Role of Essential Fatty Acids in Liver on the Conversion of Cholesterol to Bile Acid, especially Significance of Essential Fatty Acids concerning the Formation of Cholesterolstones

by

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I. INTRODUCTION

Since the nutritional significance of essential fatty acids (abbreviated as EFA) was clarified by BURR and BURR in 1930[14], it has been gradually recognized that fat is of significance not only as a caloric source, but also as the source of numerous specific nutritional factors.

The term "EFA" is given to some poly-unsaturated fatty acids: linoleic, linolenic and arachidonic acids *etc.*, which can not be synthesized in the animal body at all. Table 1. shows metabolic pathways of polyunsaturated fatty acids in animal bodies, based upon the works of MEAD and HOLMAN and their coworkers. Recently HOLMAN and MOHRHAUER showed dietary linolenate did not maintain normal gain of body weight as efficiently as did linoleate or arachidonate, and symptoms referable to fat deficiency could not be cured completely. Furthermore, they reported that increasing amounts of dietary linolenate suppressed the levels of arachidonate in tissue lipids, thus showing that the conversion of linoleate to arachidonate was inhibited by dietary linolenate. Therefore, the term "EFA" should be given to fatty acids of linoleic acid family.

HIKASA et al. have investigated the specific nutritional effects of EFA, and demonstrated that a great quantity of EFA was contained in liver, adrenals and heart muscle.

In experimental and clinical studies on hypothermia, the significance of high concentration of EFA in heart muscle was clarified by HIKASA, TOMIOKA, KUWANA and SAITO in 1961 and 1962.

At the time, MATSUDA and NAGASE observed that animals deficient in EFA showed decreased adrenocortical activity. TAMAKI reported that the adrenals of EFA deficient rats secreted smaller quantities of glucocorticoids in vivo under various stresses. HAYASHIDA, PORTMAN and SKOVSTEAD et al. obtained the same results under stimulation of ACTH.

Cholesterol can serve as a precursor of adrenal steroid hormones and a great quantity of cholesterol in the adrenal gland is mainly combined with fatty acids, especially with EFA. Administration of ACTH to rats produces a marked drop in the cholesterol ester fraction of the adrenal.

From all of these observations and facts, it has been considered that adrenal cholesterol esters, especially esterified with EFA, may be involved in steroid hormone biosynthesis.

Recently HIKASA, MURAOKA and FUKUDA have come to the conclusion that adrenal

Table 1 Metabolic pathways of polyunsaturated fatty acids in animal bodies

1) CH_3-$(CH_2)_4$-CH=CH-CH_2-CH=CH-$(CH_2)_7$-COOH
Linoleic acid (9, 12-Octadecadienoic acid) C_{18}, $\Delta_{9,12}$(18 : 2ω6)
↓ -2H
γ-Linolenic acid (6, 9, 12-Octadecatrienoic acid) C_{18}, $\Delta_{6,9,12}$(18 : 3ω6)
↓ +2C
8, 11, 14-Eicosatrienoic acid C_{20}, $\Delta_{8,11,14}$(20 : 3ω6)
↓ -2H
Arachidonic acid (5, 8, 11, 14-Eicosatetraenoic acid) C_{20}, $\Delta_{5,8,11,14}$(20 : 4ω6)
↓ +2C
7, 10, 13, 16-Docosatetraenoic acid C_{22}, $\Delta_{7,10,13,16}$
↓ -2H
4, 7, 10, 13, 16-Docosapentaenoic acid C_{22}, $\Delta_{4,7,10,13,16}$(22 : 5ω6)

2) CH_3-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH=CH-$(CH_2)_7$-COOH
Linolenic acid (9, 12, 15-Octadecatrienoic acid) C_{18}, $\Delta_{9,12,15}$(18 : 3ω3)
↓ -2H
6, 9, 12, 15-Octadecatetraenoic acid C_{18}, $\Delta_{6,9,12,15}$
↓ +2C
8, 11, 14, 17-Eicosatetraenoic acid C_{20}, $\Delta_{8,11,14,17}$(20 : 4ω3)
↓ -2H
5, 8, 11, 14, 17-Eicosapentaenoic acid C_{20}, $\Delta_{5,8,11,14,17}$
↓ +2C
7, 10, 13, 16, 19-Docosapentaenoic acid C_{22}, $\Delta_{7,10,13,16,19}$(22 : 5ω3)
↓ -2H
4, 7, 10, 13, 16, 19-Docosahexaenoic acid C_{22}, $\Delta_{4,7,10,13,16,19}$

3) CH_3-$(CH_2)_7$-CH=CH-$(CH_2)_7$-COOH
Oleic acid (9-Octadecenoic acid) C_{18}, Δ_9
↓ -2H
6, 9-Octadecadienoic acid C_{18}, $\Delta_{6,9}$(18 : 2ω9)
↓ +2C
8, 11-Eicosadienoic acid C_{20}, $\Delta_{8,11}$
↓ -2H
5, 8, 11-Eicosatrienoic acid C_{20}, $\Delta_{5,8,11}$(20 : 3ω9)
↓ +2C
7, 10, 13-Docosatrienoic acid C_{22}, $\Delta_{7,10,13}$

4) CH_3-$(CH_2)_5$-CH=CH-$(CH_2)_7$-COOH
Palmitoleic acid (9-Hexadecenoic acid) C_{16}, Δ_9
↙ -2H ↘ +2C
6, 9-Hexadecadienoic acid — Vaccenic acid (11-Octadecenoic acid)
C_{16}, $\Delta_{6,9}$ ↘ +2C — C_{18}, Δ_{11} ↙ -2H
8, 11-Octadecadienoic acid C_{18}, $\Delta_{8,11}$(18 : 2ω7)
↓ -2H
5, 8, 11-Octadecatrienoic acid C_{18}, $\Delta_{5,8,11}$(18 : 3ω7)
↓ +2C
7, 10, 13-Eicosatrienoic acid C_{20}, $\Delta_{7,10,13}$(20 : 3ω7)
↓ -2H
4, 7, 10, 13-Eicosatetraenoic acid C_{20}, $\Delta_{4,7,10,13}$(20 : 4ω7)

cholesterol esterified with EFA, especially with arachidonic acid, may be the main precursor of adrenocortical hormones. And also they have assumed that when cholesterol is esterified with EFA, especially with arachidonic acid, it may acquire the metabolic activity for itself.

On the other hand, bile acid is a main end-metabolite of cholesterol in the liver. So that it is reasonable to analogize that cholesterol should be esterified with EFA, especially with arachidonic acid, prior to the conversion of cholesterol to bile acid. According to this analogical consideration, HIKASA has first surmised in 1960 that cholesterol gallstone may be due to deficiency or metabolic disturbances in EFA.

Deficiency and metabolic disturbances in EFA lead to decreased biosynthesis of bile acid. Lecithin which contains EFA as its component may be decreased naturally in these

states. Both bile acid and lecithin are significantly important substances to keep cholesterol in solution in the bile. Thus, cholesterol precipitation may occur and formation of stones may begin.

The present study was designed to clarify the role of EFA in the liver on cholesterol metabolism toward bile acid under the various dietary conditions and stresses, and also dealt with the initiating factors of cholesterol stone.

II. MATERIALS AND METHODS

I) MATERIALS

1) Clinical Experiment

The liver slices excised at operation were weighed immediately by microbalance and analysed. The patients were composed of 28 cases of cholesterol stones, 16 cases of bilirubin stones and as a control group, 18 cases of peptic ulcer, admitted at the Second Surgical Division, Kyoto University Hospital, without distinct disturbances of liver function and infection of the bile.

2) Animal Experiment

Male albino rats of Wistar strain supplied by the Animal Center of Kyoto University were used for this study. The weanling rats were fed a rat chow (a product of Oriental Yeast Ind. Co. Japan) until their body weight reached about 40 to 50g, then were divided randomly into six groups; (1) EFA diet group (2) EFA deficient diet group (3) V. B_6 deficient-EFA diet group (4) V.B_6 deficient-EFA with lard diet group (5) V. B_6 deficient-EFA with lard plus cholesterol diet group and (6) V. B_6 deficient-lard diet group.

These groups were maintained on these synthetic diets, respectively, listed in Table 2, 3, 4 and 5 *ad libitum*. Each of (1)- and (2)- diet group was fed each diet for 12 weeks. (3)-, (4)-, (5)- and (6)- diet group were fed their respective diets for 8 weeks after EFA diet for 4 weeks.

Table 2 Composition of the Diets

	EFA Diet	EFA deficient Diet	V. B_6 deficient EFA Diet	V.B_6 deficient EFA with Lard Diet	V.B_6 deficient EFA with Lard plus Cholesterol Diet	V. B_6 deficient Lard Diet
Starch	65.0% (w/w)	80.0% (w/w)	65.0% (w/w)	65.0% (w/w)	64.9% (w/w)	65.0% (w/w)
Vitamin-free Casein	16.0	16.0	16.0	16.0	16.0	16.0
Salt Mixture	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin Mixture	0.5	0.5	0.5*	0.5*	0.5*	0.5*
Cholin Chloride	0.5	0.5	0.5	0.5	0.5	0.5
Sesame Oil	15.0		15.0	5.0	5.0	
Lard				10.0	10.0	15.0
Cholesterol					0.1	
* Pyridoxine free						
Body weight at the end of the feeding period	199±15.6* g	165±13.9* g	150±17.4* g	209±31.6* g	280±25.1* g	223±32.1* g

* Standard error of the mean

Table 3 Composition of Fatty Acids in Sesame Oil and Lard

Fatty Acid	Sesame Oil	Lard
14 : 0	—(%)	2.0*(%)
15 : 0	—	0.3
16 : 0	9.5	28.1
16 : 1	0.7	3.0
17 : 0	—	1.0
18 : 0	5.1	12.5
18 : 1	34.6	41.9
18 : 2	48.9	6.7
18 : 3	0.7	0.3
20 : 0	0.5	1.0
20 : 4	—	—

* : Percentage of total area of gasliquid chromatographic elution diagram

Table 4 Vitamin Mixture per 1 g

Vit. A	2500	I.U.
Thiamine Nitrate	1.0	mg
Riboflavin	1.5	〃
Pyridoxin Hydrochloride	1.0	〃
Cyano-cobalamin	1.0	〃
Ascorbic Acid	37.5	mg
Calciferol	200	I.U.
dl-α-Tocopherol	1.0	mg
Vit. K	0.2	〃
Nicotinic Amid	10.0	〃
Pantothenic Cal.	2.5	〃
Folic Acid	0.5	〃

Tab. 5 Salt Mixture per 1 kg

NaCl	46.3g
NaH_2PO_4	92.0
K_2HPO_4	253.0
$CaH_4(PO_4)_2H_2O$	143.0
Cal. Lact.	369.0
$MgSO_4$	70.4
KI	26.3

Each gram of the casein and the starch used in this study contained, 437μg and 46μg of total fatty acids, 7μg and 7μg of linoleic acid, 3μg and 2μg of linolenic acid and no other polyunsaturated fatty acids (abbreviated as PUFA) according to gas-liquid chromatographic analysis, respectively. Therefore, a rat eating 10g of the fat deficient diet per day ingests less than 0.1mg of EFA per day.

At the end of feeding period, rats were fastened for 12 hours and under intraperitoneal anesthesia, sacrificed by bleeding from the aorta.

The liver slices were excised and weighed immediately by microbalance. The biochemical analyses were made with these liver slices.

II) METHODS

The liver lipids were extracted by the method of JINDO with minor modifications. Each of about 0.3g and about 1.2g of excised liver slices was weighed exactly. The former lipids were extracted with acidic BLOOR's solution, and the latter lipids extracted with non acidic BLOOR's solution.

Cholesterol esters of liver lipids were separated from five sixths of the latter liver lipids by columnchromatography on silicic acid according to the method of HIRSCH and AHRENS with minor modifications.

Total fatty acids were separated from the former liver lipids. Total cholesterol was separated from one-sixth of the latter liver lipids. Cholesterol ester fatty acids and cholesterol in esterified form were separated from the cholesterol ester obtained by columnchromatography.

Each of fatty acid and cholesterol was separated by the following procedures. The liver lipids were saponified with 2% ethanolic KOH at 40°C for 90 min.. After extraction of non saponifiable materials from the alkaline solution with petroleum ether, the fatty acids were removed by acidification of the aqueous solution and subsequent extraction with petroleum ether. Petroleum ether extract of non saponifiable materials was used to determine cholesterol by the method of ABELL et al..

