Effects of a nutritional deficiency of unsaturated fats on the distribution of fatty acids in rat liver mitochondrial phospholipids

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SUMMARY The fatty acid composition of liver mitochondrial phospholipids from rats rendered deficient in essential unsaturated fatty acids has been determined, and compared with that of rats fed a diet containing corn oil. In addition to marked reductions in the amounts of linoleic and arachidonic acids esterified at the β-position of ethanolamine-, inositol-, and choline glycerophosphatides, the deficiency resulted in extensive changes in the distribution of saturated acids at both the α- and β-positions. Palmitoleic and oleic acids were increased in amount in fat deficiency, and large amounts of docosatrienoic acids appeared in these three phospholipids. The fatty acids of the sphingomyelins were not altered as a result of essential fatty acid deficiency.

The data demonstrate that each phospholipid is unique in the way in which its fatty acid moieties change in response to feeding a fat deficient diet.

KEY WORDS phospholipids · liver mitochondria · rat · essential fatty acids · fat deficiency · ethanolamine glycerophosphatide · choline glycerophosphatide · serine glycerophosphatide · inositol glycerophosphatide · fatty acid composition

In the first part (1) of this study of the effects of a nutritional deficiency of the essential unsaturated fatty acids on rat liver mitochondria (Mt),[1] it was postulated that the primary effect is the development of structural lability. Among the biochemical sequelae are an uncoupling of oxidative phosphorylation and a loss of respiratory control and ATP–P_i exchange, as the Mt are aged in vitro (1, 2). Some of the observations made previously suggested an involvement of lipids in the deficiency-induced structural lability, and our interest was directed particularly to the fatty acid moieties of the phospholipids of liver Mt from fat-deficient rats. In the present study, a measurement has been made of the fatty acids esterified to the α- and β-positions of ethanolamine-, inositol-, and choline glycerophosphatides, and of the total fatty acids of another glycerophosphatide fraction[2] and of sphingomyelins of liver Mt. A preliminary account has been reported (3).

MATERIALS AND METHODS

Male Holtzman rats were weaned at 18 days of age and fed either a fat-free diet or one in which corn oil (Mazola) replaced carbohydrate at a level of 5%. The experimental diet contained 58% dextrose, 21% "vitamin-free" casein, 16% inert cellulose, 4% salt mixture U.S.P. XIV, and 1% vitamin fortification mixture (General Biochemicals, Inc.). The animals were maintained on these diets for 12–14 weeks, at which time those on the fat-deficient diet showed weight loss and other gross symptoms of a fat deficiency. The rats were killed by exsanguination, and Mt were prepared from pooled livers from either two or five animals (see footnotes, Table 1). Two such samples were analyzed from each group of rats. The Mt were prepared in a

[1] The abbreviation Mt will be used for mitochondria. The terms deficient Mt and normal Mt will refer to liver mitochondria prepared from fat deficient and normal rats, as described herein, respectively.

[2] The glycerophosphatide fraction referred to is the first to be eluted from a silicic acid column by the methods described. Its composition is not identified in the present study, but it will be referred to as a cardiolipin plus phosphatidic acid fraction (9–11).

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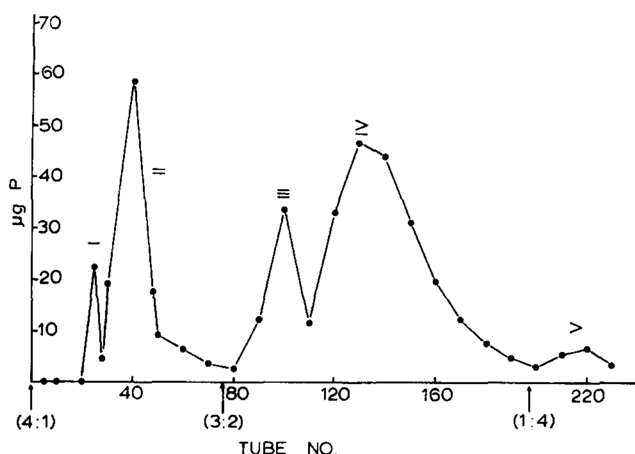

