LIPID METABOLISM IN THE DEVELOPMENT OF CHOLESTEROL GALLSTONES IN HAMSTERS
IV. THE EFFECT OF ESSENTIAL PHOSPHOLIPIDS AND PLANT STEROLS ON THE BILIARY LIPIDS*)

By

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ABSTRACT

Female golden hamsters were fed either with a lithogenic diet alone (72. 3% glucose diet according to Dam's[1] prescription) or in combination with 0. 7 ml/2days of 5 g/dl essential phospholipids (EPL) or 2. 9% plant sterols for 30 days.

Hamsters fed with the lithogenic diet were found to have produced a high level of cholesterol (lithogenic) in bile as well as formation of gallstone in the gallbladder.

Analysis of the bile lipids proved that EPL very slightly (NS) and plant sterols significantly lowered the lithogenic index of bile preventing gallstone formation. EPL increased not only biliary phospholipid but also cholesterol and total bile acid concentrations. But the increased total bile acids seemed to be due to the increased deoxycholic acid contaminated in an EPL solution as a detergent. Fatty acid analysis of biliary phospholipids revealed that the increased phospholipids contained a large amount of C 18 : 2 and C 18 : 3.

The administration of plant sterols decreased not only biliary cholesterol but also phospholipids accompanied by alteration of their fatty acid composition. It also decreased chenodeoxycholic and its secondary bile acid (lithocholic acid) concentrations diminishing their pool sizes.

However, the administration of plant sterols did not alter the glycine/taurine ratio which is often elevated in various hepatobiliary diseases.

EPL and plant sterols improve lithogenic bile and prevent gallstone formation not only by affecting biliary cholesterol, phospholipid and bile acid concentrations but also by altering the fatty acid composition of phospholipids and some of bile acid concentration and their pool sizes.

INTRODUCTION

The results of our previous experiments[2-4] suggested that not only the ratio of biliary bile acids to cholesterol but also the ratio of biliary lecithin to cholesterol plays an important

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role in the lithogenesity of bile in hamsters and that biliary lipids are closely related to serum lipids and lipoproteins in these animals. To increase biliary lecithin without increasing biliary cholesterol or to decrease biliary cholesterol without decreasing biliary lecithin and bile acids will be useful for the prevention of gallstone formation and development. Essential phospholipids and plant sterols which affect the serum lipid concentration by mutually different mechanism of action, are able to influence the lipid composition of bile during the development of gallstone.

In the present experiment the effect of EPL and plant sterols on the bile lipid composition was investigated in hamsters fed with a lithogenic diet containing 72.3% glucose.

MATERIALS AND METHODS

I. Experimental procedures:

1. Fifteen female golden hamsters were fed with a lithogenic diet containing 72.3% glucose prescribed by Dam[1]. for 30 days. Five of them were orally infused with 0.7 ml/2days of EPL solution (5 g essential phospholipids–70% linoleate C18 : 2 and 30% linoleniate C18 : 3 and others–2.3 g deoxycholic acid and vitamines in 100 ml solution, Nippon Shoji Co.–Nattermann) using a polyethylene cannula. Another five were given 2.9% plant sterols (55.2% β–sitosterol, 38.8% campesterol and 6.0% stigmasterol, Sigma Chemical Co.) mixed in a lithogenic diet mentioned above. Remaining five were given a lithogenic diet alone as a control group.

Thirty days after feeding, the abdomen of animals was opened under light anesthesia, a 0.61 mm O.D. polyethylene cannula was inserted into the common bile duct after ligation of cystic duct and bile was collected for an hour. At the same time the presence or absence of gallstone formed in the gallbladder in all the animals was recorded.