For fatty acid analysis, two different methods were used; alkaline isomerization for

PUFA content and gas-liquid chromatography for fatty acid composition of these materials. PUFA of total lipids was determined by ultraviolet spectrophotometry after alkaline isomerization for 20 min. at 180°C, using 21% KOH in ethylene glycol according to the method of JINDO, which was a modification of HOLMAN and HAYES. Absolute amount of tetraen in cholesterol ester was calculated as follows:

$$M_{20:4} = \frac{\text{Est. Chol}}{387} \times \frac{n_{20:4}}{\sum \frac{n_t}{N_t}}$$

$M_{20:4}$: Absolute amount of 20 : 4 acid in cholesterol ester in liver
Est. Chol. : Absolute amount of cholesterol in esterified form
387 : Molecular weight of cholesterol
$n_{20:4}$: Percentage of 20 : 4 acid in cholesterol ester fatty acids which is obtained by gas-liquid-chromatography
n_t : Percentage of each fatty acid in cholesterol ester
N_t : Molecular weight of each fatty acid in cholesterol ester

The residual fatty acids from total lipids and cholesterol esters were methylated by using 2% methanolic sulfuric acid at 70°C for 90 min.. The methyl esters of the fatty acids were analyzed in a SHIMADZU Gas Chromatograph Model GC 1-B equipped with a hydrogen flame ionization detector. A 150cm U-shaped stainless-steel column of 6mm i. d., packed with 25% diethylene glycol succinate coated on Shimalite-Q, 60~80 mesh was used at 210°C. Nitrogen was the carrier gas, and the flow rate was 30 ml/min. at an inlet pressure of 3 kg/cm². The inlet heater was kept at 300°C and the detector cell at 235°C. The individual esters were identified by carbon number and by internal standards wherever feasible. Authentic methyl esters of fatty acids, obtained from the National Institute of Health (U. S. A.) and Hormel Institute (U. S. A.), were used as internal standards. Quantification was carried out by triangulation. The fatty acid composition was reported as area percent, and the area percent of mixed methyl esters of standard saturated C_8~C_{22} fatty acids well agreed with their weight percent.

APPENDIX

The lipids, especially phospholipids are extracted most efficiently by FOLCH'S method, in which chloroform is used. In this study, PUFA of the total lipids was determined by the spectrophotometric method. HOLMAN advised that trace of chloroform in the sample might later confuse the ultraviolet spectral measurements. So that the total lipids in liver were extracted by acidic BLOOR'S solution, which was more excellent than non acidic solution in the extraction of lipids. The total cholesterol was extracted by non acidic BLOOR'S solution, because it seemed likely that trace of HCl in the sample inhibited the LIEBERMANN-BRUCHARD reaction. Cholesterol ester fraction of the liver lipids was extracted by non acidic BLOOR'S solution for the reason that the cholesterol ester was soluble very easily in the solution and petroleum ether, and trace of HCl might later confuse the columnchromatography on silicic acid.

III. RESULTS

Generally speaking, the lipid contents of the liver show respectably the physiological

fluctuation. So that practically it is often difficult to distinguish strictly between physiological fluctuation and pathological fluctuation. Furthermore, it is important to distinguish whether the fluctuation of the lipid contents is the reflection of changes in lipid fractions or fatty acid composition or both. And also, biosynthesis of cholesterol in the liver is controlled by feedback mechanism.

Table 6 Liver-Lipids of Cow (KAUCHER)

	% of Dry-weight
Total lipid	20 ～25
Neutral fat	5.5～ 6.0
Free cholesterol	0.4～ 0.5
Cholesterol-ester	0.4～ 0.6
Phospholipid	14 ～19
Cephalin	5.0～ 8.5
Lecithin	8.0～10.0
Sphingomyelin	0.7～ 0.8

Cholesterol ester fraction shows a very low content (about 2 %) in the total liver lipid (Table 6). Therefore, it seems not to affect the total liver lipid content. However, it is regarded as the source of serum cholesterol ester. So that cholesterol ester in the liver is considered to have an important physiological significance.

In general, many saturated fats of either animal or vegetable origin raise the serum-cholesterol level, and some highly unsaturated fats (oils) from marine or vegetable sources tend to lower it. Linoleic acid is easily esterified with cholesterol and lowers the serum-cholesterol level remarkably.

These observations show that PUFA, especially EFA is closely concerned with cholesterol metabolism. Based on this reason, the ratio of cholesterol esterified with EFA to esterified cholesterol (abbreviated as $Chol._{EFA}$/Est. Chol.) was calculated. It may be regarded as an indicator of the metabolic activity of esterified cholesterol.

A) Clinical Experiment

It is necessary, in the first place, to know whether the fatty acid composition, tetraen content and cholesterol content in the liver of patients with gallstones are peculiar to cholelithiasis or not. The results of the analyses are listed in Table 7. In the liver of patients with gallstones, especially cholesterol stones, the following metabolic tendencies were observed ;

(1) Fatty acid composition and tetraen content

a) Total lipid

Palmitoleic acid (16 : 1), Oleic acid (18 : 1), 5, 8, 11-eicosatrienoic acid (20 : 3) increased, stearic acid (18 : 0), arachidonic acid (20 : 4), 4, 7, 10, 13, 16, 19-docosahexaenoic acid (22 : 6) decreased, Palmitic acid (16 : 0), Linoleic acid (18 : 2) were not so different from those of control patients, in proportion respectively, and the ratio of (20 : 3) acid to (20 : 4) acid was elevated, besides total tetraen content decreased.

b) Cholesterol ester fraction

(16 : 0), (16 1) acids increased, (18 : 2), (20 : 4) acids decreased, (18 : 0), (18 : 1) acids did not show any remarkable change, in proportion, and cholesterol ester tetraen content increased very slightly.

(2) Cholesterol content

Total and esterified cholesterol contents and the ratio of esterified cholesterol to total cholesterol (abbreviated as E. C./T. C.) increased, $Chol._{EFA}$/Est. Chol. decreased, respectively.

Table 7 The Composition of Liver Lipids of the Patients with Gallstones

		Control group		Cholesterol stone group		Bilirubin-Pigment stone group	
		Total	Ch-ester	Total	Ch-ester	Total	Ch-ester
Fatty Acid Composition	~14 :	0.4±0.1**	1.3±0.2	0.8±0.1	2.0±0.4	1.0±0.2	1.2±0.2
	15 : 0	0.2±0.0	0.3±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.3±0.1
	16 : 0	23.7±0.8	15.7±0.7	23.5±1.3	18.1±1.9	22.8±0.7	*20.5±2.2
	1	2.7±0.3	5.0±0.7	* 4.7±1.0	5.8±0.6	* 4.5±0.6	6.3±1.2
	17 0	0.5±0.1	0.5±0.1	0.7±0.1	0.5±0.1	0.6±0.2	0.5±0.2
	1	0.2±0.1	0.3±0.1	0.2±0.0	0.3±0.1	0.2±0.1	0.3±0.2
	18 : 0	16.3±0.4	3.7±0.6	*13.7±1.0	3.7±0.4	14.2±1.2	4.5±1.0
	1	13.1±1.3	29.7±1.5	*18.7±1.7	30.2±1.2	*19.3±1.4	27.9±2.2
	2	15.6±0.7	31.5±1.0	15.8±0.5	*26.4±1.7	14.9±0.6	*26.3±1.3
	3	0.5±0.1	0.6±0.1	0.6±0.2	0.7±0.1	0.6±0.2	0.8±0.2
	20 : 1	0.3±0.1	0.7±0.1	0.3±0.1	0.7±0.1	0.4±0.0	0.6±0.4
	2	0.2±0.0	0.3±0.0	0.2±0.0	0.3±0.1	0.2±0.1	0.2±0.0
	3	1.0±0.2	0.7±0.1	1.1±0.1	0.8±0.3	1.2±0.2	0.7±0.1
	4	10.5±0.7	5.9±0.5	* 8.2±0.6	5.0±0.7	* 8.2±0.7	5.8±0.9
	22 : 0	2.1±0.4	1.5±0.3	1.4±0.3	1.6±0.3	1.4±0.1	2.1±0.6
	3	0.1±0.1	0.4±0.1	0.1±0.0	0.2±0.0		
	4	0.1±0.0	0.2±0.0	* 0.1±0.0	0.1±0.0		
	5	0.8±0.2	0.7±0.3	1.5±0.7	0.3±0.1	1.1±0.4	
	6	12.6±1.0	2.7±0.5	* 9.5±1.0	2.5±0.4	*10.2±0.6	4.0±0.8
	20 : 3/20 : 4	0.10±0.02	0.15±0.01	0.13±0.02	0.14±0.0	0.16±0.0	0.17±0.0
Cholesterol(mg/100mgliver)		0.327±0.031	0.051±0.010	0.354±0.020	0.065±0.012	0.344±0.027	0.061±0.005
E.C/T.C (%)		15.9±2.7		18.1±1.3		18.1±1.5	
Chol$_{EFA}$/Est. Chol (%)		36.0±1.5		* 30.6±1.9		* 31.2±1.7	
Chol$_{20\ 4}$/Est. Chol (%)		5.5±0.6		4.6±0.6		5.2±0.8	
Tetraen (mg/100mgLiver)		0.234±0.012	0.0022±0.0004	0.209±0.023	0.0023±0.0003	0.218±0.019	0.0025±0.0005
Fatty Acid(mg/100mgLiver)		2.24	0.037	2.56	0.046	2.65	0.043
Mean cholesterol content of stone (%w/w)				91.8		11.8	

** Standard error of the mean * Significant change ($P<0.05$)

On the other hand, MARUYAMA has observed that the reduced biliary excretion of total bile acid and total fatty acid, the decreased ratio of total bile acid to cholesterol (abbreviated as B/C), and the increased ratio of dihydroxycholanic acid to trihydroxycholanic acid (abbreviated as Di/Tr) in the bile of patients with gallstones. And also he has reported that about 95 to 99% of total fatty acids in the bile is combined with lecithin. Therefore, licithin in the bile of patients with gallstones has been reduced.

From these observations, following conclusions are obtained.

(i) In the fatty acid composition in the liver of patients with gallstones, PUFA of oleic and palmitoleic acid families increased and those of linoleic acid family decreased, though cholesteryl oleate did not show any remarkable change and total linoleic acid did not decrease, in proportion respectively. Thus, it is presumed that the formation of human gallstones may be attributed to not the deficiency but the metabolic disturbances of EFA.

(ii) No significant difference is found between the liver lipid constituents of the patients with cholesterol stones and with bilirubin stones.

(iii) Both reduced ratios of Chol.$_{EFA}$/Est. Chol. and Chol.$_{20:4}$/Est. Chol. in the liver of patient with gallstones were observed. Under these circumstances, therefore, the conversion of cholesterol to bile acid may be suppressed. In fact, total bile acid and lecithin in the bile decreased, thus total and esterified cholesterol in the liver of patients with gallstones increased.

B) Animal Experiment

In clinical experiment, the metabolic pattern which is peculiar to cholelithiasis has been obtained. In present study, some experiments were designed to produce the metabolic patterns bearing a close resemblance to the changes in the fatty acids, bile acids, and cholesterol in the liver and bile of the patients with gallstones as shown in Fig. 1 by means of rearing rats on various dietary compositions and exposing them to stresses or both. The changes in the bile were studied by YOSHINAGA and MARUYAMA.

a) Resting State

Mean body weights of the six diet groups at the end of feeding period are shown in Table 2. During the course of the feeding period, an EFA deficient diet group exhibited the signs of EFA deficiency; scaly paw and tails, loss of hair on the back, on the neck and around the face, and stunted growth. In V. B_6 deficient-EFA diet group, mild dermatitis and retardation of growth were observed. But three groups fed V. B_6 deficient diet with lard did not show the retardation of growth.

(1) EFA diet group

In present study, this group was taken as control (Table 8).

(2) EFA deficient diet group

The fatty acid compositions of total lipids in the liver of this group exhibited higher proportions of 16 : 0, 16 : 1, 18 : 1, 20 : 3, 22 : 3 acids, and lower proportions of 18 : 0, 18 : 2, 20 : 4, 22 : 4 acids as compared with control group. Therefore, the ratio of 20 : 3/20 : 4, which was proved to be an useful parameter for describing linoleate metabolism by HOLMAN, was remarkably elevated.

The fatty acid composition of cholesterol ester in the liver of this group showed similar metabolic patterns to those of total lipids, but C_{22} acids were detected only trace.

Tetraen content was reduced strikingly in cholesterol ester as well as in total lipid.

According to MOHRHAUER and HOLMAN, eicosatetraenoic acid (20 : 4) from rat liver lipids consists mainly of arachidonic acid (5, 8, 11, 14-eicosatetraenoic acid) and small amounts of 4, 7, 10, 13-eicosatetraenoic acid as determined by reductive ozonolysis. In fat-deficient animals, the latter increases to almost one-quarter of the 20 : 4 acid. The eicosatrienoic acid (20 : 3) in liver lipids of fat deficient rats consists mainly of 5, 8, 11-eicosatrienoic acid with small amounts of the 7, 10, 13-isomer. These facts have to be recollected when proportions of 20 : 3 and 20 : 4 acids are evaluated.

From the Table 8, it is obvious that increased fatty acids in EFA deficient diet group are those of the endogenously derived oleic and palmitoleic acid families. While, in EFA diet group (control group), an increase is found in linoleic acid, which can never be synthesized in animal bodies, and in fatty acids of linoleic acid family.

