 1 shows the infrared spectra of both kind of stones. They were composed mostly of cholesterol but contained small amounts of proteins (**Table 4**). Lecithin and bilirubin were not found.

Lipid composition in gallbladder bile

The gallbladder weight including bile, the lipid composition in the gallbladder bile, and bile acid composition are given in **Table 5.** The diabetic mice were divided into two groups, gallstone-positive (+) and -negative (−) without regard to the experimental periods, since no significant difference was found between the groups of 5 and 10 weeks, except slightly higher values for cholesterol in the 10-week diabetic mice.

The gallbladder bile weight was about three times higher in the gallstone-negative diabetic mice than in the normal mice. When gallstones developed in the diabetic mice fed either the ordinary diet or the cholesterol diet, the gallbladder bile weight decreased.

The concentration of biliary lipids, especially bile acids and phospholipids, increased in the diabetic mice. The cholesterol also increased or showed a trend to increase in the diabetic mice, but there was a large increase in the gallstone-positive mice, which would be due to the fact that the cholesterol levels included cholesterol in gallstones. After feeding the cholesterol diet, however, the concentrations of bile acids and phospholipids in the gallstone-positive mice decreased.

In regard to biliary bile acid composition, cholic acid and β-muricholic acid were two major constituents in normal mouse bile, and each comprised over 40% of the total bile acids. Deoxycholic, α-muricholic, chenodeoxycholic, hyodeoxycholic, and some keto bile acids were also found but their amounts were small. In the diabetic mice, cholic acid markedly increased to about 80% while β-muricholic acid decreased to a few percent, without any relation to development of gallstones. Deoxycholic acid increased slightly but the other bile acids remained unchanged. Cholesterol feeding to normal mice resulted in an increase in chenodeoxycholic acid and a decrease in cholic acid, but the changes were very slight. In the diabetic mice, cholesterol feeding produced almost no change except a slight increase in α-muricholic acid. Although the diabetic mice showed such remarkable changes in the bile acid composition, there was no difference between gallstone-positive and gallstone-negative mice.

Fecal sterols and bile acids

The analyses were performed on five randomly selected mice in each group. As shown in **Table 6,** the diet intake and dry weight of feces almost doubled in the diabetic mice. The fecal sterols, coprostanol and cholesterol, and bile acids also increased in the diabetic mice but the increase in the sterols was more predominant. Cholesterol feeding increased not only sterols but also bile acids in both normal and diabetic mice. In the fecal bile acids,

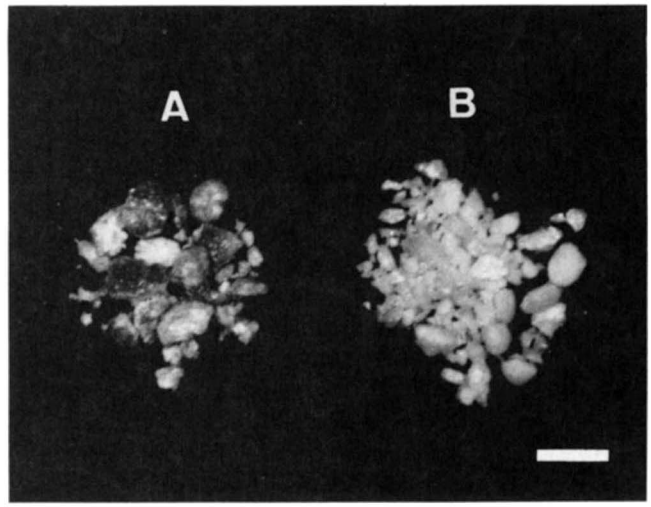

Fig. 2. Gallstones found in an alloxan-diabetic mouse given cholesterol (A) and in a normal mouse given cholesterol and sodium cholate (B). Bar marker indicates 1 millimeter (magnification × 9).

