Compounds X——A mixture of XI and corresponding amine was heated on a water-bath, evaporated to dryness. The residue was recrystallized from MeOH or EtOH.

3-Hydroxy-4-amino-6-methylpyridazine——XI (1 g) in 200 ml of MeOH was hydrogenated over 0.4 g of 20% Pd-C. The catalyst was filtered, the filtrate was evaporated to dryness. The residue was recrystallized from EtOH to give 0.53 (73%) of colorless cubes, mp 257.5—259°. *Anal.* Calcd. for $C_5H_7ON_3$: C, 48.00; H, 5.64; N, 33.58. Found: C, 48.09; H, 5.61; N, 33.65.

Reaction of 3-Methoxy-4-chloro-6-methylpyridazine 1-Oxide(XII) with Methylamine——a) In an Aqueous Solution: A mixture of 0.5 g of XII, 1.7 g of 30% methylamine and 2 ml of MeOH was heated in a sealed tube at 150—160° for 7 hours. The reaction mixture was evaporated to dryness and the residue was dissolved in $CHCl_3$, poured on an alumina column for chromatography. The solvent was removed to dryness from the elution. To the residue, diluted HCl was added, evaporated to dryness and the residue was recrystallized from EtOH to give 0.21 g of colorless needles, mp 227—228° (decomp.), which was identified with an authentic sample of XIII.

b) In an Ethanolic Solution: A mixture of 0.4 g of XII, 20 ml of ethanolic methylamine was heated in a sealed tube at 150—160° for 7 hours. Solvent was evaporated to dryness, the residue was recrystallized from benzene to give 0.31 g of yellow needles, mp 168—169°. *Anal.* Calcd. for $C_6H_8ON_3Cl$ (XIV): C, 41.50; H, 4.61; N, 24.21. Found: C, 41.45; H, 4.62; N, 24.97.

3-Hydroxy-4-chloro-6-methylpyridazine 1-Oxide(XIII)——A mixture of 0.3 g of XII and 7 ml of 18% HCl was refluxed for 5 hours. Reaction mixture was evaporated to dryness *in vacuo* and the residue was recrystallized from EtOH to give 0.24 g of colorless needles, mp 227.5—228° (decomp.). *Anal.* Calcd. for $C_5H_5O_2N_2Cl$: C, 37.40; H, 3.14; N, 17.45. Found: C, 37.48; H, 3.12; N, 17.50.

[Chem. Pharm. Bull. 20(1) 170—174 (1971)]

UDC 547.854.5.09 : 577.15.04

Effects of Phenobarbital, Ethanol and Ethionine on the Content and Fatty Acid Composition of Hepatic Microsomal Phospholipids[1]

Toshihiko Ariyoshi and Eigo Takabatake

Faculty of Pharmaceutical Sciences, Nagasaki University[2]

(Received April 14, 1971)

The drug metabolizing enzyme activities in the hepatic microsomes were induced by various drugs as well known.[3] The hepatic microsomes consist of fragments of the endoplasmic reticulum and contain higher proportion of phospholipids than any other fraction of cell.[4] The properties of microsomal membrane might be regulated in part by the phospholipid component.[5] The role of phospholipids in the hepatic microsomal drug metabolizing system has been recently investigated in enzymatic or spectral view.[6,7] The content of phospholipid was increased by phenobarbital, a potent inducer of drug metabolizing enzyme.[8]

1) A part of this work was presented at the 90th Annual Meeting of Pharmaceutical Society of Japan, Sapporo, July 1970.
2) Location: *Bunkyo-machi, Nagasaki.*
3) A.H. Conney, *Pharmacol. Rev.*, **19**, 317 (1967).
4) P. Favarger, "The Liver and Lipid Metabolism," ed. by C. Roullier Academic Press, New York, 1963, p. 549.
5) L.B. Krischner, *J. Gen. Physiol.*, **42**, 231 (1958); J.F. Hoffman, J.H. Schulman and M. Eden, *Federation Proc.*, **18**, 70 (1963).
6) H.W. Strobel, A.Y.H. Lu, J. Heidema and M.J. Coon, *J. Biol. Chem.*, **245**, 4851 (1970).
7) M.D. Chaplin and G.J. Mannering, *Mol. Pharmacol.*, **6**, 631 (1970).
8) H. Remmer and H.J. Merker, *Science*, **142**, 1657 (1963); S. Orrenius, J.L.E. Ericsson and L. Ernster, *J. Cell Biol.*, **25**, 627 (1965).

