Stimulative Effect of Dietary Glucose on Hepatic Cholesterol Biosynthesis and Formation of Cholesterol Gallstones in Hamsters

by

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INTRODUCTION

DAM and his co-workers reported a high incidence of cholesterol gallstones in hamsters maintained on a fat-free and high-glucose diet[1]. The stones were not produced when glucose was replaced by starch or when unsaturated fatty acids were added to the diet[2)3)]. HIKASA and his collaborators[4] also found that cholesterol stones were formed in the gallbladders of hamsters fed on a high-glucose diet deficient in essential fatty acids or on a high-glucose diet supplemented with animal oil such as butter. Extending these observations, the present study was undertaken to explore the correlation between cholesterol metabolism in the liver and the formation of cholesterol gallstones in hamsters. Experiments to be described here show that there is a close correlation between increased biosynthetic activity of cholesterol in the liver and the formation of cholesterol gallstones. Furthermore, compared with starch, glucose feeding markedly enhances both the incidence of cholesterol gallstones and the activity of cholesterol synthesis from acetate as assayed with liver slices, as well as homogenates. The high activity of cholesterol synthesis appeared to be due not to the increased level of NADPH but mainly to the increase of enzymic activities before mevalonate in the biosynthetic pathway of cholesterol.

MATERIALS AND METHODS

Materials. Golden hamsters were obtained from our stock colony. Rat chow (CLEA chow) was purchased from Central Laboratories of Experimental Animals, Tokyo. Ethyl linoleate was obtained from Ono Pharmaceutical Co., Japan, and ethyl palmitate, from Tokyo Chemical Industry Co. Casein (vitamin-free) and crystalline tomatine were purchased from Nutritional Biochemical Corporation ; acetate-2-^{14}C and cholesterol-4-^{14}C, from the Radiochemical Centre, Amersham ; mevalonate-2-^{14}C, from California Biochemical Research Corporation ; and ATP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and NADP, from Sigma Chemical Company. All other chemicals were of reagent grade.

Treatment of animals. Golden hamsters, ranging in body weight from 40 to 60 g, were divided into three groups and were maintained for 28 days *ad libitum* on the following diets. The first group received a fat-free diet containing glucose, 73.5 % ; casein,

20.0 %; salt mixture*, 5.0 %; vitamin mixture**, 1.0 %; and choline chloride, 0.5 % ("glucose diet"). The second group received the same fat-free diet except that glucose was replaced by potato starch ("starch diet"). The third group received a commercially available rat chow which contained wheat and corn, 60%; fat, 4%; crude casein, 24%; cellulose, 4%; and mixtures of vitamins and salts, 8 %. In another set of experiments, ethyl linoleate or ethyl palmitate (5%) was added to the glucose diet. The average body weights after the feeding period of 28 days were 57 g in the glucose diet group, 69 g in the starch diet group, and 75 g in the rat chow group.

Incubation procedures. Animals were sacrificed by decapitation and the livers were quickly removed and chilled in an ice-cold Krebs-Ringer phosphate buffer, pH 7.4. Liver slices of approximately 0.5 mm in thickness were prepared at 4° with a thin razor blade. The slices (500 mg) were incubated in a 50 ml flask containing 5 ml of Krebs-Ringer phosphate buffer, pH 7.4, and either 6 μmoles of sodium acetate-2-^{14}C (1.6×10^6 cpm) or 0.2 μmole of mevalonate-2-^{14}C (1.8×10^5 cpm). Incubation was carried out for 2 hours at 37° with shaking under oxygen. Liver homogenates were prepared by the method of BUCHER[5] and were centrifuged at 800 g for 10 minutes. A 2 ml aliquot of the supernatant fraction was incubated in a Warburg flask containing 1.2 μmoles of sodium acetate-2-^{14}C (2.3×10^6 cpm), 1.0 μmole of $MgCl_2$, and 1.8 μmoles of ATP in a final volume of 2.5 ml. As an NADPH generating system, 20.0 μmoles of glucose 6-phosphate, 2.0 μmoles of NADP, and 0.04 unit of glucose 6-phosphate dehydrogenase were added to the reaction mixture. Incubation was carried out for 2 hours at 37° with shaking.

Estimation of cholesterol formation. The reaction was terminated by the addition of 0.05 ml of 10 *N* H_2SO_4 and the $^{14}CO_2$ evolved was trapped with a 1 *M* Hyamine 10-X solution. Cholesterol fraction was extracted and saponified by the method of KABARA[6]. The total cholesterol was isolated as a tomatinide complex, and its radioactivity was determined with a Tri-Carb liquid scintillation spectrometer[6]. Radioactive cholesterol thus obtained was identified by thin-layer chromatography (petroleum ether : ether : acetic acid = 74 : 15 : 1)[7].

Analysis of bile acid formation. Radioactive bile acid produced under the experimental conditions described above was extracted by the method of MOSBACH et al.[8], and was identified by paper chromatography (*iso*-amyl acetate : *n*-heptane : acetic acid = 3 : 5 : 0.8).

Liver cholesterol level. Total cholesterol from 0.5 g of fresh tissue was isolated as a tomatinide complex[6], and was determined by a modification of the LIEBERMANN-BURCHARD reaction[9].

Examination of cholesterol gallstone formation. The formation of cholesterol gallstones was examined macroscopically. Any visible stone, no matter how small or how few, was registered as a positive test. The stones were analyzed for cholesterol by a modification of the LIEBERMANN-BURCHARD reaction[9].

Collection of bile and determination of radioactivity of biliary cholesterol following intravenous administration of cholesterol-4-^{14}C. Animals were maintained on each diet

* The salt mixture contained NaCl, 4.62%; $MgSO_4$, 7.10%; $NaH_2PO_4\cdot2H_2O$, 9.23%; K_2HPO_4, 25.44%; $CaH_4(PO_4)_2\cdot H_2O$, 14.40%; Fe-citrate, 3.15%; Ca-lactate, 34.67%; and KI, 1.39%.

** One g of the vitamin mixture contained vitamin A, 2,500 I. U.; thiamine, 1.0 mg; riboflavin, 1.5 mg; pyridoxine, 1.0 mg; folic acid, 0.5 mg; vitamin B_{12}, 1.0 μg; ascorbic acid, 37.5 mg; vitamin D, 200 I. U.; tocopherol, 1.0 mg; niacin, 10.0 mg; pantothenic acid, 5.0 mg; and inositol, 10.0 mg.

as indicated for 28 days. The common bile duct was ligated, the gallbladder was removed, and the hepatic duct was cannulated with a fine polyethylene tube under nembutal anesthesia. Bile was collected for the 24 hours following intravenous administration of 5 μmoles of cholesterol-4-^{14}C (8×10^5 cpm) dissolved in saline with the aid of Tween-20. After mounting an aliquot of bile directly on the planchet, the radioactivity recovered in bile was assayed by counting the dried sample with a Nuclear Chicago gas-flow counter. Cholesterol was extracted from bile with petroleum ether as described by SIPERSTEIN et al.[10], and its radioactivity was assayed with a gas-flow counter.

