Serum Phospholipids of Pigs Given Different Amounts of Linoleic Acid

2. LOCATION OF EICOSATRIENOIC ACID IN LECITHIN

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Essential-fatty acid deficiency is characterized by the accumulation in tissue lipids of an eicosatrienoic acid derived from oleic acid (Fulco & Mead, 1959). In an experiment in which six pigs had been reared on diets containing different amounts of linoleic acid, it was shown that about 65% of the serum eicosatrienoic acid was located in the lecithin fraction (Leat, 1964). Collins (1962) has reported that there is an increased turnover of lecithins in the livers of essential-fatty acid-deficient rats and has suggested that the lecithins containing eicosatrienoic acid are less stable than those containing arachidonic acid. This difference in turnover rate between lecithins containing either arachidonic acid or eicosatrienoic acid may be governed by some characteristic of the fatty acid itself or by a difference in the position of attachment of the fatty acid to the lecithin molecule. The lecithins from normal animals contain almost

exclusively unsaturated fatty acids at the β -position (Tattarie, 1959; Hanahan, Watts & Pappajohn, 1960), but it is possible that the increased turnover rates of lecithins in essential-fatty acid-deficient animals may be due to the attachment of eicosatrienoic acid to the α -position. Lands (1960) has shown that the fatty acids of the α - and β -positions of lecithin undergo turnover and metabolism independently of each other. The α -group may have a higher turnover rate than the β -group, since in triglycerides the primary hydroxyl group (α) is more reactive than the secondary hydroxyl group (β) (Brandner & Birkmeier, 1960).

The object of the present investigation was to determine the position of attachment of arachidonic acid and eicosatrienoic acid in the serum lecithins of pigs in various stages of essential-fatty acid deficiency by using phospholipase A from *Crotalus adamanteus*. This enzyme is known to

Table 1. *Fatty acids of the serum lecithins and lysolecithins from three pigs of differing essential-fatty acid status*

Experimental details are given in the text. Some minor fatty acids have been omitted for clarity. The carbon no. was calculated by the method of Woodford & van Gent (1960). The designation of the fatty acids follows the system recommended by Farquhar, Insull, Rosen, Stoffel & Ahrens (1959). Percentage composition is calculated as: $\frac{\text{peak area of fatty acid}}{\text{total peak area}} \times 100$.

Carbon no. on poly(ethylene glycol adipate)	Designation	Lecithin	Fatty acid released by phospholipase A	Lysolecithin remaining	Lysolecithin of serum
Pig no. 2. Percentage composition of fatty acids					
14.0	14:0	0.3	0.3	0.6	0.5
15.0	15:0	0.2	0.3	0.3	0.4
16.0	16:0	17.8	2.4	22.9	28.0
16.3	16:1	1.7	1.3	3.0	2.8
18.0	18:0	22.3	0.5	50.0	28.2
18.3	18:1	33.7	49.5	16.0	28.5
18.9	18:2	5.3	10.0	1.0	2.7
19.6	18:3	0.3	0.4	0.5	.
20.0	20:0	0.3	1.1	0.4	0.6
20.6	20:2	1.0	1.5	0.3	0.7
21.0	20:3	9.7	18.6	2.0	4.4
21.2	?	0.8	2.2	Trace	0.2
21.5	20:4	3.1	6.2	0.6	1.2
22.3	22:1	0.3	0.5	.	.
23.6	?	0.7	1.0	.	.
23.8	?	0.3	0.5	.	.
24.3	24:1	1.0	1.8	.	.
24.6	?	0.6	0.8	.	.
	$\frac{20:3}{20:4}$ ratio	3.1	3.0	3.3	3.5
Total unsaturated acids		58.3	95.4	24.3	41.2

Table 1. (cont.)

Carbon no. on poly(ethylene glycol adipate)	Designation	Lecithin	Fatty acid released by phospholipase A	Lysolecithin remaining	Lysolecithin of serum
Pig no. 4. Percentage composition of fatty acids					
14.0	14:0	0.2	0.2	0.9	0.4
15.0	15:0	0.2	0.1	0.3	0.3
16.0	16:0	18.6	2.5	27.4	32.7
16.3	16:1	2.0	0.8	2.3	2.9
18.0	18:0	22.2	0.4	45.9	29.2
18.3	18:1	29.7	43.4	14.5	24.0
18.9	18:2	9.2	16.7	0.7	3.1
19.6	18:3	0.4	0.6	0.2	0.3
20.0	20:0	0.1	0.2	0.1	0.1
20.6	20:2	0.5	0.7	.	0.3
21.0	20:3	5.4	12.2	1.0	1.9
21.2	?	0.8	2.8	.	0.4
21.5	20:4	5.9	12.7	0.9	1.8
22.3	22:1	0.6	0.9	.	.
23.6	?	0.7	0.7	.	.
23.8	?	0.6	0.3	.	.
24.3	24:1	1.0	1.8	.	.
24.6	?	0.4	1.0	.	.
	$\frac{20:3}{20:4}$ ratio	0.91	0.96	1.1	1.0
Total unsaturated acids		57.7	96.5	23.2	35.5
Pig no. 6. Percentage composition of fatty acids					
14.0	14:0	0.2	0.2	0.7	0.4
15.0	15:0	0.2	0.2	0.3	0.3
16.0	16:0	16.2	2.0	26.0	29.9
16.3	16:1	2.7	0.9	2.0	2.3
18.0	18:0	22.2	0.4	43.0	29.0
18.3	18:1	30.0	36.0	17.0	22.5
18.9	18:2	13.6	28.0	1.5	3.9
19.6	18:3	0.4	0.8	.	0.5
20.0	20:0	0.1	.	0.3	0.4
20.6	20:2	0.2	.	.	0.3
21.0	20:3	1.8	4.0	.	0.4
21.2	?	0.7	2.0	.	0.2
21.5	20:4	8.4	19.0	0.5	1.6
22.3	22:1	0.4	0.8	.	.
23.6	?	0.4	0.7	.	.
23.8	?	.	.	.	.
24.3	24:1	0.7	2.0	.	.
24.6	?	0.6	1.0	.	.
	$\frac{20:3}{20:4}$ ratio	0.2	0.2	.	0.2
Total unsaturated acids		60.3	96.7	28.6	35.2

