

## Initiating Factors in Formation of Cholesterol Gallstones

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Received for Publication April 30, 1978

### Introduction

According to the previous epidemiological studies on cholelithiasis in our country, the incidence of cholesterol cholelithiasis was higher in the inhabitants of large cities than in those living in the country<sup>19)</sup>, though the over-all morbidity of cholesterol cholelithiasis was far less than that of Europeans and Americans. The majority of gallstone consisted chiefly of pigment stones before World War II. Biliary calculi containing cholesterol, however, tended to increase in Japan after the war, as though they occurred in parallel with improvement of the national sanitary conditions and Euro-americanization of our traditional dietary composition as well<sup>34)</sup>, whereas cholelithiasis of pigment stones decreased gradually<sup>22)</sup>. The statistical data in relation to localization of gallstones in the biliary tract as shown in Fig. 1~3, revealed that the ratio of cholecystolithiasis to choledocholithiasis was as low as 0.6 to 1.6, but it increased to reach the same higher level of 10.7 as that of Europeans and Americans. This historical fact shows that an experiment has been performed in very natural way in the Japanese in order to teach us where to find the pathogenetic factors of cholesterol cholelithiasis. It seems to us, indeed, that providence of nature gave us valuable suggestions in this field of investigation.

Based on these suggestions, we have performed basic experiments, reaching the conclusion that cholesterol cholelithiasis can be initiated by pure dietary factors. The details of our serial experiments are described in order.

#### 1. *Difference in dietary composition between Japanese and Euro-americans, and alteration of Japanese dietary composition.*

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Key words : Dietary lithogenic factor, Formation of gallstones, Scanning electron microscopy, X-ray microanalyzer,  $\beta$ -glucuronidase activity.

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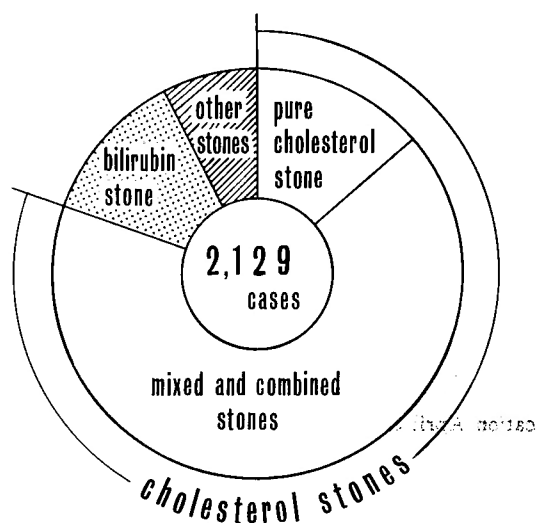


Fig. 1. Kinds of gallbladder stones in Japan (1975-77).

The most characteristic difference in dietary composition between Euro-americans and Japanese is that although the former are used to an intake of large amounts of fat and protein than the latter do, the former ingest a very small amount of carbohydrate. Paying attention to the constituents of carbohydrate, it is true that Europeans and Americans consume more purified and highly absorbable sugar than the Japanese do, while Japanese are used to an intake of a carbohydrate-rich diet consisting of unpurified, indigestible and less absorbable starch (containing much residue), and vegetable fibers.

Recent alterations in the Japanese eating habits are striking, indicating clearly that the consumption of animal fat and of sugar has increased remarkably in parallel with enrichment of the national economy<sup>10)</sup>. On the contrary, consumption of indigestible and less absorbable carbohydrate has decreased gradually. In short, the most predominant

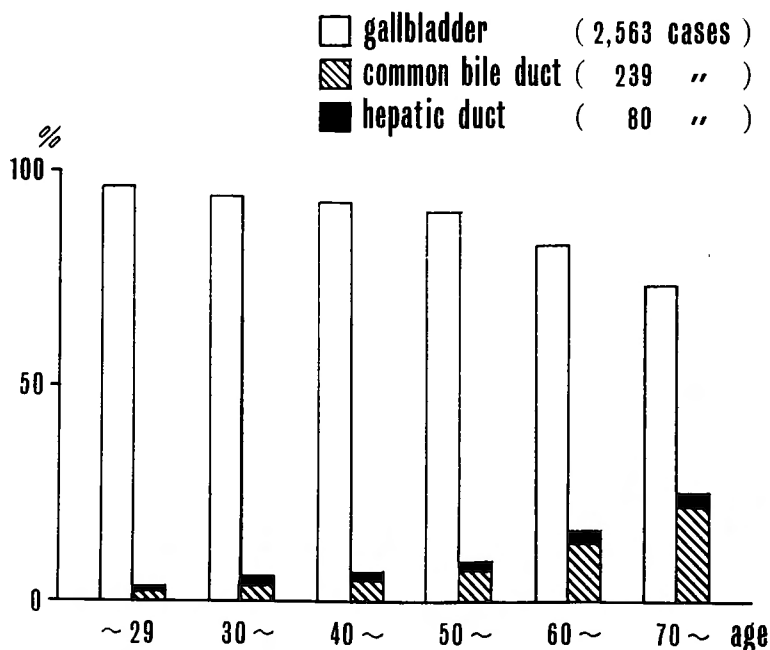


Fig. 2. Age and kinds of gallstones in Japan (1975-77).

