Fatty acid synthesis during early linoleic acid deficiency in the mouse

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SUMMARY The capacity of liver to synthesize long-chain fatty acids is greatly enhanced during early linoleate deficiency in mice. The enzymes catalyzing the synthesis of fatty acids from acetate or malonyl CoA were measured directly in soluble fractions isolated from liver.

The period of dietary restriction of linoleate is also accompanied by a rapid fall in the relative linoleate content of the liver; by marked changes in the composition of the fatty acids in liver; and by an accumulation of liver triglyceride.

KEY WORDS liver · fatty acid biosynthesis · linoleic acid · linoleic acid deficiency · fatty acid synthetase · fatty acid composition · acetyl CoA carboxylase · fatty liver · mouse

PREVIOUS REPORTS from this laboratory have emphasized the variation in activity of the enzymes catalyzing the synthesis of long-chain fatty acids from acetate or malonyl CoA in liver during periods of alloxan diabetes (1–3), starvation (2–4), and lipid-free alimentation (3–5). The enzyme determinations were made in partially purified, soluble fractions of liver cytoplasm such that the influence of ancillary enzyme systems which may support fatty acid synthesis in the intact cell (e.g., TPNH generation) was avoided (although the effect of enzyme-bound cofactors or inhibitors was not excluded). By this means it was established that in starvation and diabetes the levels of the enzymes directly catalyzing the synthesis of saturated fatty acids were greatly depressed, but that the synthetic activities exceeded normal values 10-fold in livers of animals maintained on a fat-free diet. These changes in enzyme activity reflected the capacity of intact liver tissue to incorporate acetate into fatty acids (6–9).

In the present investigation it was found that fatty acid synthesis from acetate or malonyl CoA by soluble isolated fractions of liver was greatly accelerated in young mice maintained on a fat-free diet (3, 10–12). Under conditions of ad lib. feeding, limited supplementation of the fat-free diet with saturated triglycerides and cholesterol did not restore the normal enzyme levels, whereas supplementation with fats containing linoleic acid did. Elevation of the activity levels of the enzymes catalyzing fatty acid synthesis in liver was coincident with a profound relative depletion of linoleate in the fatty acids of liver lipids (12). The rapid onset of the latter changes in liver indicated that these may be among the earliest metabolic alterations in linoleic acid deficiency.

METHODS

Animals

Male mice, 4 weeks of age and weighing 15–20 g, (C57Bl/6J strain from Jackson Memorial Laboratory, Bar Harbor, Maine) were fed a balanced stock diet for several days after arrival. The mice were then separated into groups and fed the various diets described in the text. Food and water were accessible ad lib.

Two principal diets were employed: the balanced stock diet (Rockland, A. E. Staley Mfg. Co., Decatur, Ill.) and the fat-free diet (Nutritional Biochemicals Co., Cleveland, Ohio). The latter was employed directly or supplemented with various lipids. The composition of the fat-free diet is given in Table 1. The stock diet contained approximately 4% fat by weight. The fatty acid composition of the stock diet, the corn oil, and coconut oil used for supplementation to the fat-free diet are presented in Table 2. The amount of supplementary lipid is expressed in terms of percentage by weight of the dry

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TABLE 1 COMPOSITION OF FAT-FREE DIET

Fat-free, vitamin-free casein	21.10%
Alphacel cellulose	16.45%
Sucrose	58.45%
Salt mixture USP XIV	4.00%
Vitamin Supplement:	
Choline chloride	6.05 mg/kg
Nicotinic acid	0.605 "
Inositol	0.305 "
Vitamin A concentrate (200,000 units/g)	10.0 μg/kg
α-Tocopherol	22.7 "
Menadione	2.3 "
Thiamine hydrochloride	22.2 "
Pyridoxine hydrochloride	22.2 "
Riboflavin	22.2 "
Calcium pentothenate	45.5 "
Ascorbic acid*	900 mg/kg
Folic acid*	2 "
Vitamin B_{12}*	27 μg/kg

* Vitamins added to commercial diet.

fat-free diet. During the relatively short experimental periods employed in this study there were no consistent differences in the body weights of the mice (of the same age) fed the fat-free or the linoleic acid-free diet (with or without supplementation), in comparison with each other or with the mice receiving the balanced stock diet (see Table 3).

