Effect of Sex and Gonadal Hormones on Rat Plasma Lipids During the Development of an Essential Fatty Acid Deficiency

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(Received 2 August 1965)

1. Male, female and castrated rats treated with oestradiol (30 μ g./week) or testosterone (2mg./week) were given an essential fatty acid-deficient diet containing 10% of hydrogenated coconut oil for 9 weeks. The concentrations and fatty acid composition of plasma phospholipids, cholesteryl esters and triglycerides were determined. 2. Between the second and third weeks of the deficiency, concentrations of plasma cholesteryl esters, phospholipids and triglycerides decreased, then remained relatively constant. There were no significant differences between males and females, but oestradiol caused a significant rise in plasma phospholipids and triglycerides as compared with testosterone-treated animals. 3. During the first 2 weeks of the deficiency, linoleic acid in the plasma lipids of all groups decreased to low concentrations and changed very little thereafter. 4. Female rats maintained higher percentages and concentrations of arachidonic acid and stearic acid in plasma phospholipids and arachidonic acid in cholesteryl esters than did males. Males had higher proportions of eicosatrienoic acid and oleic acid. There was no sex difference in the fatty acid composition of plasma triglycerides. 5. Oestradiol-treated rats had concentrations of cholesteryl and phospholipid arachidonate comparable with those of female rats and higher than the testosterone-treated group. Eicosatrienoic acid in the oestradiol-treated rats was high and resembled that of the male rats, apparently because of the higher concentration of plasma phospholipids in this group. 6. Supplementation of the essential fatty acid-deficient rats with linoleate restored plasma cholesteryl and phospholipid linoleate and arachidonate nearly to normal concentrations in a single day. The increase in arachidonic acid in these fractions was accompanied by a similar quantitative decrease in eicosatrienoic acid. 7. These sex differences appear to be related to the smaller size of the female rat and to a more direct influence of oestradiol on the formation or maintenance of phospholipids rich in arachidonic acid.

There is ample evidence that sex hormones influence lipid metabolism in mammals (Gyorgy, Rose & Shipley, 1947; Fillios, Kaplan, Martin & Stare, 1958; Okey & Lyman, 1956; Olson & Vester, 1960) and the fatty acids of tissue and blood lipids (Coleman, Chen & Alfin-Slater, 1958; Morton & Horner, 1961; Horner & Morton, 1961; Aftergood & Alfin-Slater, 1965; Bowers & Schelly, 1965). Okey, Ostwald, Shannon & Tinoco (1962) reported that female rats on diets containing cholesterol had higher proportions of arachidonic acid and stearic acid in their plasma phospholipids than similarly treated male rats; the difference occurred principally in the lecithins (Lyman, Shannon, Ostwald & Miljanich, 1964b). Monsen, Okey & Lyman (1962) showed that neither cholesterol nor diets varying widely in linoleic acid influenced this sex difference

in plasma phospholipid fatty acid composition. Oestradiol injected into castrated male rats produced a comparable increase in the stearic acid and arachidonic acid of the lecithins. These results suggested that the female rat may be able to mobilize or to utilize linoleic acid in the conversion of arachidonic acid better than the male. Consequently, an experiment was conducted with male and female rats, in which carcass linoleate and liver-lipid fatty acids were analysed during the development of an essential fatty acid deficiency (Ostwald, Bouchard, Miljanich & Lyman, 1965). The results failed to show any sex differences in the total amount of linoleic acid mobilized or total amount of arachidonic acid in liver-lipid fractions, but female rats maintained somewhat higher concentrations of linoleate in their adipose tissue and

higher proportions of arachidonic acid and stearic acid in their liver phospholipids than males. The effects of sex and the gonadal hormones, oestradiol and testosterone, on linoleate and arachidonate in plasma phospholipids, cholesteryl esters and triglycerides during the development of EFA* deficiency were investigated on the same animals.

MATERIALS AND METHODS

Details of the diet, treatment of animals and design of the experiment were presented by Ostwald *et al.* (1965). Intact female and male rats and castrated male rats, injected with the hormone vehicle (hydrogenated coconut oil), oestradiol (30 µg./week) and testosterone (2 mg./week), were given for 64 days a diet free of essential fatty acids but containing 10% of hydrogenated coconut oil. Some of the intact male and female animals were kept on this EFA-deficient diet for a total of about 17 weeks (119 days), after which groups of rats of both sexes were given the basic diet but with the 10% of hydrogenated coconut oil replaced by an equivalent amount of safflower oil.

Plasma-lipid extraction, fractionation and analyses. The blood was centrifuged (at 5°) immediately, and the plasma obtained was extracted by adding it dropwise to 10 vol. of 95% ethanol containing 1 mg. of quinol. Plasma from each animal (3.5–5.0 ml.) was treated individually except those in the zero-time group, where pools of plasma (5–5 ml.) from males or females were analysed in duplicate. The ethanol extract was then filtered and the residue was re-extracted twice with 10 vol. of 95% ethanol–diethyl ether (3:1, v/v). The combined filtrates were concentrated on a rotary vacuum evaporator below 50° to about 1 ml. The aqueous residue was extracted three times with light petroleum (b.p. 30–60°) at room temperature, and stored at 0° until analysed. The residue was extracted further with three rinses of warm chloroform and three rinses of cold chloroform (Stevan & Lyman, 1963). The combined chloroform extracts were also stored at 0° until analysed.

