Influence of Sex and Gonadal Hormones on Rat-Liver and Carcass Lipids during the Development of an Essential Fatty Acid Deficiency

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1. Groups of intact male and female rats and castrated rats injected with oestradiol or testosterone were given a diet containing hydrogenated coconut oil for 9 weeks, and at intervals the amounts and fatty acid compositions of the carcass and liver lipids were determined. 2. Male rats grew faster and larger, and exhibited typical external essential fatty acid deficiency symptoms sooner than did females. Testosterone-treated castrated male rats were similar to males, and oestradiol-injected castrated male rats resembled females. 3. Intact females maintained a higher linoleic acid concentration in their carcass than did males. Total amounts of carcass linoleic acid remained similar for all groups, only 200mg. being removed in 9 weeks regardless of body size. 4. The amounts of total cholesteryl esters were independent of liver size. They were higher in males and testosterone-treated castrated male rats than in females and oestrogen-treated castrated male rats. 5. Phospholipids represented about 80% of the liver lipids. The total amounts of the phospholipid linoleic acid and arachidonic acid were similar for all groups regardless of liver size, and were not affected appreciably by the deficiency. Females and oestrogen-treated castrated male rats maintained a higher proportion of phospholipid arachidonic acid for longer periods than did their male counterparts. Both the total amounts and the proportions of eicosatrienoic acid and palmitic acid were higher in males than in females. 6. Supplementation of the essential fatty acid-deficient diet with linoleic acid caused a rapid loss of eicosatrienoic acid and palmitic acid with a concomitant increase in stearic acid and arachidonic acid. 7. There were no obvious differences in the way that the essential fatty acids were metabolized or mobilized from adipose tissue of male or female rats during essential fatty acid deficiency. 8. The results indicated that the greater growth rate of the male rats caused them to require and synthesize more phospholipids than did the females. In the absence of adequate amounts of arachidonic acid, eicosatrienoic acid was substituted into the additional phospholipid. The earlier symptoms of essential fatty acid deficiency in the male rat could therefore be ascribed to the higher tissue concentrations of this unnatural phospholipid and its inability to perform the normal metabolic functions of phospholipids.

Sex differences in the lipid metabolism of rats deficient in EFA* have interested investigators for a number of years. Early studies by Loeb & Burr (1947) showed that female rats made deficient in EFA by means of a fat-free diet stored more fat than did comparable males; they suggested that the female rat had a more efficient system for utilizing its EFA. Greenberg, Calbert, Savage & Deuel (1950) demonstrated that the requirement for linoleic acid in the female animals was only about one-third (or less) that of the males, suggesting possible sex differences in the intermediary metabolism of the lipids. Coleman, Chen & Alfin-Slater (1958) demonstrated sex differences for the rat in the requirement for EFA as regulators of cholesterol esterification. Gonadectomy of EFA-deficient male rats appeared to favour esterification of liver cholesterol with saturated fatty acids. Morton & Horner (1961) showed that female rats had less liver triglyceride and more cholesteryl tetraenoate than males. When male and female rats were given dietary cholesterol while being depleted of EFA (Horner & Morton, 1961) female rats grew better and longer and showed milder dermal symptoms. Females also had a higher proportion of dienoic acids and tetraenoic acids in their liver fat but male rats tended to accumulate more liver cholesterol, especially cholesteryl dienoate. It appeared, therefore, that female rats utilized available linoleic acid

*Abbreviation: EFA, essential fatty acids.

more efficiently. Rats given dietary cottonseed oil or coconut oil also exhibited sex differences in the metabolism of the unsaturated fatty acids (Okey, Shannon, Tinoco, Ostwald & Miljanich, 1961; Okey, Ostwald, Shannon & Tinoco, 1962) that could be similarly interpreted. Ostwald, Okey, Shannon & Tinoco (1962) reported that female rats were better able to mobilize linoleic acid from their adipose tissue when subjected to diets low in linoleic acid.

In the present paper we report the amounts and fatty acid compositions of cholesteryl esters, phospholipids and triglycerides in the liver and carcass of weanling male and female rats at intervals during the development of an EFA deficiency. If differences in the utilization of EFA occurred between sexes, they would be expected to be more evident during the early stages of the deficiency than at a later date, when concentrations of the EFA would have decreased and approached similar values in both sexes. In addition, groups of castrated male rats injected with oestradiol benzoate or testosterone propionate were included.

MATERIALS AND METHODS

Animals. Male and female weanling rats of the Long–Evans strain, weighing about 50g., were fed on a semi-synthetic diet (Monsen, Okey & Lyman, 1962) for 1 week. At the end of that time, groups of ten males and ten females were killed, and the remaining animals were then fed on the EFA-deficient diet (Table 1). Groups of five or six rats of each sex were killed 16 and 21 days after being placed on the EFA-deficient diet. During the first 2 weeks on the EFA-deficient diet some of the males were castrated and assigned to groups to be treated with hormones. Thus there were five experimental groups: intact females and males, and castrated males injected with oestradiol, testosterone or the vehicle oil used to dissolve the hormones. The hormones were dissolved in hydrogenated coconut oil, and 0·1ml. was injected subcutaneously three times a week to provide a total weekly dose of 30μg. of oestradiol benzoate or 2mg. of testosterone propionate and 0·3ml. of hydrogenated coconut oil. The dosages of hormones were the same as those used by Lyman, Shannon, Ostwald & Miljanich (1964). Since food consumption, growth and adrenal weights of the EFA-deficient rats given oestradiol or testosterone compared well with those of the intact female and male groups, it was assumed that a reasonably physiological hormone dose had been maintained. Six rats from each of the five groups were then killed after 32, 46 and 64 days on the EFA-deficient regime. This experimental design provided results for five time-intervals of EFA deficiency for the intact male and female animals and for three time-intervals for the three groups of castrated rats. Food intake and weekly gain records were kept for all animals throughout the experiment.

A few of the intact male and female animals were continued on the EFA-deficient diet for a total of about 17 weeks. These rats were then allowed to eat the basic diet in which the hydrogenated coconut oil had been replaced by an equivalent amount of safflower oil. Five rats of each sex were killed at 1 day and at 3 days after supplementation with linoleic acid. The average intake of linoleic acid after 1 day was 1560mg./male rat and 1470mg./female rat. After 3 days the males had consumed 3210mg. and females 2930mg. of linoleic acid. One male and two females that had remained on the EFA-deficient diet were killed at the same time to obtain an approximate measure of the lipid composition of that age group.

Table 1. *Composition of basal diet*

The fatty acid composition of the hydrogenated coconut oil was (% by wt.): C_{10} acids, 13; C_{12} acids, 49; C_{14} acids, 18; C_{16} acids, 9; C_{18} acid, 8; $C_{18:1}$ acid, 3·3; $C_{18:2}$ acid, < 0·1. In some experiments (see the text) the hydrogenated coconut oil was replaced by safflower oil, which contained 77% (by wt.) of $C_{18:2}$ acid. The vitamin B mix contained (mg./100g. of diet): folic acid, 0·2; pyridoxine hydrochloride, 0·2; calcium pantothenate, 0·45; thiamine hydrochloride, 0·3; riboflavine, 0·3; nicotinic acid, 0·45; biotin, 0·15; inositol, 50; ascorbic acid (as antioxidant), 10·0. The vitamins A, D, E and K mix (Nopco Chemical Co., Richmond, Calif., U.S.A.) contained (per 100g. of diet): vitamin A (Nopcay type I), 1700 i.u.; vitamin D (Super Nopex, type II), 500i.u.; α-tocopherol (Nopvite), 15mg.; menadione, 33mg.