Enzyme Preparation

At selected time intervals, mice were killed by a blow on the head and exsanguinated. The freshly extirpated livers from a group of 3–20 identically treated mice were pooled and homogenized with 3–4 volumes of 0.1 M potassium phosphate buffer (pH 7.5) in a Teflon–glass homogenizer for a period of less than 1 min at 0–4°. The homogenate was centrifuged at 54,000 × *g* for 100 min (Spinco model L ultracentrifuge). The clear supernatant fraction was removed by pipette in such a way that contamination with the sediment or the overlying lipid layer was avoided. The whole supernatant solution or the precipitate obtained from it at 40% saturation with ammonium sulfate (the P0–40 fraction) (2, 13) was used in the enzyme assays. The enzymes catalyzing the synthesis of the saturated long-chain fatty acids (acetyl CoA carboxylase and fatty acid synthetase) are quantitatively recovered in these fractions (13, 17). The fatty acid product in this assay contains over 80% of palmitic acid (16). Myristic, lauric, and stearic acids, in that order, make up the rest. Unsaturated fatty acids are not synthesized, nor are fatty acids oxidized (16) by these soluble liver preparations. Protein concentration was determined by the biuret reaction (14).

TABLE 2 FATTY ACID COMPOSITION OF DIETS

Fatty Acid	Stock Diet*	Corn Oil†	Coconut Oil‡
		%	
8:0			7.2
10:0	<0.1	5.0	6.8
12:0			19.7
14:0	0.8	0.6	17.2
16:0	21.4	11.7	15.4
16:1	1.5	<0.1	0.5
18:0	4.4	1.2	24.2
18:1	31.2	26.6	8.8
18:2	39.3	54.5	<0.1
18:3	1.3	<0.1	<0.1
20:4	<0.1	<0.1	<0.1

* Rockland, A. E. Staley Mfg. Co., Decatur, Ill.
† Mazola brand, Corn Products, Argo, Ill.
‡ Durkee brand (Hydrol 110), Chicago, Ill.

Enzyme Assays

Fatty acid synthesis was determined by one or more of the following assays: the rate of acetate-1-C^{14} incorporation into long-chain fatty acids (the rate is linear over the first hour of incubation), using the whole undialyzed supernatant fraction (15) (see Fig. 4); malonyl-2-C^{14} CoA incorporation into fatty acids and malonyl CoA-dependent TPNH oxidation (4 min incubation, or initial reaction velocity, respectively), using the P0–40 fraction or the whole supernatant fraction (2, 4, 16) (see Figs. 1, 2, 3A, and 3B); and net incorporation of pyruvate-2-C^{14} into fatty acids by adipose tissue (see Fig. 5). Incubations were terminated by adding 10% alcoholic KOH. The mixture was saponified and then extracted with pentane to remove unsaponifiable material. The remaining aqueous phase was acidified and the long-chain fatty acids were extracted with pentane (17). After washing the pentane phase with dilute acetic acid, aliquots of the pentane extracts were placed directly in glass vials and evaporated to dryness. Each residue was finally dissolved in 10 ml of 0.4% 2,5-diphenyloxazole in toluene for counting in a scintillation counter (Packard) for 10 min. Radioactivity measurements were routinely well within 2% reproducibility limits. The counting efficiencies of acetate-C^{14} and C^{14}-fatty acids were identical, as measured in the presence of standard benzoic acid-C^{14} or toluene-C^{14}.

The enzyme activities of the soluble enzyme preparations, as determined from the rates of incorporation of acetate or malonyl CoA into fatty acids or from the initial rate of malonyl CoA-dependent TPNH oxidation, were usually expressed in terms of yield of enzyme activity per liver, instead of activity per gram of tissue or activity per milligram of protein in the isolated soluble tissue fraction. The observed activity measurements were covariant for each of these expressions of enzyme activity level.