Estimation of glucose 6-phosphate (and 6-phosphogluconate) dehydrogenase activity in liver. Animals were maintained on each diet as indicated for 28 days and were sacrificed by decapitation. The livers were quickly removed and were homogenized in a Potter-Elvehjem type homogenizer with 5 volumes of ice-cold water for a minute. The resultant supernatant fluids after centrifugation of the liver homogenates at 105,000 g for 30 minutes at 4° were used for the assay of enzyme activity. The assay system consisted of 0.05 ml of liver supernatant, 0.4 ml of 0.1 *M* $MgCl_2$, 0.5 ml of 0.25 *M* glycylglycine buffer, pH 7.5, 0.2 ml of 1.5×10^{-3} *M* NADP, and 0.1 ml of 0.02 *M* glucose 6-phosphate in a total volume of 3.0 ml[11]. The activity was measured spectrophotometrically by following the initial rate of change in absorbance at 340 mμ with a Cary recording spectrophotometer.

RESULTS

Effect of diet on the formation of cholesterol gallstones. In confirmation of the results obtained by DAM and his co-workers, a high incidence (about 85%) of gallstones was found in animals fed on the glucose diet, whereas no gallstone was formed in those animals fed on either the starch diet or rat chow as shown in Table 1, Experiment 1. The gallstones found within gallbladders were usually white or yellow and round or mulberry-shaped. Chemical analysis revealed that the stones were composed mainly of cholesterol (50-75%). The addition of ethyl linoleate to the glucose diet completely prevented the formation of cholesterol gallstones as shown in Table 1, Experiment 2, although in some few cases dark brown stones were produced. These dark brown stones, designated as pigmented stones, contained almost no cholesterol. The addition of ethyl palmitate to

Table 1 Incidence of cholesterol gallstones and formation of cholesterol from acetate-2-^{14}C in liver slices of hamster

Incubation mixture contained 500 mg of liver slices, 6 μmoles of acetate-2-^{14}C (1.6×10^6 cpm) and 5 ml of Krebs-Ringer phosphate buffer, pH 7.4. Incubation was carried out for 2 hours at 37° with shaking under oxygen. Numbers of animals examined are shown in parentheses.

Diet		No. of animals with stones	Radioactivity recovered as cholesterol	
			(cpm)	(%)[a]
Expt. 1	Glucose diet	43 (50)	25,000± 5,000[b]	1.56 (12)
	Starch diet	0 (42)	6,700± 900	0.41 (8)
	Rat chow	0 (36)	1,400± 400	0.08 (5)
Expt. 2	Glucose diet	7 (7)	38,600± 2,200	2.41 (7)
	Glucose diet + linoleate	0 (25)	10,100± 2,400	0.63 (9)
	Glucose diet + palmitate	12 (14)	35,700±10,300	2.23 (7)

[a] : Percentage of radioactivity recovered as cholesterol. [b] : Standard error of mean.

the glucose diet did not prevent the formation of cholesterol gallstones, and 85% of animals fed on this diet developed cholesterol gallstones. The cholesterol gallstones, observed in animals on the glucose diet supplemented with ethyl palmitate, were similar to those observed in animals on the glucose diet. No significant difference was observed between the two sexes with respect to the incidence of cholesterol gallstones. Thus the type of both dietary carbohydrate and lipid strongly influenced the formation of cholesterol gallstones. The results indicated that in hamsters fed on the fat-free diet, an easily absorbable dietary carbohydrate such as glucose enhanced the formation of cholesterol gallstones, whereas a complex carbohydrate such as starch did not. Furthermore, in contrast with a saturated fatty acid such as ethyl palmitate, an unsaturated fatty acid such as ethyl linoleate prevented the formation of cholesterol gallstones even when dietary carbohydrate was supplied in the form of easily absorbable glucose.

Cholesterol synthesis from acetate. In order to elucidate the relationship between the metabolism of liver cholesterol and the formation of cholesterol gallstones, the activity of cholesterol synthesis from acetate was examined with liver slices obtained from animals on various diets. The activity of cholesterol synthesis from acetate in liver slices under the experimental conditions was linear with the incubation time as shown in Fig. 1. The cholesterol thus obtained was identified by thin-layer chromatography as shown in Fig. 2. As shown in Table 1, Experiment 1, when the glucose diet was given, the radioactivity of acetate-2 ^{14}C incorporated into cholesterol was about 18-fold that of animals fed on

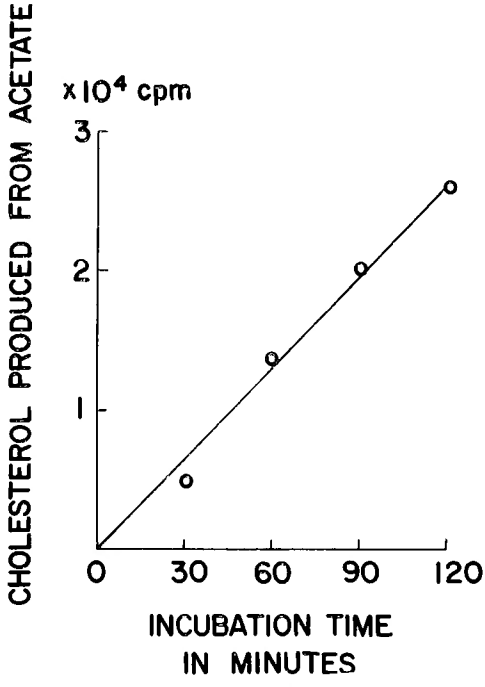

Fig. 1 Time course of the synthetic activity of cholesterol from acetate in liver slices of hamsters. The activity was assayed as described in the text. Liver slices were obtained from hamsters maintained on the glucose diet for 28 days.