Table 8 The Composition of Lipids in the Liver of Rats fed various Diets

		EFA diet group		EFA deficient diet group		V.B6 deficient EFA diet group		V.B6 deficient EFA with Lard diet group		V.B6 deficient EFA with Lard plus Cholesterol diet group		V.B6 deficient Lard diet group	
		Total	Ch-ester	Total	Ch-ester	Total	Ch-ester	Total	Ch-ester	Total	Ch-ester	Total	Ch-ester
Fatty Acid Composition	16 : 0	17.0	25.7	19.4	29.0	16.1	23.0	16.1	26.1	19.7	21.0	16.8	45.6
	1	1.4	3.7	7.0	13.8	1.6	3.2	2.3	4.1	2.4	5.8	1.5	2.7
	18 : 0	23.8	22.4	14.3	10.5	24.8	20.2	22.5	14.8	20.2	12.9	24.7	22.0
	1	10.7	22.3	30.8	40.4	11.7	22.9	21.8	28.6	20.2	38.4	18.6	16.1
	2	14.6	12.2	2.2	1.2	14.5	12.2	12.7	7.9	9.1	6.5	10.1	4.1
	3	0.2	0.6	0.4	0.4	0.5	0.4	0.5	0.5	0.5		0.1	
	20 : 3	0.2	0.1	11.8	1.7	0.4				0.2		1.4	
	4	26.9	12.5	8.1	1.5	24.1	16.3	20.6	15.7	23.3	12.1	21.6	7.3
	22 . 3			0.3	0.2								
	4	0.8	0.1			1.1	0.4	0.3		0.2		0.4	
	5	1.1	0.4	0.8	0.1	1.9		0.3		0.6		0.5	
	6	2.2	1.0	2.9	0.2	1.4	0.4	1.9	0.2	1.6		3.0	
	20 : 3/20 : 4	0.01		1.46		0.02				0.01		0.07	
Cholesterol (mg/100mgLiver)		0.254	0.024	0.339	0.084	0.259	0.018	0.278	0.029	0.286	0.038	0.294	0.028
E.C/T.C (%)		9.5		25.0		7.4		10.5		13.2		9.5	
$Chol_{EFA}$/Est. Chol (%)		21.9		2.5		28.2		22.0		17.4		10.4	
$Chol_{20:4}$/Est.Chol (%)		13.1		1.3		15.5		14.3		11.0		6.5	
Tetraen (mg/100mgLiver)		0.651	0.0027	0.212	0.0010	0.578	0.0025	0.538	0.0030	0.760	0.0033	0.765	0.0014
Fatty Acid(mg/100mgLiver)		2.34		2.62		2.30		2.57		3.23		4.07	

5 rats in each group

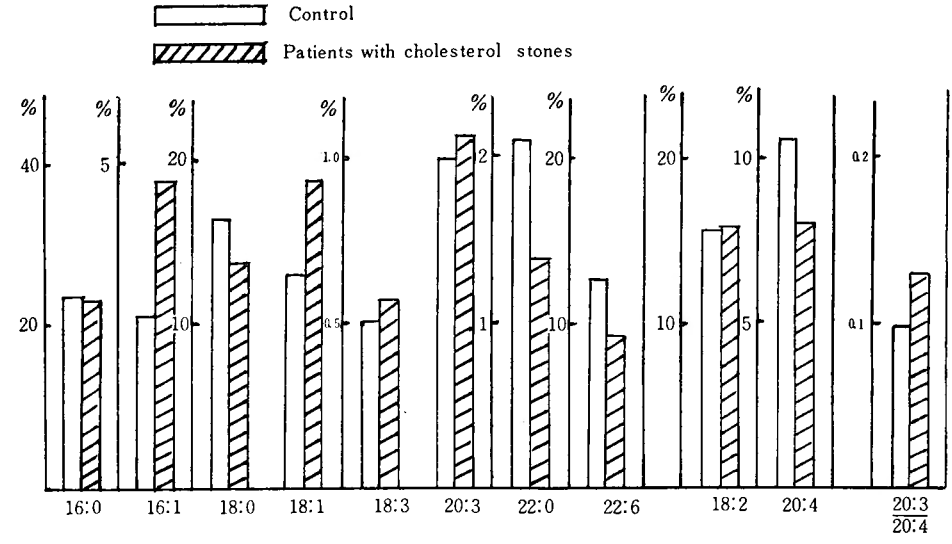
Both ratios of $Chol._{EFA}$/Est. Chol. and $Chol_{20:4}$/Est. Chol were reduced remarkably in EFA deficient diet group. So that the conversion of cholesterol to bile acid should be suppressed and then cholesterol content in the liver should be elevated. In fact, significant elevation was observed in total and esterified cholesterol contents in the liver and in the ratio of E. C./T. C.

There are some reports that unsaturated fat diets stimulate cholesterol biosynthesis more than do saturated fat diets, and fat free diets will also inhibit cholesterolgenesis. If they are so, in this experiment, the increase of cholesterol in the liver of EFA deficient diet group reveals that the cholesterol is far less converted to bile acid in this group than in EFA diet group.

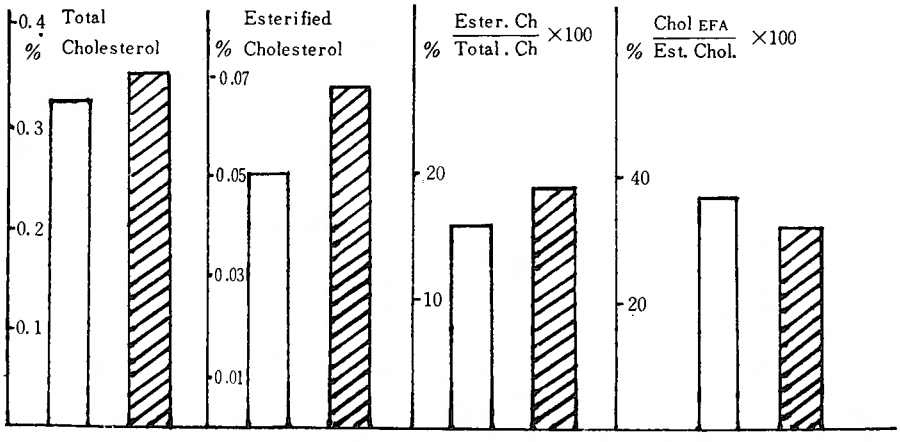
On the other hand, in the bile of EFA deficient rats, YOSHINAGA observed that total bile acid and B/C decreased significantly, Di/Tr increased moderately, cholesterol showed a tendency to increase, lecithin decreased moderately but its fatty acid constituents showed "EFA deficiency pattern." Furthermore, MARUYAMA observed marked decrease in total bile acid, and elevated ratio of Di/Tr in the liver slices of EFA deficient rats.

From these observations, it can be easily recognized that a close resemblance is found

Fig. 1 Percentage of each Fatty Acid in the Liver of the Patients with Cholesterol Stones



Cholesterol in the Liver of the Patients with Cholesterol Stones



Bile Components of the Patients with Gallstones

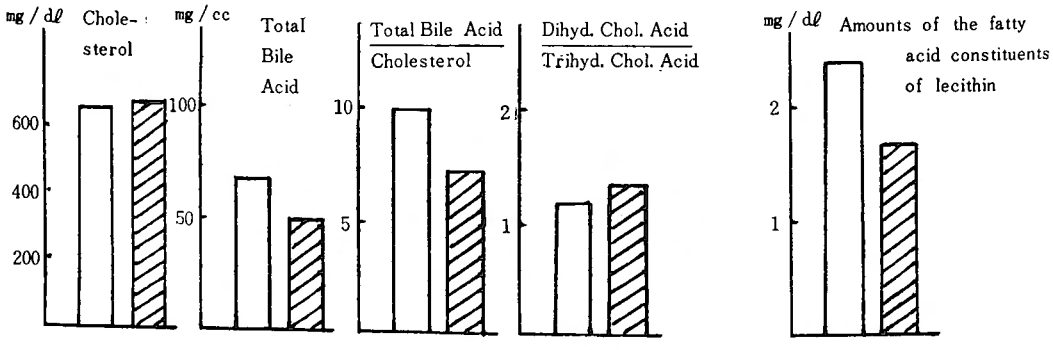
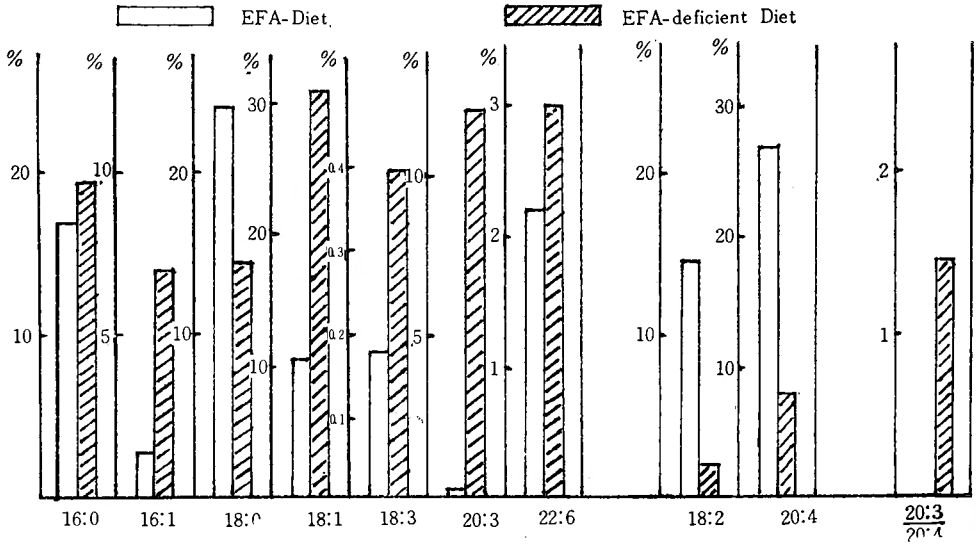
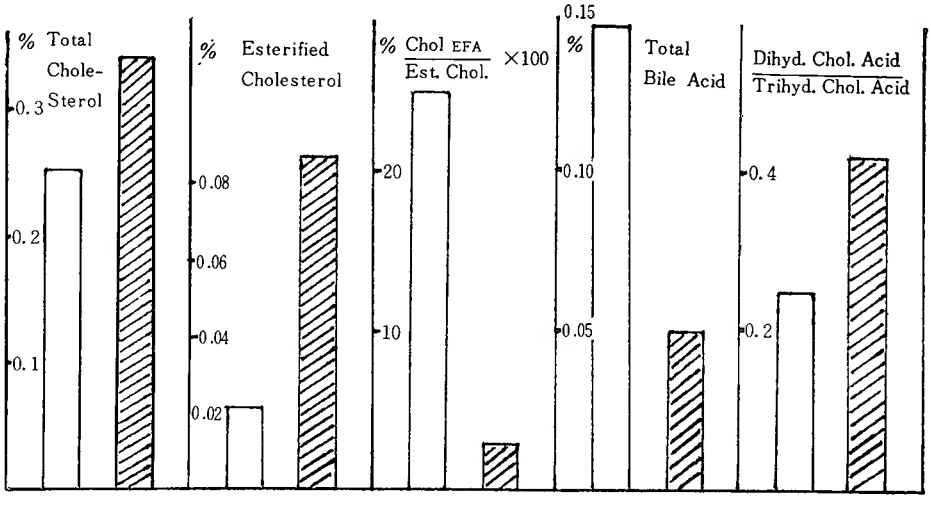


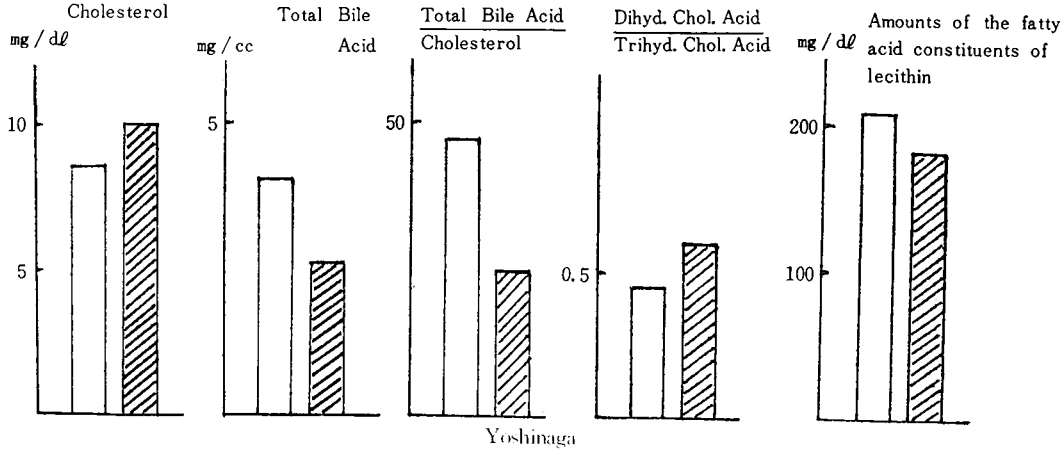
Fig. 2 Percentage of Each Fatty Acid in the Liver of Rats Fed EFA Diet and EFA-deficient Diet



Cholesterol and Bile Acid in the Liver of Rats Fed EFA Diet and EFA-deficient Diet



Bile Components of Rats Fed EFA diet and EFA-deficient Diet



Yoshinaga

between the metabolic patterns of liver lipids and bile in EFA deficient rat and those in the patients with gallstones, especially cholesterol stones. Of course, it is clear that EFA diet group of rat corresponds to control group of human.

