EFFECTS OF RIBOFLAVIN DEFICIENCY ON THE LIPIDS OF RAT LIVER MITOCHONDRIA AND MICROSOMES

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Summary Weanling male rats were fed a riboflavin deficient diet for 5 weeks and enlargement of the liver mitochondria and discontinuity of the outer membrane were observed. The content of phospholipids was slightly increased in the deficient mitochondria, and decrease in phosphatidylcholine and increase in phosphatidylethanolamine were shown respectively. In comparison with the mitochondria, the content and distribution of phospholipids in microsomes were not affected. The fatty acid composition of phosphatidylcholine in mitochondria and microsomes was remarkably altered by the deficiency, and increases in palmitic and linoleic acids and decrease in arachidonic acid were demonstrated. The incorporation of ³²P into diphosphatidylglycerol in mitochondria was reduced by the deficiency. The incorporation into other phospholipids was not significantly altered, whereas the incorporation into the subspecies of phosphatidylcholine was variously affected.

By the intraperitoneal injection of riboflavin to the deficient rats, normalization of the mitochondrial size and fatty acid composition of liver mitochondrial lipids was observed. However, decreased incorporation of ³²P into diphosphatidylglycerol in mitochondria was not recovered completely at 40 hours after the injection, and in the mitochondrial lipids linoleic acid was higher and arachidonic acid was lower than respective controls at 60 hours.

Riboflavin deficiency is accompanied by decrease in flavin enzymes, resulting in degeneration of energy production. In addition to the decline in rate of respiration, changes of intracellular microstructure of the livers were revealed in the riboflavin-deficient rats (1) or mice (2–3). It was reported in our earlier paper that riboflavin

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deficiency exerted effects upon fatty acid composition of rat liver lipids and decrease in arachidonic acid and increase in linoleic acid were observed (4).

In the present paper, changes in the structure and lipids of the liver mitochondria were studied in riboflavin-deficient rats. Hence arachidonic acid is a predominant constituent of phospholipids in mitochondrial membrane, it was presumed that decrease in arachidonic acid would affect the membrane structure of the mitochondria.

Weanling rats were fed a riboflavin-deficient diet for 5 weeks, and enlargement of the liver mitochondria, diminution of the inner membrane profiles and discontinuity of the outer membrane were revealed by electron microscopy as reported previously by others (1-3). Fatty acid composition of the liver mitochondria was remarkably altered and incorporation of ^{32}P into the respective subspecies of phosphatidylcholine of the mitochondria was variably changed by the deficiency, whereas the content of phospholipids in the mitochondria was slightly altered. The incorporation of ^{32}P into diphosphatidylglycerol diminished in the liver mitochondria of the deficient rats.

The exchange of phospholipids between mitochondria and microsomes was reported by WIRTZ and ZILVERSMIT (5) and McMURRAY and DAWSON (6), therefore the microsomal lipids of the riboflavin-deficient rat liver were studied simultaneously with determination of mitochondrial lipids. The alterations in fatty acid composition of phospholipids and incorporation of ^{32}P into the subspecies of phosphatidylcholine were found in microsomes as well as in mitochondria.

EXPERIMENTAL

1. *Animals.* Twenty weanling male rats, Wistar-King strain, 21-days-old were fed a riboflavin-free basal diet and maintained for 5 weeks as reported previously (4).³ As the control, 10 rats were fed the diet to which 20 mg of riboflavin per kg of the basal diet had been added. The experiments were performed three times. Observations of electron microscopy and analysis of lipids were repeated twice and results of the lipid analysis were shown as average of the two experiments. Incorporation of ^{32}P and analysis of subfractions of phospholipids were carried out with the third experiment. In each experiment, rats were taken at random from the deficient and control groups.

For the recovery experiment, riboflavin was dissolved in sterilized 0.9% NaCl (w/v) solution as 5 mg of riboflavin per ml, and injected intraperitoneally to the deficient rats at the concentration of 10 mg per 100 g of body weight under light ether anaesthesia, while control rats were injected the sterilized NaCl solution. The

³ Composition of the basal diet: sucrose, 58.15%; vitamin-free casein (Nutritional Biochemical Corporation), 28.1%; salts (McColumn's salt mixture No. 185), 3.1%; vitamin mixture in casein (inositol, 200 mg; thiamine hydrochloride, 10 mg; pyridoxine hydrochloride, 10 mg; niacin, 40 mg; calcium pantothenate, 40 mg; menadione, 6 mg; folic acid, 5 mg; ascorbic acid, 20 mg; vitamin A acetate, 0.86 mg; vitamin D₃, 6.3 μg ; vitamin-free casein to 1 g), 0.5%; Crisco, 10.0%, and choline chloride, 0.15%.

riboflavin was supplemented to each rat daily when the recovery experiment was continued for a few days.