The light-petroleum extracts were centrifuged, and the residue was washed with chloroform. The light-petroleum and chloroform extracts were then combined and evaporated to dryness. The lipids from a single plasma sample were dissolved in 10 ml. of light petroleum. Total cholesterol (Sperry & Webb, 1950) and phosphorus (Sumner, 1944) were determined on 2 ml. of this solution. The remainder of the extract was then fractionated into cholesteryl esters, triglycerides, free cholesterol and phospholipids by the method of Lis, Tinoco & Okey (1961). Recoveries of cholesterol and phospholipid were never less than 90% and mostly were higher.

A known quantity of heptadecanoic acid was added to the lipid fractions as an internal standard, so that the amount of each fatty acid in a fraction could be determined (Tinoco, Shannon, Miljanich, Lyman & Okey, 1962). Where independent measurements of cholesterol or phosphorus could be made, such as in the esterified cholesterol or the phospholipid fractions, the internal standard was used mainly to check the total recovery of the fatty acids. Plasma triglycerides, however, were determined completely by means of the internal standard. Fatty acids from the lipid

fractions were methylated and then determined by gas-liquid chromatography (Lyman *et al.* 1964b). Plasma concentrations for the phospholipid fatty acids were calculated on the basis of the analyses of phosphorus in the phospholipid fraction $\times 17.3 \times \text{weight (\%)} of the fatty acid determined by gas-liquid chromatography. Esterified cholesterol fatty acid concentrations were calculated from cholesterol determined in the cholesteryl ester fractions $\times 0.73 \times \text{weight (\%)} of fatty acids in the fraction. Carbon-chain lengths and number of double bonds in the eicosatrienoic acids were established by hydrogenation and by comparison with known fatty acids.$$

Statistical comparisons of the results were made by the *t* test (Snedecor, 1956).

RESULTS

Concentrations of plasma cholesterol, phospholipid and triglyceride. Fig. 1 shows the concentrations of plasma cholesterol, phospholipid and triglyceride in the experimental groups during the development of EFA deficiency. Apart from the slight fall between 16 and 21 days in the free and esterified cholesterol of intact females and males, the EFA deficiency had very little effect on the concentration of either free or esterified cholesterol in any of the groups. Although male and testosterone-treated rats appeared to have consistently less free cholesterol than did females and castrated rats injected with oestradiol, the differences were not significant owing to wide variations in individual values. In intact rats there was an initial increase and then a fall in phospholipid concentration in both sexes between the second and third weeks of the EFA deficiency. Phospholipid concentrations in males appeared to decrease slightly during the deficiency, whereas in females phospholipids remained about the same from the fourth week of the deficiency to the termination of the experiment at 9 weeks. However, after 17 weeks, both sexes had greatly elevated amounts of phospholipids, which decreased when linoleic acid was fed (Fig. 1c). The changes in the phospholipid concentrations in testosterone-treated rats were similar to those of the male animals. However, oestradiol caused a sharp increase in the plasma phospholipid, which was maintained significantly above the concentrations of the testosterone-treated group and those of the intact animals.

Plasma triglycerides in female rats declined in the first 3 weeks of the EFA deficiency, then remained at about 20–30 mg./100 ml. of plasma over the 9-week interval. Males showed only a slight decrease in this lipid during the onset of the EFA deficiency. Again, as with the phospholipid, oestradiol treatment increased plasma triglycerides significantly above the value in the intact females and in the group injected with testosterone. The concentrations in the latter group were similar to

* Abbreviation: EFA, essential fatty acids.

Fig. 1. Plasma cholesterol, phospholipids and triglycerides in rats during development of EFA deficiency. (a) Intact males (■), females (○). (b) Castrated males treated with testosterone (■), oestradiol (○) or the hormone vehicle (Δ). Castrations were performed and hormone injections begun approximately 2 weeks after animals were placed on the EFA-deficient diet. (c) Results of re-feeding linoleate to male and female rats after 17 weeks on the EFA-deficient diet. Intervals are for 1 and 3 days after feeding with a diet containing safflower oil. Vertical lines on Figures are half standard error of the mean from five or more rats/group/time period.

those of the intact males. Castrated rats injected only with the oil used to dissolve the hormone maintained concentrations of cholesterylesters, phospholipids and triglycerides that were generally between the values of the oestradiol- and testosterone-treated rats. Therefore plasma lipids of this group usually were not significantly different from those of the other two groups.

After 17 weeks, all plasma-lipid fractions in rats on the deficient diet had increased above the 9-week values in both sexes (Fig. 1c). Feeding with linoleic acid caused an abrupt decrease in those concentrations, so that after 3 days they were as low as or

lower than at the beginning of the experiment. In EFA deficiency therefore linoleic acid significantly decreased not only plasma cholesterol but also plasma phospholipids and triglycerides.

Phospholipid fatty acids. Table 1 shows the percentages of the unsaturated fatty acids in the plasma phospholipids. Initially, linoleic acid and arachidonic acid were the principal unsaturated fatty acids in this fraction. By the ninth week of the deficiency, however, the percentages of linoleic acid and, to a less extent, arachidonic acid, had decreased, whereas oleic acid and eicosatrienoic acid had greatly increased. Males generally had

Table 1. *Effect of EFA deficiency in male, female and hormone-treated rats on the unsaturated fatty acids of plasma phospholipids*

Fatty acids are expressed as percentages of total methyl esters determined. Longer-chain fatty acids represented about 4% of the total fatty acids. Means $\pm$ S.E.M. are from five or more rats/group/time period.

