Arch. Jap. Chir. 38(1), 107~124, Jun. 1969

Initiating Factors of Gallstones, Especially Cholesterol Stones (III)

by

Yorinori Hikasa, Susumu Matsuda, Masao Nagase, Michio Yoshinaga, Takayoshi Tobe, Izumi Maruyama, Ryuzo Shioda, Hiroshi Tanimura, Ryusuke Muraoka, Hirohisa Muroya and Minoru Togo

From the Second Surgical Division, Faculty of Medicine, Kyoto University
(Director : Prof. Dr. Chuji Kimura)

Received for Publication Oct. 10, 1968

INTRODUCTION

Although many studies on the pathogenesis of cholelithiasis have been made up to the present time, they have not succeeded in unveiling it entirely.

In our laboratory, we have long investigated the initiating factors of cholelithiasis experimentally and clinically[1)-11)]. As far as cholesterol stone formation is concerned, recently we have reached the conclusion that the dietary factors are of importance for it.

EXPERIMENTAL PRODUCTION OF CHOLESTEROL STONES

When an essential fatty acid-free diet (EFA-free diet) containing starch (if not mentioned, starch is always in beta form) and another containing monosaccharide or disaccharide, e.g. glucose and sucrose, were given to the animals, difference was observed in the cholesterol contents of liver between these two diets, suggesting that the hepatic biosynthesis of cholesterol enhanced in the group of animals fed on the latter as shown in Fig. 1[12)]. In the preliminary experiments, designed for investigating cholesterol catabolism, therefore, we used starch as the source of carbohydrates in a basic diet.

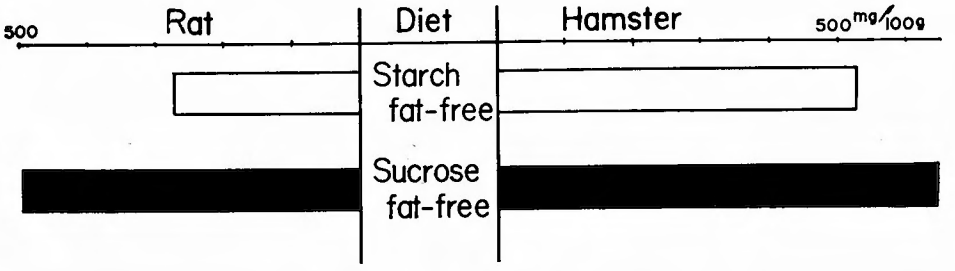

Fig. 1 Cholesterol level in the liver

Fig. 1 shows diagrammatically difference in cholesterol levels in the livers of hamsters and rats between starch and sucrose.

In general, the patients with cholesterol stones showed a characteristic pattern in their liver fatty acid composition[13)] ; a decrease in polyenoic acids derived from linoleic acid, such

as 5, 8, 11, 14-eicosatetraenoic acid (arachidonic acid), 7, 10, 13, 16-docosatetraenoic acid, 4, 7, 10, 13, 16-docosapentaenoic acid etc. Deficiency in linoleic acid, however, has not been found in the patients with cholesterol stones, suggesting that EFA could not be physiologically metabolized. The polyenoic acids derived from oleic and palmitoleic acid showed a relative increase in the fatty acid composition of the livers of the patients at the expense of the other polyenoic acids derived from linoleic acid.

To produce a similar change in the pattern of fatty acid composition in the experimental animals to that of the patients with cholesterol stones, even though they are receiving a diet supplemented with linoleic acid as the source of EFA (intake of EFA is generally via food in the form of linoleic acid), the animals must be maintained on a diet containing animal fats and kept in a relative deficient state in pyridoxine at the same time [14)15)]. The animals treated as above showed a very close pattern in the fatty acid distribution to that of the patients with cholesterol stones. They also revealed increases in total, esterified and free cholesterol, and in the ratio of dihydroxycholanic to trihydroxycholanic acid in bile and decreases in total bile acids, in the ratio of total bile acids to cholesterol, and in lecithin and fatty acid concentrations in bile in much the same way as the patients with cholesterol stones, as shown in Fig. 2. These findings appeared to be compatible with induction of a condition facilitating cholesterol stone formation in the animals. However, no stones was observed among them since we used rats which had no gallbladder. Following are other reasons for this; 1) deficiency in pyridoxine was not so remarkable as to disturb EFA metabolism probably because of the bacterial production of the vitamins, though pyridoxine was completely removed from the diet, and 2) no enhancement in the hepatic biosynthesis of cholesterol occurred since starch was used as the source of carbohydrates in the diet. So far as the animals are fed on a diet containing starch, it is in general difficult to induce them a distinct deficient state in pyridoxine, for indigestible starch becomes good nutrients for the intestinal flora so that they may produce vitamins such as B complex and K, supplying them to some extent to their host. Following were the dites used for the experiments; one was EFA-free diet, and the other was supplemented with animal fats such as butter fat but otherwise the same composition as the former. The vitamins the intestinal flora could synthesize, such as pyridoxine, biotin, inositol, folic acid, vitamin

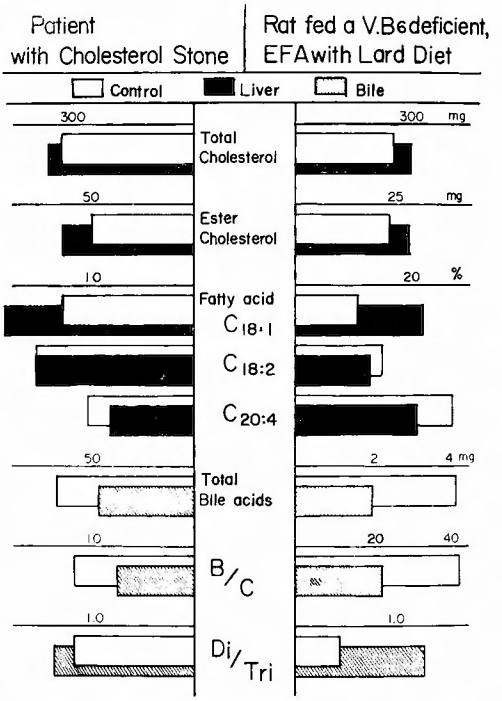

Fig. 2 Fig. 2 demonstrates an excellent similarity in changes of cholesterol and fatty acid composition in liver, and of total bile acids and ratios of total bile acids to cholesterol and dihydroxycholanic acid to trihydroxycholanic acid in bile between the patients with cholesterol stones and the rats maintained on a vitamine B_6-free diet supplemented with linoleic acid and animal fat.

K etc., were removed from them. Glucose or sucrose was used as the source of carbohydrates. When the hamsters were maintained on either diet, cholesterol stones were observed in the gallbladders as quickly as expected.

Supplementation of the same EFA-free diet as above with CMC (carboxyl-methyl-cellulose) or agar-agar lowered to some extent the incidence of cholesterol stones[16] while the same tendency was observed when laying straw at the bottom of animal cages[17] (Fig. 3). On the other hand, an experiment was made in parallel with the main ones; when the preliminarily cholecystectomized animals were fed on an EFA-free diet containing glucose or sucrose we observed that they were almost entirely relieved of cholesterol stones, assuring clearly that the gallbladders were sites necessary for stone formation.