2. *Preparation of mitochondria and microsomes.* In each experiment, the rats were sacrificed by decapitation at 9 to 10 o'clock, and the livers were removed immediately and placed in a cold 0.25 M sucrose solution (0–2°C). The livers were homogenized in 10 volumes of the 0.25 M sucrose solution containing 10^{-4} M EDTA, pH 7.4 (0–2°C), and mitochondria were prepared essentially by the same method as described by JOHNSON and LARDY (7). The homogenate was centrifuged at $600 \times g$ for 15 min, and the upper suspension was saved. The pellets were suspended in 5 volumes of the sucrose solution, and centrifuged at $600 \times g$ for 10 min. The upper fractions were combined with the former, and mitochondria were sedimented at $8,000 \times g$ for 20 min. The pellets were resuspended in the sucrose solution by homogenization and centrifuged at $10,000 \times g$ for 10 min. The subsequent mitochondrial pellets were suspended by hand-operated homogenization in the sucrose solution as 1 ml per g of the original tissue, and preserved at 0 to 2°C until for use.

The upper suspension after sedimentation of mitochondria were centrifuged at $15,000 \times g$ for 10 min, and the upper fractions were centrifuged at $105,000 \times g$ for 60 min, and subsequent pellets of microsomes were suspended by homogenization in 1 ml of 1.15% KCl (w/v) per g of the original tissue.

3. *Electron microscopy.* Immediately after removal, small pieces of the liver were fixed in ice cold 1% osmium tetroxide buffered with 0.1 M tetramethyldiarsine solution, pH 7.4, for 2 hours. The specimens were dehydrated in ethanol, and embedded in epoxy resin.

For electron microscopy of the isolated mitochondria, a small portion of mitochondrial pellets after the second centrifugation was fixed, and embedded in the same way as mentioned above. The sections from both tissues and mitochondrial pellets were made with glass knives on a Porter-Blum microtome, stained with lead tartarate solution and examined in a Hitachi HU 12 A electron microscope.

4. *Preparation and analysis of lipids.* The suspension of mitochondria or microsomes which corresponded to 2 g of the original liver was mixed in 20 volumes of chloroform and methanol (2/1 = v/v) and the lipids were extracted with FOLCH's method (8) as described previously (4). The lipid extract was evaporated to dryness under N_2 gas and lipid residues were dissolved in 5 ml of chloroform containing 1% of methanol (v/v). Phospholipids were determined with 0.1 ml of the lipid solution, after digestion with 70% perchloric acid by the method of COLLINS and WHEELDEN (9).

The lipid solution of 0.5 to 1 ml was taken for the analysis of fatty acid composition, and the lipids were saponified with methanolic KOH. The fatty acids were methylated with BF_3 methanol complex (10), and analyzed by gas chromatography as described in our previous paper (4).

For analysis of individual phospholipids, 0.5 ml of the lipid solution were applied on a thin layer plate of silica gel H (Merck), and the solvent of

chloroform/methanol/acetic acid = 60/25/4 (v/v/v) was used. The phospholipids were detected with 0.2% dichlorofluorescein under ultraviolet light. Each fraction was transferred into a digestion flask, and phosphorus of phospholipid was determined after digestion with perchloric acid.

The composition of fatty acids in individual phospholipids was analyzed after separation of the phospholipids by thin layer chromatography, using 2 to 3 ml of the lipid solution. The silicic acid area corresponding to phospholipid was transferred into a centrifuge tube with sintered stopper, and the phospholipid was eluted from the silicic acid with 5 ml of chloroform/methanol/acetic acid/water = 50/39/1/10 (v/v/v/v). The extraction was repeated three times, followed by centrifugation each time. The extracts were combined and then washed with 5 ml of 4M NH_4OH solution according to the method of FEX (11). The upper phase was discarded, and the lower phase was further washed three times with 5 ml of 50% methanol in water (v/v) containing 0.5% NaCl (w/v). The lipid extract was evaporated and saponified, and fatty acids were analyzed as described above. For analysis of subspecies of phosphatidylcholine, having fatty acids of different number of desaturation, the lipid was fractionated by thin layer chromatography with silica gel H containing AgNO_3 (15 g per 50 g of silica gel H). The plate was activated at 190°C for 1 hour before use. The solvent of chloroform/methanol/water = 60/30/5 (v/v/v) was used. The individual subspecies of the phospholipid were extracted as described above, and the extracts were used for analysis of phosphorus or radioactivity when incorporation of ^{32}P into phospholipids was performed.

5. *Incorporation of ^{32}P .* The *in vivo* incorporation of ^{32}P into phospholipids of the liver was carried out by intraperitoneal injection of inorganic phosphate. The ^{32}P in HCl solution was obtained from Japan Radio Isotope Association, Tokyo. As a carrier of inorganic phosphate, 0.1 M phosphate buffer of pH 7.4 which had been sterilized was added to the ^{32}P solution at the final concentration of 1 mCi/1 mg of phosphorus, and pH of the solution was adjusted to 7.4 with 0.1 N KOH. To the solution, sterilized aqueous 0.9% NaCl was added, and the solution was injected to rats at 1 μCi per 100 g of the body weight, while under light ether anaesthesia. The rats were sacrificed 15 hours after the injection of ^{32}P , and the mitochondria and microsomes were prepared. The lipids in each organelle were extracted and the content of phosphorus and radioactivities of phospholipids were determined.

