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The effect of dose level of essential fatty acids upon fatty acid composition of the rat liver*

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SUMMARY

Rats were fed various levels of either ethyl linoleate, ethyl arachidonate, or ethyl linolenate. Weight gain, fat-deficiency status, and fatty acid composition of the liver lipids were determined.

Dietary linoleate, fed in excess of 1% of calories, maintained good growth and cured fat deficiency. Increasing amounts of dietary linoleate were stored in the liver lipids and converted into fatty acids of the linoleate family—20:4 and 22:4. The concentration of 20:3 was decreased. Dietary arachidonate cured fat deficiency three times more effectively than linoleate. Increasing amounts of dietary arachidonate were stored in liver lipids and converted to 22:5ω6. The level of 20:3 was lowered three times more effectively than when linoleate was fed. No fatty acids of the linolenate family were synthesized from linoleate or arachidonate.

Dietary linolenate did not support weight gain as efficiently as did linoleate or arachidonate. Fat-deficiency symptoms could not be cured completely. Increasing amounts of dietary linolenate increased the levels of fatty acids of the linolenate family; linolenic acid was stored, and 20:5, 22:5ω3, and 22:6 were synthesized from linolenate. The level of 20:3 was lowered in the same fashion as when linoleate or arachidonate was fed. The level of 20:4 was decreased with increasing amounts of dietary linolenate.

Experiments with radioactive labeled compounds have been of great value in studies concerned with the metabolism of essential fatty acids (EFA). Several basic metabolic conversions in this field, however, have been elucidated in feeding experiments with unlabeled materials. Early investigators have shown, by feeding of linoleate concentrates to rats, that linoleate is converted to arachidonate (1). Proof for this conversion was provided by Mead and coworkers (2) using C^{14}-labeled linoleic acid. Eicosatrienoic acid was found to appear in fat-deficient rats (1); and Fulco and Mead (3) showed that, in the absence of dietary EFA, eicosatrienoic acid was synthesized from C^{14}-labeled oleate.

The conversion of linolenic acid to the highly unsaturated fatty acids of 20 and 22 carbon atoms was established by Widmer and Holman (4) by feeding experiments. This study also showed that arachidonate cannot be synthesized from linolenate. The latter finding was confirmed by Mead and coworkers (5), who used C^{14}-labeled linolenate, and also by Klenk and Oette (6). The intermediate steps in the synthesis of docosahexaenoic acid from linolenic acid via eicosapentaenoic acid and docosapentaenoic acid were elucidated by investigations of Klenk and Mohrhauer (7), who fed synthetic C^{14}-labeled compounds to rats.

Most of the investigators quoted above studied the metabolism of EFA by making comparisons between animals fed fat-free diets and those fed single levels of an EFA. The analytical tools employed in some of the older studies, like alkaline isomerization and ozonolysis of mixtures of polyunsaturated fatty acids, do not allow one to distinguish between certain individual fatty acids of similar structure.

The present study was conducted to show the influence of various levels of intake of linoleic, arachidonic, and linolenic esters upon the fatty acid composi-

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tion of liver lipids of the rat. Gas-liquid chromatography (GLC), which permits a discriminating analysis of the entire range of tissue fatty acids, was employed in this work. Changes in concentrations of tissue fatty acids under the influence of varying amounts of single dietary EFA are interpreted in terms of fatty acid metabolism. For example, the concentration of a certain fatty acid of the liver lipids might increase with increasing amounts of dietary linolenate, but does not undergo any changes when linoleate or arachidonate are fed. Thus, the appropriate conclusion seems to be that the fatty acid in question is synthesized in the rat liver from linolenate.

EXPERIMENTAL METHODS

One hundred and eighty-four weanling, 27-day-old, male rats of the Sprague-Dawley strain were kept on a basic fat-free diet, the composition of which is given in Table 1. The daily intake of basic diet was recorded for each animal.

Ten groups of rats received daily supplements of highly purified ethyl linoleate (The Hormel Foundation, Austin, Minnesota) varying from 0.01 to 5% of total calories (Table 2). Analysis by GLC of the ethyl linoleate indicated it to be 98.3% pure. The only contaminant was 1.7% oleate.

Ten groups of rats were given diets supplemented with synthetic (9) ethyl arachidonate (20:4), varying from 0.01 to 5% of total calories. This product was obtained from Hoffmann La Roche, Nutley, New Jersey. It contained 81.8% arachidonate, and 8.8% "short-chain" and 9.7% "long-chain" impurities according to GLC analysis. Calculation of dietary arachidonate was based on the actual 20:4 content of the preparation. Santoquine (0.25%) was added as antioxidant.

Thirteen groups of rats were given diets supplemented with ethyl linolenate (The Hormel Foundation, Austin, Minnesota), varying from 0.01 to 10% of total calories. This preparation was found to be 97.3% pure. It contained less than 0.4% linoleate and five other minor unidentified impurites according to GLC analysis.

The ethyl esters of the fatty acids were administered orally by microsyringe. The amount fed was calculated in percentage of calories of the diet consumed by each animal. Groups fed more than 1% of calories as EFA were restricted in size to two to four animals because supplies of EFA were limited. All other groups consisted of six rats. Weight gain, dermal score (10), and fatty acid composition of the liver lipids were determined for each single animal. The results are reported as averages per group of rats. There are more groups in the low-calorie-intake range because previous experiments had shown that the most dramatic changes occurred in this region. Twenty-four animals were kept as controls without any supplements.

