The Influence of Low Casein Diet on the *in vivo* Incorporation of ^{32}P -Orthophosphate into Individual Phosphatides of Rat Liver Mitochondria

By Naoko HARADA and Toshiaki SUZUKI*

*Chukyo Women's University, Ohbu, Chita-gun, Aichi-ken
and Department of Agricultural Chemistry, Nagoya
University, Nagoya, Aichi-ken*

Received September 12, 1967

The influence of low casein diet on the pattern of *in vivo* incorporation of ^{32}P -orthophosphate into individual phosphatides of rat liver mitochondria was investigated.

The composition of the mitochondrial phosphatides showed no difference between low casein and high casein diet fed rats. On the contrary, incorporation of ^{32}P into lecithin was greater in rats fed a low casein diet than that in rats fed a high casein diet. The mean value was about three fold higher.

In recent years phospholipids were noticed as having important roles in mitochondrial function.^{1~7)}

In preceding report, it was described that liver mitochondria were easily induced to swell when they were fed a low casein diet.⁸⁾ Further, it was reported that the ratio of the amount of lipids to that of protein in rat liver cell fractions became higher in mitochondrial fraction in rats fed a low casein diet than that in rats fed a high casein diet.⁹⁾

From these facts, it was supposed that nature and function of lipoprotein or phospholipids themselves of mitochondria might be varied on low casein diet.

In the following, as a first step in mitochondrial lipid research, *in vivo* incorporation of ^{32}P -orthophosphate into individual phosphatides was investigated and it was found that an incorporation of ^{32}P into lecithin increased in rats fed a low casein diet.

EXPERIMENTAL

Animals and diets. Male rats of Donryu strain, weighing from 100 to 180 g, were used. They were housed in individual cages in a temperature regulated room at about 22°C.

As the test diets, low and high casein diets were used. Compositions were the same as in previous report.⁸⁾ The low and high casein diets contained 4 and 25% casein respectively.

Rats were divided into two groups; low casein diet was given to one group, and high casein diet to another. They were fed ad libitum on the test diets for 40 to 60 days, and thereafter used for the experiments.

Preparation of mitochondria. Method of preparation of mitochondria was the same as in previous report.⁸⁾

* Present Address: Control Department, Banyu Pharmaceutical Co., Ltd., Okazaki, Aichi-Ken.

1) S. Fleischer and H. Klouwen, *Biochem. Biophys. Res. Commun.*, **5**, 378 (1961).

2) P. Jurtshuk, Jr., I. Sekuzu and D. E. Green, *ibid.*, **6**, 76 (1961).

3) P. Jurtshuk, Jr., I. Sekuzu and D. E. Green, *J. Biol. Chem.*, **238**, 3595 (1963).

4) R. L. Lester and S. Fleischer, *Biochim. Biophys. Acta*, **47**, 358 (1961).

5) S. Fleischer, G. P. Brierley, H. Klouwen and D. B. Slautterback, *J. Biol. Chem.*, **237**, 3264 (1962).

6) G. P. Brierley and A. J. Merola, *Biochim. Biophys. Acta*, **64**, 205 (1962).

7) G. P. Brierley, A. J. Merola and S. Fleischer, *Biochim. Biophys. Acta*, **64**, 218 (1962).

8) N. Harada, *J. Nutrition*, **93**, 263 (1967).

9) N. Harada and S. Mogi, *Agr. Biol. Chem.*, **30**, 274 (1966).

Extraction of mitochondrial lipids. Mitochondrial lipids was extracted by Folch's method¹⁰ using chloroform-methanol (2:1). In the extraction procedure, precaution was taken not to raise the temperature above room temperature.

Paperchromatography of the phosphatides. Paperchromatography of the phosphatides was made by Marinetti's method¹¹ using silicic acid impregnated paper, which was cut into 40×3 cm pieces, then about 300 μg of phospholipids was applied to the paper in the shape of line, and the applied paper was developed in diisobutylketone-acetic acid-water (40:20:3) at 5°C for 16 hr.

Detection and identification of the phosphatides on paper chromatograms. The phosphatides were detected and identified by three kinds of color reactions, namely, Rhodamine 6G test,¹² Schiff reagent test and Dragendorff test.¹³

Fig. 1. Fractionation of the Chromatogram of Rat Liver Mitochondrial Phosphatides into Five Fractions According to the Positions of the Spots.

Quantitative estimation of the phosphatides.

Quantitative estimation of the phosphatides was made according to the method of Marinetti *et al.*¹² After papers were developed and the phosphatides were detected by Rhodamine 6G treatment, positions of spots were marked with pencil. As shown in Fig. 1, according to the positions of the spots, papers were cut into the five fractions from origin to the top. On each fraction, phosphatides were extracted and digested by the method of Marinetti *et al.*¹² Phosphorus was determined by the modified method of Chen *et al.*¹⁴

Injection of ^{32}P -orthophosphate and determination of radioactivity.