A portion of the lipid extract was taken to a vial and evaporated. To the vial, 10 ml of scintillator solution containing 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis(5-phenyloxazol-2 yl) was added, and radioactivity was counted with Hitachi-Horiba scintillation spectrometer (Model LS 500). The radioactivity of individual phospholipids was determined after separation by thin layer chromatography as described above. The spots on the plate were detected with iodine vapour. Each area was scraped and transferred into a vial, and radioactivity was counted after the decolorization of iodine. The radioactivities of subspecies of phosphatidylcholine were determined after separation by thin layer chromatography. The lipids were extracted as described above, and a portion of the extracted solution was used for

counting. The recovery of the radioactivity was about 85 to 90% of the original lipid solution which was applied on the thin layer chromatograms. The final count of radioactivity was adjusted to the initial value at the date of injection, using the recovery ratio and the time of decay of ^{32}P . The specific radioactivities were expressed as cpm/ μg of inorganic phosphorus in phospholipids.

Concentrations of protein in mitochondria and microsomes were determined by LOWRY's method (12) with bovine albumin fraction V as the standard.

RESULTS

1. *Electron microscopy of the liver mitochondria*

Enlargement of the mitochondria was seen in the hepatocytes of the deficient rat as shown by others (1-3). Mitochondria of various sizes were observed in the deficient rats, and giant mitochondria occurred in the hepatocytes of the rat accompanied by severe symptoms of riboflavin deficiency such as dermatitis of the lip and nose, appearance of oily and rough hair, and reduced rate of growth. In addition to the enlargement of mitochondria, discontinuity of the outer membrane was often observed especially in the mitochondria which were contiguous to others (Fig. 1).

Similar discontinuity of the outer membrane was also observed in the deficient liver mitochondria of isolated preparations (Fig. 2), though the sizes of mitochondria were smaller than those shown in the liver tissue preparation. This might be partly due to the method of preparation by which large organelles were sedimented at $600 \times g$, and volume of mitochondria might be reduced in the medium of 0.25 M sucrose solution used for isolation.

At 20 and 40 hours after riboflavin injection, normalization and constriction of mitochondria were observed (Fig. 3). Although the normalization in size of mitochondria suggested division as shown by TANDLER *et al.* (14), dividing mitochondria with membranous septum were not observed in this experiment.

2. *Phospholipids in mitochondria and microsomes*

The contents of total phospholipids in mitochondria of the deficient rat livers were shown to be increased in terms of μg of phosphorus in phospholipids per mg of protein, but the change in microsomes was not statistically significant (Table 1). The ratios of individual phospholipids to total phospholipids altered slightly in mitochondria of the deficient livers, while those in microsomes were not changed significantly. A significant decrease in phosphatidylcholine and a slight increase in phosphatidylethanolamine appeared in the deficient mitochondria, as expressed in the ratio of the respective phospholipid-phosphorus to total phospholipid-phosphorus. The content of diphosphatidylglycerol was not altered at this stage of the deficiency.

The change in the ratios of individual phospholipids to total phospholipids were slight in microsomes of the deficient livers in comparison with mitochondria,

regardless of the fact that most of the phospholipids were shown to be synthesized in the endoplasmic reticulum and then transferred into the mitochondria (5, 6).

3. *Fatty acids in mitochondria and microsomes*

The composition of fatty acids in the lipids of mitochondria and microsomes was analyzed with six deficient and five control rats of which contents of the phospholipids were determined as shown in Table 1. The composition of fatty acids of mitochondria and microsomes was similar to the control as shown in Table 2, but the respective ratio of linoleic and arachidonic acids to the total fatty acids was slightly different. In mitochondria, the ratio of linoleic acid was higher and that of arachidonic acid was slightly lower than those of microsomes respectively.

The change of the fatty acid composition by the deficiency was similar to the total liver lipids of the deficient rats as shown in our previous report (4), and increase in myristic and linoleic acids and decrease in arachidonic acid were shown in mitochondria and microsomes. The increment in linoleic acid and decrement in arachidonic acid were somehow intensified in microsomes, compared with those of mitochondria.

By intraperitoneal injection of riboflavin to the deficient rats, fatty acid composition was restored to the control, and increase in arachidonic acid and decreases in myristic and linoleic acids were shown. However, arachidonic acid in microsomes and linoleic acid in mitochondria were statistically different from those of control at 60 hours after the injection (Fig. 4).

4. *Fatty acids in phospholipids*

The change in the composition of fatty acids in individual phospholipids was investigated in order to clarify which phospholipid was most affected by the deficiency. Fatty acids in phosphatidylcholine, phosphatidylethanolamine and diphosphatidylglycerol were determined respectively with the lipids of mitochondria and microsomes whose fatty acids have been analyzed above, after separation of the phospholipids by thin layer chromatography.