FIG. 1. Curve showing the elution pattern from a silicic acid column of phospholipids of normal rat liver Mt. Figures in parenthesis indicate addition, as eluting solvent, of chloroform–methanol, in the proportions given. I, "Cardiolipin plus phosphatidic acid"; II, ethanolamine-, III, inositol-, IV, choline glycerophosphatides; V, sphingomyelins.

medium of 0.25 M sucrose and 1 mM EDTA as described earlier (1). Mitochondrial lipids were extracted with ethanol for 1 hr, followed by three extractions with ethanol–ether 3:1. The extracts were combined, evaporated to dryness, and taken up in a minimum volume of chloroform, and the phospholipids were precipitated with acetone (4). The phospholipid mixture was then separated into five fractions by column chromatography on silicic acid (5, 6). The elution pattern of the phospholipids of normal mitochondria, shown in Fig. 1, is the same as that described in earlier studies (6).

Each fraction was rechromatographed repeatedly in the same manner, using the entire peak, until a single band could be eluted from the column. Fractions II–IV (numbered in order of elution) were then examined for homogeneity by thin-layer chromatography (TLC) on Silica Gel G, employing the solvent system $CHCl_3$–methanol–acetic acid–H_2O 50:25:8:4 (v/v/v/v). Fractions II and IV appeared homogeneous, but Fraction III, obtained as a single band by column chromatography, always contained a small quantity of material not containing inositol. Consequently, this fraction was purified and finally isolated by preparative TLC using $CHCl_3$–methanol–H_2O 50:25:5. The principal phospholipids occurring in peaks II through V have been identified by Hanahan et al. (5), and confirmed by ourselves by comparison of their behavior with that of known lipids, as phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl choline, and sphingomyelin, respectively. Peak I is a mixture of phospholipids, which is presumed to include at least cardiolipin and phosphatidic acid (7–9). The elution pattern obtained with phospholipids from Mt of essential fatty acid (EFA)-deficient rats was very similar to that shown in Fig. 1, and is not shown.

Fractions II, III, and IV were dissolved in diethyl ether, and subjected to enzymatic hydrolysis by snake (*Crotalus adamanteus*) venom, according to the method of Marinetti et al. (10). The fatty acids thus removed from the β-positions were dissolved in redistilled petroleum ether. The residues (lysophosphatides) were taken up in water, and hydrolyzed chemically (11) to remove the fatty acids esterified at the α-positions. The latter were transferred finally to petroleum ether. Fractions I and V were chemically hydrolyzed (11) without prior treatment with snake venom, and the fatty acids were transferred to petroleum ether.

The fatty acids thus collected were converted to silver salts, which were then reacted with iodomethane to prepare the methyl esters (12). The compositions of the methyl ester mixtures were determined by gas–liquid chromatography (GLC) on a Barber-Colman model 20 gas chromatograph, equipped with disk integrator and capillary column. The column consisted of copper tubing, $^1/_8$ inch o.d. and 12 ft long, packed with 20% LAC-4R-777 on Gas Chrom CLA 60–80 mesh (Applied Science Labs., Inc., State College, Pa.). The operating temperatures were 220° for the column, 280° for the hydrogen flame detector, and 300° at the point of sample injection. The nitrogen gas pressure was 30 psi at the inlet of the column. Standard methyl esters[3] used for identification were (chain length:no. of double bonds) 12:0, 13:0, 14:0, 14:1, 15:0, 16:0, 16:1, 17:0, 18:0, 18:1, 18:2, 18:3, 19:0, 20:0, 20:4, 20:5, 22:0, 22:1, 22:6, 24:0, and 24:1.

RESULTS

Cardiolipin Plus Phosphatidic Acid Fraction

Relatively small amounts of cardiolipin–phosphatidic acid (Fraction I) were available, and no attempt was made to examine, individually, the acids which might be esterified at the α- and β-positions. Linoleic acid (18:2) was the principal fatty acid found in the fraction obtained from normal Mt (Table 1). Other acids occurring in significant amounts were oleic, palmitoleic, and palmitic. In the cardiolipin plus phosphatidic acid fraction of EFA-deficient Mt, the content of linoleic acid was markedly decreased, and the contents of oleic and palmitoleic were markedly increased. The proportions of 18:2 remaining in this fraction in the fat-deficient Mt were, interestingly, approximately the same as those

[3] We gratefully acknowledge a gift of samples of methyl esters including 20:5 and 22:6 methyl esters from Dr. Edward H. Gruger, Jr. The other esters were obtained from commercial sources.