TABLE 4. Composition of gallstones in mice

Gallstones	Dry Weight	Cholesterol	Protein	Lecithin	Bilirubin
	mg/mouse				
A[a]	2.4	86.7%	13.3%	nd	nd
B[b]	3.1	96.8%	3.2%	nd	nd

Mean values in two animals; nd, not detectable.
[a]Gallstones found in alloxan-diabetic mice given cholesterol.
[b]Gallstones found in normal mice given cholesterol and sodium cholate.

deoxycholic and cholic acids increased but β-muricholic acid decreased in the diabetic mice. On the other hand, cholesterol feeding increased β-muricholic acid and decreased deoxycholic acid in the normal mice, but it increased deoxycholic acid in the diabetic mice. No significant difference was found between gallstone-positive and -negative mice.

Plasma and liver lipids

The diabetic mice showed marked increases in plasma glucose, cholesterol, and phospholipid levels and liver cholesterol level, with a decrease in body weight and an increase in liver weight (**Table 7**). Cholesterol feeding caused no significant change in plasma lipid levels but showed a tendency to increase the liver triglyceride concentration in both normal and diabetic mice. No significant difference was found between gallstone-positive and gallstone-negative mice.

Cholesterol absorption

Fig. 3 shows the changes in plasma radioactivities after oral administration of [^{14}C]cholesterol (1.0 μCi/mouse). The radioactivities increased almost in parallel in both the groups up to 8 hr after the administration of cholesterol, but the radioactivities in the normal mice decreased gradually thereafter while those in the diabetic mice remained high, with the highest value at 12 hr.

Radioactivities of ^{14}C and ^{3}H (derived from oral [^{14}C]cholesterol and intravenous [^{3}H]cholesterol) in the plasma, liver, bile, and feces are shown in **Table 8.** The cholesterol absorption calculated from the corrected plasma ^{14}C/^{3}H ratio was 20.3% in the normal mice and 32.1% in the diabetic mice. Fecal excretion of ^{14}C-labeled sterols decreased in the diabetic mice. Cholesterol absorption rate calculated from the fecal ^{14}C-labeled sterols was 26.2% in the normal mice and 38.2% in the diabetic mice. These values are very close to those obtained by the plasma ^{14}C/^{3}H ratio method.

DISCUSSION

The present experiments demonstrated that cholesterol gallstones developed in alloxan-diabetic mice by cholesterol feeding.

The experimental formation of gallstones in mice was first reported by Tepperman et al. (2). They demonstrated that the feeding of cholesterol and cholic acid produced cholesterol gallstones in mice, and withdrawal of either cholic acid or cholesterol (2), or replacement of cholic acid with chenodeoxycholic acid (5) failed to cause development of stones. As for the mechanisms through which the stones develop, Pedreira and Tepperman (30) and Caldwell, Levitsky, and Rosenberg (31) showed that biliary