TABLE 1. COMPOSITION OF FAT-FREE DIET

Component	Weight
	%
Vitamin test casein	16
α-Cellulose	4
Sucrose	74
Wesson salt mixture	4
Vitamin* mixture	1
Choline chloride*	1

* Vitamins D_2, K, B_1, B_2, B_6, B_{12}, Ca-pantothenate, niacin, inositol, p-amino-benzoic acid, folic acid, biotin, and choline chloride were mixed into vitamin test casein in doses recommended by Glaxo Laboratories (8). One kilogram of the diet was mixed with 100 ml of a solution of 4 mg vitamin A acetate (1,000,000 i.u/g) and 280 mg vitamin E (α-tocopherol) in ether. The ether was evaporated before using the diet. All components of the diet but sucrose were obtained from Nutritional Biochemicals Corporation, Cleveland 28, Ohio.

After 100 days the animals were sacrificed by ether anesthesia. The livers were quickly removed and kept in saline solution at −20° until analyzed. The livers were homogenized and extracted with chloroform–methanol 2:1 according to Folch et al. (11). The lipids were transesterified by refluxing with 30 volumes of a 5% solution of HCl in methanol. All operations were conducted under nitrogen. The methyl esters were analyzed by GLC, using a Barber-Coleman Model 10 apparatus with argon-ionization detector. A 210-cm glass column of 5 mm i.d., packed with 20% ethylene-glycol-succinate polyester[1] (EGS) coated on Gaschrom P, 80–100 mesh[1] was used. The flow rate was 60 ml argon/min at an inlet pressure of 16 psi.

The inlet heater was kept at 270° and the detector cell at 250°. The esters with retention times shorter than that of 18:3 were chromatographed at 180° column temperature. Approximately 5 μl of a 10% solution of the methyl esters in petroleum ether was injected. The long-chain esters were analyzed on a second run at 200° by injecting approximately 30–50 μl into the chromatographic column.

The individual esters were identified by carbon number (12) and by internal standards wherever feasible. Authentic methyl esters of fatty acids (purity 95–99%), obtained from The Hormel Foundation,

[1] Obtained from Applied Science Laboratories, State College, Pennsylvania.

were used as internal standards. The mixtures of methyl esters were rechromatographed with the added standard, and the peak increased by the standard was identified. Quantification was carried out by triangulation. The fatty acid composition is reported as area per cent, a procedure justified in cases, such as this, where changes in concentration but not the absolute composition are to be measured.

RESULTS AND DISCUSSION

The average weight gain (Fig. 1) and average dermal score of fat deficiency (10) (Fig. 2) for each group of rats were plotted vs dietary EFA. The weight-gain curves and dermal-score curves permitted comparison of the results of the fatty acid analyses in this experiment with older investigations in which weight-gain data and deficiency scores were used as the only parameter to measure fat deficiency. Weight gain increased in almost the same pattern with increasing amounts of all three EFA, although the values were slightly higher for arachidonate and linoleate than for linolenate. An optimum for all three EFA with respect to weight gain seemed to be between 1 and 1.5% of calories. All rats given diets supplemented with EFA in excess of 1.5% of calories showed no further weight-gain increase. In fact, there seemed to be a slight decrease in weight gain, which, however, was inconsistent.

The fat-deficiency signs (i.e., necrotic tail and scaly feet) appeared in increasing severity in all animals fed linoleate at less than 0.6% of calories and arachidonate at less than 0.25% of calories. None of the animals given diets supplemented with linolenate was free of deficiency symptoms. Animals fed levels of EFA at 5 and 10% of calories developed severe dermal symptoms similar to those of fat-deficient rats. This was most striking in rats fed the highest level of arachidonate. It is likely that massive oral doses of polyunsaturated fatty acids exceed the capacity of the antioxidant content of the diet and are subject to oxidation before absorption. The resultant vitamin-E deficiency and the effect of the fatty acid oxidation products (13) could account for the development of dermal symptoms. This reversal of phenomenon is not reflected in the fatty acid composition of tissue.

In general, both weight-gain data and dermal scores obtained in this experiment confirm and extend what is known about the influence of EFA upon fat-deficiency symptoms since the experiments of Burr and Burr (14). Fat-deficiency symptoms are cured by less than 0.5% of calories of dietary linoleate, and by even smaller levels of arachidonate—about 0.25% of

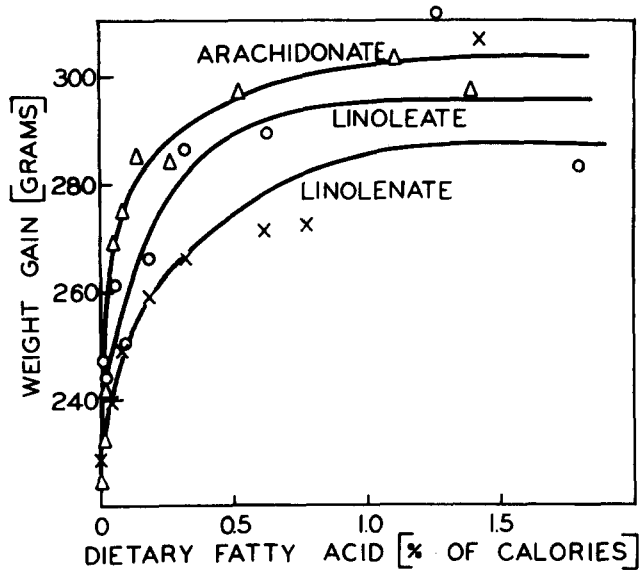

FIG. 1. The effect of varying levels of dietary EFA upon weight gain of rats during the 100-day period of the experiment.