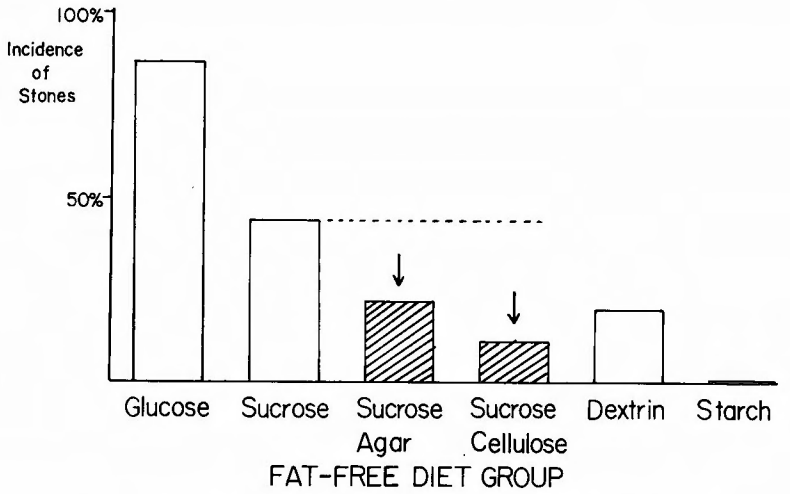

Fig. 3 Effect of indigestible substances on cholesterol stone formation

Fig. 3 illustrates relations between the incidence of cholesterol stones and types of carbohydrates used in an EFA-free diet.

We have placed great emphasis on deficiency in vitamins and their possible changed activities in relation to cholesterol stone formation since whenever the animals received a diet containtng starch they were free of stones while they showed a high incidence of cholesterol stones as they were fed on a diet containing glucose or sucrose. Thus, we made an experiment that each or all of the vitamins, such as pyridoxine, biotin, inositol, folic acid, K_3, etc., was added to the basic diet containing glucose or sucrose and that the animals were maintained on a series of the enriched diets. In any case, however, the incidence of cholesterol stones was not lowered, suggesting that all the supplemental vitamins were destroyed while making or serving the diet or inactivated for some other reasons. Since then, we supplemented the EFA-free diet containing glucose or sucrose as the source of carbohydrates with the vitamin mixture, even though they were known to be inactive and ineffective, and used as a basic diet for the experiments.

Fats, for example, sesame oil, lard, butter fat and a combination of sesame oil and lard, were supplemented to the basic diet[16]. Occurrence of cholesterol stones was not negative but higher in the animals when they received a diet with a higher concentration of saturated fat and a larger ratio of oleic plus saturated fatty acids to EFA. However, the occurrence

was to some extent lowerd in every case when fats, whether saturated or unsaturated, were added to the basic diet, as compared with the case the animals received an EFA-free diet only. This suggests a possible inhibitory effect of fats, particularly EFA, on cholesterol stone formation, as shown in Fig. 4.

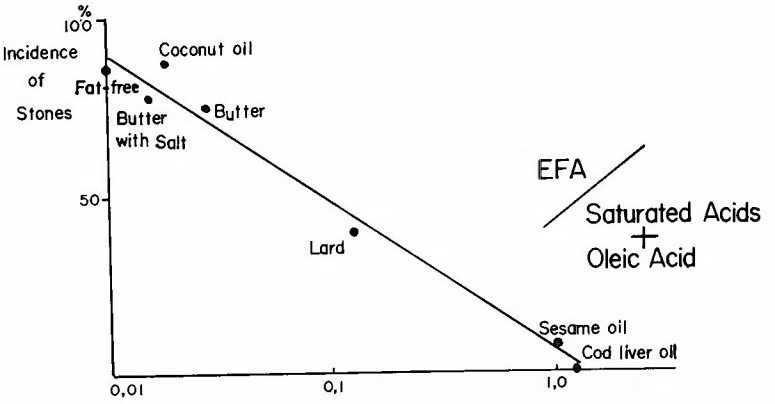

Fig. 4 Relationship between essential fatty acids in dietary fats and incidence of cholesterol stones
Fig. 4 shows a relation between the incidence of cholesternl stones and types of fat administered to the hamsters, in particular, the rate of EFA to saturated fatty acids plus oleic acid.

Although such inactive vitamins as mentioned above were not effective to protect the animals from cholesterol stones, we came cross to the parenteral administration of a vitamin, pyridoxal phosphate, which we speculated as the most effective, to the animals maintained on a sesame oil diet. We assumed that the pyridoxal phosphate would facilitate a metabolic process involved in the biosynthesis of polyenoic acids, such as 5, 8, 11, 14-eicosatetraenoic acid, so that such polyenoic acids might be more active to catabolize cholesterol, protecting the animals against cholesterol stone formation[16]. The parenteral administration of pyridoxal phosphate (1 to 2 mg per animal, every day) to two groups of animals, one of which was maintained on a sesame oil diet and the other on a lard diet, resulted in protecting both of them against cholesterol stones (Fig. 5). It is worthy to mention that despite injection of the pyridoxal phosphate in the animals fed on an EFA-free diet cholesterol stones occurred at much the same high rate as the animals received an EFA-free diet only. This suggests that two conditions are at least necessary for prevention of cholesterol stone formation; 1) linoleic acid as the source of EFA should be consistently supplied via food and 2) pyridoxal phosphate, an active form of vitamin B_6, should be sufficiently stored in a body at the same time. These results showed that the higher was the relative concentration of saturated fatty acids plus oleic acid the severer was the hindrance in a conversion process of pyridoxine to active pyridoxal phosphate. The most severe was the disturbance in case of intake of lower saturated fatty acids. KOTAKE and his coworkers reported that intake of saturated fatty acids and oleic acid induced inactivation of pyridoxine phosphate oxidase, an enzyme involved in conversion of pyridoxine phosphate to pyridoxal phosphate[18].

Reviewing difference in the results obtained from the two groups of animals, one of

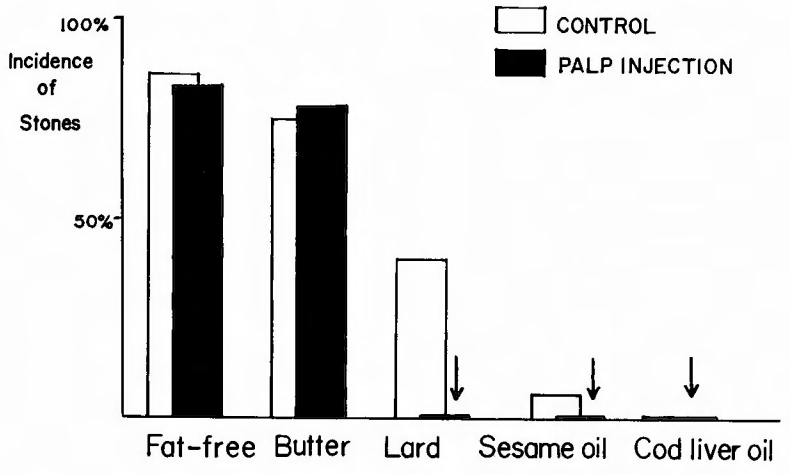

Fig. 5 Effect of PALP (Vit. B_6) on the cholesterol stone formation

Fig. 5 shows an effect of pyridoxal phosphate given to the hamsters parenterally on cholesterol stone formation.

which received an EFA-free diet containing glucose or sucrose and the other an EFA-free diet with starch as the source of carbohydrates, we observed in the former an increase in cholesterol and decreases in total bile acids and in the ratio of total bile acids to cholesterol while the latter showed a normal ratio of total bile acids to cholesterol[16)19)]. Analysis of bile lecithin, however, showed that its concentration was higher in the former than the latter. No difference was observed in the fatty acid composition of lecithin between these two groups except they equally showed only a characteristic pattern used to be found in the animals when they did not receive fats (Fig. 6).