Ingredient	Composition of diet (% by wt.)
Casein	5·0
Egg albumin	10·0
Oil	10·0
Sucrose	69·9
U.S.P. XIV salts	4·0
Vitamin B mix	1·0
Vitamins A, D, E and K mix	1·0
Choline hydrogen tartrate	0·14

Preparation of tissues. Food cups were removed from the cages 16hr. before the animals were killed, and at that time a final hormone injection was given. The rats were anaesthetized with sodium pentobarbital. Blood was collected in a heparin-treated syringe, by open-heart puncture, and placed in an ice bath. Within 2hr. after removal, the blood was centrifuged at about 4° and the plasma thus obtained was extracted. Liver, heart, adrenals and digestive tract were removed (pancreas and mesenteric fat were left with the carcass), as were the head and tail. The carcasses were weighed and placed in ethanolic KOH (25g. of KOH/100ml. of 95% ethanol) for digestion and saponification of the fat. The livers were weighed, frozen immediately on solid CO_2, freeze-dried for 24hr. and reweighed to obtain dry weights and water contents. They were then stored under N_2 at about −10° until further processing (Tinoco, Miljanich & Lyman, 1963).

Carcass. Each carcass was refluxed for 3–4hr. in 250ml. of ethanolic KOH, on a steam table, in a loosely covered Erlenmeyer flask. After this treatment only a very small amount of particulate material remained. The solution was decanted, and the residue was rinsed several times with distilled water. To a portion of this extract, containing approx. 10mg. of fatty acids, was added 1·0mg. of heptadecanoic acid as an internal standard so that the amounts of each fatty acid could be calculated when necessary (Tinoco,

Shannon, Miljanich, Lyman & Okey, 1962). The solution was then acidified and the free fatty acids were extracted into light petroleum (b.p. 30–60°). The free fatty acids were methylated by refluxing for about 30min. in 10ml. of a solution of 1% (v/v) H_2SO_4 in methanol, and analysed by gas–liquid chromatography. Because the carcass lipids represented principally triglycerides, no fractionation of the lipids was performed.

Extraction, fractionation and analysis of liver lipid. Each liver was pulverized in a mortar and its lipids were extracted with ethanol, followed by diethyl ether. The combined extracts were evaporated to dryness *in vacuo*, re-extracted with light petroleum and stored at about −10°. The extracted liver lipids were fractionated on silicic acid columns into cholesteryl esters, triglycerides, free cholesterol and phospholipids, as described by Okey *et al.* (1961). Total lipid (Bloor, 1928), total cholesterol (Sperry & Webb, 1950) and phosphorus (Sumner, 1944) were determined. The recoveries of cholesterol (that found in the cholesteryl ester plus free cholesterol fractions) and of phosphorus found in the phospholipid fraction were 98–101% of those from unfractionated extract. The recovery of total lipid, i.e. the sum of the lipid fractions obtained from the columns, was generally 95% or better of the total lipid determined in the unfractionated extract. Only in the groups supplemented with linoleic acid was the recovery as low as 70–80% of the total lipid. We have no ready explanation for this result.

The fatty acid composition of the individual fractions was determined by gas–liquid chromatography. Details of the procedures and calculations for the absolute amounts of cholesteryl esters and phospholipid fatty acids were as reported by Lyman *et al.* (1964).

Carbon chain length and number of double bonds in the eicosatrienoic acid were established by hydrogenation and by comparison with other known unsaturated fatty acids. The major component of the 18:1 acids was the Δ^9 isomer, and that of the 20:3 acids was the $\Delta^{5,8,11}$ isomer that Mead & Slaton (1956) have shown to be derived from oleic acid. The position of the double bonds was determined by oxidation with $KMnO_4$ (Tinoco & Miljanich, 1965).

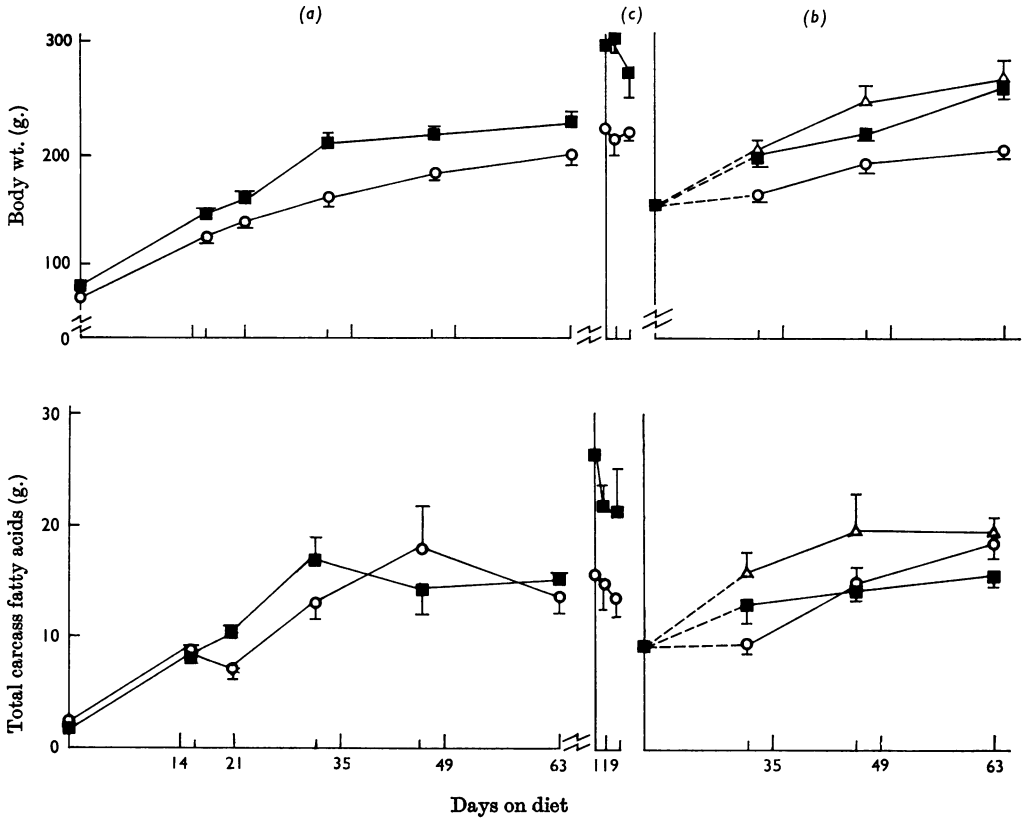

Fig. 1. Growth and carcass fat of rats during the development of an EFA deficiency: (*a*) intact males (■) and females (○); (*b*) castrated males treated with oestradiol (○), testosterone (■) or the vehicle oil (△) (castration was performed and hormone injections were begun approx. 2 weeks after the animals had been put on the EFA-deficient diet); (*c*) 17-week-deficient intact male (■) and female (○) rats re-fed with linoleic acid (the values represent 1 day and 3 days after the start of feeding with linoleic acid). Vertical lines on the Figures are half S.E.M. values of the means from five or more rats.

Statistical comparisons of the results were made by *t* test (Snedecor, 1956). Values with $P > 0.05$ were considered not to be significantly different. (Most of the statistical calculations were performed on an IBM 7090 computer at the Computer Center, University of California, Berkeley.)

RESULTS

EFA-deficiency symptoms. External symptoms (dandruff, scaly feet and tails) were relatively mild. Most of the intact males and the testosterone-injected animals, as well as a few of the oil-injected animals, showed symptoms after 46 days, whereas the females and the oestrogen-injected group did not. By 64 days most of the animals in all groups showed symptoms.

Growth rate and body weight. As expected, the males grew more than did the females, and after 64 days on the deficient diet were about 30g. heavier than the females (Fig. 1). The oestradiol-treated castrated male rats grew at a rate comparable with that of the intact females, whereas the growth of the testosterone-treated and the oil-injected castrated animals was similar to that of the intact male rats.