Palmitic, stearic, oleic, linoleic, arachidonic and docosaheptaenoic acids were the principal fatty acids in mitochondrial phosphatidylcholine, and these acids were all affected by the deficiency except oleic and docosaheptaenoic acids. Palmitic and linoleic acids were increased by the deficiency, and stearic and arachidonic acids were decreased (Table 3). The changes in fatty acid composition of microsomal phosphatidylcholine were similar to those of mitochondria; however stearic acid was not reduced in microsomes.

After supplementation of riboflavin to the deficient rats, the composition of

Fig. 1. Electron micrograph of the liver from a rat which had been fed a riboflavin-deficient diet for 5 weeks. The marker bar represents 1μ .

Fig. 2. Electron micrographs of liver mitochondria from (A) control, and (B) the rat which had been fed a riboflavin-deficient diet for 5 weeks. The marker bar represents 1μ .

Fig. 1.

Fig. 2.

Fig. 3. Electron micrographs of the livers from rats which had received riboflavin by intraperitoneal injection at (A) 20 hours and (B) 40 hours before killing. The marker bar represents 1μ .

fatty acids of the phosphatidylcholine in mitochondria was restored nearly to the control values at 60 hours after the injection; however the ratio of arachidonic acid to the total fatty acids was statistically different from the control value and a slight increase in oleic acid appeared in mitochondria and microsomes (Fig. 5).

The changes in fatty acid composition in the deficient livers were slight in the phosphatidylethanolamine of mitochondria and microsomes compared with those in phosphatidylcholine. In phosphatidylethanolamine, a statistically significant increase in linoleic acid of mitochondria, and a decrease in arachidonic acid of

Table 1. Contents of phospholipids in mitochondria and microsomes of rat livers.

Phospholipids	Mitochondria		Microsomes	
	Riboflavin-deficient (8) (%)	Control (5) (%)	Riboflavin-deficient (8) (%)	Control (5) (%)
Phosphatidylinositol	10.7 ± 0.4	9.3 ± 1.0	15.1 ± 2.1	14.4 ± 2.2
Phosphatidylcholine	36.8 ± 1.1*	40.7 ± 0.7	61.4 ± 1.9	62.5 ± 1.5
Phosphatidyl-ethanolamine	34.1 ± 0.8**	32.2 ± 3.8	22.0 ± 1.9	19.9 ± 1.8
Diphosphatidylglycerol	16.3 ± 1.5	16.9 ± 1.1	—	—
P _i -phospholipid (μg)/protein (mg)	3.9 ± 0.3**	3.5 ± 0.1	11.2 ± 2.9	10.7 ± 1.5

Values are expressed as average ± S.D. of percent of phosphorus of each phospholipid to phosphorus of total phospholipids. Numbers in parentheses are the number of rats. Contents of phospholipids are expressed as μg of phosphorus of phospholipids per mg protein of mitochondria or microsomes.

Significance of difference between the deficient and control is indicated as * ($p < 0.01$) and ** ($p < 0.05$).

Table 2. Fatty acid composition of total lipids in rat liver mitochondria and microsomes.

Fatty acids	Mitochondria		Microsomes	
	Riboflavin-deficient (6)	Control (5)	Riboflavin-deficient (6)	Control (5)
14:0 ^a	0.5 ± 0.1*	0.1 ± 0.0	0.9 ± 0.1*	0.2 ± 0.0
16:0	13.8 ± 1.2	12.1 ± 0.7	15.3 ± 1.6	14.0 ± 0.8
16:1	1.2 ± 0.2	1.6 ± 0.5	1.3 ± 0.4	1.0 ± 0.3
18:0	17.2 ± 1.1	17.8 ± 1.5	18.0 ± 1.1	19.9 ± 2.3
18:1	12.8 ± 1.1	12.5 ± 1.8	15.3 ± 1.3	13.0 ± 2.2
18:2	17.2 ± 0.7*	13.5 ± 0.5	13.1 ± 1.1*	7.6 ± 0.7
20:4	22.6 ± 0.8*	28.2 ± 1.2	23.0 ± 1.0*	32.0 ± 1.8
22:4	2.5 ± 0.7	2.7 ± 0.7	2.4 ± 0.6	2.4 ± 0.9
22:5	0.7 ± 0.3	0.5 ± 0.1	0.8 ± 0.2	0.5 ± 0.2
22:6	8.0 ± 1.3	8.7 ± 1.3	7.0 ± 1.1	8.2 ± 1.1

Values are average ± S.D. of weight percent of fatty acids.

^a Number of carbon atoms; number of double bonds. Numbers in parentheses are the number of rats.

* Significantly different from control ($p < 0.01$).

Fig. 4. Changes of oleic, linoleic and arachidonic acids of total lipids of liver mitochondria (A) and microsomes (B), after intraperitoneal injection of riboflavin to the rats which had been fed a riboflavin-deficient diet for 5 weeks. Each point and bar represent average and standard deviation of weight percent of fatty acids. $\odot$ myristic, $\bullet$ oleic, $\bullet$ linoleic, and $\circ$ arachidonic acids. Time (hours) indicates the time after the injection of riboflavin. Number of rats are deficient: 6, at 20 hours recovery: 5, 40 hours: 6 and 60 hours: 4, and control: 5. Significantly different from control * ($p < 0.01$) and ** ($p < 0.05$).