The fact that cholesterol stones occurred highly in the former but no stones was formed in the latter suggests that unbalance between biosynthesis and degradation of cholesterol in liver is probably responsible for cholesterol stone formation. Decreases in total bile acids and in the ratio of total bile acids to cholesterol in bile appear to be major factors in cholesterol stone formation. Parenteral administration of pyridoxal phosphate to the animals receiving an EFA-free diet containing glucose or sucrose did neither increase total bile acids and lecithin in bile nor prevent cholesterol stone formation. On the other hand, when pyridoxal phosphate was given parenterally to the animals receiving a sesame oil or lard diet, total bile acids and lecithin showed significant increases, a slight decrease in cholesterol concentration in bile, and cholesterol stone formation was completely prevented at the same time. These data showed clearly that pyridoxal phosphate was playing an important role in EFA metabolism in vivo. Despite supplementation of the basic diet with EFA, inactive pyridoxine did not show an inhibitory effect on cholesterol stone formation as did pyridoxal phosphate injection since saturated fatty acids or oleic acid contained in the dietary fats might inactivate the pyridoxine phosphate oxidase. Thus, large intake of saturated fatty acids, in particular, lower saturated ones hindered probably conversion of pyridoxine to pyridoxal phosphate and induced unbalance between synthesis and degradation of cholesterol in liver. This unbalance would induce an increase in the biliary excretion of chole-

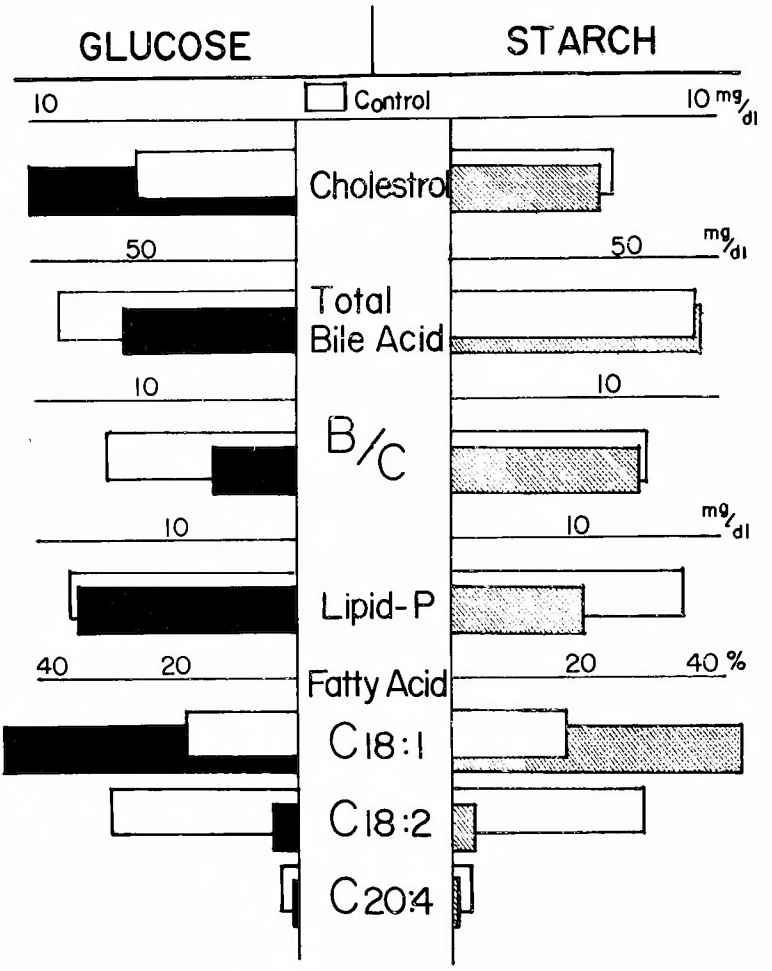

Fig. 6 Effect of carbohydrate on bile components (Fat-free diet group)

Fig. 6 is showing difference in cholesterol, bile acids, phospholipids, fatty acids and ratio of total bile acids to cholesterol (B/C) in bile between glucose and starch used as the source of carbohydrates in an EFA-free diet (Hamsters).

sterol, a decrease in the concentration of total bile acids, resulting in a qualitative change of bile where cholesterol precipitated to form a stone in the gallbladder.

We have observed that cortison showed a remarkable inhibitory effect on cholesterol stone formation in the animals receiving an EFA-free diet containing glucose or sucrose[20]. Under the physiological condition, EFA is amply stored in the adrenal glands[21]. This is not incompatible with the fact that EFA is playing an important role in the physiological maintenance of the adrenocortical functions. Large intake of lower saturated fatty acids inducing a deficient state in pyridoxal phosphate may hinder EFA metabolism. It is assumed, therefore, that the adrenocortical functions are secondarily impeded to some extent, so that cholesterol stone formation may be promoted.

According to the results that intake of saturated fat, in particular, lower saturated fatty

acids impeded remarkably the activity of pyridoxine phosphate oxidase, we made an experiment for clarifying the relationship between pyridoxal phosphate and saturated fat. When the animals were maintained on a diet supplemeted with butter fat containing a large proportion of short-chain fatty acids, 1 to 2 mg of pyridoxal phosphate injection was entirely powerless to protect them from cholesterol stone formation, indicating that the activity of pyridoxal phosphate oxidase was extremely impeded and pyridoxal phosphate was not supplied enough to satisfy their demand for it (Fig. 7). We also observed that the ratios of total bile acids to cholesterol and of lecithin to cholesterol decreased to the greatest extent.

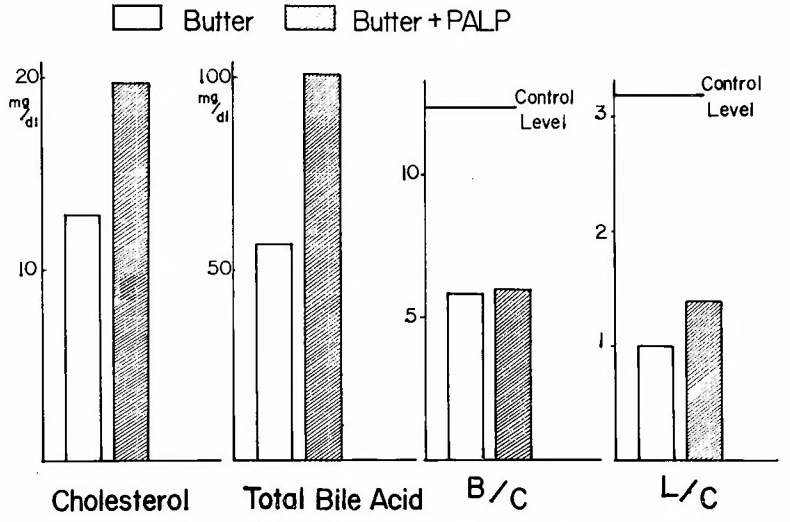

Fig. 7 Effect of vitamin on bile components

Fig. 7 shows an effect of pyridoxal phosphate given parenterally to the animals on cholesterol, bile acids, ratios of total bile acids to cholesterol (B/C) and total phospholipids to cholesterol (L/C) in the livers of the animals maintained on a butter fat diet (Hamsters).