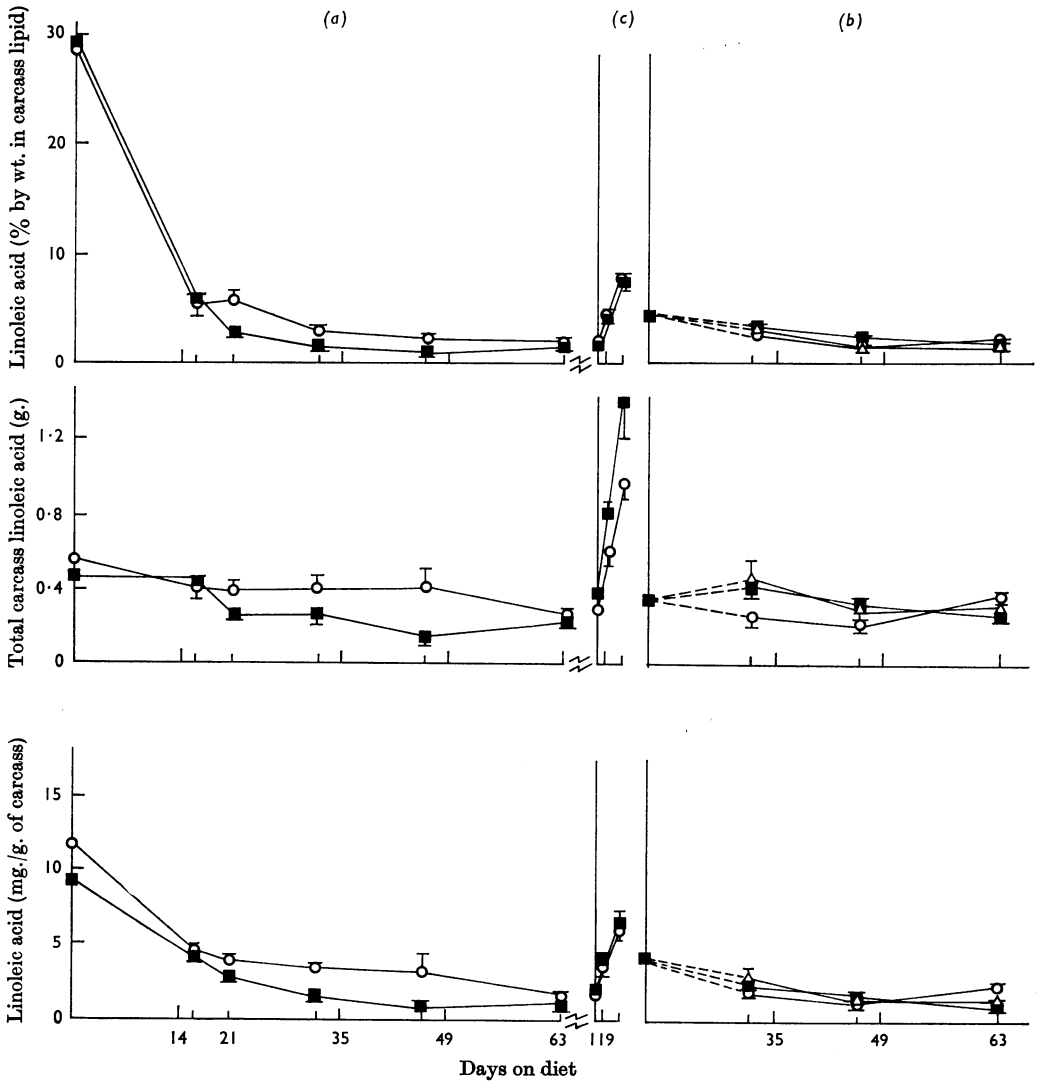

Fig. 2. Linoleic acid content of carcass fat of rats during the development of an EFA deficiency: (*a*) intact males (■) and females (○); (*b*) castrated males treated with oestradiol (○), testosterone (■) or the vehicle oil (△); (*c*) 17-week-deficient intact male (■) and female (○) rats re-fed with linoleic acid. For further details see Fig. 1.

Fat accumulated in both sexes during the first 32 days of the deficiency, then remained at about 10% of carcass weight. Although the percentage of linoleic acid in the carcass fat of both males and females (Fig. 2) fell abruptly from 30% to about 5% in the first 15 days of the experiment, total linoleic acid decreased only slowly throughout the deficiency period from about 500 mg. to 300 mg. Intact females maintained a significantly higher linoleic acid concentration ($P < 0.05$ or better at 32 and 46 days). The major fatty acids of the carcass lipid were oleic acid and palmitic acid, the sum of which represented over 50% of the total fatty acids. About 20% of the fatty acids had a chain length of less than C_{16}, and were undoubtedly derived from coconut oil. Palmitoleic acid and stearic acid made up the remainder of the main carcass acids. The patterns in fat accumulation and linoleic acid concentration in the hormone-treated animals were similar to those in the intact male and female

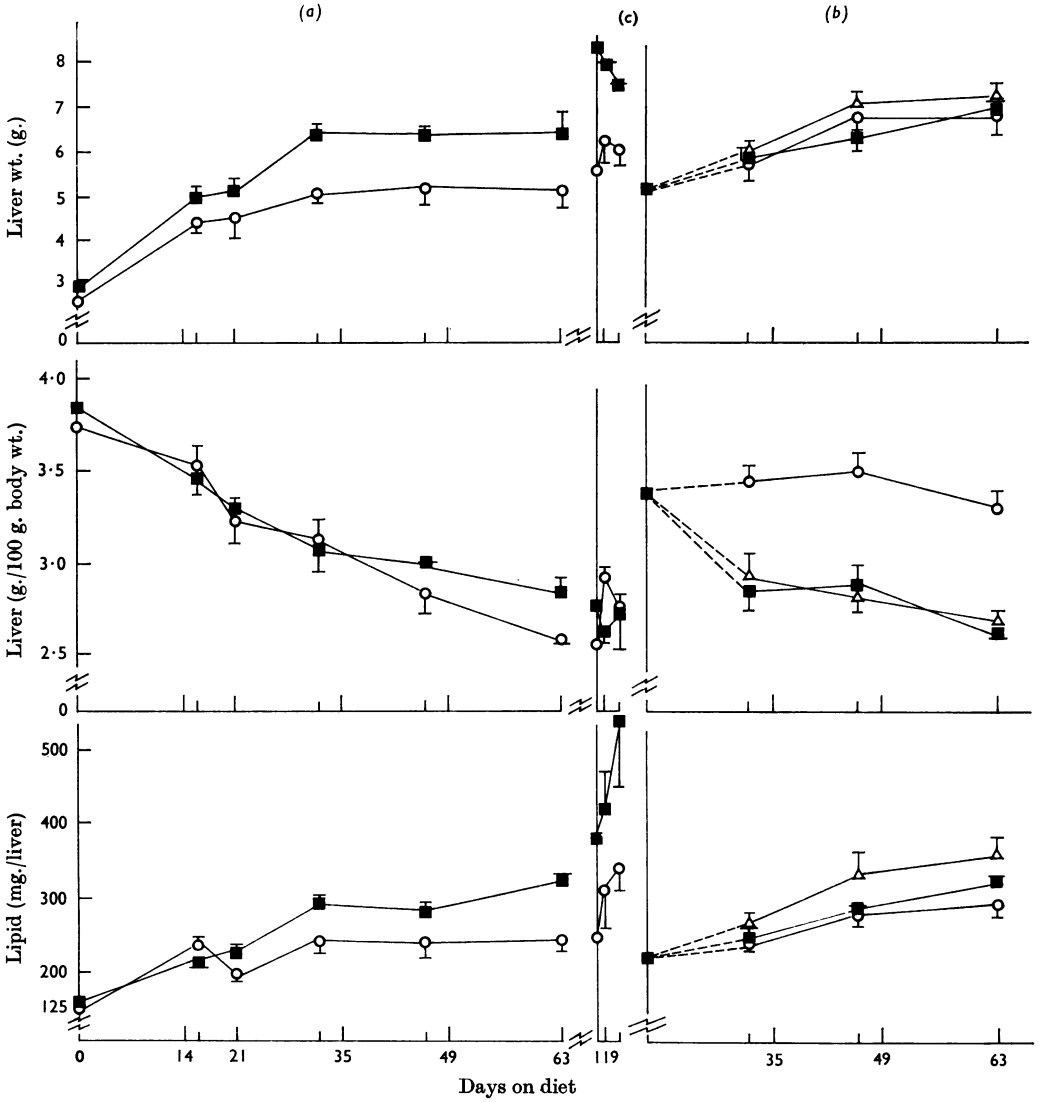

Fig. 3. Liver weights and total lipid content of rats during the development of an EFA deficiency: (*a*) intact males (■) and females (○); (*b*) castrated males treated with oestradiol (○), testosterone (■) or the vehicle oil (△); (*c*) 17-week-deficient intact male (■) and female (○) rats re-fed with linoleic acid. For further details see Fig. 1.