Glucose-6-phosphate dehydrogenase activity in the whole supernatant fraction was determined according

to the spectrophotometric method of Glock and McLean (18) (see Table 4).

The large number of experimental variables tested and the task of separating the enzyme quantitatively from many samples of liver simultaneously precluded an examination of the variation among individual like specimens of liver. Consequently, the livers of 3–20 animals of the same kind were homogenized together, such that the average value of enzyme activity yield was directly determined. The values for the control animals kept on a balanced stock diet served as a satisfactory baseline for comparing the experimental values. Where possible, "t-values" for the experimental points were determined from the following derived equation (51)

$$t = (\text{experimental value} - \text{control mean})/\left(S^2 \cdot \frac{n+1}{n}\right)^{1/2}$$

S^2 is the variance of n control values, and n the number of control values used for comparison. The P values were read directly from statistical tables ($n-1$ degrees of freedom). In the first three figures (Figs. 1, 2, 3A), the variance among the 15 control values was employed. For experimental values 3 times greater than the control mean, P values less than 0.001 were obtained.

Tissue Fatty Acid Analysis

Two methods were used in the isolation of tissue fatty acids. In the first method (19) the tissue was homogenized in 30 volumes of ethanol–ether (3:1) containing 5% by volume of HCl. The residue remaining after filtration was washed with the same solvent and finally with pentane. The filtrates were combined, reduced in volume to about one-fourth, and transferred with pentane to a separatory funnel. The extract was washed with water and dried with anhydrous sodium sulfate. Aliquots of the extracts were evaporated to dryness and saponified with 10% alcoholic KOH. After the unsaponifiable fraction was removed with pentane, the aqueous phase was acidified and fatty acids were extracted into pentane. In the second method whole tissue or tissue homogenates were saponified directly in 2 ml of 30% KOH per g of tissue. Unsaponifiables and fatty acids were extracted with pentane as before.

Methyl esters of the fatty acids were prepared by the method of Metcalfe and Schmitz (20). An aliquot of the pentane extract containing fatty acids was evaporated to dryness with nitrogen, and 1 ml of boron trifluoride–methanol was added per 100 mg of fatty acids. The solution was heated in a boiling water bath for 2 min. To the cooled solution 5 ml of distilled water and 10 ml of pentane were added and the mixture was shaken vigorously for several min. The pentane phase was removed and concentrated in a stream of nitrogen to about 0.1 ml or less. If necessary, methyl esters were stored under nitrogen at −20° until analyzed.

The composition of the methyl ester fraction was analyzed with a Research Specialties Company (Richmond, Cal.) model 600 gas–liquid chromatograph. A 6 ft stainless steel column (0.25 inch o.d.) containing diethylene glycol succinate polyester (20%) on Chromosorb W (acid washed, 80–100 mesh) was employed. The column temperature was maintained at 190°, and the argon ionization detector at 230°. Fatty acid composition was calculated by determining the area of the peaks by means of a disk integrator. The concentration of total methyl ester in the initial mixture was determined as the acyl hydroxamate (21).

Analysis of Lipid Components

Weighed aliquots of liver were homogenized with 9 volumes of 0.066 M sodium phosphate buffer (pH 7.0) (22). One portion of the homogenate was extracted with a chloroform–methanol mixture according to Folch et al. (23), for determination of phospholipids. The second portion was mixed with chloroform and zeolite (Doucil, obtained from W. A. Taylor Co., Baltimore, Md., activated by heating at 125° for 4 hr), which sepa-

TABLE 3 VARIATION OF BODY WEIGHT WITH DIET

Days	Normal	Linoleate-Deficient	Deficient Diet Supplementation		
			Palmitate	Oleate	Linoleate
1	19.4 ± 1.62	16.0 ± 1.89			
4	24.6 ± 0.80	22.8 ± 2.13			
8	20.4 ± 1.20	20.4 ± 1.00			
14	19.8 ± 0.74	22.4 ± 1.02			
18	20.6 ± 1.02	22.4 ± 0.80			
20			23.0 ± 1.09	22.6 ± 1.49	24.0 ± 1.89
22			23.4 ± 0.49	23.0 ± 0.89	23.8 ± 1.16
24			22.4 ± 0.49	22.8 ± 1.72	24.4 ± 1.68
26	21.6 ± 1.02	25.6 ± 0.80	22.2 ± 0.98		25.4 ± 0.80

The animals and diets are the same as those indicated in the legend to Fig. 3. Each number is the average body weight in grams and the deviation from the mean for separate groups of five mice.