According to HIKASA's primary presumption, the metabolic pattern in EFA deficient rats is considered to be ideal for the formation of cholesterol stones. Therefore, in accordance with this experimental results, SHIODA has observed the regular occurrence of gallstones with high cholesterol content in hamsters of which composition of bile acids may be similar to human ones, when placed on an EFA-deficient diet for two months or three. Almost all the gallstones produced in the experimental animals were cholesterol stones, but some of them were pigment stones in which cholesterol content was very low.

It is noteworthy, however, that linoleic acid was reduced remarkably in the liver lipids and the lecithin composing fatty acids in the bile of the EFA deficient rats, but on the other hand, this phenomenon was never observed in the patients with gallstones. Only at this point, the patterns of the data obtained from the EFA deficient rats don't coincide with those from the patients with gallstones. It is evident that the formation of human gallstones is not attributed to only a complete deficiency in EFA. If the conditions causing the pathologic patterns peculiar to cholelithiasis could be found out in the experimental animals with sufficient administration of linoleic acid, those would be the true initiating factors in gallstone formation.

(3) V B_6 deficient-EFA diet group

Pyridoxine has been suggested by many investigators to have an important relationship to lipid metabolism. HOLMAN reported that pyridoxine was concerned with the conversion of linoleic acid to arachidonic acid. In fatty acid composition of total lipids in the liver of patients with gallstones, the proportion of linoleic acid was kept in a normal proportion but that of arachidonic acid was lowered. Thus, to know the effects of pyridoxine on fatty acid metabolism in the liver, this group was prepared.

As compared with EFA diet group (Control), in total fatty acid composition, this group was resemble to the relation of cholelithiasis group to control group in human; namely, slight increases of 16 : 1, 18 : 1, 18 : 3, 20 : 3 acids, decreases of 20 : 4, 22 : 6 acids and no change of 18 : 2 acid.

Cholesterol ester fatty acid composition of this group, however, was not at all resemble to that of cholelithiasis group, namely, cholesteryl linoleate was not lowered and cholesteryl arachidonate was elevated. Therefore, $Chol._{EFA}$/Est. Chol. and $Chol_{20:4}$/Est. Chol were clearly elevated.

However, total cholesterol in the liver showed a slight increase. Further, cholesterol ester in the liver decreased and the ratio of E. C./T. C. decreased.

Tetraen content in the liver decreased in total lipid and slightly in cholesterol ester.

On the other hand, YOSHINAGA observed, in the bile of rats of this group, remarkable decrease of total bile acid and B/C, moderate increase of Di/Tr, a tendency to increase of cholesterol, and very slight decrease of lecithin, in concentration, respectively. However, MARUYAMA reported that bile acid in the liver slices of rats of this group, showed almost similar content to that of EFA diet group.

In these observations, most noticeable phenomenon was that the total bile acid in the

bile showed a remarkable decrease in spite of the clear elevations of both $Chol_{EFA}$/Est. Chol and $Chol_{20:4}$/Est Chol. Therefore, in pyridoxine deficient rats, the production of bile acid may be reduced according to the inhibited esterification of cholesterol. Thus, total cholesterol content in the liver showed only a slight increase.

(4) V. B_6 deficient-EFA with lard diet group

To add to V. B_6 deficiency, the effects of animal fat on the fatty acid metabolism of the liver were investigated in the EFA supplemented animals.

In the fatty acid compositions of total lipids and cholesterol ester, the same patterns as those being characteristic of cholelithiasis were obtained except only the slight elevation of cholesteryl arachidonate. And also the changes of both cholesterol and tetraen content in the liver, showed a close resemblance to those of cholelithiasis group.

Furthermore YOSHINAGA observed the same patterns in the changes of bile of this group as those of cholelithiasis group, namely, no difference in cholesterol concentration, remarkable decrease of total bile acid and B/C, moderate increase of Di Tr, and moderate decrease of lecithin.

In a word, the experimental animals in this dietary regimen, showed almost the same patterns as those of cholelithiasis group in metabolism of fatty acids, cholesterol and bile acids in the liver and bile. Therefore, this dietary regimen may be lithogenic.

(5) V. B_6 deficient-EFA with lard plus cholesterol diet group

This is prepared when 0.1% (in weight) of cholesterol is added to the V B_6 deficient-EFA with lard diet.

The fatty acid compositions in cholesterol ester as well as in total lipids showed a close resemblance to those of the patients with cholesterol stones. The changes of cholesterol also showed the same resemblance. So that this dietary regimen may be lithogenic.

Additional dietary cholesterol lowered evidently the proportion of EFA in the liver lipids, especially in cholesterol ester and invited the decreased ratios of both $Chol_{EFA}$/Est. Chol and $Chol_{20:4}$/Est. Chol, which resulted in the elevation of cholesterol, and on the other hand might suppress the conversion of cholesterol to bile acid. However, both total tetraen content and cholesterol ester tetraen content increased, especially in the former, and then total fatty acid content in the liver increased. In other words, in this dietary regimen, the liver lipids may be accumulated.

(6) V. B_6 deficient-lard diet group

In this dietary condition, adding to pyridoxine deficiency, only the animal fat was used as the source of fat. This group is in contrast with the V. B_6 deficient-EFA diet group.

In the fatty acid composition of total lipids, the proportion of 18 : 1 acid was higher and those of 18 : 2, 20 : 4 acids were lower as compared with the V. B_6 deficient-EFA diet group.

In the fatty acid composition of cholesterol ester, the remarkable differences from the V. B_6 deficient-EFA diet group were observed ; striking increase of 16 : 0 acid and marked decreases of 18 : 2, 20 : 4 acids were obtained, while 18 : 1 acid decreased.

Therefore, the ratios of both $Chol_{EFA}$/Est. Chol and $Chol_{20:4}$ Est. Chol were remarkably lowered. So that cholesterol content increased significantly. Thus, this dietary

regimen also may be lithogenic.

Tetraen content in the liver decreased in cholesterol ester, but in total lipids showed the marked increase. Then, total fatty acid content in liver increased strikingly. Therefore, in this dietary condition, the lipids accumulation in the liver should be found.

Table 9 Changes in the Composition of Lipids in the Liver of Rats fed various Diets following a single Administration of ACTH-Z 3 I. U.

		EFA diet group				EFA deficient diet group				V. B_6 deficient-EFA diet group			
	hrs. after inj.	before	2 hrs after inj.	4	6	before	2	4	6	before	2	4	6
Fatty Acid Composition of Total Lipids	14 : 0	0.2	0.2	0.4	0.6	0.7	1.1	1.2	0.8	0.2		0.4	0.2
	16 : 0	17.4	15.8	15.2	17.4	19.6	19.2	18.8	17.5	17.3	15.0	16.0	18.8
	1	1.7	1.9	2.1	2.1	7.6	10.7	8.7	7.3	1.7	5.7	2.4	2.2
	18 : 0	31.7	32.7	32.2	35.8	13.3	3.6	6.0	3.7	21.4	17.4	17.3	22.4
	1					30.0	41.4	40.4	46.2	14.3	19.5	23.4	15.8
	2	16.5	18.3	20.6	17.5	3.3	0.2	0.3	0.4	12.4	15.6	18.2	13.0
	3									0.7		0.8	0.5
	20 : 1	0.5	0.2	0.5	0.5	0.3	0.3	0.4	0.7	0.1	0.1	0.2	0.2
	2	0.4		0.5	0.9					0.2		0.2	0.2
	3					10.0	8.6	11.0	11.5	0.4	0.2		
	4	24.5	25.2	22.3	19.2	8.4	4.8	8.7	7.0	26.2	22.5	18.2	23.2
	22 : 3					0.2	0.1	0.3	0.3	1.0	0.9	0.7	0.8
	4	1.0	0.9	0.7	1.2					2.0	1.7	1.0	1.7
	5	1.2	0.6	0.6	1.1	0.9	1.7	0.9	0.7				
	6	4.4	3.7	4.3	3.1	3.4	7.6	2.6	3.0	1.5	0.9	0.9	1.2
Fatty Acid Composition of Cholesterol Ester	14 : 0	0.2	0.2	0.2	0.2	0.5	0.6	0.7	0.2	0.4	0.4	0.6	0.7
	16 : 0	17.5	20.8	17.9	19.3	25.8	22.3	25.4	29.7	21.6	25.0	23.6	18.2
	1	6.1	1.8	8.5	3.0	16.8	19.3	15.8	15.0	3.1	3.5	3.4	4.5
	18 : 0	47.8	36.6	43.4	42.4	3.3	4.7	2.3	2.2	27.4	28.6	20.0	18.2
	1					48.6	48.1	48.8	48.5	16.1	11.5	17.7	19.9
	2	16.7	10.8	16.8	16.6	0.4	0.2	0.2	0.4	8.7	7.6	9.8	11.3
	3									0.3	0.6	0.9	1.2
	20 : 1	0.2	0.4	0.7	0.3	0.3	0.4	0.3					
	3					1.5	1.8	3.3	2.0				
	4	10.8	27.5	11.7	17.6	1.5	0.7	2.7	1.6	20.4	21.6	22.8	24.2
	22 : 6	0.6	0.6	0.5	0.3	0.2	0.2	0.2					
Total Cholesterol (mg/100mg liver)		0.301	0.261	0.288	0.262	0.349	0.382	0.350	0.318	0.278	0.268	0.250	0.274
esterified Cholesterol	〃	0.068	0.027	0.070	0.032	0.103	0.134	0.106	0.079	0.017	0.015	0.013	0.017
E.C/T.C	(%)	22.5	10.3	24.2	12.0	29.6	35.1	30.3	24.8	6.1	5.4	5.1	6.3
$Chol_{EFA}$/Est.Chol	(%)	26.3	36.2	26.2	33.0	1.7	0.8	2.6	2.2	27.4	27.4	30.6	33.6
Chol $_{20:4}$/Est.Chol	(%)	9.8	25.4	10.2	16.2	1.3	0.6	2.4	1.4	18.8	19.8	20.9	22.3
Total Tetraen (mg/100mg liver)		0.681	0.689	0.765	0.648	0.283	0.194	0.262	0.214	0.726	0.686	0.802	0.620
Cholesterol Ester Tetraen (mg/100mg liver)		0.0052	0.0054	0.0056	0.0040	0.0010	0.0006	0.0020	0.0009	0.0025	0.0023	0.0021	0.0030
No. of rats		5	5	5	5	5	5	5	5	5	5	5	5

b) States exposed to Stresses

(1) A single administration of ACTH

Table 9 shows the changes in liver lipids of EFA diet group, EFA deficient diet group and V.B_6 deficient-EFA diet proup, following a single administration of ACTH-Z in a dose of 3 I. U. into the back muscles.

i) Fatty acid composition of total lipids

In EFA diet group, the fluctuations were comparatively limited. 18 : 2 acid began to increase gradually following ACTH injection, reached its maximum value after 4 hours, and after 6 hours returned to the value before the injection. 20 : 4 acid decreased gradually since 2 hours after ACTH injection.

In EFA deficient diet group, 18 : 0, 18 : 2, 20 : 4 acids showed clear decrease 2 hours after injection, and only 20 : 4 acid of them returned to the starting level since 4 hours after injection. 18 : 2 acid almost disappeared since 2 hours after injection. 18 : 1 acid showed striking increase, which might be attributed to the mobilization of dépot fat.

In V.B_6 deficient-EFA diet group, 16 : 0, 18 : 0, 20 : 4 acids decreased 2 to 4 hours after ACTH injection, returned to the starting level after 6 hours. 18 : 1, 18 : 2 acids began to increase gradually after ACTH injection, reached its maximum value after 4 hours, and returned to the starting level after 6 hours.

ii) Fatty acid composition of cholesterol ester

In EFA diet group, 20 : 4 acid strikingly increased after ACTH injection, reached its maximum value after 2 hours, whereas, 18 : 2, 18 : 0+18 : 1, 16 : 1 acids decreased.

In EFA deficient diet group, 18 : 0, 18 : 1, 18 : 2, 20 : 4 acids showed a very limited fluctuation.

In V.B_6 deficient-EFA diet group, 18 : 2, 20 : 4 acids increased gradually.

In general, 18 : 0 and 18 : 1 acids changed in the same way as 16 : 0 and 16 : 1 acids compensated each other.

The changes peculiar to each diet group in fatty acid composition of the liver lipids following ACTH-Z injection appear after 2 to 4 hours in total lipids, and after 2 hours in cholesterol ester. So that it may be most reasonable to take the data 2 hours after the charge in order to know the changes of fatty acid composition of liver lipids following ACTH injection.

