Studies on Essential Fatty Acid Deficiency

EFFECT OF THE DEFICIENCY ON THE LIPIDS IN LIVER MITOCHONDRIA AND OXIDATIVE PHOSPHORYLATION

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1. Dietary deficiency of essential fatty acids results in a twofold increase in the neutral lipid content of liver mitochondria as compared with the corresponding value for stock-fed rats. 2. Deficiency produces changes in the pattern of the constituent fatty acids of the main phospholipid fractions of liver mitochondria which are similar to those previously reported for the lipids of whole liver. There is a fall in the content of $C_{18:2}$ acid and to a smaller extent of $C_{20:4}$ acid associated with a rise of $C_{16:1}$, $C_{18:1}$ and $C_{20:3}$ acids. 3. Deficiency results in small decreases in the phosphorylation quotients of liver mitochondria during oxidation of succinate and pyruvate, but the values lie within the range reported for normal mitochondria. Mitochondrial respiration with succinate is decreased as a result of deficiency but no change was observed with pyruvate as substrate.

The fatty acid composition of liver mitochondrial lipids of stock-fed rats is similar to the composition of lipids extracted from whole liver (Getz, Bartley, Stirpe, Notton & Renshaw, 1962; Macfarlane, Gray & Wheeldon, 1960).

The composition of whole tissue lipids is very markedly changed as a result of deficiency of essential fatty acids (Mohrhauser & Holman, 1963*a,b*; Biran, Bartley, Carter & Renshaw, 1964). In the present study the composition of the mitochondrial lipids in rats deficient in essential fatty acids and in those supplemented with linseed oil was investigated.

Some experiments were also carried out on the capacity for oxidative phosphorylation by liver mitochondria from deficient and supplemented animals. Klein & Johnson (1954) reported that in mitochondria isolated from the livers of rats deficient in essential fatty acids there was an impairment in phosphorylation coupled with oxidation of α -oxoglutarate, malate and glutamate, whereas during oxidation of succinate no uncoupling of phosphorylation was observed. They concluded that deficiency of essential fatty acids resulted in a defect of the electron-transport system at or near the level of NADH oxidation. In later work Levin,

Johnson & Albert (1957) reported a marked increase in the oxygen consumption of 'deficient' mitochondria, an observation which may be correlated with the increased oxygen consumption of the whole animal suffering from deficiency of essential fatty acids first noted by Burr & Beber (1937). The changes in the respiratory activity observed by Levin *et al.* (1957) were associated with morphological changes in the 'deficient' mitochondria.

EXPERIMENTAL

Male rats used in the present study were treated as described by Biran *et al.* (1964). Rats in the supplemented group (group B) received daily 100 mg. of linseed oil in addition to the vitamin supplements received by the deficient animals (group A). After about 90 days on the experimental diet, when the deficient animals were showing well-marked signs of deficiency, they were killed.

Analytical methods. Liver mitochondria were prepared as described by Werkheiser & Bartley (1957) and suspended in sucrose solution (0.25 M) in a concentration of 70–90 mg. wet wt. of mitochondrial pellet/ml. Total mitochondrial lipids were extracted as described by Biran *et al.* (1964) and the total lipid extract was fractionated on silicic acid columns. The phospholipids were fractionated with a series of mixtures of chloroform and methanol (Table 1). The eluate was collected in 10 ml. portions and the content of lipid in each portion was measured by the method of Berenblum & Chain (1938), as modified by Bartley (1953). The elution pattern was practically identical for lipids from deficient animals and those from the supplemented group and resembled each other in the proportions of various lipids

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Table 1. *Sequence of eluents used for the fractionation of phospholipids from rat-liver mitochondria*

The chloroform used contained 2% of ethanol as stabilizer.

