

Initiating Factors of Gallstones, Especially Cholesterol Stones (II)

by

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I. INTRODUCTION

Various concepts concerning the initiating factors of gallstone formation have been originated in an inflammatory theory advocated by NAUNYN⁴⁸⁾ and in a bile stasis theory by ASCHOFF-BACMEISTER²⁾. LICHTWITZ³³⁾, SCHADE et al.⁵³⁾ have tried to elucidate the details of gallstone formation with their colloid chemical theory. Many other investigators have declared that the formation of gallstone might be attributed to disturbances of metabolism⁴¹⁾, dysfunctions of the endocrine system⁵⁷⁾, disorders in the autonomic nervous system⁴⁾,⁶⁴⁾ and abnormalities of constitution⁵⁾. Nowadays mechanisms of gallstone formation remains obscure, though cholelithiasis has been often encountered clinically.

In our laboratory, various effects of essential fatty acids (EFA) in vivo have been extensively studied over longer period²¹⁾,²²⁾, demonstrating that adrenals, liver and heart muscle contained greater quantities of EFA more than did the other organs (Fig. 1). In the case of EFA deficiency in vivo, dienoic acid and tetraenoic acid decreased, respectively,

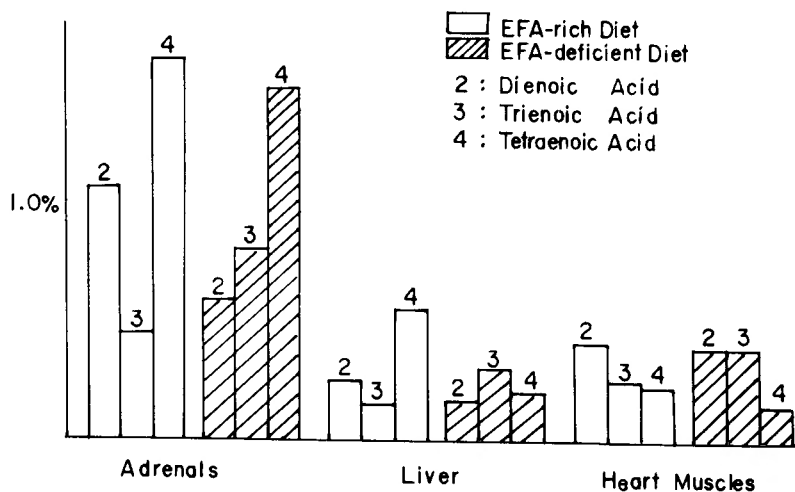


Fig. 1 Polyenoic Acids in the Tissues of the Rat

whereas trienoic acid increased in those organs³⁷⁾⁵⁹⁾, demonstrating that adrenocortical capacity of the organisms had been already to a greater extent weakened so that they could not produce enough glucocorticoid to meet their demand by themselves, when they were exposed to the various sorts of stress²³⁾²⁴⁾²⁵⁾.

In adrenals cholesterol metabolism has indispensable relations with the coexisting EFA. Adrenal functions are not controlled by the quantity of cholesterol in adrenals but controlled by that of fatty acids, tetraens, esterified with cholesterol (Figs. 2~5)⁴⁵⁾⁵⁸⁾. It seems most likely that the formation of gallstones, especially cholesterol stones may be attributed to EFA deficiency and/or metabolic disturbances in EFA, since analogical relationship was found between the degradation of cholesterol to glucocorticoid and to bile acids, reviewing the fact that cholesterol is a precursor of glucocorticoids and at the same time it is a precursor of bile acids as well (Fig. 6). Thus, several sorts of experiments have been designed in order to get more knowledge about the mechanism of gallstone formation.

For the first place, the adrenocortical capacity and the concentration of tetraens in blood of the patients with cholesterol stones were determined. And both of them decreased remarkably as compared with normal human subjects. This suggests that the patients with

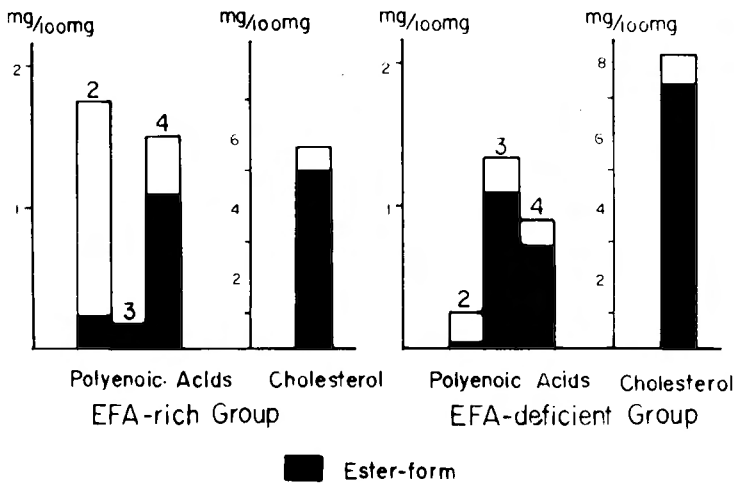


Fig. 2 a Polyenoic Acids and Cholesterol in the Adrenal Glands of the Rat

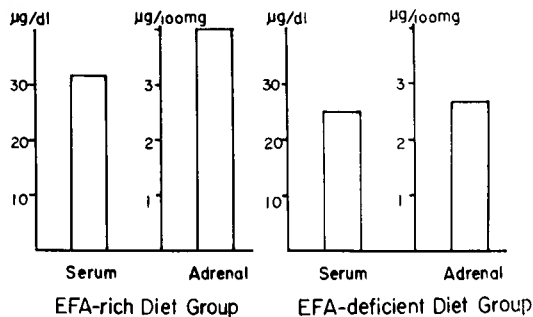


Fig. 2 b Corticosterone in Serum and Adrenal Glands of the Rat

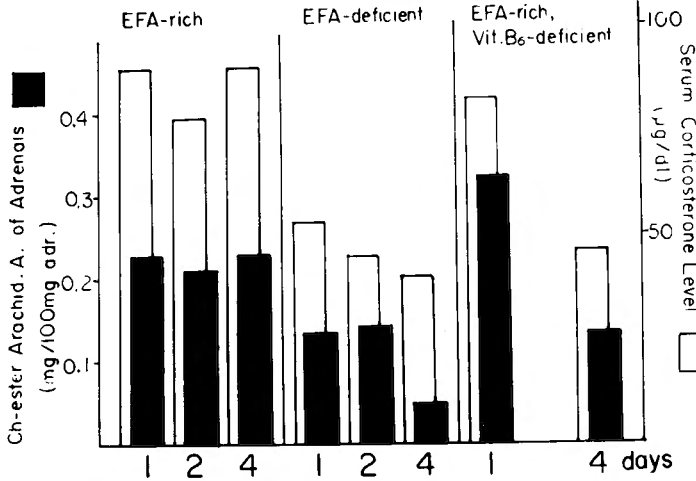
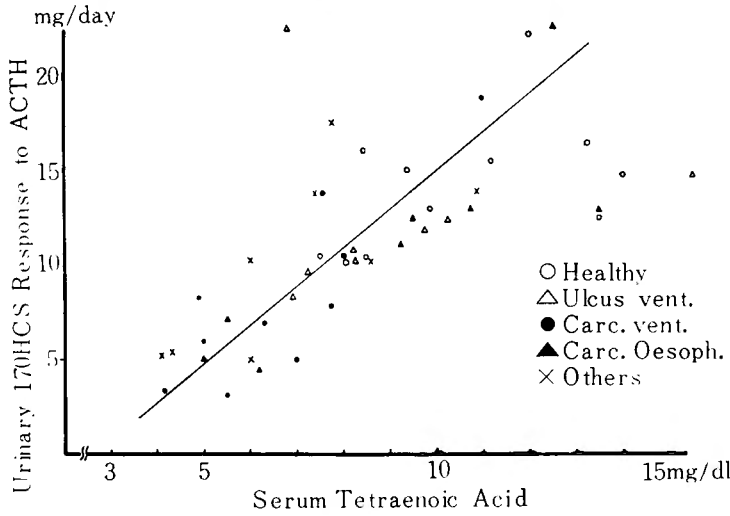


Fig. 3 Effect of ACTH on Serum Corticosterone and Arachidonic Acid Esterified with Cholesterol in the Adrenals of Rats



cholesterol stones have already fallen into a latent critical condition referable to EFA deficiency and/or its metabolic disturbances (Figs. 7~8)¹⁹⁾.

Here is another importance that we must take into consideration in order to investigate a lots of initiating factors of gallstone formation. It is that since CALDWELL⁹⁾, LYNEN³⁶⁾, WIELAND et al⁶³⁾, have advocated HMG•CoA-cycle in their enzymological studies on the biosynthesis of cholesterol, many investigators have concentrated their attentions upon the relations between cholesterolgenesis and ketogenesis (Fig. 9). These two geneses are always counteracting one another on account of variations of the conditions. We must notice the

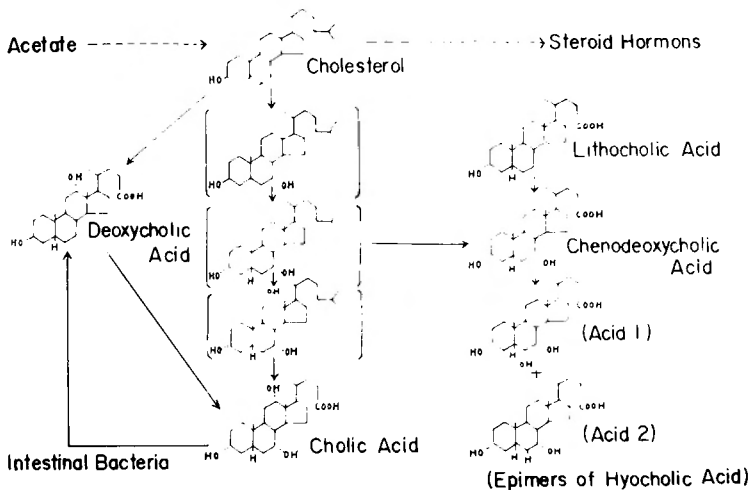


Fig. 6 Conversion of Cholesterol to Bile Acids (Cook, 1958)¹²⁾

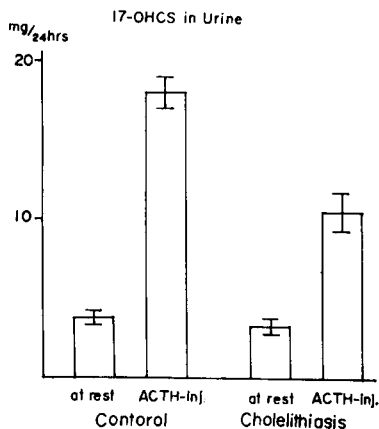


Fig. 7 Adrenocortical Capacity in the Patients with Gallstones

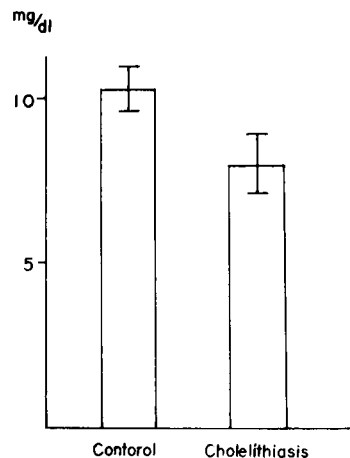


Fig. 8 Serum Tetraenoic Acid in the Patients with Gallstones

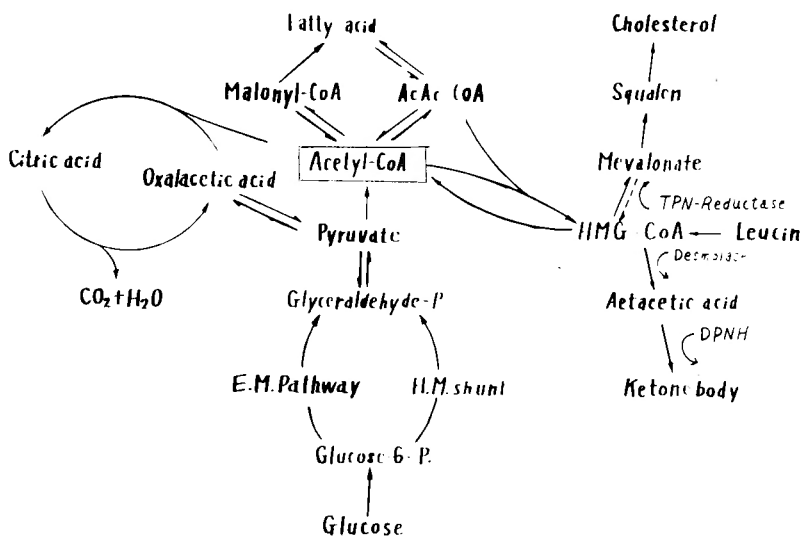


Fig. 9 Biosynthesis of Cholesterol (Wieland et al. 1960)