2. Additional ten animals were fed with the same lithogenic diet but half of them were given the lithogenic diet supplemented with 2.9% plant sterols for 30 days. After the animals were kept in fast overnight, the abdomen was opened and a 0.61 mm O.D. polyethylene cannula was indwelt for 7–10 hours during when bile was collected entirely. The pool size of bile acids was determined according to

the washout technique described by Mok et al[5]. The operation was deliberately timed for performance from 9 : 00 to 10 : 00 a.m. to avoid fluctuations in the diurnal rate of bile and cholesterol syntheses.

II. Analytical procedures:

Bile was collected into 0.5 ml plastic test tubes surrounded with crushed ice and was kept at $-20°C$ until analysis.

Individual bile acids and cholesterol were analyzed by a Shimadzu GC–6A gaschromatograph with 2.5% OV–1 as described earlier[6,7]. Biliary phospholipids (lecithin) were determined by an enzyme method[8].

The fatty acid composition of biliary phospholipids was analyzed by a Shimadzu GC–4A gaschromatograph as described earlier[1-3].

Analysis of conjugated and unconjugated forms of bile acids was also performed by a high performance liquid chromatograph (HPLC) and with immobilized 3α–hydroxysteroid dehydrogenase in column form[9] (Nippon Bunko HPLC–Trirotar II with solvent programmer GP–A30 and fluorometer UVIDEC–100III). The operating conditions were as follows: mobile phase, A–CH_3CN/10 mM KH_2PO_4 (pH 7.8) : 40/80 v/v, B–CH_3CN/30 mM KH_2PO_4 (pH 8.0) : 20/80 v/v, gradient B/A ratio 0/100 at 0 time and 55/45 at 64 min. detection, fluorescense, $\lambda_{ex}=365$ nm, $\lambda_{ex}=470$ nm.

The lithogenic index was calculated according to the methods described by Admirand and Small[10] and Holzbach[11] and modified by Thomas[12] et al. from which the saturation index was drawn.

RESULTS

A. Gallstone formation and biliary lipid composition in hamsters fed with the lithogenic diet alone, or in combination with EPL or plant sterols:

1. Gallstone formation:

Two of the five hamsters had gallstones in their gallbladder as a result of 30–day administration of lithogenic diet but no stone was formed in hamsters fed with a lithogenic diet in combination with EPL or plant sterols. (Fig. 1)

Stones formed were soluble in chloroform, methanol or diethylether and gaschromatographic analysis revealed that they were composed of 99% free cholesterol.

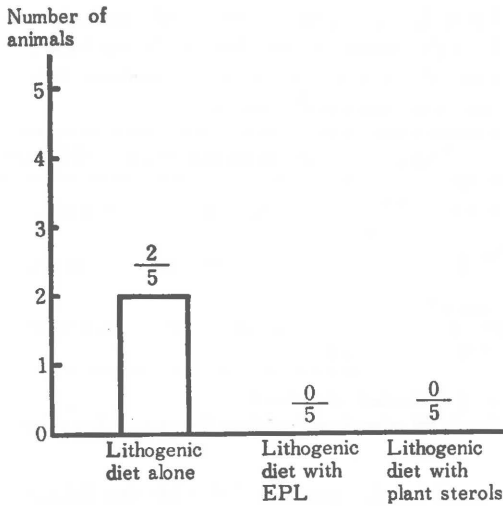2. Concentration and percent composition of

Number of animals

Fig. 1. Number of animals with gallstones

: Lithogenic diet alone [5]

: Lithogenic diet with EPL [5]

: Lithogenic diet with plant sterols [5]
N. S. : not significant

Fig. 2. Concentration of biliary cholesterol, phospholipids and total bileacids in hamsters fed with lithogenic diet alone or in combination with EPL or plant sterols.

biliary cholesterol, phospholipids and total bile acids:

As shown in Table 1, Figures 2 and 3, EPL significantly increased biliary cholesterol, phospholipid and total bile acid concentrations (p< 0.01), and EPL hardly altered the percent composition of these three biliary constituents. Plant sterols decreased cholesterol (p<0.01) and phospholipid concentrations (p<0.05). But the change in the total bile acid concentration was not significant as shown in Figure 2. Therefore, the percent composition revealed a relative increase in total bile acid proportion as shown in Figure 3.