Composition of eluent	Vol. of eluent (ml.)
Chloroform	250
Chloroform-methanol (19:1, v/v)	100
Chloroform-methanol (11:1, v/v)	100
Chloroform-methanol (9:1, v/v)	100
Chloroform-methanol (7:1, v/v)	100
Chloroform-methanol (4:1, v/v)	200
Chloroform-methanol (3:2, v/v)	600
Chloroform-methanol (1:4, v/v)	240
Methanol	400

Table 2. *Sequence of eluents used for re-fractionating the amino phosphatides of rat-liver mitochondrial phospholipids*

The chloroform contained 2% of ethanol as stabilizer. Lipids were put on the column as a solution in chloroform.

Composition of eluent	Vol. of eluent (ml.)
Chloroform	50
Chloroform-methanol (11:1, v/v)	100
Chloroform-methanol (10:1, v/v)	100
Chloroform-methanol (9:1, v/v)	100
Chloroform-methanol (8:1, v/v)	100
Chloroform-methanol (7:1, v/v)	100
Chloroform-methanol (4:1, v/v)	100
Methanol	100

present in them. The 10 ml. portions were pooled so that each pooled fraction corresponded to a single well-separated peak of lipid P concentration. The lecithin eluted was divided into three sequential fractions with approximately the same content of P so as to provide information on the degree of correlation between the fatty acid composition of the phosphatides and their order of elution from the silicic acid column. Fraction 4, which contained two overlapping peaks in both deficient and supplemented groups, was refractionated with the sequence of eluents shown in Table 2 to give five subfractions 4a-4e. Fraction 4a contained insufficient lipid for analysis. The composition of the other fractions is shown in Table 3. The lipids eluted in the various fractions were identified by thin-layer chromatography on silicic acid (Wagner, Hörhammer & Wolff, 1961). A portion of each fraction was saponified and the liberated fatty acids were extracted and methylated with diazomethane. The mixture of methyl esters so obtained was estimated by the method of Rapport & Alonzo (1955) as modified by Biran & Bartley (1961) and the proportions of individual fatty acids were measured by gas chromatography.

The content of lipid amino N was measured in some fractions by the method of Cocking & Yemm (1954) and the content of lipid P in the lipid fractions was measured by

Table 3. *Fatty acid content of phospholipid fractions obtained by silicic acid chromatography of lipids from rat-liver mitochondria.*

Fatty acids were measured as methyl esters after saponification.

Fraction	Main component	Percentage of total phospholipid fatty acids	
		Group A	Group B
2	Cardiolipin	8.5	9.5
3	Amino phosphatides	4.3	1.9
4b	Serine phosphatide	3.4	5.4
4c	Ethanolamine phosphatide	3.0	5.2
4d	Ethanolamine phosphatide	8.7	9.3
4e	Amino lysophosphatides	2.8	6.2
5	Amino phosphatides	4.2	3.3
6	Inositol phosphatides	5.5	4.6
7	Lecithin	14.0	12.5
8	Lecithin	17.5	18.5
9	Lecithin	14.0	11.3
10	Lecithin, lysolecithin	3.2	2.4
11	Sphingomyelin, lecithin, lysolecithin	3.7	2.5
12	Sphingomyelin, lysolecithin	2.0	2.4