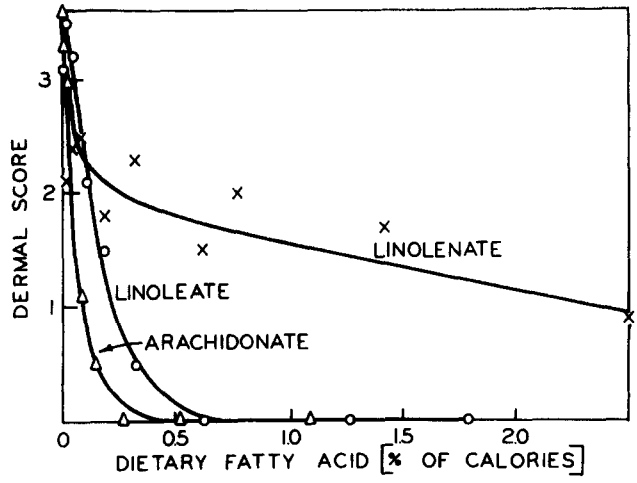

FIG. 2. The effect of varying levels of dietary EFA upon dermal score (the degree of fat-deficiency symptoms on feet and tail of the rats [10]).

calories. Linolenate has some beneficial influence upon dermatitis, but cannot cure fat deficiency completely. However, normal weight gain can be observed when any of the three EFA is fed at a level of approximately 1.5% of calories.

The fatty acid composition of the liver lipids as analyzed by GLC is given in Table 2. The saturated fatty acids (myristic (14:0), palmitic (16:0), and stearic (18:0) acids) were identified by carbon number and comparison with authentic standards. Throughout the experiment, no consistent change was observed in the concentration of these saturated acids, indicating that their synthesis is not significantly affected by

TABLE 2. FATTY ACID COMPOSITION OF

Dietary EFA (% of Cal.)	No. of Animals	14:0	16:0	16:1	18:0	18:1	18:2	18:3
None	24	0.94 ± 0.04	27.22 ± 0.43	11.54 ± 0.42	9.56 ± 0.29	40.30 ± 0.58	1.07 ± 0.08	<0.01
Linoleate								
0.009	6	0.91 ± 0.18	26.04 ± 2.16	11.01 ± 1.56	8.75 ± 1.36	40.89 ± 1.79	1.31 ± 0.26	<0.01
0.02	6	0.83 ± 0.20	25.36 ± 0.97	11.98 ± 1.73	9.50 ± 1.92	41.91 ± 1.22	1.22 ± 0.45	<0.01
0.05	6	0.92 ± 0.12	24.12 ± 1.93	12.06 ± 1.93	10.38 ± 1.85	40.32 ± 2.74	1.44 ± 0.36	<0.01
0.10	6	0.94 ± 0.43	23.76 ± 2.89	12.18 ± 2.58	10.34 ± 2.27	41.49 ± 2.09	2.03 ± 0.33	<0.01
0.18	6	0.78 ± 0.22	23.02 ± 2.18	9.42 ± 1.80	14.78 ± 2.22	35.73 ± 3.44	2.20 ± 0.24	<0.01
0.32	6	0.74 ± 0.21	23.76 ± 3.05	9.84 ± 1.37	11.13 ± 1.80	40.24 ± 2.85	2.25 ± 0.42	<0.01
0.61	6	1.28 ± 0.22	25.59 ± 2.24	10.85 ± 1.94	10.99 ± 1.55	38.42 ± 2.00	2.66 ± 0.64	<0.01
1.26	2	1.26	25.64	9.90	12.50	34.50	3.02	<0.01
1.79	2	1.38	27.81	10.83	12.14	27.95	4.28	<0.01
4.87	2	1.00	25.46	7.95	13.60	27.54	5.70	<0.01
Arachidonate								
0.007	6	0.78 ± 0.30	24.14 ± 1.68	8.63 ± 2.58	11.17 ± 1.75	41.01 ± 3.11	1.36 ± 0.19	0.01
0.019	6	0.62 ± 0.16	24.65 ± 2.87	10.56 ± 1.40	10.71 ± 1.69	41.39 ± 2.49	0.88 ± 0.38	<0.01
0.04	6	1.27 ± 0.49	26.24 ± 1.95	10.57 ± 0.84	10.23 ± 0.75	39.07 ± 2.11	1.40 ± 0.24	<0.01
0.08	6	0.86 ± 0.21	24.24 ± 1.52	10.21 ± 1.00	11.19 ± 1.10	38.01 ± 1.70	1.08 ± 0.22	<0.01
0.14	6	0.88 ± 0.58	23.43 ± 1.96	9.44 ± 2.32	11.61 ± 2.99	38.37 ± 3.99	1.20 ± 0.35	<0.01
0.27	6	0.99 ± 0.17	23.50 ± 2.79	9.20 ± 2.14	13.92 ± 2.72	32.91 ± 1.55	1.00 ± 0.51	<0.01
0.52	6	0.99 ± 0.27	25.20 ± 2.28	9.67 ± 1.31	12.53 ± 2.81	33.08 ± 3.75	0.96 ± 0.17	<0.01
1.08	2	0.70	25.26	9.04	12.57	26.64	0.48	<0.01
1.39	2	0.56	25.06	7.53	13.30	21.20	0.42	<0.01
3.75	2	0.50	23.14	5.06	13.34	16.97	0.54	<0.01
Linolenate								
0.009	6	0.62 ± 0.18	24.98 ± 0.86	11.05 ± 1.83	10.88 ± 1.89	41.20 ± 2.91	0.52 ± 0.09	0.01
0.02	6	0.90 ± 0.09	25.16 ± 0.96	11.80 ± 1.34	10.79 ± 1.93	40.53 ± 2.78	0.55 ± 0.17	0.02 ± 0.01
0.04	6	1.00 ± 0.24	24.89 ± 2.11	13.01 ± 2.28	10.45 ± 2.22	40.69 ± 1.96	0.47 ± 0.13	0.02 ± 0.01
0.08	6	1.11 ± 0.26	26.31 ± 3.32	14.18 ± 2.37	9.85 ± 1.65	39.30 ± 4.24	0.44 ± 0.13	0.03 ± 0.01
0.18	6	0.85 ± 0.20	23.62 ± 1.99	13.24 ± 1.35	12.56 ± 2.08	39.08 ± 2.07	0.49 ± 0.10	0.02 ± 0.01
0.32	6	1.10 ± 0.32	23.86 ± 1.39	13.93 ± 1.54	12.23 ± 1.61	38.44 ± 1.84	0.41 ± 0.05	0.21 ± 0.09
0.61	6	0.96 ± 0.26	25.61 ± 1.63	14.82 ± 1.43	11.18 ± 2.47	37.41 ± 3.40	0.42 ± 0.10	0.22 ± 0.06
0.77	4	0.92 ± 0.11	28.85 ± 2.14	12.93 ± 1.96	12.16 ± 2.99	35.03 ± 2.79	0.40 ± 0.16	0.30 ± 0.16
1.42	4	0.78 ± 0.07	30.00 ± 1.90	11.93 ± 0.65	9.74 ± 1.05	36.36 ± 1.40	0.50 ± 0.20	1.03 ± 0.19
2.56	4	0.79 ± 0.18	29.40 ± 3.26	12.26 ± 1.04	12.33 ± 1.07	28.64 ± 2.29	0.39 ± 0.08	1.57 ± 0.56
3.56	4	0.69 ± 0.04	29.55 ± 0.52	10.53 ± 1.29	13.94 ± 0.12	24.60 ± 1.33	0.38 ± 0.12	2.00 ± 0.91
4.14	4	0.68 ± 0.10	27.37 ± 2.94	10.67 ± 0.87	11.64 ± 0.25	28.00 ± 2.45	0.30 ± 0.06	5.40 ± 3.72
9.42	2	0.51	30.30	7.85	13.42	18.23	0.27	5.72

