Effect of Essential Fatty Acids and Pyridoxine on the Formation of Gallstones, Especially Cholesterol Stones

by

IZUMI MARUYAMA

From the 2nd Surgical Division, Kyoto University Medical School
(Director : Prof. Dr. CHUJI KIMURA)
Received for Publication Nov. 10, 1964

I INTRODUCTION

The fundamental studies on the mechanisms of gallstone formation were established by NAUNYN and ASCHOFF. In general, it is believed that local factors such as biliary infections, bile stasis, damage to the gallbladder wall and so on lead to the decrease of cholesterol holding power of gallbladder bile so that cholesterol precipitation may occur and stones begin to form. However, cholecystitis without stones or cholelithiasis without biliary infection are frequently encountered clinically. Many other investigators have tried to elucidate the initiating factors of gallstone formation with disturbances of metabolism, dysfunctions of the endocrine system, dysharmonies of the autonomic nervous system and abnormalities of constitution. But the etiology of gallstone yet remains obscure.

In our laboratory, HIKASA et al.[10)14)16)37)] investigated the specific nutritional effects of essential fatty acids (EFA) and recently have reached to the conclusion that cholesterol esterified with EFA especially with tetraenoic acid, i.e. metabolically active cholesterol, is the direct precursor of adrenocortical hormones. On the other hand, bile acid is a main end-product of cholesterol[2)3)5)49)]. So that it is reasonable to suppose that cholesterol should be metabolically active, i.e. it should be esterified with tetraenoic acid, for the biosynthesis of bile acid. According to this supposition, HIKASA[13)] has first surmised in 1960 that gallstone may be due to deficiency or metabolic disturbances in EFA. Deficiency and/or metabolic disturbances in EFA lead to decreased biosynthesis of bile acid. Lecithin, which contains EFA as its component, may be decreased in these states. So that cholesterol precipitation may occur and stones begin to form.

The present study was designed to clarify the influences of EFA upon the gallstone formation.

II MATERIALS AND METHODS

MATERIALS

1) Clinical Experiment

Gallbladder biles obtained aseptically during surgery were analysed. The patients were composed of 39 cases of gallstones and as a control group 21 cases of peptic ulcer, admitted at the Second Surgical Division, Kyoto University Hospital. They were all without distinct disturbances of liver functions. Further, the hepatic biles from the common bile duct through the draining tube were analysed.

2) Animal Experiment

Male albino rats of Wistar strain supplied by the Animal Center in Kyoto University were housed in single, screen-bottom cages at 20°C. They were fed a rat chow until their body weight reached about 40 to 50 mg, then they were divided randomly into three groups: EFA diet, EFA-deficient diet and pyridoxine-deficient EFA diet groups (5 to 9 animals in each group). The composition of each diet is listed in Table 1, 2 and 3. As the source of EFA a purified and peroxide free sesame oil was used. The sesame oil contained 48.9% linoleic acid, 0.7% linolenic acid, and no other polyunsaturated fatty acids.

Table 1 The Composition of the Diet

	EFA Diet	EFA-deficient Diet	Pyridoxine-deficient EFA Diet
Starch	60%	80%	60%
Vitamin-free Casein	16	16	16
Sesame Oil	20	0	20
Salt Mixture	3	3	3
Vitamin Mixture	0.5	0.5	0.5*
Choline Chloride	0.5	0.5	0.5

* Pyridoxine free.

Table 2 Vitamin Mixture (in 1 g)

Vitamin A	2500 I.U.
B_1	1.0mg
B_2	1.5mg
B_6	1.0mg
B_{12}	1.0γ
C	37.5mg
D	200 I.U.
E	1.0mg
K	0.2mg
Niacin	10.0mg
Pantothenic Acid	2.5mg
Folic Acid	0.5mg

Table 3 Salt Mixture (in 1000 g)

NaCl	46.3gm
$NaHPO_4$	92.0
K_2HPO_4	253.0
$CaH_4(PO_4)_2 \cdot H_2O$	143.0
Cal. Lactate	369.0
$MgSO_4$	70.4
KI	26.3

All rats were thus maintained for about 3 months. During the course of feeding period, EFA-deficient and pyridoxine-deficient EFA diet groups exhibited the characteristic signs of EFA-deficiency and pyridoxine-deficiency. At the end of this period, all rats were fastened for 12 hours and sacrificed by bleeding from the aorta. The liver slices were excised and weighed immediately by microbalance. The biochemical analysis were made with these liver slices.

METHODS

1) Extraction and Determination of Bile Acid in Bile

Bile acids were extracted by a modification of the procedure reported by MOSBACH[36]. Twenty volumes of 99% ethanol was added to the bile (or homogenated liver slices), and the mixture was heated on a steam bath for 1 hour. The mixture was filtered and the residue was washed twice with 5 ml of petroleum ether (bp. 30° to 50° C) to remove neutral lipids. This solution was then acidified with 3N-HCl and further

extracted with petroleum ether to remove fatty acids. The acidic alcohol phase was then evaporated on the steam bath under a current of air. The residue was dissolved in 5% NaOH and hydrolysed at 120°C for 3 hours. The hydrolysate was acidified with 3N-HCl, and extracted 4 times with 10 ml of ethyl ether. The ether extract was washed with distilled water to remove chloride and dried over anhydrous sodium sulfate. A suitable aliquot of this extract was pipetted into a test tube, and evaporated on the steam bath. Five ml of 65% H_2SO_4 were added and after heating 60±1 C for 15 min., the optical density was read at 3200 and 3850 A with the BECKMAN DU quartz spectrophotometer.