The authors suggested previously that ethanol and ethionine cause the qualitative and quantitative changes in the smooth endoplasmic reticulum and secondarily the changes of the activities of drug metabolizing enzyme.[9,10] In order to elucidate these changes, the present work was attempted in comparing microsomal phospholipids and their fatty acid composition after phenobarbital, ethanol and ethionine treatment.

Material and Method

Female Wistar rats weighing 100—150 g were divided into 5 groups of 6 each and fed Clea rat chow for a week prior to all experiments. Phenobarbital was injected intraperitoneally in a dose of 80mg/2 ml/kg once a day for 3 days and the animals were sacrificed 24 hr after the last injection. Ethanol was given orally in a dose of 5.0g/25 ml/kg 16 hr before sacrifice. *d,l*-Ethionine dissolved in 0.9% NaCl (25 mg/ml) was injected-intraperitoneally in 3 divided doses within 2 hr in a total dose of 750 mg/kg 24 hr before sacrifice.

The livers were homogenized with 4 volumes of 0.25 M sucrose containing 1 mM EDTA and the microsomes were prepared by the procedures described previously.[9] Total microsomal lipids were extracted with chloroform-methanol (2: 1, v/v). The 96—97% of lipids was extracted with this usual solvent and no significant differences were observed in the lipid contents from various treatments. The extracts were washed according to Folch, *et al.*,[11] concentrated in an atmosphere of nitrogen and stored in chloroform under nitrogen. Microsomal phospholipids were separated from the lipid mixture by silicic acid chromatography[12] and each fraction of phospholipid was identified by means of the thin layer chromatography using the solvent system of Abramson, *et al.*[13] Phosphorus analyses were performed on aliquots of each fraction.[9] In the elution pattern of this method, there is a fraction which may contain glycerides, pigments, sterols and sterol glycoside, but the content of phosphorus in this fraction was less than 0.1% of the total phosphorus. Average recovery from the columns was 85%. The fatty acids of each fraction were methylted by a modification of the method of Stoffel, *et al.*[14] and analyzed by gas chromatography on a 5% diethyleneglycol succinate-1% H_3PO_4 on Chromosorb W, 60—80 mesh, column using a Shimadzu GC-1C Chromatograph equipped with a hydrogen flame ionization detector. The fatty acids were identified by comparison of retention times with those of known standards. The peaks were evaluated by triangulation. Statistical comparisons were made using the Student *t*-test.

Result

Effect on Phospholipid Content and Composition

The phospholipid fractions of hepatic microsomes obtained from female rats pretreated with phenobarbital or ethanol and/or ethionine were analysed and shown in Table I. Major phospholipids of microsomes were phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Phenobarbital treatment increased the total phospholipid content with the increases of PE and PC content. On the contrary, the administration of ethanol or ethionine in the dose used decreased slightly total phospholipid and PC content but increased PE significantly. The percentage composition of microsomal phospholipids was remained unchanged by phenobarbital, but the ratio of PE: PC was increased by ethanol and/or ethionine.

Effect on Fatty Acid Composition of Phospholipid

In Table II, the effects of phenobarbital or ethanol and/or ethionine on fatty acid composition of each phospholipid fraction were summarized. The major fatty acids were 16: 0, 18: 0, 18: 1, 18: 2, 20: 4 and 22: 6. The proportion of saturated acids was higher in minor phospholipids, sphingomyelin (S) and phosphatidylinositol (PI), than in PE and PC. Either amount of fatty acid composition of minor phospholipids did not show significant differences

9) T. Ariyoshi, E. Takabatake and H. Remmer, *Life Sci.*, **9**, Part II, 361 (1970).
10) T. Ariyoshi and E. Takabatake, *Life Sci.*, **9**, Part II, 371 (1970).
11) J.F. Folch, M. Lee and G.H. Sloane-Stanley, *J. Biol. Chem.*, **226**, 497 (1957).
12) D.J. Hanahan, J.C. Dittmer and E.A. Warashina, *J. Biol. Chem.*, **228**, 685 (1957); R.J. Morin, *Cancer Res.*, **25**, 118 (1965).
13) D. Abramson and M. Blecher, *J. Lipid Res.*, **5**, 628 (1964).
14) W. Stoffel, F. Chu and E.H. Ahrens, *Anal. Chem.*, **31**, 307 (1959).