Days on diet	Fatty acids (% by wt.)				
	Females	Males	Castrated rats		
			Oil	Oestradiol	Testosterone
			16:1		
0	1.4	2.4	—	—	—
16	1.7 $\pm$ 0.2	2.0 $\pm$ 0.2	—	—	—
21	1.7 $\pm$ 0.2	2.6 $\pm$ 0.9	—	—	—
32	2.1 $\pm$ 0.2	2.8 $\pm$ 0.4	2.2 $\pm$ 0.2	1.5 $\pm$ 0.3	2.2 $\pm$ 0.08
46	2.1 $\pm$ 0.2	2.7 $\pm$ 0.2	2.5 $\pm$ 0.1	2.0 $\pm$ 0.5	2.4 $\pm$ 0.3
64	1.9 $\pm$ 0.9	2.6 $\pm$ 0.1	2.3 $\pm$ 0.3	1.7 $\pm$ 0.3	2.1 $\pm$ 0.1
			18:1		
0	7.5	7.5	—	—	—
16	11.8 $\pm$ 1.1	12.6 $\pm$ 0.7	—	—	—
21	12.8 $\pm$ 0.9	16.3 $\pm$ 0.8	—	—	—
32	15.0 $\pm$ 0.7	15.8 $\pm$ 0.6	15.3 $\pm$ 0.5	13.5 $\pm$ 0.4	15.2 $\pm$ 0.8
46	14.8 $\pm$ 1.8	18.3 $\pm$ 0.6	16.5 $\pm$ 0.6	15.4 $\pm$ 0.7	17.1 $\pm$ 0.7
64	15.6 $\pm$ 0.4	19.3 $\pm$ 0.6	18.2 $\pm$ 0.8	13.7 $\pm$ 0.6	16.0 $\pm$ 1.6
			18:2		
0	13.2	12.5	—	—	—
16	6.7 $\pm$ 0.4	7.6 $\pm$ 0.1	—	—	—
21	6.4 $\pm$ 0.4	7.3 $\pm$ 0.8	—	—	—
32	5.6 $\pm$ 0.7	5.3 $\pm$ 1.0	7.0 $\pm$ 0.7	6.4 $\pm$ 0.8	8.7 $\pm$ 0.4
46	5.1 $\pm$ 0.2	3.8 $\pm$ 0.5	4.6 $\pm$ 0.4	4.4 $\pm$ 0.7	6.2 $\pm$ 1.0
64	4.8 $\pm$ 0.3	4.6 $\pm$ 0.8	4.0 $\pm$ 0.5	4.5 $\pm$ 0.1	5.3 $\pm$ 0.4
			20:4		
0	18.9	16.4	—	—	—
16	26.8 $\pm$ 2.6	23.4 $\pm$ 1.0	—	—	—
21	28.9 $\pm$ 3.0	13.6 $\pm$ 1.2	—	—	—
32	22.6 $\pm$ 2.3	14.1 $\pm$ 1.2	19.3 $\pm$ 1.4	19.2 $\pm$ 2.9	16.1 $\pm$ 2.1
46	21.4 $\pm$ 3.4	8.2 $\pm$ 0.8	13.1 $\pm$ 1.2	15.0 $\pm$ 2.0	13.6 $\pm$ 1.4
64	18.5 $\pm$ 2.0	10.6 $\pm$ 1.9	12.3 $\pm$ 1.7	18.6 $\pm$ 0.7	10.6 $\pm$ 1.0
			20:3		
0	—	—	—	—	—
16	5.4 $\pm$ 1.6	6.1 $\pm$ 0.9	—	—	—
21	5.1 $\pm$ 1.4	12.6 $\pm$ 0.9	—	—	—
32	12.5 $\pm$ 3.2	19.8 $\pm$ 2.4	14.0 $\pm$ 2.2	14.3 $\pm$ 3.4	12.3 $\pm$ 1.1
46	12.7 $\pm$ 2.0	24.9 $\pm$ 2.0	20.1 $\pm$ 1.8	19.7 $\pm$ 1.9	18.9 $\pm$ 3.1
64	17.3 $\pm$ 1.6	21.8 $\pm$ 2.1	20.2 $\pm$ 1.2	20.1 $\pm$ 1.1	22.2 $\pm$ 0.8

higher proportions of oleic acid and eicosatrienoic acid than females, whereas females maintained significantly higher proportions of arachidonic acid than males ($P < 0.02$ at 21, 32 and 46 days; $P < 0.05$ at 64 days). The major decrease in the percentage of linoleic acid in this lipid fraction occurred during the first 2 weeks of the deficiency, with only a slight change thereafter. Palmitoleic acid was a relatively minor component of the phospholipids, and its proportion was unaffected by sex or by EFA deficiency. In general, the proportions of plasma phospholipid fatty acids in the oestradiol- or testosterone-treated animals resembled those in the respective intact controls. However, the differences between percentages of arachidonic acid in the

oestrogen- and androgen-treated groups were not so large as those between female and male rats, and values were significant only at 64 days ($P < 0.01$). Further, the proportion of eicosatrienoic acid was higher in the oestradiol group than in the females, and was therefore nearly identical with that in the testosterone-injected animals.