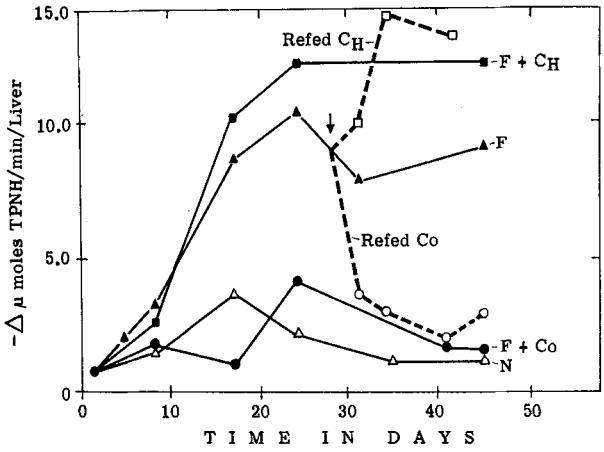

FIG. 1. Fatty acid biosynthesis in soluble liver preparations from mice maintained on a fat-free diet or a fat-free diet supplemented with corn oil or cholesterol.

The ordinate represents the total yield of fatty acid synthetase per liver expressed in terms of the initial rate of malonyl CoA-dependent TPNH oxidation. The soluble enzyme preparation was the P0–40 fraction of the liver supernatant solution (see Methods).

The abscissa shows the number of days on the indicated diet. Mice were fed a normal stock diet (N), a fat-free diet (F), a fat-free diet with 2% cholesterol added (F + C_H), or a fat-free diet with 10% corn oil added (F + Co) for 45 days. After 28 days (arrow) some of the mice on the fat-free diet received 2% cholesterol (Refed C_H) or 10% corn oil (Refed Co) for 17 days. Each point represents a group of 3–4 mice.

The enzyme assay system consisted of potassium phosphate buffer (pH 6.5), 50 mM; versene, 10 mM; 2-mercaptoethanol, 2.5 mM; acetyl CoA, 0.175 mM; TPNH, 0.25 mM; malonyl-2-C^{14} CoA, 0.175 mM; and 0.1–0.5 mg of enzyme in a total volume of 0.40 ml. The assay systems were incubated in the spectrophotometer at 37°.

In comparison with a mean value of 1.503 for all of the normal controls in Figs. 1, 2, and 3*A*, all points in this experiment with values of 3.7 or higher were significantly greater than controls ($P<0.01$). All other experimental points were not significantly different from the control (see Methods).

rated a fraction containing triglycerides and cholesterol (phospholipids are adsorbed on the zeolite) (22).

Liver phospholipid. The residue of the Folch extract, after evaporation of solvent, was digested with 5 N sulfuric acid and the liberated phosphate was determined by the molybdate colorimetric procedure of Fiske and Subbarow (24). Moles of lipid phosphorus were converted to moles of phospholipid by the factor of 25.

Total liver cholesterol. Aliquots of the chloroform–zeolite extract of the whole liver homogenate were evaporated to dryness and redissolved in isopropanol. Total cholesterol was determined by Leffler's modification (25) of the Zlatkis-Zak ferric chloride color reaction (26).

Liver triglyceride. Aliquots of the chloroform–zeolite extract were saponified, the resultant glycerol was oxidized to formaldehyde, and the formaldehyde estimated colorimetrically with chromotropic acid as outlined by Butler (22). The method is a modification of the procedure of Van Handel and Zilversmit (27).

