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Metabolic Interactions among Polyunsaturated Fatty Acids in Response to an Atherogenic Diet[1] (38737)

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A critical factor in the formation of atherosclerotic lesions is the severity of diet-induced hyperlipemia (1). That qualitative changes in the lipids and lipoproteins may likewise be an important factor is apparent from the observation that hyperlipemia, induced in dogs by the non-ionic detergent, Triton WR 1339, does not produce arteriosclerotic lesions (2). Arterial lesions were induced in dogs maintained on a cholesterol-supplemented hydrogenated coconut oil diet for over a year (3). Subsequently, we reported the occurrence of severe arteriosclerotic lesions in all animals maintained on the same diet for 12–16 mo (4). Even after only 4 mo all dogs showed ultrastructural changes in medial smooth muscle cells (4). We have also reported alterations in lipid composition which occur in arterial tissue (5), leukocytes (6), plasma lipids and lipoproteins (7) and liver (8) from dogs fed the essential fatty acid (EFA) deficient diet with or without added cholesterol.

Many observations support the theory that dietary lipids play a significant role in human arterial disease (9) though the exact mechanisms by which EFA deficiency and cholesterol in the diet cause metabolic abnormalities leading to atherosclerosis are still obscure. This report deals with the diet-induced changes in hepatic polyunsaturated fatty acid (PUFA) composition in the above mentioned dogs, maintained on the atherogenic diet for 4 mo, with emphasis on the $\omega 9$ and $\omega 6$ isomers of eicosatrienoic acid (20:3). The metabolic changes accompanying EFA deficiency alone were compared to those resulting from supplementation of the EFA deficient diet with cholesterol.

Materials and Methods. Eighteen adult male mongrel dogs were studied as described previously (4). Six dogs were placed on the atherogenic diet containing 16% hydrogenated coconut oil and 5% cholesterol (Diet I). A second group of six dogs was fed the same diet except that the cholesterol was replaced with nonnutritive cellulose (Diet II). Both of these diets were devoid of essential fatty acids and contained, in addition to the above mentioned components, 29% sucrose, 20% casein, 12% cellulose, 12% silicic acid, 3% talc and a 3% mixture of salts, choline chloride, inositol, *p*-aminobenzoic acid and vitamins. The remaining six dogs were maintained on a nutritionally adequate commercially available diet (7) consisting of approximately 63% carbohydrate, 28% protein and 9% fat excluding ash and crude fiber content. The three groups of dogs were maintained on these diets for four months. The dogs were then sacrificed and samples taken from liver and other tissues for light and electron microscopy, the results of which have been reported previously (4).

Subcellular fractions of liver were obtained from all dogs by differential centrifugation. Samples were homogenized in 0.25 *M* sucrose and centrifuged at 600 *g* for 10 min to remove nuclei and cell debris. Mitochondria were isolated by sedimentation at 4500 *g* for 10 min. Microsomes were isolated by centrifugation at 100,000 *g* for 1 hour. An intermediate fraction consisting of light mitochondria and heavy microsomes was removed by centrifugation at 12,500 *g* and was not processed further.

Lipids were extracted by the Folch procedure (10). Lipid fractionation and determination of fatty acid composition by gas chromatography were performed as described earlier (6). Major fatty acids were

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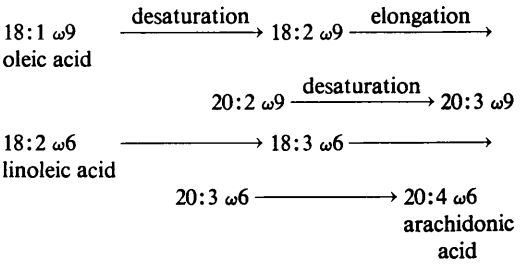
identified by comparison with commercially available standards. Identification of the ω9 and ω6 isomers of eicosatrienoic (20:3) acid was established by separation of the fatty acid methyl esters, according to their degree of unsaturation, by thin-layer chromatography on ammoniacal silver nitrate-silica gel by the method of Wood and Snyder (11). The trienes were extracted from the silica gel into chloroform and separated further by gas chromatography. Equivalent chain length values of the resulting peaks were then calculated (12). The 20:3 ω9 fatty acid is known to emerge ahead of the ω6 isomer in ethylene glycol succinate polyester columns (13).

Results and Discussion. The expected decrease in the content of the essential fatty acids, linoleic acid (18:2) and arachidonic acid (20:4) was observed in almost all the hepatic lipid fractions analyzed from the experimental animals when compared to EFA levels in the normal chow-fed dogs. This decrease was, however, consistently greater in the cholesterol supplemented (Group I) EFA deficient dogs than in those with EFA deficiency alone (Group II). Oleic acid (18:1), which increased in simple EFA deficiency, showed an even greater increase with cholesterol supplementation (Tables I to III).