3. Lithogenic indices

Figure 4 shows lithogenic indices by the methods reported by two investigators. The average index only slightly moved to the right-lower area in the coordinate by administration of 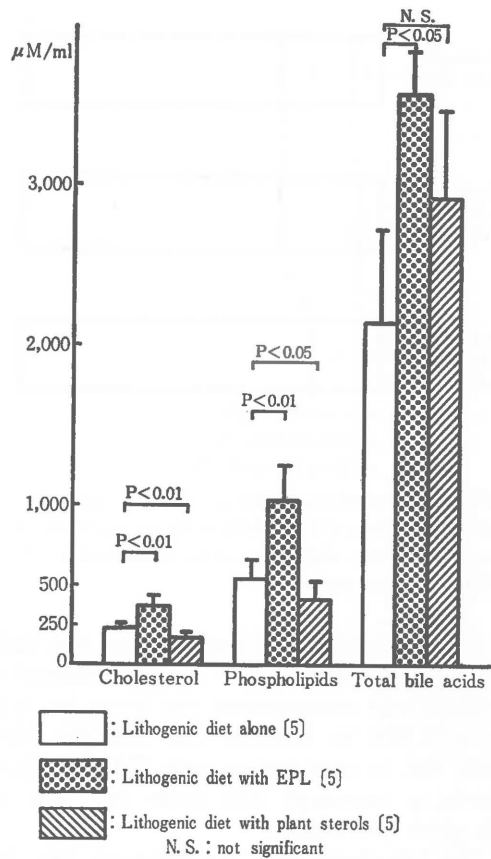EPL. On the contrary it moved significantly to the left-lower area resulting in decreasing the lithogenesity of bile in animals treated with plant sterols.

4. Concentration and percent composition of individual bile acids:

Table 1. Concentration and percent composition of biliary cholesterol, phospholipids and total bile acids in hamsters fed with lithogenic diet alone and in combination with EPL or plant sterols

Group	Cholesterol μM/ml(%)	Phospholipids μM/ml(%)	Total bile acids μM/ml(%)
Lithogenic diet alone [5]	232.8±28.1 (8.18±1.51)	542.8±124.7 (19.00±4.99)	2140.2±586.8 (72.79±6.35)
Lithogenic diet with EPL [5]	385.5±63.6** (7.75±1.57)	1033.4±220.9** (20.61±3.78)	3574.6±267.0** (71.61±4.48)
Lithogenic diet with plant sterols [5]	172.0±31.1** (5.12±0.79)	408.2±115.0* (12.51±3.77)	2918.2±558.2 (82.53±3.52)

[ ] : Number of animals

*p<0.05 **p<0.01 (significant against lithogenic diet alone)

a : Cholesterol (%)
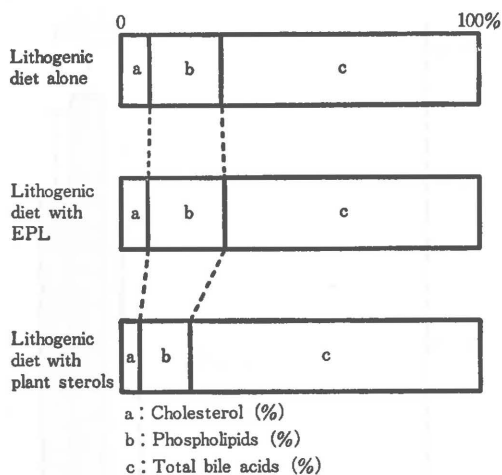b : Phospholipids (%)
c : Total bile acids (%)

Fig. 3. Percent composition of biliary cholesterol, phospholipids and total bile acids in hamsters fed with lithogenic diet alone or in combination with EPL or plant sterols