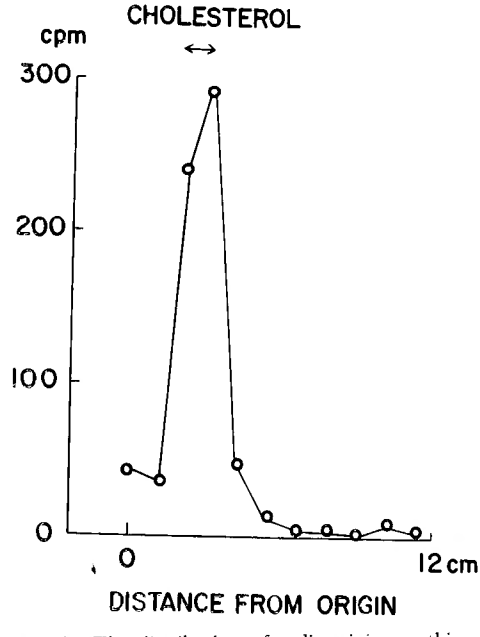

Fig. 2 The distribution of radioactivity in thin-layer chromatography of tomatine precipitated sterols. Solvent system was petroleum ether : ether : acetic acid = 74 : 15 : 1. Silica Gel G was used as an adsorbent.

rat chow. When glucose was replaced by potato starch, however, the rate of incorporation was only about 5-fold that of the animals fed on rat chow. Even if dietary carbohydrate was supplied in the form of glucose, the addition of ethyl linoleate to the glucose diet completely counteracted the effect of the glucose diet in stimulating the incorporation of acetate-2-^{14}C into cholesterol (Table 1, Experiment 2). The addition of ethyl palmitate to the glucose diet did not counteract the glucose diet in stimulating the incorporation of acetate into cholesterol, and showed almost the same activity as the glucose diet alone. The formation of radioactive bile acid was almost negligible under the experimental conditions employed. No significant difference was observed in the formation of $^{14}CO_2$ from acetate under these dietary conditions. Thus, as in the formation of cholesterol gallstones, glucose in the fat-free diet enhanced the activity of cholesterol synthesis much more than did starch in the fat-free diet. Furthermore, in contrast with ethyl palmitate, ethyl linoleate counteracted the glucose effect in stimulating the synthesis of cholesterol from acetate, as in the formation of cholesterol gallstones. The results mentioned above strongly suggested a correlation between the increased activity of cholesterol synthesis in the liver and the formation of cholesterol gallstones. The effect of the duration of glucose feeding on the activity of cholesterol synthesis from acetate is shown in Fig. 3. The results indicate that in animals on a fat-free diet cholesterol synthesis from acetate is enhanced by dietary glucose more than by starch. This enhancement was observed after at least 4 days of administration of glucose and continued to increase during the 21 days on this diet.

Similar results were obtained with liver homogenates. As shown on the left column in Table 2, the incorporation of acetate-2-^{14}C into cholesterol was approximately 2-fold when starch was replaced by glucose. Although the addition of an NADPH generating system to the reaction mixture stimulated the incorporation of acetate-2-^{14}C into cholesterol, the incorporation in the glucose diet group was again 2-fold that in the starch diet group. Thus in liver homogenates obtained from animals fed on the fat-free diet, cholesterol synthesis from acetate was enhanced more by dietary glucose than by starch as was observed in liver slices. The results also suggested that the level of NADPH may not be a factor responsible for the difference in the activity of cholesterol synthesis from acetate between these dietary groups.

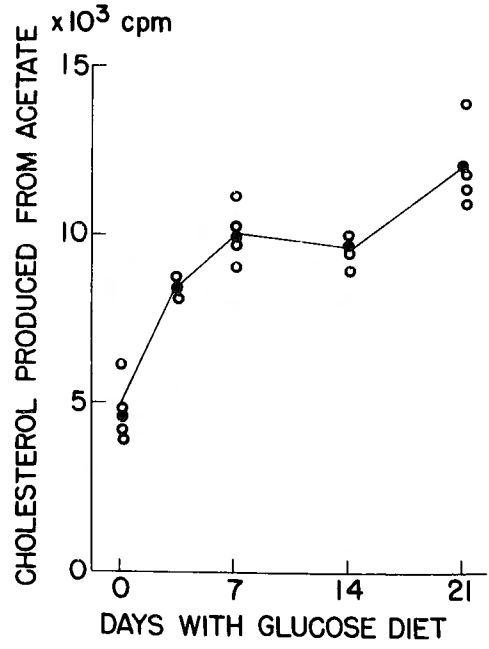

Fig. 3 The synthetic activity of cholesterol from acetate as a function of duration with the glucose diet. The activity was assayed with liver slices as described in the text. The animals were maintained on the starch diet and then transferred to the glucose diet. The total feeding period on these diets was 21 days for each animal. The abscissa represents the duration after substitution of the starch diet by the glucose diet. Open circles represent the experimental values and solid circles the average values.

Table 2 Effect of NADPH on incorporation of acetate-2-^{14}C into cholesterol in liver homogenates

Liver homogenates were prepared by the method of Bucher[5] and were centrifuged at 800 g for 10 minutes. A 2 ml aliquot of the supernatant fraction was incubated in a Warburg flask containing 1.2 μmoles of sodium acetate-2-^{14}C (2.3×10^6 cpm), 1.0 μmole of $MgCl_2$ and 1.8 μmoles of ATP in a final volume of 2.5 ml. As an NADPH generating system, 20.0 μmoles of glucose 6-phosphate, 2.0 μmoles of NADP and 0.04 unit of glucose 6-phosphate dehydrogenase were added to the reaction mixture. Incubation was carried out for 2 hours at 37° with shaking. Numbers of animals examined are shown in parentheses.

Diet	Cholesterol produced (cpm)	
	−NADPH	+NADPH
Glucose diet	1,640±160[a] (7)	2,610±630 (5)
Starch diet	850±110 (7)	1,160± 70 (5)

[a] : Standard error of mean.