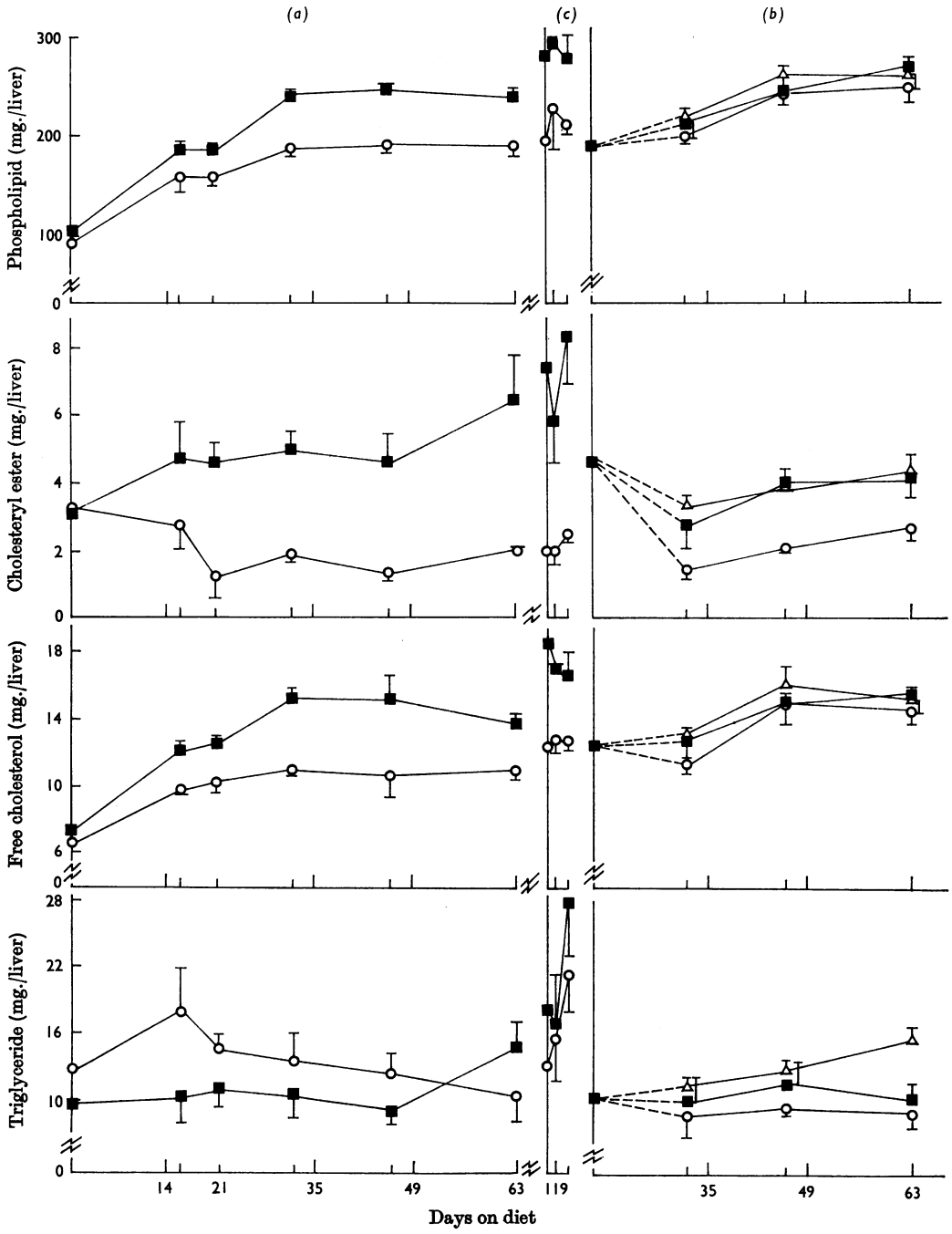

Fig. 4. Composition of liver lipids of rats during the development of an EFA deficiency: (*a*) intact males (■) and females (○); (*b*) castrated males treated with oestradiol (○), testosterone (■) or the vehicle oil (△); (*c*) 17-week-deficient intact male (■) and female (○) rats re-fed with linoleic acid. For further details see Fig. 1.

groups. When linoleic acid was re-fed to the intact rats, there was a 2-fold increase in carcass linoleic acid after the first day of supplementation, and by 3 days its amount was 3–4 times that of the deficient animals. Male rats appeared to accumulate more linoleic acid than did the females. Little or no arachidonic acid was detected in the carcass lipids.

Liver size and total lipid. The oestradiol-treated animals had larger livers in relation to their body weight than did the remaining groups (Fig. 3). The moisture content of the livers in all groups was very consistent, and was in the range 69–71%.

Because the amount of total liver lipid was a function of the liver weight, intact males had more lipid in their livers after 32 days on the deficient diet than did females ($P < 0.05$ or better). Differences in liver lipid content between the castrated groups were not significant. Liver fat in both sexes increased appreciably when linoleic acid was re-fed to the rats for 1 and 3 days ($P < 0.05$) (Fig. 3). Lipid accumulation during that time seemed to be greater in males than in females.

Composition of liver lipid. Fig. 4 shows changes in amounts of liver phospholipids, cholesterol and triglyceride as the EFA deficiency progressed. By far the greatest contribution to liver lipid came from the phospholipids, which represented 80% or more of the total lipid. The quantity of phospholipid and unesterified cholesterol in the livers of both sexes was closely related to the size of the livers; thus after 32 days of the deficiency males had significantly greater amounts of these liver lipids than did females (at 21 days, $P < 0.05$; at 32 days, $P < 0.01$). Liver cholesteryl esters, on the other hand, did not increase with increasing liver weights, but remained unchanged in males and even decreased in the females (0-time versus 21 days, $P < 0.01$). This led not only to smaller amounts of liver cholesteryl esters in females but also to a lower concentration (mg./g. of liver) (at 21 days, $P < 0.05$; at 32 days, $P < 0.01$). No significant sex differences in the amount of liver triglycerides were observed throughout the course of the experiment.

Similarly, amounts and concentrations of liver cholesteryl esters in the oestradiol-treated castrated rats were consistently lower than those of the testosterone-treated groups (differences were significant at 46 days, $P < 0.01$). Total liver phospholipids and unesterified cholesterol were comparable for all groups and at a level resembling that of the intact male. Feeding linoleic acid to these EFA-deficient animals did not produce any appreciable change in phospholipids or cholesteryl esters. Amounts of triglycerides, however, did increase considerably in both sexes (64 days versus 3-day re-fed, $P < 0.02$).