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found in the ethanolamine, inositol, or choline glycerophosphatides of normal Mt.

Ethanolamine Glycerophosphatides

The principal fatty acid found esterified at the α-position of ethanolamine phosphatide of normal Mt was palmitic acid (Table 1, Fraction IIα). Stearic and oleic acids were also present in significant quantities, while only traces of other acids were found. In the fat-deficient animals, the 16:0 was decreased and 18:1 and 18:0 were increased in amount in the α-position, and significant amounts of 18:2 appeared esterified at this position.

The predominant acid esterified in the β-position of ethanolamine phosphatides of normal Mt was arachidonic acid. There were also significant amounts of linoleic, stearic, oleic, palmitic, and docosahexaenoic acids, and an acid whose methyl ester had a retention time, relative to methyl palmitate, of 11.3. With the onset of fat deficiency, 20:3 predominated in the β-position, and the content of 18:1 was increased. There was a marked reduction in 20:4; despite this, however, relatively large amounts still remained. There were also reductions in the amounts of palmitic, stearic, and linoleic acids, until only small amounts of these acids remained in the β-position.

Inositol Glycerophosphatides

Approximately three-quarters of the fatty acid esterified in the α-position of the inositol phosphatides in normal Mt was stearic acid (Table 1, Fraction IIIα). This observation is in keeping with the observations of others, who have reported large amounts of stearic acid in the inositol glycerophosphatides of brain and liver (13, 14). The remaining acids found in significant amounts esterified to the α-carbon atom were 16:0 and 18:1. With the onset of fat deficiency, the amount of 16:0 in

TABLE 1 Fatty Acid Composition of Fractionated Phospholipids from Rat Liver Mitochondria of Normal (N) and Unsaturated-Fat Deficient (D) Rats*

Relative Retention Time	Fatty Acid	I		II α		II β		III α		III β		IV α		IV β		V	
		N	D	N	D	N	D	N	D	N	D	N	D	N	D	N	D
0.517							0.7										
0.539	12:0	0.9	0.5	Tr.	Tr.	1.2	Tr.			0.5	Tr.			0.1	Tr.		
0.604			0.0			Tr.				Tr.				0.0			
0.636				Tr.		0.1	0.4		Tr.	0.2	Tr.	Tr.		0.1	0.2		
0.715	14:0	1.3	0.5	0.1	0.3	1.2	0.8	0.3	0.4	1.1	0.8	0.4	1.0	0.3	0.6	0.6	1.7
0.838		0.3	0.2	0.1	0.1	0.2			0.4	0.2	0.1	0.4	0.2	0.1	0.2	Tr.	Tr.
1.0	16:0	7.8	5.3	58.8	25.6	7.9	3.9	21.7	10.3	10.1	9.3	54.5	36.7	20.6	22.1	9.3	12.0
1.13	16:1	5.8	33.4	1.0	3.2	1.2	5.0			1.2	1.3	1.6	5.8	2.1	9.1	1.3	0.9
1.20				0.5				0.8	0.5								
1.33				0.4										0.1			
1.47	18:0	2.3	1.2	29.1	36.9	4.3	1.5	72.4	80.4	16.9	38.1	28.7	19.9	16.4	11.6	6.0	5.4
1.66	18:1	19.1	43.1	8.8	25.6	5.6	19.0	4.0	8.0	7.0	8.9	9.6	27.7	9.8	37.1	6.9	5.4
1.81											1.5						
1.95	18:2	56.4	9.1	0.5	6.5	11.1	2.9	0.4		6.8	2.4	2.4	2.0	14.1	2.7	0.5	0.2
2.23	20:0					0.2	1.3			0.2	0.7			0.3	0.4		
2.48	18:3	2.2	1.1	0.3	1.2	0.2		0.2		0.2	0.5	0.4	1.4	0.5		3.1	1.9
2.77			1.2				0.7				3.2						
3.11		1.6		0.2	0.6	0.1						0.9		0.3			
3.39	20:3	1.2				0.5	31.1			1.1	20.0		3.9	0.7	11.4		
3.89	20:4	1.1		0.2		48.2	19.5	0.2		30.7	1.7	1.1	1.4	27.2	0.8		
4.46	20:5									0.4						8.8	10.8
4.95							2.1				0.6						
5.43			0.2								2.0					3.8	
6.18	24:0		2.0			2.2				0.8				0.9			
6.98	24:1					3.2	2.3			0.6				1.1			
7.65						1.1											
8.91	22:6					4.2	3.7			0.4				1.1			
9.47			2.2														
11.3						6.7	5.1			21.6	8.9			4.2	3.8		
14.3						0.6											
20.4†																59.7†	61.7