DIETARY INFLUENCES ON DAILY HEPATIC OUTPUT IN BILE OF LIPIDS AND STEROIDS

KINSELL and his coworkers have recently reported that daily excretion of bile acids and sterols increased in the stool when human subjects were maintained on diets rich in polyunsaturated, as compared to saturated, fat. This finding suggested investigation of any changes in daily hepatic output of bile components in relation to different types of fat.

A 34-year-old woman and a 54-year-old man, each with cholelithiasis, were studied on the Metabolic Ward, Highland General Hospital during several months on well-defined isocaloric formula diets containing 25% of total calories as either coconut oil or trilinolein. During cholecystectomy, a BALDWIN T-tube had been inserted into the common bile duct. This T-tube has a separate side arm through which water or air may be injected to inflate a balloon which effectively blocks the lumen of the common duct. Thus, the hepatic bile flow to the duodenum may be completely blocked at will, the bile being externalized through the long arm of the T-tube. After partial bile drainage was stopped, 100 mic-

rocuries of 1-^{14}C-cholesterol was given intravenously to the male patient, and 20 microcuries to the female patient, to label the body pools of cholesterol and bile acids and facilitate analysis. A total of 17 complete 24-hour bile collections were made in the male patient, after he had fully recovered from surgery. The bile volume was measured after each 2-hour collection period, and a 1% aliquot was taken for analysis. The remaining bile was returned to the duodenum via a nasal tube to minimize disturbance of the enterohepatic circulation. Statistical treatments were made on the data obtained from six studies during intake of coconut oil and on the data of nine studies during intake of trilinolein. Two out of the seventeen complete studies were eliminated as they were felt to be in "unsteady states". A total of 16 24-hour bile collections were carried out in the second patient under similar circumstances; four studies during intake of coconut oil and six studies during intake of trilinolein were treated statistically.

Table 1 shows 24-hour output of bile, total solids, total bile acids, total phospholipids, and cholesterol with means and standard deviations on the two diets in the two patients. A significant increase was observed in 24-hour volume of bile on the trilinolein diet as compared to the coconut oil diet. In the second patient a significant but smaller increase in daily bile volume was also observed. Both patients showed a very significant increased daily output of bile total solids on trilinolein. On substituting polyunsaturated for saturated fat, the output of total bile acids and of total phospholipids increased significantly in both patients. The output of cholesterol about 1 gram per day showed a moderate increase on transfer to the trilinolein diet; this increase was significant in one subject.

Table 1. Mean 24-hour outputs of bile, total solids and three major bile components with standard deviations and p values (two subjects on two diets)

Dietary Fat		Coconut Oil		Trilinolein		
Nos. of 24-hour Bile Collection	(F)	(4)		(6)		
	(M)	(6)		(9)		
		Mean	SD	Mean	SD	p
24-Hour Output of Bile (ml)	(F)	633	30	862	47	<0.005
	(M)	787	44	847	45	<0.005
Total Solids (gm)	(F)	22.9	1.9	31.0	4.5	<0.005
	(M)	25.3	1.3	31.9	2.6	<0.005
Total Bile Acids as Free (gm)	(F)	7.2	0.7	10.9	1.0	<0.005
	(M)	9.3	1.1	12.6	1.1	<0.005
Total Phospholipids (gm)	(F)	4.4	0.7	5.0	0.7	<0.05
	(M)	3.5	0.3	4.4	0.4	<0.005
Cholesterol (gm)	(F)	1.2	0.1	1.3	0.2	ns*
	(M)	0.9	0.1	1.2	0.3	<0.005

*not significant

Table 2 shows the 24 hour output of individual bile acids in the two subjects on two diets. Large and significant increases in the output of cholic acid, and moderate but significant increases in chenodeoxycholic acid were observed on exchanging fats. However,

no significant change was found in the daily output of deoxycholic acid. It is clear that transfer to a polyunsaturated fat diet from a saturated fat diet has produced an increase in the proportion of primary cholic and chenodeoxycholic acids in bile and a decrease in the proportion of secondary deoxycholic acid. It seems most likely that this change is brought about by an increased rate of synthesis of primary bile acids in the liver.

Table 2. Mean 24-hour outputs of cholic, chenodeoxycholic and deoxycholic acid with standard deviations and p values in two subjects (F ; female, M ; male) on two diets

Dietary Fat		Coconut Oil		Trilinolein		
Nos. of 24-Hour Bile Collection	(F)	(4)		(6)		
	(M)	(6)		(9)		
		Mean	SD	Mean	SD	p
Cholic Acid (gm)	(F)	2.5	0.3	5.3	0.7	<0.005
	(M)	3.4	0.9	5.2	0.7	<0.005
Chenodeoxycholic Acid (gm)	(F)	3.0	0.5	4.1	0.5	<0.005
	(M)	4.9	0.7	6.1	0.5	<0.005
Deoxycholic Acid (gm)	(F)	1.7	0.4	1.5	0.1	ns
	(M)	1.0	0.4	1.2	0.3	ns

Bile acids exist in bile as either glycine or taurine conjugates. In both patients, glycine conjugates were dominant at all times. Table 3 shows the mean daily output in gram for individual conjugated bile acids on two diets in the female patient. Glycocholic, glycochenodeoxycholic and taurocholic acids increased significantly on exchanging fats. Taurodeoxycholic acid showed a significant decrease on transfer to trilinolein while glycodeoxycholic and taurochenodeoxycholic acids showed no significant changes. In the male patient glycocholic and glycochenodeoxycholic acids showed a significant increase on transfer to trilinolein. However, the other conjugated bile acids did not show any changes.

Table 3. Mean 24-hour outputs of individual conjugated bile acids with standard deviations and p values in the female subject on two diets

Dietary Fat		Coconut Oil		Trilinolein		
Nos. of 24-Hour Bile Collection		(4)		(6)		
		Mean	SD	Mean	SD	p
Glycocholic Acid	(gm)	1.71	0.33	3.96	0.54	<0.005
Glycochenodeoxycholic Acid	(gm)	1.99	0.25	2.84	0.43	<0.005
Glycodeoxycholic Acid	(gm)	1.16	0.42	1.10	0.10	ns
Taurocholic Acid	(gm)	1.16	0.32	2.11	0.34	<0.005
Taurochenodeoxycholic Acid	(gm)	1.54	0.55	1.85	0.34	ns
Taurodeoxycholic Acid	(gm)	0.75	0.10	0.61	0.06	<0.005

Table 4 shows ratios—cholic acid to cholesterol, chenodeoxycholic acid to cholesterol, and deoxycholic acid to cholesterol—on two diets in both patients. The ratio of cholic

acid to cholesterol showed a significant increase on transfer to trilinolein, while the ratio of chenodeoxycholic acid to cholesterol showed a less significant increase and the ratio of deoxycholic acid to cholesterol showed a moderate decrease; this decrease was insignificant in one subject.