The fatty acids of special interest in these experimental animals were the eicosatrienoic acid isomers, 20:3 ω9 and 20:3 ω6, since both of them increased from negligible to significant amounts with EFA deficiency (Table I). Even higher percentages were observed with cholesterol supplementation. However, in the cholesterol supplemented group, the increase in 20:3 ω6 (linoleic acid family) was far greater than the increase in the 20:3 ω9 isomer (oleic acid family). This fact is brought forth clearly when the ratios of 20:3 ω6/20:3 ω9 are compared (Table IV) The accumulation of 20:3 ω6 in hepatic lipid fractions from dogs fed the cholesterol supplemented diet is seen still more distinctly when the relative amounts of 20:3 ω6 and its metabolic product, arachidonic acid (20:4 ω6), are expressed as a ratio (Table IV).

The metabolism of polyunsaturated fatty acids has been reviewed by many investigators (14–16). When the positions of the double bonds are designed by numbering from the terminal methyl group, the long chain unsaturated fatty acids fall into two major families: the oleic acid family (ω9) and the linoleic acid family (ω6). The linolenic acid family (ω3) is present in mammalian liver only in trace amounts and is not discussed here. The chain elongation and desaturation pathways starting from the 18-carbon fatty acids take similar routes.



The enzymes affecting the conversion of the polyunsaturated fatty acids to longer, more unsaturated homologues show lack of specificity and consequently lead to the phenomenon of competitive inhibition (16). Thus, the increase in the 20:3 ω9 in EFA deficiency has been considered to be a result of the release of inhibition by linoleic acid of the desaturation of oleic acid since less linoleic acid would be present to compete with oleic acid in the initial desaturation reaction of the ω9 biosynthetic pathway. This conversion of 18:1 ω9 to 18:2 ω9 has recently been shown to be the rate limiting and principal regulatory step in the biosynthesis of 20:3 ω9 in rat liver microsomes (17). Conversely, large amounts of dietary oleic acid have also been shown to successfully compete for the enzyme systems and produce symptoms of EFA deficiency (18). Accumulated 20:3 ω9 could, in turn, then compete for the desaturation system that leads to the formation of arachidonic acid. The result would be an increase in 20:3 ω6, the precursor of arachidonic acid. Lowry and Tinsley (19) found an inhibition of the conversion of 18:2 ω6 to 20:4 ω6 and an accumulation of 20:3 ω6 in rat liver by increasing the amounts of oleate administration. Morin *et al.* (20) found a similar phenomenon in rats upon addition of choles-

TABLE I. UNSATURATED FATTY ACID DISTRIBUTION IN HEPATIC LIPIDS.*

		18:1[a]	18:2	20:3 ω9	20:3 ω6	20:4
Total liver	C[b]	11.4	19.0	0.05	0.7	22.1
	1[c]	16.2 ↑[e]	11.5 ↓[e]	2.7 ↑	3.4 ↑	11.3 ↓
	11[d]	15.4 ↑	14.7 ↓	2.0 ↑	1.5 ↑	14.0 ↓
Mitochondrial	C	15.3	15.3	0	0.3	20.3
lipids	1	23.5 ↑	10.1 ↓	2.4 ↑	2.9 ↑	7.4 ↓
(total)	11	18.7 ↑	11.8 ↓	2.2 ↑	1.4 –	12.6 ↓
Microsomal	C	13.6	15.9	0	0.3	21.9
lipids	1	20.3 ↑	13.1 ↓	3.6 ↑	3.4 ↑	10.6 ↓
(total)	11	16.8 ↑	11.7 ↓	2.1 ↑	1.4 –	15.4 ↓

* Individual fatty acids are given as a percentage of the total of all saturated and unsaturated fatty acids combined. Results are expressed as mean of six dogs per group.

[a] Carbon chain length: number of double bonds.

[b] Dogs maintained on the commercial meat:chow diet.

[c] EFA deficient, cholesterol supplemented group.

[d] EFA deficient, cholesterol free group.

[e] ↑ = Significant increase, ↓ = Significant decrease (both $P < 0.05$), – = No significant change.

TABLE II. UNSATURATED FATTY ACID DISTRIBUTION IN HEPATIC MITOCHONDRIAL LIPIDS.