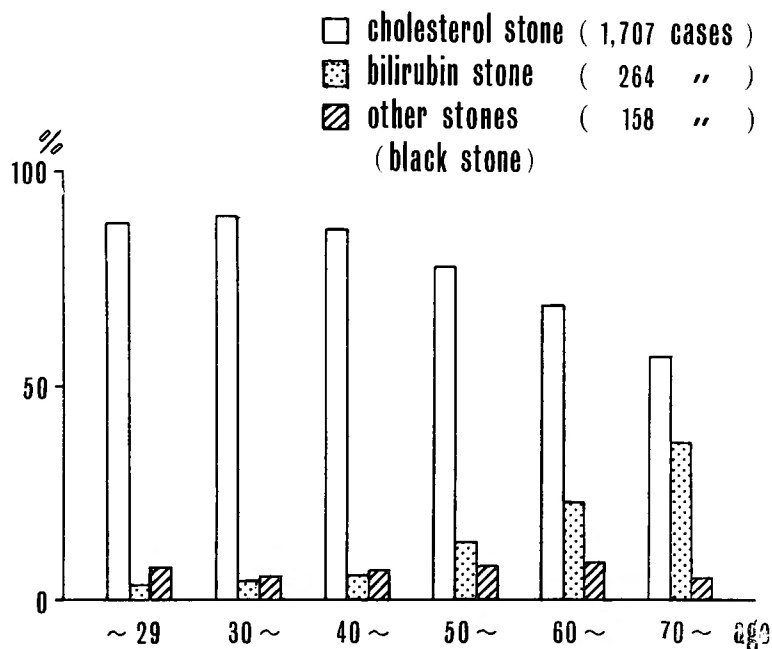


Fig. 3. Age and localization of gallstones in Japan (1975-77).

change in recent Japanese eating habits is nothing but a tendency toward Euro-americanization.

#### II. Selection of animals in experimental production of cholelithiasis.

It is essentially important in investigation of the pathogenesis of cholelithiasis in animal models that animals in which the anatomy of the liver and the biliary system as well as the composition of bile and metabolism of bile acids are quite similar to those of human beings should be selected and that they can live on a well defined synthetic diet and they can be easily tamed at the same time<sup>37)</sup>. We chose hamsters as an ideal animal for our experiments since they showed a striking resemblance to human subjects in all these aspects. REDINGER and SMALL proved this fact by making use of their triangle coordinates in which they found differences in biliary composition among a variety of animals<sup>25)</sup>. For selecting animals, however, the most important factor is that the metabolism of the bile acids must be similar to that of human subjects even though various conditions are given to them.

It is well known that the primary bile acids, cholic and chenodeoxycholic acid, which are produced as physiological metabolites by degradation of cholesterol in the liver are excreted into the intestinal lumen, but the majority of them is reabsorbed as primary bile acids, while the minority is converted to the secondary bile acids, deoxycholic and lithocholic acid, by the action of the intestinal flora and reabsorbed, followed by reexcretion of the

primary and secondary bile acids<sup>5)</sup>. Thus, bile acids are retained in the physiological enterohepatic circulation but some of them are lost via fecal excretion. The liver must, therefore, synthesize bile acids to supply fecal loss in order to maintain the total amount of circulating bile acids which is defined as "pool size" constant<sup>23)</sup>. Cholesterol is also excreted from the liver in a form of swollen micelles in which bile acids and lecithin are playing important roles.

First of all, we selected partial ileal bypass as a condition which could disturb the metabolism of bile acids in order to know whether hamsters would show the same changes in the composition and metabolism of bile acids as those of human subjects with ileal bypass or not. The results<sup>14)15)</sup> were that hamsters provided with partial ileal bypass showed quite similar changes to those of human subjects as shown in Fig. 4. We have reached, therefore, the conclusion that hamsters are the most appropriate animal for production of experimental cholelithiasis. Squirrel monkeys have been used for this type of experiment<sup>24)</sup> but we hesitated to select them because of the remarkable difference in the attitude of liver mitochondria from that of human subjects. Baboons are very difficult to obtain and to tame<sup>3)</sup>, though they appear to be ideal for experimental cholelithiasis.

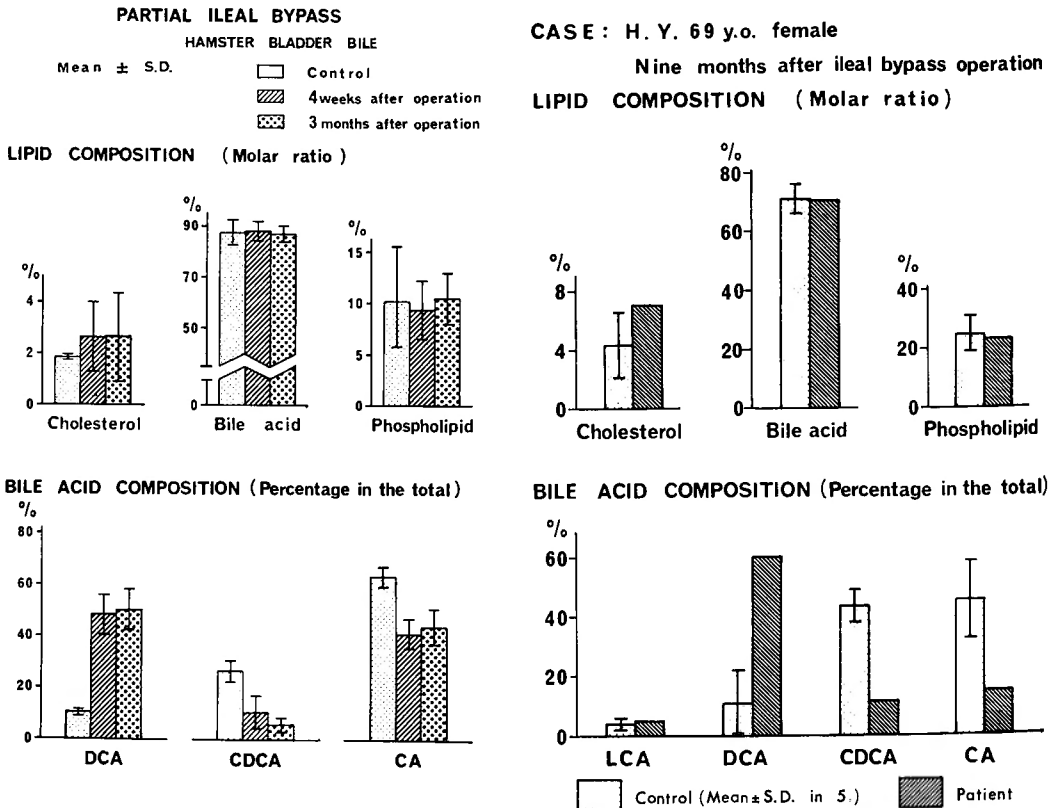


Fig. 4. Effect of partial ileal bypass on biliary lipids in hamsters and human subjects.