Fatty acid composition of phospholipids. The fatty acid composition of the liver phospholipids is shown in Table 2. The proportion of linoleic acid in both sexes decreased to about 5% in the first 15 days of the deficiency, then remained at about that level. The decrease in the percentage of arachidonic acid was slower, and was compensated by a nearly equivalent increase in eicosatrienoic acid. Proportions of oleic acid and palmitoleic acid increased as the deficiency developed, whereas no consistent changes in palmitic acid or stearic acid were evident.

As the deficiency progressed, females maintained higher proportions of arachidonic acid and stearic acid as compared with males ($P < 0.05$, except at 32 days), whereas the latter had higher proportions of eicosatrienoic acid ($P < 0.01$). After 64 days, however, these differences had mostly disappeared. The compositions of the phospholipids in the oestradiol- and testosterone-treated groups were similar to those of their respective intact controls, as were the differences between these two groups.

During the period of rapid growth and formation of structural phospholipids the amounts of all phospholipid fatty acids, except linoleic acid, increased (Figs. 5 and 6). During this time the linoleic acid decreased slowly and at a similar rate for all groups. The amount of arachidonic acid increased in both sexes during the first 15 days of the deficiency, then remained relatively constant in all groups. Therefore, although male rats had about 60–70mg. more phospholipids in their livers than did the females, both groups of animals had essentially the same amounts of linoleic acid and arachidonic acid. The additional phospholipids in male rats contained principally palmitic acid and eicosatrienoic acid (male versus female, $P < 0.01$ for both 20:3 acid and 16:0 acid after 15 days on the deficient diet).

The amounts of liver phospholipid fatty acids in the three hormone-treated castrated groups resembled those of the intact male animals.

Figs. 5 and 6 show that feeding linoleic acid to the rats for as short a time as 1 day resulted in an immediate reversal of the effects of the EFA deficiency. Linoleic acid, arachidonic acid and stearic acid increased, whereas eicosatrienoic acid, oleic acid and palmitoleic acid abruptly decreased. A rapid conversion of linoleic acid into arachidonic acid in this lipid fraction was evident, since arachidonic acid appeared to have reached a maximum after only 1 day of re-feeding.

Fatty acid composition of cholesteryl esters. Cholesteryl esters in the EFA-deficient animals represented about 2% of the total liver lipid in males and only 0·6% in females. Quantitatively therefore this lipid fraction did not contribute significant amounts of linoleic acid or arachidonic acid to the total liver lipids. The percentages and amounts of the major fatty acids present as

Table 2. *Phospholipid fatty acids in livers of rats during the development of an EFA deficiency*

Results are given as percentages of total methyl esters measured. Only the major fatty acids from C_{16} to C_{20} are reported. Means are of five or more animals/group at each time period. The maximum S.E.M. was less than 10%.

	Fatty acid (% by wt.)				
			Castrated males		
Days on diet	Females	Males	Vehicle-injected	Oestrogen-injected	Testosterone-injected
$C_{18:2}$ acid					
0	13·6	12·5	—	—	—
15	5·3	7·0	—	—	—
21	6·5	6·3	—	—	—
32	4·8	4·2	5·7	5·3	6·3
46	4·1	3·0	3·6	3·7	4·9
64	4·0	4·2	3·5	3·7	4·0
$C_{20:4}$ acid					
0	27·4	27·1	—	—	—
15	30·1	27·0	—	—	—
21	29·4	21·5	—	—	—
32	23·8	17·8	20·6	24·1	22·3
46	23·2	12·4	18·0	18·9	17·1
64	18·0	16·8	16·9	19·6	14·9
$C_{20:3}$ acid					
0	0	0	—	—	—
15	4·1	4·9	—	—	—
21	4·7	11·9	—	—	—
32	9·9	16·4	10·7	11·2	10·6
46	10·4	21·8	17·2	19·1	17·2
64	13·0	17·8	17·4	14·6	19·6
$C_{18:1}$ acid					
0	4·9	4·5	—	—	—
15	9·7	10·2	—	—	—
21	11·5	12·7	—	—	—
32	13·8	14·0	13·3	11·6	12·2
46	13·2	17·5	14·0	13·2	15·2
64	16·6	16·7	17·6	14·6	17·4
$C_{16:1}$ acid					
0	0·7	0·6	—	—	—
15	1·7	1·9	—	—	—
21	1·7	2·5	—	—	—
32	2·7	3·0	2·9	1·9	2·6
46	2·5	3·6	2·7	1·8	2·6
64	2·9	3·4	3·1	2·2	3·0
$C_{16:0}$ acid					
0	17·1	17·4	—	—	—
15	14·2	16·5	—	—	—
21	14·0	16·6	—	—	—
32	14·8	16·5	17·1	14·3	16·1
46	12·7	13·8	14·2	13·7	13·6
64	13·8	14·4	14·1	13·7	14·6
$C_{18:0}$ acid					
0	26·0	26·3	—	—	—
15	27·0	23·0	—	—	—
21	25·6	22·4	—	—	—
32	27·4	23·4	25·6	28·1	23·8
46	26·8	22·9	24·0	27·3	23·2
64	26·6	21·7	22·9	26·6	23·2

cholesteryl esters are shown in Table 3. The proportion of cholesteryl linoleate in both males and females fell within the first 15 days of the deficiency to about 3–4% and remained at that level. This decrease was compensated by increases in the proportions primarily of palmitic acid, palmitoleic acid and eicosatrienoic acid. The proportion of cholesteryl arachidonate decreased promptly in males, whereas females maintained the percentage of arachidonic acid initially present until late in the development of the deficiency. Although the proportion of eicosatrienoic acid increased as the deficiency progressed, its contribution to the cholesteryl ester composition was much less than to that of the phospholipid.

Both sexes and the three groups of castrated rats had similarly low total amounts of cholesteryl linoleate and arachidonate after the first 21 days