TABLE 5. Biliary lipids and bile acid composition in normal and diabetic mice

		Normal Mice		Diabetic Mice[a]			
				Ordinary Diet		Cholesterol Diet	
		Ordinary Diet	Cholesterol Diet	Gallstones (−)	Gallstones (+)	Gallstones (−)	Gallstones (+)
No. of mice		19	16	20	3	4	12
Gallbladder bile (mg)		28 ± 2.4[b]	28 ± 2.8	96 ± 7.0[c]	42 ± 12.3[c]	52 ± 11.9	19 ± 4.6[d,e]
Cholesterol (mg/g)		3.8 ± 0.27	5.5 ± 0.43[c]	4.8 ± 0.35[c]	20.5 ± 4.88[e]	7.6 ± 0.88	27.1 ± 3.61[d,e]
Phospholipids (mg/g)		18.2 ± 1.44	16.4 ± 0.67	41.4 ± 1.59[c]	36.9 ± 6.13	51.8 ± 3.41[d]	33.7 ± 2.32[e]
Bile acids (mg/g)		39.5 ± 2.72	39.8 ± 2.85	77.4 ± 4.21[d]	71.4 ± 11.92	78.5 ± 5.84[d]	44.5 ± 8.12[e]
Lithocholic acid	(%)	<1	<1	<1	<1	<1	<1
Deoxycholic acid	(%)	2 ± 0.3	3 ± 0.3	6 ± 1.3	3 ± 0.7	7 ± 3.3	6 ± 1.3
α-Muricholic acid	(%)	3 ± 0.2	4 ± 0.2	2 ± 0.1	3 ± 0.4	4 ± 1.1	4 ± 0.3
Chenodeoxycholic acid	(%)	1 ± 0.3	4 ± 0.4[c]	3 ± 0.2[c]	3 ± 0.9	3 ± 0.3	3 ± 0.3
Hyodeoxycholic acid	(%)	2 ± 0.2	3 ± 0.2	2 ± 0.2	2 ± 0.5	2 ± 0.8	2 ± 0.2
Ursodeoxycholic acid	(%)	<1	1 ± 0.2	1 ± 0.1	1 ± 0.5	1 ± 0.3	1 ± 0.1
Cholic acid	(%)	42 ± 0.4	37 ± 0.5[c]	77 ± 1.6[c]	78 ± 2.7[c]	74 ± 3.3[d]	75 ± 2.1[d]
β-Muricholic acid	(%)	43 ± 0.4	43 ± 0.4	4 ± 0.4[c]	6 ± 0.2[c]	5 ± 0.7[d]	5 ± 0.4[d]
Keto bile acids	(%)	3 ± 0.5	2 ± 0.3	2 ± 0.4	2 ± 0.6	1 ± 0.3	3 ± 0.8
Others	(%)	2 ± 0.2	3 ± 0.3	3 ± 0.4	2 ± 0.9	3 ± 0.7	2 ± 0.4

[a]A dose of 50 mg/kg of alloxan was injected intravenously 5 or 10 weeks before.
[b]Mean ± SEM.
[c]Statistically significant compared to normal mice on ordinary diet ($P < 0.05$).
[d]Statistically significant compared to normal mice on cholesterol diet ($P < 0.05$).
[e]Statistically significant compared to gallstone (−) mice ($P < 0.05$).

TABLE 6. Diet intake, feces dry weight, and fecal sterols and bile acids in normal and diabetic mice

	Normal Mice		Diabetic Mice[a]		
			Ordinary Diet	Cholesterol Diet	
	Ordinary Diet	Cholesterol Diet	Gallstones (−)	Gallstones (−)	Gallstones (+)
No. of mice	10	10	10	3	7
Diet intake (g/day)	5.1 ± 0.18[b]	5.2 ± 0.31	11.8 ± 0.32[c]	11.8 ± 1.17[d]	12.2 ± 0.76[d]
Feces dry weight (g/day)	1.39 ± 0.062	1.32 ± 0.060	3.15 ± 0.172[c]	3.38 ± 0.205[d]	3.32 ± 0.225[d]
Total sterols (mg/day)	3.64 ± 0.112	13.38 ± 1.201[c]	7.28 ± 0.862[c]	38.86 ± 4.922[d]	39.91 ± 2.609[d]
Coprostanol (mg/day)	0.22 ± 0.060	1.18 ± 0.290[c]	2.07 ± 0.410[c]	4.64 ± 1.859	8.64 ± 2.221[d]
Cholesterol (mg/day)	3.44 ± 0.138	12.20 ± 1.178[c]	5.21 ± 0.661[c]	34.22 ± 6.193[d]	31.27 ± 2.710[d]
Bile acids (mg/day)	1.04 ± 0.070	1.96 ± 0.146[c]	1.77 ± 0.224[c]	3.65 ± 0.998	3.18 ± 0.240[d]
Lithocholic acid (%)	8 ± 0.7	6 ± 0.7	5 ± 0.8	4 ± 0.7	9 ± 3.4
Deoxycholic acid (%)	25 ± 1.2	20 ± 0.6[c]	34 ± 2.1[c]	44 ± 2.6[d]	46 ± 1.6[d]
α-Muricholic acid (%)	2 ± 0.2	4 ± 0.4	<1	2 ± 0.5	2 ± 0.2
Chenodeoxycholic acid (%)	2 ± 0.4	<1	1 ± 0.2	<1	<1
Hyodeoxycholic acid (%)	<1	<1	1 ± 0.3	<1	2 ± 0.5
Ursodeoxycholic acid (%)	2 ± 0.3	2 ± 0.4	<1	<1	2 ± 0.5
Ursocholic acid (%)	1 ± 0.3	2 ± 0.3	<1	<1	<1
Cholic acid (%)	2 ± 0.6	2 ± 0.6	9 ± 2.1	7 ± 1.3[d]	4 ± 0.7
β-Muricholic acid (%)	13 ± 1.3	19 ± 1.6[c]	5 ± 0.5[c]	4 ± 1.1[d]	3 ± 0.4[d]
ω-Muricholic acid (%)	21 ± 1.0	24 ± 1.5	18 ± 1.3	20 ± 2.4	16 ± 1.7[d]
Keto bile acids (%)	15 ± 1.1	12 ± 0.9	20 ± 1.9	15 ± 0.9	15 ± 1.6
Others (%)	8 ± 1.1	8 ± 0.6	4 ± 1.3[c]	4 ± 0.2	3 ± 0.4[d]