the intake of EFA below 5% of calories. Dhopeshwarkar and Mead (15) reported a significant increase in the concentration of stearic acid in tissues from guinea pigs fed a high percentage of corn oil. The same observation was made for mice by Tove and Smith (16). In both cases the content of linoleic acid in the diet exceeded the amounts fed in the present experiment.

Palmitoleic (16:1) and oleic (18:1) acids were identified by internal standards. The concentration of oleic acid showed a significant increase with decreasing amounts of all three dietary EFA, whereas there was only a slight parallel increase in the level of palmitoleic acid at low EFA intakes (Figs. 3, 4). However, all changes were most pronounced in the experiment with dietary arachidonate and were least significant for dietary linolenate. Increased amounts of monoenoic fatty acids in livers of fat-deficient rats have been reported by Mead (17). It seems that the accelerated synthesis of monoenoic fatty acids under these conditions maintains a certain degree of total unsaturation and physical properties of the tissue lipids that, under normal conditions, are provided by dietary EFA and their metabolites.

Linoleic (18:2) and linolenic (18:3) acids have also been identified by internal standards. All livers analyzed, even from animals on the fat-free diet, contained 18:2, which probably is a mixture of isomers

LIVER LIPIDS* (area percentage on GLC)

Dietary EFA (% of Cal.)	No. of Animals	20:3	20:4	20:5	22:2	22:5ω6	22:5ω3	22:6
None	24	6.54 ± 0.34	1.79 ± 0.13	<0.10	0.24 ± 0.02	0.23 ± 0.03	<0.01	0.30 ± 0.02
Linoleate								
0.009	6	7.61 ± 2.07	2.36 ± 0.66	<0.10	0.26 ± 0.10	0.42 ± 0.14	<0.01	0.42 ± 0.17
0.02	6	6.60 ± 1.81	1.87 ± 0.52	<0.10	0.15 ± 0.07	0.27 ± 0.16	<0.01	0.27 ± 0.12
0.05	6	7.12 ± 1.37	2.54 ± 0.81	<0.10	0.28 ± 0.13	0.42 ± 0.16	<0.01	0.41 ± 0.19
0.10	6	5.56 ± 1.30	2.82 ± 0.47	<0.10	0.16 ± 0.05	0.34 ± 0.19	<0.01	0.15 ± 0.08
0.18	6	7.75 ± 1.33	4.99 ± 1.00	<0.10	0.32 ± 0.12	0.66 ± 0.26	<0.01	0.33 ± 0.26
0.32	6	5.80 ± 1.96	4.80 ± 1.23	<0.10	0.21 ± 0.13	0.72 ± 0.36	<0.01	0.30 ± 0.16
0.61	6	3.44 ± 0.79	5.46 ± 0.73	<0.10	0.22 ± 0.05	0.79 ± 0.31	<0.01	0.31 ± 0.08
1.26	2	3.08	8.46	<0.10	0.16	0.97	<0.01	0.38
1.79	2	1.17	9.59	<0.10	0.34	2.07	<0.01	0.41
4.87	2	0.84	14.70	<0.10	0.05	2.24	<0.01	0.20
Arachidonate								
0.007	6	8.79 ± 2.36	2.81 ± 0.88	<0.10	0.35 ± 0.06	0.48 ± 0.11	<0.01	0.50 ± 0.10
0.019	6	7.64 ± 2.22	2.33 ± 0.73	<0.10	0.27 ± 0.08	0.45 ± 0.12	<0.01	0.34 ± 0.13
0.04	6	7.10 ± 1.08	2.98 ± 0.43	<0.10	0.28 ± 0.04	0.60 ± 0.22	<0.01	0.26 ± 0.04
0.08	6	6.88 ± 0.89	5.50 ± 0.84	<0.10	0.28 ± 0.06	1.28 ± 0.34	<0.01	0.44 ± 0.22
0.14	6	6.12 ± 2.55	6.92 ± 0.99	<0.10	0.39 ± 0.08	1.26 ± 0.46	<0.01	0.36 ± 0.15
0.27	6	4.79 ± 1.21	11.25 ± 1.43	<0.10	0.20 ± 0.07	1.98 ± 0.50	<0.01	0.32 ± 0.15
0.52	6	2.13 ± 0.72	12.71 ± 4.39	<0.10	0.38 ± 0.24	1.90 ± 0.67	<0.01	0.44 ± 0.16
1.08	2	0.61	20.04	<0.10	0.06	2.22	<0.01	0.18
1.39	2	0.42	26.22	<0.10	0.04	2.87	<0.01	0.18
3.75	2	0.23	32.46	<0.10	0.11	2.31	<0.01	0.14
Linolenate								
0.009	6	7.23 ± 1.89	1.97 ± 0.65	0.09 ± 0.02	0.22 ± 0.07	0.27 ± 0.05	<0.01	0.46 ± 0.13
0.02	6	6.09 ± 0.72	2.16 ± 0.65	0.13 ± 0.05	0.17 ± 0.01	0.35 ± 0.10	0.01	0.76 ± 0.30
0.04	6	5.46 ± 1.60	1.74 ± 0.68	0.18 ± 0.09	0.14 ± 0.05	0.19 ± 0.07	0.03 ± 0.32	1.15 ± 0.46
0.08	6	4.86 ± 1.23	1.28 ± 0.64	0.30 ± 0.14	0.11 ± 0.05	0.16 ± 0.10	0.03 ± 0.05	1.50 ± 0.65
0.18	6	4.10 ± 0.64	1.08 ± 0.23	0.74 ± 0.37	0.06 ± 0.02	0.10 ± 0.07	0.22 ± 0.19	3.02 ± 1.09
0.32	6	3.24 ± 0.66	0.73 ± 0.15	1.20 ± 0.30	0.06 ± 0.01	0.11 ± 0.06	0.25 ± 0.23	3.62 ± 0.88
0.61	6	1.88 ± 0.70	0.65 ± 0.20	1.78 ± 0.55	0.05 ± 0.03	0.08 ± 0.05	0.45 ± 0.24	3.96 ± 1.68