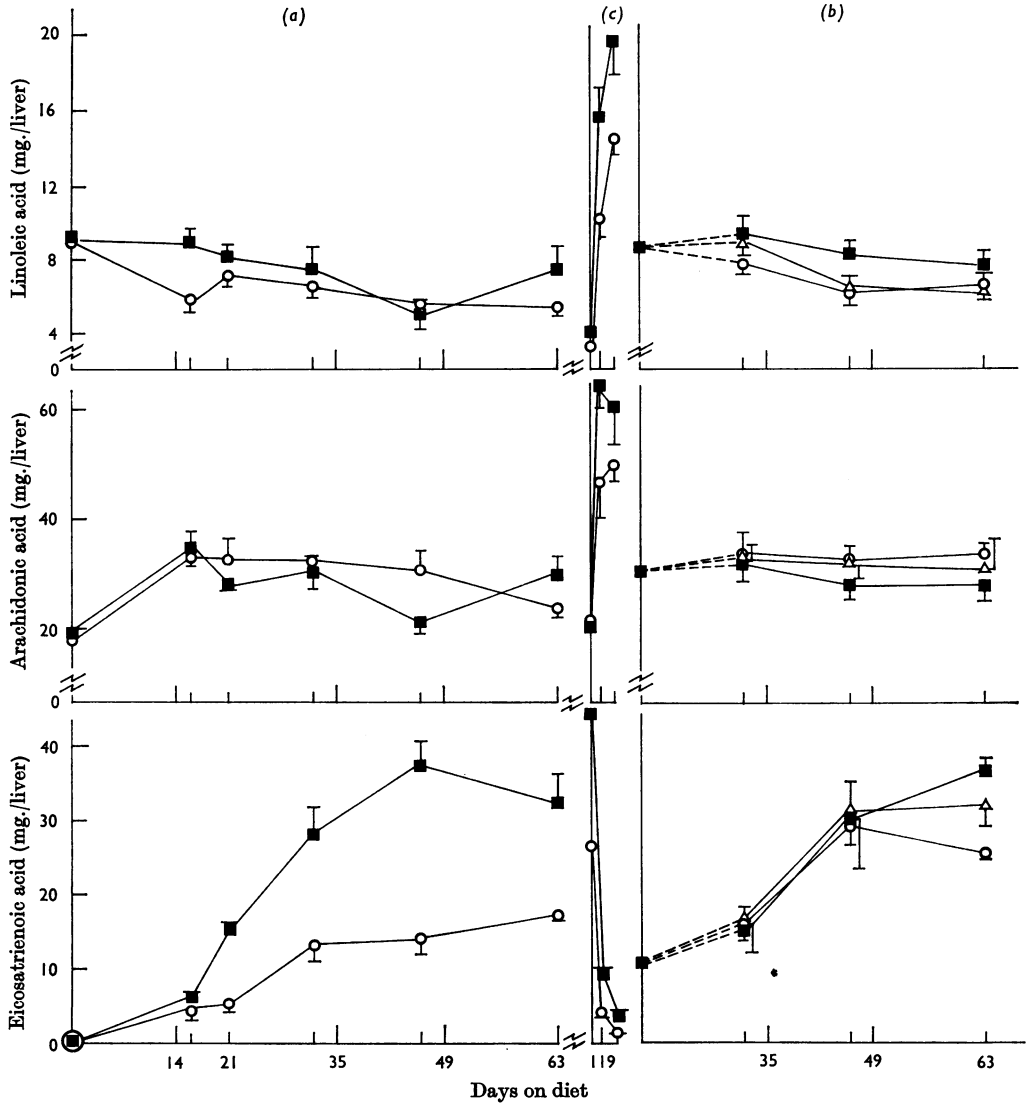

Fig. 5. Linoleic acid, arachidonic acid and eicosatrienoic acid contents of liver phospholipids from rats during the development of an EFA deficiency: (*a*) intact males (■) and females (○); (*b*) castrated males treated with oestradiol (○), testosterone (■) or the vehicle oil (△); (*c*) 17-week-deficient intact males (■) and females (○) re-fed with linoleic acid. For further details see Fig. 1.

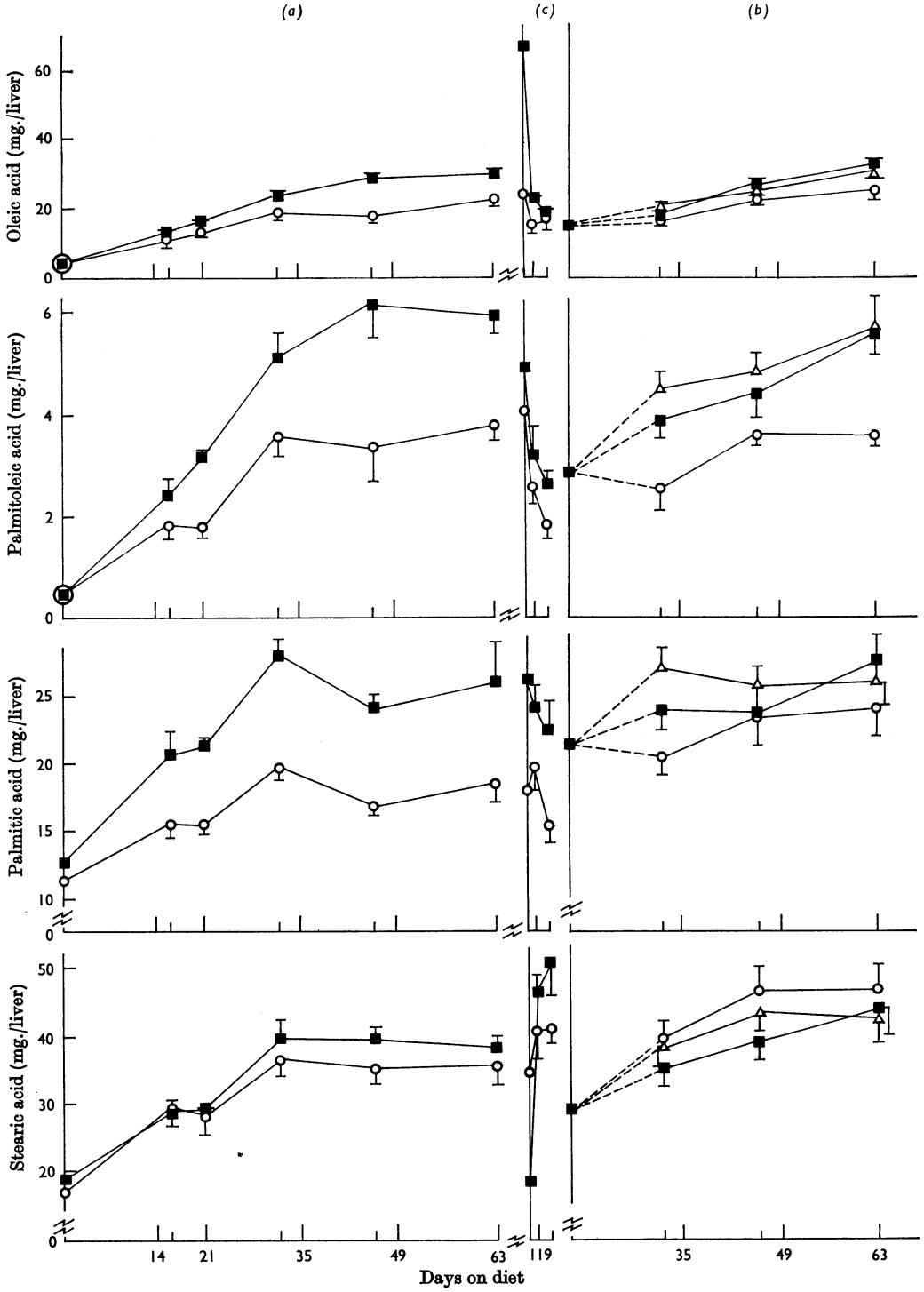

Fig. 6. Oleic acid, palmitoleic acid, palmitic acid and stearic acid contents of liver phospholipids of rats during the development of an EFA deficiency: (*a*) intact males (■) and females (○); (*b*) castrated males treated with oestradiol (○), testosterone (■) or the vehicle oil (△); (*c*) 17-week-deficient intact male (■) and female (○) rats re-fed with linoleic acid. For further details see Fig. 1.

Table 3. *Cholesteryl ester fatty acids in livers of rats during the development of an EFA deficiency*

Results are given as percentages of total methyl esters measured, and (in parentheses) as mg. of fatty acid/liver. Only the major fatty acids from C_{16} to C_{20} are reported. Means are of five or more animals/group at each time period. The maximum S.E.M. was less than 10% for the percentages by weight, and less than 20% for the weights/liver.