[a] A dose of 50 mg/kg of alloxan was injected intravenously 5 or 10 weeks before.
[b] Mean ± SEM.
[c] Statistically significant compared to normal mice on ordinary diet ($P < 0.05$).
[d] Statistically significant compared to normal mice on cholesterol diet ($P < 0.05$).

secretion of cholesterol increased after feeding a lithogenic diet that contained 1% cholesterol and 0.5% cholic acid, and suggested that the secretion of cholesterol-rich bile from the liver and the absorption of bile acids from the gallbladder are direct causes of the formation of gallstones in mice.

How cholesterol-rich bile is formed is another question but, since cholic acid markedly increases cholesterol absorption from the intestine (13, 14), the increase in cholesterol absorption and the subsequent accumulation of cholesterol in the liver would increase biliary secretion of cholesterol. Chenodeoxycholic acid shows almost no

TABLE 7. Plasma and liver lipid levels in normal and diabetic mice

	Normal Mice		Diabetic Mice[a]			
			Ordinary Diet		Cholesterol Diet	
	Ordinary Diet	Cholesterol Diet	Gallstones (−)	Gallstones (+)	Gallstones (−)	Gallstones (+)
No. of mice	20	20	20	3	5	12
Body weight (g)	38 ± 0.7[b]	40 ± 0.8	28 ± 0.6[c]	26 ± 3.8	29 ± 1.4[d]	29 ± 0.9[d]
Plasma cholesterol (mg/dl)	115 ± 5.0	117 ± 5.6	276 ± 12.3[c]	182 ± 24.9	249 ± 17.6[d]	249 ± 20.4[d]
Plasma phospholipids (mg/dl)	198 ± 7.3	193 ± 8.9	312 ± 13.6[c]	204 ± 31.4[c]	285 ± 27.5	316 ± 27.4[d]
Plasma triglycerides (mg/dl)	57 ± 4.5	56 ± 4.5	36 ± 2.8[c]	45 ± 5.3	38 ± 3.6	39 ± 3.6
Plasma glucose (mg/dl)	174 ± 11.4[f]	217 ± 16.9[f]	862 ± 22.6[cf]	777 ± 56.0[c]	885[g]	894 ± 23.1[d]
Liver weight (g)	1.8 ± 0.06	1.9 ± 0.05	2.1 ± 0.07[c]	1.8 ± 0.45	2.5 ± 0.22	2.4 ± 0.12[d]
Liver cholesterol (mg/g)	3.6 ± 0.12	6.7 ± 0.33[c]	6.2 ± 0.34[c]	9.3 ± 2.24[c]	12.1 ± 2.87	14.0 ± 2.37[d]
Liver phospholipids (mg/g)	50.0 ± 1.05	49.1 ± 0.52	46.3 ± 1.01[c]	43.8 ± 2.71	46.0 ± 1.32	45.6 ± 1.17
Liver triglycerides (mg/g)	9.5 ± 0.54	17.7 ± 3.30[c]	8.8 ± 1.49	23.5 ± 18.58	31.0 ± 11.64	32.3 ± 6.90[d]