TABLE I. Effects of Phenobarbital, Ethanol and Ethionine on Compositions of Hepatic Microsomal Phospholipids

A) Phosphorus content, μg/g of liver

Fraction[a]	Control	Phenobarbital	Ethanol	Ethionine	Ethanol+ ethionine
PE	64.1±2.0	87.8±2.0[b]	67.7±3.4	75.8± 3.0[b]	92.3± 2.9[b]
S	11.0±1.3	13.9±2.4	11.8±2.4	10.9± 1.3	10.0± 0.8
PC	116.9±2.3	144.6±4.8[b]	100.2±5.3	92.7±12.3	100.4±15.1
PI	14.3±2.0	12.3±1.9	11.5±2.4	13.5± 1.3	15.1± 1.5
Total	206.3±4.8	258.6±7.1[b]	191.7±7.6	192.9±10.9	217.7±13.1

B) Per cent phosphorus of total phosphorus

Fraction[a]	Control	Phenobarbital	Ethanol	Ethionine	Ethanol+ ethionine
PE	31.5±0.4	33.4±1.0	35.6±1.7[c]	40.0±3.2[c]	43.5±3.6[c]
S	5.3±0.5	5.3±0.8	6.1±1.0	5.8±0.8	4.7±0.6
PC	56.8±0.7	55.9±1.0	52.4±1.5[c]	47.1±3.9[c]	44.9±4.3[c]
PI	6.9±0.9	4.8±0.8	6.0±1.2	7.1±0.7	7.0±0.6
PE/PC	0.55	0.60	0.68	0.84	0.97

a) PE: phosphatidylethanolamine, S: sphingomyelin PC: phosphatidylcholine, PI: phosphatidylinositol
The values are mean ± standard error of 6 rats per group.
b) $P<0.01$
c) $P<0.05$

between treatment and control values excepting for the reduction of percentage of 16: 0 in S by ethanol treatment. In PE fraction, phenobarbital reduced the percentage of saturated acids, especially 18: 0. On the contrary, the treatment with ethanol and/or ethionine caused the small increase of 18: 0 and the marked decrease of 16: 0 and increase of polyunsaturated acids.

Discussion

Many drugs induce hepatic microsomal drug metabolizing enzymes. Phenobarbital, a typical inducer, increases aminopyrine demethylase and aniline hydroxylase activities, cytochrome P_{450} concentration, and spectral change of P_{450} with substrates.[3,8,15]
Ethanol or ethionine enhanced apparent aniline hydroxylase activity but did not change aminopyrine demethylase. No correlation of enzyme activity with P_{450} concentration or spectral change was observed.[9,10] These facts suggest that the mechanisms of ethanol or ethionine on drug metabolizing enzymes differ from that of phenobarbital.

The importance of phospholipids in the mechanism of drug metabolizing enzyme system has been recognized. Strobel, *et al.*[6] showed the PC requirement in the hydroxylation of drugs by a solubilized microsomal system containing cytochrome P_{450}. Chaplin, *et al.*[7] treated microsomes with phospholipase C and provided the evidence that the type I binding site is associated with membrane phospholipids.

According to Schulze, *et al.*,[16] there is a close correlation between the changes in phospholipid content and those in P_{450} content in various conditions of animals, and P_{450} was suggested to be a lipoprotein.

15) H. Remmer, R.W. Estabrook, J. Schenkman and H. Greim, *Arch. Exptl. Pathol. Pharmakol.*, **259**, 98 (1968).
16) Hu. Schulze and Hj. Staudinger, *Z. Physiol. Chem.*, **351**, 184 (1970).