2) Extraction of Lipids and Determination of Other Lipids in Bile

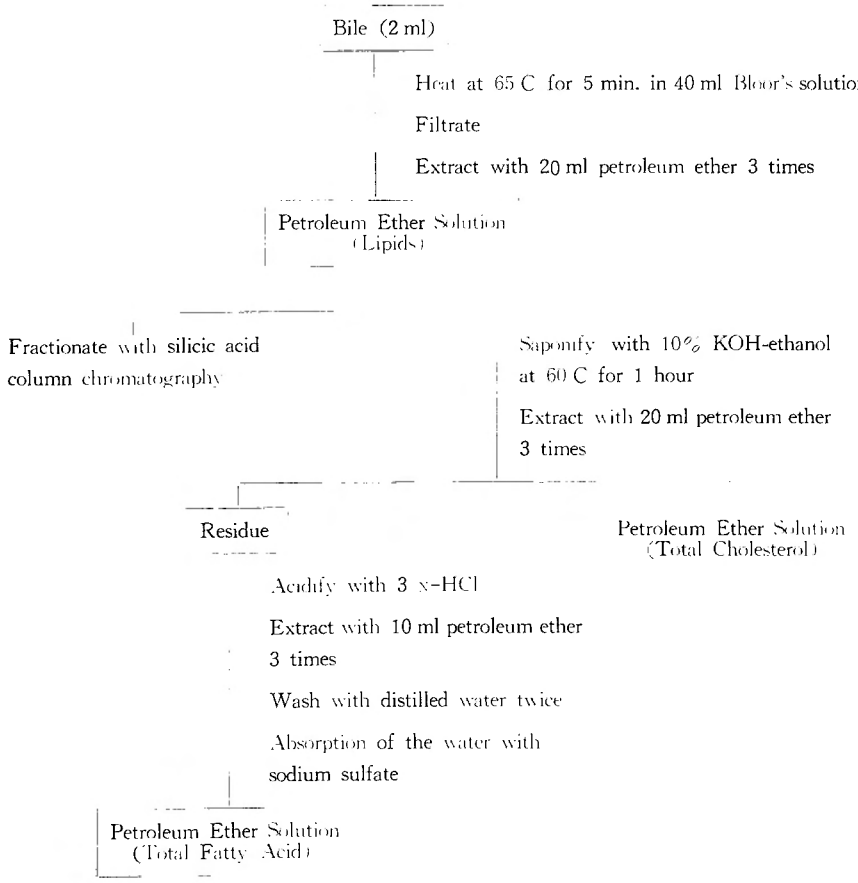
a) The extraction of lipids is illustrated in Fig. 1.

b) Fractionation of Bile Lipids with Silicic Acid Column Chromatography

Fractionation of bile lipids were performed by a modification of HIRSCH and AHRENS method[19]. Petroleum ether solution containing 1% (80 ml), 10% (100 ml) and 20% (150 ml), were employed to elute successively cholesterol esters, triglycerides and free cholesterol. Phospholipids (lecithin) were subsequently eluted with 300 ml methanol.

Fractionated bile lipids were then saponified with 10% KOH-ethanol at 60 C for 1

Fig. 1 Extraction of Bile Lipids



hour. After extraction of nonsaponifiable materials (esterified and free cholesterol) from the alkaline solution with petroleum ether, fatty acids contained in cholesterol esters, glycerides and phospholipids were removed by acidification with 3N-HCl and subsequent extraction with petroleum ether, respectively. The extracts were washed with distilled water and dried over anhydrous sodium sulfate.

c) Determination of Cholesterol

Esterified, free and total cholesterol were determined by KINGSLEY's method[25] of LIEBERMANN-BURCHARD reaction.

d) Determination of Fatty Acids

Fatty acids from cholesterol esters, glycerides and phospholipids, and total fatty acids were weighed by microbalance, after the addition of hydroquinone and evaporation to dryness under nitrogen.

3) The Gas-liquid Chromatographic Analysis of Fatty Acids

The extracted fatty acids were methylated with freshly prepared 2% H_2SO_4-methanol at 70°C for 90 min.. The methyl esters of the fatty acids were dissolved into petroleum ether and chromatographed in a SHIMADZU Seisakusho Model GC-1-B instrument equipped with a hydrogen frame ionization detector. A 150 cm × 6 mm i. d. column packed with 25% diethylene glycol succinate polyester coated on Shimarite (60 to 80 mesh) was used. The flash heater was maintained at 300°C, the column at 210°C, and the detector at 240°C ; the nitrogen flow rate was 30 ml/min. × 2, the gas pressure 3 Kg/cm². The fatty acids from phospholipids and the total fatty acid were analysed by gas-liquid chromatography. Each component of the fatty acids separated by gas-liquid chromatography was identified with the standard samples offered from National Institute of Health and Hormel Institute in U. S. A.. The details of this procedure were reported previously[15].

4) The Analysis of Gallstones

The analysis of gallstones were performed by a modification of the method reported by NISHIMURA[39]. The gallstones containing over 70% of cholesterol were grouped into cholesterol stones.

III RESULTS

1) Clinical Experiments

Gallbladder biles were tested microbiologically, and the patients were divided into the groups of with or without biliary infection. The results of the experiments are listed in Table 4.

a) Cholesterol

About 98% of cholesterol in bile was free and the esterified cholesterol was detected only traces. Total cholesterol in bile did not significantly increase statistically in cholesterol stones when biliary infection was absent, while it decreased significantly in the presence of biliary infection.

b) Bile Acid

Total bile acid decreased moderately in the bile with cholesterol stones when without biliary infection, while it did strikingly with biliary infection. Moreover, change in composition of bile acids, that is the ratio of dihydroxycholanic acid to trihydroxycholanic acid

Table 4 Biochemical Analysis of Human Gallbladder Bile

	Control	Cholesterol Stones without Biliary Infection	Cholesterol Stones with Biliary Infection	Pigment Stones without Biliary Infection
No. of Patients	21	20	6	6
Total Cholesterol mg/dl	608±36	681±85	261±65	671±181
Total Bile Acid mg/ml	67.8±3.1	47.9±4.0	23.1±6.0	42.8±6.3
Dihydroxy. Acid/Trihydroxy. Acid	1.18±0.08	1.39±0.14	0.90±0.10	1.10±0.09
Bile Acid/Cholesterol	11.68±2.06	7.75±1.67	10.20±3.04	9.67±2.51
Total Fatty Acid mg/ml	24.39±2.01	17.11±2.18	7.94±1.90	19.57±1.62

Numbers after ± are standard errors.

(Di Tri), was not so different from that of control group in the cases of gallstones without biliary infection, on the contrary, it decreased markedly when with biliary infection. Thus change in bile acids in the cases of cholesterol stones with biliary infection is clearly different from that of without biliary infection.

c) The Ratio of Bile Acid to Cholesterol (B C)

As shown in Table 4, the ratio of B C was decreased significantly in the bile with cholesterol stones when without biliary infection. The ratio was less than 8. This value is important because MIYAKE[31] has reported that the ratio of B/C more than 8 was necessary to keep cholesterol in solution.

d) Fatty Acids

When lipid composition of bile was analysed by silicic acid column chromatography, it was found that about 95 to 99% of total fatty acid was contained in lecithin. Therefore, quantitative change in total fatty acid was parallel with that in lecithin. Further, changes in total fatty acid composition were almost identical to those of lecithin composing fatty acids, as shown in Table 5.