attitude of acetoacetate in the liver of the patients with cholesterol stones. It has been now well demonstrated that the hepatic biosynthesis of acetoacetate in those patients decreased as compared with normal individuals. It is surmised, therefore, that the biosynthesis of cholesterol may be stimulated either due to the decreased activity of desmolase or due to the increased activity of TPN-reductase in the liver of the patients with cholesterol stones⁶²⁾. It seems most likely that the formation of cholesterol stones is within the range of possibility, when the hepatic biosynthesis of cholesterol is to a great extent stimulated and the capacity of bile keeping cholesterol in solution is simultaneously weakened. According to the experimental results that we have just obtained in studying the effects of EFA upon adrenocortical capacity, we have started some experiments to find out what sorts of conditions might affect the bile to lower its capacity keeping cholesterol in solution.

II. PRELIMINARY EXPERIMENTS

Since the physiological significance of EFA was insisted by BURR and BURR⁷⁾ in 1929, it has been generally accepted that the experimental animals had to be placed on an EFA deficient diet with sucrose or glucose as the main source of carbohydrate throughout the whole experimental feeding period in order to know the effects of EFA on the animals of going without it. When we are dealing with the catabolic process of cholesterol into bile acids in the liver that is not only a great store of EFA but also a major site of the biosynthesis and degradation of cholesterol, it is desirable to investigate only the catabolic process of cholesterol under such a condition, as simpler as possible, as cholesterol biosynthesis is not at all stimulated, in order to record the results of the experiments more naturally and with accuracy. We have then made comparative studies of hepatic cholesterol by using rats receiving three kinds of EFA deficient diets with starch, sucrose, glucose as carbohydrate component, respectively. Hepatic cholesterol content increased markedly in case of feeding sucrose or glucose, as compared with the animals received starch. The similar changes were observed even in the case of supplementation of the same diets with EFA (Fig. 10)¹⁸⁾.

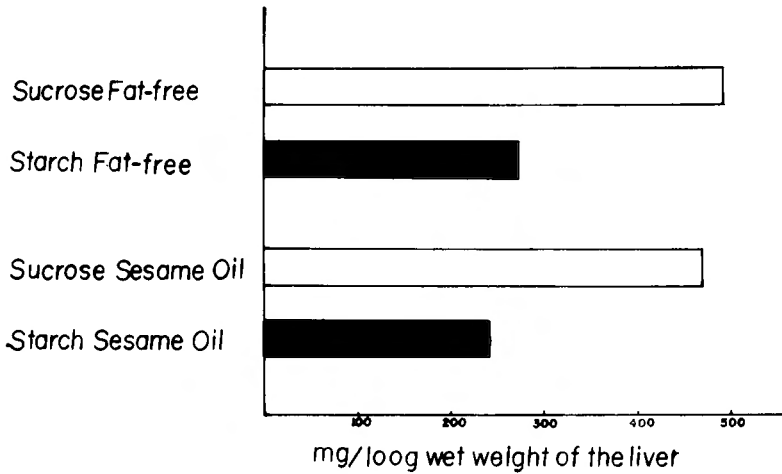


Fig. 10 Total Cholesterol level in the Liver of Rats Fed Various Diets

These are thought to suggest the possibility that the biosynthesis of cholesterol may be alimentarily stimulated in liver by only rearing the animals on the diets containing sucrose or glucose as the sole source of carbohydrate. In the experiments that we have designed for the purpose of clarification of the catabolic process of cholesterol to bile acids in rat's liver, we have purposely made use of starch as the main source of carbohydrate in the synthetic diet.

In fact, it is said that starch contains often EFA, however, it is in a negligible amount. Although starch was used as carbohydrate source, if none of EFA were added to the diet, gas-liquid chromatographic separation of the fatty acids of each organ revealed also even in case of feeding starch the same typical patterns referable to EFA deficiency as in case of feeding sucrose or glucose (Fig. 1). This favoured us to think that it was not always necessary to use only sucrose or glucose as the source of carbohydrate.

III. THE EFFECTS OF EFA DEFICIENCY AND/OR ITS METABOLIC DISTURBANCES UPON BILE CONSTITUENTS IN RATS^{(26), (27)}

We have carried out the main experiments, after finishing the above-mentioned preliminary ones. Liver and aseptic bladder bile from the patients with cholesterol stones were analysed biochemically for fatty acids, bile acids, cholesterol, lecithin and its fatty acid constituents, respectively, demonstrating that there was a close resemblance between the results from human subjects with cholesterol stones and those from rats fed an EFA deficient diet (Figs. 11~17)^{(30) (38) (68)}. Fatty acids that were esterified with cholesterol so as to activate the metabolism of cholesterol and to be utilized ultimately as EFA in vivo at the same time, for example, 5, 8, 11, 14-eicosatetraenoic acid, 7, 10, 13, 16-docosatetraenoic acid, 4, 7, 10, 13, 16-docosapentaenoic acid, decreased to a great extent quantitatively. On the other hand, oleic, palmitoleic and eicosatrienoic acid (most of them could be synthesized vigorously from the other kinds of foodstuffs in vivo) increased as if they would compensate for deficiency of those essential fatty acids. In parallel with the changes of those fatty acids, both total and esterified cholesterol increased in liver, total bile

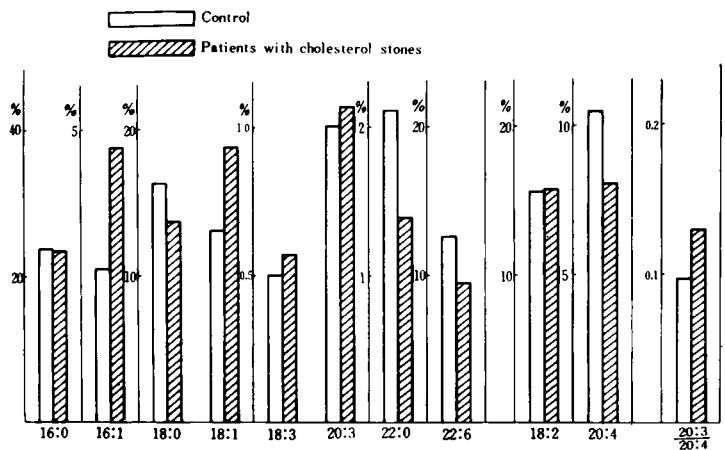


Fig. 11 Fatty Acid Composition in the Liver of the Patients with Cholesterol Stones

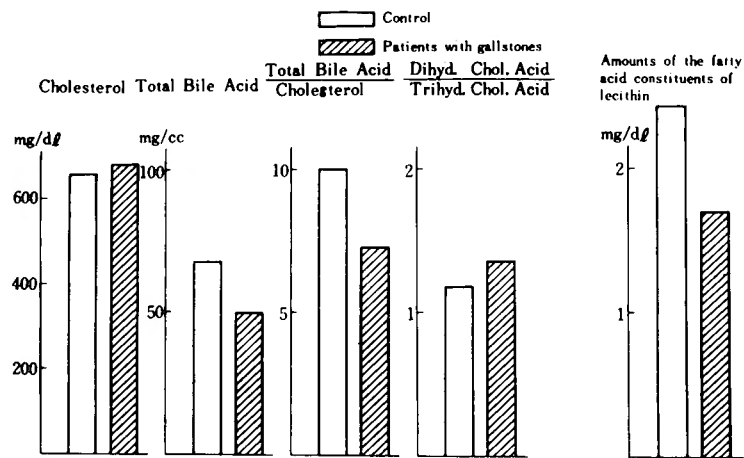


Fig. 12 Bile Composition of the Patients with Gallstones

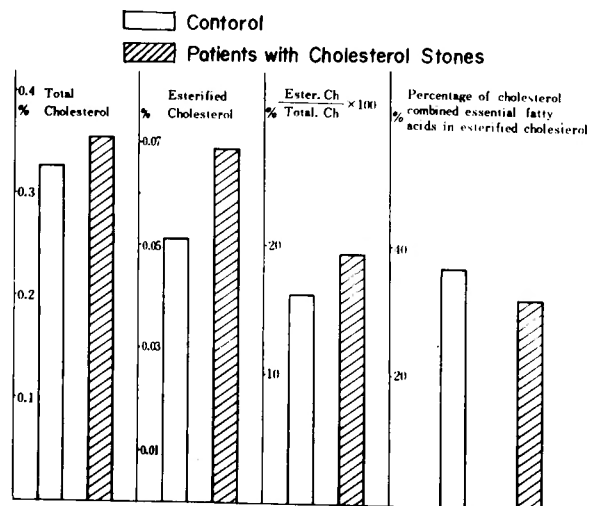


Fig. 13 Liver Cholesterol level in the Patients with Cholesterol Stones

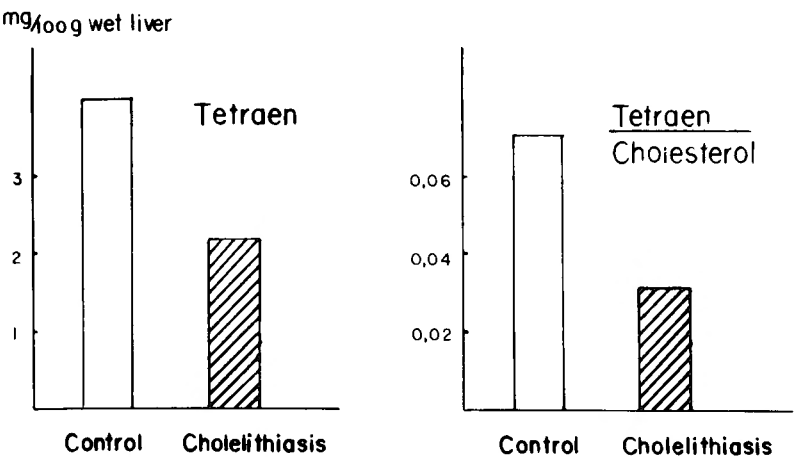


Fig. 14 Tetraenoic Acid Esterified with Cholesterol in the Liver of the Patients with Cholesterol Stones