Table 3. Fatty acid composition of phosphatidylcholine of rat liver mitochondria and microsomes.

Fatty acids	Mitochondria		Microsomes	
	Riboflavin-deficient (6)	Control (5)	Riboflavin-deficient (6)	Control (5)
14:0 ^a	0.5 $\pm$ 0.2	0.3 $\pm$ 0.1	0.4 $\pm$ 0.1*	0.1 $\pm$ 0.0
16:0	21.5 $\pm$ 3.3**	16.7 $\pm$ 1.5	19.5 $\pm$ 1.8*	15.8 $\pm$ 1.4
18:0	15.6 $\pm$ 2.2**	19.4 $\pm$ 2.7	20.3 $\pm$ 1.6	22.0 $\pm$ 2.2
18:1	13.5 $\pm$ 0.5	12.5 $\pm$ 1.6	12.6 $\pm$ 0.7	11.9 $\pm$ 1.6
18:2	13.1 $\pm$ 1.1*	7.8 $\pm$ 1.6	11.6 $\pm$ 1.1*	7.7 $\pm$ 1.5
20:4	23.4 $\pm$ 1.4*	31.9 $\pm$ 2.0	25.1 $\pm$ 2.1*	33.5 $\pm$ 1.3
22:4	1.8 $\pm$ 0.5	2.1 $\pm$ 0.5	1.7 $\pm$ 0.2**	1.4 $\pm$ 0.2
22:5	0.5 $\pm$ 0.3	0.3 $\pm$ 0.1	0.4 $\pm$ 0.2	0.2 $\pm$ 0.1
22:6	7.0 $\pm$ 0.7	6.7 $\pm$ 0.8	6.2 $\pm$ 0.6	5.4 $\pm$ 0.8

Significance of difference between the deficient and control is indicated as * ($p < 0.01$) and ** ($p < 0.05$). Others are same as listed in Table 2.

Fig. 5. Changes of oleic, linoleic and arachidonic acids of phosphatidylcholine of liver mitochondria (A) and microsomes (B), after intraperitoneal injection of riboflavin to the deficient rats. Marks and others are same as shown in Fig. 4.

Table 4. Fatty acid composition of phosphatidylethanolamine in rat liver mitochondria and microsomes.

Fatty acids	Mitochondria		Microsomes	
	Riboflavin-deficient (6)	Control (5)	Riboflavin-deficient (6)	Control (5)
14:0 ^a	0.1 ± 0.0	0.7 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
16:0	12.7 ± 0.9	13.4 ± 1.9	15.9 ± 1.3	14.3 ± 0.5
18:0	23.7 ± 1.3	22.4 ± 2.5	22.8 ± 1.5	20.8 ± 1.7
18:1	8.2 ± 1.2	8.5 ± 1.3	9.3 ± 1.2	9.8 ± 1.7
18:2	5.4 ± 0.8*	3.3 ± 0.8	6.0 ± 1.1	4.8 ± 0.9
20:4	31.5 ± 2.0	32.6 ± 2.6	26.6 ± 1.0*	30.7 ± 1.3
22:4	3.2 ± 0.9	2.9 ± 0.6	3.1 ± 0.8	3.1 ± 0.8
22:5	0.7 ± 0.3	0.4 ± 0.2	0.7 ± 0.3	0.6 ± 0.2
22:6	12.2 ± 1.8	13.2 ± 0.9	13.3 ± 1.1	14.2 ± 2.0

Values and others are same as listed in previous tables.

microsomes were produced by the deficiency as shown in Table 4.

Fatty acids in diphosphatidylglycerol in the liver mitochondria consisted principally of linoleic and oleic acids. Others were palmitic, palmitoleic, stearic and minor acids. The composition of fatty acids in this phospholipid differed from that of phosphatidylcholine or phosphatidylethanolamine (Tables 3 and 4), and it

Table 5. Fatty acid composition of diphosphatidylglycerol in rat liver mitochondria.

Fatty acids	Riboflavin-deficient (6)	Control (5)
14:0 ^a	0.7 ± 0.2**	0.4 ± 0.2
16:0	5.6 ± 1.6	4.5 ± 0.7
16:1	2.2 ± 0.3	4.9 ± 1.8
18:0	2.4 ± 0.5	2.0 ± 0.7
18:1	12.9 ± 0.9	16.2 ± 4.9
18:2	64.5 ± 2.3**	56.6 ± 4.9
18:3	2.3 ± 1.3	2.7 ± 0.9
20:4	1.3 ± 0.4	2.1 ± 1.1
22:5	1.5 ± 0.5	1.5 ± 0.3
22:6	0.6 ± 0.2**	1.2 ± 0.5

Values and others are same as listed in previous tables.

differed also from that of triglyceride of the rat liver which consisted mainly of palmitic, oleic and linoleic acids (24, 39 and 16% respectively as shown in our previous paper(4)). In the deficient livers, a quantitatively small but significant decrease in palmitoleic acid and an increase in linoleic acid were demonstrated in diphosphatidylglycerol (Table 5). The content of oleic acid, another main constituent, was not affected by the deficiency.