Table 4. Mean ratios of cholic acid to free cholesterol, chenodeoxycholic acid to free cholesterol and of deoxycholic acid to free cholesterol with standard deviations and p values in two subjects on two diets

Dietary Fat		Coconut Oil		Trilinolein		
Nos. of 24-Hour Bile Collection	(F)	(4)		(6)		
	(M)	(6)		(9)		
		Mean	SD	Mean	SD	p
Cholic Acid / Cholesterol	(F)	2.18	0.27	4.32	1.00	<0.005
	(M)	3.89	0.31	4.13	0.61	<0.005
Chenodeoxycholic Acid / Cholesterol	(F)	2.66	0.33	3.35	0.70	<0.01
	(M)	5.67	0.87	5.16	0.54	<0.01
Deoxycholic Acid / Cholesterol	(F)	1.46	0.36	1.20	0.23	<0.05
	(M)	1.09	0.43	1.01	0.42	ns

In summary remarkable consistent increases in volume of bile secreted per day were observed on exchange of fats, although the increase was small in the male patient. A considerable increase in daily output of total bile acids-due to increases in output of the two primary bile acids, cholic and chenodeoxycholic acid-were consistently found. On substituting trilinolein for coconut oil, glycocholic and glycochenodeoxycholic acids showed very significant increases in both subjects. The increase in the ratio of cholic acid concentration to cholesterol concentration observed in the bile of both subjects on transfer to trilinolein, may favor the formation of a more stable micellar solution[22], with reduced risk of cholesterol precipitation and gallstone formation.

This study was carried out by R. SHIODA[23)-26)], H. WEIZEL, P. WOOD[27)] and L. KINSELL[28)29)] and reported at the Subspecialty Meetings Under Joint Sponsorship of the Western Section, American Federation for Clinical Research and the Western Society for Clinical Research, February 1, 1968.

EXPERIMENTAL INVESTIGATION ON BIOSYNTHESIS OF CHOLESTEROL, WHETHER STIMULATED OR NOT, IN CHOLESTEROL STONE FORMATION

Difference was observed in cholesterol contents of liver between the two groups of hamsters fed on either an EFA-free diet containing starch or another containing monosaccharide or disaccharide as the source of carbohydrates, as mentioned earlier. The group of animals received the latter diet showed higher content of liver cholesterol as compared with those received the former diet, as shown diagrammatically in Fig. 1.

To investigate the detailed aspects in the enhanced hepatic biosynthesis of cholesterol, an experiment was carried out by using acetate-2-^{14}C or mevalonate-2-^{14}C in cooperation with Dr. HAYAISHI, Prof. of Medical Chemistry, Faculty of Medicine, Kyoto University[30)31)].

When animals were maintained on an EFA-free diet containing glucose or sucrose, the hepatic biosynthesis of cholesterol showed a large increase and almost all of them produced cholesterol stones in their gallbladders. However, when the EFA-free diet containing starch was given to the animals, they were not only lower in the rate of the hepatic synthesis of cholesterol but also free of cholesterol stone formation, as shown in Fig. 8. When the EFA-free diet containing starch as the source of carbohydrate was given to the animals initially and then changed to the other EFA-free diet containing glucose or sucrose, the synthetic rate of cholesterol in liver began to increase in four days and continued to increase during 21 days on this diet. This gave us an evidence that enhancement in the hepatic biosynthesis of cholesterol had occurred before cholesterol stones were formed, as shown in Fig. 9. When the EFA-free diet containing glucose or sucrose was supplemented with either ethyl-palmitate or ethyl-linoleate, the animals received the diet with ethyl-palmitate showed a high rate in the hepatic biosynthesis of cholesterol and a high incidence of cholesterol stones, as shown in Fig. 10. To explain the reason why the animals showed larger increases in the hepatic synthesis of cholesterol when they were maintained on the FEA-free diet containing glucose or sucrose, as compared to the animals received the EFA-free diet containing starch, β-hydroxy-β-methylglutaryl-CoA reductase must be considered as an important factor. Since it has been well known that NADPH was of importance as a co-enzyme to the β-hydroxy-β-methylglutaryl-CoA reductase in cholesterol biosynthesis and SIPERSTEIN et al. reported that NADPH generating system through hexose monophosphate shunt enhanced

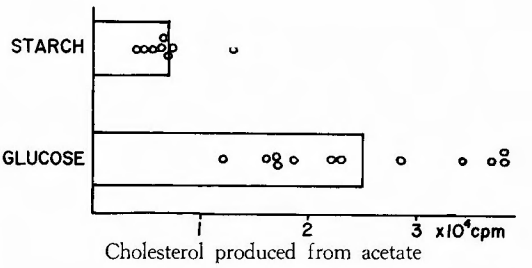

Fig. 8

Fig. 8 shows difference in the hepatic biosynthesis rate of cholesterol from acetate-2-^{14}C between glucose and starch in the EFA-free diet ; determination of the biosynthesis rate was made on the liver slices.

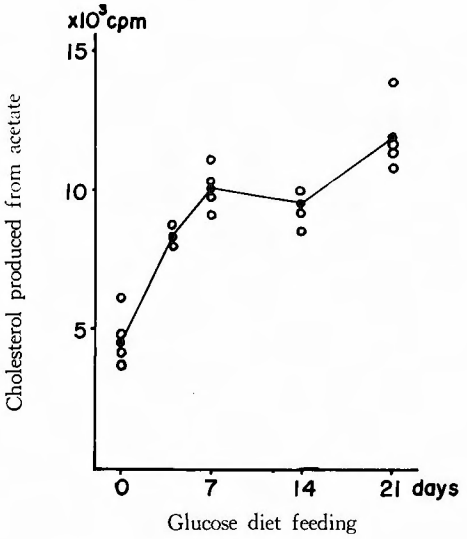

Fig. 9

Fig. 9 shows the cholesterol synthsized from acetate-2-^{14}C after the EFA-free diet containing glucose as the source of carbohydrate was given to the animals.

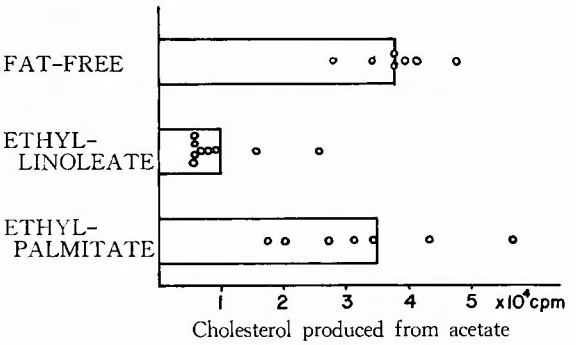

Fig. 10

Fig. 10 shows the effects of supplementation of an EFA-free diet containing glucose with ethyl palmitate or ethyl linoleate on the hepatic biosynthesis of cholesterol from acetate-2-^{14}C.

cholesterol biosynthesis[32]. We investigated the activities of glucose-6-phosphate and of glucose-6-phosphogluconate dehydrogenases, representatives in NADPH generating system, by adding glucose-6-phosphate and NADP to the liver homogenates. No difference was observed in the activities between the two groups of animals, one of which received the EFA-free diet containing glucose or sucrose and the other containing starch. The addition of an NADPH generating system to the liver homogenate of animals fed on the EFA-free diet containing starch enhanced to some extent cholesterol synthetic rate. This enhancement, however, did not exceed an increase in the biosynthetic rate observed in the animals fed on the EFA-free diet containing glucose or sucrose, as shown in Table 5.

Table 5. shows the effect of NADPH on hepatic cholesterol biosynthesis from acetate-2-^{14}C

Diet	Cholesterol produced	
	−NADPH (c.p.m.)	+NADPH (c.p.m.)
Glucose diet	1,640±160 *(7)	2,610±630 (5)
Starch diet	850±110 (7)	1,160± 70 (5)

* : Standard error of mean.