Fig. 2 shows the concentrations of linoleic acid, arachidonic acid and eicosatrienoic acid in the plasma phospholipids of the five groups of animals. Throughout the experiment linoleic acid decreased only about 5 mg./100 ml., and to the same value in both sexes. Sex hormones did not influence its ultimate concentration. The concentration of arachidonic acid was significantly higher in females

Fig. 2. Concentrations of linoleic acid, arachidonic acid and eicosatrienoic acid in plasma phospholipids of rats during development of EFA deficiency. For explanation of symbols see Fig. 1.

Table 2. *Palmitic acid and stearic acid in plasma phospholipids, esterified cholesterol and triglycerides of EFA-deficient male, female and hormone-treated rats*

Fatty acids are expressed as percentages of total methyl esters determined. Lauric acid and myristic acid represented less than 2% of phospholipid, 3% of esterified cholesterol and 6% of triglyceride fatty acids. Means of palmitic acid or stearic acid from five or more rats/group/time period are shown. Standard errors for palmitic acid and stearic acid in the phospholipids were generally less than 10% of the mean.

Days on diet	Females		Males		Castrated rats					
					Oil		Oestradiol		Testosterone	
	(%, by wt.)	(mg./ 100 ml.)	(%, by wt.)	(mg./ 100 ml.)	(%, by wt.)	(mg./ 100 ml.)	(%, by wt.)	(mg./ 100 ml.)	(%, by wt.)	(mg./ 100 ml.)
Phospholipid										
16:0										
0	25.5	—	28.5	—	—	—	—	—	—	—
16	19.0	—	22.4	—	—	—	—	—	—	—
21	18.2	—	22.0	—	—	—	—	—	—	—
32	16.2	(13.0)	18.8	(14.8)	17.2	(14.0)	16.0	(16.5)	19.7	(15.4)
46	15.8	(13.4)	17.8	(13.2)	17.9	(15.1)	16.9	(18.8)	18.4	(14.5)
64	15.2	(13.2)	18.0	(13.8)	17.9	(16.2)	14.4	(15.8)	17.6	(12.0)
18:0										
0	28.9	—	28.9	—	—	—	—	—	—	—
16	25.2	—	21.0	—	—	—	—	—	—	—
21	24.3	—	20.7	—	—	—	—	—	—	—
32	21.3	(17.4)	19.4	(16.0)	21.2	(16.9)	25.5	(25.7)	20.4	(15.4)
46	23.1	(19.4)	18.9	(14.3)	20.2	(17.0)	22.5	(25.1)	19.2	(14.9)
64	22.5	(19.6)	18.9	(14.5)	19.9	(17.9)	22.8	(24.6)	20.8	(14.2)
Esterified cholesterol										
16:0										
0	11.3	—	11.4	—	—	—	—	—	—	—
16	8.8	—	10.9	—	—	—	—	—	—	—
21	8.8	—	11.1	—	—	—	—	—	—	—
32	8.0	—	9.2	—	9.0	—	8.6	—	10.9	—
46	9.0	—	9.8	—	8.7	—	8.5	—	10.3	—
64	7.8	—	10.0	—	9.7	—	9.5	—	10.4	—
18:0										
0	2.9	—	3.4	—	—	—	—	—	—	—
16	2.5	—	3.1	—	—	—	—	—	—	—
21	3.0	—	3.1	—	—	—	—	—	—	—
32	2.4	—	2.4	—	3.0	—	4.3	—	3.3	—
46	3.0	—	3.2	—	3.2	—	5.6	—	3.9	—
64	3.1	—	3.8	—	3.8	—	6.3	—	4.3	—
Triglyceride										
16:0										
0	18.9	—	20.5	—	—	—	—	—	—	—
16	28.0	—	28.2	—	—	—	—	—	—	—
21	26.9	—	27.7	—	—	—	—	—	—	—
32	24.8	(5.3)	27.2	(5.1)	26.8	(6.9)	27.0	(10.6)	25.8	(5.3)
46	26.3	(7.2)	26.4	(5.1)	25.5	(6.7)	26.7	(14.7)	25.2	(4.2)
64	24.1	(4.5)	26.4	(5.7)	26.2	(6.9)	27.2	(15.9)	25.4	(4.6)
18:0										
0	4.9	—	6.4	—	—	—	—	—	—	—
16	5.4	—	5.4	—	—	—	—	—	—	—
21	4.9	—	4.8	—	—	—	—	—	—	—
32	5.2	(1.1)	3.8	(0.7)	4.8	(1.1)	3.5	(1.3)	4.4	(0.9)
46	4.6	(1.2)	5.3	(1.0)	4.6	(1.1)	3.2	(1.5)	4.9	(0.8)
64	5.7	(1.1)	4.9	(1.0)	4.5	(1.1)	3.7	(2.2)	5.1	(0.9)

than in males within 3 weeks after giving the EFA-deficient diet ($P < 0.02$ at 21, 46 and 64 days). Oestradiol-injected rats also maintained plasma phospholipid arachidonate at values comparable with those of the female, whereas oil or testosterone injections kept the values of this fatty acid significantly below those of either the oestradiol-treated or female rats ($P < 0.01$ at 46 days for both groups). Although the concentration of eicosatrienoic acid rose more rapidly and was consistently higher in male rats than in females, the differences between groups were significant only at 21 and 46 days

($P < 0.02$). Since treatment with oestradiol increased the concentration of phospholipid arachidonate, the greater increase of eicosatrienoic acid ($P < 0.02$ at 64 days) in this group as compared with that in the rats injected with testosterone was unexpected. Concentrations of eicosatrienoic acid in this instance more closely resembled those in intact males than in the females.