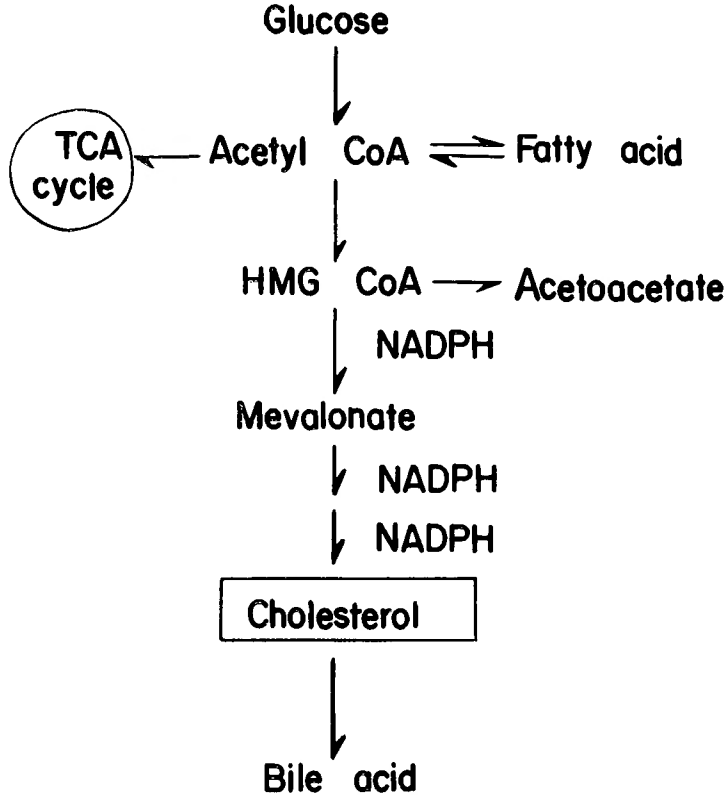

Fig. 4 A simplified diagram of the biosynthesis of cholesterol

Cholesterol synthesis from mevalonate. To obtain information on which steps of cholesterol synthesis are stimulated by dietary glucose as compared with starch, the incorporation of mevalonate-2-^{14}C into cholesterol was examined. The activity of cholesterol synthesis from mevalonate in liver slices under the experimental conditions was approximately linear with the incubation time as shown in Fig. 5. When mevalonate was employed as a substrate, the activity of cholesterol synthesis in the liver slices of animals fed on either

the glucose diet or the starch diet far exceeded that of animals fed on rat chow, as shown in Table 3. However, no marked difference in the activity of cholesterol synthesis from mevalonate was observed between animals on the glucose diet and those on the starch diet. Considering the fact that cholesterol synthesis from acetate in animals on the glucose diet far exceeded that of animals on the starch diet, the above results indicate that the enhanced activity of cholesterol synthesis from acetate in animals on the glucose diet is probably due to the increase of enzymic activities before mevalonate in the biosynthetic pathway of cholesterol.

Liver cholesterol level. The experiments with liver slices showed that the activity of cholesterol synthesis was affected by the various dietary conditions. For instance, it was enhanced by the glucose diet or by the glucose diet supplemented with ethyl palmitate. In order to elucidate whether excessively produced cholesterol accumulates in the liver or not, the level of liver cholestrol in these animals was examined. As shown in Table 4, the level of liver cholesterol was somewhat

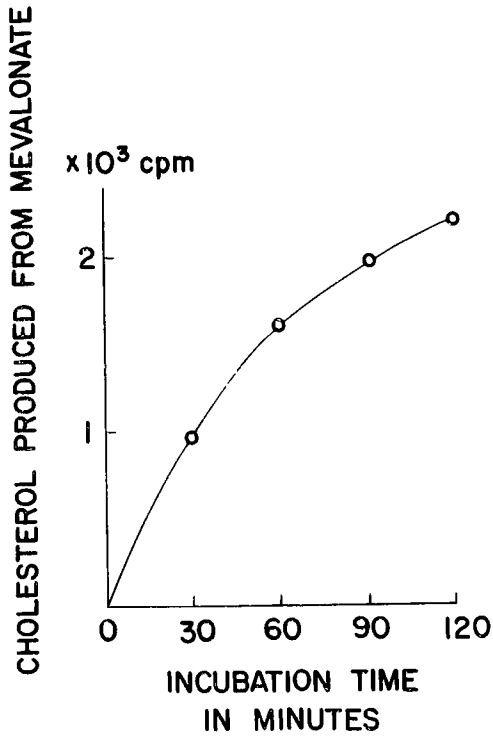

Fig. 5 Time course of the synthetic activity of cholesterol from mevalonate in liver slices of hamsters. The activity was assayed as described in the text. Liver slices were obtained from hamsters maintained on the glucose diet for 28 days.

Table 3 Incorporation of mevalonate-2-^{14}C into cholesterol in liver slices of hamster

Incubation mixture was the same as indicated in Table 1 except that 0.2 μmole of mevalonate-2-^{14}C (1.8×10^5 cpm) was employed as a substrate. Incubation was carried out for 2 hours at 37° with shaking under oxygen. Numbers of animals examined are shown in parentheses.

Diet	Radioactivity recovered as cholesterol (cpm)	(%)[a]
Glucose diet	1,600±200[b]	0.88 (3)
Starch diet	3,100± 30	1.72 (3)
Rat chow	170± 30	0.09 (3)

[a] : Percentage of radioactivity recovered as cholesterol. [b] : Standard error of mean.

Table 4 The level of liver cholesterol

Numbers of animals examined are shown in parentheses.

Diet		Liver weight (g)	Cholesterol level (mg/g liver)
Glucose diet	(20)	1.2±0.1[a]	2.53±0.3
Starch diet	(17)	1.1±0.2	2.10±0.1
Rat chow	(15)	1.3±0.2	2.30±0.0
Glucose diet + linoleate	(15)	1.3±0.1	2.30±0.3
Glucose diet + palmitate	(13)	1.3±0.1	2.43±0.2

[a] : Standard error of mean.

higher in animals fed on the glucose diet than in those on the other diets. However, no marked difference of the liver cholesterol level was observed among these dietary groups. It appears that excessively produced cholesterol does not accumulate in the liver under these experimental conditions.

Excretion into the bile of intravenously administered cholesterol. The excretion of intravenously injected cholesterol-4-^{14}C into bile was examined in hamsters fed on either the glucose diet or the starch diet. Fig. 6 shows the cumulative excretion of radioactivity into bile during 24 hours following the intravenous administration of cholesterol-4-^{14}C. About 5% of the administered dose was excreted into the bile. As shown in Table 5, no significant difference between these two dietary groups was observed in the volume of bile excreted during the 24 hours. Also no marked difference in the radioactivity of bile excreted was observed

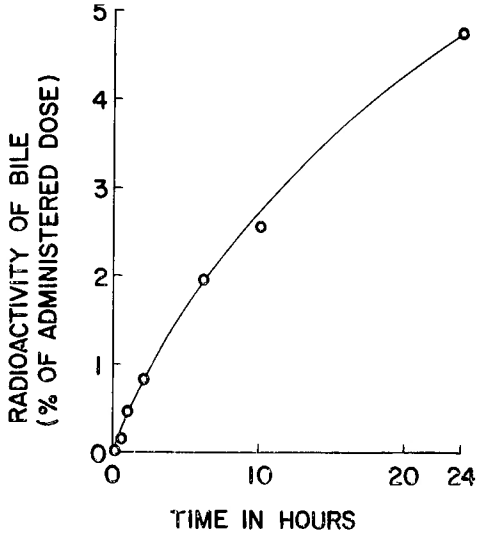

Fig. 6 Cumulative excretion of radioactivity into bile following intravenous injection of cholesterol-4-^{14}C in hamsters maintained on the glucose diet. Five μmoles of cholesterol-4-^{14}C (8×10^5 cpm) were injected intravenously. Bile was collected for 24 hours and its radioactivity was determined as described in the text.