Table 2. Lithogenic indices of bile in hamsters fed with lithogenic diet alone or in combination with EPL or plant sterols by two methods, Admirand and Small, and Holzbach

Group		Admirand and Small	Holzbach
Lithogenic diet alone	[5]	0.849 ± 0.139	1.351 ± 0.138
Lithogenic diet with EPL	[5]	0.786 ± 0.149	1.210 ± 0.229
Lithogenic diet with plant sterols	[5]	$0.601 \pm 0.117^*$	1.167 ± 0.293

[   ] : Number of animals
*$p < 0.05$ (significant against lithogenic diet alone)

The deoxycholic acid concentration was higher ($p < 0.001$ or $p < 0.01$) and the chenodeoxycholic acid concentration was lower ($p < 0.05$ or $p < 0.001$) in hamsters fed with the lithogenic diet in combination with EPL and plant sterols as compared with those fed with the lithogenic diet alone.

The lithocholic acid concentration also decreased in the animals fed with the lithogenic diet in combination with plant sterols as compared with those fed with the lithogenic diet alone ($p < 0.01$).

As shown in Figure 6 in the percent composition of these individual bile acids the cholic acid content was larger than any other bile acids in animals fed with the lithogenic diet alone. On the other hand in the animals fed with the lithogenic diet in combination with EPL, the deoxycholic acid content was the largest constituent followed by the cholic, chenodeoxycholic and lithocholic acid propor-
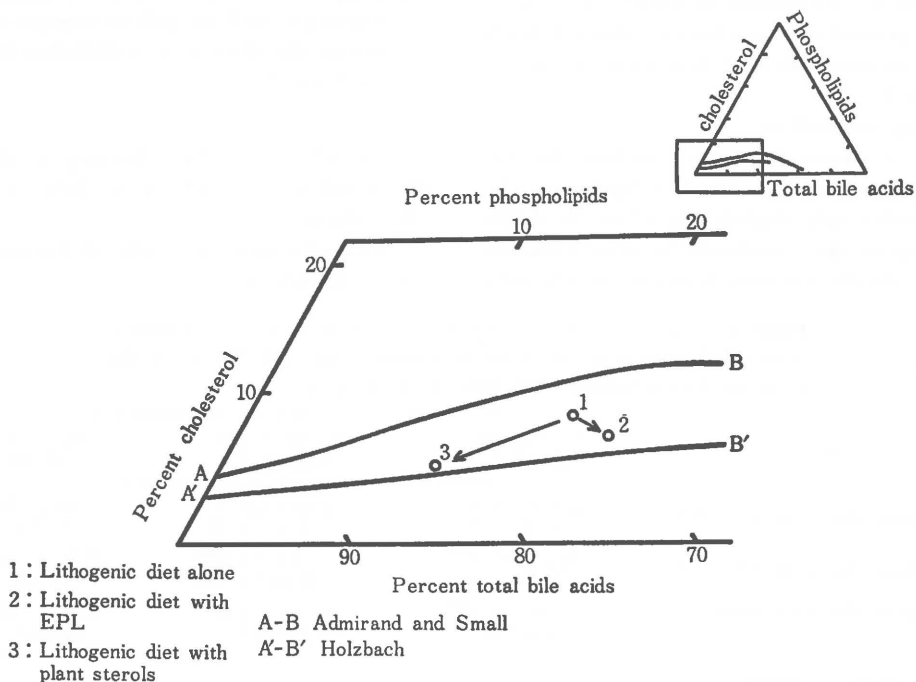

1 : Lithogenic diet alone
2 : Lithogenic diet with EPL
3 : Lithogenic diet with plant sterols

A-B Admirand and Small
A′-B′ Holzbach

Fig. 4. Lithogenic indices presented in the triangular coordinate

Table 3. Concentration and percent composition of individual bile acids in hamsters fed with lithogenic diet alone or in combination with EPL or plant sterols