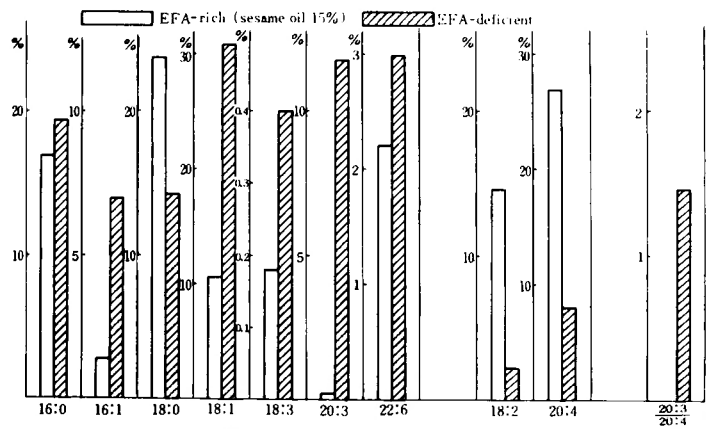


Fig. 15 Fatty Acid Composition in the Liver of Rats Fed a EFA-deficient Diet

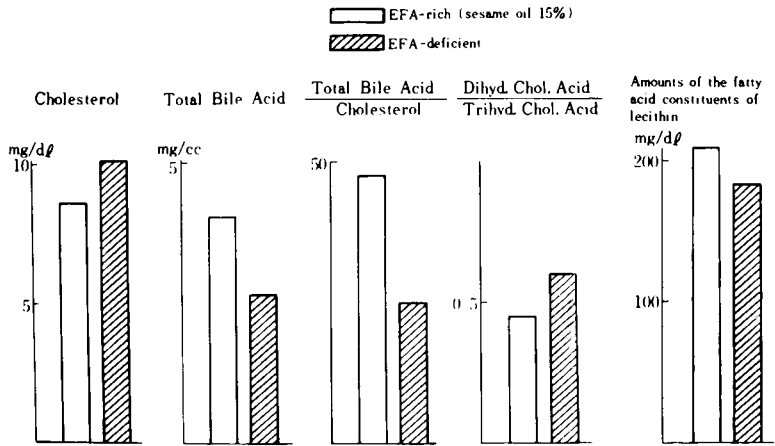


Fig. 16 Bile Composition in Rats Fed a EFA-deficient Diet

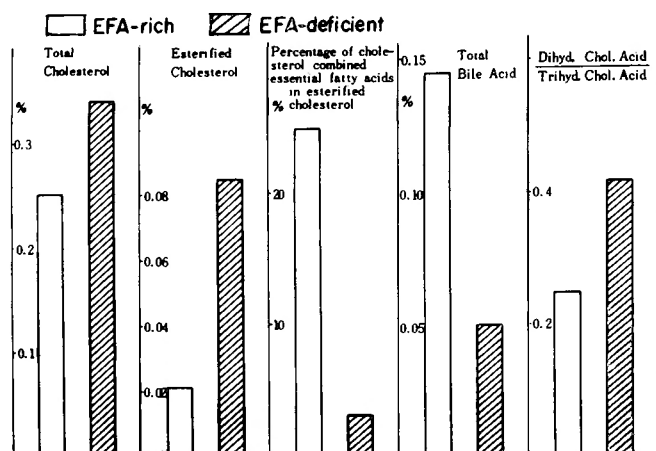


Fig. 17 Cholesterol and Bile Acid levels in the Liver of Rats Fed a EFA-deficient Diet

Metabolism of Polyenoic Fatty Acid (1)

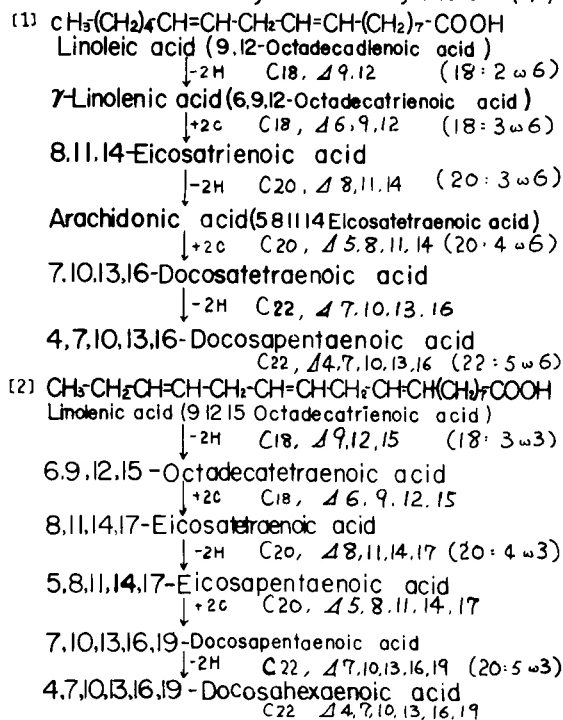


Fig. 18

Metabolism of Polyenoic Fatty Acid (2)

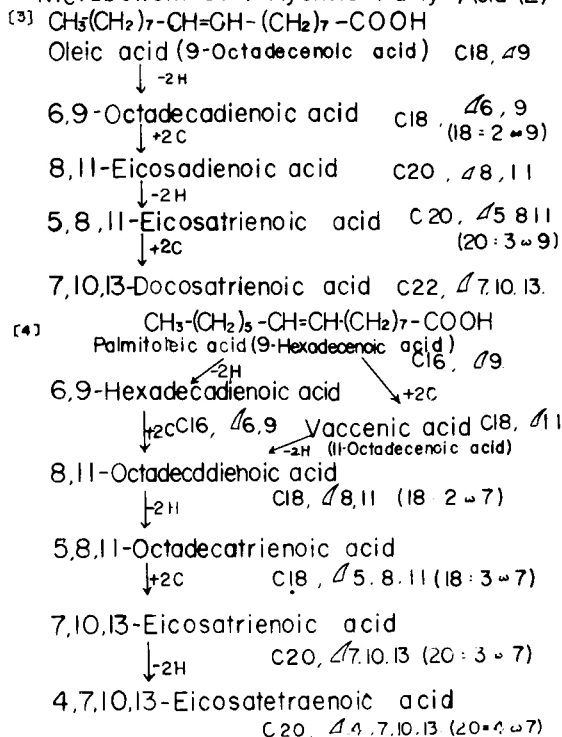


Fig. 19

Fatty Acid	Normal (13 cases)	Cholelithiasis (15 cases)
C 14:0	0.3±0.1	0.5±0.1
C 16:0	41.0±2.9	34.8±3.0
C 16:1	3.9±0.5	4.1±0.5
C 18:0	2.8±0.6	3.7±0.7
C 18:1	14.3±1.1	15.9±1.1
C 18:2	22.4±1.2	24.1±1.0
C 18:3	0.5±0.2	0.4±0.1
C 20:3	0.7±0.2	1.2±0.2
C 20:4	6.6±0.7	6.2±0.5
C 22:0	3.4±0.5	2.9±0.4
C 22:6	4.7±0.6	4.7±0.4

Fig. 20 Fatty Acid Composition of Lecithin in the Bile of the Patients with Cholesterol Stones

the ratio of total bile acids to cholesterol decreased in bile, whereas the ratio of dihydroxycholanolic acid to trihydroxycholanolic acid increased in bile. In case of EFA deficiency and/or its metabolic disturbances, the biosynthesis of lecithin may also be inhibited in liver so that lecithin and the fatty acids constituting lecithin decreased in bile. These changes are thought to have a possibility enough for crystallization and precipitation of cholesterol in bile of the patients with cholesterol stones. In the bile from them, the absolute amounts of both lecithin and fatty acids constituting of it decreased, however, there was no difference in the proportion of each fatty acid to total fatty acids constituting of lecithin, as compared with normal individuals (Fig. 20)³⁸). As a matter of fact, it is impossible to think that only EFA deficiency becomes a true cause for cholesterol stone formation, because a complete deficient

state in EFA cannot be produced in human subjects, as far as they are taking an ordinary meal. This may be also demonstrated by the following difference between the results from rats placed on an EFA deficient diet and those from the patients with cholesterol stones; in the former 9, 12-octadecadienoic acid, linoleic acid, decreased to a great extent in liver quantitatively as well as in percentage, and further linoleic acid constituting lecithin also decreased in both the absolute amount and percentage in bile, while in the latter these changes were not observed, indicating that linoleic acid deficiency had not occurred in liver of the patients with cholesterol stones. In other words, they have probably a maltransformation of linoleic acid to the fatty acids that are exhibiting specific physiological effects on them as EFA, for example, 5, 8, 11, 14-eicosatetraenoic acid, 7, 10, 13, 16-docosatetraenoic acid and 4, 7, 10, 13, 16-docosapentaenoic acid, though they are taking in EFA sufficiently from their meals^{32), 68)}. They have not fallen into a state of deficiency in EFA. They showed rather that they had been suffering from metabolic disturbances in EFA *in vivo*.

Here is a question how such metabolic disturbances in EFA would be produced *in vivo*. Difference in the rate of incidence of cholesterol stones and bilirubin stones was found between Europeans and Japanese⁴²⁾, and furthermore between townsmen and countrymen in our country³⁹⁾. Recently in Japan, the incidence of cholesterol stones has been gradually increasing in parallel with the large consumption of animal fats and proteins⁴⁰⁾. These two phenomena were thought to suggest some idea for us to find out an answer to the question. The reason why we thought so is that when greater quantities of proteins and saturated fatty acids were absorbed, they would produce deficiency in pyridoxine and disturbance in its activation *in vivo*, would cause disturbances in EFA metabolism secondarily, and would ultimately lead to a possibility of cholesterol stone formation.

By using another group of rats fallen in a relative deficient state in pyridoxine and fed of linoleic acid sufficiently at the same time, we have determined bile acids, fatty acids,

cholesterol, lecithin and its constituting fatty acids in liver as well as in bile, respectively. Total and esterified cholesterol in liver did not increase and lecithin and its constituting fatty acids in bile did not decrease, while fatty acids and bile acids in liver as well as in bile showed the same changes as those observed in the patients with cholesterol stones (Figs. 21~23). When animal fats, which were containing more or less cholesterol and chiefly consisted of many sorts of saturated fatty acids and oleic acid, were added to the same diet, changes in liver and bile constituents of the rats coincided in detail with those from the patients with cholesterol stones (Figs. 24~27). This suggests that an increase in the ratio of saturated fatty acids and oleic acid to EFA is playing an important role for this coincidence.