0.77	4	1.58 ± 0.23	0.49 ± 0.15	2.28 ± 0.82	0.05 ± 0.03	0.07 ± 0.04	0.62 ± 0.34	3.75 ± 1.37
1.42	4	0.84 ± 0.06	0.46 ± 0.12	3.30 ± 0.35	0.02 ± 0.02	0.05 ± 0.04	0.92 ± 0.24	3.41 ± 0.56
2.56	4	0.48 ± 0.15	0.69 ± 0.24	5.56 ± 1.29	0.07 ± 0.04	0.13 ± 0.07	2.25 ± 1.16	4.96 ± 1.53
3.56	4	0.47 ± 0.09	0.81 ± 0.04	7.55 ± 0.59	0.09 ± 0.03	0.14 ± 0.04	3.10 ± 0.57	5.61 ± 0.37
4.14	4	0.31 ± 0.15	0.74 ± 0.07	7.18 ± 0.32	0.06 ± 0.04	0.11 ± 0.09	2.58 ± 0.32	4.54 ± 0.81
9.42	2	0.19	1.03	12.18	0.13	0.17	3.88	5.31

* Mean values ± standard deviation.

including linoleate and dienes derived from oleic and palmitoleic acids (3, 6). The concentration of linoleate (18:2) in liver lipids was elevated only when linoleate was fed. With increasing amounts of dietary 20:4 and 18:3, a slight decrease in 18:2 levels was noted. The content of linolenate in liver lipids was extremely small, and in most cases not even measurable, in rats fed linoleate and arachidonate. Linolenate increased significantly, however, in the liver lipids of animals given diets supplemented with linolenate.

Recently, Witting et al. (18) reported very similar results for the fatty acid composition of lipids from liver mitochondria and erythrocytes of rats. 18:3 could not be detected in these tissues when diets containing linoleate were fed, whereas the 18:2 content of these tissues could be altered by different amounts of dietary linoleate. These authors found it very difficult to separate linolenic acid (18:3) from eicosaenoic acid (20:1) on an EGS column under the conditions they used. We were able to obtain a very good separation of these two components by using "Hi Eff-2b".[1]

The most striking differences in fatty acid composition of the rat liver under the influence of different amounts of the three dietary EFA occurred in the highly unsaturated acids of C_{20} and C_{22} chain lengths. Eicosatrienoic (20:3) and eicosatetraenoic (20:4) acids were isolated by preparative GLC for further identification. Analysis by GLC of the isolated sample

was carried out to assure the purity of the preparative fraction. Analysis of a hydrogenated aliquot verified the chain length of the individual ester. Reductive ozonolysis was carried out to determine the double bond position. The resulting aldehydes and half-ester aldehydes were identified by analytical GLC.[2]

The 20:3 fraction consisted mainly of 5,8,11-eicosatrienoic acid with small amounts of the 7,10,13-isomer. Both trienoic acids have been shown to appear in liver lipids of fat-deficient rats (2,6). The content of eicosatrienoic acids in liver lipids was decreased by increasing amounts of all three dietary EFA (Fig. 5). Under the influence of dietary linoleate, the amounts of 20:3 decreased rapidly, and the curve leveled off beyond 1.9% of calories. Dietary arachidonate achieved the same level of 20:3 concentration at an intake of only 0.65% of calories, and thus proved to be about three times more efficient than linoleate. This agrees well with results of Turpeinen (19), who found arachidonate to be three times more efficient than linoleate on the basis of fat-deficiency symptoms using the method of "minimum dose for maximum response." Other investigators found arachidonic acid to be less, or more than three times as effective as linoleate (20, 21, 22). The evaluation of *deficiency symptoms* in the present experiment suggests arachidonate to be two-and-a-half to three times more efficient. Our *chemical data* indicate also that arachidonate is three times more effective than linoleate as EFA. Linolenate lowered the 20:3 level in almost the same fashion as did linoleate and arachidonate; in fact, it was even *more* efficient than linoleate in depressing the level of 20:3.