[a] A dose of 50 mg/kg of alloxan was injected intravenously 5 or 10 weeks before.
[b] Mean ± SEM.
[c] Statistically significant compared to normal mice on ordinary diet ($P < 0.05$).
[d] Statistically significant compared to normal mice on cholesterol diet ($P < 0.05$).
[e] Statistically significant compared to gallstone (−) mice ($P < 0.05$).
[f] Mean ± SEM in ten mice after 10 weeks.
[g] Mean in two mice.

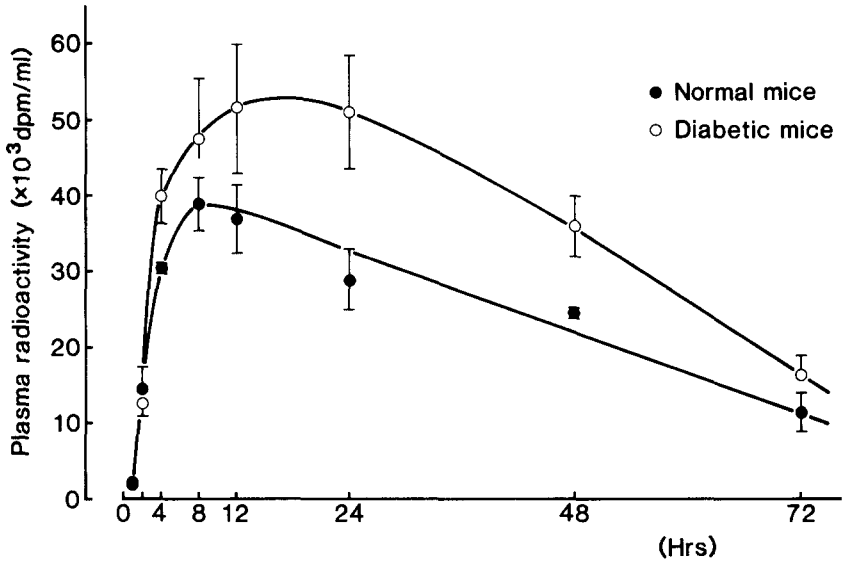

Fig. 3. Changes in plasma radioactivities after a single oral administration of [^{14}C]cholesterol (1 μCi/mouse) in normal (solid circle) and diabetic (open circle) mice. Each point and bar indicates mean ± SEM in five mice.

enhancement of cholesterol absorption (1, 14). Therefore, the role of cholic acid in the gallstone model of Tepperman et al. (2) seems to be the stimulation of cholesterol absorption.

In the diabetic mice, the gallbladder was enlarged and biliary cholesterol, phospholipid, and bile acid concentrations increased, though their molar ratios were similar to those in intact mice when cholesterol was not fed in excess. These changes were probably caused by the increase of bile acid secretion, since bile acids increased bile flow (32) and biliary cholesterol and phospholipids (33). Bile acid composition was altered in the diabetic mice; cholic acid increased and β-muricholic acid decreased, as was found in alloxan-injected rats (7). This trend was also found in the fecal bile acid composition, where deoxycholic and cholic acids increased while β-muricholic acid decreased (Table 6), suggesting that cholic acid synthesis increased but β-muricholic acid synthesis decreased in the diabetic mice. Therefore, the bile acid composition in the bile, and consequently in the intestine, of the diabetic mice resembled that in the mice given cholic acid, which would be a reason for the development of gallstones in diabetic mice by feeding cholesterol without cholic acid.