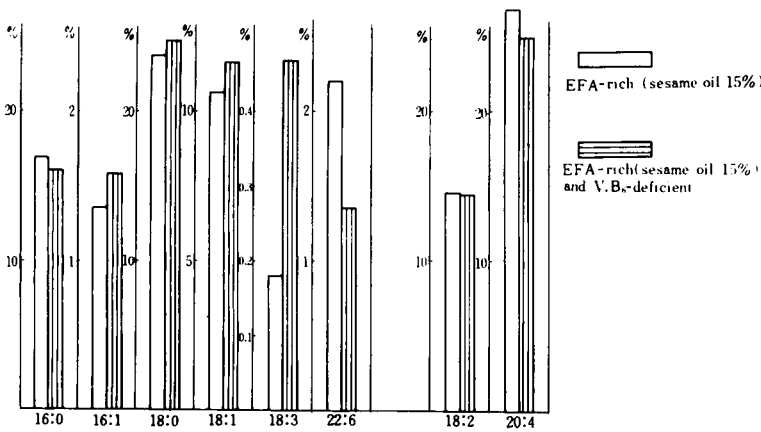


Fig. 21 Fatty Acid Composition in the Liver of Rats Fed a EFA-rich and Vit. B₆-deficient Diet

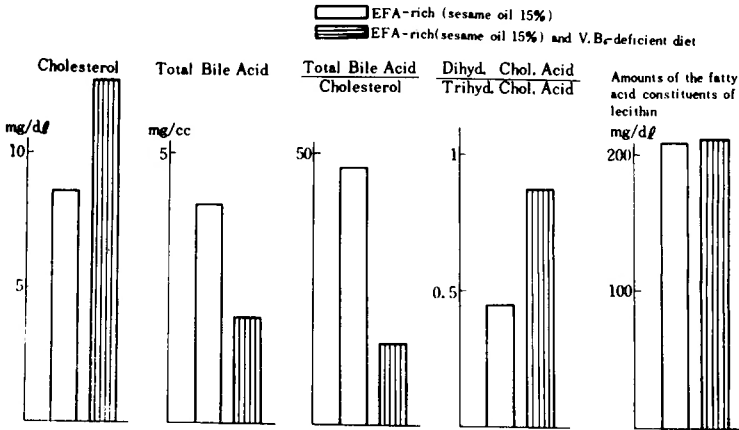


Fig. 22 Bile Composition in Rats Fed a EFA-rich and Vit. B₆-deficient Diet

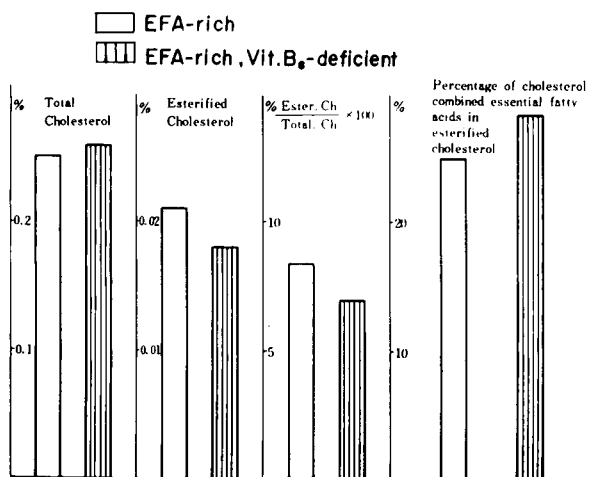


Fig. 23 Cholesterol in the Liver of Rats Fed a EFA-rich and Vit. B₆-deficient Diet

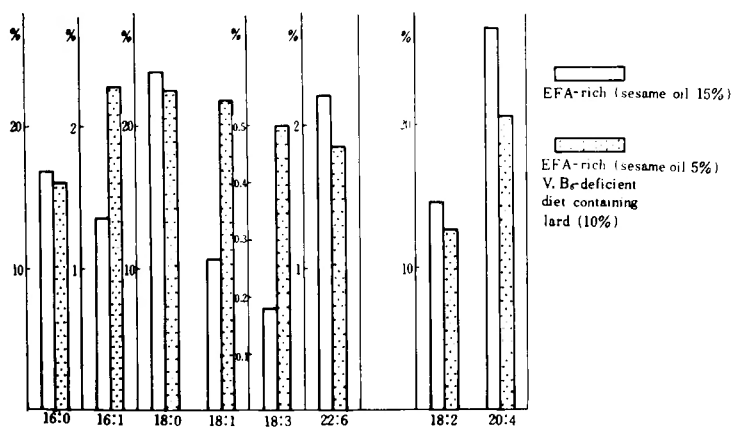


Fig. 24 Fatty Acid Composition in the Liver of Rats Fed a EFA-rich, Vit. B₆-deficient Diet Containing Lard

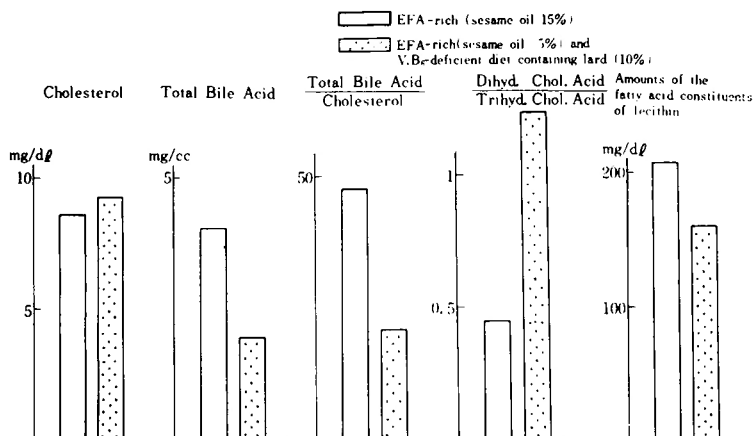


Fig. 25 Bile Composition in Rats Fed a EFA-rich, Vit. B₆-deficient Diet Containing Lard

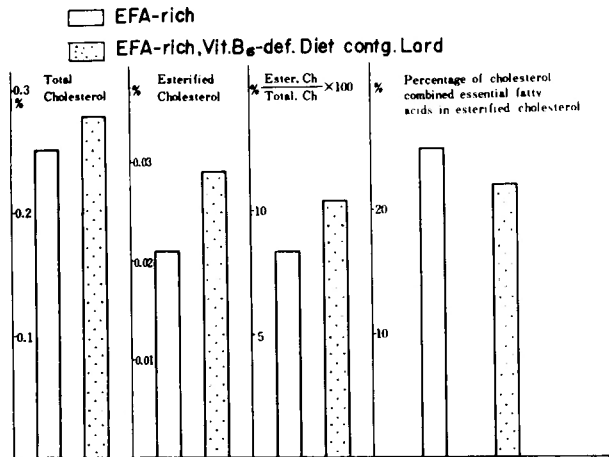


Fig. 26 Cholesterol in the Liver of Rats Fed a EFA-rich, Vit. B₆-deficient Diet Containing Lard

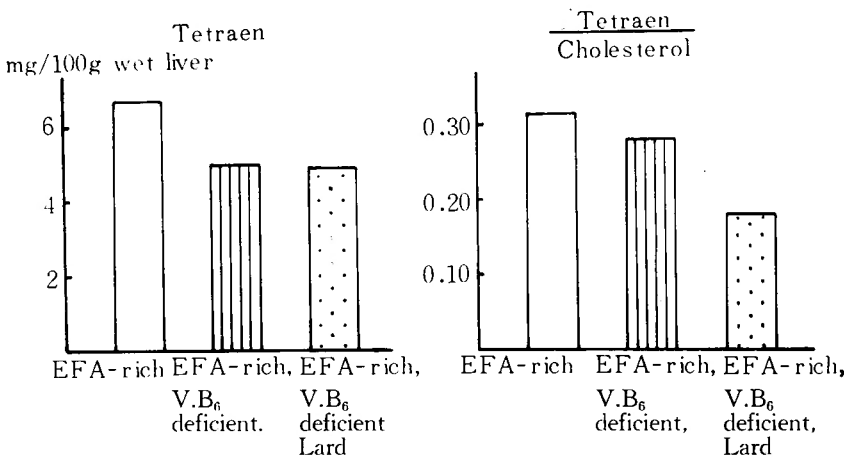


Fig. 27 Tetraenoic Acid Esterified with Cholesterol in the Liver of Rats

We have come to the following conclusions through these experiments in rats^{28), 29)}.

- 1) In human subjects, cholesterol stone formation is not attributed to the absolute deficiency in EFA, but to its metabolic disturbances.
- 2) The metabolic disturbance in EFA seems to be secondarily caused by pyridoxine deficiency and/or disturbances in its activation.
- 3) A greater absorption of animal fats containing saturated fatty acids and oleic acid is a *sine qua non* (a necessary condition) for cholesterol stone formation in human subjects.
- 4) Cholesterol will possibly begin to crystallize, precipitate, and then grow up to cholesterol stones, when the bile constituents will be changed through the metabolic disturbances in the turnover of cholesterol into bile acids as well as the disturbed biosynthesis of lecithin in the liver.
- 5) Large amounts of EFA are usually contained in adrenals, having a close relation to their functions, especially adrenocortical functions. When EFA metabolism is disturbed,

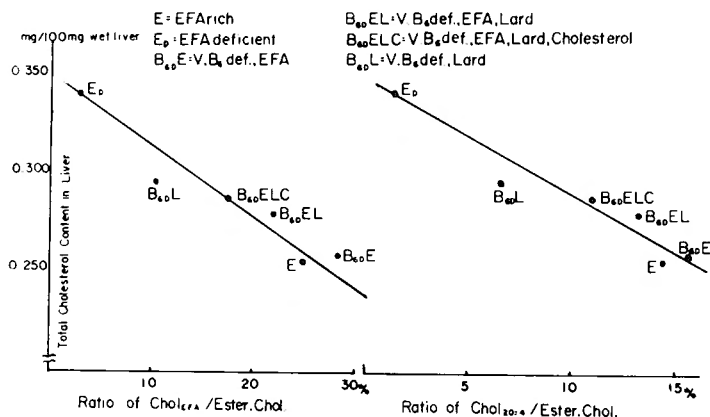


Fig. 29 The Relations between the Cholesterol Content and the Ratio of Cholesterol esterified with EFA to Esterified Cholesterol in the Rat Liver (2)

Figs. 28 and 29 show that even in liver cholesterol metabolism has indispensable relations with the coexisting EFA that was esterified with cholesterol

we have had the diet full furnished with supplementation of all these vitamins and we will expect the same results as those from the case of feeding starch, when the animals receive the diet with sucrose or glucose as carbohydrate component. Difference between vitamins was found with respect to their activities. It has been well known that there were active and inactive types of the vitamins. For instance, as far as pyridoxine may be concerned, we cannot regard the diet as a furnished, though it was sufficiently supplemented in the form of pyridoxine hydrochloride. It is quite questionable whether the pyridoxine added to the diet can be converted to the active and utilized in vivo as pyridoxal phosphate, in case of feeding sucrose or glucose. We must notice that the vitamins supplied by the intestinal flora to meet the greater parts of the host's demand are pyridoxine, biotin, inositol, folic acid and vitamin K.

In the former experiments made by using rats receiving a diet with starch as carbohydrate component, when we placed rats on an EFA deficient diet, or when we kept them receiving another diet, that was added with linoleic acid as EFA source and further added with saturated fatty acids and oleic acid at the level of high percentage, under a relative deficient state in pyridoxine, the experimental data indicated that the bile constituents changed extensively in both cases, so as to be initiating cholesterol stones. However, none of cholesterol stones was found in all rats. The reasons why the animals were kept free from cholesterol stones are summarized as follows: i) Rats have no gallbladder. ii) They have fallen into a slight and relative deficient state in pyridoxine and never fallen into a serious state, because we have got used to make use of starch as carbohydrate component. iii) In case of feeding starch, the hepatic synthesis of cholesterol was never stimulated.