The depression of 20:3 content by all three EFA indicates that the organism prefers polyunsaturated fatty acids of the linoleate or linolenate types; i.e., acids having the first double dond at the 6th or 3rd carbon atoms counting from the methyl group. There may, indeed, be a competition between exogenous linoleate or linolenate and endogenous oleate or palmitoleate for enzyme sites responsible for their conversion to more highly unsaturated fatty acids. When linoleate or linolenate are available, their affinity for the enzymatic systems effectively prevents the synthesis of 20:3. Oleate is converted into 5,8,11-eicosatrienoic acid (20:3) only when dietary EFA are limited. Similarly, 7,10,13-eicosatrienoic acid is synthesized from palmitoleate. Thus the organism is supplied with highly unsaturated fatty acids. But the progress of fat-deficiency indicates that 20:3 cannot meet all the needs of the animal for polyunsaturated fatty acids.

[2] These procedures are described by Privett and Nickell, *J. Am. Oil Chemists' Soc.*, **39**: 414, 1962.

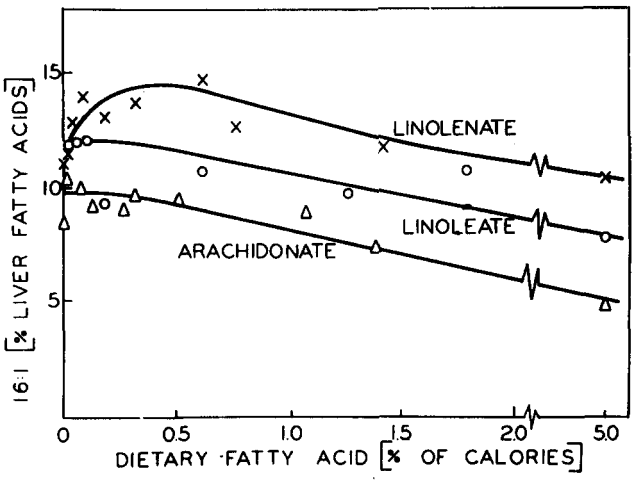

FIG. 3. The effect of varying levels of dietary EFA upon concentration of 16:1 of liver lipids.

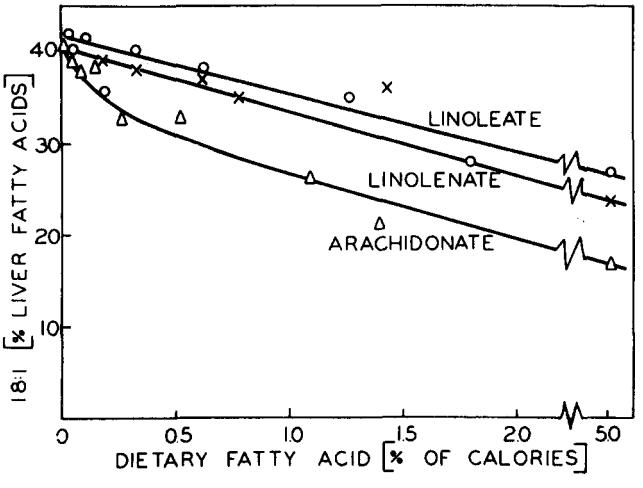

FIG. 4. The effect of varying levels of dietary EFA upon concentration of 18:1 of liver lipids.

Eicosatetraenoic acid (20:4) from liver lipids consists mainly of arachidonic acid (5,8,11,14-eicosatetraenoic acid) and small amounts of 4,7,10,13-eicosatetraenoic acid as determined by reductive ozonolysis. In fat-deficient animals, the latter increased to almost one-quarter of the 20:4. The concentration of total 20:4 increased in proportion to dietary linoleate (Fig. 6). The level of 20:4, at approximately 5% of caloric intake, exceeded the level in fat-deficient rats by a factor of eight. Arachidonate, fed at high-caloric-intake levels, was stored in the liver lipids to make up almost one-third of all liver fatty acids. With increasing amounts of dietary 18:3, the concentration of arachidonate decreased. Recently, it has been reported that linseed oil in the diet depresses the concentration of arachidonate in the lipids of chicken liver (23). The influence of dietary linolenate upon the synthesis of

arachidonate in the rat liver is currently under investigation in our laboratory.

Eicosapentaenoic acid (20:5), docosapentaenoic acid (22:5ω3), and docosahexaenoic acid (22:6) have been identified by comparison with internal standards, which were isolated from fish oils.[3] They were 5,8,11,-14,17-eicosapentaenoic acid and 7,10,13,16,19-docosapentaenoic acid from menhaden oil, and 4,7,10,13,16,19-docosahexaenoic acid from tuna oil. In all cases where linoleate and arachidonate were fed, the 20:5 and 22:5ω3 contents of the liver lipids were extremely low, and were estimated to be less than 0.1% and 0.01%, respectively. The values for docosahexaenoic acid were significantly higher but did not show any consistent changes with increasing amounts of dietary linoleate and arachidonate. Feeding linolenate, however, dramatically increased all three of the above fatty acids (Fig. 7). Approximately 10% of calories of linolenate in the diet increased 20:5, 22:5ω3, and 22:6 in liver lipids more than 100, 100, and 10 times, respectively, in comparison with the amounts present in rats not supplemented with linolenate. Thus, it has been shown that, in this experiment, these pentaenoic and hexaenoic fatty acids were synthesized only from linolenate. Their structures, therefore, can most likely be deduced by the simple rules of chain lengthening by acetyl groups and by dehydrogenation in the divinyl-methane rhythm (skipped double bonds) between the existing unsaturation and the carboxyl groups. Therefore, the pentaenes and hexaene synthesized from linolenate should be 5,8,11,14,17-eicosapentaenoic, 7,10,13,16,19-docosapentaenoic, and 4,7,10,13,16,19-docosahexaenoic acids. The polyunsaturated fatty acids of the linolenate type, exceeding in some cases the amounts of arachidonate synthesized from similar amounts of dietary linoleate, do not provide the functions of the linoleate-type polyunsaturated fatty acids; not all deficiency symptoms can be cured by dietary linolenate (Figs. 1, 2).