Materials

Malonyl-2-C^{14} CoA was prepared by the method of Trams and Brady (28). Acetyl CoA was prepared by reacting acetylthiophenol with reduced CoA (29, 30) or it was purchased from Pabst Laboratories (Milwaukee, Wis.). Acetyl-1-C^{14} CoA and other radiochemicals were obtained from New England Nuclear Co. (Boston, Mass.). TPNH, TPN^+, glucose-6-phosphate, and 6-phosphogluconate were purchased from Sigma Chemical Co. (St. Louis, Mo.). Pure methyl esters of linoleic, oleic, and palmitic acids were obtained from California Corp. for Biochemical Research (Los Angeles, Calif.). The hydrogenated coconut oil was Durkee's brand Hydrol 110 (Chicago, Ill.) and the corn oil was Mazola brand.

RESULTS

In the first experiment (Fig. 1) mice were fed a balanced stock diet (N series) or fed a fat-free diet (F series) over a 45-day period. The capacity for fatty acid synthesis in liver was measured as the yield of fatty acid synthetase activity per liver (in terms of the initial rate of malonyl CoA-dependent TPNH oxidation) in the P0–40 fraction of the liver supernatant solution.

Over a period of 21 days the yield of fatty acid synthetase in liver of the mice maintained on a fat-free diet (F) was observed to rise to levels 5–10 times as great as the control series (N). This difference in total activity continued through the remainder of the 45 day experimental period. The same relationship was obtained when the data were expressed in terms of specific enzyme activity (initial rate per milligram protein in the P0–40 fraction) instead of yield of total activity per liver.

In an attempt to ascertain if a particular lipid component in the normal diet is responsible for holding enzyme activity at a certain level, various lipid fractions were added in small amounts as supplements to the fat-free diet.

As shown in Fig. 1, corn oil (Co) when added to the fat-free diet (10% of the diet by weight) maintained normal activity levels whether the lipid was added from the beginning of the experiment or added 28 days later (at arrow). On the other hand, when the fat-free diet was supplemented to the extent of 2% by weight with cholesterol, activity levels were even slightly elevated over the average value obtained with the fat-free diet alone. In a separate study, hydrogenated coconut oil, added as a supplement to the fat-free diet (4% by weight), did not depress the greatly elevated enzyme levels, whereas the same quantity of corn oil returned the elevated

levels of fatty acid synthetase toward normal. Cholesterol and hydrogenated coconut oil, when added together in the fat-free diet, did not depress the elevated fatty acid synthetase enzyme levels in liver (Fig. 2).

Since corn oil is rich in triglycerides containing linoleic acid (50–60% of the fatty acid is linoleic acid), the effect of pure methyl linoleate was tested in comparison with corn oil as a lipid supplement to a linoleic acid-deficient diet (i.e., a fat-free diet supplemented with 1% cholesterol and 4% hydrogenated coconut oil). In Fig. 2 it is seen that methyl linoleate, like corn oil, brought the yield of synthetase toward normal over an 8 day period.

In a similar experiment (Fig. 3) the influences of pure methyl esters of palmitate, oleate, and linoleate were compared. Of these only linoleate was effective in returning synthetase levels to normal. Synthetase activity was measured as malonyl CoA-dependent TPNH oxidation (part *A* of Fig. 3), and, in the same incubation mixture, as malonyl-2-C^{14} CoA incorporation into fatty acids (part *B* of Fig. 3).

Studies of acetate-1-C^{14} incorporation into fatty acids in the series of mice presented in Fig. 3 demonstrated the same pattern of changes as indicated for TPNH oxidation and malonyl CoA incorporation. Long-chain fatty acid synthesis was followed as acetate-1-C^{14} incorporation by the whole supernatant fraction (Fig. 4). The yield of enzyme activity is expressed in terms of acetate-1-C^{14} incorporation into fatty acids per hour per liver. The limiting enzyme in this assay was acetyl CoA carboxylase (31). The specific rate and yield of acetate incorporation was covariant with synthetase activity (see Fig. 3), although the latter was much greater.