Group		L C μmol/ml(%)	D C μmol/ml (%)	C D C μmol/ml (%)	C μmol/ml (%)
Lithogenic diet alone	[5]	105.6±41.5 (4.89±1.24)	416.2±108.8 (19.59±1.53)	687.0±201.4 (32.09±4.86)	931.4±285.2 (43.40±5.18)
Lithogenic diet with EPL	[5]	106.4±69.0 (3.09±2.15)	1784.2±316.6*** (50.41±11.04)	386.6±121.6* (10.69±2.74)	1297.4±453.4 (35.56±10.44)
Lithogenic diet with plant sterols	[5]	48.8±47.1** (1.82±1.62)	888.6±313.9** (31.19±10.66)	467.8±166.7*** (16.50±4.35)	1402.4±489.7 (50.35±14.53)

[ ] : Number of animals
LC : Lithocholic acid   DC : Deoxycholic acid   CDC : Chenodeoxycholic acid
C : Cholic acid   *$p < 0.05$ **$p < 0.01$ ***$p < 0.001$ (significant against lithogenic diet alone)

tions. In the animals fed with the lithogenic diet in combination with plant sterols the bile acid proportions formed the order of cholic, deoxycholic, chenodeoxycholic and lithocholic acid.

5. Fatty acid composition of biliary phospholipids:

The percent composition of fatty acids in biliary phospholipids revealed a significant increase of C 18 : 2 ($p < 0.01$) and C 18 : 3 ($p < 0.001$) in animals fed with the lithogenic diet in combination with EPL. On the contrary, percent C 18 : 2 ($p < 0.001$) and C 20 : 4 ($p < 0.001$) decreased and percent C 16 : 1 ($p < 0.05$) and C 18 : 1 ($p < 0.001$) increased in animals fed with the lithogenic diet in combination

with plant sterohs as compared with those fed with the lithogenic diet alone.

B. Bile acid pool size and glycine/taurine ratio in hamsters fed with the lithogenic diet in combination with plant sterols:

1. Bile acid pool size:

Plant sterols decreased pool sizes of chenodeoxycholic acid ($p < 0.05$) and its secondary bile acid (lithocholic bile acid) ($p < 0.05$) and did not alter those of cholic acid and its secondary bile acid (deoxycholic acid). Total bile acid pool size, however, did not significantly decrease in animals fed with lithogenic diet in combination with plant sterols.

2. Glycine/taurine ratio:

No change was found in glycine/taurine ratio

LC : Lithocholic acid   DC : Deoxycholic acid   CDC : Chenodeoxycholic acid   C : Cholic acid
N.S. : not significant
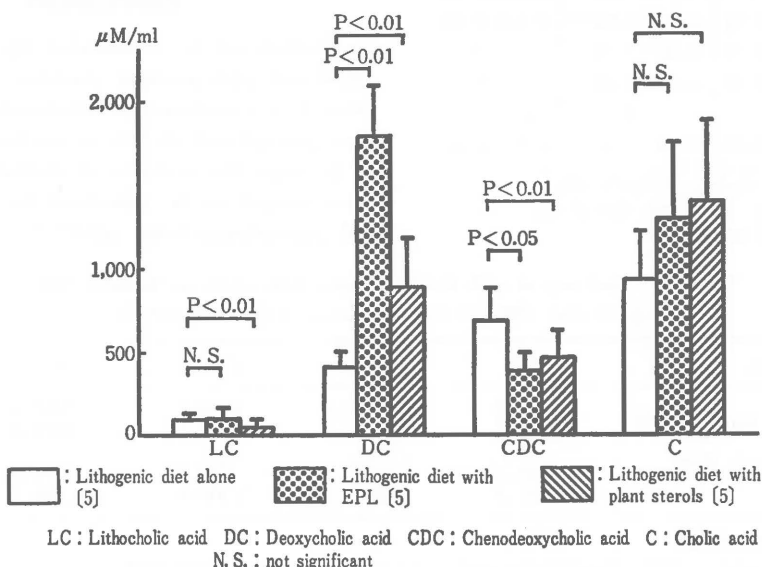
Fig. 5. Concentration of individual bile acid composition in hamsters fed with lithogenic diet alone or in combination with EPL or plant sterols
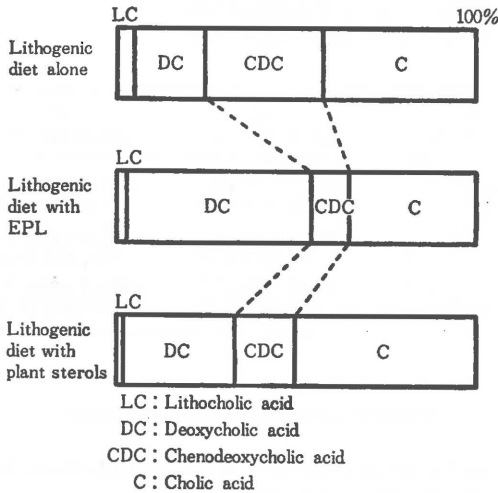
Fig. 6. Percent composition of individual bile acidsin hamsters fed with lithogenic diet alone or in combination with EPL or plant sterols.