the method of Burton & Petersen (1960), modified by increasing the volumes of all the reagents tenfold. The estimation of lipid P was done on very small amounts of lipid and may be subject to considerable error. It was therefore used only to confirm the identity of lipids as shown by thin-layer chromatography of individual fractions. From the thin-layer chromatograms it could be seen that cardiolipin (fraction 2), lecithin (fractions 7, 8 and 9) and total amino phosphatides (original fraction 4) were well separated. These fractions constituted about 90% of the total phospholipid fatty acids and in each of these fractions not more than about 10% of the fatty acids were derived from other phospholipids. Thus the main cardiolipin fraction (fraction 2) contained approx. 95 and 90% respectively of the total cardiolipin present in the mitochondrial lipids of the supplemented and deficient groups and, on the basis of analyses for lipid P and amino N, was contaminated with about 8% of nitrogenous phospholipid and possibly neutral lipid. Of the three lecithin fractions (fractions 7, 8 and 9), which accounted for 99 and 94% respectively of the total lecithin present in the mitochondrial lipids of the supplemented and deficient groups, fraction 7, containing one-fifth of the total, was contaminated with about 20% of amino phosphatides and possibly inositol phosphatide. Fractions 8 and 9 gave only one spot on thin-layer chromatograms and were assumed to be pure. The total amino phosphatides in fractions 3, 4b-e and 5 gave recoveries of 83 and 75% respectively for the mitochondrial amino phosphatides of the supplemented and deficient groups. Fractions 4c and 4d (Table 3) accounted for 54 and 62% of the total ethanolamine phosphatide and were uncontaminated by other phosphatides. Serine phosphatide gave a poor separation and no accurate estimate of its total content or composition can be made. The main inositol phosphatide fraction (fraction 6) contained only 49 and 68% respectively of the total inositol

phosphatide of the mitochondrial lipids of the supplemented and deficient groups, and this fraction was contaminated by about 25% of the amino phosphatides and lecithin.

The percentage of fatty acids in the phospholipid fractions (Table 4) was based on estimations of the methyl esters prepared from the fatty acids liberated by saponification with dilute alkali.

Oxidative phosphorylation. The procedure was based on that of Aldridge (1957) using conventional Warburg technique. Each flask contained 3.3 ml. of a medium having (final concentrations): AMP (1.15 mM), ATP (1.15 mM), KCl (0.1 M), MgCl₂ (2.8 mM), EDTA (2 mM), potassium phosphate (15 mM), sucrose (38 mM), succinate (13 mM) or pyruvate (13 mM) plus malate (1.2 mM). The side arm contained glucose (180 μ moles), hexokinase (200–400 units) and glycylglycine (50 μ moles). The final pH was adjusted to 7.1–7.2. The solution of pyruvate was prepared freshly for each experiment. After preliminary incubation for 10 min. at 25° the contents of the side arms were tipped and uptake of O₂ was measured over a period of 20 min. At intervals of 10, 15 and 20 min. flasks were removed, the contents deproteinized with trichloroacetic acid, centrifuged, and phosphate in the supernatant was estimated by the method of Fiske & Subbarow (1925).

RESULTS AND DISCUSSION

Content of mitochondrial lipids. The total lipid contents and the contents of phospholipid per unit of mitochondrial dry weight of the group deficient in

essential fatty acids (group A) and of rats receiving a daily supplement of linseed oil (group B) were the same (Table 4). The amount of neutral lipid in the mitochondria of group A was about twice as much as that found by Getz *et al.* (1962) and a daily intake of about 50 mg. of linoleic acid by group B was inadequate to prevent this increase. Morton & Horner (1961) have reported that the concentrations of cholesterol esters and triglycerides in the livers of male rats deficient in essential fatty acids were higher than in controls receiving 100 mg. of linoleic acid daily.

Table 5, which records the proportions of the main phosphatide fractions analysed, indicates that these proportions are not changed by essential fatty acid deficiency and are similar to those reported by Getz *et al.* (1962) for stock-fed rats.

Fatty acid composition of mitochondrial lipids. The effect of essential-fatty acid deficiency on the fatty acid distribution in the neutral-lipid fraction is similar to that reported by Biran *et al.* (1964) for the corresponding fraction in whole liver tissue (Table 6). The concentrations of C_{18:2} acid in this fraction of mitochondrial lipids in both groups A and B are markedly higher than in the corresponding fractions from whole liver or the other tissue examined by Biran *et al.* (1964). However, the amount of C_{18:2} acid of group A is reduced to about one-third of that

Table 4. *Lipid content of rat-liver mitochondria*

Data for stock-fed animals were taken from ⁽¹⁾ Getz & Bartley (1961) and ⁽²⁾ Getz *et al.* (1962).