hydrolyse the fatty acid from the β -position irrespective of the type of fatty acid involved (Tattrie, 1959; de Haas & van Deenen, 1961). Three samples of serum lecithin were selected, containing a high, low and intermediate percentage of eicosatrienoic acid respectively, and the fatty acids at the α - and β -positions were determined.

EXPERIMENTAL

Serum. Three samples of pig sera, containing in the lecithin (i) a high percentage of eicosatrienoic acid and a low percentage of arachidonic acid (pig. no. 2), (ii) equal amounts of these acids (pig no. 4), and (iii) a low percentage of eicosatrienoic acid and a high percentage of arachidonic acid (pig no. 6), were used (Leat, 1964).

Extraction of serum lecithin and its enzymic conversion into lysolecithin. The lipids of 50 ml. of serum were extracted and separated from non-lipid contaminants as described by Leat (1963). The total lipids were separated by chromatography on a 13 g. silicic acid column (Böttcher & van Gent, 1961; Leat, 1964). Neutral lipids were eluted with 100 ml. of chloroform, cephalins with 200 ml. of methanol-chloroform (1:4, v/v) and the lecithin-plus-sphingomyelin fraction with 300 ml. of methanol-chloroform (3:2, v/v). A sample of the lecithin-plus-sphingomyelin fraction, equivalent to 20 ml. of serum, was evaporated to dryness in a rotary evaporator at 40° and dissolved immediately in 15 ml. of anhydrous freshly distilled diethyl ether. Then 0.1 ml. of a 0.5% solution of phospholipase A (*Crotalus adamanteus*; L. Light and Co. Ltd.) in 5 mm-calcium chloride solution was added, and the solution was shaken vigorously at intervals over 20 min. and allowed to stand

overnight, when a gelatinous precipitate of lysolecithin was formed. The reaction was stopped by adding 1 ml. of ethanol. The solvents were then removed on a rotary evaporator at 40°, and the products of hydrolysis dissolved immediately in chloroform. A sample was chromatographed on silicic acid-impregnated paper and stained with Ponceau Red to confirm that all the lecithin had been converted into lysolecithin. The fatty acids, liberated by the action of phospholipase A on lecithin, were separated from the lysolecithin by chromatography on a silicic acid column, basically as described by Böttcher & van Gent (1961). The fatty acids were eluted with 50 ml. of methanol-chloroform (1:4, v/v), and the lysolecithin-plus-sphingomyelin fraction was eluted with 100 ml. of methanol-chloroform-water (20:4:1, by vol.).

The fatty acids of the lysolecithin and the original lecithin were obtained by differential hydrolysis, and the methyl esters were prepared and analysed by gas-liquid chromatography, as described by Leat (1964).

RESULTS AND DISCUSSION

The composition of the fatty acids released by the action of phospholipase A on the serum lecithins of pigs in various degrees of essential-fatty acid deficiency is depicted in Table 1. The composition of the fatty acids of the lysolecithin remaining after attack by phospholipase A is also included and compared with that of the lysolecithin occurring naturally in serum. Over 95% of the fatty acids attached to the β -position of lecithin was unsaturated, and over 95% of the linoleic acid, arachidonic acid and eicosatrienoic acid of lecithin was attached to this position, irrespective of the nutritional essential-fatty acid status of the pig. Thus about 65% of the eicosatrienoic acid of serum was found in the β -position of lecithin. All the fatty acids of lecithin having retention times greater than that of arachidonic acid were also located in the β -position, none being found in the fatty acids of the lysolecithin remaining after degradation by phospholipase A. The eicosatrienoic acid:arachidonic acid ratio in the fatty acids of the β -position was the same as in the total fatty acids of lecithin, indicating that the distribution of eicosatrienoic acid was no different from that of arachidonic acid. Therefore the increased turnover of lecithin in essential-fatty acid deficiency cannot be explained by the attachment of the eicosatrienoic acid to the lecithin molecule at a different position from arachidonic acid but rather by some property of the eicosatrienoic acid itself. These results may be compared with those of Rhodes (1958), who found that, after the feeding of highly unsaturated fatty acids to hens, these acids were found only in the β -position of the egg lecithin. It would appear, therefore, that the β -position of the lecithin is the metabolically active site of the molecule (Hanahan, 1960).