Total fatty acid was decreased in the bile with cholesterol stones in cases of without biliary infection. When accompanied with biliary infection, it decreased more strikingly. However, fatty acid composition was not so different from that of control group in either

Table 5 Composition of Total Fatty Acid and Fatty Acid Contained in Lecithin (%)

Patient	Diagnosis	Fatty Acid	C 14 : 0	C 16 : 0	C 16 : 1	C 18 : 0	C 18 : 1	C 18 : 2	C 18 : 3	C 20 : 3	C 20 : 4	C 22 : 0	C 22 : 6
Case 1	Peptic Ulcer	Total	0.2	31.5	4.2	6.1	20.0	23.3	0.3	2.1	5.4	1.5	5.0
		Lecithin	0.4	30.5	4.4	8.2	20.8	22.2	0.3	1.9	5.2	1.1	4.5
Case 2	Peptic Ulcer	Total	1.3	38.8	5.3	6.3	13.5	22.2	0.5	1.0	1.1	2.3	3.8
		Lecithin	0.9	38.2	4.9	1.0	15.2	21.9	0.2	1.1	4.6	1.8	3.9
Case 3	Cholesterol Stone without Infection	Total	0.5	36.0	5.5	6.0	14.2	22.5	0.1	1.0	4.3	2.5	6.3
		Lecithin	0.5	36.8	5.8	6.5	13.2	22.0	0.5	1.1	4.1	2.8	6.2
Case 4	Cholesterol Stone without Infection	Total	0.3	41.4	4.3	3.5	11.4	26.8	0.4	0.2	4.6	1.9	4.2
		Lecithin	0.2	40.8	3.8	7.7	12.1	23.6	0.3	0.4	4.2	1.6	4.7

case (Table 6). These results were agreed with the data reported by BLOMSTRAND[4], although several investigators have reported an increase of oleic and a decrease of linoleic acid[40)52)]. Anyway, the present results show that lecithin does not change qualitatively, but does quantitatively.

In a word, in the bile of patients with gallstones, especially with cholesterol stones and without biliary infection, decreased bile acid and lecithin were observed. When accompanied with biliary infection, however, decrease of bile acid and lecithin became more striking and qualitative change in the bile acid was observed. Therefore, when biliary infection was not present, the composition of the bile with cholesterol stones was different from when it was with biliary infection. The changes in composition of the bile with cholesterol stones may not be caused by the infection.

2) Animal Experiment

It has already been demonstrated in our laboratory that cholesterol esterified with EFA especially with tetraenoic acid was a precursor of adrenocortical hormones[10)14)16)37)]. According to this fact, it was surmised that deficiency in EFA resulted the reduced synthesis of bile acid from cholesterol in liver. Based on this presumption, the determination was done on bile acid in the liver slices of the rats fed various diets.

a) Resting State

As compared with EFA diet group, marked decrease in total bile acid and elevated ratio of Di/Tri were observed in EFA-deficient diet group. In pyridoxine-deficient EFA diet group, however, patterns of bile acid were almost similar to those of EFA diet group (Table 7).

b) Administration of ACTH-Z

After the daily intramuscular administration of ACTH-Z 3 I.U. for consecutive 4 days, total bile acid decreased markedly in pyridoxine-deficient EFA diet group. On the other hand, it decreased slightly in EFA diet group (Fig. 2).

Table 6 Fatty Acid Composition of Human Gallbladder Bile (%)

	Control	Cholesterol Stones without Biliary Infection	Cholesterol Stones with Biliary Infection	Pigment Stones without Biliary Infection
No. of Patients	13	15	6	6
C14 : 0	0.3±0.1	0.5±0.1	0.5±0.2	0.5±0.2
C16 : 0	41.0±2.9	31.8±3.0	32.1±1.1	25.1±1.2
C16 : 1	3.9±0.5	1.1±0.5	3.5±0.6	1.6±0.1
C18 : 0	2.8±0.6	3.7±0.7	3.9±0.6	5.1±0.9
C18 : 1	11.3±1.1	15.9±1.1	17.0±1.1	17.2±0.1
C18 : 2	22.1±1.2	21.1±1.0	22.1±1.0	27.3±2.7
C18 : 3	0.5±0.2	0.1±0.1	0.4±0.1	0.6±0.2
C20 : 3	0.7±0.2	1.2±0.2	2.3±0.5	0.7±0.7
C20 : 1	6.6±0.7	6.2±0.5	7.8±0.6	8.6±1.3
C22 : 0	3.4±0.5	2.9±0.4	2.9±0.4	2.8±0.3
C22 : 6	4.7±0.6	4.7±0.4	4.9±0.5	5.8±0.7

Numbers after ± are standard errors.

Table 7 Bile Acid in the Liver of Rats Fed Various Diets

	EFA Diet	EFA-deficient Diet	Pyridoxine-deficient EFA Diet
No. of Animals	10	10	6
Total Bile Acid (w/w%)	0.135±0.032	0.049±0.012	0.146±0.041
Dihydroxy. Acid / Trihydroxy. Acid	0.26±0.04	0.45±0.04	0.23±0.05

Numbers after ± are standard errors.

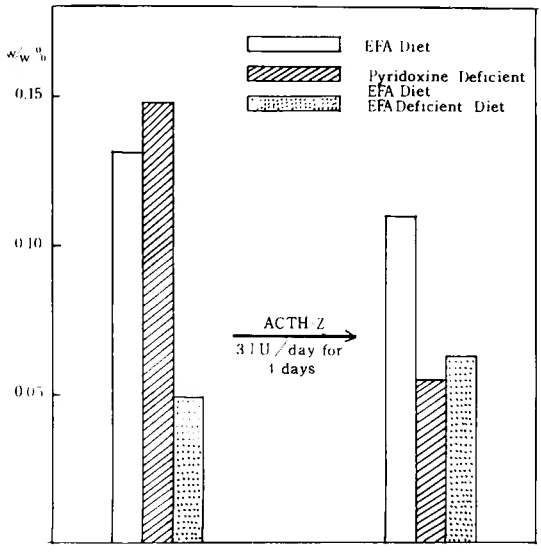


Fig. 2 Changes in the Bile Acid Levels in the Liver of Rats Fed Various Diets after the Administration of ACTH-Z

According to these results, it is evident that EFA are necessary for the synthesis of bile acid from cholesterol and that pyridoxine deficiency accompanied by the stress may affect this process.

3) Clinical Application

Patients with gallstones were selected for this experiment after surgical drainage was done from the common bile duct. They were not suffering from any distinct disturbances of liver functions. They were fed a basal diet (protein: 74.3 g, carbohydrate: 470.0 g, fat: 10.0 g, 2267 Cal.) after 7 th day of operation. Case 1 was administered both sōya lecithin 20 g and pyridoxine hydrochloride 50 mg per day. Case 2 was administered sōya lecithin 20 g per day and the other 2 patients were not administered. Twenty grams of sōya lecithin contained 8.52 g of total fatty acid: 2.004 g of palmitic acid, 0.444 g of stearic acid, 0.904 g of oleic acid, 4.468 g of linoleic acid, and 0.460 g of linolenic acid according to gas-liquid chromatographic analysis. Hepatic bile of these patients were collected for every 24 hours and analysed.