Three diabetic mice at 5 weeks and two at 10 weeks did

TABLE 8. Radioactivities of orally administered [^{14}C]cholesterol and intravenously injected [^{3}H]cholesterol in plasma, liver, bile and feces, and cholesterol absorption rate in normal and diabetic mice

	[^{14}C]Cholesterol		[^{3}H]Cholesterol	
	Normal	Diabetic[a]	Normal	Diabetic[a]
No. of mice	5	5	5	5
Plasma cholesterol (× 10^3 dpm/ml)	6.2 ± 1.18[b]	7.3 ± 0.54	15.1 ± 0.98	24.6 ± 2.63[c]
Liver cholesterol (× 10^3 dpm/g)	16.2 ± 2.84	28.5 ± 0.80[c]	45.8 ± 2.15	94.5 ± 16.01[c]
Bile cholesterol (× 10^3 dpm/g)	7.58 ± 1.91	16.17 ± 2.28[c]	22.7 ± 2.82	59.6 ± 6.30[c]
Feces sterols (× 10^3 dpm/3 days)	4867 ± 697.0	4082 ± 119.9	170.9 ± 15.40	221.5 ± 27.62
Feces sterols (× 10^3 dpm/mg sterol)	334.5 ± 50.06	152.0 ± 20.11[c]	11.7 ± 1.17	8.4 ± 1.51
Feces bile acids (× 10^3 dpm/3 days)	262.2 ± 25.09	205.4 ± 28.78	112.4 ± 7.38	69.6 ± 6.59[c]
Cholesterol absorption (%)				
Plasma ^{14}C/^{3}H ratio	20.3 ± 3.14	32.1 ± 7.68[d]		
Fecal excretion of ^{14}C-labeled sterols[e]	26.2 ± 3.81	38.2 ± 1.12[c]		

[a] A dose of 50 mg/kg of alloxan was injected intravenously 5 or 10 weeks before.
[b] Mean ± SEM in five mice.
[c] Statistically significant compared to normal mice ($P < 0.05$).
[d] The plasma ^{14}C/^{3}H ratio was calculated by the method of Zilversmit (23, 24) and corrected by the specific activity of fecal ^{14}C-labeled sterols. The corrected ^{14}C/^{3}H ratio = ^{14}C/^{3}H ratio × sp act of ^{14}C-labeled sterols in the normal mouse feces/sp act of ^{14}C-labeled sterols in the diabetic mouse feces.
[e] Cholesterol absorption rate = (1 − total activities found in feces/activities administered) × 100.

not develop gallstones though they were fed the cholesterol diet and their biliary bile acid composition was very similar to that in the gallstone-positive mice. Why they did not develop gallstones is not known but they showed enlarged gallbladders, low biliary cholesterol concentration, and high biliary bile acid concentration, compared with gallstone-positive mice. There is a strain difference in the occurrence of gallstones. When a lithogenic diet containing 0.5% cholesterol and 0.25% cholic acid was fed for 5 weeks, the occurrence was 100% in DS strain mice but it was 70 to 80% in Crj-ICR strain male mice (H. Takase et al., unpublished data). Therefore, when we used the DS strain mice in the present experiments, a high occurrence of gallstones could be expected.

Hyperphagia in diabetic animals is an important factor causing a disorder in cholesterol metabolism, perhaps including development of cholesterol gallstones. Young et al. (10) have demonstrated that prevention of hyperphagia in diabetic rats normalizes their plasma and liver cholesterol levels and HMG-CoA reductase activities in the liver and small intestine. Normal mice developed cholesterol gallstones without showing any significant increase of diet intake when sodium cholate was fed with cholesterol, as demonstrated by Tepperman et al. (2) and by the present experiments. Therefore, although it is not yet known whether hyperphagia enhances development of cholesterol gallstones, the change in bile acid metabolism, especially increase of cholic acid, may be closely related to development of gallstones in diabetic mice.