It is essential for us to make use of the following experimental materials and methods in order to be satisfied with these three conditions at a time. This was accomplished by using hamsters as the experimental animals³⁾ and making use of sucrose or glucose as carbo-

hydrate source. Thus we have planned some experiments for the purpose of experimental production of cholesterol stones, referring to the experimental results that were obtained previously by using male albino rats.

IV. EXPERIMENTAL PRODUCTION OF CHOLESTEROL STONES IN HAMSTERS

In case of using starch as carbohydrate component, cholesterol stones did not occur in hamsters as well as rats, not only when the experimental animals received an EFA deficient diet, but also when they received a diet supplemented with animal fats, that were containing greater quantities of saturated fatty acids and oleic acid as well as linoleic acid, for example lard, so that they might fall into a relative deficient state in pyridoxine because of removing it from the diet⁶⁰. On the other hand, when glucose or sucrose was chosen as carbohydrate component, cholesterol stones occurred regularly in hamsters receiving an EFA deficient diet⁵⁹. They also occurred regularly in those receiving the same diet with a modification of supplementation of butter fat⁶⁰. These facts indicate that the conditions, (ii) and (iii) as described at Chapter III, are thought to be necessary and responsible for cholesterol stone formation. The condition (i) is also thought to be essential for this, since cholesterol stones did not occur in hamsters of which the gallbladder was extirpated before the beginning of the experimental feeding period, though they received the same diets³⁴. We have got used to make use of a basal diet, from which pyridoxine, biotin, inositol, folic acid and vitamin K were all removed, so far. Therefore, we have thought whether the reason why the regular occurrence of cholesterol stones was observed in case of feeding glucose or sucrose whereas it was not in case of feeding starch depended on the specific physiological effects of these vitamins upon the cholesterol stone formation. We have performed another experiments to know whether cholesterol stones would be formed, when what sort of those vitamins ran short or was hardly activated.

Hamsters were placed on a diet, that carboxymethyl cellulose (C. M. C.), as nutrients for the intestinal flora, was added to at the level of 18%, or they received another diet with agar-agar at the level of 30%. These two EFA deficient diets protected to some extent the animals against cholesterol stones. This seemed to suggest the possibility that cholesterol stones would be possibly initiated and formed, as deficiency and inactivation took place in some of those vitamins that were produced by the intestinal flora to supply the greater parts of their host's demand, for example pyridoxine, biotin, inositol, folic acid and vitamin K (Fig. 30)⁶⁰.

Although according to the previous experiments performed by using rats, pyridoxine was the most important of those vitamins, we have added each of them or all of them to the basal diet with sucrose or glucose as carbohydrate component and have kept the animals receiving the various diets in order to know what the most effective and important was. These diets of different sorts could neither lower the incidence of cholesterol stones nor kept most animals free from them. In other words, supplementation of the basal diet with these inactive vitamins could not protect the animals against cholesterol stones, so that the regular occurrence was observed in the same way as the animals received an EFA deficient diet without any addition of those vitamins. It is a matter of no importance whether the inactive vitamins were always to be added to the basal diet. Thereafter we

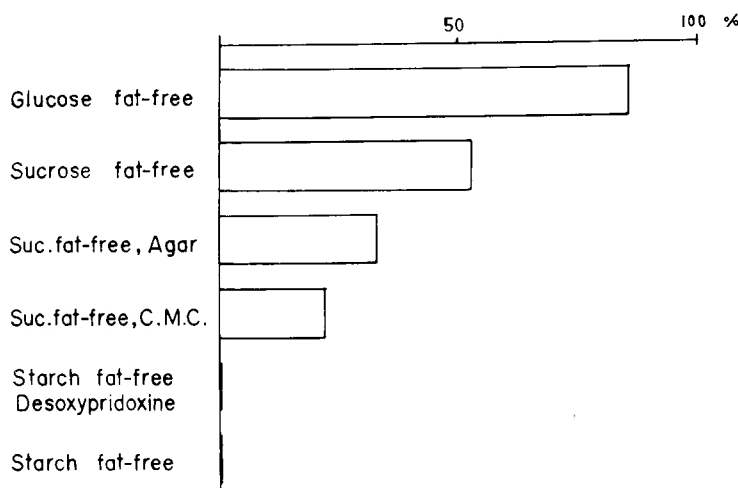


Fig. 30 Effect of Indigestible Substances on the Cholesterol Stone Formation in Hamsters

have generally made use of the basal diet which was supplemented with them, e. g. pyridoxine, biotin, inositol, folic acid and vitamin K₃.

At that time when some experiments were planned and began to start, Shioda, one of coworkers in our laboratory, found the report by DAM et al.¹³⁾ in 1952 that they had observed the regular occurrence of cholesterol stones in hamsters by means of placing them on a fat-free diet with sucrose as carbohydrate component. After receiving the references from DAM, we have been astonished to know their extensive works that in hamsters cholesterol stones would be to some extent protected by supplementation of a basal diet with lard, soybean oil, cod liver oil and so on¹⁴⁾, and starch made from grains of different sorts showed a complete protection against cholesterol stones¹⁵⁾. However, we have already planned and started some experiments by ourselves according to our own experimental hypothesis. We have thought the following two phenomena more important that the abnormal shift of the intestinal flora might disturb them to produce the different sorts of vitamins and that the hepatic biosynthesis of cholesterol would possibly increase when the animals received a diet with sucrose or glucose as carbohydrate component. An EFA deficient diet and the same diet with butter fat, that we have made use of for the first time to produce cholesterol stones in hamsters, are not containing pyridoxine, biotin, inositol, folic acid and vitamin K. In this sense, they are quite different from the lithogenic diets as those used by DAM et al.¹¹⁾ After reviewing the fact that those vitamins were quite indifferent to the occurrence of cholesterol stones in hamsters, though they were added to our own basal diet, each or together, we have made up our mind of using another basal diet of which vitamins and other components were nearly equal to the lithogenic diets used by DAM et al.

Different groups of hamsters received different sorts of diets, to which different sorts of fats and oil were added. Sesame oil, butter fat, lard, and sesame oil plus lard were added to the basal diet with sucrose or glucose as carbohydrate component and with pyridoxine, biotin, inositol, folic acid and vitamin K₃ together, respectively. According to the minimum amounts of EFA estimated by DEUEL et al.¹⁷⁾, these fat diets are thought to have

in them enough EFA needed to keep the animals alive and growing well. In case of a sesame oil-diet, the rate of absorption of EFA became far more greater than in case of a butter fat- or a lard-diet. On the other hand, in case of a butter fat-diet or a lard-diet there was a reciprocal relation in the rate between EFA and the saturated fatty acids plus oleic acid, and in case of a diet with sesame oil plus lard it was an intermediate of the two. In short, the difference was found only in the ratio of the saturated fatty acids plus oleic acid to EFA in the ingested lipids. As far as the incidence of cholesterol stones may be concerned, the greater was the ratio of the saturated fatty acids plus oleic acid to EFA in those fat diets, the higher was the incidence of cholesterol stones. However, if the animals would receive any of those fat diets, the incidence would be always to some extent lowered, as compared with the animals receiving an absolutely EFA deficient diet. This also suggests that EFA has an important relation to the initiating factors of cholesterol stones (Fig. 31 a, b, c)^{20), 60)}.

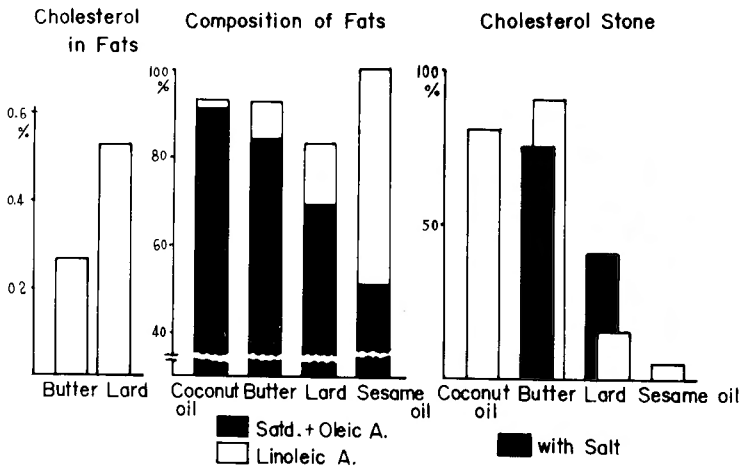


Fig. 31 a Effect of Fats on the Cholesterol Stone Formation in Hamsters

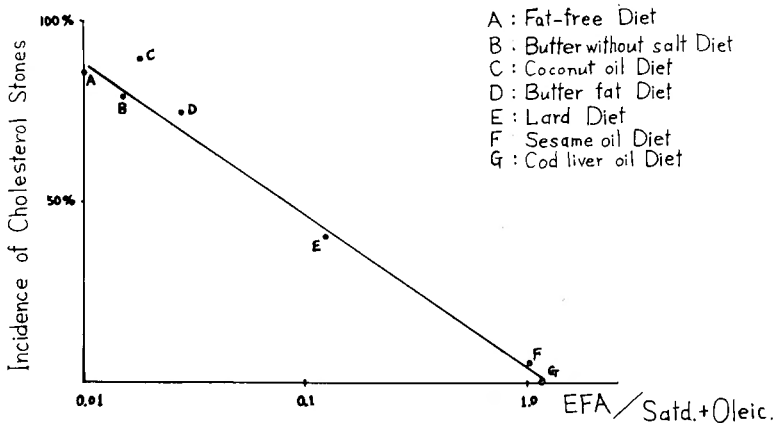


Fig. 31 b The Relationship between EFA in the Dietary Fats and the Incidence of Cholesterol Stones in Hamsters

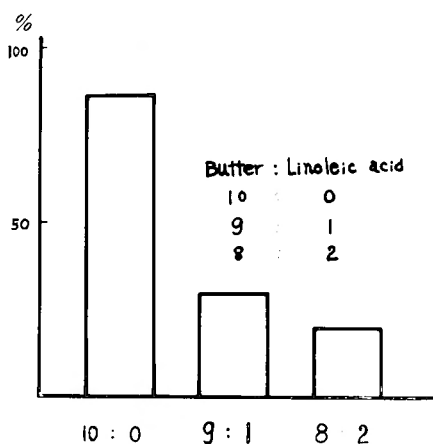


Fig. 31c Effect of Linoleic Acid on the Cholesterol Stone Formation in Hamsters Fed Glucose and Butter Fat.