	No. of measure- ments	Group A	Group B	Stock-fed
Lipid (mg./g. mitochondrial dry wt.)	1	183	181	234 ⁽¹⁾
Lipid P (μ moles/g. mitochondrial dry wt.)	4	183 $\pm$ 25	186 $\pm$ 15	157 ⁽²⁾
Fatty acid (μ moles/g. mitochondrial dry wt.)	Total	403 $\pm$ 22	405 $\pm$ 37	384 ⁽¹⁾ , 340 ⁽²⁾
	In neutral lipids	73	76	36.5 ⁽²⁾
	In phospholipids	330	329	303.9 ⁽²⁾

Table 5. *Proportions of the main phospholipid fractions of rat-liver mitochondria*

The constituent fatty acids and the lipid P in each phospholipid fraction are calculated as percentages of fatty acid and of lipid P in the total mitochondrial phospholipid. Data from ⁽¹⁾ Getz *et al.* (1962) and ⁽²⁾ Macfarlane *et al.* (1960) for stock-fed rats are given for comparison.

Fraction	Moles of fatty acid in fraction Moles of fatty acid in total phospholipid $\times 100$			Moles of lipid in fraction Moles of lipid P in total phospholipid $\times 100$
	Group A	Group B	Stock-fed ⁽¹⁾	Stock-fed ⁽²⁾
Cardiolipin	8.7	8.6	7.9	9
Total amino phosphatides	31.7	35.0	38.8	31
Inositol phosphatide	5.6	6.5	6.5	6
Lecithin	45.0	41.7	45.0	51
Sphingomyelin	0	0	1.0	

of group B and shows a fivefold decrease as compared with the value reported for stock-fed rats. This change is associated with a rise of $C_{18:1}$ and

$C_{20:3}$ acids. The amounts of $C_{20:4}$ acid for both groups are markedly lower than the corresponding value for stock-fed rats. In general, the pattern of distribution of the fatty acids of group B more closely approximates to that of group A than to that of stock-fed rats, whose daily intake of linoleic acid (160 mg.) is over three times as great as that of group B (Biran *et al.* 1964).

Table 7 records the fatty acid distribution of the main cardiolipin fraction. Subject to the reservation that this fraction was contaminated with about 8% of nitrogenous phospholipid and possibly neutral fat, the distribution for both groups A and B was similar to the corresponding pattern found by Biran *et al.* (1964) in whole liver. In both the supplemented and deficient groups, however, the amount of $C_{18:2}$ acid in mitochondrial cardiolipin was about double that found for whole liver. The deficient group showed a 77% decrease in the amount of $C_{18:3}$ acid as compared with group B and this was associated with a tenfold increase of $C_{20:3}$ acid. It may be noted that in both groups A and B the amounts of $C_{16:1}$ and $C_{18:1}$ acids are markedly higher than the values for stock-fed animals, a feature which is also evident for the neutral-lipid fraction (Table 6). There was no significant change in the amounts of $C_{20:4}$ acid in the two groups.

Table 8 records the fatty acid distribution of the lecithin, fractions 8 and 9, and of the ethanolamine phosphatide, fractions 4c and 4d, all of which on chromatographic evidence were uncontaminated by other components. The patterns of fatty acid distribution in these nitrogenous phosphatides was modified as a result of deficiency by a marked fall in the amount of $C_{18:2}$ and $C_{22:6}$ acids, a slight decrease of $C_{20:4}$ acid and a rise in the amounts of $C_{16:1}$, $C_{18:1}$ and $C_{20:3}$ acids.

The fraction containing inositol phosphatide (fraction 6) was contaminated with other phospho-

Table 6. *Fatty acid composition of neutral lipids from rat-liver mitochondria*

Data for stock-fed rats are from Getz *et al.* (1962).