Table 5 Elimination of intravenously administered cholesterol-4-^{14}C into the bile

Animals were maintained on each diet for 28 days as indicated. After the intravenous injection of 5 μmoles of cholesterol-4-^{14}C (8×10^5 cpm), bile was collected for 24 hours and the ratio of radioactivity of biliary cholesterol to total radioactivity of bile was determined as described in the text. Numbers of animals examined are shown in parentheses.

Diet	Volume of bile excreted (ml)	Total radioactivity of bile excreted (cpm)	$\frac{\text{Radioactivity of cholesterol in bile}}{\text{Total radioactivity of bile}}\times100$
Glucose diet	6.8±0.3[a]	43,520±3,800	12.8±1.5 (7)
Starch diet	7.1±0.3	40,280±5,800	9.8±1.1 (7)

[a] : Standard error of mean.

between these two dietary groups. When the radioactivity recovered as biliary cholesterol was compared, however, it was slightly higher in aninals on the glucose diet than in those on the starch diet. The ratio of radioactivity of biliary cholesterol to total radioactivity of bile was 12.8% in the glucose diet group and 9.8% in the starch diet group. Studies on the metabolism of cholesterol in animals with a bile fistula may not reflect the true metabolic changes which take place in normal animals, since the bile fistula completely interrupts the enterohepatic circulation of bile and excludes the modification of bile in the intestine by intestinal microorganisms. In addition, there are some questions concerning the exchange of intravenously administered cholesterol with metabolically active

cholesterol in such a short period as 24 hours. However it appeared from the present results that in animals on the glucose diet, cholesterol was more preferentially excreted into the bile than in animals on the starch diet.

Glucose 6-phosphate (and 6-phosphogluconate) dehydrogenase activity. It was suggested that an NADPH generating system through hexose monophosphate shunt may enhance the cholesterol synthesis in the liver[12]. In order to ascertain whether the difference in the activity of cholesterol synthesis, observed between the animals on the glucose diet and those on the starch diet, is due to changes in the level of NADPH which might be caused by different activities of glucose 6-phosphate (and 6-phosphogluconate) dehydrogenase, the activity of this NADPH generating enzyme was studied in the livers of animals fed on either the glucose diet or the starch diet. However, no appreciable difference in the activity of glucose 6-phosphate (and 6-phosphogluconate) dehydrogenase was observed between these dietary groups, as shown in Table 6.

Table 6 Activity of glucose 6-phosphate (and 6-phosphogluconate) dehydrogenase in the liver of hamster

The activity of glucose 6-phosphate (and 6-phosphogluconate) dehydrogenase was measured spectrophotometrically by following the initial rate of change in absorbance at 340 mμ with a Cary recording spectrophotometer under the following assay condition. The assay system consisted of 0.05 ml of liver supernatant, 0.4 ml of 0.1 *M* $MgCl_2$, 0.5 ml of 0.25 *M* glycylglycine buffer, pH 7.5, 0.2 ml of 1.5×10^{-3} *M* NADP, and 0.1 ml of 0.02 *M* glucose 6-phosphate in a total volume of 3.0 ml. The reaction was started by the addition of 0.05 ml of liver supernatant to the reaction mixture. A unit of enzyme activity was defined as the quantity of enzyme which, at 20° and at pH 7.5, changes in optical density of 1,000/minute, based on the readings over the first one minutes. Numbers of animals examined are shown in parentheses.

Diet	Activity of glucose 6-phosphate (and 6-phosphogluconate) dehydrogenase (unit per mg protein $\times 10^3$)
Glucose diet	21.06±2.04[a] (5)
Starch diet	22.50±2.19 (4)

[a] : Standard error of mean.

DISCUSSION

Although the alimentary production of cholesterol gallstones in young hamsters was reported first by DAM and his co-workers[13], and has been confirmed by several investigators[14)-17], the exact mechanism of the formation of cholesterol gallstones under these experimental conditions has not been completely elucidated.

The present studies with liver slices of hamsters strongly indicated a correlation between the increased biosynthetic activity of cholesterol in the liver and the formation of cholesterol gallstones. This correlation was observed under the various dietary conditions shown in Table 1. For instance, the livers of hamsters fed on either the glucose diet or the glucose diet supplemented with ethyl palmitate showed high activities of cholesterol synthesis, and cholesterol gallstones were formed in almost all animals in these dietary groups. On the other hand, the livers of hamsters fed on the starch diet, or on the glucose diet supplemented with ethyl linoleate, or on rat chow showed low activities of cholesterol synthesis, and no cholesterol gallstone was formed in these dietary groups. In general, enhanced activities of cholesterol synthesis in the liver were observed under the

dietary conditions which led to cholesterol gallstones formation[18)19)], whereas the activities of cholesterol synthesis in the liver were low under the dietary conditions which did not lead to cholesterol gallstones formation.

VILLA et al.[20)] reported increased concentrations of cholesterol and decreased concentrations of acetoacetate in the livers of patients with cholesterol gallstones. They suggested that the competition between keto- and cholesterol-genesis which occurs at the β-hydroxy-β-methylglutaryl CoA level would be modified in favour of cholesterol synthesis in patients with cholesterol stones and that this metabolic error might play a role during the formation of the cholesterol stones. The present results appear to support the assumption of VILLA et al.

Of the several dietary factors which affect the formation of cholesterol gallstones in hamsters, the source of dietary carbohydrate is a particularly interesting one. Compared with a fat-free diet in which starch is the sole carbohydrate, a similar diet with glucose as the sole carbohydrate enhanced both the formation of cholesterol gallstones and the biosynthetic activity of cholesterol in the liver. The results are consistent with those obtained by JENSEN et al.[21)] who have shown *in vivo* that radioactive acetate administered intraperitoneally is incorporated into the whole body cholesterol in much higher quantities in hamsters maintained on a fat-free and high-glucose diet than in those on a fat-free and high-starch diet. The increased activity of cholesterol synthesis in the liver caused by the glucose diet was established within 4 days after initiating this diet feeding schedule, and continued to increase gradually during the period on this diet (Fig. 3). Very rarely the formation of cholesterol gallstones occurred as early as within 2 weeks in hamsters on the high-glucose diet, but in almost all cases cholesterol gallstones were formed during the 4th week on this diet. It appears that the increase of cholesterol synthesis in the liver precedes the formation of cholesterol gallstones in the gallbladder.