Not all the fatty acids attached to the β -position of lecithin molecule were unsaturated, as evidenced by the small amount (3–4%) of palmitic acid and stearic acid. If phospholipase A from *Crotalus adamanteus* specifically hydrolyses the fatty acid of the β -position, as appears to be the case (Robertson & Lands, 1962), then this position is not entirely specific for unsaturated acids. Moore & Williams (1963) have presented evidence for the occurrence of saturated acids at the β -position of lecithin.

About 25% of the fatty acids of the lysolecithin remaining after attack by phospholipase A on the lecithin was unsaturated. This was expected as over 50% of the acids in lecithin was unsaturated. There were no acids in this lysolecithin having retention times greater than that of arachidonic acid and no more than 5% having retention times greater than that of oleic acid. These acids may be attached to the α -position of lecithin or may originate from any lecithin not attacked by phospholipase A. Although no lecithin was detected by chromatography on silicic acid-impregnated paper there are reports that up to 8% of egg lecithin is not attacked by phospholipase A (Moore & Williams, 1963). The sum of the individual fatty acids released by phospholipase A and those of the remaining lysolecithin agree well with the original lecithin, except for palmitic acid. This discrepancy may be due to the palmitic acid peak being very narrow with resultant errors in triangulation.

Comparison of the fatty acid composition of the naturally occurring lysolecithin of serum with that of the lysolecithin produced by the action of phospholipase A on lecithin shows that the latter is more saturated, containing more stearic acid and less oleic acid and polyunsaturated acids. The relationship between the lecithin and lysolecithin of serum is unknown but, if the same distribution of fatty acids occurs in lysolecithin as in lecithin, it would appear that some of the fatty acids of lysolecithin are attached to the β -position of the molecule but that most are attached at the α -position.

SUMMARY

1. The composition of the fatty acids attached at the α - and β -positions of the serum lecithin and the fatty acids of lysolecithin of pigs suffering from essential-fatty acid deficiency have been examined.

2. About 95% of the eicosatrienoic acid and arachidonic acid in lecithin was located at the β -position of the molecule.

3. It is concluded that the increased turnover rate of lecithins in essential-fatty acid deficiency cannot be explained by the eicosatrienoic acid being attached to the lecithin at a position different from that of arachidonic acid.

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The Active Transport of Potassium and Sodium Ions in Respiratory-Deficient Mutants of *Saccharomyces cerevisiae*

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Stiehler & Flexner (1938) first clearly expressed the view that ion transport could be linked directly with the respiratory systems of the cell. Lundegårdh (1939) further developed this view in connexion with the active transport of anions through the root hairs of plants. Conway & Brady (1948) put forward a redox theory for the secretion of hydrochloric acid by the oxyntic cells of the gastric mucosa. This theory was expanded into the more general 'redox pump' theory applicable to the active transport of inorganic cations across cell membranes (Conway, 1951). The evidence for the theory is particularly strong for the active transport of K^+ and Na^+ ions in yeast. Conway & Kernan (1955) found that very small concentrations of redox dyes could exercise a considerable influence on the uptake of K^+ ions and the excretion of Na^+ ions by yeast, and on the basis of this and other findings they concluded that the active transport of these ions across the yeast cell membrane is directly concerned with redox systems acting as carriers and functioning as the immediate suppliers of the energy necessary for the transport. Considerable evidence has been presented (Conway, 1953, 1955, 1963) to show the validity of the 'redox pump' concept also in relation to skeletal muscle.

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The use of respiratory-deficient mutants of yeast as described in the present paper has given further confirmation of the operation of such a redox mechanism for the transport of cations. The accumulation and secretion of K^+ and Na^+ ions in normal and mutant yeasts are compared, as are also the effects of redox dyes. These observations are considered in the light of the general metabolism and of the deficiency of the cytochrome system of the mutant yeast.

EXPERIMENTAL

Yeast cultures. The yeasts employed were a pure strain of brewer's yeast (Guinness 521 A) and an acriflavine-produced respiratory-deficient mutant derived from it.

The yeasts were grown in 2 l. conical flasks containing a 'natural medium' consisting of inorganic salts, yeast extract and glucose (Slonimski, 1953, p. 7). The flasks were incubated, with occasional shaking, at 30°, and growth was allowed to continue for 48–62 hr. The crop was harvested by centrifugation and was twice washed with tap water. The yield was about 15 g./l. for the normal yeast and 10 g./l. for the mutant yeast.

After being harvested, the yeasts were usually suspended in tap water acidified to pH 5 with HCl and bubbled with oxygen for up to 24 hr., both to deplete their high K^+ ion content and to induce the development of the cytochrome system of the cells.

Sodium ion-rich yeasts. These were prepared by fermenting 1 part of yeast in 20 vol. of 0.2 M-trisodium citrate