Giving linoleic acid to these animals (Fig. 2c) resulted in a sudden increase in plasma-phospholipid linoleate and arachidonate in just 1 day. During the same time, eicosatrienoic acid decreased

Table 3. *Effect of EFA deficiency in male, female and hormone-treated rats on the unsaturated fatty acids of plasma esterified cholesterol*

Fatty acids are expressed as percentages of total methyl esters determined. Longer-chain fatty acids represented only about 3% of the total fatty acids. Means $\pm$ S.E.M. are from five or more rats/group/time period.

Days on diet	Fatty acid (% by wt.)				
	Females	Males	Castrated rats		
			Oil	Oestradiol	Testosterone
			16:1		
0	4.8	4.4	—	—	—
16	8.6±0.8	8.6±0.6	—	—	—
21	6.9±0.8	10.4±0.6	—	—	—
32	9.4±0.7	10.9±0.9	9.8±0.4	6.7±0.3	8.8±0.5
46	10.6±0.9	11.4±1.0	9.8±0.5	8.2±0.7	9.2±0.7
64	8.9±0.8	11.2±0.6	10.2±0.7	7.2±0.8	9.0±0.8
			18:1		
0	10.1	10.0	—	—	—
16	11.6±0.7	16.1±1.3	—	—	—
21	11.4±1.1	20.1±1.5	—	—	—
32	13.4±0.4	17.2±0.5	15.8±0.9	12.8±0.4	18.3±1.8
46	14.0±0.7	20.9±0.8	16.7±0.6	15.7±1.3	19.3±1.0
64	13.8±1.0	19.7±0.8	18.0±0.7	14.2±0.6	20.0±1.7
			18:2		
0	15.6	15.4	—	—	—
16	4.9±0.3	6.3±0.5	—	—	—
21	5.6±1.2	6.7±0.2	—	—	—
32	5.7±0.3	5.7±0.8	6.4±0.5	6.1±0.4	7.8±0.4
46	4.9±0.4	4.3±0.4	4.8±0.5	4.6±0.6	5.8±0.6
64	4.5±0.5	5.1±0.2	4.4±0.1	4.6±0.2	5.1±0.5
			20:4		
0	40.4	46.5	—	—	—
16	49.7±4.1	42.7±1.8	—	—	—
21	49.6±4.3	26.8±2.3	—	—	—
32	41.9±4.5	26.4±3.8	36.0±3.2	39.2±3.9	31.6±3.9
46	37.2±2.0	14.7±1.6	27.1±2.1	29.4±4.0	23.8±3.1
64	35.9±3.9	19.0±1.1	23.7±2.7	32.2±3.2	18.9±2.7
			20:3		
0	—	—	—	—	—
16	6.8±1.7	6.5±1.0	—	—	—
21	6.8±1.4	15.8±1.6	—	—	—
32	12.5±2.8	20.7±2.3	13.4±2.3	16.6±4.3	12.1±1.2
46	13.7±2.6	26.8±1.8	21.9±1.7	21.4±2.8	18.9±2.7
64	17.5±1.6	22.0±2.1	22.4±1.6	20.4±1.3	23.9±0.6

to less than 5mg./100ml. The rates at which these changes occurred apparently were not influenced consistently by sex of the animals.

Palmitic acid and stearic acid were the major saturated fatty acids present in the plasma phospholipids (Table 2). The proportions of both decreased rapidly during the first 2 weeks of the deficiency, with a much slower decline thereafter. The only sex difference in these fatty acids was a higher proportion of stearic acid in females than in males ($P < 0.01$ at 16, 21, 46 and 64 days). Oestradiol apparently produced a similar but less pronounced effect on the proportion of phospholipid stearic acid. After the first 3 weeks of the EFA-deficient diet, the concentration (values in parentheses, Table 2) of both fatty acids remained essentially

constant in all groups. Although palmitic acid concentrations were similar for both sexes, stearic acid concentrations in females and oestradiol-treated rats were significantly higher than in males and testosterone-injected rats (females versus males, $P < 0.05$ at 16, 46 and 64 days; oestradiol versus testosterone, $P < 0.05$ at 32, 46 and 64 days). In this respect therefore stearic acid behaved in a manner similar to that of arachidonic acid (Fig. 2).

Cholesteryl ester fatty acids. The percentages of the unsaturated fatty acids in plasma cholesteryl esters are shown in Table 3. The esterified cholesterol fraction was only about one-third the size of the phospholipid fraction and had higher proportions of palmitoleic acid and arachidonic acid. The changes in the proportions of oleic acid, linoleic

Fig. 3. Concentrations of linoleic acid, arachidonic acid and eicosatrienoic acid in plasma esterified cholesterol of rats during development of EFA deficiency. For explanation of symbols see Fig. 1.

acid and eicosatrienoic acid as the EFA deficiency progressed were similar to those for the phospholipids. Although the percentages of linoleic acid were the same for both sexes, females had higher proportions of arachidonic acid than males ($P < 0.02$ at 21, 46 and 64 days; $P < 0.05$ at 32 days). Oestradiol had a similar effect on arachidonic acid when administered to castrated rats. Males had significantly larger proportions of oleic acid ($P < 0.02$ at all periods) and eicosatrienoic acid ($P < 0.05$ at 32

days; $P < 0.01$ at 46 days). Testosterone significantly increased the proportion of oleic acid ($P < 0.05$ at 32 and 46 days; $P < 0.02$ at 64 days). However, no differences in eicosatrienoic acid resulted from hormone treatment.