### III. *Production of pure cholesterol gallstones.*

#### (1). Experiments using a fat-free diet.

When weanling hamsters were reared on a fat-free diet in which the source of carbohydrate was glucose or sucrose, the content of hepatic cholesterol showed an abnormal increase, resulting in a marked development of cholesterol gallstones in their gallbladders<sup>33</sup>. In rats born without gallbladders, cholesterol gallstones were never produced, even though they were reared on the same fat-free diet as hamsters received<sup>18)49</sup>. It is interesting enough that cholesterol cholelithiasis did not occur in the cholecystectomized hamsters, even if they were reared on the same lithogenic diet as healthy hamsters<sup>38</sup>. These experimental results indicate clearly that the gallbladder is essential for the development of cholesterol gallstones in animals as well as probably in human subjects.

DAM et al. found unexpectedly the development of cholesterol gallstones in the gallbladder of weanling hamsters reared on a fat-free diet in their studies on the experimental production of muscular dystrophy<sup>1</sup>. They excluded even a trace of fat from their fat-free diet, using glucose or sucrose as the main source of carbohydrate, while they never used alpha-starch, our daily main source of carbohydrate. The animals which were reared on such a diet as they used showed unusual symptoms, though not recognized in human subjects with cholesterol cholelithiasis, such as cirrhotic change of the liver, acanthosis, other skin manifestations, prolapse of the anorectum and colon, loss of body weight, malaise and death<sup>35</sup>. This denotes, therefore, that hamsters reared on such a fat-free diet are not suitable as an animal model for human cholesterol cholelithiasis.

#### (2). Changes in lipids and steroids of patients with cholesterol cholelithiasis.

We established the fact that total and esterified cholesterol increased, but total bile acids decreased in the liver of patients with cholesterol cholelithiasis<sup>11</sup>. This suggests that the biosynthesis of cholesterol is accelerated abnormally while degradation of cholesterol to bile acids is inhibited in the liver of patients with cholesterol gallstones. The advanced investigations on lipid metabolism clarified that the normal liver in which bile acids were synthesized was containing a fairly large amount of polyunsaturated fatty acids, e.g. polyenoic acids, proving that about 1 g of linoleic acid was necessary for degradation of cholesterol (1 to 2 g) to the primary bile acids<sup>12</sup>. By referring to these changes, the metabolism of polyunsaturated fatty acids was investigated in the livers obtained from patients with cholesterol gallstones. The results showed that dienes did not change while tetraenes decreased remarkably. Trienes showed a marked compensatory increase in the liver of those with cholesterol gallstones<sup>6)7</sup>. In brief, these indicate a preponderance in the impaired metabolism of essential fatty acids in patients with cholesterol cholelithiasis.

Cholesterol increased while total bile acids decreased in the hepatic bile of the hamsters when they were reared on a fat-free diet in which glucose or sucrose was used as carbohydrate, hence, the ratio of total bile acids to cholesterol decreased<sup>33</sup>. These changes

were quite similar to those of patients with cholesterol cholelithiasis except for diens which decreased in the animals but they maintained a constant level in patients with cholesterol gallstones<sup>8)</sup>. It is true that deficiency of essential fatty acids and/or their disturbed metabolisms are assumed to be of fundamental importance for the development of cholesterol gallstones<sup>2)</sup>.

We have extended our studies to investigate the influences of essential fatty acids on the metabolism of bile acids in human subjects. Followings are results of measuring the daily output of bile and of total bile and lecithin in a group of patients with cholesterol gallstones receiving a diet in which the sole source of fat was coconut oil consisting chiefly of saturated fatty acids, such as caprylic, capric, lauric, myristic, palmitic acid, etc. and of a trace amount of unsaturated fatty acid which was lower than that of the other group receiving trilinolein. We found that an increase in the total bile acids in case of administration of trilinolein was due to an increase in the biosynthesis of primary bile acids, cholic and chenodeoxycholic acid, precisely, due to conjugated primary bile acids with taurin and glycine; glycocholic, taurocholic, glycochenodeoxycholic and taurochenodeoxycholic acids<sup>29)30)</sup>. It is said, in general, that the daily output of bile in women is smaller than in men. It is also well known that the hormonal slackening effect on connective tissue which becomes prominent in pregnancy may delay transport of bile through the enterohepatic circulation, causing biliary stasis. We could not deny, therefore, that the possible hormonal influence may contribute to the higher morbidity of cholesterol cholelithiasis in females as compared to males.

(3). Changes in the rate of hepatic cholesterol biosynthesis in animals receiving a lithogenic diet.

It is well known that cholesterol is synthesized from acetate via mevalonate with the aid of HMG-CoA reductase in the liver. The rate of cholesterol biosynthesis from acetate was determined in liver slices obtained from weanling hamsters which were reared on a fat-free lithogenic diet containing glucose or sucrose as the source of carbohydrate. It was highly accelerated under this condition<sup>20)</sup>. On the other hand, supplements of linoleic acid to the same lithogenic diet showed an inhibitory effect on the biosynthetic rate of cholesterol, whereas supplementation of the lithogenic diet with saturated fatty acids, e.g. palmitic acid, did not show any inhibitory effect on cholesterol biosynthesis<sup>21)</sup>. When we gave a fat-free diet of which the main carbohydrate source was glucose or sucrose after rearing the same group of hamsters on a fat-free diet containing beta-starch as the main source of carbohydrate, the livers of the animals synthesized cholesterol vigorously from acetate, followed by acceleration of cholesterol synthesis for a certain period of time. The biosynthesis of cholesterol from mevalonate showed, however, a completely inverse change against that from acetate. This denotes, therefore, that the cause of the abnormally accelerated hepatic biosynthesis of cholesterol in the animals receiving a fat-free lithogenic diet containing glucose or sucrose as the main carbohydrate source should be

searched for in the metabolic process of cholesterol antecedent to mevalonate. Although cholesterol gallstones were produced in the gallbladder of weanling hamsters when they were reared on a fat-free diet in which glucose or sucrose was used as the main source of carbohydrate, no cholesterol gallstones were produced when we reared them on a diet, though fat-free, in which alpha- or beta-starch was substituted for glucose or sucrose<sup>1)</sup> To join these observations together, we cannot help assuming finally that the development of pure cholesterol gallstones may be due to an unbalance between the two processes ; one is the intrahepatic biosynthesis of cholesterol and the other its degradation. Thus, as mentioned in the beginning, we anticipated the excessive intake or increased consumption of sugar to be a pathogenetic factor for high morbidity of cholesterol cholelithiasis among Europeans and Americans and for its remarkably increasing tendency in parallel with Euro-americanization of dietary composition in modern Japan. We have succeeded through our serial experimental studies in verifying that the excessive intake of sugar might be a contributory factor for cholesterol gallstones.