From the view-point of clinical practice, every human subjects, as Europeans, Americans or Japanese, are thought to be taking in enough EFA (it is found in both vegetable foodstuffs and animal). In fact, the biochemical analysis in our laboratory revealed that the liver of the patients with cholesterol stones had sufficiently linoleic acid in it³⁰⁾. It is quite useless, therefore, only to research on the initiating factors of cholesterol stones, that can be easily produced as the animals have fallen into a state of complete deficiency in EFA. In other words, it is impossible to think that EFA deficiency would be an only cause for cholesterol stone formation. We must find out the details in the mechanism of cholesterol stone formation in the animals

receiving those fat diets. It is thought to be necessary for us to apply them to a case of cholesterol stones in human subjects. It is as evident as described above that in hamsters none of the vitamins, as pyridoxine, biotin, inositol, folic acid and vitamin K₃, did not show any protective properties against the development of cholesterol stones, though they were thought to have at least any important influences upon cholesterol stone formation. Here we must notice that all of the vitamins added to the basal diet in the former experiments were those as known as inactive forms. It is of no use, therefore, that those inactive vitamins are added to the basal diet, because it is said that the intestinal flora can produce active vitamins to meet the ordinary demand of their host. We have already demonstrated in the previous experiments in rats that pyridoxine had the most important influence upon the metabolisms of EFA and bile acids. Thus we have administered one or two mg of pyridoxal phosphate to each of one group of hamsters receiving a sesame oil diet every day and have succeeded, for the first time, in keeping the animals free from cholesterol stones. Although pyridoxal phosphate was administered to the animals, when they received a diet, from which fats, especially EFA, were completely removed, the incidence of cholesterol stones was as high as they received no treatment of pyridoxal phosphate. It has become evident that we had to let the animals to keep enough EFA and pyridoxal phosphate at the same time in their bodies in order to protect them against cholesterol stones (Fig. 32). This also indicates that pyridoxal phosphate are playing an important role in EFA metabolism in vivo^{46), 63)}. When we treated the other group of hamsters receiving a lard diet with the same dosis of pyridoxal phosphate every day, we could also keep all of them free from cholesterol stones.

In view of these facts, we have next placed hamsters on an EFA-and pyridoxine-deficient diet, in which starch was chosen as carbohydrate component and to which desoxypyridoxine was added as antipyridoxine, but we have failed to produce cholesterol stones (Fig. 30). When the abnormal shift of the intestinal flora, *dysbacteria*, may be induced in the animals by keeping them to receive a diet with sucrose or glucose as carbohydrate component, they will not be able to produce any more pyridoxal phosphate, so

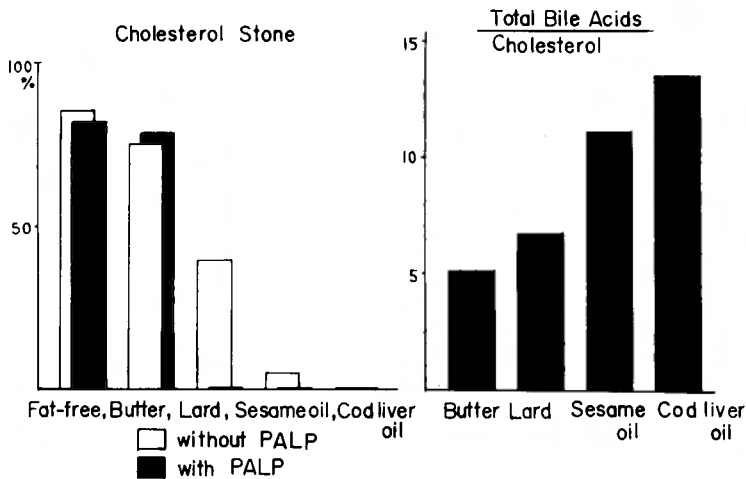


Fig. 32 Effect of PALP (Pyridoxal phosphate) on the Cholesterol Stone Formation in Hamsters

that they will become short of it. If this were truth, it would make the bile to lower its capacity keeping cholesterol in solution through the disturbance in EFA metabolism. However, cholesterol stones have never been formed by this mechanism only. Thus, it appears that another factor is at least necessary for cholesterol stone formation, besides the above-mentioned conditions, as deficiency in pyridoxal phosphate, disturbances in pyridoxine activity and so on.

After reviewing the actions of the main vitamins formed by the intestinal flora, we have found that vitamin K_1 was the most important among them in the next place of pyridoxal phosphate. Vitamin K that we have got used to add to the basal diet was vitamin K_3 . We have taken notice that there was a difference between vitamin K_3 , K_4 and vitamin K_1 , K_2 , because it was said that the formers were synthetic but the latters were natural. Then one or two mg of vitamin K_1 was subcutaneously administered instead of pyridoxal phosphate to the different another groups of hamsters receiving an EFA deficient diet, a sesame oil diet, a lard diet, respectively (each of these diets contained sucrose or glucose as carbohydrate component and contained pyridoxine, inositol, biotin and folic acid as well), in the same way as the former groups were treated with pyridoxal phosphate. Cholesterol stones were completely protected in each of the groups (Fig. 33)²⁰⁾. The reasons why cholesterol stones were frequently formed only in case of feeding sucrose or glucose and why not any formed in case of feeding starch, though the conditions might be changed, are now thought as follows; the bacterial production of pyridoxal phosphate and vitamin K_1 , K_2 will be possibly minimized through *dysbacteria* in the former case, because the diets with sucrose or glucose as carbohydrate component don't contain the indigestible substances needed as nutrients for the intestinal flora to keep their multiplication normal at the lower part of the intestine, while in the latter case the intestinal flora will escape *dysbacteria* and will be able to produce pyridoxal phosphate and vitamin K_1 , K_2 as much as they do under the normal condition to meet their host's demand for the vitamins. It seems most likely from the present experiments that cholesterol stones

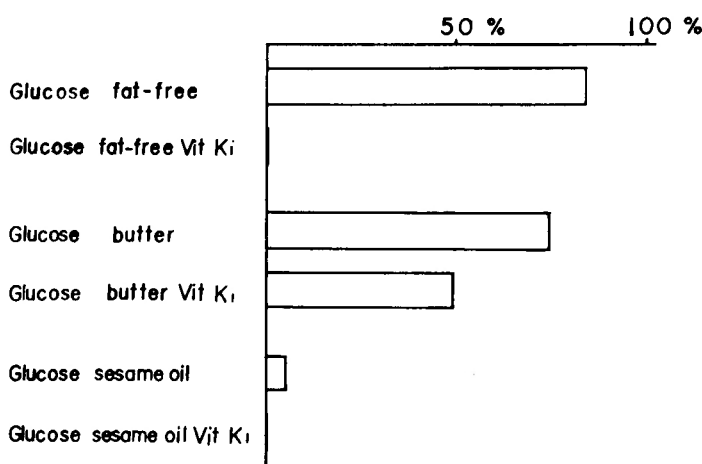


Fig. 33 Effect of Vitamin K₁ on the Formation of Gallstones

may be formed more frequently in those who are taking in large amounts of proteins and fats, especially those who are taking in more sucrose at the same time, as Europeans and Americans. According to the reports by YUDKIN⁸⁹⁾, some of them were taking in more than 130 to 140 g of sucrose for one day. It can be thought, therefore, that some of cholesterol stones would have been formed by the same mechanism as described above in Europeans and Americans⁸⁾. It cannot be neglected the possibility that cholesterol stones also will be possibly formed by prolonged administration of antibiotics^{16), 35)}. However, the abnormal shift of the intestinal flora produced by antibiotics is not the same as that by the difference between carbohydrates. It is quite uncertain whether the prolonged administration of antibiotics would be always accompanied with cholesterol stone formation. As it may be true in some of Europeans and Americans, we can hardly regard this as an initiating factor of cholesterol stones in our Japanese patients. As it is evident from this, the etiology of cholesterol stones is far more complex than it is and it will remain obscure. We have next had a question whether there were any other factors producing consequently the same condition as *dysbacteria*. When we have kept the experimental animals receiving the various diets with sucrose or glucose as carbohydrate component as well as with vitamins, pyridoxine, biotin, inositol, folic acid and vitamin K₃, for example a butter fat diet, a lard diet and a sesame oil diet, they revealed the fact that the greater was the ratio of saturated fatty acids plus oleic acid to EFA, the higher was the incidence of cholesterol stones. We could not help paying our attention to the difference in characters of the lipids taken up from the diets and have performed a number of experiments so as to get more knowledge about that.

According to the results from our previous experiments dealing with interrelation between incidence of diabetes mellitus and compositions of the ingested lipids⁵²⁾ we have decided ourselves to make use of butter fat which had in it great quantities of lower saturated fatty acids and salt more than did the other kinds of animal fats. Thus another experiment was made by using three groups of hamsters. In the first group the animals received a butter fat diet only (Fig. 31 c), the second received the same diet and was treated with one or two mg of pyridoxal phosphate at the same time every day, and the

third receiving the same diet was also treated with one or two mg of vitamin K_1 instead of pyridoxal phosphate every day. Cholesterol stones occurred at the level of high percentage in each of the three groups. On the other hand, when the animals received a diet to that lard, another sort of animal fats, was added instead of butter fat, and further they were treated with pyridoxal phosphate or vitamin K_1 at the same time, none of cholesterol stones was observed in all of the animals (Figs. 32 and 33)²⁰. These two experimental results seemed to indicate that there was a true cause for cholesterol stone formation in the difference between compositions of butter fat and lard. The difference was summarized as follows; (i) Butter fat has in it great quantities of lower saturated fatty acids whereas lard hardly has any of them in it. (ii) There is difference between butter fat and lard in relation to cholesterol content. (iii) Butter fat contains salt at the level of ca. 2%, but lard does not. In short, judged from the experimental results, we have come to the conclusion that cholesterol stones would be possibly initiated, as the absorption of lower saturated fatty acids increased, in other words, as the animals received a diet with animal fats containing greater quantities of the lower saturated fatty acids (Fig. 31 a, b). Cholesterol stones are thought to be formed by charging the animals with such a dietary condition, though they had a great store of pyridoxal phosphate or vitamin K_1 , K_2 in vivo. It is well known from the clinical reports that in Japan recently cholesterol stones are apt to occur far more frequently in city dwellers than countrymen in parallel with the improvement of dietary compositions (*Westernization*!) and that there is a causative relation between cholesterol stone formation and the quantitative and qualitative changes of animal fats.

We are going to work harder to find out the true cause for cholesterol stone formation according to our concepts that we must first of all solve a number of questions concerning the lower saturated fatty acids. In fact, we have found that even in case of using starch (mashed potato) as carbohydrate component, cholesterol stones occurred frequently in hamsters when they received a diet with animal fat, for example butter fat, that was containing greater quantities of lower saturated fatty acids (Fig. 34)²⁰.

As shown in Fig. 34, cholesterol stones were formed with high rates, when starch,

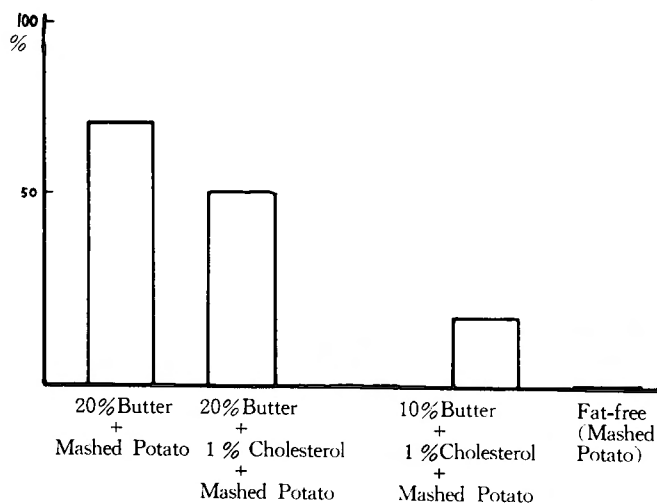


Fig. 34 Incidence of the Cholesterol Stone Formation in Hamsters Fed a Starch Diet

in the form of mashed potato as a carbohydrate source, was given to hamsters with butter alone and with both butter and cholesterol. As is generally known, untreated raw starch, such as potato starch, is not soluble in cold water and is not degraded by any digestive enzyme. It is therefore of β -form. But when this β -form starch is heated in water to a certain degree of temperature (cooked), its chemical micelle is suddenly separated, swells, is gelatinized, and makes α -form starch which is easily digested and absorbed. The mashed potato is this α -form starch and human beings usually take a carbohydrate in the form of α -starch.