The concentrations of linoleic acid, arachidonic acid and eicosatrienoic acid in the cholesteryl esters are shown in Fig. 3. Of these three fatty acids, only arachidonic acid showed significant sex differences. Females maintained significantly higher concen-

Table 4. *Effect of EFA deficiency in male, female and hormone-treated rats on the unsaturated fatty acids of plasma triglycerides*

Fatty acids are expressed as percentages of total methyl esters determined. Fatty acids above C₂₀ in this fraction constituted less than 3% of the total. Means of the percentages or concentrations of fatty acids from five or more rats are shown.

			Castrated rats					
Days on diet	Females (%, by wt.)	Males (%, by wt.)	Oil		Oestradiol		Testosterone	
			(%, by wt.)	(mg./100 ml.)	(%, by wt.)	(mg./100 ml.)	(%, by wt.)	(mg./100 ml.)
16:1								
0	1.6	2.2	—	—	—	—	—	—
16	8.0	7.7	—	—	—	—	—	—
21	6.5	7.7	—	—	—	—	—	—
32	7.6	8.8	8.1	(2.0)	7.1	(2.8)	7.0	(1.3)
46	8.6	8.4	8.8	(2.1)	6.8	(3.9)	7.2	(1.2)
64	7.6	8.5	8.2	(2.1)	7.0	(3.8)	8.0	(1.4)
18:1								
0	16.6	19.0	—	—	—	—	—	—
16	39.1	38.2	—	—	—	—	—	—
21	39.9	44.4	—	—	—	—	—	—
32	40.6	41.8	40.8	(10.1)	45.2	(17.7)	37.3	(7.8)
46	40.6	39.9	40.1	(10.4)	47.1	(25.3)	38.4	(6.6)
64	40.3	37.7	43.3	(11.4)	47.1	(27.7)	42.0	(7.9)
18:2								
0	23.6	24.8	—	—	—	—	—	—
16	6.1	6.8	—	—	—	—	—	—
21	6.7	4.1	—	—	—	—	—	—
32	3.8	3.2	4.3	(1.2)	3.5	(1.4)	4.3	(0.9)
46	4.3	3.5	2.7	(0.6)	2.5	(1.4)	4.0	(0.6)
64	4.9	4.3	2.7	(0.7)	2.5	(1.4)	3.1	(0.5)
20:4								
0	10.5	14.0	—	—	—	—	—	—
16	2.5	2.9	—	—	—	—	—	—
21	3.4	0.3	—	—	—	—	—	—
32	1.6	1.4	0.8	(0.3)	1.4	(0.6)	1.7	(0.4)
46	2.1	0.8	2.0	(0.5)	1.8	(1.1)	3.1	(0.5)
64	1.2	0.8	1.0	(0.3)	1.3	(0.4)	1.2	(0.3)
20:3								
0	—	—	—	—	—	—	—	—
16	2.8	3.8	—	—	—	—	—	—
21	5.1	4.5	—	—	—	—	—	—
32	5.8	5.3	5.3	(1.3)	5.4	(2.1)	6.1	(1.1)
46	4.6	5.1	6.7	(1.6)	6.8	(3.5)	6.7	(1.1)
64	5.7	4.4	4.8	(1.4)	4.9	(1.9)	5.5	(1.1)

trations than males ($P < 0.01$ at 21 and 46 days; $P < 0.05$ at 64 days). A less-pronounced effect on cholesteryl arachidonate was produced by oestradiol ($P < 0.01$ at 64 days only).

Although males consistently averaged more eicosatrienoic acid than females, any significant differences between them were eliminated by wide variations among the groups. Feeding again with linoleate (Fig. 3c) had an effect on linoleic acid, arachidonic acid and eicosatrienoic acid in the cholesteryl esters similar to that produced in the phospholipids. Quantitatively, the greatest changes were in arachidonic acid (two- to three-fold increase) and eicosatrienoic acid (six- to seven-fold decrease) after only a single day of feeding with linoleate. The principal saturated fatty acids associated with the cholesteryl esters (Table 2) were palmitic acid and stearic acid, the latter representing only about 3–4% of all the esterified fatty acids in this fraction. Unlike those of the phospholipids, the proportion and concentration of these fatty acids were not influenced by sex-hormone treatment or by EFA deficiency.

Triglyceride fatty acids. Plasma-triglyceride fatty acids are shown in Table 4. The proportions of linoleic acid and arachidonic acid decreased to very low values within the first 2 weeks of the deficiency, whereas those of oleic acid, palmitoleic acid and eicosatrienoic acid increased. No qualitative sex differences were found in this lipid fraction. However, as a result of the greatly increased triglyceride concentrations in the oestradiol-treated animals (Fig. 1), the concentrations of all the triglyceride fatty acids in this group were two or three times those of the other groups. The predominating fatty acids in this fraction were oleic acid and palmitic acid (Table 2). Quantitatively therefore these fatty acids appeared to increase more than any of the others, although proportionately all fatty acids increased about the same.