(4). The effects of supplemental dietary fats.

Another characteristic change in recent Japanese food life which has close resemblance to that of Europeans and Americans is an increased consumption of animal fat, such as butter fat and milk products. We have investigated, therefore, the effects of various dietary fats on the rate of formation of cholesterol gallstones in weanling hamsters by rearing them on a basic lithogenic diet to which a variety of natural fat was supplemented. We have found that the rate of formation of cholesterol gallstones was easily influenced by qualitative differences in the fatty acid composition of the supplementary dietary fat; the higher the rate of essential fatty acids to oleic acid plus other saturated fatty acids was, the less was the development of cholesterol gallstones<sup>33)</sup>. We have found also a most important fact that the inhibitory effect of essential fatty acids on the development of cholesterol stones was completed by parenteral administration of vitamin B<sub>6</sub> in an active form of pyridoxal phosphate, not as pyridoxine, suggesting that pyridoxal phosphate might contribute to facilitate the intrahepatic metabolism of polyunsaturated fatty acids<sup>9)</sup>.

(5). The influences of indigestible and less utilizable carbohydrates.

We have already mentioned that the most common dietary carbohydrates in the older times in Japan when cholesterol cholelithiasis was rare consisted chiefly of unpurified carbohydrates containing indigestible and less absorbable constituents and vegetable fibers. In recent years, on the contrary, cholesterol cholelithiasis has increased in our country in parallel with the decreased consumption of such indigestible carbohydrates and fibers.

When weanling hamsters reared on a basic fat-free lithogenic diet containing glucose or sucrose as carbohydrate source, to which carboxyl-methyl-cellulose (CMC), agar-agar or beta-starch was supplemented, the rate of formation of cholesterol gallstones was reduced

remarkably, as compared with that of controls receiving a basic fat-free lithogenic diet alone<sup>9)</sup>. When alpha- or beta-starch was substituted for glucose or sucrose in the lithogenic diet, cholesterol gallstones were not produced<sup>38)</sup>. When animals were confined in cages in which straw was put on their screen bottoms, the development of cholesterol gallstones decreased, even though they were reared on the same lithogenic diet<sup>43)</sup>. These observations demonstrate clearly that vegetable fibers and/or carbohydrates containing indigestible and less absorbable components appeared to be contributory in inhibiting the intestinal re-absorption of lithocholic acid which was known to impede the intrahepatic metabolism of bile acids<sup>5)</sup>.

(6). Experimental production of pure cholesterol gallstones by natural diet.

Human beings are used to obtain their necessary calories from natural food stuffs. We are taking suitable amount of fat. Sugar is not always the main source of carbohydrate in our daily life. No matter how easily pure cholesterol gallstones would be produced in animals by feeding them a fat-free diet containing glucose or sucrose as the main source of carbohydrate, they should not show unusual general symptoms, as mentioned elsewhere, which are extremely different from the clinical symptoms of patients with cholesterol cholelithiasis. If we would expect experimental animals to be a completely ideal model for clinical cholesterol cholelithiasis, the diet which is given to the animals should bear a close resemblance to the human diet ; it must contain fat and alpha-starch should be used as the main source of carbohydrate. Although the animals receiving such a lithogenic diet should have quite similar general symptoms to those of patients with cholesterol cholelithiasis, cholesterol gallstones must be produced in their gallbladders at the same time.

In our daily life, we are taking starch as the main source of carbohydrate after changing crude beta-starch to alpha-starch by cooking, making it more digestible than the former. We prepared a fat-free diet in which alpha-starch processed from crude beta-starch was selected as the carbohydrate source and administered it to weanling hamsters. Pure cholesterol gallstones, however, were not produced by rearing them on this diet alone in much the same way as in the animals receiving a fat-free diet containing beta-starch as the main source of carbohydrate. When we reared weanling hamsters on the same lithogenic diet containing alpha-starch as carbohydrate by supplementing it with natural animal fat, such as butter fat consisting of short- or medium-chain saturated fatty acids, pure cholesterol gallstones were produced in their gallbladders. Such unusual general symptoms were not observed as we encountered frequently in feeding animals on a lithogenic fat-free diet containing glucose or sucrose as the main source of carbohydrate and the majority of animals receiving this new lithogenic diet survived well at the same time.

Scanning electron microscopic examination revealed that pure cholesterol gallstones which were thus produced in the animals had the same ultramicrostructures as those of human cholesterol stones<sup>26)</sup>, as shown in Fig. 5.

By referring to the above-mentioned experimental results, we elaborated a synthetic



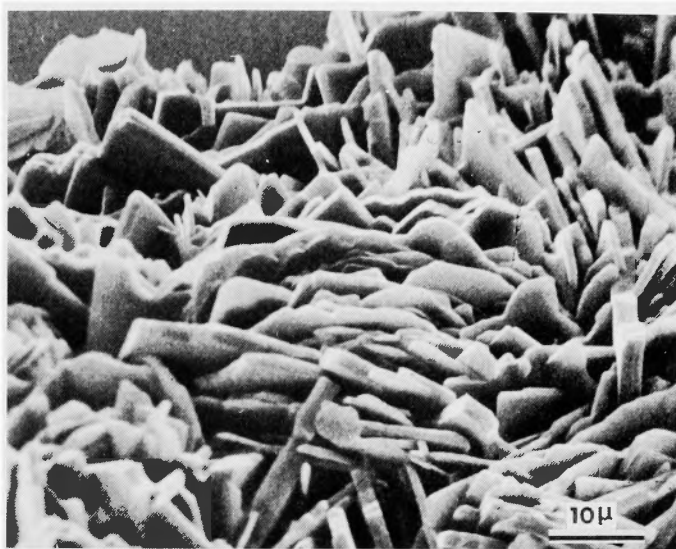


Fig. 5. Scanning electron micrograph of experimental pure cholesterol gallstone in golden hamsters.

lithogenic diet facilitating the development of pure cholesterol gallstones without unusual general symptoms. This regimen has been prevailing abroad now for experimental production of pure cholesterol gallstones<sup>47</sup>.