Cholesterol absorption increased in the diabetic mice. The absorption rate was estimated by the plasma $^{14}C/^{3}H$ ratio according to the method of Zilversmit (27, 28) and by the fecal excretion of ^{14}C-labeled sterols (29), and the diabetic mice were found to have an increased absorption rate (Table 8). At that time, we had to correct the plasma $^{14}C/^{3}H$ ratio by the specific activity of fecal sterols, since the diabetic mice ate a larger amount of diet. Even the ordinary diet contains a certain amount of cholesterol, which reduces the specific activity of orally administered [^{14}C]cholesterol. Our diet contained about 0.1% cholesterol, which is enough to affect the specific activity of [^{14}C]cholesterol. The specific activity of fecal sterols, however, decreased exponentially after a single oral administration of [^{14}C]cholesterol (**Fig. 4**) and a time difference should be considered to compare the plasma cholesterol and fecal sterols at the same time. It is not yet known what is the best way to conduct such a correction, but at least the correction by the mean value of the specific activity of fecal sterols during 3 days after the administration of [^{14}C]cholesterol gave values similar to those calculated from the fecal excretion of ^{14}C-labeled sterols.

Young et al. (11) demonstrated that cholesterol absorption increased in diabetic rats by using sitosterol as a recovery standard for cholesterol absorption. They fed [^{3}H]cholesterol and [^{14}C]sitosterol simultaneously to the animals and found that [^{3}H]cholesterol absorption was significantly increased while [^{14}C]sitosterol absorption was unaffected. Nervi, Gonzalez, and Valdivieso (6) also demonstrated the increase of cholesterol absorption in diabetic rats by directly measuring the amount of lymphatic cholesterol.

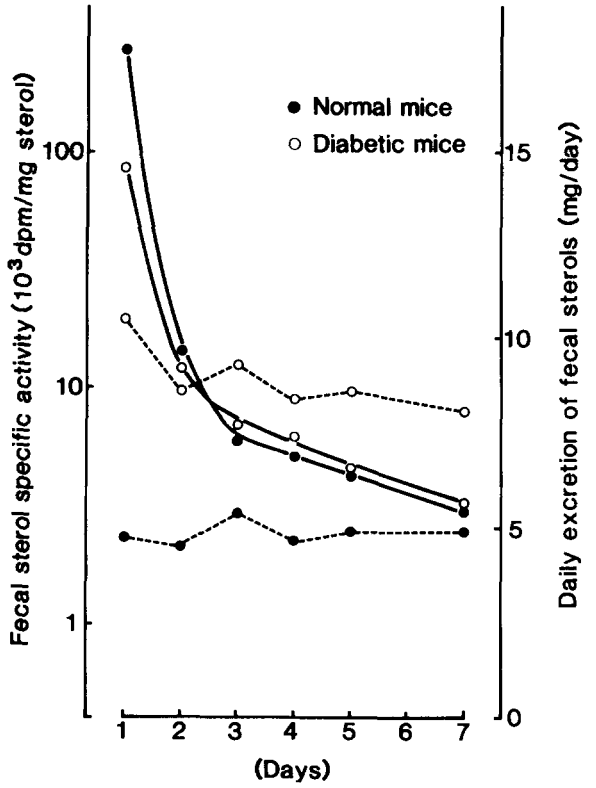

Fig. 4. Changes in fecal sterol specific activities (solid lines) after a single oral administration of [^{14}C]cholesterol (1 μCi/mouse) and daily fecal sterol excretions (broken lines) in normal (solid circle) and diabetic (open circle) mice. Each point indicates mean value in five mice.

In a steady state, the amount of fecal bile acids corresponds to the amount of bile acids synthesized in the liver, and our mice seemed to be in such a steady state. When the gallstone-positive and -negative mice were compared, although the number of mice was small, no significant difference was found in either the total amounts or the composition of fecal bile acids (Table 6), suggesting that the bile acid synthesis was not different in the gallstone-positive mice. In addition, plasma and liver lipid levels, diet intake, fecal mass, and fecal sterols were similar in both groups.

We conclude that the formation of gallstones in the diabetic mice was caused by the enhancement of cholesterol absorption, which was brought about by increases in the synthesis and secretion of cholic acid in the diabetic mice.

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