Docosadienoic acid (22:2) has been designated by carbon number only, and the identification is tentative. No significant and consistent changes in concentration of 22:2 could be observed throughout the experiment.

An ester having a calculated carbon number of 25.2 on an EGS column was tentatively identified as 22:4 by extrapolation of retention times of methyl 7,10,13,-16,19-docosapentaenoate (C = 25.7) and methyl 4,7,10,13,16,19-docosahexaenoate (C = 26.3) standards. However, when this substance was isolated and its structure determined,[4] it was found to be methyl

[3] The isolation procedures and analyses of these fatty acids will be published by Privett and Nickell of this institute.

[4] Unpublished results of J. Rahm and R. T. Holman.

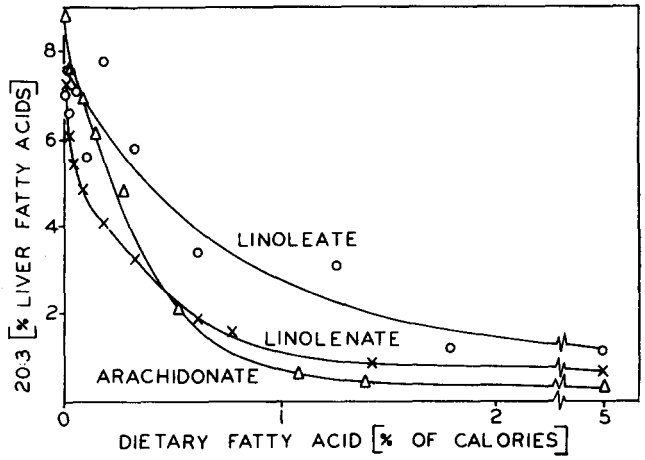

FIG. 5. The effect of varying levels of dietary EFA upon concentration of 20:3 of liver lipids.

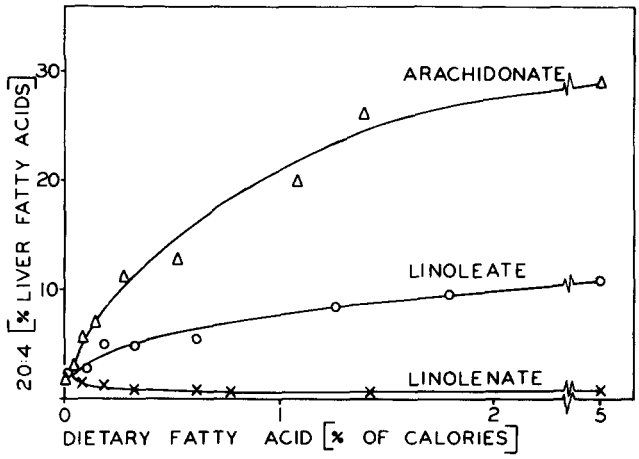

FIG. 6. The effect of varying levels of dietary EFA upon concentration of 20:4 of liver lipids.

4,7,10,13,16-docosapentaenoate, the pentaene of the linoleate family. In order to distinguish the two isomers of 22:5 we have used the suffixes ω3 and ω6 in the shorthand formulae to indicate at which carbon atom the first double bond from the ω carbon lies. Thus 22:5ω3 is the linolenate type pentaene and 22:5ω6 is the linoleate type docosapentaene. The concentrations of 22:5ω6 increased with dietary linoleate and arachidonate (Fig. 8) in almost the same pattern in liver lipid as did arachidonate (Fig. 6). Dietary linolenate did not give rise to 22:5ω6. Thus we conclude that the fatty acid is synthesized from linoleic acid via arachidonic acid by chain lengthening and dehydrogenation. This conclusion is substantiated by findings of Klenk and Mohrhauer (7), who showed that 22:5ω6 and 22:6 are synthesized from linolenate via 8,11,14,17–20:4 in analogy to the conversion demonstrated here. With increasing amounts of dietary

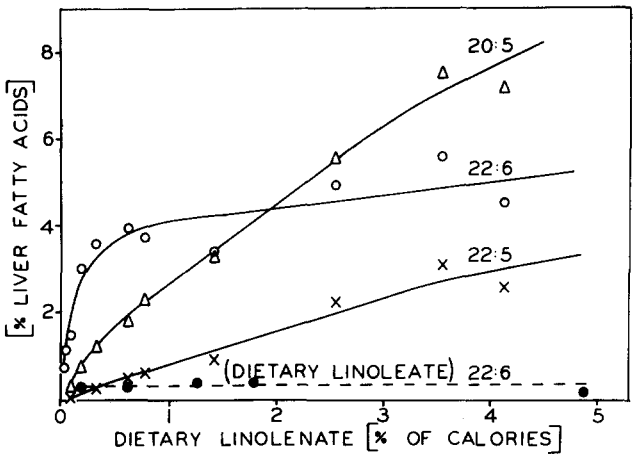

FIG. 7. The effect of varying levels of dietary linolenate upon concentration of 20:5, 22:5ω3, and 22:6 of liver lipids. The dotted line shows the effect of dietary *linoleate* upon the concentration of 22:6.