Case 1 (treated with both sōya lecithin and pyridoxine) : bile acid increased gradually while cholesterol decreased, so that the ratio of B/C was elevated (Fig. 3 a).

Case 2 (treated with sōya lecithin only) : although both bile acid and cholesterol increased, the increasing rate was higher in bile acid than in cholesterol, so that the ratio of B/C was elevated (Fig. 3 b).

Case 3 (non-treated) : change in bile acid level was slight and cholesterol was increased. The ratio diminished gradually (Fig. 4 a).

Case 4 (non-treated) : bile acid was decreased markedly and the ratio diminished (Fig. 4 b).

The ratio of B/C was elevated in all patients treated with EFA and pyridoxine, on the contrary, in non treated patients the ratio of B/C was decreased gradually. Thus EFA and pyridoxine are effective for the treatment of gallstones after surgery and have a

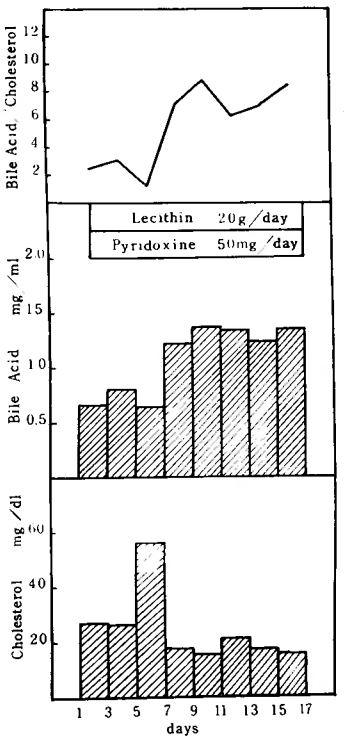


Fig. 3 (**a**) Case 1

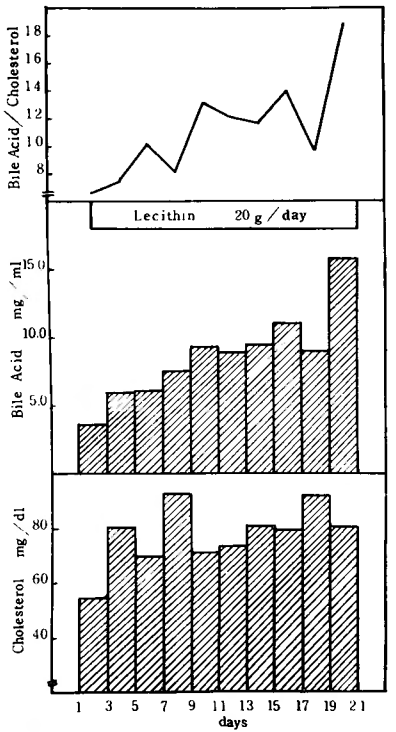


Fig. 3 (**b**) Case 2

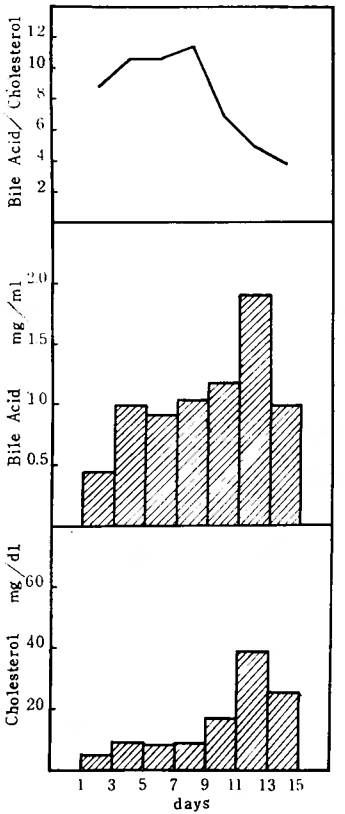


Fig. 4 (**a**) Case 3

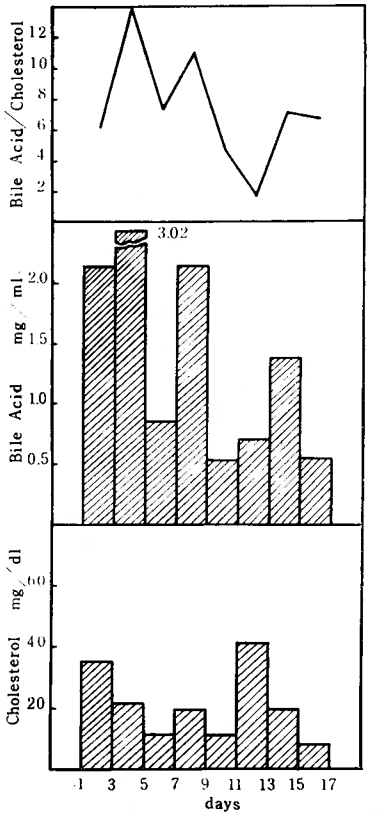


Fig. 4 (**b**) Case 4

Fig. 3 and 4 The Influences of EFA and Pyridoxine upon the Composition of Human Hepatic Bile

protective effect for the gallstone formation.

IV GAS-LIQUID CHROMATOGRAPHY OF BILE ACIDS

In the present chapter, separation of bile acids from human bile with the aid of gas-liquid chromatography has been reported.

Method. Bile acids, extracted by the method described above, are methylated with freshly prepared diazomethane. Then trifluoroacetates were prepared by heating the methyl-esters in 0.1 ml of trifluoroacetic anhydride and 0.1 ml of pyridine in a glass stoppered test tube at 30°C for 15 min.[20)50)]. The reagent was evaporated under nitrogen and extracted with ethyl ether. The ether solution was then washed with dil-HCl and then distilled water and evaporated to dryness. The residue was dissolved into acetone and chromatographed in a SHIMADZU Seisakusho Model GC-1-B instrument equipped with a hydrogen frame ionization detector. A 225 cm × 4 mm i. d. column packed with 0.7% nitrile silicone coated on Chromosorb W (60 to 80 mesh) was used[9)]. The flash heater maintained at 240°C, the column at 230°C, and the detector at 240°C; the nitrogen flow rate was 30 ml/min. × 2, the gas pressure 3 Kg/cm².

Trihydroxy compounds gave somewhat lower responses with the detector, therefore, it was necessary to use correction factors determined with a testmixture of known composition.