* Values, in g/100 g of total fatty acid in the mixture, are means of two individual determinations. For each determination, livers from two animals were pooled to obtain the mitochondrial lipids in fractions II, III, and IV, and livers from five animals were pooled to obtain fractions I and V. Fraction I, "cardiolipin plus phosphatidic acid"; II, ethanolamine glycerophosphatides; III, inositol glycerophosphatides; IV, choline glycerophosphatides; V, sphingomyelins. α and β indicate the position of esterification on the glycerolipid molecule.

† Considering the nature of the column, it is possible that this fraction is not a fatty acid ester.

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this position was decreased somewhat, and there were small increases in the amounts of 18:0 and 18:1.

Arachidonic acid predominated in the β-position in the inositol glycerophosphatides of normal Mt. There was, also, a relatively large amount of the fatty acid with a relative retention time of 11.3. Other acids found in the β-position in significant amounts were 16:0, 18:0, 18:1, and 18:2. With the onset of fat deficiency, the arachidonic acid almost completely disappeared, and stearic acid predominated in the β-position. The fatty acid having a retention time of 11.3 was reduced in amount, and 20:3 appeared in the β-position, in the amount of about 20% of the total.

Lecithins

Palmitic acid was the predominant fatty acid esterified at the α-position of the lecithins of normal Mt, and 18:0 and 18:1 were also present at this position in significant amounts. In Mt from fat-deficient animals, the oleic acid increased to a considerable extent at the α-position; there was a decrease in the amount of palmitic and stearic acids, and eicosatrienoic acid appeared in small amounts.

The fatty acids esterified at the β-position in lecithins from normal Mt were palmitic, stearic, oleic, linoleic, and arachidonic acids, and the acid having a relative retention time of 11.3. With the onset of fat deficiency, the acid occurring in largest amounts in the β-position of lecithin was oleic acid. In addition, there was also an increase in the amount of palmitoleic acid in this position, a small but significant decrease in stearic acid, and a marked reduction in linoleic and arachidonic acids. Eicosatrienoic acid appeared at a level of about 11% of the total fatty acid.

Sphingomyelins

Relatively little change was observed in the proportions of the fatty acids of the sphingomyelins as the animals were rendered EFA-deficient. In both the normal and deficient Mt, the predominant acid in this fraction was one having a relative retention time of 20.4. The other acids occurring in significant amounts were an acid with a relative retention time of 5.4, eicosapentaenoic, octadecatrienoic, oleic, stearic, and palmitic acids.

DISCUSSION

Other workers have measured the fatty acids in total lipid extracts of normal or fat-deficient liver and liver Mt (15–19), and in some of the phospholipids of normal and fat-deficient livers (20–21). The data in Table 1 describing the fatty acids esterified at the α- and β-positions of individual liver mitochondrial phospholipids are in general agreement with these observations, and indicate further that each phospholipid is unique in the manner in which its fatty acid pattern responds to feeding a fat-deficient diet.

The fatty acid composition of the mitochondrial phospholipids of the young rats, at the time they were placed on the experiment, is not known. In view of the fact, however, that fatty acid patterns of mitochondrial lipids respond readily to changes in dietary fatty acids (18, 22), and also that the fat-deficient diet was fed for at least 12 weeks, it seems safe to conjecture that the fatty acid pattern described here in Mt phospholipids is representative of a fatty acid deficiency. Under similar considerations, the phospholipid fatty acid patterns observed in the control mitochondria undoubtedly are representative only of animals fed the particular sample of corn oil contained in the diet. In the absence of a dietary source of unsaturated fatty acids, such as that found in corn oil, the rat will synthesize mitochondrial phospholipids containing relatively large amounts of unsaturated fatty acids—approximately the same weight percentage as are found in phospholipids of the normal Mt. Thus, Table 2 shows that Fraction I phospholipids from deficient Mt contain similar percentages of unsaturated fatty acids to those in the controls, but they consist largely of 16:1 and 18:1 acids, whereas the control Mt fatty acids are predominantly 18:2.