(7). Criticism on experimental cholesterol cholelithiasis from the view point of syntropism.

It has long been indicated clinically that there is a relationship of syntropism between cholesterol cholelithiasis and diabetes mellitus as well as acute pancreatitis. It is essentially important for us to select an ideal animal model for experimental production of pure cholesterol gallstones in order that we should investigate whether animals would show abnormal carbohydrate metabolism or not, or whether they might have predisposition to acute pancreatitis or not. It should be emphasized that such animal models are not necessary ideal unless they would have a relationship of syntropism to abnormal carbohydrate metabolism or to acute pancreatitis<sup>39</sup>.

In patients with cholesterol cholelithiasis the glucose tolerance test revealed that the majority of them had abnormal carbohydrate metabolism, such as diabetic, possible diabetic and oxyhyperglycemia<sup>36</sup>. The animals in which cholesterol gallstones were produced by the above mentioned means showed also a similar abnormal carbohydrate metabolism to that of patients with cholesterol cholelithiasis. In vitro permeability tests on the membranes of pancreatic acinar cells revealed that the resistability of the cellular membranes against trypsin and bile was reduced remarkably in the animals with cholesterol gallstones<sup>48</sup> as compared with that of controls in much the same way as in patients with cholesterol cholelithiasis<sup>16</sup>. This indicates that both animals and patients with cholesterol cholelithiasis had a definite predisposition to pancreatitis. The most interesting thing is that this predispo-

sition is not always irreversible since it can be restored by altering the dietary composition<sup>41)</sup>.

When a minor additional condition (a factor inducing acute pancreatitis) producing only a very slight macroscopic edema in the healthy controls was applied to the animals receiving a lithogenic diet, the latter showed the typical microscopic and macroscopic changes of acute pancreatitis, such as accumulation of ascites, hemorrhage and necrosis, while they showed higher values of amylase in serum, urine and ascites and their intraperitoneal depot fat revealed higher lipolytic activity than did those of the controls<sup>32)</sup>. These observations suggest that acute pancreatitis occurring frequently among patients with cholesterol cholelithiasis can be initiated by the same mechanism as that of cholesterol cholelithiasis. Taking into consideration the fact that the cellular membrane consists of lipoprotein complex, it seems most likely that a deficiency of essential fatty acids and/or their impaired metabolisms are responsible for the above mentioned phenomena. In fact, we have found that the reduced permeability of cell membranes of pancreatic acinar cells returned to normal and cholesterol gallstones would never be formed at the same time when we switched from a lithogenic and pancreatitis-inducing diet to a normal one. Increasing morbidity of cholesterol cholelithiasis and acute pancreatitis is predicted in Japan<sup>42)</sup>, so that an epidemiological survey should be performed to prove that our anticipations are true.

#### *IV. Experimental production of mixed and combination cholesterol gallstones.*

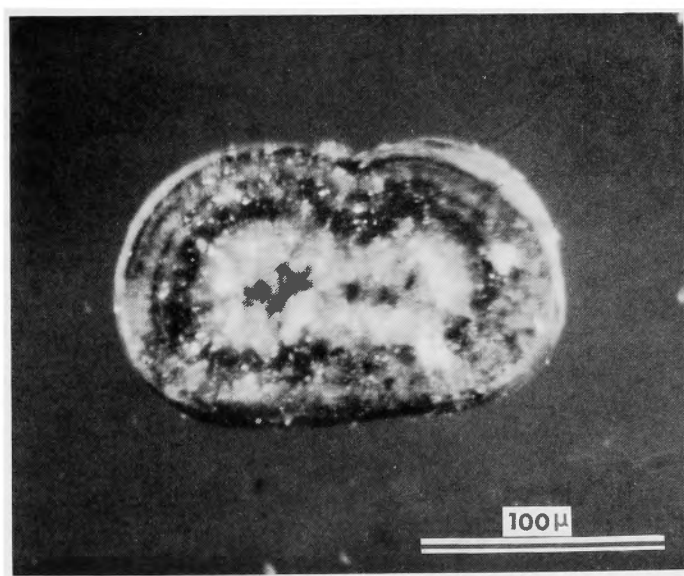
Classification of cholesterol gallstones is variable. The pure cholesterol stone contains more than 95 per cent cholesterol and a trace amount of bilirubin with sections showing a radiating array of cholesterol crystals. There are also mixed and combination stones.

Cholesterol gallstones which were produced in weanling hamsters were pure cholesterol stones. However, majority of cholesterol gallstones which we have encountered clinically recently in Japan are mixed stones, i. e. Cholesterin-Pigment-Kalkstein according to a german classification<sup>22)</sup>. The mixed stones consist of more than 70 per cent cholesterol and of bilirubin and calcium; the latter two substances, in particular bilirubin, renders a yellowish, brownish or greenish color to them. Their sections always reveal cholesterol crystals. It is interesting to mention here that microorganisms are detected more frequently from the bile of patients with this mixed type of stone than in that of other cholesterol gallstones<sup>17)</sup>.