5. Incorporation of ³²P into phospholipids

Specific radioactivities of total phospholipids in mitochondria and microsomes were not affected by the deficiency in terms of cpm per μ g of phosphorus in phospholipids (Table 6). However, incorporation of ³²P into individual phospholipid was variously affected. Incorporation of ³²P into microsomal phosphatidylethanolamine increased, whereas that of mitochondria was not changed significantly. The incorporation into phosphatidylcholine of mitochondria and microsomes was not changed. The incorporation of ³²P into diphosphatidylglycerol decreased remarkably, though the content of this phospholipid was not altered as shown in Table 1.

By supplement of riboflavin to the deficient rats, the specific radioactivities of diphosphatidylglycerol in mitochondria and phosphatidylethanolamine in microsomes were shown to be increased toward the control values. However, the specific radioactivity of diphosphatidylglycerol did not recover completely at 60 hours after the riboflavin injection.

6. Incorporation of ³²P into subspecies of phosphatidylcholine

The composition of fatty acids in phosphatidylcholine was affected remarkably by the deficiency, while the content and the incorporation of ³²P were affected slightly (Table 7). In order to distinguish the effect of the deficiency on the structure

Table 6. Incorporation of ^{32}P into phospholipids of rat liver mitochondria and microsomes.

	Phospholipids	Riboflavin-deficient (7)	Riboflavin supplemented 20 hours (4)	40 hours (3)	Control (5)
Mitochondria	Phosphatidyl- inositol	$1,010 \pm 142$	$1,044 \pm 160$	$1,014 \pm 93$	$1,113 \pm 109$
	Phosphatidyl- choline	$1,747 \pm 205$	$1,874 \pm 267$	$1,801 \pm 276$	$1,702 \pm 190$
	Phosphatidyl- ethanolamine	$1,481 \pm 209$	$1,680 \pm 153$	$1,487 \pm 168$	$1,527 \pm 153$
	Diphosphatidyl- glycerol	$165 \pm 33^*$	$292 \pm 21^*$	$280 \pm 34^*$	394 ± 50
	cpm/Phospholipids- $\text{P}_i(\mu\text{g})$	$1,436 \pm 108$	$1,330 \pm 60$	$1,320 \pm 170$	$1,414 \pm 153$
Microsomes	Phosphatidyl- inositol	$1,393 \pm 235$	$1,574 \pm 304$	$1,279 \pm 69$	$1,414 \pm 199$
	Phosphatidyl- choline	$1,816 \pm 161$	$1,707 \pm 320$	$1,674 \pm 336$	$1,990 \pm 105$
	Phosphatidyl- ethanolamine	$1,707 \pm 211^*$	$1,252 \pm 30$	$1,467 \pm 165$	$1,370 \pm 106$
	cpm/Phospholipids- $\text{P}_i(\mu\text{g})$	$1,651 \pm 213$	$1,669 \pm 101$	$1,622 \pm 193$	$1,672 \pm 163$

Values are average $\pm$ S.D. of specific radioactivities of phospholipids, cpm/phospholipid-phosphorus.

* Significantly different from control ($p < 0.01$). Numbers in parentheses are rats. 20 and 40 hours are time after supplement of riboflavin to the deficient rats.

Table 7. Incorporation of ^{32}P into subspecies of phosphatidylcholine of rat liver mitochondria and microsomes.

Fraction ^a	Mitochondria		Microsomes	
	Riboflavin-deficient (%)	Control	Riboflavin-deficient (%)	Control
1	13.7 ± 3.1	12.8 ± 1.3	14.1 ± 2.6	13.5 ± 0.7
2	4.3 ± 1.0	4.0 ± 1.0	5.3 ± 1.2	4.3 ± 1.0
3	$24.1 \pm 4.4^*$	52.0 ± 2.3	$32.6 \pm 2.5^*$	51.2 ± 2.5
4	4.5 ± 0.4	3.3 ± 0.7	4.1 ± 1.0	3.1 ± 0.7
5	$43.2 \pm 2.6^*$	27.5 ± 1.3	$43.5 \pm 2.5^*$	27.9 ± 1.4

Values are average $\pm$ S.D. of percent of cpm of respective subfraction to cpm of total phosphatidylcholine with 4 deficient and 4 control rats.

^a Fractions separated on thin layer chromatography and numbered from 1 to 5, according to the development from the original spot to the solvent front.

* Significantly different from control ($p < 0.01$).

Table 8. Composition of fatty acids in subfractions of phosphatidylcholine of rat liver mitochondria.