P^{32} -Orthophosphate was dissolved into the 0.001 M phosphate buffer at pH 7.2. In experiment I, 66 μC of ^{32}P per 100 g body weight was injected interperitoneally. In experiment II, 100 μC of ^{32}P per 100 g body weight was injected. Ninety minutes after injection, rats were killed, and liver mitochondria were separated, and lipids were extracted from them.

Paperchromatography was applied on extracted lipids as described above. After papers had been developed and dried, their radioactivity was measured with low background automatic radio paperchromatograph counter (Nihon Musen Irigaku Kenkyujo Co., JPC-105).

In experiment II, after measurement of radioactivity, papers were immersed in Rhodamine 6G solution, and phosphatides were detected and marked. Spots corresponding to lecithin and phosphatidylethanolamine were cut off, and after extraction and digestion, amount of phosphorus was determined. Specific activity on lecithin and phosphatidylethanolamine fraction was then calculated.

RESULTS

Identification of mitochondrial phosphatides on silicic acid impregnated paper.

When the papers were treated with Rhodamine 6G solution and observed under ultraviolet light, the spot nearest to the origin appeared purple, upper two spots appeared yellow or orange. The spot showed purple color is acidic phosphatide, whereas spots exhibited yellow or orange color are neutral phosphatides. When the papers were treated with Schiff reagent, positions of the spots appeared were the same as in Rhodamine 6G treatment. The upper two spots gave red-

10) J. Folch, M. Lees and G. H. Sloanestanley, *J. Biol. Chem.*, **226**, 497 (1957).

11) G. V. Marinetti, *J. Lipid Res.*, **3**, 1 (1962).

12) G. V. Marinetti, M. Albrecht, T. Ford and E. Stotz, *Biochim. Biophys. Acta*, **36**, 4 (1959).

13) H. Wagner, L. Hörhammer and P. Wolff, *Biochem. Z.*, **334**, 175 (1961).

14) P. S. Chen, Jr., T. Y. Toribara and H. Warner, *Anal. Chem.*, **28**, 1756 (1956).

dish brown color with Dragendorff reagent. Authentic samples of lecithin and phosphatidylethanolamine were developed together with mitochondrial lipids. It was found that, with respect to the upper two spots, the *R_f* value of the one nearer to the origin was the same as that of lecithin and the other showed the same as phosphatidylethanolamine.

Composition of mitochondrial phosphatides.

The final body weight, liver weight and the number of rats per group used in the determination of the composition of the mitochondrial phosphatides are shown in Table I.

TABLE I. FINAL BODY WEIGHT, LIVER WEIGHT AND NUMBER OF RATS PER GROUP USED IN THE DETERMINATION OF MITOCHONDRIAL LIPID COMPOSITION

Group	Initial body weight (g)	Final body weight (g)	Final liver weight (g)	Number of rats per group
4% Casein	103 ± 4 ¹⁾	105 ± 8	3.2 ± 0.2	4
25% Casein	101 ± 10	242 ± 14	8.6 ± 1.0	4

¹⁾ Standard deviation

In Table II, are shown the value of phosphorus content of each fraction fractionated as shown in Fig. 1 expressed as percentage of total lipid phosphorus. As described above, fraction III was lecithin and fraction IV phosphatidylethanolamine. From Table II, it is seen that the percentage of each phosphatide was varied somewhat in both groups, but the values of the standard deviations were large, so that the differences were not significant in all the fractions.

TABLE III. FINAL BOBY WEIGHT, LIVER WEIGHT AND NUMBER OF RATS PER GROUP USED IN THE EXPERIMENTS OF ³²P INCORPORATION INTO MITOCHONDRIAL PHOSPHATIDES

Group	Initial body weight (g)	Final body weight (g)	Final liver weight (g)	Number of rats per group
Experiment I				
4% Casein	145 ± 14 ¹⁾	158 ± 3	4.6 ± 0.2	5
25% Casein	153 ± 16	279 ± 14	10.3 ± 0.4	5
Experiment II				
4% Casein	170 ± 15	179 ± 13	4.9 ± 0.5	5
25% Casein	166 ± 20	304 ± 18	10.7 ± 0.8	5

¹⁾ Standard deviation

TABLE II. MITOCHONDRIAL LIPID COMPOSITION

Group	Fraction No.				
	I (%)	II (%)	III (%)	IV (%)	V (%)
4% Casein	5.3 ¹⁾ ± 6.0 ²⁾	15.1 ± 3.6	45.3 ± 8.6	26.4 ± 3.2	8.0 ± 7.8
25% Casein	3.8 ± 3.4	18.4 ± 12.1	39.7 ± 14.9	23.4 ± 4.4	15.8 ± 11.3

¹⁾ Expressed as percentage of total lipid phosphorus

²⁾ Standard deviation

TABLE IV. PERCENTAGE OF ³²P INCORPORATION AND SPECIFIC ACTIVITY OF LECITHIN AND PHOSPHATIDYLETHANOLAMINE

Group	Percentage of ³² P Incorporation ¹⁾		Specific Activity	
	Lecithin (%)	Phosphatidylethanolamine (%)	Lecithin (count/min/μg P)	Phosphatidylethanolamine (count/min/μg P)
Experiment I				
4% Casein	64.1 ± 3.2 ²⁾	23.6 ± 1.7		
25% Casein	37.9 ± 5.2	46.0 ± 6.2		
Experiment II				
4% Casein	70.7 ± 3.5	20.2 ± 4.1	1462 ± 303	581 ± 184
25% Casein	39.3 ± 7.1	48.4 ± 6.7	448 ± 251	817 ± 408

¹⁾ Expressed as percentage of ³²P incorporation into total lipid phosphorus

²⁾ Standard deviation

Incorporation of ^{32}P -orthophosphate into mitochondrial phosphatides.