TABLE II. Effects of Phenobarbital, Ethanol and Ethionine on Fatty Acid Compositions of Hepatic Microsomal Phospholipids

A) Phosphatidylethanolamine

Fatty acids	Control	Phenobarbital	Ethanol	Ethionine	Ethanol+ ethionine
16:0	22.3±0.7	19.3±0.7	17.4±0.8[a]	16.0±0.6[a]	16.1±0.5[a]
16:1	1.0±0.1	0.5±0.1	0.4±0.1	0.5±0.1	0.4±0.1
18:0	26.3±0.8	21.8±0.8[b]	29.9±1.2[b]	28.8±0.6[b]	31.7±2.0
18:1	15.1±0.3	16.0±0.5	12.4±0.6	11.3±0.3	12.1±0.5
18:2	7.8±0.6	9.7±0.4	7.3±0.1	8.6±0.7	8.0±0.4
(20:3)[c]	1.2±0.2	3.5±0.3	2.4±0.2	2.8±0.1	2.6±0.3
20:4	16.8±1.0	18.7±1.2	20.0±0.8[a]	20.4±0.4[a]	19.5±0.4[a]
22:6	9.2±0.2	11.4±1.6	13.0±0.9[a]	13.6±1.5[b]	12.3±1.3[b]

B) Sphingomyelin

Fatty acids	Control	Phenobarbital	Ethanol	Ethionine	Ethanol+ ethionine
16:0	33.5±1.5	31.2±1.8	26.2±0.8[b]	32.4±0.7	25.2±3.1[b]
18:0	28.1±2.7	32.2±0.3	25.1±1.3	31.2±0.8	25.3±3.4
18:1	5.6±0.7	3.6±0.6	4.7±0.9	6.9±2.5	4.4±1.5
18:2	2.1±0.6	1.8±0.1	1.1±0.2	4.0±2.8	2.1±0.8
(20:3)[c]	8.6±1.0	6.7±2.7	6.7±1.6	2.9±0.3	6.9±0.9
20:4	4.3±0.7	6.7±2.0	5.4±0.9	5.2±1.2	6.9±3.4
(20:5)[c]	5.0±0.4	4.2±0.9	5.4±0.2	1.8±0.4	5.6±2.1
( )[d]	4.3±1.5	4.2±1.6	4.1±1.4	4.0±0.7	2.2±0.1
( )[d]	—	5.5±0.3	10.5±0.3	3.3±1.5	10.2±1.1
22:6	8.5±1.8	6.1±2.0	11.3±3.2	8.7±2.2	11.4±2.6

C) Phosphatidylcholine

Fatty acids	Control	Phenobarbital	Ethanol	Ethionine	Ethonol+ ethionine
16:0	21.6±0.4	19.2±0.4	19.3±1.0	19.7±0.2	19.5±1.0
16:1	0.5±0.1	0.8±0.1	0.4±0.1	0.5±0.1	0.4±0.1
18:0	24.6±0.3	24.0±0.8	24.9±0.8	24.6±0.7	25.1±0.7
18:1	12.3±1.0	12.7±1.2	11.4±1.1	11.4±0.8	11.4±0.7
18:2	13.8±0.4	14.2±0.6	11.0±0.4[b]	17.0±1.3[b]	17.0±0.7[b]
(20:3)[c]	—	—	—	2.3±0.1	2.0±0.8
20:4	19.1±0.8	20.3±1.4	22.5±0.6[b]	17.3±1.2	17.1±0.9[b]
(20:5)[c]	0.4±0.1	0.9±0.1	1.1±0.3	1.3±0.2	1.3±0.4
22:6	7.0±0.3	7.0±0.6	8.5±0.5	5.9±0.8	5.8±0.2