DISCUSSION

The relatively greater resistance of female than of male rats to EFA deficiency has been observed by many investigators (Loeb & Burr, 1947; Greenberg, Calbert, Savage & Deuel, 1950; Morton & Horner, 1961; Aftergood & Alfin-Slater, 1965). Ostwald *et al.* (1965) sought reasons for this sex difference by following the distribution of linoleic acid and arachidonic acid in the carcass and liver lipids of female and male rats during the development of EFA deficiency. The results showed that, although no sex differences in linoleate were evident, female rats had a higher proportion of arachidonic acid in their liver phospholipids than males. Total arachidonic acid in this lipid fraction (99% of liver

arachidonic acid was in the phospholipids) was similar for both sexes, however. Males had larger livers, more liver phospholipid and more eicosatrienoic acid than the females. It was concluded that the need for more structural phospholipid by the faster-developing male rat necessitated synthesis of a phospholipid in which eicosatrienoic acid replaced the normal arachidonic acid. The early increase in concentration of this abnormal phospholipid in the tissues of the EFA-deficient male rat could account for the earlier appearance and more severe deficiency symptoms observed in these animals.

In the present study, plasma phospholipids and cholesteryl ester fatty acids from the same animals also revealed no sex differences in linoleic acid. Females, however, had significantly higher proportions and concentrations of arachidonic acid in both lipid fractions as compared with male rats. Therefore, during this experiment, in which the total liver linoleate and arachidonate were similar in both sexes, female rats maintained higher concentrations of arachidonic acid in plasma phospholipids and cholesteryl esters than did the males. It is generally recognized that plasma phospholipids are derived from the liver (Zilversmit & Bollman, 1956); therefore the higher proportions of arachidonic acid in the plasma phospholipids of females and eicosatrienoic acid in males would have resulted if both sexes secreted lipoproteins having similar proportions of the phospholipid sub-classes, because liver phospholipids of males had higher proportions of eicosatrienoic acid, whereas those of females maintained higher percentages of arachidonic acid. Comparison of the proportions of the liver-phospholipid fatty acids of both sexes (Ostwald *et al.* 1965) with their proportions in the plasma (Table 1) shows a considerable similarity in fatty acid composition in the two compartments. The small but consistent difference between plasma-phospholipid concentrations in male and female rats as the deficiency progressed would further accentuate the quantitative sex differences in the concentrations of the arachidonic acid (Fig. 1).

The absence of sex differences in proportion and concentration of linoleic acid as well as its low concentration in the lipid fractions of the EFA-deficient rats indicates that any linoleic acid available from adipose-tissue stores was converted into arachidonic acid so rapidly that little or no linoleic acid accumulated. The sudden increase in plasma phospholipid and cholesteryl arachidonate after only 1 day of feeding with linoleate (Figs. 2c and 3c) also illustrated the speed of the conversion and the avidity of these lipid fractions for the deficient fatty acid. The reciprocal decrease in eicosatrienoic acid in the phospholipid seems to support the contention of Brenner & José (1965) that arachidonic acid

competes with and replaces the eicosatrienoic acid in the phospholipids.

In general, the proportions of fatty acids in the plasma phospholipids of the oestradiol- and testosterone-treated rats were comparable with those of the intact female and male animals. In these EFA-deficient rats, however, oestradiol seemed to have a specific effect on plasma phospholipids (Fig. 1b), which elevated their concentrations to 1.5 times those of the other groups. Although the concentration of phospholipid was increased, the concentration of arachidonic acid did not rise above that of the intact female rat (Fig. 2b), whereas concentrations of eicosatrienoic acid, oleic acid and stearic acid, i.e. those fatty acids readily synthesized, increased. Therefore, when supplies of arachidonic acid are insufficient, synthesis of additional phospholipid seems to be accomplished by substituting eicosatrienoic acid for the unavailable arachidonic acid. Why oestradiol has such an effect on the plasma phospholipids of the EFA-deficient rats is not known. At the amounts administered, oestradiol maintained growth similar to that of the intact female rat, but the livers in these animals were enlarged to such an extent that they resembled the livers of males (Ostwald *et al.* 1965). The quantity of the hormone administered (30 $\mu\text{g./week}$) was chosen because, in castrated rats on normal diets, it maintained food consumption, growth and liver and adrenal weights at values comparable with those of intact female rats. Apparently EFA-deficient rats may have a greater sensitivity to oestrogen than have normal animals. Such a possibility has been discussed by Klein, Finerty, Panos & Wall (1957). In man, oestradiol increases serum high-density lipoproteins, which contain abundant quantities of phospholipid (Russ, Eder & Barr, 1955; Oliver & Boyd, 1958). Borden, Wissler & Hughes (1964) showed that, in the rat given high-fat diets and large doses of oestradiol (333 $\mu\text{g./day}$), serum low-density (β -) and high-density (α -) lipoproteins, both of which contained relatively large amounts of phospholipid, were greatly raised. Quantitatively, the increase in cholesterol concentration produced by the oestradiol was much less than the phospholipids. It may be that, in EFA-deficient rats, increased sensitivity to oestradiol causes a similar synthesis and secretion of phospholipid-rich lipoproteins. The increase in β -lipoprotein might also explain the rise in triglyceride induced in the castrated rats by oestradiol (Table 4).