As mentioned earlier, Japanese were accustomed to taking a diet consisting of large amounts of crude alpha-starch and a very small amount of fat and protein. We prepared, therefore, another low protein and high carbohydrate intake by modifying Tanimura's synthetic lithogenic diet to rear weanling hamsters<sup>40)</sup> as shown in Table 1. The percentage of glucose and casein was reduced but CMC was increased, while alpha-starch was substituted for the reduced amount of glucose. The animals produced, as we expected, mixed or combination stones. By examining their appearance and sections we ascertained that they belonged to the category of mixed or combination stones as shown in Fig. 6. Then we investigated whether such mixed or combination stones as were thus produced

**Table 1.** Composition of Experimental Lithogenic Diets

|              | Lithogenic Diet                     |       |                                          |               | Chow Diet          |
|--------------|-------------------------------------|-------|------------------------------------------|---------------|--------------------|
|              | Pure Chol. Stone<br>(Tanimura 1965) |       | Mixed Stone<br>(Tanimura, Setoyama 1975) |               |                    |
| Carbohydrate | glucose                             | 60.0% | glucose<br>$\alpha$ -starch              | 50.0%<br>20.0 | wheat & corn 59.3% |
| Protein      | casein                              | 20.0  | casein                                   | 8.5           | crude casein 22.6  |
| Fat          | butter fat                          | 10.0  | sesame oil                               | 5.0           | vegetable oil 0.4  |
| Vitamins     |                                     | 1.5   |                                          | 1.5           | 0.4                |
| Minerals     |                                     | 5.0   |                                          | 5.0           | 6.8                |
| Cellulose    |                                     | 3.5   |                                          | 10.0          | 4.5                |

**Fig. 6.** An experimental combination gallstone in golden hamsters.

were identical with those of human subjects or not by the scanning electron microscopic and crystallographic analyses<sup>28)</sup>. The mixed stones which were newly produced in weanling hamsters showed features quite different from those of pure cholesterol stones consisting of laminar or columnar cholesterol crystals alone. The majority of them consisted partly of laminar or columnar cholesterol crystals and partly of hemispherical granular structures. Scanning electron microscopical examination revealed that they had the same ultramicrostructures as those of human mixed stones, as shown in Fig. 7. We verified that these hemispherical structures were identical to those of the chemical compounds of bilirubin and calcium. In this connection, we also ascertained that the hemispherical granular

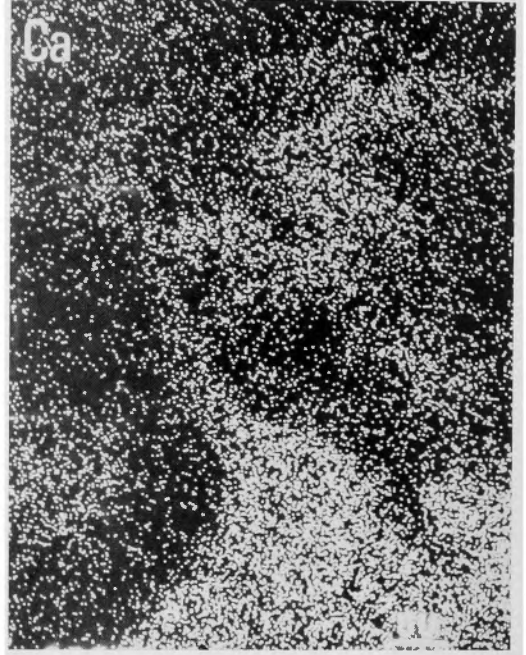
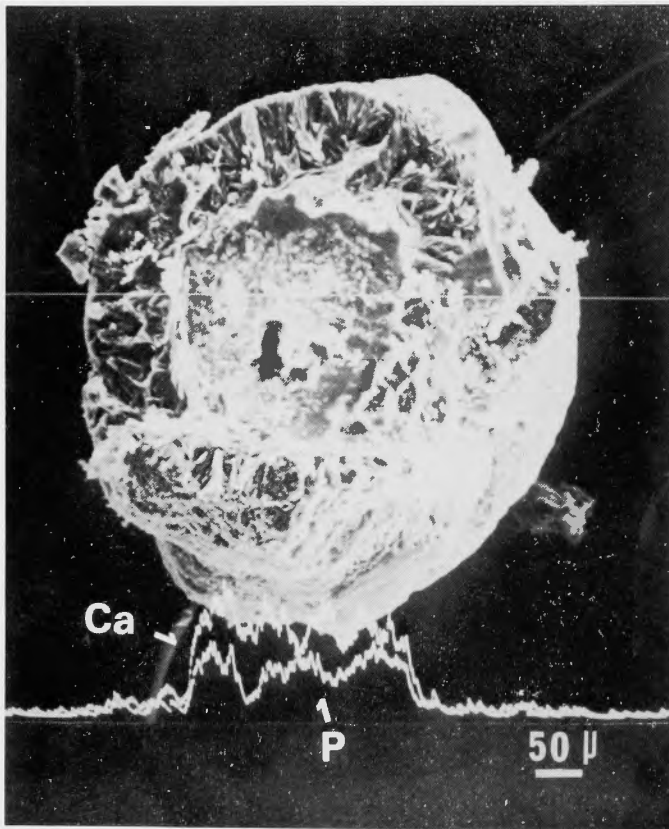


Fig. 7. Scanning electron micrograph of experimental mixed gallstone in golden hamsters and calcium (Ca.) distribution in 2-dimensional X-ray microanalysis of the same area.



←Scanning line

Fig. 8. Scanning electron micrograph of experimental combination gallstones in golden hamsters and distribution of calcium(Ca.) and phosphorus (P.) in line X-ray microanalysis on the scanning line.

structures were entirely different from those compounds made between bilirubin and iron, copper or other metallic elements. Two dimensional analysis of the sections of the mixed stones by the scanning electron microscope provided with an X-ray microanalyzer revealed that calcium was a major component and bilirubin was another one of the hemispherical granular structures, demonstrating that these structures were definitely composed of calcium bilirubinate as shown in Fig. 8. We have succeeded in producing the mixed stones in weanling hamsters by rearing them on the new diet. We would like, therefore, to designate the diet which facilitated the development of mixed stones in weanling hamsters as a synthetic lithogenic diet for production of experimental mixed stones.