D) Phosphatidylinositol

Fatty acids	Control	Phenobarbital	Ethanol	Ethionine	Ethanol+ ethionine
16:0	28.5±4.1	28.6±4.5	26.0±4.1	27.1±4.2	26.0±3.3
18:0	35.1±0.8	35.7±3.2	35.8±2.5	37.9±3.8	36.0±3.5
18:1	7.4±1.0	7.4±0.8	7.0±0.5	8.5±0.1	8.0±0.7
18:2	3.4±1.2	4.5±0.2	3.7±1.2	3.8±1.8	4.6±2.1
( )[d]	2.2±0.3	—	2.4±0.3	1.1±0.2	2.2±0.2
(20:3)[c]	7.4±4.3	7.0±3.0	7.0±4.5	6.7±4.1	6.7±4.5
20:4	6.9±2.3	7.1±3.0	7.4±3.2	7.8±3.6	6.0±2.3
(20:5)[c]	0.8±0.1	—	0.5±0.1	—	0.4±0.1
( )[d]	—	1.7±0.2	3.2±0.2	—	3.3±1.3
22:6	8.2±2.6	7.8±1.8	7.1±1.6	6.6±1.2	6.7±1.9

a) $P<0.01$
b) $P<0.05$
c) Not identified comparing with authentic sample but estimated from retention time.
d) unidentified
The values are expressed as percentage of total fatty acids detected. Mean ± standard error of 6 rats per group.

Although the phospholipid levels increase in hepatic microsomes treated by phenobarbital or other drugs, little information is available about the effect of drugs on the fatty acid composition of phospholipids. According to Holzman, *et al.*,[17] phenobarbital pretreatment enhanced synthesis of microsomal phospholipid in male rats but not in females and reduced the catabolism of phospholipid in both male and female rats. Schulze, *et al.*[16] demonstrated that the qualitative composition of microsomal phospholipids remained unchanged, although the content of phospholipids was increased by phenobarbital treatment.

Mendenhall, *et al.*[18] observed that the chronic ethanol administration increased hepatic phospholipid content, principally PC, and that ethanol altered fatty acetyl-CoA lysophosphatide transferase activities *in vitro* in male rat liver microsomes. Sato[19] reported a slight decrease in PC and an increase in PE of female rat liver *in vivo* and *in vitro* by ethionine and suggested that this phenomenon may result from the inhibition of transmethylation in liver microsomes.

The present study showed that phenobarbital did not change the composition of microsomal phospholipid but decreased the proportion of stearic acid in PE. On the other hand, the increase of PE and the decrease of PC were observed in the ethanol and/or ethionine induced fatty liver. Thus, the ratio of PE to PC was increased by ethanol and /or ethionine treatment. The alteration in the fatty acid compositions of phospholipids were also observed in female rat liver microsomes following ethanol/or ethionine treatment. Especially, the increase of polyunsaturated fatty acid such as arachidonic and docosahexaenoic acid and the decrease of saturated acid such as palmitic acid in PE fraction were noteworthy.

Kaschnitz[20] recently reported that the aniline hydroxylase activity was reduced in the essential fatty acid deficient rat liver microsomes which contain very low proportion of arachidonic acid. In other work,[21] thioacetamide treatment reduced markedly aniline hydroxylase activity, cytochrome P_{450} content and spectral change of P_{450} with aniline. Furthermore, we observed also the decrease of percentage of arachidonic acid in total fatty acids of PE and PC in microsomal phospholipids. These facts suggest that polyunsaturated fatty acid in phospholipids might play some important role in aniline hydroxylase system.

It is still not obvious how these changes in fatty acid composition relate to the drug metabolizing enzyme activities. However, it is not unreasonable that the changes of fatty acid composition result in the changes of conformation and function of endoplasmic reticulum membrane and secondarily the enhancement of drug metabolism.

17) J.L. Holtzman and J.R. Gillette, *Biochem. Biophys. Res. Commun.*, **24**, 639 (1966); *idem*, *J. Biol. Chem.*, **243**, 3020 (1968).
18) C.L. Mendenhall, R.H. Bradford and R.H. Furman, *Biochim. Biophys. Acta*, **187**, 501, 510 (1970).
19) R. Sato, *Sapporo Med. J.* (Japan), **31**, 41 (1967).
20) R. Kaschnitz, *Z. Physiol. Chem.*, **351**, 771 (1970).
21) T. Ariyoshi, Y. Toshinaga and E. Takabatake, Meeting of Kyushu Branch, Pharmaceutical Society of Japan, Fukuoka, February 1971.