Fatty acid	Percentage of methyl ester in measurable peaks		
	Group A	Group B	Stock-fed
$C_{16:0}$	13.8	12.7	22.6
$C_{16:1}$	18.6	18.0	2.5
$C_{18:0}$	6.5	6.2	9.6
$C_{18:1}$	44.7	36.7	21.8
$C_{18:2}$	3.4	9.9	17.9
$C_{20:3}$	2.7	0.9	1.0
$C_{20:4}$	2.4	1.3	7.2
$C_{22:6}$	+	+	3.5

Table 7. *Fatty acid composition of cardiolipin from rat-liver mitochondria*

Data for stock-fed rats are from Getz *et al.* (1962).

Fatty acid	Percentage of methyl ester in measurable peaks		
	Group A	Group B	Stock-fed
$C_{16:0}$	4.6	6.6	3.9
$C_{16:1}$	24.3	22.8	2.5
$C_{18:0}$	7.9	2.5	1.4
$C_{18:1}$	39.0	34.2	12.8
$C_{18:2}$	2.7	12.1	74.0
$C_{20:3}$	5.5	0.5	1.2
$C_{20:4}$	3.2	2.0	1.6
$C_{22:6}$	+	3.0	0.4

Table 8. *Fatty acid composition of ethanolamine phosphatide (fractions 4c and 4d) and of lecithin (fractions 8 and 9) from rat-liver mitochondria*

Fractions ... Fatty acid	Percentage of methyl ester in measurable peaks							
	Ethanolamine phosphatide				Lecithin			
	Group A		Group B		Group A		Group B	
	4c	4d	4c	4d	8	9	8	9
$C_{16:0}$	17.0	19.0	21.8	27.2	24.1	34.4	31.7	35.8
$C_{16:1}$	3.6	4.0	+	6.7	6.7	14.7	6.2	12.3
$C_{18:0}$	20.2	17.2	23.2	20.7	15.7	15.3	14.2	10.4
$C_{18:1}$	26.9	25.9	10.7	16.4	30.3	23.9	22.6	22.6
$C_{18:2}$	+	+	1.8	3.1	+	+	4.4	3.9
$C_{20:3}$	14.6	16.5	0	3.8	15.3	4.8	3.5	2.0
$C_{20:4}$	7.4	6.2	9.5	8.8	4.5	1.4	5.0	2.8
$C_{22:6}$	0	+	5.5	4.5	+	0	5.2	1.9

Table 9. *Oxidative phosphorylation by liver mitochondria from rats deficient in essential fatty acids (group A) and rats receiving daily supplements of 100 mg. of linseed oil (group B)*

For conditions of incubation see the Experimental section.

Substrate	No. of expts.	Mean P/O ($\pm$ s.d.)		Significance of difference	Q_{O_2} ($\pm$ s.d.)		Significance of difference
		Group A	Group B		Group A	Group B	
Succinate	5	1.59 $\pm$ 0.26	1.74 $\pm$ 0.22	$P < 0.05$	48.0 $\pm$ 2.2	54.6 $\pm$ 6.8	$P < 0.05$
Pyruvate (+ malate)	4	2.72 $\pm$ 0.61	3.02 $\pm$ 0.43	$P < 0.05$	38.3 $\pm$ 4.6	43.6 $\pm$ 12.6	$P < 0.4$

tides but, as with the inositol phosphatide of normal rat-liver mitochondria (Getz *et al.* 1962), ox liver (Macfarlane, 1961) and ox heart (Fauré & Morelec-Coulon, 1954), it was characterized by its high content of stearic acid. Deficiency of dietary essential fatty acids resulted in a fall in the amounts of $C_{20:4}$ and $C_{22:6}$ acids in this fraction and some slight rise of $C_{16:1}$, $C_{18:1}$ and $C_{20:3}$ acids. The content of $C_{18:2}$ acid was low in both groups A and B.