	Fatty acid (% by wt., and, in parentheses, mg./liver)				
			Castrated males		
Days on diet	Females	Males	Vehicle-injected	Oestrogen-injected	Testosterone-injected
$C_{18:2}$ acid					
0	22·1 (0·50)	22·8 (0·56)	—	—	—
15	3·6 (0·07)	4·7 (0·17)	—	—	—
21	4·9 (0·05)	2·9 (0·09)	—	—	—
32	3·4 (0·05)	3·3 (0·12)	3·8 (0·10)	3·4 (0·03)	4·2 (0·08)
46	3·4 (0·04)	1·7 (0·06)	2·9 (0·07)	2·4 (0·04)	3·0 (0·09)
64	2·9 (0·04)	2·1 (0·12)	2·6 (0·08)	2·5 (0·04)	2·1 (0·06)
$C_{20:4}$ acid					
0	8·7 (0·18)	10·2 (0·22)	—	—	—
15	10·1 (0·20)	7·0 (0·20)	—	—	—
21	11·1 (0·12)	4·0 (0·13)	—	—	—
32	6·9 (0·13)	3·7 (0·14)	5·0 (0·12)	9·8 (0·09)	4·3 (0·08)
46	12·0 (0·14)	2·4 (0·11)	6·2 (0·14)	6·4 (0·09)	3·2 (0·10)
64	3·7 (0·06)	3·1 (0·17)	2·1 (0·06)	3·7 (0·06)	1·4 (0·05)
$C_{20:3}$ acid					
0	0·0 (0·0)	0·0 (0·0)	—	—	—
15	0·7 (0·0)	1·6 (0·05)	—	—	—
21	1·2 (0·0)	2·8 (0·09)	—	—	—
32	5·6 (0·08)	4·1 (0·14)	2·4 (0·05)	6·5 (0·06)	2·8 (0·06)
46	4·5 (0·05)	3·5 (0·14)	5·4 (0·11)	4·0 (0·06)	3·0 (0·09)
64	2·6 (0·04)	3·9 (0·22)	4·1 (0·11)	4·8 (0·09)	4·2 (0·11)
$C_{18:1}$ acid					
0	22·5 (0·55)	23·0 (0·56)	—	—	—
15	25·7 (0·56)	32·8 (1·23)	—	—	—
21	20·4 (0·22)	35·6 (1·19)	—	—	—
32	25·5 (0·38)	33·3 (1·27)	31·2 (0·79)	21·6 (0·22)	28·9 (0·58)
46	22·5 (0·25)	32·3 (0·89)	25·6 (0·82)	24·6 (0·36)	34·9 (1·09)
64	31·0 (0·46)	36·2 (2·06)	32·8 (1·02)	31·1 (0·56)	34·3 (1·08)
$C_{16:1}$ acid					
0	7·7 (0·19)	7·5 (0·18)	—	—	—
15	10·8 (0·24)	11·5 (0·45)	—	—	—
21	8·4 (0·09)	12·6 (0·41)	—	—	—
32	11·2 (0·16)	13·5 (0·51)	13·4 (0·32)	8·2 (0·08)	11·3 (0·22)
46	7·1 (0·08)	11·0 (0·42)	9·7 (0·30)	8·8 (0·13)	10·1 (0·32)
64	9·3 (0·14)	12·4 (0·70)	11·1 (0·34)	9·4 (0·19)	10·8 (0·33)
$C_{16:0}$ acid					
0	20·2 (0·51)	19·2 (0·46)	—	—	—
15	26·1 (0·50)	24·1 (0·85)	—	—	—
21	28·7 (0·31)	24·4 (0·77)	—	—	—
32	27·0 (0·37)	24·5 (0·88)	25·6 (0·67)	26·2 (0·26)	28·1 (0·54)
46	23·8 (0·24)	23·6 (0·86)	22·0 (0·63)	24·2 (0·34)	23·2 (0·73)
64	29·0 (0·44)	25·6 (1·41)	27·0 (0·79)	26·2 (0·51)	27·6 (0·80)
$C_{18:0}$ acid					
0	10·2 (0·25)	10·7 (0·26)	—	—	—
15	15·7 (0·28)	12·3 (0·40)	—	—	—
21	18·2 (0·18)	12·3 (0·38)	—	—	—
32	12·9 (0·17)	11·5 (0·40)	10·7 (0·29)	14·2 (0·14)	12·4 (0·23)
46	16·9 (0·17)	11·2 (0·41)	16·1 (0·36)	14·4 (0·20)	11·9 (0·36)
64	12·9 (0·20)	9·9 (0·52)	11·5 (0·33)	12·0 (0·20)	11·5 (0·32)

of deficiency (Table 3). This was so in spite of the larger livers of the males as compared with those of the females. The amounts of cholesteryl esters of the other major fatty acids, however, were generally higher in the males than in the females. Although the sizes of the livers in the three groups of castrated rats were essentially the same, the oestradiol-treated animals, like the intact females, had less oleic acid, palmitic acid and stearic acid as cholesteryl esters than did testosterone-injected rats ($P < 0.05$). Re-feeding linoleic acid to the rats led to an immediate reversal of the changes seen during the development of the deficiency. Linoleic acid and arachidonic acid increased both in amounts and proportions, with corresponding decreases in eicosatrienoic acid, palmitic acid and oleic acid.

Fatty acid composition of triglycerides. Triglycerides also represented a relatively small proportion of the total liver lipids (approx. 5%). The fatty acid composition of this fraction responded to the EFA-deficient diet by a rapid decrease in the proportions of linoleic acid and arachidonic acid (Table 4). Oleic acid and palmitic acid increased, and represented the major fatty acids in this lipid, whereas eicosatrienoic acid and stearic acid remained minor constituents. Because of the similar amounts of triglycerides in the five groups, the quantities of the individual triglyceride fatty acids were directly proportional to their percentages. Neither hormone treatment nor sex of the animal affected either the proportion or amount of fatty acid in this liver lipid. The total amounts of linoleic acid and arachidonic acid available from this lipid were insignificant after 15 days of the deficiency.

DISCUSSION

Carcass and adipose tissue represent the major sources of linoleic acid on which the rat can draw for its metabolic needs during times when no dietary EFA are available. A sex difference in the rate of mobilization of linoleic acid from this source to other tissues could therefore contribute to the sex differences in linoleic acid requirement reported by Greenberg *et al.* (1950). The similarity between males and females in the amounts of linoleic acid removed from the carcass depots (Fig. 2) indicates that sex differences in mobilization are not a major factor. However, since female rats in the present experiment did not grow as large as the males, and maintained a higher concentration of carcass linoleic acid, they had more linoleic acid available per unit weight to meet the EFA requirements of other tissues. This could perhaps explain the higher proportions of arachidonic acid in liver phospholipids and cholesteryl esters in females as compared with those in males in the early stages of the deficiency, even though the total amounts of these fatty acids were similar for both sexes.

Changes in the amounts of liver phospholipids and of unesterified cholesterol followed patterns that were almost identical with the growth patterns of the livers in each group. Thus the amounts of these lipid components appeared to be determined by the demands of growth, whereas the amounts of liver cholesteryl esters and triglycerides seemed to be independent of liver size.

Metabolic differences exist in rats made EFA-deficient with a fat-free diet as compared with rats fed on a diet containing a hydrogenated fat free of EFA. Rats fed on a fat-free diet deposited cholesterol in their livers (Deuel, Alfin-Slater, Wells, Kryder & Aftergood, 1955), whereas an EFA deficiency produced with fat in the diet did not cause any disproportionate increase in total liver lipid or in any of its fractions after growth of the animals had 'plateaued' (Deuel *et al.* 1955; Figs. 3 and 4 in the present paper). The amount of fat in the diet appears to influence the liver lipid composition. Mukherjee, Achaya, Deuel & Alfin-Slater (1958) demonstrated that, in rats fed on a fat-free diet, cholesteryl esters composed largely of monounsaturated fatty acids accumulated in the liver, whereas rats fed on a diet containing 30% of hydrogenated coconut oil had mainly saturated fatty acids esterified with their liver cholesterol. In the present experiment the liver cholesteryl esters of rats fed on a diet containing 10% of hydrogenated oil were composed of about 50% of monounsaturated fatty acids and 40% of saturated fatty acids.

Coleman *et al.* (1958) have reported that fat-deficient male rats had more liver cholesteryl esters than did females. In their experiments, gonadectomized males had lower amounts of cholesteryl esters than did intact male rats. Morton & Horner (1961) also have shown that liver cholesteryl esters were increased and contained more trienoic acid in fat-deficient male rats than in females. Our results (Fig. 4) have further demonstrated that female rats had lower amounts of liver cholesteryl esters. This difference was only partly due to liver size; it appeared to be more a specific effect of oestradiol, because liver cholesteryl esters were as low in the oestradiol-treated castrated rats as in the intact female rats, even though the livers in this group were as large as in the testosterone-treated animals. Other investigators have shown that the sex of the rat may influence the metabolism of cholesterol. For instance, liver mitochondria from female rats synthesize and oxidize cholesterol more extensively than do those of the male (Kritchevsky, Tepper, Staple & Whitehouse, 1963; Fillios, Kaplan, Martin & Stare, 1958). There is also some evidence that esterification with the more

Table 4. *Triglyceride fatty acids in livers of rats during the development of an EFA deficiency*

Results are given as percentages of total methyl esters measured. Only the major fatty acids from C_{16} to C_{20} are reported. Means are of five or more animals/group at each time period. The maximum S.E.M. was less than 10%.