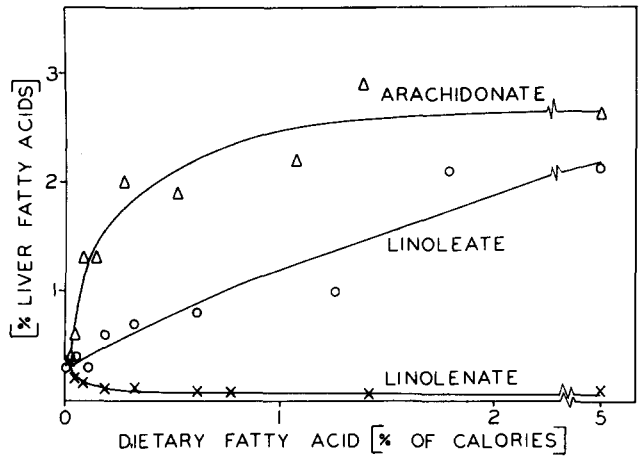

FIG. 8. The effect of varying levels of dietary EFA upon concentration of 22:5ω6 in liver lipids.

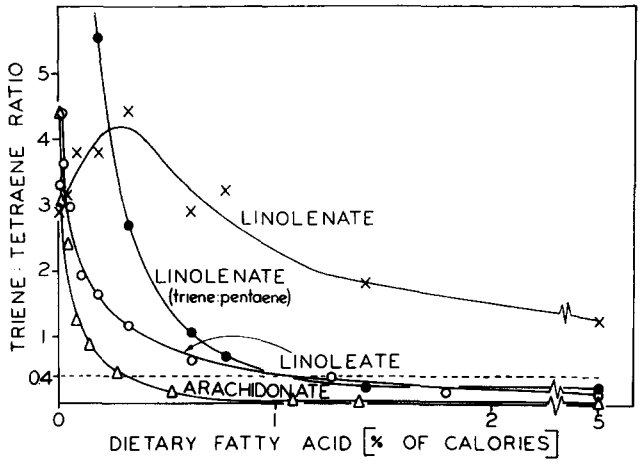

FIG. 9. The effect of varying levels of dietary EFA upon the ratio of 20:3 and 20:4 of liver lipids. Solid circles show the effect of dietary linolenate upon the ratio of 20:3 and 20:5. The dotted line represents a level of the triene/tetraene ratio below which the metabolism of EFA is considered to be normal.

linolenate, a slight decrease of 22:5ω6, in analogy to the performance of 20:4, can be noted.

It has recently been shown by Holman (24), that the ratio of trienoic to tetraenoic acids can be used to indicate the degree of EFA deficiency in rats and other species (25). In those earlier experiments, mixtures of natural fats were fed, and the analyses of tissue lipids were carried out by alkaline isomerization. That technique does not distinguish between isomeric trienes and tetraenes, nor between polyenes of different chain length. In the present experiments, highly purified single essential fatty acids were fed. The analyses of tissue fatty acids by GLC made it possible to measure specifically the pronounced changes in concentrations of 20:3 and 20:4. The curve relating the triene/tetraene ratio to dietary linoleate was also constructed from data obtained by alkaline isomerization analyses of the same liver lipids. This curve was almost identical to those found in the original experiment (24) and the one reported here (Fig. 9). Therefore, the triene/tetraene ratio, or the ratio of 20:3 to 20:4, proved to be a useful parameter for describing linoleate metabolism.

Below a triene/tetraene ratio of 0.4, no major changes in the curves occur, which corresponds to a normal EFA status in rats. This level of 0.4 is reached at a linoleate intake of 1% of calories, which is equivalent to a daily supplement of approximately 40 mg. linoleate per rat. One per cent of calories represents a level of linoleate below which the normal metabolism of polyunsaturated fatty acids no longer persists. Below this level, normal conversion of linoleate to arachidonate does not appear to take place at a sufficient rate, and the 20:3 synthesis from oleate and palmitoleate becomes predominant.

The curve relating the triene/tetraene ratio to dietary arachidonate reaches the 0.4 level at 0.3% of calories, which indicates again that arachidonate is approximately three times more effective as EFA than is linoleate. The triene/tetraene ratio reaches lower levels than in the case of dietary linoleate, but the two curves follow the same pattern.

The plot of triene/tetraene ratio vs dietary linolenate, however, is of a different type. The concentration of 20:4 does not change with increasing amounts of dietary linolenate, whereas the 20:3 decreases in the same fashion as when linoleate or arachidonate is fed. By using the ratio of eicosatrienoic acid (20:3) to eicosapentaenoic acid (20:5) instead, a curve was obtained, which is very similar to the curves relating the

triene/tetraene ratio vs dietary linoleate or arachidonate. This triene/pentaene ratio might be used to describe the metabolic status of the rat in regard to linolenate metabolism. A triene/pentaene ratio smaller than 0.4 indicates that the synthesis of the highly unsaturated fatty acids of the linolenate type is sufficient to replace the synthesis of 20:3 from oleate. The level of 0.4 is reached at a dietary intake that also supports maximum growth (Fig. 1). However, both the triene/tetraene and triene/pentaene ratios are derived for the metabolism of pure dietary linoleate or arachidonate and linolenate, respectively. Since all three EFA have similar activities in lowering the levels of 20:3 of deficient rats, the interactions of these three EFA must be studied before the proposed ratios can be used as strict indicators of the overall EFA status of an animal. Experiments to evaluate these interrelationships are currently under way in this laboratory.

The basic accomplishment of the present experiment is the description of the influence of carefully measured doses of highly purified EFA upon the composition of the liver lipid fatty acids as analyzed by employing the discriminating method of GLC. The previously known relationships between dietary EFA and weight and dermal symptoms in fat deficiency have now been related in a more precise manner to specific biochemical changes in tissue fatty acids. These relationships can be used also to estimate more precisely the EFA requirement of the rat and to develop prediction equations, which allow calculation of the dietary EFA intake from tissue analysis. The estimation of EFA requirement and the prediction equations for dietary EFA will be treated in a forthcoming publication.

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