EFA deficiency in rats developed by giving a fat-free diet has been reported to increase liver lipids and to decrease plasma cholesterol (Deuel, Alfin-Slater, Wells, Kryder & Aftergood, 1955), whereas the inclusion of hydrogenated coconut oil allows maintenance of normal liver and plasma cholesterol concentrations. In the present experiment,

EFA deficiency was induced with diets containing 10% of hydrogenated coconut oil, so that neither liver-lipid accumulation nor changes in plasma cholesterol concentrations occurred. However, the higher concentrations of plasma cholesterol occurring in female and oestradiol-treated rats (Fillios, 1957; Okey & Lyman, 1956; Aftergood & Alfin-Slater, 1965) were not so obvious in these animals. The fatty acid composition of the plasma cholesteryl esters differed from the phospholipids principally by having higher proportions of palmitoleic acid and arachidonic acid and less palmitic acid and stearic acid. As with the plasma phospholipids, however, females had significantly higher proportions (Table 3) and concentrations (Fig. 3) of arachidonic acid than males. The sex differences in arachidonic acid in the esterified cholesterol fraction cannot be explained on the basis of a direct secretion from the liver to the plasma because the fatty acid composition of the plasma cholesteryl esters differs greatly from that of the liver esters (Klein, 1958; Okey *et al.* 1962; Aftergood & Alfin-Slater, 1965; Ostwald *et al.* 1965). Glomset (1962) suggested that the high proportion of polyunsaturated fatty acids in plasma cholesteryl esters arises from a plasma transesterification reaction between the β -unsaturated fatty acids of the plasma lecithins and free cholesterol. Sugano & Portman (1965) also consider that a reaction between plasma phospholipid and cholesterol, mediated by plasma enzymes, may play a significant role in the formation of plasma cholesteryl esters; but Roheim, Haft, Gidez, White & Eder (1963), using liver-perfusion techniques, and Goodman & Shiratori (1964), in turnover studies of individual cholesteryl esters *in vivo*, concluded that the liver was the major source of plasma esterified cholesterol. The greater proportion of arachidonic acid esterified with plasma cholesterol, as compared with the liver cholesteryl esters, has been reconciled by proposing that the liver selects, preferentially, polyunsaturated cholesteryl esters for lipoprotein formation (Goodman, Deykin & Shiratori, 1964; Swell, Law & Treadwell, 1964). In our experiment there was remarkable parallelism between the proportions and concentrations of arachidonic acid and eicosatrienoic acid in the plasma cholesteryl esters and phospholipids at each interval studied. Since these fatty acids occur almost exclusively on the β -position of the phospholipids (De Tomás, Brenner & Peluffo, 1963), apparently phospholipids are contributing their β -fatty acids directly for the formation of the plasma cholesteryl esters. Although the results suggest that this transesterification could take place in the plasma, the possibility of a selective secretion of cholesteryl arachidonate or eicosatrienoate from the liver cannot be dismissed without additional experimental evidence.

This study has shown that female and oestradiol-treated rats given a diet completely devoid of essential fatty acids could maintain, for relatively long periods, higher concentrations of plasma cholesteryl and phospholipid arachidonate than male or testosterone-treated animals. Much of this sex difference may be attributed to the smaller body and organ size of the female or oestrogen-treated rats. Less of their arachidonic acid therefore is fixed in the form of structural phospholipids, and more is allowed to be associated with the circulating lipids. We believe, however, that oestrogen may have a more specific effect on the metabolism of essential fatty acids than just suppression of growth. Lyman *et al.* (1963) showed that female or oestradiol-injected rats given more than adequate amounts of linoleic acid had higher concentrations of stearic acid and arachidonic acid in their plasma lecithins than males or testosterone- or oil-injected animals. Therefore, under conditions in which no shortage of arachidonic acid would be expected to occur in either sex, females still maintained higher concentrations of lecithin arachidonate. Tinoco, Shannon & Lyman (1964) demonstrated that, in choline-deficient rats, a condition in which phospholipid metabolism is undoubtedly affected, the sex differences in plasma phospholipid stearic acid and arachidonic acid were lost with reversion to a male pattern (i.e. to relatively higher proportions of palmitic acid and linoleic acid). In the EFA-deficient rats in this study, female characteristics were also associated with maintenance of a phospholipid that contained more stearic acid and arachidonic acid compared with deficient males or testosterone-treated rats. Collins (1960) and Harris, Robinson & Getz (1960) showed that lecithins containing high proportions of stearic acid and arachidonic acid are metabolically different from lecithins rich in palmitic acid and linoleic acid. Since female hormones have been associated with various aspects of phospholipid metabolism (Stekol, Weiss, Smith & Weiss, 1953; Bowser, Henderson & Zimmerman, 1961; Natori, 1963), it is possible that oestradiol may influence essential fatty acid metabolism through an effect on formation or maintenance of phospholipids characteristically rich in arachidonic acid.

The authors thank Miss Ruth Babcock for the numerous analytical determinations, and Mr Peter Miljanich for maintenance and standardization of the gas-liquid chromatographs. The assistance of Mr Tom Bouchard and Mrs Margaret Rose in care and handling of the animals is also greatly appreciated. This investigation was supported in part by U.S. Public Health Service Grant H-6480 and Western Regional Experiment Station Project W-44R. A preliminary report of this study has been presented at the Federation of American Societies for Experimental Biology, Chicago, Ill. (Lyman, Shannon & Ostwald, 1964a).

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