On the other hand, the ethanolamine glycerophosphatides (Fraction II) produced in a fat deficiency have increased amounts of unsaturated acids at both α- and β-positions. At the α-position, this is due largely to 18:1 and 18:2 acids, and at the β-position, to 18:1 and 20:3 acids. It may be noteworthy that even in the deficient state the ethanolamine glycerophosphatides contain significant amounts of 20:4 acids. In the lecithins, one also observes an increased content of unsaturated fatty acids in deficient Mt occurring at the α-position, ac-

TABLE 2 Total Saturated and Unsaturated Fatty Acids of Liver Mitochondrial Phospholipids

	I		II				III				IV				V	
			α		β		α		β		α		β			
	N	D	N	D	N	D	N	D	N	D	N	D	N	D	N	D
Saturated acids	12.3	9.5	88.0	62.8	17.0	7.5	94.4	91.1	29.6	48.9	83.6	57.6	38.6	34.7	15.9	19.1
Unsaturated acids	85.8	86.7	10.8	36.5	74.2	83.5	4.8	8.0	48.4	34.8	15.1	42.2	56.6	61.1	20.6	19.2

Values are obtained by the summation of the values from Table 1, of all saturated acids, and of all unsaturated acids. All notations are the same as those used in Table 1. Fatty acids represented in Table 1 by relative retention times only are not included in this summation.

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counted for by 16:1, 18:1, and 20:3 acids. The inositol glycerophosphatides of the deficient Mt contain increased amounts of saturated acids at the β-position, due to an increase in 18:0 acids.

The trienoic acid appearing in Mt lipids in the fat deficiency state was not found either in the cardiolipin–phosphatidic acid fraction (Fraction I) or in sphingomyelin, but was found almost entirely at the β-position of the phospholipids in Fractions II, III, and IV. The appearance of relatively large amounts of the 20:3 acids, and the reduction in the amounts of 18:2 and 20:4 in the ethanolamine-, inositol-, and choline glycerophosphatides are clearly some of the major events associated with feeding a fat-free diet. It is quite possible, however, that other changes in structures of the phospholipid molecules, indicated in Table 1, which involve both saturated and unsaturated fatty acids, might be of real and possibly equal physiological significance. For example, the unique requirement by β-hydroxybutyric dehydrogenase of beef heart Mt for lecithin and the fact that the activity of lecithin in meeting this requirement depends on the nature of its fatty acid moiety (23, 24), suggests that the changes in the fatty acids of the phospholipids, observed in the present study, might result in extensive changes in mitochondrial physiology. It is not surprising then that altered cytochrome oxidase (25) and succinic, butyric, and glutamic dehydrogenase (26) activities occurred as a result of fat deficiency in rats.

Earlier data led to the suggestion that the mitochondrial instability in fat deficiency was associated with the lipids within the Mt (1). While the present data provide a much closer view of the fatty acid components of the mitochondrial phospholipids in the fat deficient animal, there are not yet enough supporting data to relate the changes observed to either structural instability of the mitochondria, uncoupled oxidative phosphorylation, loss of respiratory control, or altered ATP–P_i exchange (1, 2). Green and Fleischer (27) have suggested that the oxidative phosphorylation apparatus is composed of networks of alternating protein and phospholipid. Conceivably, the stability of such a structure would depend, in part, on the nature of the fatty acids of the phospholipids. In addition to contributing to structural instability, the latter may influence the activity of one or more enzymes operating within the network. Another way in which the fatty acid moieties of the mitochondrial phospholipids may influence reactions within the mitochondria is by way of altering the dielectric constant of the medium (27). It is conceivable, on this basis, that the phospholipids found in deficient mitochondria provide an environment which is unfavorable to the synthesis of high energy intermediates of oxidative phosphorylation that are unstable in water.

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