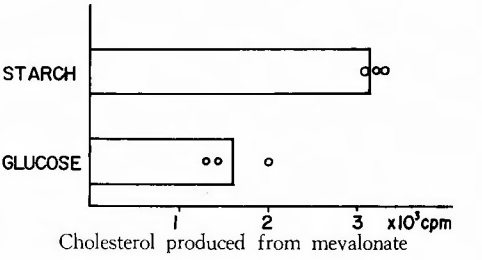

Fig. 11

Fig. 11 shows difference in cholesterol biosynthesis from mevalonate-2-^{14}C between two diets ; one is an EFA-free diet containing glucose and the other is containing starch as the source of carbohydrate.

Although the biosynthesis of cholesterol from acetate showed a greater increase in the animals maintained on the EFA-free diet with glucose or sucrose than in the animals on the EFA-free diet containing starch, the cholesterol synthesis from mevalonic acid showed a slightly higher increase in the animals fed on the EFA-free diet with starch, as compared with the animals on the EFA-free diet containing glucose or sucrose, as shown in Fig. 11.

Reviewing these data, it has been found that difference in NADPH levels was not only a factor for production of such distinct difference in the biosynthesis of cholesterol between the animals receiving the EFA-free diet containing glucose or sucrose and the others receiving the EFA-free diet with starch, though NADPH was necessary as a co-factor in cholesterol biosynthesis. Thus, it is a matter of importance where and how to find out true causes for this difference. According to the accumulated references, following are noted as the true causes. Starch is always splitted to glucose before absorption. Blood sugar levels, therefore, show a milder but prolonged rise, as compared with intake of glucose per se. The accompanying insulin release is limitted within a small range. COHEN reported that repeated ingestion of glucose resulted in an abnormal glucose tolerance test and a decrease in the activity of insulin like substance in serum at the same time[33]. This suggests that difference in insulin release between glucose and starch might be induced by enzymeproteins necessary for cholesterol biosynthesis. HOTTA et al. reported that the biosynthesis of chole-

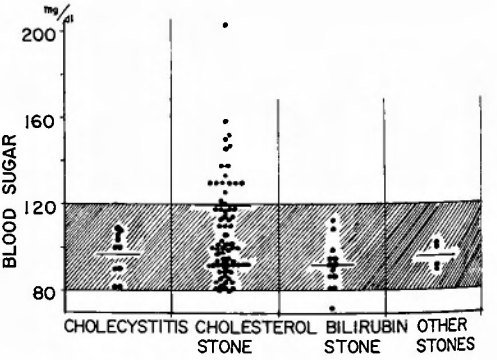

Fig. 12 Fasting blood sugar in the cholecystectomized patients.

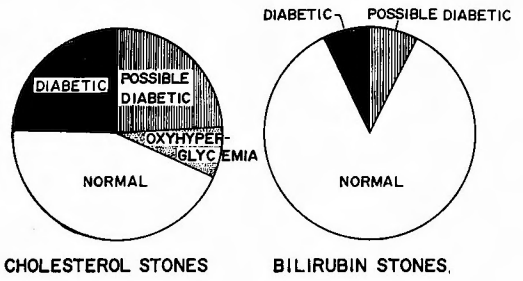

Fig. 13 Glucose tolerance test in the patients with gallstones

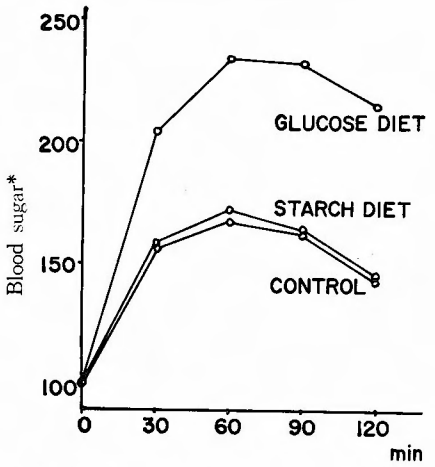

Fig. 14 Glucose tolerance test in hamsters

Fig. 14 shows glucose tolerance tests carried out on the animals maintained on three different diets ; one is a butter fat diet containing glucose, the second is containing starch in beta form and the third is a rat chow diet.

* percent to F. B. S.

sterol was enhanced in the liver slices from the diabetic rats but preliminary administration of insulin deprived the liver slices of such increased cholesterol biosynthesis[34].

From this point of view, we examined the patients with cholesterol stones (Figs. 12 and 13)[35][36] ; the results of fasting blood suger level and glucose tolerance test in the patients with cholesterol stones showed that they were latent (possible or probable) diabetic as BRAUNSTEINER reported[37]. Almost all the animals maintained on the conditions where cholesterol stones were formed, for example, administration of butter fat diet containing glucose or sucrose, revealed that they were actually in a state considered as a latent diabetic, though they did not spill sugar in urine[38] (Fig. 14). As mentioned above, we emphasized repeatedly that remarkable changes in bile composition was ultimately induced by the impeded cholesterol catabolism which was resulted from disturbances in EFA metabolism on account of deficiency in both pyridoxine and pyridoxal phosphate as a result of "dysbacteria" or deficiency in pyridoxal phosphate due to inactivation of pyridoxine phosphate oxidase as observed in large intake of animal fats containing saturated fatty acids, in particular, lower saturated ones. We assumed, therefore, that the abnormal enhancement in the hepatic biosynthesis of cholesterol, which we considered as an essential factor in cholesterol stone formation, was resulted from deficiency in pyridoxal phosphate. Meanwhile KOTAKE et al. reported in their studies on tryptophan metabolism that pyridoxal phosphate was necessary for the normal metabolism of tryptophan and that its deficiency caused a large increase in xanthurenic acid, resulting in inactivation of insulin through xanthurenic acid-insulin-complex formation[39]. Then we speculated that when deficiency in pyridoxal phosphate was induced, a latent diabetic state would be brought about by the abnormal metabolism in tryptophan, increasing the hepatic biosynthesis of cholesterol.

EXPERIMENTAL INVESTIGATION ON CHOLESTEROL STONE FORMATION: WHETHER OR NOT CHOLESTEROL STONES OCCUR IN THE ANIMALS EVEN IF THEY ARE RECEIVING A DIET CONTAINING STARCH AS THE SOURCE OF CARBOHYDRATE

From the above-mentioned results, it has been assumed that large intake of animal

fats rich in saturated fatty acids, in particular, in lower saturated ones induced deficiency in pyridoxal phosphate and produced "unbalance" between biosynthesis and degradation of cholesterol in liver which caused the increased biliary excretion of cholesterol and qualitative and quantitative changes in bile, resulting in initial precipitation of cholesterol and final development of cholesterol gallstones in the gallbladders.