		Mitochondria				
		18:1[a]	18:2	20:3 ω9	20:3 ω6	20:4
Neutral Lipids						
Total neutral lipids	C[b]	24.8	11.8	0.05	0.4	13.5
	1[c]	39.1 ↑[e]	4.4 ↓[e]	2.0 ↑	3.1 ↑	3.8 ↓
	11[d]	33.8 –	6.3 ↓	2.3 ↑	1.0 –	6.7 –
Cholesterol esters	C	39.7	16.2	0.05	0.3	7.0
	1	58.3 ↑	8.7 ↓	1.0 ↑	1.1 –	3.3 ↓
	11	38.5 –	16.1 –	1.7 ↑	1.1 –	5.6 –
Triglycerides	C	32.8	13.0	0.1	0.4	13.3
	1	41.4 ↑	2.6 ↓	0.3 –	0.4 –	0.9 ↓
	11	40.0 ↑	5.0 ↓	0.4 ↑	0.2 –	1.1 ↓
Free fatty acids	C	20.4	8.1	0.1	0.1	8.8
	1	33.5 ↑	4.3 ↓	2.0 –	1.0 –	2.2 ↓
	11	32.1 ↑	5.5 –	0.9 ↑	0.5 ↑	2.3 ↓
Phospholipids						
Total phospholipids	C	11.6	16.0	0	0.6	17.6
	1	19.2 ↑	14.7 –	3.3 ↑	3.7 ↑	10.5 ↓
	11	14.0 ↑	15.3 –	2.4 ↑	1.7 ↑	13.7 ↓
Cardiolipin	C	7.4	62.9	0.1	1.1	22.0
	1	19.0 ↑	48.5 ↓	1.1 –	1.6 –	5.8 ↓
	11	16.3 –	52.5 ↓	1.0 –	0.6 –	11.1 ↓
Phosphatidyl ethanolamine	C	10.9	9.1	0.2	0.2	31.8
	1	14.2 ↑	12.1 –	2.0 ↑	2.3 ↑	24.5 ↓
	11	11.3 –	18.0 –	2.4 ↑	1.7 ↑	27.3 ↓
Phosphatidyl choline	C	13.3	13.1	0.5	1.3	18.3
	1	20.4 ↑	13.1 –	4.3 ↑	4.9 ↑	6.3 ↓
	11	13.0 –	11.8 –	2.6 ↑	1.5 –	13.1 ↓

Superscripts same as for Table I.

TABLE III. UNSATURATED FATTY ACID DISTRIBUTION IN HEPATIC MICROSOMAL LIPIDS.

		Microsomes				
		18:1[a]	18:2	20:3 ω9	20:3 ω6	20:4
Neutral Lipids						
Total neutral lipids	C[b]	24.1	13.2	0.03	0.4	16.7
	1[c]	36.5 ↑[e]	5.2 ↓[e]	2.6 –	2.7 –	3.5 ↓
	11[d]	35.2 ↑	7.8 ↓	1.4 ↑	1.1 –	4.6 ↓
Cholesterol esters	C	25.2	15.5	0.04	0.2	19.2
	1	40.3 ↑	17.6 –	2.2 ↑	2.4 ↑	5.3 ↓
	11	34.7 ↑	21.2 ↓	1.4 ↑	1.0 ↑	5.9 ↓
Triglycerides	C	33.9	14.5	0	0.1	14.3
	1	41.4 ↑	6.1 ↓	0.7 ↑	0.7 –	2.0 ↓
	11	32.1 –	7.6 ↓	1.5 ↑	1.1 –	3.9 ↓
Free fatty acids	C	22.2	7.8	0.04	0.8	8.8
	1	28.4 –	6.1 –	2.6 ↑	2.8 –	6.7 –
	11	32.8 ↑	6.3 –	2.3 ↑	1.6 –	3.6 –
Phospholipids						
Total phospholipids	C	15.7	21.8	0.05	0.4	19.1
	1	33.5 ↑	11.2 ↓	3.5 ↑	4.7 ↑	5.8 ↓
	11	28.9 ↑	13.7 ↓	4.7 ↑	3.2 ↑	9.1 ↓
Phosphatidyl ethanolamine	C	10.5	11.8	0.2	1.2	32.8
	1	16.4 ↑	11.8 –	1.8 ↑	2.4 ↑	21.8 ↓
	11	11.0 –	10.8 –	2.3 ↑	1.8 –	25.9 ↓
Phosphatidyl choline	C	13.2	13.6	1.1	1.3	20.0
	1	17.9 ↑	14.0 –	3.3 ↑	4.1 ↑	9.0 ↓
	11	15.0 –	14.1 –	3.6 ↑	3.5 ↑	10.6 ↓

Superscripts same as for Table I.