It is noteworthy that the proportion of cholesteryl arachidonate after 2 hours following ACTH injection shows the most remarkable increase in EFA diet group, the moderate increase in V.B_6 deficient-EFA diet group, and the decrease in EFA deficient diet group.

iii) Cholesterol in the liver

The changes of total cholesterol were almost parallel with those of esterified cholesterol, and after 2 hours following ACTH injection showed the minimum in EFA diet group, the maximum in EFA deficient diet group and the slight decrease in V.B_6 deficient-EFA diet group in which the minimum occurred after 4 hours, respectively. It seems likely that the changes of $Chol._{EFA}$/Est. Chol. or $Chol._{20:4}$/Est. Chol. is roughly in a negative correlation with those of total cholesterol, especially in EFA diet group. Further, it is noticeable that the ratio of esterified cholesterol to total cholesterol is remarka-

bly lowered in V.B_6 deficient-EFA diet group.

iv) Tetraen content

Total tetraen content in the liver following ACTH injection showed almost similar changes to one another in three diet groups; namely, after 2 hours, slightly increased in EFA diet group and decreased in other two diet groups, however, in these three diet groups after 4 hours, reached each maximum value and after 6 hours showed a decrease again. It is considered that the decreases of total tetraen content in the liver after 2 hours following ACTH injection may be due to increasing demand under the stress and the increases after 4 hours may be affected by mobilization of dépot fat.

Cholesterol ester tetraen content in the liver following ACTH injection showed the changes similar to those of total tetraen content except V.B_6 deficient-EFA diet group. In this diet group, it decreased following ACTH injection, reached the minimum value after 4 hours and showed a considerable increase after 6 hours. From this observation, it is surmised that in V.B_6 deficient-EFA diet group, the conversion of the esterified cholesterol combined with tetraen to further metabolite may be suppressed since 4 hours after ACTH injection.

(2) Successive administration of ACTH for 4 days

Three diet groups (EFA diet, EFA deficient diet, V.B_6 deficient-EFA diet) of the rats were injected with ACTH-Z in a dose of 3 I. U. into back muscles on 4 successive

Table 10 The Composition of Lipids in the Liver of Rats fed various Diets before and after Administration of ACTH-Z 3.0 I.U. for 4 Days

		EFA diet group				EFA deficient diet group				V. B_6 deficient EFA diet group			
		before		after		before		after		before		after	
		Total	Ch-ester	Total	Ch-ester	Total	Ch-ester	Total	Ch-ester	Total	Ch-ester	Total	Ch-ester
Fatty Acid Composition	16 : 0	18.4	25.5	22.6	24.0	20.6	32.2	23.0	25.6	16.6	21.2	19.3	13.8
	1	1.3	3.2	3.1	4.9	6.1	10.8	8.1	11.8	2.0	4.5	3.0	4.7
	18 : 0	25.0	21.0	10.1	13.1	17.9	17.6	13.3	23.5	25.8	10.8	12.8	3.4
	1	10.1	23.3	27.5	20.4	31.8	32.2	35.4	30.4	9.0	30.1	21.7	36.8
	2	14.6	10.7	17.3	14.6	2.1	1.9	2.6	2.4	13.9	13.0	17.5	17.1
	3	0.4	0.6	0.1		0.3	0.4	0.4	0.3	0.1	0.1	0.0	
	20 : 3	0.2				11.8	1.9	7.2	1.7				
	4	25.8	11.4	14.9	19.3	6.0	1.5	5.5	2.0	25.2	17.5	19.8	19.2
	22 : 4	0.8	0.1	0.8	0.3	0.3	0.2	0.2	0.1	1.4	0.4	1.8	0.4
	5	1.4	0.2	1.0	0.4					2.3		1.5	0.3
	6	1.6	0.1	1.3	0.8	1.8	0.2	1.6	0.4	1.9	0.4	1.1	0.4
Cholesterol (mg/100mgLiver)		0.260	0.029	0.184	0.018	0.309	0.087	0.286	0.066	0.254	0.019	0.278	0.042
E.C/T.C (%)		11.5		9.8		28.2		22.9		7.4		15.0	
$Chol_{EFA}$/Est.Chol (%)		25.2		32.2		3.1		4.1		29.0		35.8	
$Chol_{20:4}$/Est. Chol (%)		12.8		17.6		1.3		1.8		16.0		18.2	
Tetraen (mg/100mgLiver)		0.710	0.0030	0.442	0.0025	0.140	0.0009	0.177	0.0009	0.570	0.0023	0.520	0.0060
Fatty Acid (mg/100mgLiver)		2.68		2.82		2.24		3.10		2.14		2.40	
No. of rats		5		5		5		5		5		5	

days. In the course of this experiment, animals were fed the diets *ad libitum*, but an overnight fast was forced before sacrifice. Two hours after the injection on the fourth day, rats were sacrificed and liver was analyzed for lipids. The results are listed in Table 10.

i) Fatty acid composition of total lipids

After 4 days, the proportions of 16 : 0, 18 : 1, 18 : 2 acids in all three diet groups showed clear increases which might be due to the mobilization of dépot fat, on the contrary, 18 : 0, 20 : 4 acids decreased evidently in all groups.

ii) Fatty acid composition of cholesterol ester

After 4 days, the proportions of 18 : 2, 20 : 4 acids in all groups increased evidently, so that the ratio of $Chol._{EFA}$/Est. Chol. or $Chol._{20:4}$/Est. Chol. showed marked increase.

iii) Cholesterol content

After 4 days, both EFA diet group and EFA deficient diet group showed clear decreases of cholesterol content in esterified as well as in total form, and of the ratio of E. C./T. C., whereas, $V.B_6$ deficient-EFA diet group showed evident increases of them, especially of esterified cholesterol.

In resting state, $V.B_6$ deficient-EFA diet group showed the lowest ratio of E. C./T. C. in all three diet groups, but after 4 days this group exceeded EFA diet group in the ratio.

iv) Tetraen content

After 4 days, both total and cholesterol ester tetraen contents decreased evidently in EFA diet group, and increased slightly in EFA deficient diet group. In $V.B_6$ deficient-EFA diet group, total tetraen content decreased, but cholesterol ester tetraen content increased remarkably and exceeded it in EFA diet group.

It is noticeable that, after 4 days, $V.B_6$ deficient-EFA diet group has not shown the decreased cholesterol content in spite of the greatest ratio of $Chol._{EFA}$/Est. Chol. or $Chol._{20:4}$/Est. Chol. and the greatest tetraen content of cholesterol ester in all three diet groups, on the contrary EFA deficient group has shown completely the inverse phenomenon.

On the other hand, MARUYAMA analyzed the bile acid in the liver slices in the same experimental condition, and reported that after 4 days, total bile acid decreased markedly in $V.B_6$ deficient-EFA diet group, while it decreased slightly in EFA diet group in comparison with before the administration of ACTH.

From these observations, on the cholesterol metabolism in the liver of $V.B_6$ deficient-EFA diet group, the same metabolic tendencies as observed in preceding experiment were obtained again. Namely, it is considered that the conversion of esterified cholesterol combined with tetraen to bile acid may be suppressed in this experimental condition.

(3) Successive administration of ACTH for 15 days

The rats of $V.B_6$ deficient-EFA diet group were injected with ACTH-Z in a dose of 1 I. U. into back muscles on 15 successive days. In the course of this experiment, animals were fed the diet *ad libitum*, but an overnight fast was forced before sacrifice. Two hours after the injection on the fifteenth day, rats were sacrificed and liver lipids were analyzed. The results are listed in Table 11.

i) Fatty acid composition of total lipids

Table 11 Changes in the Composition of Lipids in the Liver of Rats fed V. B_6 deficient EFA diet following daily Administration of ACTH-Z 1 I. U. for 15 Days

		before		on the 10th Day		on the 15th Day	
		Total	Ch-ester	Total	Ch-ester	Total	Ch-ester
Fatty Acid Composition	16 : 0	17.3	21.6	16.1	29.0	19.9	30.0
	1	1.7	3.1	2.7	1.8	0.7	1.7
	18 0	21.4	27.4	22.4	35.8	18.9	40.1
	1	14.3	16.6	16.5	11.1	15.1	10.9
	2	12.4	8.7	15.3	6.4	13.8	5.8
	3	0.7	0.3	0.7		0.6	0.5
	20 : 3	0.4		0.2		0.3	
	4	26.2	20.4	21.6	14.0	25.7	9.4
	22 : 4	1.0		0.8		0.9	
	5	2.0		1.6		1.8	
	6	1.5		1.0		1.1	
Cholesterol (mg/100mgliver)		0.278	0.017	0.262	0.013	0.252	0.013
E.C/T.C (%)		6.1		5.0		5.0	
Chol EFA/Est. Chol (%)		27.4		19.0		14.2	
Chol 20 : 4/Est. Chol (%)		18.8		12.8		8.5	
Tetraen (mg/100mgliver)		0.696	0.0025	0.543	0.0013	0.762	0.0008
No. of rats		5		5		5	

As the days rolled on, in total liver lipids, the proportions of 16 : 0, 18 : 1, 18 : 2 acids showed the increases and those of 18 : 0, 20 : 4 acids did the slight decreases.

ii) Fatty acid composition of cholesterol ester

The changes of this composition were characteristic. The proportions of saturated fatty acids, *i. e.* 16 : 0, 18 : 0 acids showed the marked increases, on the contrary, those of unsaturated fatty acids, *i. e.* 16 : 1, 18 : 1, 18 : 2, 20 : 4 acids showed the decreases, especially marked decrease of 20 : 4 acid was noticeable.

iii) Cholesterol content

As the days rolled on, the ratio of Chol.$_{EFA}$/Est. Chol. or Chol.$_{20:4}$/Est. Chol. decreased significantly. Especially marked decrease of Chol.$_{20:4}$/Est. Chol. was noticeable. These results were the inversion on those as presented in the preceding experiment. However, it may be understood that according to the increasing demands to Chol.$_{EFA}$ or Chol.$_{20:4}$ following the prolonged ACTH administration and the disturbances of esterification of cholesterol under the V.B_6 deficiency, Chol.$_{EFA}$ especially Chol.$_{20:4}$ has decreased remarkably.

On the other hand, YOSHINAGA analyzed the bile of rats under the same experimental conditions and reported that total bile acids and B/C ratio decreased slightly after 14 days as compared with before the administration of ACTH.

Both total and esterified cholesterol, nevertheless, decreased slightly against expectation. Under these experimental conditions, the biosynthesis of cholesterol in liver may be suppressed. Because, the adrenal cortex will be exhausted under the prolonged successive

administration of ACTH and become unable to maintain homeostasis in the excretion of glucocorticoids, thus the gluconeogenesis in liver will be reduced.

(4) Starvation

Rats of both EFA diet group and EFA deficient diet group were fasted for a period. Water was supplied *ad libitum*. Every two days, rats of each group were sacrificed and their liver lipids were analyzed. The results were listed in Table 12. The survival period of fasting rats was 10 days in EFA diet group and 8 days in EFA deficient diet group, respectively.

i) Fatty acid composition of total lipids

Table 12 Changes in the Composition of Lipids in the Liver of Rats fed EFA Diet and EFA deficient Diet during Fasting

		EFA diet group						EFA deficient diet group				
	Days	before	2	4	6	8	10	before	2	4	6	8
Fatty Acid Composition of Total Lipids	16 : 0	20.4	15.4	11.8	17.4	14.2	8.4	19.6	14.9	17.5	13.4	14.8
	1							7.6	6.2	2.7	4.2	3.7
	18 : 0	34.5	30.8	34.4	31.3	31.1	28.8	13.3	17.1	15.7	12.7	15.9
	1							30.0	25.4	21.6	31.1	32.2
	2	15.8	20.6	20.2	19.5	17.1	23.8	3.3	1.4	6.0	4.6	6.0
	3							0.6	0.6	0.7	0.3	0.4
	20 : 3	0.2	0.4	0.2	0.2	0.1	0.1	10.0	10.9	7.6	8.7	5.0
	4	23.7	26.6	21.5	24.2	28.9	28.3	8.4	12.1	17.4	16.3	15.1
	22 : 3							0.2	0.1	0.1	0.1	0.1
	4	1.8	1.2	0.8	1.3	1.2	0.8					
	5							0.9	0.9	1.6	1.0	0.5
	6	2.1	1.7	4.7	5.7	7.0	9.4	3.4	5.1	7.5	5.7	4.4
Fatty Acid Composition of Cholesterol Ester	16 : 0	21.3	22.4	25.1	28.2	25.7	28.9	25.8	22.4	28.0	22.6	20.2
	1							16.8	14.3	8.0	5.0	6.8
	18 : 0	51.0	46.2	45.7	35.4	45.0	39.7	3.3	3.2	2.5	1.9	1.7
	1							48.6	49.9	53.4	63.5	64.0
	2	11.8	11.6	11.7	16.6	13.6	11.9	0.4	0.5	0.5	0.4	0.4
	3											
	20 : 3	0.1			0.3	0.5	0.5	1.5	3.0	1.5	1.3	1.0
	4	12.9	16.6	10.9	14.6	11.9	11.3	1.5	4.0	4.0	4.1	4.5
	22 : 6	1.3	2.6	3.0	4.1	1.2		0.2	0.1	1.2	0.6	0.5
Total Cholesterol (mg/100mgliver)		0.255	0.276	0.229	0.291	0.362	0.307	0.349	0.288	0.349	0.459	0.454
esterified Cholesterol		0.023	0.030	0.018	0.022	0.031	0.035	0.100	0.055	0.082	0.168	0.170
E.C/T.C	(%)	9.2	11.0	8.0	7.6	8.6	11.4	28.5	19.2	23.4	36.6	37.4
Chol EFA/Est. Chol	(%)	23.8	26.8	24.5	24.5	21.4	27.8	1.7	4.0	1.2	4.1	4.4
Chol 20 : 4 /Est. Chol	(%)	11.9	15.3	9.9	13.4	10.9	13.0	1.3	3.6	3.6	3.7	4.0
Total Tetraen (mg/100mgliver)		0.610	0.550	0.422	0.342	0.565	0.336	0.254	0.282	0.342	0.334	0.289
Cholesterol Ester Tetraen	(〃)	0.0022	0.0037	0.0014	0.0023	0.0027	0.0036	0.0010	0.0016	0.0023	0.0049	0.0054
Total Fatty Acid (mg/100mgliver)		2.57	2.07	1.72	1.41	1.95	1.19	3.03	2.33	1.97	2.05	1.92
No. of rats		5	5	5	1	4	3	5	5	4	4	3

As the days rolled on, in EFA diet group, 16 : 0+1, 18 : 0+1 acids decreased remarkably, 18 : 2, 20 : 4 acids increased evidently and 22 : 6 acid increased remarkably, in proportion, respectively.