Table 4. Fatty acid composition of biliary phospholipids in hamsters fed with lithogenic diet alone or in comibnation with EPL or plant sterols

Fatty acids	Lithogenic diet alone [5]	Lithogenic diet with EPL [5]	Lithogenic diet with plant sterols [5]
C_{14}	2.04±0.84	2.54±4.00	0
C_{16}	33.02±6.84	33.92±3.49	33.75±4.23
$C_{16:1}$	2.70±1.06	2.14±1.18	4.23±0.22*
C_{18}	8.75±2.67	6.14±10.06	7.33±3.74
$C_{18:1}$	27.04±3.56	22.08±9.87	39.55±2.58***
$C_{18:2}$	20.96±2.16	28.58±4.56**	13.03±1.75***
$C_{18:3}$	0.02±0.04	0.28±0.04***	2.63±3.54
C_{20}	1.30±0.83	0.98±1.59	0
$C_{20:2}$	0.36±0.48	0.06±0.09	0
$C_{20:3}$	0	0	0
$C_{20:4}$	4.18±0.94	3.54±2.37	0.86±0.61***

*p<0.05,   **p<0.01,   ***p<0.001
(significant against lithogenic diet alone)
[ ] : Number of animals

Fig. 7. Bile acid pool size of bile in hamsters (n=5)

of individual bile acids between animals fed with the lithogenic diet alone and those with the lithogenic diet in combination with plant sterols.

DISCUSSION

Cholesterol is surrounded by adequate bile acid and phospholipid (lecithin) molecules and therefore waterinsoluble cholesterol is stable and not precipitated in bile of normal men.

In man, the majority of cholesterol gallstones was found to be dissolved by administration of chenodeoxycholic acid[13,14]. The fact that

Table 5. Pool size of individual and total bile acids in hamster fed with lithogenic diet alone or in combination with plant sterols

Groups		L C	D C	C D C	C	T B A
Lithogenic diet alone	[5]	403.9 ±113.2	1462.8 ±352.4	1988.9 ±357.4	3431.4 ±1572.0	7632.1 ±2134.5
Lithogenic diet with plant sterols	[5]	113.5* ±81.8	1575.3 ±468.2	766.0* ±300.0	2974.5 ±368.7	5730.2 ±853.3