Oxidative phosphorylation in liver mitochondria. Table 9 records the mean P/O ratios for the oxidation of succinate or pyruvate (plus catalytic amounts of malate) by mitochondria of deficient and supplemented rats. With succinate the P/O ratio for group A is slightly but significantly lower than for group B. It may be noted, however, that the ratios given by both groups lie between 1.5, reported by Levin *et al.* (1957), and 2.2 (Greengard, Minnaert, Slater & Betel, 1959) for liver mitochondria from stock-fed rats. These variations may be attributed to variations in the concentration of succinate employed and to the possible effects of mitochondrial damage. Klein & Johnson (1954) found a ratio 1.8 for liver mitochondria both from normal and deficient rats during the oxidation of succinate. The present experiments confirm their conclusion that there is no impairment in the phosphorylation quotient for succinate as a result of essential fatty acid deficiency. The data in Table 9 also indicate that with succinate as substrate the respiratory activity (Q_{O_2}) of the 'deficient' mitochondria is lower than that of the supplemented group. This finding is at variance with the data of Levin *et al.* (1957), who reported an increased respiration by liver mitochondria from deficient rats oxidizing α -oxoglutarate, succinate, malate and NADH as compared with controls. With pyruvate as substrate there was a small but significant lowering of the P/O ratio by the deficient mitochondria but no impairment of respiratory activity. The change is less marked than that reported by Klein & Johnson (1954) for the NAD-linked dehydrogenase systems for α -oxoglutarate, glutamate and malate. As with succinate, it is concluded that there is no material impairment of phosphorylation by liver mito-

chondria oxidizing pyruvate as a result of essential fatty acid deficiency.

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REFERENCES

- Aldridge, W. N. (1957). *Biochem. J.* **67**, 423.
 Bartley, W. (1953). *Biochem. J.* **54**, 677.
 Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
 Biran, L. A. & Bartley, W. (1961). *Biochem. J.* **79**, 159.
 Biran, L. A., Bartley, W., Carter, C. W. & Renshaw, A. (1964). *Biochem. J.* **93**, 492.
 Burr, G. O. & Beber, A. J. (1937). *J. Nutr.* **14**, 553.
 Burton, K. & Petersen, G. B. (1960). *Biochem. J.* **75**, 17.
 Cocking, E. C. & Yemm, E. W. (1954). *Biochem. J.* **58**, xii.
 Fauré, M. & Morelec-Coulon, M. J. (1954). *C.R. Acad. Sci., Paris*, **238**, 411.
 Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
 Getz, G. S. & Bartley, W. (1961). *Biochem. J.* **78**, 307.
 Getz, G. S., Bartley, W., Stirpe, F., Notton, B. M. & Renshaw, A. (1962). *Biochem. J.* **83**, 181.
 Greengard, P., Minnaert, K., Slater, E. C. & Betel, I. (1959). *Biochem. J.* **73**, 637.
 Klein, P. D. & Johnson, R. M. (1954). *J. biol. Chem.* **211**, 103.
 Levin, E., Johnson, R. M. & Albert, S. (1957). *J. biol. Chem.* **228**, 15.
 Macfarlane, M. G. (1961). *Biochem. J.* **78**, 44.
 Macfarlane, M. G., Gray, G. M. & Wheeldon, L. W. (1960). *Biochem. J.* **77**, 626.
 Mohrhauer, H. & Holman, R. T. (1963a). *J. Lipid Res.* **4**, 161.
 Mohrhauer, H. & Holman, R. T. (1963b). *J. Lipid Res.* **4**, 346.
 Morton, R. A. & Horner, A. A. (1961). *Biochem. J.* **79**, 631.
 Rapport, M. M. & Alonzo, N. (1955). *J. biol. Chem.* **217**, 193.
 Wagner, H., Hörhammer, L. & Wolff, P. (1961). *Biochem. Z.* **334**, 175.
 Werkheiser, W. C. & Bartley, W. (1957). *Biochem. J.* **66**, 79.