The reasons that the activity of cholesterol synthesis in the liver is enhanced more by the glucose diet than by the starch diet are of interest. Cholesterol synthesis appears to be very sensitive to various experimental conditions. Reports from several laboratories have shown that cholesterol synthesis in the liver increases under various experimental conditions, including X-irradiation[22)-24)], triton injection[24)25)], and the removal of bile from the enterohepatic circulation via a bile fistula[26)]. In addition, cholesterol synthesis was reported to decrease when animals were fasted[24)25)27)] or when either cholesterol[24)28)-30)] or cholic acid[31)] was given. Enzymic analyses of these experimental animals revealed that the great alteration was concerned with the synthesis of mevalonate from acetate[24)-26),28)-30)], presumably by the activity of β-hydroxy-β-methylglutaryl CoA reductase, which was the major limiting step in cholesterol synthesis[25)29)30)32)]. NADPH plays an important role as a co-factor of β-hydroxy-β-methylglutaryl CoA reductase in cholesterol biosynthesis. SIPERSTEIN et al.[12)] suggested that NADPH which was generated through the hexose monophosphate shunt increased cholesterol synthesis in the liver. When the effect of dietary glucose on cholesterol synthesis was compared with that of starch, the present studies revealed that the increase of cholesterol synthesis observed in hamsters maintained on the glucose diet may be due not to a higher level of NADPH but to increased enzymic activities before mevalonate in the biosynthetic pathway of cholesterol. This is supported by the following findings: 1. No appreciable difference in glucose 6-phosphate dehydro-

genase activity, a most important NADPH generating system, was observed in the liver between animals on the glucose diet and those on the starch diet (Table 6). Furthermore, the activity of cholesterol synthesis in liver homogenates of animals on the glucose diet was higher than that of animals on the starch diet both in the presence and absence of an NADPH generating system (Table 2), although the addition of an NADPH generating system to the liver homogenates stimulated the activity of cholesterol biosynthesis to some extent. These findings suggest that the level of NADPH is not the sole factor responsible for the difference in the biosynthetic activity of cholesterol between these dietary groups. 2. When acetate was employed as a substrate, the activity of cholesterol synthesis in animals on the glucose diet far exceeded that in animals on the starch diet, whereas when mevalonate was employed as a substrate, no marked difference in the activity of cholesterol synthesis was observed between these dietary groups (Table 1 and Table 3). The higher activity of cholesterol synthesis in the livers of animals on the glucose diet than in those on the starch diet may be due to the higher activity of β-hydroxy-β-methylglutaryl CoA reductase, which is very sensitive to several conditions.

The means by which the enzymes catalyzing cholesterol synthesis become more active during glucose feeding than starch feeding may be explained by the following possible mechanisms.

1. Different insulin response. Since starch, which must be hydrolyzed to glucose before it is absorbed from the gut, is absorbed more slowly than glucose, there is a mild but prolonged increase in blood glucose concentration with a small insulin response compared to that which occurs following the ingestion of glucose, which is absorbed more rapidly. COHEN et al.[33] reported that repeated ingestion of a high-carbohydrate diet in which glucose (or sucrose) was substituted for starch resulted in a significant impairment of glucose tolerance and diminished serum insulin-like activity in the rat. Such variation in the insulin-secretion system might induce enzymes which catalyze cholesterol synthesis in animals fed on a glucose diet. HOTTA et al.[34] reported a definite increase in cholesterol formation in the diabetic liver and it was further shown that as a result of insulin injection in a diabetic rat, slices subsequently prepared from its liver had a reduced capacity to synthesize cholesterol. BRAUNSTEINER et al.[35] reported that in patients with cholelithiasis, the average fasting blood glucose level was higher than in normal persons and that many of these patients were found to be prediabetic by the tolbutamide test. From these findings they suggested that a latent diabetic state might play an important role in the pathogenesis of cholelithiasis. In the present experiments, no glucosuria was observed in animals on the glucose diet.

2. Difference in intestinal flora. A variation in intestinal flora resulting from the feeding of different carbohydrates[36] might affect the synthetic activity of cholesterol by influencing the bile acids cycle. During the enterohepatic circulation of bile, the primary bile acids are modified by intestinal microorganisms in the caecum and colon, and some of the modified bile acids are poorly reabsorbed[37] Indeed, a decreased turnover rate of bile acids was reported in germ-free rats[38] or in conventional rats treated with antibiotics[39]. Recently KELLOGG et al.[40] suggested that the presence, due to incomplete digestion, of carbohydrate in the lower gut would prevent bacterial modification of steroids. They reported that fecal bile acids in animals on starch diets resembled qualitatively those of

germ-free animals. There is a possibility that in animals on the glucose diet, a great modification of bile acids by the intestinal flora might reduce the reabsorption of bile acids, causing a diminished level of bile acids in the portal vein, and resulting in stimulation of cholesterol biosynthesis by releasing a feedback regulation by bile acids[41].

3. Deficiency of some nutrients. The possibility cannot be excluded that the difference in biosynthetic activity of cholesterol between animals on the glucose diet and those on the starch diet may be due to a deficiency of some essential nutrients, for instance, vitamins[42]. However it seems difficult to differentiate whether the change in cholesterol synthetic activity is due to the direct effect of vitamin-deficiency or to an indirect effect resulting from inanition, since a number of vitamin-deficiency states are associated with inanition. In the present experiments, no symptom of vitamin-deficiency was observed in animals in these dietary groups, though the weight gain during the experimental period was somewhat greater in animals on the starch diet than in those on the glucose diet. Furthermore, when the formation of CO_2 from acetate was analyzed with liver slices as an indicator of basal metabolism, no marked difference was observed between these dietary groups.