In EFA deficient diet group, 16 : 0, 16 : 1 acids decreased, 18 : 0, 18 : 1, 18 : 2 acids increased, 20 : 3 acid decreased, 20 : 4 acid increased remarkably, and 22 : 6 acid increased, in proportion, respectively. This composition is considered to be affected by the mobilization of dépot fats.

ii) Fatty acid composition of cholesterol ester

In EFA diet group, 16 : 0+1, 18 : 2, 20 : 4 acids increased evidently, 18 : 0+1 acids decreased; in EFA deficient diet group, 16 : 0, 16 : 1, 18 : 0 acids decreased, 18 : 1 acid increased remarkably, 18 : 2 acid did not show any remarkable change, and 20 : 4 acid increased remarkably, in proportion, respectively. It was noteworthy that on the 8th day, 18 : 1 acid increased to get at 64% in the fatty acid composition of cholesterol ester.

The proportion of 20 : 4 acid showed marked increase since the early stage of fasting (since the second day) in the two diet groups, especially in EFA deficient diet group. The proportion of 18 : 2 acid in cholesterol ester of EFA deficient diet group did not increase so much as that of 20 : 4 acid.

iii) Cholesterol content

Both EFA diet group and EFA deficient diet group showed the increased cholesterol content in esterified as well as in total form, and the latter group exceeded the former group. In EFA deficient diet group, esterified cholesterol increased strikingly, so that the ratio of E. C./T. C. increased remarkably and reached 37.4% on the 8th day. Namely it is considered that cholesterol in EFA deficient diet group has combined with 18 : 1, 16 : 0, 16 : 1 acids, especially with 18 : 1 acid and accumulated in the liver.

In EFA diet group, the ratio of E. C./T. C. fluctuated but did not show any detectable changes.

The ratio of $Chol._{EFA}$/Est. Chol. or $Chol._{20:4}$/Est. Chol. showed marked increase since the early stage of fasting (since the second day) in the two diet groups, especially in EFA deficient diet group, in which, however, the ratio was extremely lowered. So that the conversion of cholesterol to bile acid should be suppressed in EFA deficient diet group.

iv) Tetraen content

In EFA deficient diet group, according to the marked increase of esterified cholesterol, tetraen content in esterified cholesterol increased in spite of the lowered ratio of $Chol._{20:4}$/Est. Chol. and exceeded it in the EFA diet group on the 8th day. However, the marked increase of esterified cholesterol in the liver of EFA deficient diet group was observed. So that the ratio of $Chol._{20:4}$/Est. Chol. is considered to be more suitable than tetraen content in cholesterol ester as an indicator of metabolic activity of esterified cholesterol in the liver.

IV. DISCUSSION

A) As described at the introduction, HIKASA et al. have investigated systematically the specific nutritional and physiological significance of EFA, and have come to the pre-

sumption that cholesterol esterified with EFA, especially with arachidonic acid should be the direct precursor of bile acid and that deficiency or metabolic disturbances in EFA should be responsible for gallstone formation.

FUKUDA showed that adrenocortical capacity and serum tetraenoic acid in the patients with gallstones decreased more than those in the control patients, and the same results were observed in the rats with EFA deficiency or its metabolic disturbance. These observations suggest that EFA deficiency or its metabolic disturbance will be concerned with the formation of gallstones.

On the other hand, it has been reported by several investigators who engaged in the studies on hypercholesterolemia or atherosclerosis that highly unsaturated fatty acids from vegetable oils promote cholesterol catabolism and bile acid excretion and lower the serum cholesterol level. These observations are advantageous to HIKASA'S presumption.

In order to establish HIKASA'S presumption, the attention should be directed to relations among esterified cholesterol in liver combined with EFA, cholesterol in liver, and bile acid in liver and bile. In present study, the relations have been already described in detail. The relations between the cholesterol content and the ratio of cholesterol esterified with EFA to esterified cholesterol in the liver of rats under various dietary conditions are illustrated in Fig. 3, 4.

1) Resting State

From Fig. 3, 4, it can be recognized that the cholesterol content in liver is in a complete negative correlation with the ratio of cholesterol esterified with EFA to esterified cholesterol, except esterified cholesterol content in the liver of $V.B_6$ deficient-lard diet group. On the other hand, MARUYAMA observed the bile acid contents in the liver slices of rats in EFA diet group, EFA deficient diet group, and $V.B_6$ deficient-EFA diet group, as shown in Fig. 5. As compared with MARUYAMA'S observation, those relations which are obtained from Fig. 3, 4 show that the increasing ratio of cholesterol esterified with EFA to esterified cholesterol results in the more conversion of cholesterol to bile acid and the less content of cholesterol in liver and suggest that cholesterol esterified with EFA may be the precursor of bile acid.

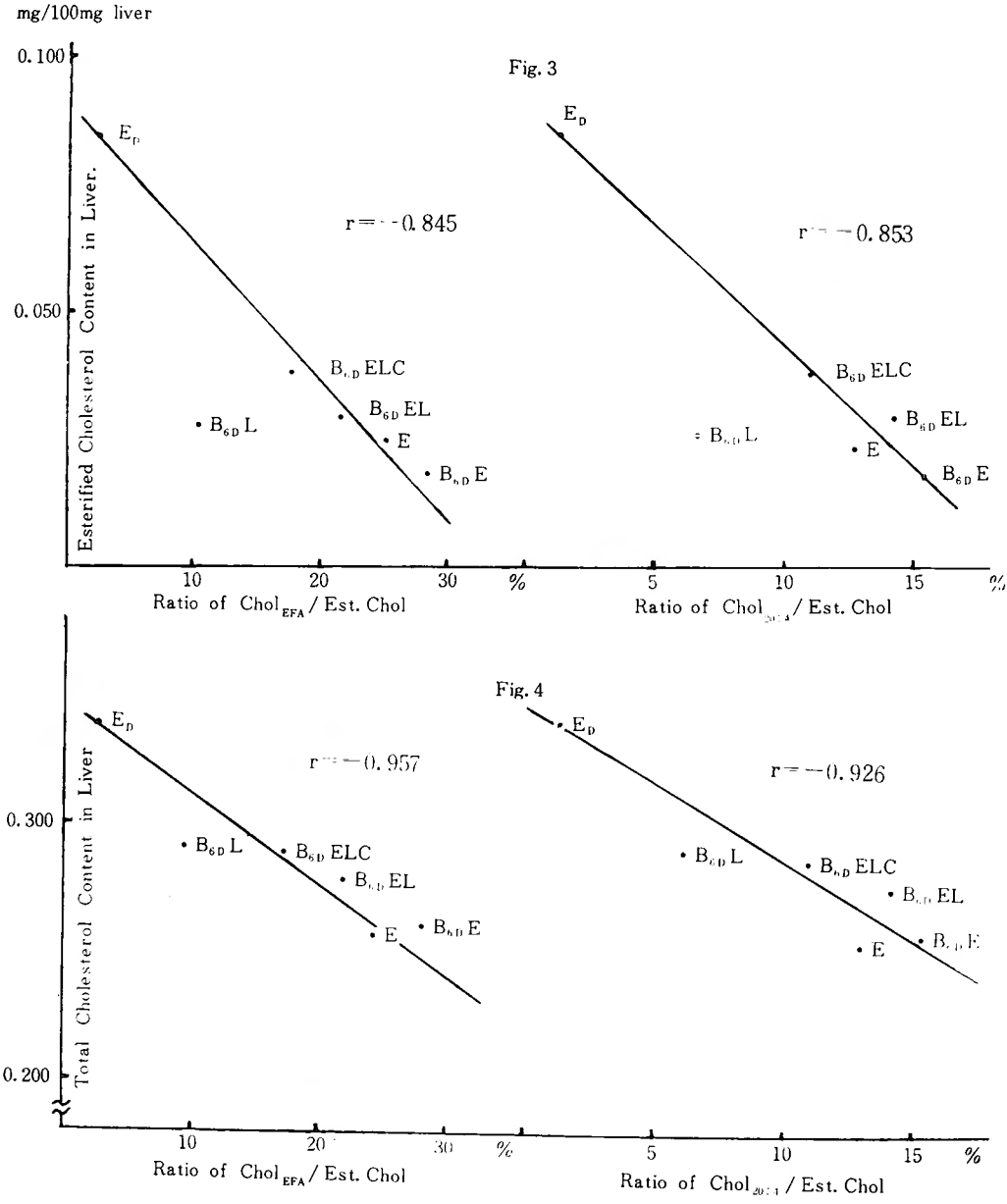
2) States exposed to stresses

Fig. 6, 7, show the relations between the same variables as described above when three diet groups (EFA diet, EFA deficient diet, $V.B_6$ deficient-EFA diet) of the rats have been injected with ACTH-Z in a dose of 3 I. U. into back muscles on 4 successive days. The arrows in figures indicate the course of this experiment. The relations in both EFA diet group and EFA deficient diet group showed negative correlation again, but in $V.B_6$ deficient-EFA diet group showed positive correlation.

From these correlations, following inferences are drown that after the administration of ACTH for 4 days, total bile acid in liver slices will increase in EFA diet group and EFA deficient diet group, and will decrease in $V.B_6$ deficient-EFA diet group.