As mentioned above, it is generally accepted that the bile specimens obtained from patients with mixed gallstones show the highest detection of microorganisms among those with cholesterol cholelithiasis. This suggests, therefore, the possibility that the bacterial beta-glucuronidase is playing a major role in the development of mixed stones in weanling hamsters. On the contrary, when we cultured the bile specimens from the weanling hamsters surviving 40 days after the initiation of mixed stones, no microorganism was detected, denying the possibility of hydrolysis of bilirubin glucuronide by bacterial beta-glucuronidase<sup>13</sup>). On the other hand, we have proven that beta-glucuronidase which was detected in the bile of hamsters with mixed gallstones was not derived from a bacterial origin but from the organ per se, by determining the pH profile and  $K_m$  values of beta-glucuronidase as shown in Fig. 9. Meanwhile, it took from 7 to 20 days till the mixed stones were formed in their gallbladders when weanling hamsters were reared on the mixed-stone-producing diet. Although once mixed stones were formed and they began to grow, we found that the daily activity of organ-derived beta-glucuronidase in the bile specimen obtained from the animals receiving the mixed-stone-producing diet did not show any striking difference from that of the controls during the period of formation of mixed

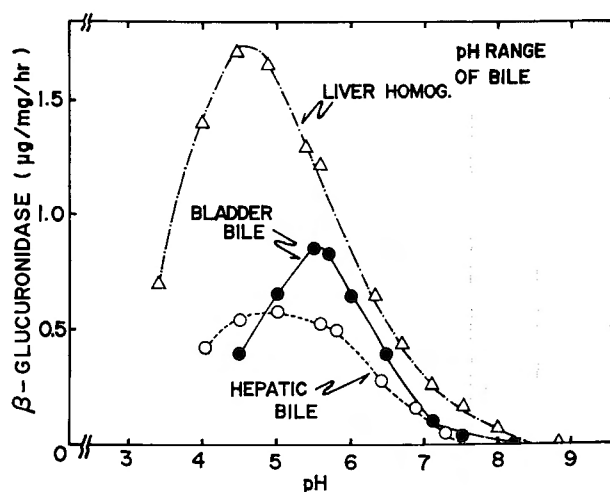


Fig. 9. PH profile of  $\beta$ -glucuronidase in bile of hamsters.

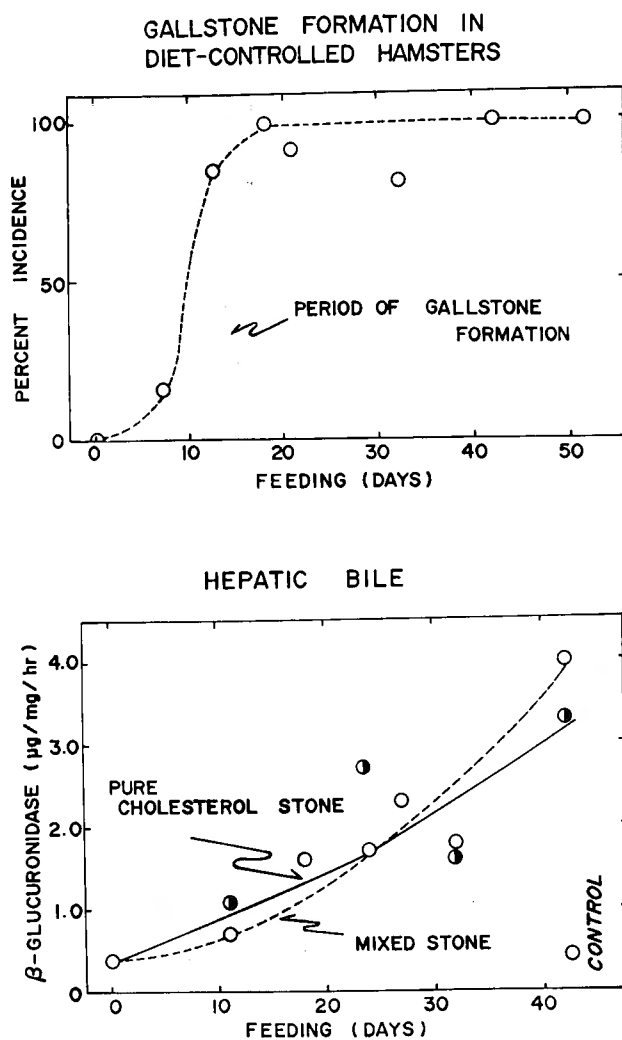


Fig 10. Glucuronidase and formation of mixed stones in hamsters.

cholesterol gallstones as shown in Fig. 10. This suggests that precipitation of bilirubin occurring in the process of development of the mixed stones is not caused by deconjugation of bilirubin glucuronide which was believed in the past to be brought about by the action of bacterial beta-glucuronidase. We have reached, therefore, the conclusion that bacterial infection of bile in patients with mixed stones, though occurring at a considerably higher rate, was merely the secondary phenomenon accompanied with the development of mixed stones.

Combination stones resembling those in humans were produced by chance in young hamsters when they were reared on the same mixed-stone-producing diet. The fact that young hamsters produced mixed or combination types of cholesterol gallstones according to

time and circumstance, even though they were kept on the same condition, suggests that the hourly or daily changes of biliary composition may be contributory to it. However, we cannot help assuming that both types of gallstones were initiated by the same pathogenetic factors. In this connection, we examined a combination stone which was produced in the young hamster's gallbladder. It consisted of hemispherical granular structures inside and of cholesterol crystals outside. Examinations by the scanning electron microscope with an X-ray microanalyzer detected calcium and phosphorus in the hemispherical granular structures (line analysis), demonstrating that the structures contained calcium bilirubinate and phosphate, as shown in Fig. 8. We have furthered our ultramicrostructural and microphysicochemical analyses to get the results that calcium bilirubinate was a major component of these granular structures and the experimentally produced combination stones were identical to those of humans. Meanwhile, point analysis also showed that inorganic substances of the granular structures were calcium and phosphorus. We have also found formation of another type of combination stones in the experimental animals and human subjects as well. It contained cholesterol crystals interiorly as a nucleus and hemispherical granular structures covering it as an outer shell.