A qualitatively similar pattern was also obtained in the same set of mice for pyruvate-2-C^{14} incorporation into fatty acids by intact epididymal fat pads (Fig. 5). These results with adipose tissue, although less clear-cut than with liver, would indicate that the changes in enzyme activity brought about by linoleate restriction are not limited to liver tissue.

The data in Table 4 show that glucose-6-phosphate dehydrogenase of liver fluctuates in response to dietary linoleic acid restriction in a manner virtually identical with that of the enzymes synthesizing fatty acid. Although not obligatory for fatty acid formation, this dehydrogenase does facilitate lipogenesis in the intact cell by generating reduced TPN (9).

Alimentary restriction of linoleate brought about an increase in the liver enzymes which catalyze fatty acid synthesis in less than a week (see Fig. 3 and 4), and it was of interest to determine if the apparent enzyme adaptation was coincident with or preceded a fall in tissue linoleate content. First of all, it was found that liver promptly reflected the absence of linoleate in the diet.

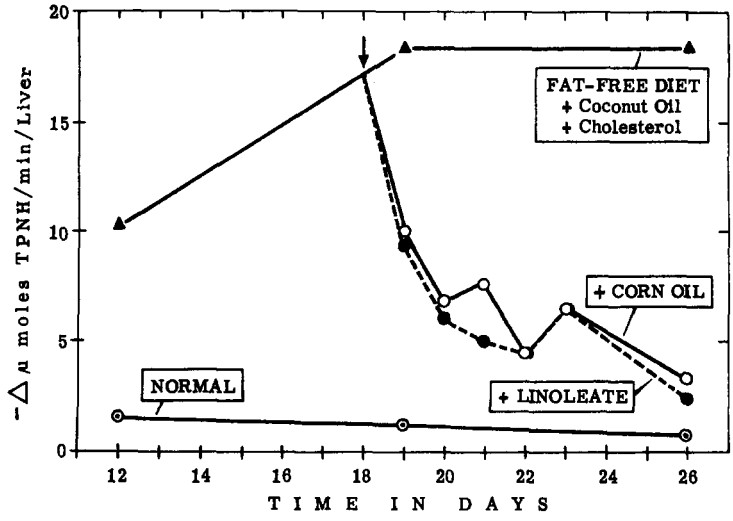

FIG. 2. Liver fatty acid synthetase activity in mice receiving a linoleate-deficient diet with and without supplementation with corn oil or methyl linoleate.

Mice were fed a balanced stock diet (normal) or a fat-free diet plus 4% hydrogenated coconut oil and 1% cholesterol (as indicated) for 26 days. After 18 days some of the mice on the linoleate-deficient diet were given 4% corn oil in place of the coconut oil (+ corn oil) or were given 2% methyl linoleate in place of half of the coconut oil (+ linoleate) for 8 days. Each point represents a group of five mice. The determination of yield of fatty acid synthetase in the P0–40 fraction of liver supernatant solution is described in Fig. 1.

Significant differences as in legend to Fig. 1.