In fact the inferences agreed with MARUYAMA'S observation except EFA diet group, in which total bile acid in liver slices decreased in slightly. However, YOSHINAGA reported that total bile acid in bile increased in EFA diet group at the same experimental conditions. In $V.B_6$ deficient-EFA diet group, total bile acid in liver slices decreased markedly

Fig. 3. 4 The Relations between the Cholesterol Content and the Ratio of Cholesterol esterified with EFA to Esterified Cholesterol in Liver



E : EFA diet group
E_D : EFA deficient diet group
$B_{6D}E$: V. B_6 deficient EFA diet group
$B_{6D}EL$: V. B_6 deficient EFA with Lard diet group
$B_{6D}ELC$: V. B_6 deficient EFA with Lard plus Cholesterol diet group
$B_{6D}L$: V. B_6 deficient Lard diet group
r : Correlation coefficient

Fig. 5 Changes in the Bile Acid Levels in the Liver of Rats Fed various Diets after the Administration of ACTH-Z

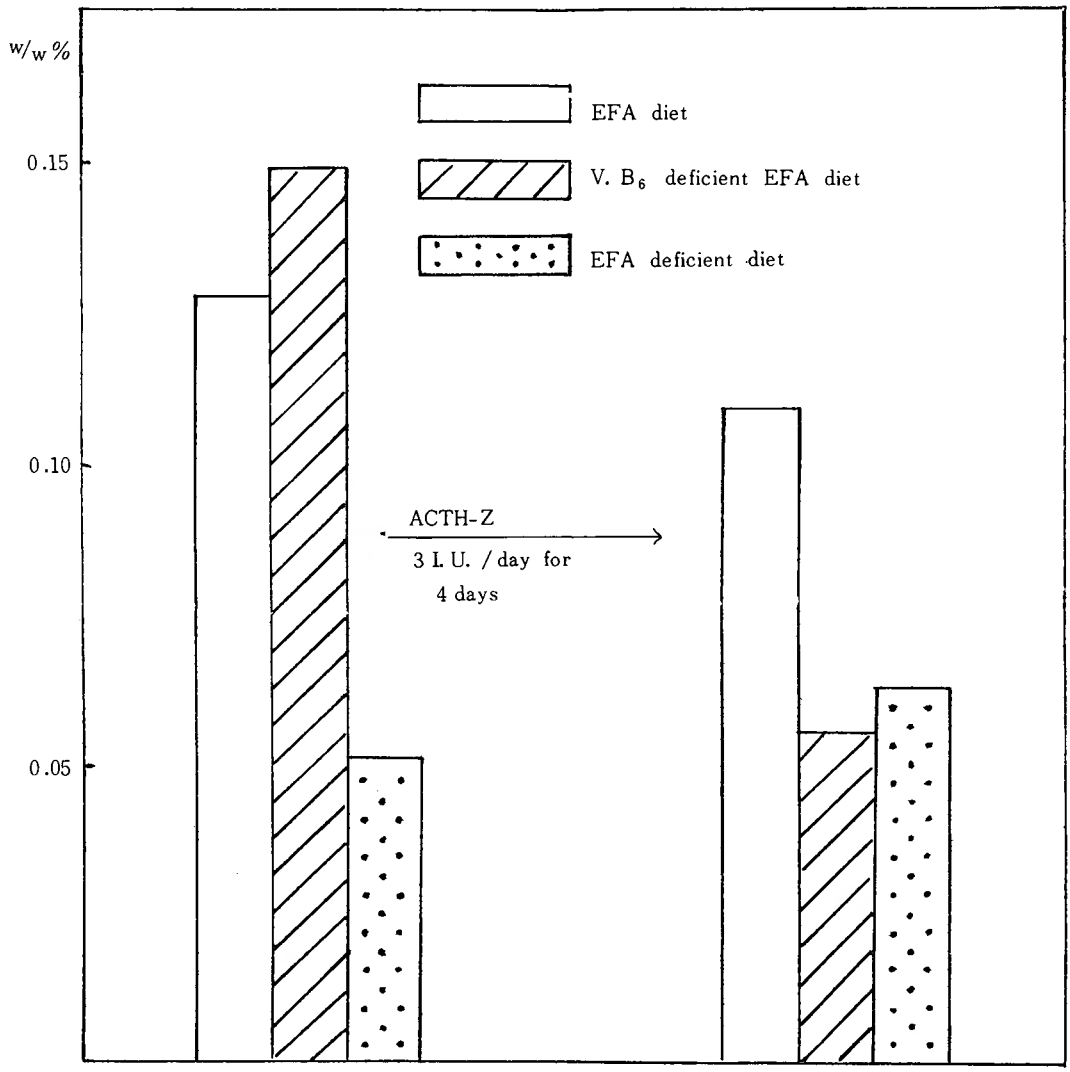


Fig. 5

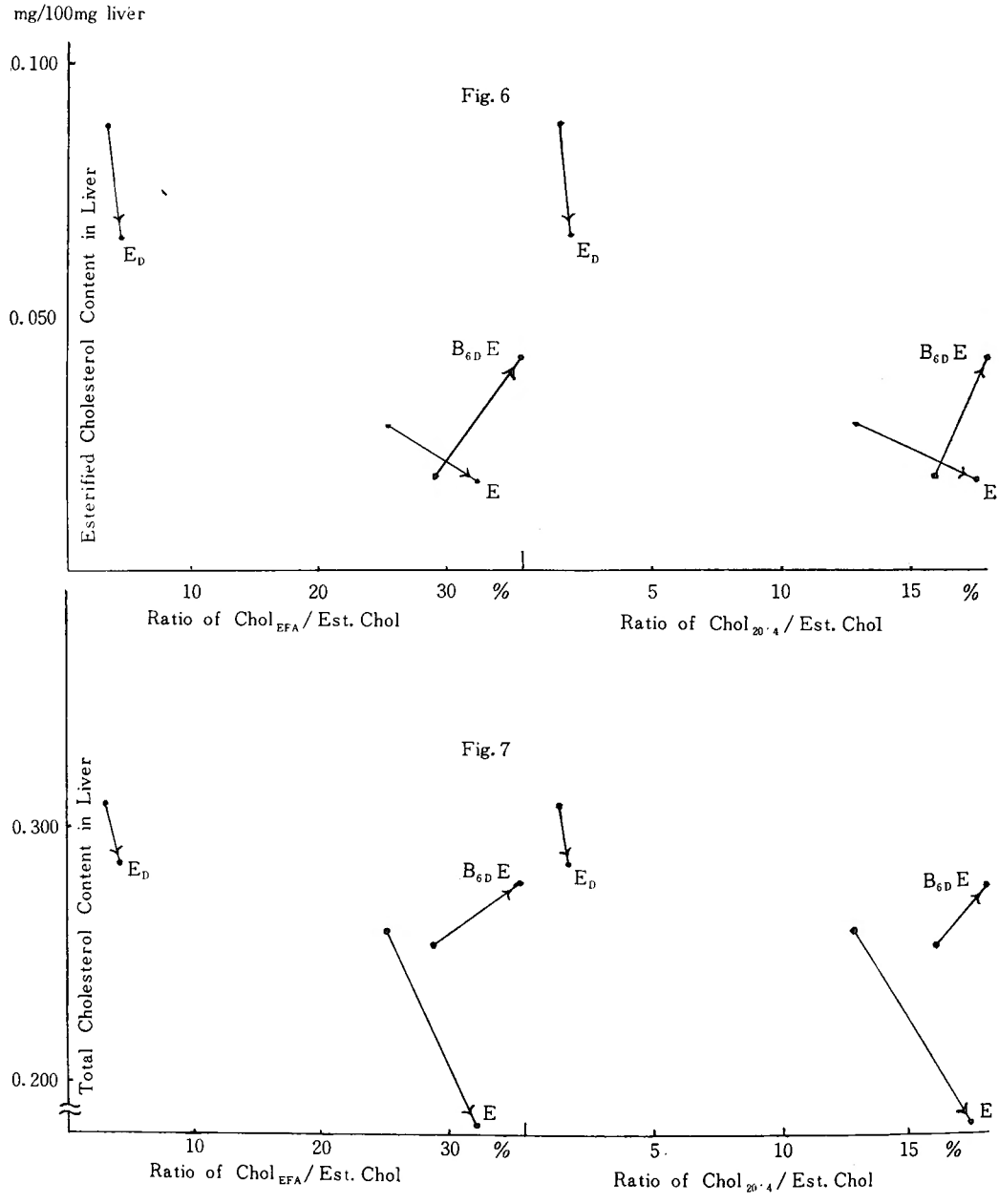
MARUYAMA

to the level of it in EFA deficient diet group.

From this observation it may be considered that in $V.B_6$ deficient-EFA diet group the conversion of cholesterol esterified with EFA to bile acid is suppressed, on the contrary, in $V.B_6$ supplemented diet groups is not affected, by the administration of ACTH, respectively.

Furthermore, in $V.B_6$ deficient-EFA diet group, by the successive administration of ACTH for 14 days, cholesteryl arachidonate showed specifically marked decrease in spite of sufficient amount of total arachidonic acid and bile acid in bile decreased (YOSHINAGA).

Fig. 6. 7 The Changes of Relations between the Cholesterol Content and the Ratio of Cholesterol esterified with EFA to Esterified Cholesterol in Liver of Rats after the daily Administration of ACTH-Z 3 I. U. for 4 Days



E : EFA diet group
E_D : EFA deficient diet group
$B_{6D}E$: V. B_6 deficient EFA diet group

And also, in starvation, EFA deficient diet group showed marked increase of cholesterol, especially esterified cholesterol and marked decrease of bile acid in bile (TANIMURA).

From these observations, it is evident that EFA is necessary for the synthesis of bile acid from cholesterol and in pyridoxine deficiency this process may be disturbed by stress and that cholesterol esterified with EFA especially with arachidonic acid may be a precursor of bile acid. Further, it is noteworthy that the conversion of cholesterol to bile acid can be explained more generally, as described already, by the ratio of $Chol_{EFA}$/Est. Chol or $Chol_{20:4}$/Est. Chol rather than by tetraen content in the liver.

B) Up to date, many attempts to clarify the mechanisms of gallstone formation have been done. However, the mechanisms still remain obscure. And many reports setting value on the local factors have been accepted commonly.

A colloid chemical unbalance of bile constituents has been well known as a direct cause of gallstone formation. So far as cholesterol stones are concerned, it is doubtless that the stability or solubility of cholesterol in bile would be the most important in the formation of gallstones. This expectation will be supported by the facts that human bile is always saturated or almost completely saturated with cholesterol unlike to the bile of other animals, and also has been evidenced by solubility-test of human gallstones in vivo or in vitro. But it was reported by MARUYAMA and YOSHINAGA that the increased concentration of cholesterol in bile itself could not be a primary factor for gallstone formation.

It has been demonstrated by many investigators that bile acid and lecithin in bile are important to keep cholesterol soluble in bile, and in fact, are decreased markedly in the bile of patients with gallstones. ISAKSSON has reported that lecithin forms, with part of the bile salts, a stable complex *i. e.* lecithin bile salts system (L. B. S.) and cholesterol is more soluble in L. B. S. than it is in bile salts alone. In fact, L. B. S. and ratio of L. B. S. to cholesterol decrease in bile of patient with gallstones (ISAKSSON). This observation has been confirmed by the solubility studies of JOHNSTON and NAKAYAMA.

MIYAKE has also shown that both the ratio of lecithin to cholesterol and of bile acid to cholesterol (B/C) are important to protect cholesterol precipitation in bile. Further, he observed marked decrease in total bile acid, appearance of free bile acid, relative increase in dihydroxycholanic acid and decrease in lecithin and he emphasized that biliary infection was an important factor for these changes.

On the other hand, MATSUO emphasized the importance of systemic factors on the formation of gallstones. ANDERSON has described that gallstones accompanied with cholecystitis are not pure cholesterol or bilirubin stones but always mixed stones. In other words, pure gallstones are never accompanied with cholecystitis and gallstone formations are due to disturbances of metabolism or of liver function rather than to an inflammatory reaction. In fact, we can often encounter with cholecystitis without gallstones.

Moreover, MARUYAMA observed the decreases in total bile acid and lecithin, and the other changes of biliary constituents in the bile of patients with gallstones even if biliary infection was not present.

These facts indicate that it is necessary to reperceive the importance of metabolic disturbances as an initiating factor of gallstones.

In the first place, biochemical analyses were made with the liver slices and aseptic

bladder bile removed from the patients with gallstones and with peptic ulcer (control group) without distinct disturbances of liver functions and infection of the bile. And then it was found that the experimental results obtained from the patients with gallstones were peculiar to cholelithiasis and similar to those from the experimental animals fed an EFA deficient diet, except some differences.

Based on the fact, SHIODA has succeeded in producing gallstones experimentally at the rate of a high occurrence by means of rearing hamsters on an EFA deficient-diet for two months or three. Hamster has been selected in this experiment for the reason that he has a gallbladder and his bile is resemble to human one in composition and constituents. Almost all the gallstones produced in the experimental animals were cholesterol stones, but some of them were pigment stones in which cholesterol content was very low.

YOSHINAGA observed decreases in total bile acids, B/C-ratio and total fatty acids, namely lecithin, and an increase in Di/Tri-ratio in bile of EFA deficient rats.

MARUYAMA observed the same patterns in bile of patients with cholesterol stones as described already.

Thus, it was considered that these changes in biliary constituents caused reduced lecithin-bile salt complex and lowered capacity of bile keeping cholesterol in solution, and then cholesterol gallstones were initiated. In other words, cholesterol stones will be initiated when these changes in biliary constituents are caused in bile ultimately. In present study, the changes of fatty acids and cholesterol in liver which form the foundation of these changes in biliary constituents were investigated.

In the liver of rats fed EFA deficient diet, fatty acids of linoleic acid family were extremely reduced quantitatively. From this fact, decreases of lecithin and its composing fatty acids are explained. And also the composition of fatty acids in bile showed patterns of EFA deficiency (YOSHINAGA). While fatty acids of both palmitoleic and oleic acid families increased remarkably. So that the ratio of $Chol_{EFA}$/Est. Chol or $Chol_{20:4}$/Est. Chol decreased extremely. Thus the conversion of cholesterol to bile acid was reduced and at the same time, cholesterol in esterified as well as in total increased in the liver.

However, it is sure that EFA deficiency itself is not the true cause in cholesterol gallstone formation in human beings. Because, in the liver of patients with cholesterol stones the decrease in total linoleic acid could not be observed in proportion as well as in absolute amount. The composition of fatty acids in bile of the patients did not show patterns of EFA deficiency. Namely, the disturbance in conversions of linoleic acid to further metabolites in linoleic acid family is considered as a main factor of human cholesterol stone formation. Therefore, the conditions were investigated by which the metabolic patterns similar to those in the patients with cholesterol stones were caused in the experimental animals with sufficient administration of linoleic acid.