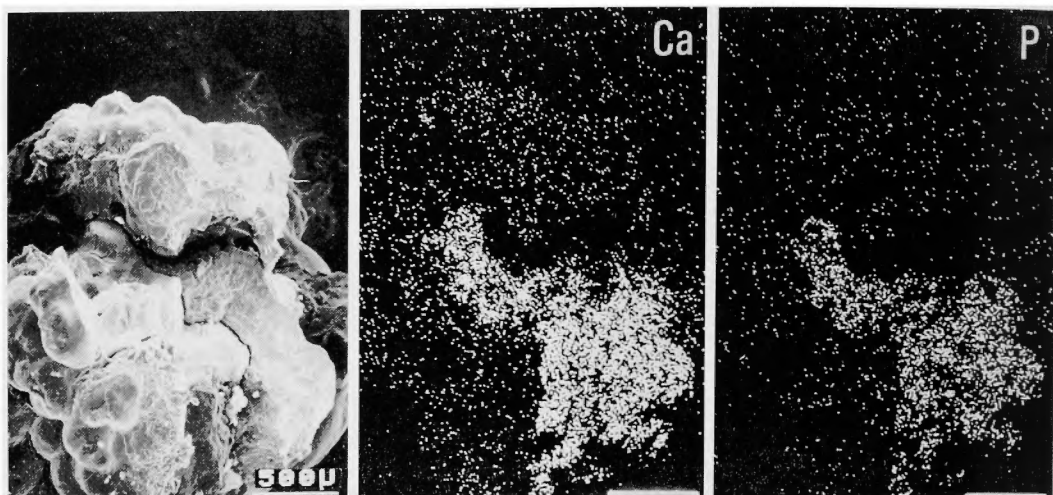
#### *V. Experimental production of black pigment stones.*

The characteristics of human pure pigment stones are that they should be black in color and contain abundant inorganic substances. Although it has been reported that the majority of biliary calculi which were formed in patients with an abnormal metabolism of bilirubin, such as hemolytic anemia, malaria, etc., pigment stones are not always encountered in these diseases alone.

The reason why we have dared to mention the pathogenesis of pigment stones is derived from the following experimental observation that when aged hamsters were reared on a lithogenic diet prepared for production of pure cholesterol gallstones 20 per cent of them developed black stones while 60 per cent produced pure cholesterol gallstones.

The composition of lithogenic diet initiating pure cholesterol gallstones is assumed to be quite similar to that of the ordinary diet of Europeans and Americans. Meantimes, SOLOWAY, TROTMAN, et al. reported that pigment stones were found at the rate of 27 per cent of all the patients with biliary calculi recently in America, commenting that their incidence rate was not so low as reported in the past and they occurred chiefly in aged patients. It is interesting to speculate a possible connection between our assumption and their report<sup>31) 44) 46)</sup>. It is unknown, however, whether they designated their pigment stones as pure pigment stones, whether they classified calcium bilirubinate stones as pigment stones, or whether they called both types of colored stones pigment stones.

Scanning electron microscopic examinations of the pigment stones which were produced in the aged hamsters receiving the lithogenic diet prepared for pure cholesterol gallstone formation revealed that they were amorphous as those in humans. Simultaneous two dimensional analysis using an X-ray microanalyzer attached to the electron microscope



**Fig. 11.** Scanning electron micrograph of human black stone and distribution of calcium (Ca.) and phosphorus (P.) in 2 dimensional X-ray microanalysis of the same area.

demonstrated that they were composed chiefly of calcium and phosphorus as shown in Fig. 11, proving that the distribution of each element was identical<sup>27)</sup>. On the other hand, some pigment stones consisting of calcium phosphate and carbonate were discovered. The latter showed a dense distribution of calcium alone on two dimensional analysis. This suggests that such pigment stones may also be produced in hamsters under the same conditions except for aging of the animals. It is easily assumed in the light of the above-mentioned experimental observations that the incidence of such pigment stones will increase among people of the younger generation eating a diet resembling that of Europeans and Americans in the future when they grow old. We should like to expect, therefore, a future epidemiological survey in this respect.

### Conclusion

We have reached the following conclusions through trials on experimental production of various types of biliary calculi by making use of hamsters as experimental animals.

- 1) There are three major factors in relation to the formation of pure cholesterol gallstones. Although they are produced by interrelated actions of these factors, in brief, they are formed by dietary origin. The followings are the major factors ; (1) an excessive intake of purified and highly absorbable carbohydrate, such as sugar, (2) a large intake of animal fats, such as butter fat and other milk products, and (3) a small intake of indigestible, less absorbable and unpurified carbohydrate and vegetable fibers containing a lot of residue.
- 2) When a low protein diet is added to the conditions producing pure cholesterol gallstones, mixed or combination stones are formed.
- 3) When another condition of aging of the animals is added to the pure cholesterol gallstone producing conditions, black pigment stones are produced.



- 4) All the experimentally produced gallstones have been examined in comparing them to human counterparts by means of ultrastructural and physicochemical analyses.
- 5) Various types of human biliary calculi are produced by the same mechanism that we have here explored in hamsters.

This research was supported by National Grant in aid for Scientific Research No. 244050, 1977.

Parts of this paper were presented at 19th Autumnal Meeting of Japanese Gastroenterology (1977) and at 6th World Congress of Gastroenterology (Madrid, 1978)

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## 和文抄録

## コレステロール系胆石の成因について

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われわれは試獣としてハムスターを使用することによって各種の実験的胆石の作製実験を試み、次のような結論に到達した。

(1) 実験的純コレステロール胆石の成因としては次のような三つの要因が与っており、それらの要因が相俟って純コレステロール胆石は形成される。すなわち、純コレステロール胆石は純食餌性に形成されるものといえる。その要因としては、(1)精製された高利用性の糖質である砂糖の大量摂取、(2)バター脂の如き乳製品をはじめとする動物性脂質の大量摂取、(3)不消化な非利用性の成分（残渣）をも含む未糖製の糖質や植物繊維摂取量の減少、を挙げることが出来る。

(2) 純コレステロール胆石を生ずる条件に低蛋白なる条件が付与されるとそこに純食餌性に混合石あるいは混成石が実験的に形成され得る。

(3) 純コレステロール胆石を生ずる条件に老齢化する条件が付与されると黒色素石が実験的に形成され得る。

(4) 此処に実験的に作製し得られた胆石はそのいずれもが微細構造学的、化学成分的にみて、人体のそれに相当するものであることが確認された。

(5) 従って、人体におけるそれら各種胆石もハムスターによって確認されたと同じ要因によって形成されるに至るものと思われる。