Fatty acids	Fraction number							
	1		3		4		5	
	Riboflavin-deficient	Control	Riboflavin-deficient	Control	Riboflavin-deficient	Control	Riboflavin-deficient	Control
14:0 ^a	1.6±0.5*	2.3±0.2	0.6±0.3	0.9±0.5	1.2±0.6	1.9±0.9	3.7±0.8**	4.9±0.4
16:0	27.6±5.3	30.0±1.8	16.4±3.0**	22.5±3.8	24.9±3.2**	30.5±2.4	32.8±4.1	32.4±0.9
16:1	2.6±1.0	3.4±0.7	0.8±0.4	1.4±0.8	2.0±0.5*	5.4±1.7	5.8±0.7	7.6±1.4
18:0	16.3±2.1	16.2±2.3	23.2±1.8	20.6±1.2	13.7±1.5	11.9±1.8	16.4±2.7	13.7±1.9
18:1	8.5±1.8	6.5±1.1	7.8±0.9	8.2±2.0	24.9±2.9	23.0±5.8	11.7±3.1	13.3±1.9
18:2	1.5±0.4	1.7±0.8	1.1±0.5	1.6±1.1	24.5±6.3	22.8±2.0	3.3±1.3	2.5±0.3
20:4	3.8±1.6	3.2±0.7	42.3±3.7**	35.7±2.2	—	—	—	—
20:5	2.3±1.1	5.4±3.6	—	—	—	—	—	—
unknown	—	—	—	—	6.5±0.7	8.1±1.4	6.0±2.3	6.9±1.9
unknown	—	—	—	—	1.4±1.2	4.6±3.1	—	—
22:4	—	—	2.2±0.9	2.7±1.7	—	—	—	—
22:5	1.2±1.1	0.7±0.3	—	—	—	—	—	—
22:6	25.1±6.9	25.1±5.3	1.4±1.1	3.3±1.7	—	—	—	—

Values are average ± S.D. of weight percent of fatty acids of the subfractions which were separated in Table 7. Significance of difference between the deficient and control is indicated as * ($p < 0.01$) and ** ($p < 0.05$). Others are same as listed in Table 2.

Table 9. Composition of fatty acids in subfractions of phosphatidylcholine of rat liver microsomes.

Fatty acids	Fraction number							
	1		3		4		5	
	Riboflavin-deficient	Control	Riboflavin-deficient	Control	Riboflavin-deficient	Control	Riboflavin-deficient	Control
14:0 ^a	1.2±0.3*	0.4±0.2	1.3±0.3*	0.3±0.1	1.1±0.1*	0.4±0.2	1.5±0.3*	0.7±0.2
16:0	20.9±0.9*	15.8±1.7	18.9±1.7**	15.1±2.4	25.9±1.3*	20.2±0.7	34.4±2.8	35.8±2.8
16:1	1.8±0.1	1.2±0.8	1.8±0.9	1.6±0.6	1.7±0.2**	3.2±0.9	2.0±0.4**	3.1±0.9
18:0	16.7±1.2	17.3±2.8	24.3±0.8*	28.3±0.3	16.2±0.9*	19.3±1.1	9.1±0.6**	12.0±1.6
18:1	10.7±1.1*	6.5±0.3	7.7±0.7	6.5±0.7	12.0±2.6	15.0±1.3	38.6±2.6	40.6±4.6
18:2	5.3±1.6**	2.4±1.0	2.8±0.4*	0.9±0.4	33.6±5.8	34.7±1.9	4.6±0.6*	1.8±0.5
20:4	8.2±4.1	14.7±4.8	30.1±7.0	40.1±6.0	0.9±0.4	1.2±0.8	0.6±0.2	0.5±0.1
20:5	2.5±0.5*	—	—	—	—	—	—	—
22:5	1.9±0.3*	3.3±0.4	—	—	—	—	—	—
22:6	24.0±5.2	24.9±9.0	—	—	—	—	—	—

Values and other numbers are same as in Table 8.

of phosphatidylcholine, the lipid was separated into subspecies by thin layer chromatography with silica gel H containing silver nitrate. The phosphatidylcholine of mitochondria or microsomes was separated into three main and two minor subspecies, according to the degree of the desaturation of fatty acids. In the deficient animals, significant reduction of labeling of ^{32}P was shown in the fraction 3 in which unsaturated fatty acids consisted mainly with tetraenoic acid. Labeling in the fraction 5 was increased by the deficiency contrary to the decrease of fraction 3.

7. *Fatty acid composition of subspecies of phosphatidylcholine*

The fatty acid composition of the subspecies of phosphatidylcholine was analyzed with mitochondria and microsomes, since labeling of the subspecies with ^{32}P was variously affected by the deficiency (Table 7). (For fraction 2 which was quantitatively small, the analysis was not carried out.) The composition of fatty acids in each subfraction was similar in the deficient and control animals. The incorporation of ^{32}P into fraction 3 of the phosphatidylcholine of either mitochondria or microsomes was reduced by the deficiency, in which arachidonic acid was found as major unsaturated fatty acid (Tables 8 and 9). In fraction 3, the ratio of arachidonic acid to the total fatty acids was higher in the deficient than control, and thus the reduction in arachidonic acid of phosphatidylcholine (Table 3) might be due to the decrease in the amount of the subspecies of fractions 3. The increase in linoleic acid in phosphatidylcholine could be due to increases in fractions 4 and/or 5.