As a matter of fact, our daily supply of carbohydrates did neither depend on glucose or sucrose nor on uncooked starch. As it has been well known, raw starch in beta-form was not easily soluble in water but also resistive to the action of digestive enzymes. The starch used, therefore, is usually changed to an easily digestible one, alpha-form, by cooking it. For example, our traditional cooking way of rice starch is nothing but alpha formation. Then, the following new experiment were carried out. The two forms of starch were used as the source of dietary carbohydrates in the basic diet and butter fat containing lower saturated fatty acids was added. When the animals were fed on the butter fat diet containing starch in beta-form they were free of cholesterol stones. However, when they were maintained on the butter fat diet containing starch in alpha-form cholesterol stone formation was as high as they received the EFA-free diet containing glucose or sucrose (Fig. 15). Through this experiment, we observed an interesting phenomenon ; the higher was the supplementary rate of the butter fat diet containing starch in alpha-form with exogenous cholesterol, the lower was the incidence of cholesterol stones. We have been encouraged by a success in the first alimental production of cholesterol stones in the animals, even though only starch was used as the source of carbohydrates.

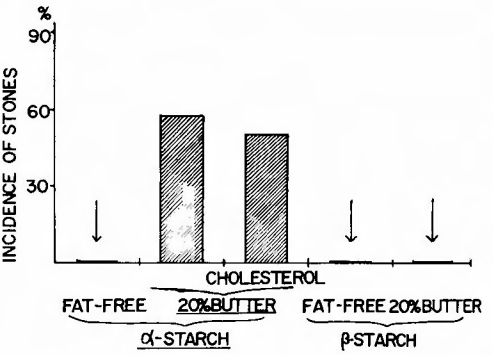

Fig. 15

Fig. 15 shows interrelations of the incidence of cholesterol stones, fats, whether containing cholesterol or not, and types of starch.

It is worthy to mention that incidence of cholesterol stones is higher in the people living in the urban area than in the country side and is higher in Europeans and Americans than in Japanese. It is interesting enough to know an increasing tendency in the occurrence of cholesterol stones in our country in accordance with changes in the dietary composition. From these facts and the results obtained throughout the experiments, we have found a very close relationship between intake of animal fats as the source of saturated fatty acids, in particular, lower saturated ones and cholesterol stone formation, paying a considerable attention to the quality and quantity of carbohydrates in relation to stone formation.

SUMMARY

From the experimental and clinical studies we have reached the following conclusions:

1) As far as cholesterol stones are concerned, initiating factors of stone formation must be searched among the systemic factors.

2) It is at least essential for cholesterol stone formation that disturbance in the degradation process of cholesterol to bile acids in liver is induced in step with the increased

hepatic biosynthesis of cholesterol.

3) So long as our daily intake of carbohydrates depends mainly on starch in alpha-form, the disturbed cholesterol catabolism with the increased cholesterol synthesis is probably produced, provided the subjects intake large amount of animal fats relatively rich in saturated fatty acids, particularly, in lower saturated ones.

4) Development of such condition may be facilitated by deficiency in the bodily reservoir of pyridoxal phosphate-due to inactivation of pyridoxine phosphate oxidase following large intake of saturated fatty acids, in particular, lower saturated acids.

5) Deficiency in pyridoxal phosphate impedes the catabolism of cholesterol to bile acids in liver through disturbances in the metabolism of EFA, inducing qualitative and quantitative changes in bile composition while it probably produces greatly xanthurenic acid through the abnormal metabolism in tryptophan as KOTAKE et al. reported. The excessive xanthurenic acid lowers insulin activity so that the subjects are driven to a latent diabetic state, resulting in an abnormal increase in the hepatic biosynthesis of cholesterol.

6) Rich food means a high fat and high protein diet (a high tryptophan diet). Relationship between the increased proportion of protein in such diet and pyridoxal phosphate must be kept in mind, regarding with cholesterol stone formation.

7) The above-mentioned conditions seem most likely to induce the qualitative and quantitative changes in bile composition, such as an increase in the biliary excretion of cholesterol and a decrease in the production of total bile acids. Thus, the changes occurred in bile facilitate crystallization and precipitation of cholesterol in the gallbladders, initiating cholesterol stones. On the other hand, a decrease in bile lecithin and changes in the fatty acid composition of lecithin do not seem so important for crystallization of cholesterol in the gallbladders as do changes in bile acids.

8) The gallbladders are essential for cholesterol stone formation, presenting a hotbed for it.

9) The hypofunction of the adrenal cortex containing EFA sufficiently appears to facilitate cholesterol stone formation as well.

10) From all the results obtained throughout the present study, we have reached good understanding for the facts that the occurrence of cholesterol stones is higher in the people in the urban than in the rural area and is higher in Europeans and American than in Japanese. In our clinic, we have recently seen an increasing tendency in the incidence of cholesterol stones; this appears to be brought about partly by changes in the dietary composition, suggesting a close relationship between rich food and cholesterol stone formation.

11) Interrelations of cholesterol stone formation, diabetes mellitus, arteriosclerosis, obesity and of sex would be solved by our above-mentioned ways of investigation.

REFERENCES

1) Hikasa, Y. et al.: Initiating factors of gallstones. Surgical Therapy (Tokyo) **9** : 658, 1963 (Jap).
2) Hikasa, Y. et al. : Initiating factors of gallstones, especially cholesterol stones. Arch. jap. Chir., **33** : 601, 1964.
3) Hikasa, Y. et al. : Initiating factors of gallstones, especially cholesterol stones (II). Arch. jap. Chir., **34** : 1430, 1965.
4) Hikasa, Y. et al. : Some problems on lipid metabolism in the surgical field. Jap. J. Clin. Med., **22** : 509, 1964 (Jap).