TABLE IV. RATIOS OF THE CONTENT OF 20:3 ω6 TO THAT OF 20:3 ω9 AND 20:4 ω6 IN THE EXPERIMENTAL DIET DOGS.*

Fraction	20:3 ω6/20:3 ω9		20:3 ω6/20:4 ω6	
	Diet 1[a]	Diet 11[b]	Diet 1	Diet 11
Total lipids	1.4	0.8	0.3	0.1
Total mitochondrial lipids	1.2	0.6	0.4	0.1
Total microsomal lipids	1.1	0.7	0.3	0.1
Mitochondria				
Total neutral lipids	1.6	0.4	0.8	0.1
Cholesterol esters	1.1	0.6	0.3	0.2
Triglycerides	1.3	0.5	0.4	0.2
Free fatty acids	0.5	0.6	0.5	0.2
Total phospholipids	1.1	0.7	0.4	0.1
Cardiolipin	1.5	0.6	0.3	0.1
Phosphatidyl ethanolamine	1.2	0.7	0.1	0.1
Phosphatidyl choline	1.1	0.6	0.8	0.1
Microsomes				
Total neutral lipids	1.0	0.8	0.8	0.2
Cholesterol esters	1.1	0.7	0.5	0.2
Triglycerides	1.0	0.7	0.4	0.3
Free fatty acids	1.0	0.7	0.4	0.4
Total phospholipids	1.3	0.7	0.8	0.4
Phosphatidyl ethanolamine	1.3	0.7	0.1	0.1
Phosphatidyl choline	1.2	1.0	0.5	0.3

* Results are expressed as mean of six dogs per group.
[a] EFA deficient, cholesterol supplemented group.
[b] EFA deficient, cholesterol free group.

terol alone to a nutritionally adequate semi-synthetic diet containing 20% cottonseed oil.

In the present study, the pattern of EFA deficiency was accentuated by cholesterol supplementation. Changes in hepatic lipids are shown in Table I. Oleic acid content increased from 11.4% of the hepatic total fatty acids in chow-fed animals to 15.4% in EFA deficiency (Group II) and to 16.2% in the EFA deficient, cholesterol supplemented animals (Group I). The 20:3 ω9 acid increased from negligible amounts in dogs maintained on the commercially available chow to 2.0% in Group II and 2.7% in Group I. The 20:3 ω6 acid likewise increased from trace amounts in the liver of chow-fed animals to 1.5% in the cholesterol free group and to 3.4% in the EFA deficient, cholesterol supplemented group. Cholesterol supplementation was thus shown to induce an additional increase in 20:3 ω6 of approximately 100%, a pattern also seen in the mitochondrial and microsomal fractions (Table I) and in most of the individual lipid classes (Table II and III). The 20:3 ω9 isomer did not respond in a similar fashion as seen by the higher 20:3 ω6/20:3 ω9 ratio in fractions from dogs in Group I compared to those in Group II (Table IV). The additional increase in 20:3 ω6 with cholesterol supplementation was, therefore, apparently too high to be explained solely by competitive inhibition of the desaturation reaction by the 20:3 ω9 isomer. It has been suggested that exogenous cholesterol could evoke an increase in arachidonic acid synthesis in rat liver due to the increased need for it in cholesterol transport (20). The resulting accelerated biosynthesis from linoleic acid coupled with the possibility of a limitation in the rate of the final enzymatic dehydrogenation was thought to be responsible for the observed elevation of 20:3 ω6. Perhaps the disproportionate increase in 20:3 ω6 compared to that of 20:3 ω9 in the cholesterol supplemented group may in part be due to an additional inhibitory effect of cholesterol itself on the desaturation of 20:3 ω6 to 20:4 ω6.

These observations may relate to the pathogenesis of human atherosclerosis insofar that eicosatrienoic acids are known to be present in lipids extracted from fatty streaks of human arteries (9), the major isomer (20:3 ω6) being identical to the one shown here to accumulate in hepatic tissue from dogs fed an EFA deficient, cholesterol supplemented, atherogenic diet.

The relative amounts of 20:3 ω6 and 20:3 ω9 in the arterial tissue lipids of these dogs unfortunately were not determined, rendering a direct comparison between changes in the distribution of these isomers in hepatic subcellular lipids and arterial lipids impossible at present. The role of dietary cholesterol supplementation on the occurrence of ω6 and ω9 isomers in arterial lesion lipids will be investigated in future experiments with animals fed an atherogenic diet.

Summary The distribution of fatty acids in hepatic lipids of dogs fed a diet containing hydrogenated coconut oil as the only source of lipid, changed in the manner characteristic of essential fatty acid deficiency. Cholesterol supplementation of this diet accentuated these changes resulting in further increases in oleic and eicosatrienoic acids and decreases in the distribution of linoleic and arachidonic acids. Two eicosatrienoic acid isomers, 20:3 ω9, derived from oleic acid and 20:3 ω6, an intermediate in the biosynthesis of arachidonic acid from linoleic acid, were identified. The increase of the 20:3 ω6 isomer was found, somewhat unexpectedly, to be greater than that of 20:3 ω9, the isomer normally associated with EFA deficiency. The increase in 20:3 ω6 was probably due in part, but not completely, to competitive inhibition by the increased concentration of 20:3 ω9 on the desaturation reaction whereby 20:3 ω6 is converted to arachidonic acid.

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