DISCUSSION

A slight increase in the content of phospholipids was observed in the riboflavin-deficient liver mitochondria, as expressed in terms of phosphorus in phospholipids per mg of mitochondrial protein. The increase in phospholipids might be partly due to the decrease in protein content of mitochondria. Discontinuities of the outer mitochondrial membrane were demonstrated in the riboflavin-deficient rats, thus increase in the release of protein would be feasible. It was shown by CREMEL *et al.* (13) that the temperature-dependent intramitochondrial release of protein was affected by fatty acid constituents in the phospholipids of mitochondrial membranes. In their study, the release was slightly increased in the rat liver mitochondria which had been fed a lipid-free diet, and in the liver lipids of those rats, arachidonic acid was lower and oleic acid was higher than the respective fatty acids of the control rats. In our study, it was shown that the ratio of arachidonic acid to total fatty acids was lower and that of linoleic acid was higher than those of the controls respectively. The release of mitochondrial protein of the riboflavin-deficient rats would be affected in the similar way as that of the rats fed the lipid-free diet.

In the liver mitochondria of riboflavin-deficient mice, an increase in phosphatidylcholine and a decrease in diphosphatidylglycerol were reported (3). The differences from our results of a decrease in phosphatidylcholine and no change

in diphosphatidylglycerol might be due to the species difference and duration of the deficient diet, 8 weeks for the former and 5 weeks for our study with rats.

According to McMURRAY and DAWSON (6), the distribution of phosphatidylcholine of the rat liver mitochondria was higher in the outer membrane and that of phosphatidylethanolamine was lower in the inner membrane respectively. In our experiments, discontinuities of the outer membrane of mitochondria were observed in the deficient rat liver both in intact cell specimens and isolated preparations; thus the decrease in phosphatidylcholine and the increase in phosphatidylethanolamine produced by the deficiency might be due to a decrease in the constituents of outer membrane.

Fatty acid composition of the lipids was remarkably altered by the riboflavin deficiency in mitochondria and microsomes, and the change was especially intensified in phosphatidylcholine. The distribution of the subspecies of phosphatidylcholine seemed to be changed as the result of alterations in fatty acid composition, and the increase in the subspecies of phosphatidylcholine with mono- and dienoic acids and the decrease in that with tetraenoic acid were demonstrated in mitochondria and microsomes by incorporation of ^{32}P . The changes in distribution of subspecies of phosphatidylcholine could bring about the alteration of lipid and protein interactions in mitochondrial membrane, and thus could cause changes in physiological function, such as permeability and binding capacity with ions.

However, alteration in lipid constituent in the membrane alone would not explain the morphological change of mitochondria. Enlarged mitochondria of the riboflavin-deficient mouse started to normalize at 6.5 hours (14) or 12 hours (3) after administration of riboflavin. Though the budding or segmentation of mitochondrial membrane was not shown in this experiment as observed by TANDLER *et al.* (14), constriction of mitochondria which suggested division of mitochondria was observed at 20 hours after injection of riboflavin, and the size of mitochondria was nearly normal at 40 hours. The time to recover to the control values of each fatty acid in phospholipids seemed to require a relatively prolonged period comparing with normalization of the mitochondrial structure. The ratio of arachidonic acid to the total fatty acids in phosphatidylcholine of the deficient mitochondria was statistically different from the control at 60 hours after riboflavin injection. The incorporation of ^{32}P into diphosphatidylglycerol of the deficient mitochondria had not recovered to the control value at 40 hours after riboflavin injection (Table 6). Thus, the change in the mitochondrial structure by riboflavin deficiency would be attributable to various factors such as decrease in electron transfer which was shown in the increase in the ratios of NADH to NAD^+ and NADPH to NADP^+ (15), the increased formation of lipid peroxide (16), which would give a damage on the membrane, and other deteriorations in addition to the alteration in phospholipids of the mitochondrial membrane.

The fatty acid composition was similar in mitochondria and microsomes, but linoleic acid was slightly higher in mitochondria than in microsomes, both in control and deficient rats. Since linoleic acid is a major constituent in diphosphatidylgly-

cerol which is specifically contained in mitochondria, the higher content of linoleic acid in mitochondria than in microsomes might be due to the diphosphatidylglycerol.

Fatty acid composition of phosphatidylcholine in control mitochondria was similar to that in microsomes. In the deficient livers, stearic acid in phosphatidylcholine in mitochondria decreased, but the change was not significant in microsomes. Arachidonic acid in phosphatidylethanolamine in microsomes was decreased by the deficiency, whereas no significant change was observed in mitochondria.

It was suggested from the above results that riboflavin deficiency produced variable effects on fatty acid composition of phospholipids in mitochondria and microsomes, and the changes in fatty acid content might alter membrane function of the organelles in riboflavin deficiency.

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