1) In the first place, $V.B_6$-deficiency was considered as one of the conditions. Because, the important role of $V.B_6$ in fatty acid metabolism, especially in conversion of linoleate to arachidonate, has been demonstrated by many investigators. In present study, using rats fed a diet containing 15% of sesame oil under a relatively deficient state in $V.B_6$, the fatty acid composition of total lipid in liver was similar to that of patients with cholesterol stones. However, the reduced proportion of EFA in cholesterol ester and the

elevated esterified cholesterol content in liver were not observed.

YOSHINAGA observed that, in this case, the bile constituents were very similar to those of the patients with cholesterol gallstones, except the absence of decreased total fatty acids (*i. e.* lecithin).

2) In the second place, feeding of animal fat was considered. Because, animal fat is composed of chiefly several kinds of saturated fatty acids, oleic acid and a little EFA. Using rats fed a diet containing 10% of lard and 5% of sesame oil under a relatively deficient state in $V.B_6$, the metabolic patterns of fatty acid, cholesterol and bile acid in liver and bile were exactly similar to those of patients with cholesterol gallstones as compared with the control group.

3) In the third place, feeding of cholesterol was considered. Because, cholesterol is contained in animal food stuffs and accelerates the deficiency of EFA in vivo. When 0.1% of cholesterol was added to the diet in preceding experiment, the metabolic patterns of fatty acid and cholesterol in liver showed a close resemblance to those of the patients with cholesterol stones, and additional dietary cholesterol lowered evidently the proportion of EFA in the liver lipids, especially in cholesterol ester and elevated the cholesterol content in liver.

4) In the fourth place, exposure to stress was considered. MARUYAMA showed that after the daily administration of ACTH-Z 3 I. U. for 4 days, total bile acid in liver slices decreased markedly in only $V.B_6$ deficient-EFA diet group, although any significant difference was not observed between this diet group and EFA diet group in resting state. In present study, it is considered that, in $V.B_6$ deficient state, the conversion of cholesterol esterified with tetraen to bile acid may be suppressed by the stress described above. YOSHINAGA observed that after the daily administration of ACTH-Z 1 I. U. for 14 days, total bile acid in bile decreased in the same diet group. Thus, the composition of bile may be aggravated to pathological state by repeated exposure to stresses in the $V.B_6$ deficient state.

From these experiments, it is evident that dietary conditions affect the fatty acid composition and cholesterol content in liver conclusively. Thus, it is considered that, to say nothing of EFA deficiency, even in the state of sufficient administration of linoleic acid, the $V.B_6$ deficiency, the feeding of animal fat and cholesterol and the exposure to stress will produce a condition predisposing to cholesterol gallstone formation, namely bring about the decreased concentration of total bile acid and lecithin in bile ultimately.

On the contrary, MARUYAMA observed that only when patients were treated with sōya-lecithin containing excessive linoleic acid and V. B_6, in drainaged bile, total bile acid increased while cholesterol decreased, so that the ratio of B/C showed a great increase as compared with non-treated.

This observation supports such concept of process of cholesterol stone formation and can be applied in treatment and prevention of cholesterol gallstone disease.

On the other hand, it has been pointed out that a large absorption of saturated fatty acids causes $V.B_6$ deficiency, and the biosynthesis of $V.B_6$ is counteracted by the administration of sulfa amino acids, especially methionine and cystine. Production of $V.B_6$ by the intestinal flora is reduced with animal foods containing a large amount of protein and fat. In general, animal fat and protein contain less $V.B_6$ than vegetables. Thus, animal

food dieting is liable to $V.B_6$ deficiency.

SCHROEDER reported that the American diet, containing a large amount of animal fat and hydrogenated vegetable fat and protein, might possibly be marginal with respect to this vitamin. In fact, gallstones in European and American are composed chiefly of cholesterol.

The frequent occurrences of cholesterol stones have been observed in the city dwellers in our country who are apt to take a diet high in protein and animal fat with higher standard of living. Moreover, it is interesting that they suffer stresses more frequently than countrymen. Recently, with the elevation of standard of living in Japanese people the consumption of animal protein and fat has increased remarkably.

MATSUKURA reported that the cases with cholesterol stones have increased markedly and with infection in their bile decreased, in the patients of cholelithiasis admitted at his clinic since 1956.

These facts are advantageous to the concept of process of cholesterol stone formation in this study.

YOSHINAGA and MARUYAMA emphasized the role of metabolic disturbances in EFA due to $V.B_6$ deficiency upon cholesterol gallstone formation. However, the excessive dieting of animal fat and cholesterol themselves may be also important for cholesterol stone formation.

As a great quantity of EFA is also contained in adrenals, the hypofunction in this organ may be brought about in the deficiency or metabolic disturbances of EFA. Thus, it can not be neglected that the function of adrenals may be concerned with gallstone formation.

V. SUMMARY AND CONCLUSION

The role of EFA in the liver on cholesterol metabolism toward bile acid was discussed under the various dietary conditions (EFA diet, EFA deficient-diet, $V.B_6$ deficient-EFA diet, $V.B_6$ deficient-EFA with lard diet, $V.B_6$ deficient-EFA with lard plus cholesterol diet, $V.B_6$ deficient-lard diet) and stresses (administration of ACTH, starvation).

At the same time, the present study is dealing with the initiating factors of cholesterol gallstone.

A)

1) EFA are necessary for the synthesis of bile acid from cholesterol and, in relative pyridoxine-deficient state, this process may be disturbed by stresses.

2) The conversion of cholesterol to bile acid can be explained more generally by the ratio of cholesterol esterified with EFA or with arachidonic acid to esterified cholesterol rather than by tetraen content in the liver. Namely, in resting state, the increasing ratio of cholesterol esterified with EFA to esterified cholesterol results in the more conversion of cholesterol to bile acid and the less content of cholesterol in liver of rats in any dietary conditions.

3) From these observations it may be concluded that cholesterol esterified with EFA, especially with arachidonic acid is the precursor of bile acid.

B)

1) The results of biochemical analysis obtained from the patients with gallstones, especially with cholesterol stones were peculiar to cholelithiasis and similar to those from the experimental animals fed an EFA deficient diet, except some differences. Namely, EFA especially arachidonic acid in the liver which combined with cholesterol to activate its metabolic degradation was reduced. And fatty acids of both palmitoleic and oleic acid families synthesized in vivo increased in proportion, as if they had compensated for want of EFA. Total bile acids were reduced in the bile. Cholesterol in esterified as well as in total increased in the liver. Lecithin and its composing fatty acids decreased naturally to lower lecithin-bile salt complex in the bile. The ratio of total bile acids to cholesterol was lowered to weaken the capacity of bile keeping cholesterol in solution. The ratio of dihydroxycholanic acid to trihydroxy-cholanic acid was elevated.

These changes should cause the precipitation of cholesterol in the bile and initiate the cholesterol gallstones.

In fact, SHIODA has succeeded in producing cholesterol gallstones experimentally by means of rearing hamsters on an EFA deficient diet.

2) However, it is sure that EFA deficiency itself is not the true cause in cholesterol gallstone formation in human beings. Because, in the liver of patients with cholesterol stones the decrease in total linoleic acid was not observed in proportion as well as in absolute amount, and the composition of fatty acids in bile of the patients did not show patterns of EFA deficiency. Namely, the disturbance in conversions of linoleic acid to further metabolites in linoleic acid family is considered as a main factor of human cholesterol stone formation.

3) Therefore, the conditions were investigated by which the metabolic patterns similar to those in the patients with cholesterol stones would be caused in the experimental animals with sufficient administration of linoleic acid.

4) From the investigations described above, it is considered that to say nothing of EFA deficiency, even in the state of sufficient administration of linoleic acid, the $V.B_6$ deficiency, the feeding of animal fat and cholesterol and the exposure to stress will produce a condition predisposing to cholesterol gallstone formation, namely bring about the decreased concentration of total bile acid and lecithin in bile ultimately.

These conditions agree with the fact that the cholesterol stones occur frequently in those who are used to take a delicious diet containing excessive animal fat, protein and cholesterol.

5) Feeding of excessive linoleic acid and $V.B_6$ will be applied in treatment and prevention of cholesterol gallstone.

6) In present study, the conditions to reduce the capacity of bile keeping cholesterol in solution are investigated. Further, the critical conditions to precipitate cholesterol in bile should be investigated. On the other hand, the factors to promote cholesterolgenesis in liver should be investigated, too.

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和文抄録

コレステロールの胆汁酸への転換に関する肝臓中必須脂肪酸の役割及びそのコレステロール系胆石形成に対する意義

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平野穣

日笠は不可欠脂酸（EFA）の栄養学的並びに特殊生理学的意義に関する系統的研究を行ないそれが確かに副腎のコレステロール代謝に密接不可分の関係を有し副腎中のコレステロールは Tetraenoic Acid とエステル結合して初めて代謝活性となり，ステロイドホルモン就中 Glucocorticoids へと代謝されて行くものである事を実験的に明らかならしめた．こうなると，EFA を多量に保有し又コレステロール代謝の主要な場である肝臓に於ても analogical な関係が成立するのではないかとの臆測が生れて来る．即ち肝臓中のコレステロールもまた EFA 特に Tetraenoic Acid と結合して初めて円滑にその正常末梢代謝産物である胆汁酸へと代謝されて行くのではなかろうかと類推される．然りとすれば EFA の欠乏ないしはその代謝障碍は当然胆汁中胆汁酸の減少とレシチンの減少をもたらすものと考えられるところから，日笠は1960年にかかる方面からする胆石特にコレステロール系結石の成因究明を行なう必要性のある事を指摘，提唱した．まづ予備的に福田は胆石症患者の副腎皮質予備能力及びその血中 Tetraen 濃度の何れもが対照に比して明らかに低下している事を明らかにしたが，この事実は胆石症患者に EFA の欠乏ないしはその代謝障碍の存在する事を暗示しているものと言えよう．

本研究ではラッテを6群に即ち，1) EFA 投与群（対照群とする），2) EFA欠乏群，3) V. B_6欠乏EFA投与群，4) V. B_6欠乏・EFA・ラード投与群，5) V. B_6欠乏・EFA・ラード・コレステロール添加食投与群，6) V. B_6欠乏・ラード投与群に別ち，その安静時及びストレス負荷（ACTH-Z注射，飢餓）時に於ける肝脂質につき生化学的分析を行なつた．更に消化性潰瘍患者を対照として，胆石症患者の肝脂質についても生化学的に分析した．而して A〕肝臓中 EFA のコレステロールから胆汁酸への移行に占める役割を検討すると共に B〕併せてコレステロール系胆石の成因

についても討究した．その結果，次の様な事実を明らかならしめ得たのである．即ち，

A〕 1) EFA はコレステロールから胆汁酸への異化的代謝に際して不可欠な物質で且つこの過程は EFA の欠乏により或いは相対的V. B_6欠乏時にはストレスにより，強く障碍されるに至る．

2) 安静時には食餌の種類の如何に拘らず，肝臓中エステル型コレステロールの中で EFA 特にアラキドン酸と結合したものの占める割合—いわばエステル型コレステロールの活性化率—が大なれば大なる程益々肝臓中の胆汁酸は増量しそれに反して肝の含有コレステロールは減量する．

3) 以上の結果より EFA 特にアラキドン酸と結合したコレステロールが胆汁酸の前駆物質であろうと推測し得た．

B〕 1) 胆石特にコレステロール胆石患者について行ない得た生化学的分析結果は EFA 欠乏ラッテから得られたそれに極めて近似していた．即ちコレステロールと結合してそれを代謝活性ならしめる肝臓中の EFA 特にアラキドン酸は明らかに減少して居り体内で合成可能なパルミトオレイン酸系，オレイン酸系の脂酸が増加していた．それに基いて肝の総及びエステル型コレステロールの増量，胆汁中総胆汁酸量，レシチン及びその構成脂酸の減少，総胆汁酸量対コレステロール量の比の減少，Dihydroxycholan 酸量対 Trihydroxycholan 酸量の比の増大といつた一連の現象が招来され，そこに必然的にコレステロールの胆汁中での析出，沈澱が惹起されて然るべき病像を呈するに至つていた．事実塩田は EFA 欠乏食でハムスターを飼育し，コレステロール結石の形成に成功している．

2) しかしコレステロール結石患者に於ては肝臓中の総リノール酸及び胆汁中レシチンの構成に与つているリノール酸は共に含有比率，絶対量の何れに於ても減少を示して居らず，胆石症患者は EFA の絶対的欠乏下にあるものではなくしてその生体内代謝障碍下にあるものと考えるべきである．

3) よつてリノール酸を充分投与し乍らもコレステロール結石患者に近似せる代謝的病像を呈せしめるには如何にすればよいかを更に検討し我々はV. B_6欠乏下にあるものに対し，必然的にコレステロールや飽和酸を多量に含有する脂質であるところの動物性脂質を併せ投与したり或いはストレス暴露を行なうことがその様な状態下に個体を至らしめる大きな因子であることを明らかにする事が出来た．