[ ] : Number of animals
LC : Lithocholic acid   DC : Deoxycholic acid   CDC : Chenodeoxycholic acid
C : Cholic acid   TBA : Total bile acids
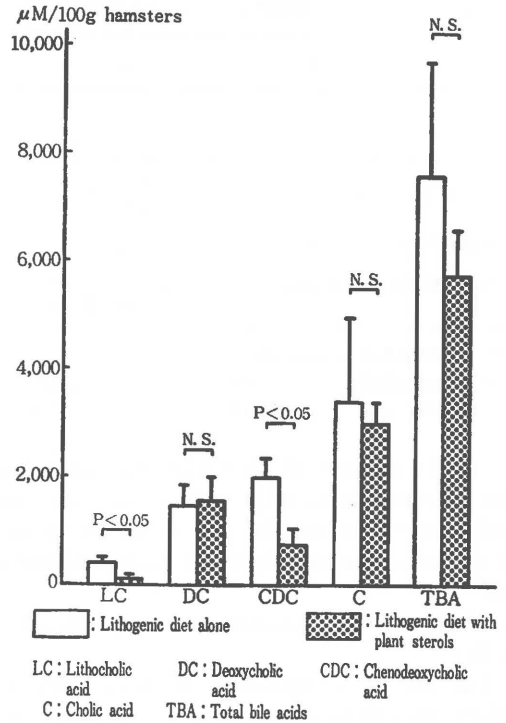*p<0.05 (significant against lithogenic diet alone)

Table 6. Glycine/Taurine ratio of individual and total bile acids in hamsters fed with lithogenic diet alone or in combination with plant sterols

	L C	D C	C D C	C	T B A
Lithogenic diet alone [5]	3.070	2.519	4.558	1.973	2.502
	±0.404	±0.642	±1.200	±0.677	±0.601
Lithogenic diet with plant sterols [5]	1.640N.S.	3.223N.S.	4.881N.S.	2.850N.S.	2.993N.S.
	±0.643	±2.761	±3.051	±1.559	±1.704

[ ] : number of animals
LC : Lithocholic acid   DC : Deoxycholic acid   CDC : Chenodeoxycholic acid
C : Cholic acid   TBA : Total bile acids
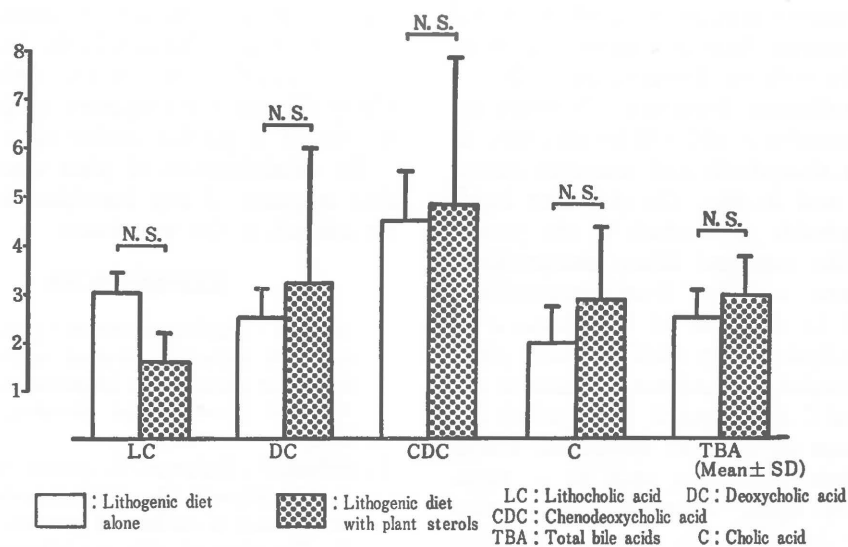N. S. : No significance against group of lithogenicdiet alone.