V. INTERRELATION BETWEEN CHOLESTEROL STONE FORMATION AND BILE CONSTITUENTS OF HAMSTERS

Hepatic bile was collected from each of hamsters of different groups kept under the same experimental conditions by using a small polyethylene tube placed in the biliary duct. Bile acids, cholesterol, lecithin and its fatty acid constituents were determined, respectively and the interrelation between the rate of incidence of cholesterol stones and those bile constituents was examined as well. We have analysed principally hepatic bile instead of analysing bladder bile^{49),50)}, since we have devoted ourselves to investigate the biosynthesis of cholesterol and its degradation into bile acids in liver. At first we have compared the animals receiving an EFA deficient diet with sucrose or glucose as carbohydrate component (pyridoxine, biotin, inositol, folic acid and vitamin K₃, though they were known as inactive, were all added to the diet) with those fed another EFA diet with starch. We have found that in the former total bile acids decreased in the drained hepatic bile whereas cholesterol increased to a great extent so that the ratio of total bile acids to cholesterol decreased (Figs. 35 and 36). There were no differences between the two groups in the ratio of lecithin to cholesterol as well as in the fatty acids that were constituting lecithin, especially in their percentage. EFA decreased to the same extent in two groups (Figs. 37 and 38). This seemed to be reflecting only a dietary condition of a fat-free diet. The hepatic fatty acids of normal hamsters were compared with those from normal rats. In hamsters analysis of the hepatic fatty acids showed an interesting finding that eicosatetraenoic acid was lower in percentage than in rats¹⁸⁾. In the sucrose- or glucose-group cholesterol stones occurred fre-

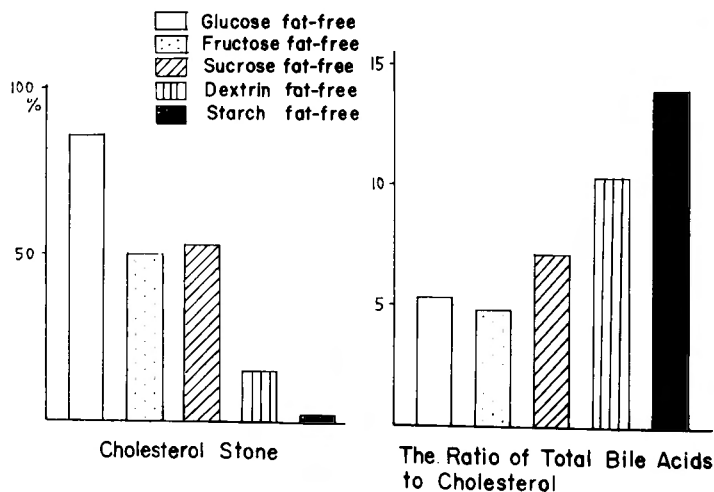


Fig. 35 Effect of Carbohydrates of the Cholesterol Stone Formation in Hamsters

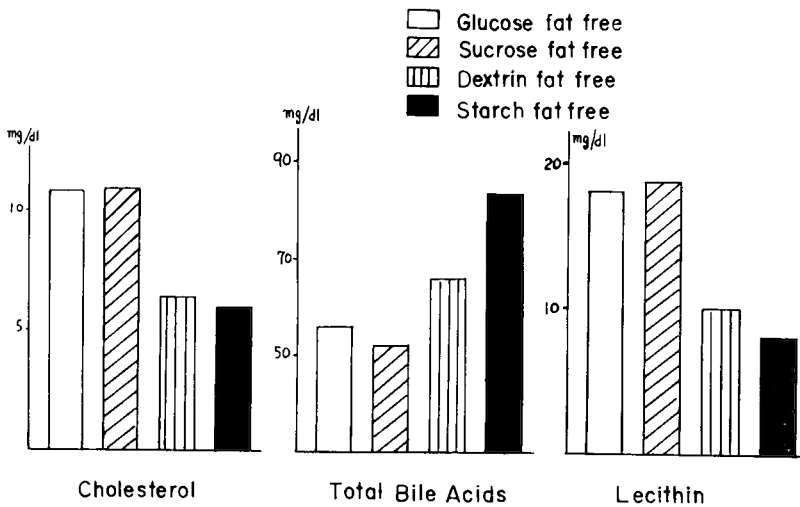


Fig. 36 Effect of Carbohydrates on Bile Components in Hamsters

quently in hamsters, but in the starch-group none of them occurred (Fig. 37). And in the latter lecithin in drained hepatic bile was lower in content than in the former (Fig. 36). It seemed most likely that cholesterol stone formation may be due to the unbalance between the biosynthesis and degradation (assimilation and dissemilation) of cholesterol in the liver. The decrease of total bile acids and the decreased ratio of total bile acids

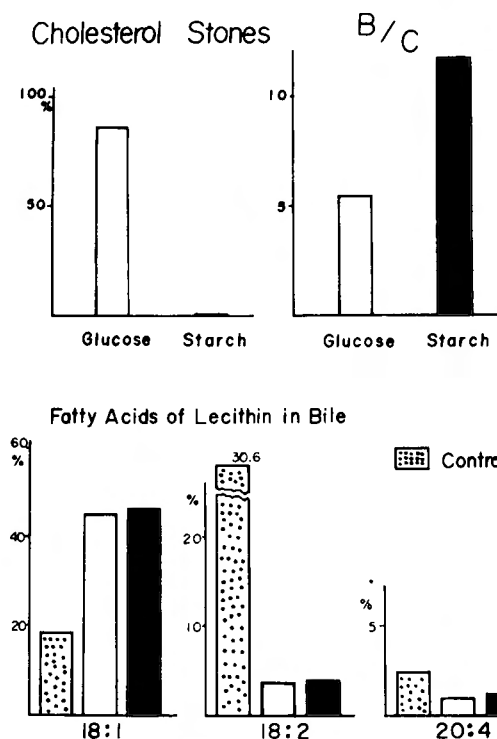


Fig. 37 Differences of Effects on the Cholesterol Stone Formation between a Glucose and a Starch Diet.

to cholesterol in bile are thought to be responsible for crystallization and precipitation of cholesterol. Ultimately we have come to the conclusion that the changes in the ratio of total bile acids to cholesterol were the most important of all the other wellknown factors. We have already reported in the previous experiments by using rats that the decrease of total bile acids and that of lecithin were equally effective to let cholesterol to crystallize and precipitate in bile. This depends on the following reasons why we have kept the animals receiving a diet with starch as carbohydrate component, have noticed only the difference between supplementation of the diet with sesame oil and non supplementation, and have never taken notice the difference between carbohydrates.

Although hamsters received an EFA deficient diet with sucrose or glucose as carbohydrate component, and at the same time they were treated with pyridoxal phos-

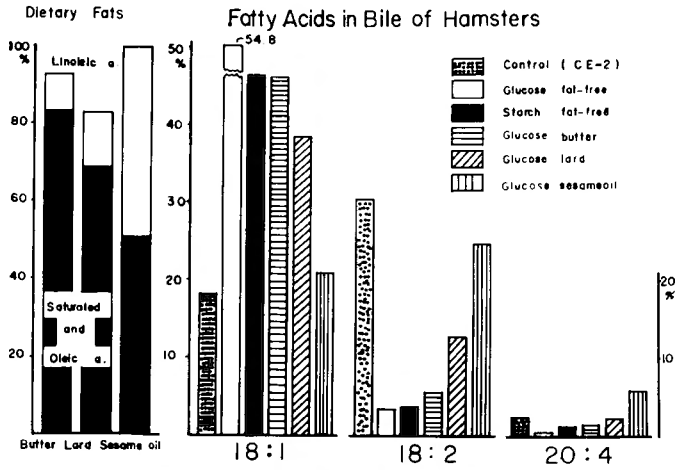


Fig. 38 Effect of Dietary Fats on the Fatty Acid Composition in the Bile of Hamsters.

phate, cholesterol stones could not be completely protected yet. However, they were not kept free from cholesterol stones until they received the same diet with minor modification such as the supplementation of sesame oil and were treated with pyridoxal phosphate at the same time. In the former case, the effect of pyridoxal phosphate was not so remarkable as to increase total bile acids and lecithin in bile, while in the latter case, there was a marked increase in both total bile acids and lecithin. And pyridoxal phosphate would possibly have an inclination to decrease the biliary excretion of cholesterol in the latter case, too. This also indicate that pyridoxal phosphate has a close relation to EFA metabolism in vivo. When the animals received the diet supplemented with lard instead of sesame oil and were treated with pyridoxal phosphate as well, total bile acids and lecithin also increased to a great extent in bile and cholesterol was also inclined to increase in a way different from the case of supplementation of the diet with sesame oil. In this case most animals were kept free from cholesterol stones. The increase of cholesterol in bile would be in part due to the fact that lard had more cholesterol in it than sesame oil, or in part due

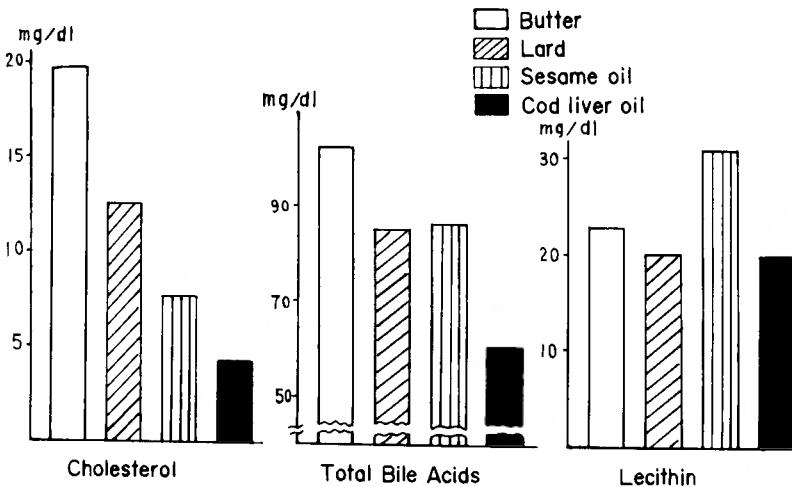


Fig. 39 Effect of PALP on Bile Components in Hamsters

to the difference in quality between the absorbed lipids (Figs. 39 and 32). If the animals would absorb any sort of lipids, that the ratio of saturated fatty acids plus oleic acid to EFA was greater, the hepatic biosynthesis of cholesterol would be possibly stimulated. Although lard had more cholesterol in it than butter fat, cholesterol concentration of bile was higher in case of feeding a butter fat diet than a lard diet, as we compared the results from the animals receiving the butter fat diet with those from the animals on the lard diet. This seemed to indicate that the hepatic biosynthesis of cholesterol would be inclined to increase extremely, as the animals would take in any of the lipids with the higher ratio of saturated fatty acids plus oleic acid to EFA, and that the ratio would have a greater effect upon the hepatic biosynthesis of cholesterol than did the concentration of cholesterol in the diet. As it was evident from the experimental results, pyridoxal phosphate had not any influence upon the biosynthetic process of cholesterol in the liver but had an influence upon the catabolic process of cholesterol into bile acids by affecting indirectly the proper metabolic process of EFA. It can be thought, therefore, that deficiency in pyridoxal phosphate will possibly invite the disturbances in the hepatic biosynthesis of bile acids through the metabolic disturbances in EFA. We have again carried out the same sort of experiments by using vitamin K₁ instead of pyridoxal phosphate, have known that vitamin K₁ was an essential substance to keep up cholesterol biosynthesis normal in the liver^{8,11}, and have also obtained the following result that deficiency in vitamin K₁ would possibly increase to a great extent the hepatic biosynthesis of cholesterol (Figs. 40 and 33). It has been now well known that vitamin K₁ was also greatly concerned in the formation of dehydrogenases¹⁰. The reason why cholesterol stones were liable to develop more frequently in case of feeding sucrose or glucose than in case of feeding starch is thought to be as follows: in the former the abnormal shift of the intestinal flora, *dysbacteria*, was produced by short-coming of the indigestible substances that the intestinal flora made use of as their nutrients at the lower part of the intestine, so that deficiency in many sorts of vitamins that the intestinal flora were used to supply a greater part of their host's demand, for example, deficiency of pyridoxal phosphate and vitamin K₁, K₂ caused an extraordinary increase in the hepatic