	Fatty acid (% by wt.)				
			Castrated males		
Days on diet	Females	Males	Vehicle-injected	Oestrogen-injected	Testosterone-injected
$C_{18:2}$ acid					
0	36·1	35·7	—	—	—
15	6·1	6·5	—	—	—
21	6·3	2·7	—	—	—
32	2·4	1·6	3·4	2·8	3·9
46	2·2	0·8	2·2	2·0	2·1
64	0·9	0·7	1·2	0·8	0·5
$C_{20:4}$ acid					
0	8·9	10·8	—	—	—
15	2·0	1·7	—	—	—
21	2·2	0·4	—	—	—
32	1·8	0·0	0·0	2·8	2·0
46	0·0	0·0	0·0	0·6	0·0
64	0·0	0·6	0·5	0·9	0·5
$C_{20:3}$ acid					
0	0·0	0·0	—	—	—
15	2·5	2·6	—	—	—
21	2·2	2·2	—	—	—
32	2·1	0·0	0·0	2·3	2·2
46	2·1	2·8	0·0	2·9	2·5
64	2·0	1·4	1·3	0·0	1·3
$C_{18:1}$ acid					
0	21·4	20·7	—	—	—
15	39·5	41·9	—	—	—
21	45·4	44·6	—	—	—
32	49·5	45·6	45·4	47·5	43·1
46	51·2	49·1	49·9	52·0	48·0
64	49·0	49·7	51·5	51·6	51·7
$C_{16:1}$ acid					
0	2·7	2·6	—	—	—
15	10·3	7·5	—	—	—
21	7·5	9·4	—	—	—
32	7·3	9·0	8·9	5·6	7·4
46	8·8	9·5	9·6	6·8	8·5
64	8·7	9·0	8·4	6·6	7·6
$C_{16:0}$ acid					
0	21·2	21·2	—	—	—
15	31·0	30·4	—	—	—
21	27·0	31·1	—	—	—
32	27·7	31·4	30·4	29·1	29·7
46	28·0	29·4	28·1	28·1	30·2
64	29·3	29·5	29·0	30·5	28·8
$C_{18:0}$ acid					
0	3·3	3·7	—	—	—
15	2·5	3·1	—	—	—
21	3·2	3·1	—	—	—
32	3·4	3·8	3·4	4·7	4·0
46	2·9	2·9	3·1	3·6	3·0
64	4·4	2·6	2·7	4·4	3·3

highly unsaturated fatty acids influences the turnover time of cholesterol (Boyd & Mawrer, 1959; Pinter, Miller & Hamilton, 1964). Possibly the greater availability of linoleic acid to the female rats owing to the greater concentration of carcass linoleic acid facilitates the removal of cholesterol out of the liver. It was the esters of oleic acid and palmitic acid and, to a smaller extent, of palmitoleic acid and eicosatrienoic acid that contributed to the higher amounts of liver cholesteryl esters in the male rat (Table 3). The appearance of much larger amounts of cholesteryl arachidonate in the plasma of these female rats compared with the males (R. L. Lyman, unpublished work) supports this hypothesis. Aftergood & Alfin-Slater (1965), on the basis of results with rats fed on a fat-free diet, have suggested that oestradiol may enhance esterification of cholesterol with the more unsaturated fatty acids and thus promote cholesterol transport and metabolism.

Liver cholesteryl ester amounts and fatty acid patterns of castrated rats given oil only resembled those of the male or testosterone-treated rats. There was no evidence that gonadectomy, under the conditions of our experiments, increased the esterification of cholesterol with saturated fatty acids, as indicated by Coleman *et al.* (1958) with animals fed on a fat-free diet.

Because of the small quantities of linoleic acid and arachidonic acid in the liver cholesteryl esters and triglycerides, these lipids would be of little importance to the animal as principal sources of EFA. Phospholipids, on the other hand, represented the largest part of the liver lipids (Fig. 4) and probably require and supply a large part of the arachidonic acid available to the animal (Fig. 5). The decrease in the amount of linoleic acid in this lipid fraction during the development of the EFA deficiency was negligible, and arachidonic acid actually increased slightly from initial levels. Since the quantity of this fatty acid, in contrast with its proportion, was the same for all groups, and was independent of liver and body size, it must represent a maximum amount of arachidonic acid converted from the limited stores of linoleic acid available from adipose tissues. The greater amounts of eicosatrienoic acid and palmitic acid in liver phospholipids of male rats seemed to arise therefore from increased need for phospholipids to support the faster growth and larger size of the male. The eicosatrienoic acid substituted principally for the arachidonic acid to meet the unsaturated fatty acid requirements of the additional phospholipids. That growth and the need for greater phospholipid synthesis rather than a direct hormonal effect were responsible for the greater increase in eicosatrienoic acid in the males is supported by the results from the castrated groups. The livers of the oestrogen-treated castrated rats were as large as those of intact males, and the amounts of eicosatrienoic acid and palmitic acid were also high and in the range of those of the male animals. In rapidly growing male rats a similar substitution of eicosatrienoic acid for arachidonic acid would be expected in other organs and tissues. The earlier onset of EFA-deficiency symptoms in male rats, as compared with females, could therefore be attributed to the more rapid growth of the male rat, with greater formation of unnatural phospholipids and subsequent impairment of normal cell-membrane functions. Formation of abnormal phospholipids during rapid growth could also explain the ease with which EFA deficiency may be induced in weanling rats, whereas adult animals are refractory.

The reasons for the larger livers of the oestradiol-treated castrated rats are not known. A similar dose of the hormone administered for 3 weeks to castrated males on normal diets did not cause any liver enlargement (Lyman *et al.* 1964). It may be that EFA deficiency makes this organ more sensitive to oestradiol. Consequently, even though growth of our hormone-treated groups was comparable with that of their respective intact controls, it is obvious that the hormone-injected animals were not entirely equivalent to intact males and females.

Administration of linoleic acid for only 1 day to either intact male or female rats resulted in almost complete reversal of the biochemical deficiency symptoms, including large increases in carcass linoleic acid and replacement of the liver phospholipid eicosatrienoic acid with arachidonic acid and of palmitic acid with stearic acid (Fig. 5). Apparently the eicosatrienoic acid or the phospholipids containing it are unstable in the presence of arachidonic acid. It cannot be decided on the basis of these findings, of course, whether the entire molecule or just the fatty acids are replaced. However, Collins (1960) and Harris, Robinson & Getz (1960) have reported two metabolically different types of lecithins in rat liver: one that contained predominantly palmitic acid and linoleic acid incorporated ^{32}P more rapidly than did a lecithin containing principally stearic acid and arachidonic acid. Collins (1962) has also shown that EFA-deficient rats incorporated nearly twice the amount of ^{32}P into liver lecithins as did control animals, and suggested that this might have been due to loss of the more stable lecithin containing stearic acid and arachidonic acid. In the present study, the eicosatrienoic acid seems to be associated more with palmitic acid than with stearic acid, in contrast with the naturally occurring arachidonic acid-containing lecithin. Thus it resembles the more metabolically active palmitic acid- and linoleic acid-containing lecithin.

It would be interesting to know if it is this phospholipid fraction that turns over so rapidly during EFA deficiency and is lost so quickly when arachidonic acid is made available.

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