Results. Fig. 5 shows the separation of a testmixture. Although lithocholic acid has almost the same retention time as deoxycholic acid, a good separation is obtained between deoxycholic, chenodeoxycholic and cholic acid. Fig. 6, 7 and 8 show gas-liquid chromatograms from various patients. As illustrated in Table 8, bile acid composition of gallbladder bile is not so different between the bile of cholesterol stones without biliary infection and that of control group. Relative decrease in cholic acid was observed in the bile with pigment stones.

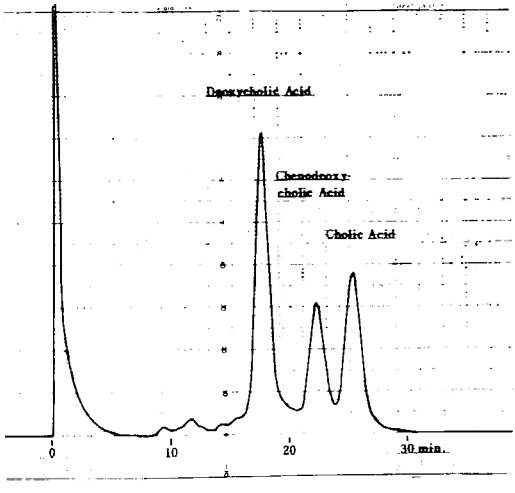


Fig. 5 Gas-liquid Chromatogram of a Test Mixture of Bile Acids

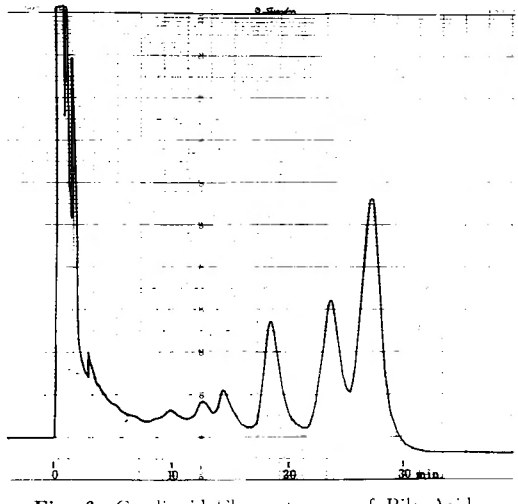


Fig. 6 Gas-liquid Chromatogram of Bile Acids in Human Gallbladder Bile. Case 1. Peptic Ulcer

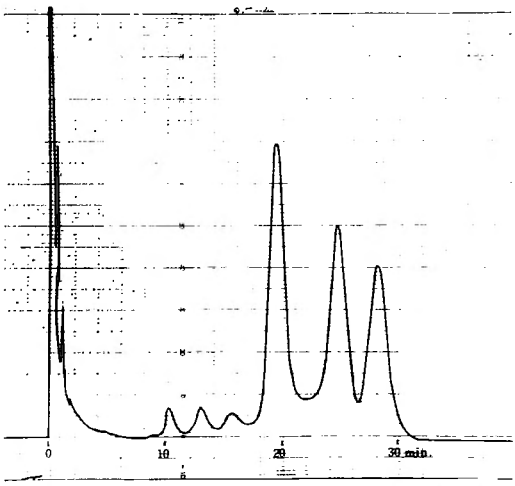


Fig. 7 Gas-liquid Chromatogram of Bile Acids in Human Gallbladder Bile
Case 5 : Cholesterol Stone

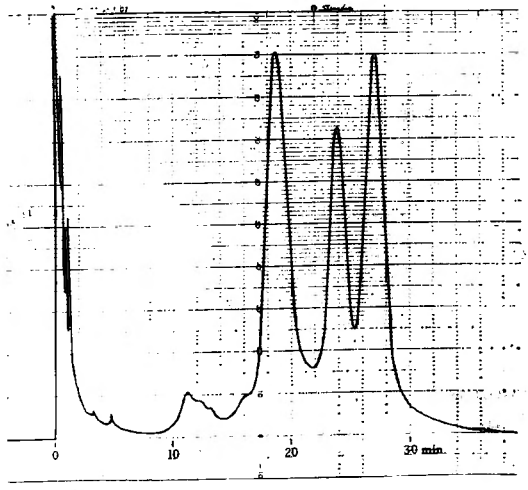


Fig. 8 Gas-liquid Chromatogram of Bile Acids in Human Gallbladder Bile
Case 11 : Pigment Stone

Table 8 Gas-liquid Chromatography of Human Bile Acids

Patient	Diagnosis	Total Bile Acid (mg/ml)	Ratio Deoxy. : Chenodeoxy. . Chol.
Case 1	Peptic Ulcer	73.1	1 : 1.98 : 3.34
Case 2	〃	83.1	1 : 1.06 : 0.91
Case 3	〃	77.1	1 : 1.08 1.46
Case 4	〃	76.9	1 : 2.97 : 2.21
	Mean	77.6	1 : 1.75 : 1.98
Case 5	Cholesterol Stone without Infection	21.0	1 : 1.19 : 0.96
Case 6	〃	49.9	1 : 1.96 : 2.03
Case 7	〃	30.8	1 : 1.63 : 1.58
Case 8	〃	36.7	1 : 0.62 : 1.00
Case 9	〃	51.5	1 : 3.70 : 2.79
Case 10	〃	54.1	1 2.64 : 3.71
	Mean	41.7	1 : 1.96 : 2.01
Case 11	Pigment Stone without Infection	56.9	1 : 1.33 : 1.59
Case 12	〃	44.2	1 : 1.88 : 1.62
Case 13	〃	23.8	1 1.19 : 1.26
	Mean	41.6	1 : 1.10 : 1.12

V DISCUSSION

Although many informations on the mechanisms of gallstone formation have been reported, the etiology of gallstone still remains obscure.

So far as cholesterol stones are concerned, it is reasonable to assume that cholesterol solubility would be the most important in the formation of gallstones[31]. It has been demonstrated by many investigators that bile acid and phospholipid (lecithin) which are present in bile are important to keep cholesterol in solution[12)21)22)28)30]. Further, ISAKSSON[23] has demonstrated that a lecithin-bile salt complex plays the actual mechanism to keep cholesterol in solution in human bile. This observation has been confirmed by the solubility studies of JOHNSTON and NAKAYAMA[24]. In our country, MIYAKE[34] has also shown that the ratio of lecithin to cholesterol should be more than 6.6 and at the same time the ratio of B/C should be more than 8.0 to protect cholesterol precipitation in bile.