In Table III, are shown the final body weight, liver weight and the number of rats used in experiments I and II.

In Table IV, are shown the value of counts per minute of lecithin and phosphatidylethanolamine fraction expressed as percentage of counts per minute of total phosphatides. In experiment II, quantitative phosphorus determination was made on lecithin and phosphatidylethanolamine fractions, in order to calculate the value of specific activity of these fractions.

From Table IV, it is seen that the incorporation of ^{32}P into lecithin fraction increased greatly in rats fed a low casein diet. The difference in specific activity of lecithin fraction was statistically significant between low casein and high casein diet fed rats ($P < 0.01$). It was about three fold higher in rats fed a low casein diet than in rats fed a high casein diet.

On the contrary, specific activity in phosphatidylethanolamine fraction was slightly higher in rats fed a high casein diet.

Fig. 2. Typical Curve of ^{32}P Incorporation into Rat Liver Mitochondrial Phosphatides.

—: 4% ----: 25%

In Fig. 2, typical curve of ^{32}P incorporation into phosphatides of liver mitochondria from low casein and high casein diet fed rats are shown. The difference in lecithin fraction was evident.

DISCUSSION

The experiments reported here, were designed to examine the pattern of *in vivo* ^{32}P -orthophosphate incorporation into each mitochondrial phosphatide in rats fed a low casein diet.

Previously, in the *in vitro* experiment on isolated mitochondria, rapid incorporation of ^{32}P during the first 5 min of incubation period into phosphatidylinositol was reported by Garbus *et al.*¹⁵⁾ and by Galliard and Hawthorne.¹⁶⁾

The results of *in vivo* experiments of ^{32}P -orthophosphate incorporation into individual phosphatides of rat liver were reported by Gurr *et al.*¹⁷⁾ and by Ulsamer and Glenn.¹⁸⁾ Gurr *et al.*¹⁷⁾ reported that the ^{32}P incorporation rate was higher in phosphatidylethanolamine and lecithin fraction than in other fractions. In our experiments, the increase of ^{32}P incorporation into lecithin was found in rats fed a low casein diet. On the contrary, the pattern of the incorporation in rats fed a high casein diet resembled the results of Gurr *et al.*,¹⁷⁾ that is, the incorporation rate is slightly higher in phosphatidylethanolamine fraction than in lecithin fraction. Ulsamer and Glenn¹⁸⁾ reported that ^{32}P incorporation into lecithin was increased after ethionine administration.

In our experiments, when the compositions of high casein and low casein diets were compared, the content of choline was the same, but the content of methionine was higher in high casein diet, so that, despite of lower methyl group content, the incorporation rate of ^{32}P into lecithin was higher in rats fed a low casein diet.

In the *in vivo* experiments, lecithin might

15) J. Garbus, H. F. DeLuca, M. E. Loomans and F. M. Strong, *J. Biol. Chem.*, **238**, 59 (1963).

16) T. Galliard and J. N. Hawthorne, *Biochim. Biophys. Acta*, **70**, 479 (1963).

17) M. I. Gurr, C. Prottey and J. N. Hawthorne, *ibid.*, **106**, 357 (1965).

18) A. G. Ulsamer and J. L. Glenn, *ibid.*, **125**, 525 (1966).

be synthesized in other fractions, and thereafter, transported to mitochondria. It was pointed out by Wilgram and Kennedy¹⁹⁾ that synthesis of lecithin requires a microsome-mediated step.

Collins²⁰⁾ reported that the turnover rate of lecithin varied according to its fatty acid composition. In our experiments, the dilution effect of ^{32}P orthophosphate was not measured, so that the turnover rate of lecithin increased in rats fed a low casein diet cannot be decided, but it is clear that the pattern

of ^{32}P incorporation into individual phosphatides of rat liver mitochondria varied greatly, and ^{32}P incorporation into lecithin was increased in comparison with the other phosphatides. From our present knowledge, the reason of increase in ^{32}P incorporation into lecithin by low casein diet is not clear. The physiological and biochemical significance of this phenomenon is the future problem.

Acknowledgements. We are grateful to Dr. Kiyoshi Ashida for the helpful advice, and to Dr. Ziro Suzuoki and Takao Matsuo for kindly providing the authentic samples of lecithin and phosphatidylethanolamine.

19) G. F. Wilgram and E. P. Kennedy, *J. Biol. Chem.*, **238**, 2615 (1963).

20) F. D. Collins, *Nature*, **186**, 366 (1960).