The quality of dietary fatty acid as well as the source of dietary carbohydrate affected both the formation of cholesterol gallstones and the biosynthetic activity of cholesterol in the liver (Table 1). In parallel with complete prevention of the formation of cholesterol gallstones, the addition of ethyl linoleate to the high-glucose diet counteracted the glucose effect in accelerating the activity of cholesterol synthesis in the liver. In contrast, neither the formation of stones nor the activity of cholesterol synthesis was reduced by ethyl palmitate. Many recent reports have emphasized the influence of dietary fats on cholesterol metabolism, particularly the serum cholesterol level. Some investigators[43] have suggested that the ability of a diet high in polyunsaturated fatty acids to reduce plasma cholesterol may be mediated by the increased fecal excretion of cholesterol and its metabolites. Since the ingestion of unsaturated fatty acids increases the amount of liver cholesterol esterified with fatty acids[44], some investigators have suggested that unsaturated fatty acids may facilitate the transportation of cholesterol by forming cholesterol esters. BOYD[45] proposed that cholesterol derivatives in which the 3 β-hydroxyl was esterified by linoleic acid might be preferentially converted to bile acids. HIKASA et al.[4] reported the possibility that arachidonic acid esterified with cholesterol might play an important role in the conversion of cholesterol to bile acids and corticosteroid hormones. Indeed, SWELL et al.[46] reported that cholesterol esterified with linoleic acid or arachidonic acid was metabolized more rapidly than other cholesterol esters. The effect of the chain length or the unsaturation of dietary fatty acids on cholesterol synthesis has also been studied by several investigators [44][47]-[49]. In the present experiments, ethyl linoleate reduced the cholesterol synthesis from acetate, whereas ethyl palmitate did not. The results are consistent with those obtained by REISER et al.[50] who have shown *in vivo* that trilinolein depressed markedly the cholesterol synthesis from acetate in the liver, while tripalmitin did not. KRITCHEVSKY et al.[51] reported that no appreciable difference in the synthesis of cholesterol from mevalonate was observed between animals maintained on saturated and unsaturated fatty acid. It may be possible that as well as dietary carbohydrate, dietary lipid also influences cholesterol synthesis in the liver in the pre-mevalonic steps, presumably at β-hydroxy-β-methyl-

glutaryl CoA reductase. The present experiments do not explain, however, whether the depression of cholesterol synthesis by linoleic acid is due to the specific action of linoleic acid or to some of its metabolic derivatives. Prostaglandins, which are mainly synthesized from linoleic and other higher polyunsaturated fatty acids[52], may affect cholesterol synthesis directly or indirectly.

Low activities of cholesterol synthesis from both acetate and mevalonate were observed in liver slices of hamsters maintained on rat chow. One possible interpretation for these low activities is that cholesterol synthesis is inhibited in both steps before and after mevalonate by the cholesterol included in this rat chow. GOULD et al.[30] reported that dietary cholesterol inhibited the cholesterol synthesis in the steps between mevalonate and cholesterol as well as in pre-mevalonic steps. In fact, the rat chow used in the present studies contained 0.07% of cholesterol. Complex carbohydrates and some unsaturated fatty acids included in this rat chow also appear to influence cholesterol synthesis.

Thus the present studies revealed a close correlation between the synthetic activity of cholesterol in the liver and the formation of cholesterol gallstones. However, it cannot be concluded simply that the increased activity of cholesterol synthesis in the liver is a direct factor in the formation of cholesterol gallstones. Cholesterol is thought to be excreted from the liver mainly via three pathways[53]. Some of the cholesterol is eliminated into the blood stream, some into the bile in the form of cholesterol, and a large amount is excreted into the bile in the form of bile acids. Therefore, the correlation between the synthetic activity of cholesterol in the liver and the formation of cholesterol gallstones must be considered in relation with dynamic changes of the cholesterol metabolism in

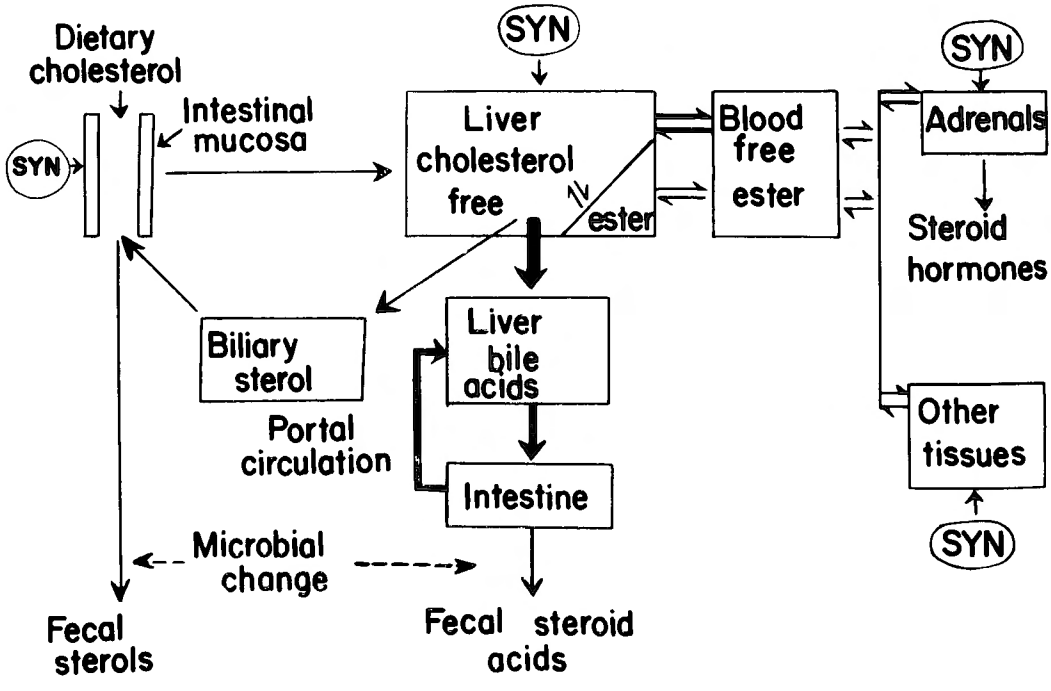

Fig. 7 A diagram of the metabolism of cholesterol (Gould and Cook[53], 1958). SYN, biosynthesis.

these three pathways.

1. Blood cholesterol level. DREWS[14] reported that no appreciable difference in serum cholesterol level was observed between hamsters maintained on a lithogenic diet (for instance, fat-free and high-glucose diet) and on a non-lithogenic diet. There is no evidence that a high cholesterol level in the serum is important for the formation of cholesterol gallstones.

2. Liver cholesterol level. The cholesterol content in the livers of hamsters maintained on a lithogenic diet showed only a slight tendency to increase in comparison with that in hamsters on a non-lithogenic diet (Table 4), although biosynthetic activity of cholesterol in the liver was markedly higher under the former dietary conditions than under the latter (Table 1). The findings mentioned above indicate that cholesterol which is synthesized excessively in the liver of hamsters maintained on lithogenic diets is excreted into the bile as cholesterol or bile acids.