Fig. 8. Glycine/Taurine ratio of bile acid in hamsters (n=5)

gallstone patients had higher cholesterol and lower bile acid production as compared with normal men was proved by accelerated HMG CoA reductase and inhibited cholesterol 7α–hydroxylase activities which are limiting enzymes for cholesterol and bile acid synthesis[15]. Therefore, according to the recent concepts, inhibition of cholesterol synthesis and enlargement of bile acid pool size by chenodeoxycholic acid administration dissolve cholesterol gallstones in man[16]. On the other hand in hamsters the administration of chenodeoxycholic acid is not effective for cholesterol gallstone dissolution and, furthermore, it enhances gallstone development[17,18].

This discrepancy between human subjects and hamsters seems to be caused by phospholipids (lecithin) which has another detergent action for cholesterol solubility in bile. In the previous experiment[1] analysis of bile lipids proved that lithogenic bile was caused by the relative decrease in phospholipids and not by that in bile acid to cholesterol in hamsters.

In the present experiment, therefore, EPL was given to animals to increase biliary phospholipids during the period of lithogenic diet feeding. No gallstone was formed in the gallbladder of the animal treated with EPL but analysis of the bile lipids proved that not only biliary phospholipids but also cholesterol as well as total bile acids significantly increased by the administration of EPL and therefore only a very small decrease in the lithogenic index occurred in the Admirand and Small

and Holzbach's coordinates as shown in Figure 4 and Table 2. The increase in total bile acids seems to be caused by increased biliary deoxycholic acid because EPL contains 2.3 g/dl deoxycholic acid. At the same time chenodeoxycholic acid was significantly cecreased in bile of animals treated with EPL. It is possible to estimate that the decrease of chenodeoxycholic acid is caused by increased cholesterol, phospholipids or deoxycholic acid which are capable of exerting an effect on bile acid metabolism in the animal liver.

But the previous serial experiments[2,3] indicated that cholesterol and phospholipids were not likely to decrease biliary chenodeoxycholic acid and moreover administration of cholesterol seemed to increase chenodeoxycholic acid when they were fed with the lithogenic diet. But further investigation including administration solely of deoxycholic acid will be necessary to confirm that deoxycholic acid decreases chenodeoxycholic acid in bile. On the other hand, it will be possible to conclude in the present study that the increased biliary phospholipids came, without a doubt, from phospholipids administered in the form of EPL because analysis of individual fatty acids of biliary phospholipids revealed a significant increase in the proportion of C 18 : 2 and C 18 : 3 which are the contituents of EPL as mentioned above.

Plant sterols have been used as a hypocholesterolemic agent. Its mechanism of action was usually considered to be the prevention of intestinal cholesterol absorption.

The present experiment clarified that the plant sterols significantly decreased biliary cholesterol, lowered a lithogenic index, and prevented gallstone formation in the animal gallbladder. The Dam's prescription increased cholesterol due to accelerated synthesis in the liver of animals fed with the lithogenic diet and not due to exogenous or dietary cholesterol in this experiment. The decreased cholesterol, therefore, should be due to the inhibited cholesterol enterohepatic circulation caused by the preventing action of intestinal cholesterol absorption by plant sterols.

The present experiment also revealed that the administration of plant sterol decreased biliary phospholipids and altered their fatty acid composition. It is well accepted that the parallel changes in cholesterol and phospholipid

concentrations are usually observed in animal and human sera. An identical or similar mechanism in the liver may regulate cholesterol and phospholipid levels both in serum and bile.

Plant sterols also affected the bile acid composition and decreased the chenodeoxycholic acid concentration and its secondary bile acid (lithocholic acid) without affecting the total bile acid concentration.

The decreased pool size of chenodeoxycholic acid and ilthocholic acid determined by the washout technique supported the above results. Decreased cholesterol as a substitute may have an adequate 12-α-hydroxylation reaction in the liver microsome to form cholic acid resulting in a relative decrease in chenodeoxycholic acid formation. Changes in the individual bile acid composition seen in the various hepatobiliary diseases are frequently accompanied by the change in glycine/taurine ratio.

The administration of plant sterols did not alter the ratio of any individual bile acids of the animals in this experiment.

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