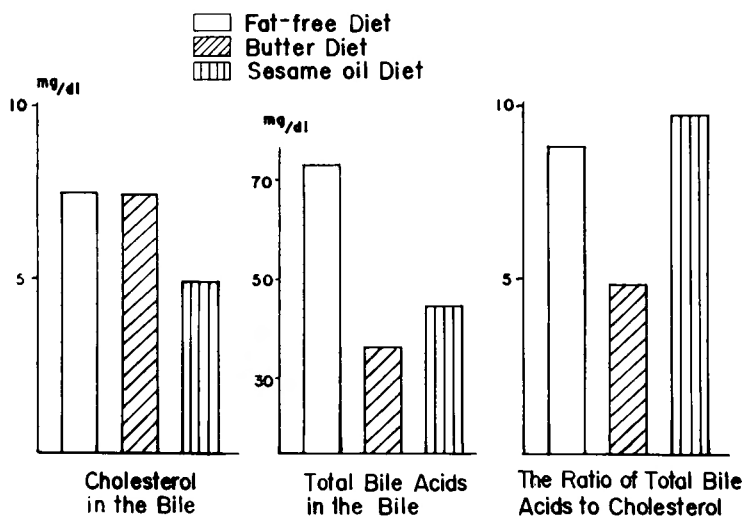


Fig. 40 Effect of Vit. K₁ on Bile Composition in Hamsters

biosynthesis of cholesterol and disturbed the catabolic process of cholesterol in the liver at the same time. Cholesterol excreted excessively in bile began to crystallize and precipitate on account of the decrease of total bile acids as well as qualitative changes of compositions of bile, and then cholesterol stones would be possibly initiated. Moreover it has been well known that gallbladder is necessary for cholesterol stone formation. The reason why cholesterol stones were not formed in case of feeding starch is that there was not any of such remarkable changes and the bile constituents have never changed extremely so as to initiate cholesterol stones.

We have never kept the animals free from cholesterol stones by means of treating them with pyridoxal phosphate only. We have succeeded in keeping them free from cholesterol stones not only by keeping them to receive a diet supplemented with lipids containing large amount of EFA instead of the lipids containing lower saturated fatty acids, but also by treating them with pyridoxal phosphate at the same time, though sucrose or glucose were chosen as the main source of carbohydrate. Cholesterol stones have been also protected completely only by treating the animals with vitamin K_1 . This seemed to indicate, therefore, that there was difference between vitamin K_1 and pyridoxal phosphate in their protective properties against cholesterol stone formation. Although we have been able to keep the animals, receiving an EFA deficient diet, free from cholesterol stones by using vitamin K_1 only, we have failed to do it by treating them with pyridoxal phosphate only. It seems most likely from this fact that EFA had none of any physiological effects upon the hepatic biosynthesis of cholesterol. However, when butter fat was added to the basal diet with sucrose or glucose as carbohydrate component and with pyridoxine, biotin, inositol, folic acid and vitamin K_3 , cholesterol stones were observed in all of the animals, though they were treated with vitamin K_1 or pyridoxal phosphate, total bile acids decreased, but cholesterol increased in bile, so that the ratio of total bile acids to cholesterol decreased to a great extent and the ratio of lecithin to cholesterol also decreased at the same time. It has been now fairly well known that the animals would be fallen into the same condition as *dysbacteria*, regardless of deficiency in vitamin K_1 and pyridoxal phosphate, as they would absorb the greater quantities of saturated fatty acids, especially lower saturated fatty acids. In hamsters cholesterol will possibly crystallize promptly, will precipitate, and then will grow up to cholesterol stones, when the ratio of total bile acids to cholesterol becomes at least lower than 6.0. It has been estimated by comparing the lowest ratio from the animals placed on an EFA deficient diet with the ratio from those on a lard diet and treated with vitamin K_1 or pyridoxal phosphate at the same time.

VI. SUMMARY AND CONCLUSION

We have carried out a number of experiments by using rats and hamsters and have obtained the following conclusions.

- 1) Cholesterol stone formation may be attributed to the unbalance between the biosynthesis and degradation of cholesterol in the liver.

- 2) Deficiency in pyridoxal phosphate and vitamin K_1 , K_2 produced by the abnormal shift of the intestinal flora, *dysbacteria*, will be thought to be responsible for causing the unbalanced condition. However only absorption of the greater quantities of saturated fatty acids, especially lower saturated fatty acids will possibly keep up the experimental animals

under the same condition as *dysbacteria* regardless of deficiency in pyridoxal phosphate and vitamin K_1 , K_2 .

3) Quantitative and qualitative changes of the total bile acids in bile will be far more important for causing cholesterol to crystallize and precipitate than those of lecithin and its constituting fatty acids.

4) Therefore, it will restrict us to find an adequate measure necessary for preventing cholesterol stones.

5) Pyridoxal phosphate has a closer relation to EFA metabolism. It has also an important influence upon the hepatic biosynthesis of bile acids through EFA metabolism. Simultaneous administration of EFA and pyridoxal phosphate is always necessary for getting the smoother biosynthesis of bile acids in the liver and only one of them is inadequate for it.

6) Simultaneous administration of the higher unsaturated fatty acids, as cod liver oil, and the active vitamins have also showed a good protection against cholesterol stones. However, in human body, main unsaturated fatty acids are linoleic acid, linolenic acid, oleic acid, palmitoleic acid and etc. As far as the etiology of human cholesterol stones may be concerned, it will be possibly sufficient for us to discuss about those unsaturated fatty acids only.

7) We have come to the supposition that most of cholesterol stones would be formed by taking in excess animal fats containing the greater quantities of lower saturated fatty acids, though few of them would be initiated through *dysbacteria*.

8) As we have already reported that the greater quantities of EFA was found in adrenals and EFA had a closer relation to their functions, the metabolic disturbances in EFA will possibly lead to the development of adrenocortical hypofunction. We have also succeeded in keeping the animals free from cholesterol stones by using glucocorticoids. This seemed to indicate that we would have to take into consideration the adrenocortical hypofunction induced by the metabolic disturbances in EFA before we discussed the initiating factors of cholesterol stones. It has been well known that the adrenocortical hypofunction had also an important influence upon the mechanism of cholesterol stone formation through the metabolic disturbances in EFA (Fig. 41).

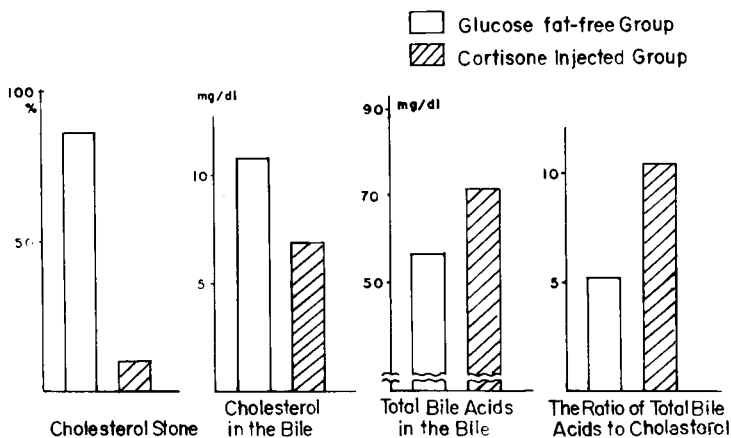


Fig. 41 Effect of Cortisone on the Cholesterol Stone Formation in Hamsters²⁰⁾

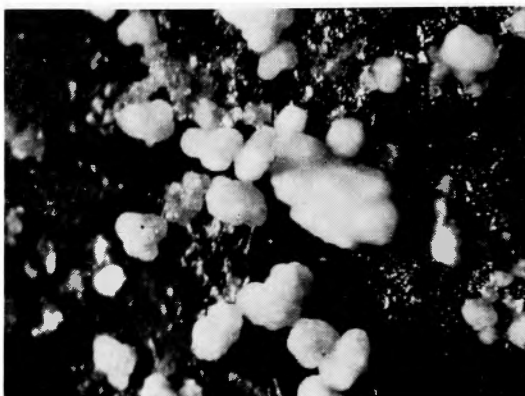


Fig. 42 Cholesterol Stones in Hamsters Fed
a Glucose Butter Diet

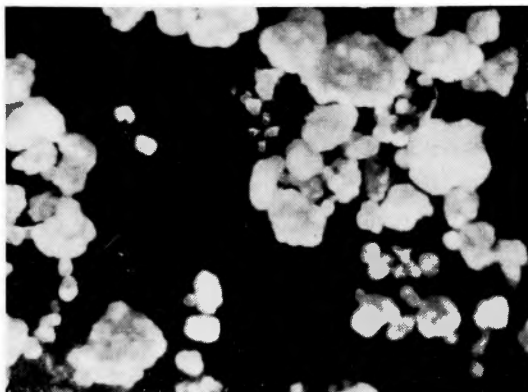


Fig. 43 Cholesterol Stones in Hamsters Fed
a Mashed Potato Butter Diet

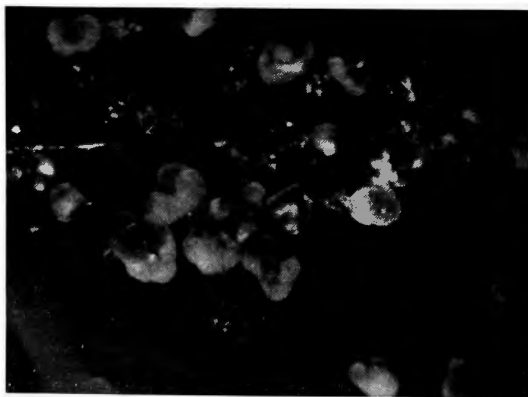


Fig. 44 Cholesterol Stones in Hamsters Fed
a Glucose Fat-free Diet

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