In general, bile acid and lecithin, which are important for holding cholesterol in solution, are decreased markedly in the bile with gallstones. MIYAKE[33)34)35] observed marked decrease in total bile acid, appearrance of free bile acid, relative increase in dihydroxycholanic acid and decrease in lecithin and he emphasized that biliary infection was an important factor for these changes. It has been reported by several investigators that selective change in permeability of gallbladder wall due to infection and inflammation lead to the decrease of bile acid and lecithin and on the contrary, the increase of cholesterol in bile[42)51]. Thus it is generally accepted that the regional factors such as infection, inflammation, bile stasis and so on are responsible for cholesterol stone formation. On the other hand, MATSUO[32] emphasized the importance of systemic factors on the formation of gallstones. Further, ANDERSON[1] has described that pure gallstones are due to disturbances of metabolism or of liver function rather than to an inflammatory reaction and that pure gallstones may form the nucleus of the combined gallstones. The results in the present study show decrease in bile acid and lecithin, though biliary infection is not present. They indicate that the composition of the bile with gallstones when unassociated with biliary infection is different from that associated with biliary infection. When accompanied with biliary infection, decrease in bile acid and lecithin are more striking than when without biliary infection and, in addition, qualitative change in bile acid and decrease in cholesterol are observed. These facts indicate that the decrease in bile acid and lecithin in bile can be occurred by some causes other than biliary infection. Therefore, it is necessary to pay attention to the importance of metabolic disturbances again.

It has already been demonstrated in our laboratory that cholesterol esterified with EFA especially with tetraenoic acid is a precursor of adrenocortical hormones and that the adrenocortical capacity of the individuals postulated in deficiency or metabolic disturbances in EFA are reduced[16]. According to these facts, HIKASA[13] has first surmised in 1960 that cholesterol esterified with tetraenoic acid should be the direct precursor of bile acid and that deficiency or metabolic disturbances in EFA are responsible for gallstone formation.

It has been reported by several investigators that highly unsaturated fatty acids from vegetable oils promote cholesterol catabolism and bile acid excretion[6)11)29)41)44]. Animal experiment and clinical application in the present study show the same phenomenon also. Bile acid in liver is markedly reduced in the rats fed a EFA-deficient diet, further, bile acid and the ratio of B/C in hepatic bile in man are gradually increased by the administration of both sōya lecithin and pyridoxine. FUKUDA[10] observed a decrease in adrenocortical capacity in patients with gallstones. HIRANO[18] observed that in the patients' bile with

gallstones, fatty acid composition showed metabolic disturbances in EFA and that cholesterol esterified with tetraenoic acid was decreased. Further, YOSHINAGA[56] and HIRANO[18] observed that decreased bile acid and lecithin in hepatic bile coincided with decreased cholesterol esterified with tetraenoic acid in liver were occurred in EFA-deficient rats. Therefore, cholesterol esterified with tetraenoic acid may be a direct precursor of bile acid. The biosynthesis of lecithin may be reduced in deficiency or metabolic disturbances of EFA. As a matter of fact, lecithin is reduced in the patients' bile with gallstones. Recently, SHIODA[48] succeeded in the production of cholesterol stones in hamsters, when they were put on an EFA-deficient diet for 2 to 3 months.

Cholesterol stone formation, however, is not due to the absolute deficiency in EFA, because the fatty acid composition of the bile with gallstones does not indicate EFA deficiency and the composition is not so different from that of control group. HIRANO[18] also failed to observe EFA deficiency in the liver of the patients with gallstones, he observed metabolic disturbances and disturbed utilization of EFA.

It has been reported by many investigators that pyridoxine is involved in the metabolism of fatty acids[26][27][38][53]. In accordance with these reports, bile acids were analysed with the liver slices of another group of rats which were fed a diet containing enough linoleic acid under deficiency in pyridoxine. Although any significant difference was observed between EFA diet and pyridoxine-deficient EFA diet groups in resting state, marked decrease in total bile acid in the latter was observed when the animals were administered ACTH-Z. Thus the composition of bile may be aggravated to pathological state by repeated exposure to stresses and finally, cholesterol precipitation may occur.

It is now generally agreed that pyridoxine is involved in the metabolism of amino acids and fatty acids. Pyridoxine deficiency syndrome can be observed when individuals are fed a diet high in protein[46][47] and saturated fat[26][27]. This is particularly evident when the diet is high in methionine and cystine[7][8][43]. Production of pyridoxine by the intestinal flora is reduced with animal foods containing a large amount of protein and fat[54]. In general, animal fat and protein contain less pyridoxine than vegetables. Thus animal foods dieting are liable to pyridoxine deficiency. Actually, SCHROEDER[45] reported that the American diet, containing a large amount of animal fat and hydrogenated vegetable fat and protein, might possibly be marginal with respect to this vitamin. As a matter of fact, gallstones in European and American compose chiefly of cholesterol. Recently the frequent occurrence of cholesterol stones has been observed in the city dwellers in our country who are apt to take a diet high in protein and animal fat with higher standard of life[55]. Moreover, it is interesting that they meet with stresses more frequently than countryman.

In accordance with these facts, it is evident that the metabolic disturbances in EFA due to pyridoxine deficiency play a very important role upon gallstone formation especially that of cholesterol stone. The total process of cholesterol stone formation in man is illustrated in the schema described by HIKASA et al.[17] in 1964. It should be emphasized that the initiating factor of cholesterol stone formation is attributed to the systemic one.

VI SUMMARY AND CONCLUSION

1) Bile acid and lecithin were decreased in the bile of patients with cholesterol stones, though biliary infection was not present.

2) The composition of the bile with cholesterol stones in the cases of without biliary infection was different from that with biliary infection.

3) Therefore, changes in the bile with cholesterol stones may not be caused by infection.

4) The resting levels of total bile acid in the rats' liver were clearly higher in EFA diet and pyridoxine-deficient EFA diet groups than in EFA-deficient diet group. After the administration of ACTH-Z, however, the levels of total bile acid reduced markedly in pyridoxine-deficient EFA diet group.

5) The ratio of bile acid to cholesterol in human hepatic bile was elevated gradually by the administration of EFA and pyridoxine.

6) Cholesterol esterified with EFA may be the direct precursor of bile acid and pyridoxine may be involved in the biosynthesis of bile acid from cholesterol.

7) The metabolic disturbances in EFA due to pyridoxine deficiency play a very important role upon gallstone formation in man and stress affects on this process.

8) Bile acids from human bile were analysed by gas-liquid chromatography.

The author wishes to express his sincere gratitude to Assistant Professor YORINORI HIKASA for his generous guidance throughout the course of this experiment, and to Dr. MICHIO YOSHINAGA for his kind cooperation. A part of this work was reported at 63rd and 64th General Meeting of Japanese Surgical Society.