5) Hikasa, Y. et al. : Pathophysiological problems on lipid metabolism in the surgical field. Jap. J. Clin. Med., **22** : 2158, 1964 (Jap).
6) Hikasa, Y. et al. : Cholelithiasis, with reference to the formation of cholesterol gallstones (I). Saishin Igaku, **21** : 187, 1966 (Jap).
7) Hikasa, Y. et al. : Cholelithiasis, with reference to the formation of cholesterol gallstones (II). Saishin Igaku, **21** : 375, 1966 (Jap).
8) Hikasa, Y. et al. . Initiating factors of gallstones, especially cholesterol stones. Recent Advances in Gastroenterology, Proc. 3rd World Cong. Gastroenterology, IV : 208, 1967.
9) Hikasa, Y. et al. : Clinical problems of polyenoic fatty acid from the surgical aspect. (3) Experimental and clinical studies on the formation of cholesterol gallstones and the role of adrenal lipid. Saishin Igaku, **22** : 1227, 1967 (Jap).
10) Hikasa, Y. et al. : Vitamin B_6 and development of cholesterol gallstones. Shinryo, **20** : 2179, 1967 (Jap).
11) Hikasa, Y. : Initiating factors in cholesterol gallstone formation. Arch. jap. Chir., **37** : 1, 1968 (Jap).
12) Eguchi, T. : Studies on pathogenesis of cholesterol gallstones, especially with respect to behaviour of cholesterol metabolism. Arch. jap. Chir., **34** : 1181, 1965.
13) Hirano, Y. : Role of essential fatty acids in liver on the conversion of cholesterol to bile acid, especially significance of essential fatty acids concerning the formation of cholesterol stones. Arch. jap. Chir., **34** : 939, 1965.
14) Maruyama, I. : Effect of essential fatty acids and pyridoxine on the formation of gallstones, especially cholesterol stones. Arch. jap. Chir., **34** : 19, 1965.
15) 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.
16) Tanimura, H. : Experimental studies on the etiology of cholelithiasis. Arch. jap. Chir., **34** : 1160, 1965.
17) Togo, M. : To be published in Arch. jap. Chir.
18) Kotake, Y. : Significance of Vitamin B_6 on the occurence of diabetes mellitus. Shinryo, **20** : 2001, 1967 (Jap).
19) Shioda, R. : Experimental studies on gallstone formation. Arch. Jap. Chir., **34** : 571, 1965.
20) Hashimoto, K. : Experimental studies on gallstones in hamsters. Arch.. jap. Chir., **35** : 981, 1966.
21) Hikasa, Y. et al. : The role of essential fatty acids on adrenocortical function. A collective review. Arch. jap. Chir., **35** : 381, 1966.
22) Juniper, K., Jr. : Physicochemical characteristics of bile and their relation to gallstone formation. Amer. J. Med., **39** : 98, 1965.
23) Shioda, R., H. Weizel, P. Wood and L. Kinsell : Dietary influences on daily hepatic output in bile of lipids and steroids. Clinical Research, **16** : 160, 1968.
24) Shioda, R., P. D. S. Wood, and L. W. Kiasell : Human biliary output of lipids in relation to diet. 51st Annual Meeting Federation of American Societies for Experimental Biology, 0268, 1967.
25) Shioda, R., P. Wood, and L. Kinsell : Dietary effects on human bile composition. Clinical Research, **14** : 138, 1967.
26) Shioda, R.. P. D. S. Wood, and L. W. Kinsell : A method for determination of individual conjugated bile acids in human bile. in press.
27) Wood, P. D. S., R. Shioda, and L. W. Kinsell : Dietary regulation of cholesterol metabolism. The Lancet, September **17**, 604, 1966.
28) Kinsell, L. W., P. D. S. Wood, R. Shioda, G. Schlierf, and Y. L. Lee : Effects of diet upon plasma, bile and fecal steroids. Progress in Biochemical Pharmacology, **4** : 59, Karger, Besel/New York, 1968.
29) Kinsell, L. W., P. D. S. Wood, and R. Shioda : Relationship to dietary fatty acids to plasma lipids and atherogenesis, including some vascular disease, 228, Appleton-Century-Crofts, Division of Meredith Publishing Company, New York, 1967.
30) Muroya, H., R. Suzue, and Y. Hikasa : Stimulation of hepatic cholesterol biosynthesis by dietary glucose, and its relation to cholesterol gallstone formation in the hamster. Arch. Biochem. Biophys., **124** : 12, 1968.
31) Muroya, H. : Stimulative effect of dietary glucose on hepatic cholesterol biosynthesis and formation of cholesterol gallstones in hamsters. Arch. jap. Chir., **37** : 76, 1968.
32) Siperstein, M. D., and V. M. Fagan : Studies on the relationship between glucose oxidation and intermediary metabolism. I. The influence of glycolysis on the synthesis of cholesterol and fatty acid in normal liver. J. Clin. Invest., **37** : 1185, 1958.

33) Cohen, A. M. and A. Teitelbaum : Effect of dietary sucrose and starch on oral glucose tolerance and insulin-like activity. Amer. J. Physiol., **206** : 105, 1964.
34) Hotta, S., and I. L. Chaikoff : Cholesterol synthesis from acetate in the diabetic liver. J. Biol. Chem., **198** : 895, 1952.
35) Tobe, T. et al. : Clinical observation of cholelithiasis. (1) Especially on preoperative and postoperative glucose metabolism. The 100th Kinki Surgical Meeting (Kyoto) in 1966.
36) Tobe, T. et al. : Glucose tolerance test at the pre- and post-cholecystectomy. The 10th Jap. Diabet. Ass. in 1967.
37) Braunsteiner, H., R. Di. Pauli, S. Sailer, und F. Sandhofer : Cholelithiasis und latent diabetische Stoffwechsellage. Schweiz. Med. Wschr., **96** : 44, 1966.
38) Tanimura, H. : To be published.
39) Kotake, Y., T. Sotokawa, E. Murakami, A. Misatake, M. Abe, and Y. Ikeda : Studies on the xanthurenic acid-insulin complex. (II) Physiological Activities. J. Biochemistry (Tokyo) **63** : 578, 1968.

和文抄録

胆石，就中コレステロール系結石の成因に就ての基礎的並びに臨床的研究 (III)

京都大学医学部外科学教室第2講座（指導：木村忠司教授）

日笠頼則・松田　晉・長瀬正夫・戸部隆吉
吉永道生・丸山　泉・塩田隆三・谷村　弘
村岡隆介・室家大久・東郷　実

われわれは胆石，就中コレステロール系結石の成因に就て基礎的並びに臨床的に考究し，次のような結論に到達した．

1) コレステロール系結石に関する限り，その成因は全身的要因に求められて然るべきである．

2) コレステロール系結石が形成されるためには，当該個体の肝臓に於けるコレステロールから胆汁酸への分解過程が阻害されると同時にコレステロールの合成能が異常に亢進しているという二つの条件の共存していることが必要且つ不可欠である．

3) このような状態は，われわれが糖質補補給源として日常々澱粉を摂取している限り，飽和酸，就中低級飽和酸を比較的豊富に含有する動物性脂質の大量摂取が併せ行なわれた際に初めて現出される．

4) このような状態が現出される理由として，飽和酸，就中低級飽和酸の摂取によつて招来されるピリドキシン活性化の障害にもとずくピリドキサール燐酸の体内欠乏にそれを求めることが出来るようである．

5) ピリドキサール燐酸の体内欠乏は不可欠脂酸の体内代謝障害を介してコレステロールから胆汁酸への肝臓に於ける異化的代謝即ち分解を阻害し，総胆汁酸の減少を初めとする胆汁の量的並びに質的変化を発来する一方，他方に於ては恐らく古武教授の提唱されるようにトリプトファン代謝の異常を介してキサントウレン酸の生成能をも異常に亢進せしめ，インシュリン活性の低下を招来，当該個体をして Probable(possible) diabetic state の状態に陥らしめ，ひいては当該個体の肝臓に於けるコレステロールの合成能を異常に増大せしめるに至るものと考えられる．

6) 美食の摂取は必然的に高脂質・高蛋白質食（高トリプトファン食）となり，それが摂取量が増大するのに反して，糖質摂取量はそれに伴ない相対的に減少することになろう．

7) 斯くして発来する胆汁中のコレステロール含量の増大と総胆汁酸量の減少を初めとする胆汁酸組成の質的並びに量的変化は必然的に胆嚢中に過剰のコレステロールを析出し，沈澱せしめ，ひいてはコレステロール系結石の形成へと発展せしめる．なお，胆汁中のレチティンの減量やレチティン構成脂酸比の変化といつたものは，コレステロールの胆嚢中に於ける析出に対して，胆汁酸に於ける変化程重要な意義を有するものではない．

8) 胆嚢がコレステロール系結石形成の場を提供している．

9) 平素から不可欠脂酸を充分に保有している副腎皮質の機能低下もコレステロール系結石の形成を助長するものと思われる

10) 以上の事実を以てする時は，日常の臨床に於いて，田舎の人に較べて都会人に，本邦人に較べて欧米人に，また本邦に於ても摂取する食餌組成の変遷に伴ないコレステロール系結石が多発する傾向にあるということもよく理解出来且つ美食（高脂質・高蛋白質食）摂取との因果関係も此処によく理解され得るに至つたものと考える．

11) また，コレステロール系結石の形成と糖尿病，動脈硬化症，肥満体更には女性といつた諸条件との因果関係も，以上のような考え方を以てすれば，合理的に説明し得られるであろう．