3. Level of cholesterol and bile acids in bile. In hamsters, the increased level of cholesterol in the bile with a concomitant decrease in the ratio of bile acid concentration to cholesterol was found as the conditions for the formation of cholesterol gallstones were approached[54]. Indeed, radioactivity recovered as biliary cholesterol during the 24 hours following the intravenous administration of cholesterol-4-^{14}C was slightly higher in hamsters maintained on a lithogenic diet (for instance, fat-free and high-glucose diet) than in hamsters on a non-lithogenic diet (for instance, fat-free and high-starch diet) (Table 5). These findings appear to support the assumption that cholesterol is held in solution in bile by bile acids or lecithin[55,56] acting as a detergent, and that the formation of cholesterol gallstones does not occur until the ratio of detergent to cholesterol diminishes to some critical figure.

The present experiments revealed that the activity of cholesterol synthesis is stimulated in the liver of hamsters more by lithogenic diets than by non-lithogenic diets. It is conceivable, therefore, that the cholesterol which is excessively produced in the liver is preferentially excreted into the bile, bringing about an increased level of cholesterol in the bile, and is probably provided for the formation of cholesterol gallstones.

SUMMARY

1. Cholesterol gallstones were formed at a high incidence (86%) in hamsters maintained on a fat-free and high-glucose diet for 28 days, while no cholesterol stone was formed when dietary glucose was replaced by starch.

2. When the fat-free and high-glucose diet was given, the radioactivity of acetate-2-^{14}C incorporated into cholesterol in liver slices was 4-fold that in liver slices of hamsters on the fat-free and high-starch diet.

3. The radioactivity of acetate-2-^{14}C incorporated into cholesterol in the liver homogenates of hamsters on the fat-free and high-glucose diet was higher than that of the liver homogenates of hamsters on the fat-free and high-starch diet both in the presence and absence of an NADPH generating system.

4. No marked difference was observed in the radioactivity of mevalonate 2-^{14}C incorporated into cholesterol in liver slices between the hamsters on the fat-free and high-glucose diet and those on the fat-free and high-starch diet.

5. The ratio of radioactivity of biliary cholesterol to total radioactivity of bile excreted during 24 hours following intravenous administration of cholesterol-4-^{14}C was slightly but significantly higher in hamsters on the fat-free and high-glucose diet than in those on the fat-free and high-starch diet.

6. The addition of ethyl linoleate to the fat-free and high-glucose diet prevented the increase in the radioactivity of acetate-2-^{14}C incorporated into cholesterol in liver slices as well as the formation of cholesterol stones. In contrast, neither the incorporation nor the formation of cholesterol stones was prevented by ethyl palmitate.

7. There seems to be a close correlation between the increased activity of cholesterol synthesis in the liver and the formation of cholesterol gallstones. Furthermore, the higher activity of cholesterol synthesis in the liver induced by dietary glucose than by dietary starch appears to be due not to the increased level of NADPH, but mainly to the increase of enzymic activities before mevalonate in the biosynthetic pathway of cholesterol.

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和文抄録

ハムスターにおけるグルコース食による肝コレステロール生合成の亢進とコレステロール胆石の生成について

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近年コレステロール胆石症は増加の傾向にあるといわれ，又本症が他の多くの疾病の原因になるにもかかわらず，コレステロール胆石形成の原因については尚不明の点が多かつた．日笠等，Dam等は，ハムスターにおけるコレステロール胆石の形成には食餌因子が重要な役割を演じる事を見出だした．従つてこの様な実験モデルを用いてのコレステロール代謝機構の研究は，物質代謝という広い視野からのコレステロール胆石形成機序の解明に重要な知見を与えるものと考えられる．

著者は，ハムスターを用いて肝におけるコレステロール代謝，殊にコレステロール生合成とコレステロール胆石形成との関係を，食餌中の炭水化物源および脂質源との関連において解明し，更に炭水化物源としてのグルコースのコレステロール生合成におよぼす促進効果のメカニズムについても考察を加えた．

1. 無脂質食で飼育した場合，炭水化物源としてグルコースを用いると高率に（86%）コレステロール胆石の形成を認めたが，澱粉を用いると胆石の形成は全く認められなかつた．又高率に胆石を生じる無脂質グルコース食でも，リノール酸エチルの添加（5%）はコレステロール胆石の形成を完全に抑止したが，パルミチン酸エチルの添加ではこの様な効果は認められず，85%の高率に胆石を生じた．

2. これらの食餌条件下でのコレステロール生合成を肝切片を用いて調べると，無脂質グルコース食では無脂質澱粉食の約4倍に亢進していた．又無脂質グルコース食にリノール酸エチルを添加した場合，コレステロールの生合成は添加しない場合の70%も抑制されたが，パルミチン酸エチルの添加ではこの様な効果は殆んど認められなかつた．つまり肝のコレステロール生合成とコレステロール胆石形成との間には密接な相関々係があり，コレステロール胆石を生じる食餌条件下では肝のコレステロール生合成の亢進が認められた．

3. 肝コレステロール量は，コレステロール胆石を生じる食餌条件下では軽微な増加傾向を示したが，著明な増加は認められなかつた．

4. 胆汁瘻ハムスターで調べると，コレステロール胆石を生じる食餌条件下では胆石を生じない食餌条件下に比べて，血管内投与された放射性コレステロールの胆汁コレステロール分画への排泄は多かつた．

5. コレステロール胆石を生じる食餌条件下では，胆汁中コレステロールの増加とそれに伴う胆汁酸対コレステロール比の低下がある事，血清コレステロール値の上昇が認められない事等と本実験結果とを考えあわせると，肝で過剰に生合成されたコレステロールは血液や肝に蓄積されずにコレステロールのままで胆汁中に排泄され，胆汁中のデタージェント対コレステロール比の低下というアンバランスを通じてコレステロール胆石形成に関与するものと考えられる．

6. 炭水化物源としてのグルコースは澱粉に比べて，肝切片での酢酸からのコレステロール生合成を著明に亢進するがメバロン酸からのそれは亢進しない事，NADPH生成系添加の有無にかかわらず肝ホモジネートでも酢酸からのコレステロール生合成を亢進する事，NADPH生成系である肝のグルコース6燐酸脱水素酵素活性に差が認められないこと等から，グルコースによるコレステロール生合成の亢進は補酵素であるNADPHレベルの変動によるものとは考えにくく，むしろコレステロール生合成系におけるメバロン酸より前の酵素活性の上昇によるものと考えられた．

従来コレステロール胆石形成の原因は主として局所要因による胆汁組成の膠質化学的変化にあると考えられていたが，本論文ははじめてそのような胆汁組成の変化が食餌組成，殊に炭水化物ならびに脂質と関連した肝におけるコレステロール生合成の亢進による事を実験的に明らかにし，コレステロール胆石症の原因解明の上で新らしい重要な知見が提供された．