REFERENCES

1) Anderson, W. A. D. : Pathology, St. Louis, 1957, C. V. Mosby Co.
2) Bergstroem, S and Norman, A. : Metabolic products of cholesterol in bile and feces of rat. Proc. Soc. Exp. Biol. Med., **83** : 71, 1953.
3) Bloch, K., Berg, B.N. and Rittenberg, D. . The biological conversion of cholesterol to cholic acid. J. Biol. Chem., **149** : 511, 1943.
4) Blomstrand, R. and Ekdall, P. : Fatty acid pattern of human bile under normal and pathological conditions. Proc. Soc. Exp. Biol. Med., **104** :205, 1960.
5) Byers, S. O and Biggs, M. W. : Cholic acid and cholesterol. Studies concerning possible interconversion. Arch. Biochem. Biophys., **39** : 301, 1952.
6) Byers, S.O. and Friedman, H. : Bile acid metabolism, dietary fats, and plasma cholesterol levels. Proc. Soc. Exp. Biol. Med., **98** : 523, 1958.
7) Cerecedo, L. R., Foy, J. R. and De Renzo, E. C. : Protein intake and vitamin B_6 deficiency in the rat. The effect of supplementing a low-protein, pyridoxine-deficient diet with cystine or with methionine. Arch. Biochem., **17** : 397, 1948.
8) De bey, H. J., Snell, E. E. and Baumann, C. A. : Studies of the interrelationship between methionine and vitamin B_6. J. Nutrition, **46** : 203, 1952.
9) Ellin, R. I., Mendeloff, A. I. and Turner, D. A : Quantitative determination of 3, 7, 12-triketocholanic acid in biological fluids by gas-liquid chromatography. Analytical Biochem., **4** : 198, 1962.
10) Fukuda, H. : personal communication.
11) Gordon, H., Lewis, B., Eales, L. and Broch, J. F. . Dietary fat and cholesterol metabolism. Faecal elimination of bile acids and other lipids. Lancet, **II** : 1299, 1957.
12) Hammarsten, O. : Zur Chemie der Galle. Ergeb. Physiol,. **4**:1, 1905.
13) Hikasa, Y. et al. : Intravenous administration of a fat emulsion. (in Japanese) Geka-shinryō, 2 : 1650, 1960.
14) Hikasa, Y. et al. : Nutritional effect of fat. (in Japanese) Sōgo-igaku, **19** : 95, 1962.
15) Hikasa, Y. et al. : Practical application of gas-liquid chromatographic analysis of fatty acids. (in Japanese)

Saishin-igaku, **18** : 921, 1963.
16) Hikasa, Y. et al.: Roles of EFA on adrenocortical capacity. (in Japanese) Nihon-rinshō, **22** : 142, 1964.
17) Hikasa, Y. et al.: Initiating factors of gallstones, especially cholesterol stones. Arch. Jap. Chir., **33** : 601, 1964.
18) Hirano, Y.: personal communication.
19) Hirsch, J. and Ahrens, E. H.: The separation of complex lipid mixture by the use of silicic acid chromatography. J. Biol. Chem., **233** : 311, 1958.
20) Ikegawa, N.: Gas-liquid chromatographic analysis of steroids and alkaloids. (in Japanese: ステロイドおよびアルカロイドのガスクロマトグラフィー) Kagaku-no-ryōiki, **15** : 449, 1961.
21) Isaksson, B.: On the lipid constituents of normal bile. Acta Soc. Med. Upsal., **56** : 177, 1952.
22) Isaksson, B.: On the lipid constituents of bile from human gallbladder containing cholesterol gallstones. Acta Soc. Med. Upsal., **59** : 277, 1954.
23) Isaksson, B.: On the dissolving power of lecithin and bile salts for cholesterol in human bladder bile. Acta Soc. Med. Upsal., **59** : 296, 1954.
24) Johnston, C. G. and Nakayama, F.: Solubility of cholesterol and gallstones in metabolic material. A. M. A. Arch. Surg., **75** : 436, 1957.
25) Kingsley, G. R. and Schaffert, R. R.: Determination of free and total cholesterol by direct chloroform extraction. J. Biol. Chem., **180** : 315, 1949.
26) Kotake, Y.: Triptophan metabolism and experimental diabetes. (in Japanese: トリプトファン代謝と実験的糖尿病) Proc. 16th General Assembly Jap. Med. Congress, **1** : 299, 1963.
27) Kotake, Y.: Recent problems on the fat-nutrition. (in Japanese: 脂質栄養における最近の問題) Proc. 16th General Assembly Jap. Med. Congress, **1** : 450, 1963.
28) Large, A. M.: On the formation of gallstones. Surgery, **54** : 928, 1963.
29) Lewis, B. B.: Effect of certain dietary oils on bile acid secretion and serum cholesterol. Lancet, I : 1090, 1958.
30) Long, J. H. and Gephart, F.: On the behavior of lecithin with bile salts and the occurrence of lecithin in bile. J. Am. Chem. Soc., **30** : 1312, 1908.
31) Lutton, R. G. and Large, A. M.: Gallstones: Solubility studies. Surgery, **42** : 488. 1957.
32) Matsuo, I.: Gallstones and diseases of biliary tracts. (in Japanese: 胆石と胆道の疾患) 1947, Daigado Co.
33) Miyake, H.: Gallstone disease. (in Japanese) Nihon-geka-zensho, Tokyo, 1957, Nankōdo Co.
34) Miyake, H.: On the formation of gallstones. (in Japanese: 胆石生成機序に関して) Saishin-igaku, **14** : 3088, 1959.
35) Miyake, H.: Pathogenesis and therapy of gallstones. (in Japanese: 胆石の生成機序と治療) Proc. 16th General Assembly Jap. Med. Congress, **1** : 733, 1963.
36) Mosbach, E. H., Kalinsky, H. J., Halpern, E. and Kendall, F. E.: Determination of deoxycholic and cholic acids in bile. Arch. Biochem. Biophys., **51** : 402, 1954.
37) Muraoka, R.: Experimental study on the role of essential fatty acids and pyridoxine on adrenocortical function. Arch. Jap. Chir., **34** : 35, 1965.
38) Mueller, J. F. and Iacono, J. M.: Effect of desoxypyridoxine induced vitamin B_6 deficiency on polyunsaturated fatty acid metabolism in human beings. Am. J. Clin. Nutr., **12** : 358. 1963.
39) Nishimura, M.: Ueber die Chemische Zusammensetzung der Gallensteine. J. Biochem., **28** : 265, 1938.
40) Nishimura, M.: Gas-liquid chromatographic analysis of fatty acids in human bile. (in Japanese: ガスクロマトグラフィーによる人胆汁中脂肪酸の分析) Saishin-igaku, **19** : 215, 1964.
41) Ōji, K. and Matsuo, S.: Essential fatty acids and cholesterol metabolism. (in Japanese: 必須脂肪酸とコレステロール代謝) Sōgō-rinshō, **7** : 2296, 1958.
42) Riegel, C., Ravdin, I.S. and Johnston, C.: Studies of gallbladder function VI. The absorption of bile salts and cholesterol from the bile free gallbladder. Am. J. Physiol., **99** : 656, 1932.
43) Sakurai, Y. and Hara, H.: Effect of sulfur-amino acids on vitamin B_6. (in Japanese: B_6作用に及ぼす含硫アミノ酸の影響) Vitamin, **5** : 415, 1952.
44) Sato, J.: Studies on the effects on unsaturated fatty acid administration upon biliary components. (in Japanese) J. Kurume Med. Assoc., **24** : 1800, 1961.
45) Schroeder, H. A.: Is atherosclerosis a conditioned pyridoxal deficiency? J. Chron. Dis., **2** : 28, 1955.
46) Schweigert, B. S., Sauberlich, H. E., Elvehjem, C. A. and Baumann, C. A.: Dietary protein and the vitamin B_6 content of mouse tissue. J. Biol. Chem., **165** : 187, 1964.

47) Sheppard, E. C. and Mc Henry, E. W.: The pyridoxine content of tissues of rats fed various diets. J. Biol. Chem., **165**: 649, 1946.
48) Shoda, R.: personal communication.
49) Siperstein, M. D. and Chaikoff, I. L.: C^{14} cholesterol. III. Excretion of carbons 4 and 26 in feces. Urine and bile. J. Biol. Chem., **198**: 93, 1952.
50) Sjoevall, J.: Qualitative analysis of bile acids by gas chromatography. Bile acids and steroids 121. Acta Chem. Scand., **16**: 1761, 1962.
51) Uraki, J.: Bile and cholesterol. (in Japanese: 胆汁とコレステロール) Sogo-rinsho, **7**: 2255, 1958.
52) Watanabe, N.: Fatty acids in bile. (in Japanese: 胆汁の脂肪酸) Nihon-rinshō, **22**: 541, 1964.
53) Witten, P. W. and Holman, R. T.: Polyethenoid fatty acid metabolism. VI. Effect of pyridoxine on essential fatty acid conversion. Arch. Biochem., **41**: 266, 1952.
54) Yano, M.: Effects of cellulose upon the synthesis of vitamin B_6 by intestinal flora. (in Japanese: 人体腸内細菌によるB_6の合成とセルロースの影響) Vitamin, **10**: 166. 1956.
55) Yoshida, M.. Clinical Studies on gallstone disease. (in Japanese: 胆石症の臨床的研究) J. Jap. Surg. Soc., **60**: 599, 1959.
56) Yoshinaga, M.: Experimental studies on the initiating factor of cholesterol gallstones, especially on the influence of essential fatty acids and pyridoxine on the bile constituents. Arch Jap. Chir., **34**: 1,, 1965.

和文抄録

不可欠脂酸及びピリドキシンの胆石，殊にコレステロール系結石の形成に対する意義

京都大学医学部外科学教室第2講座（指導：木村忠司教授）

丸山　泉

胆石症の成因に関しては，Naunyn 及び Aschoff 以来，内外に於て幾多の研究業績が発表されて来たが，現在尚不明の点が多い．従来，一般に胆道感染，胆汁鬱滞等の局所性因子が重視されて来たが，胆道感染の認められない純コレステロール結石や，無石胆嚢炎等が日常の臨床に於て屢々みられる点からすると，胆石症の成因を局所性因子のみで説明する事は出来ない．従つて，新陳代謝の異常，内分泌機能の失調，肝機能の不全等の全身性要因を重視する人もある．我々の教室では，日笠等が夙に不可欠脂酸の特殊生理学的意義に着目し，その探求につとめて来たが，最近に至り，副腎皮質ホルモン，殊に Glucocorticoid の前駆物質は，不可欠脂酸，殊に Tetraenoic acid とエステル結合したコレステロールであり，不可欠脂酸欠乏時には，副腎皮質ホルモンの産生能が低下する事を知り得るに至つた．一方，コレステロールの終末代謝産物は胆汁酸である点に鑑み，胆汁酸の生合成に際しても，亦コレステロールは不可欠脂酸とエステル結合していなければならないということが充分推測され得る．このような考え方からすれば，不可欠脂酸の欠乏，乃至はその代謝障害の存在する場合には，当然胆汁酸の産生は障害され，胆汁中への胆汁酸分泌能も低下し，これが胆汁の不安定化を招き，結石形成に重大な影響を及ぼすものと考えられる．そこで不可欠脂酸及び，その代謝に重要な役割を果す，ピリドキシンの胆汁組成に及ぼす影響をみるために，以下のような臨床的及び，基礎的実験を施行した．即ち，手術時無菌的に採取した，胆石症患者及び，対照群として，消化性潰瘍患者の胆嚢胆汁を夫々分析すると同時に，更に総胆管ドレナージを施した患者に，不可欠脂酸及びピリドキシンを投与して，肝胆汁組成の変動を経日的に分析，検討した．更に，ウイスター系雄性ラットを不可欠脂酸食，不可欠脂酸欠乏食，ピリドキシン欠乏・不可欠脂酸食，にて夫々，2～3ヵ月に亙り飼育し，その肝臓に含有される，胆汁酸をも分析し次のような結果を得た．

(1)　コレステロール系結石患者の胆嚢胆汁では，肝機能障害や胆道感染が認められなくても，総胆汁酸量及びレチティン量の減少が認められる．

(2)　胆道感染の認められない，コレステロール系結石患者の胆汁組成は，胆道感染の認められるコレステロール系結石患者のそれと，明らかに異つている．

(3)　従つて，コレステロール系結石にみられる胆汁組成の変化は，感染に起因するものとは考え難い．

(4)　ラット肝臓中に含有される胆汁酸量は，安静時に於て，不可欠脂酸欠乏食群では著減している．一方，ストレスを加えると，不可欠脂酸食群ではその肝臓含有胆汁酸量に著変が認められないのに対し，ピリドキシン欠乏・不可欠脂酸食群では著減が認められた．

(5)　総胆管ドレナージを施した患者の肝胆汁では，不可欠脂酸及びピリドキシンの投与により，胆汁酸量が増加し，胆汁酸対コレステロールの比(B/C 比)の上昇が認められた．

(6)　共同研究者，吉永，平野等の実験成績を併せ考えると，胆汁酸の前駆物質は，不可欠脂酸，殊に Tetraenoic acid とエステル結合した，コレステロールであると考えられる．

(7)　ピリドキシン欠乏に起因する不可欠脂酸の代謝障害は，胆石，殊にコレステロール系結石の形成に重要な役割を果し，ストレスがこれを助長するものと考えられる．

(8)　人胆汁中に含有される胆汁酸をガスクロマトグラフィーで分析する事にも成功した．