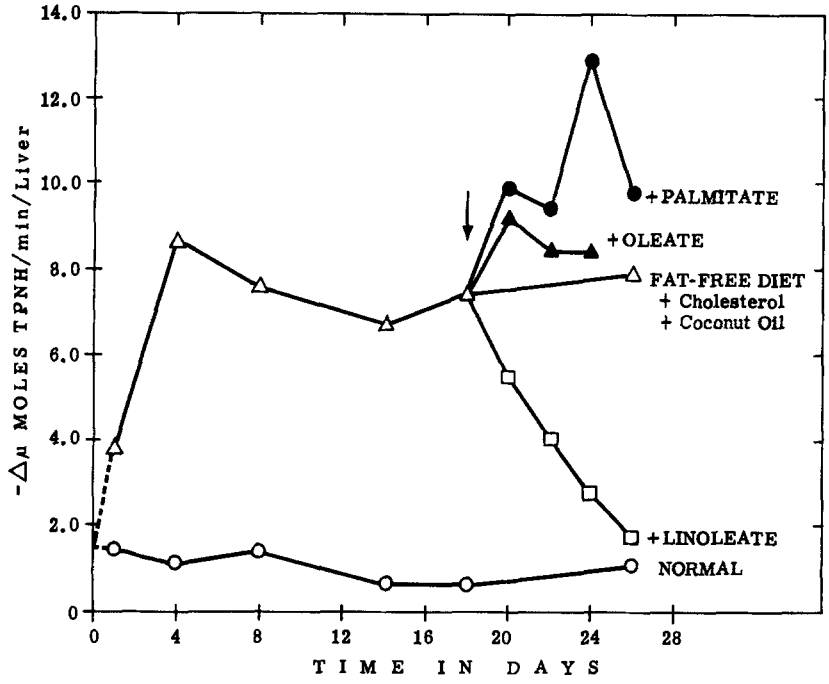

Fig. 3*A*

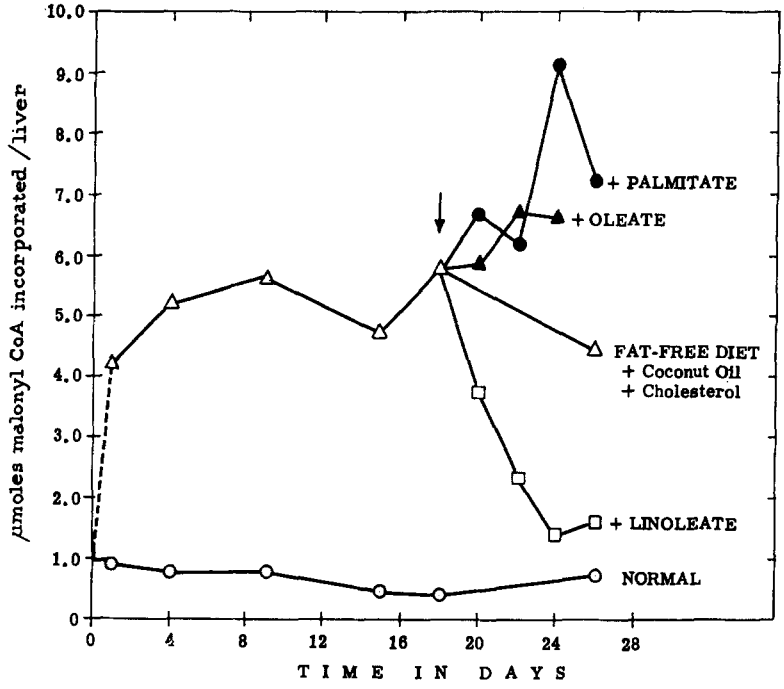

Fig. 3*B*

FIG. 3. Liver fatty acid synthetase activity in mice receiving a linoleate-deficient diet supplemented with methyl linoleate, methyl palmitate, or methyl oleate.

Mice were fed a balanced stock diet (normal) or a fat-free diet plus 2% hydrogenated coconut oil and 1% cholesterol (linoleic acid-deficient diet as indicated) for 26 days. After 18 days (arrow) the hydrogenated coconut oil in the linoleate-deficient diet was replaced by 2% methyl palmitate (+ palmitate), 2% methyl oleate (+ oleate), or 2% methyl linoleate (+ linoleate). The methyl esters were over 99% pure. Each point represents a group of five mice.

In Fig. 3*A*, the ordinate represents the total yield of fatty acid synthetase expressed in terms of the initial rate of malonyl CoA-dependent TPNH oxidation. The assay conditions are given in the legend to Fig. 1. Significance of differences as in legend to Fig. 1.

In Fig. 3*B*, the ordinate represents the total yield of fatty acid synthetase expressed in terms of malonyl-2-C^{14} CoA incorporation into atty acids. The incorporation was determined in the same samples used for measuring TPNH oxidation. In comparison with the mean value of 0.691 for the six normal control determinations, all experimental points in excess of 1.6 were significantly higher ($P < 0.01$).

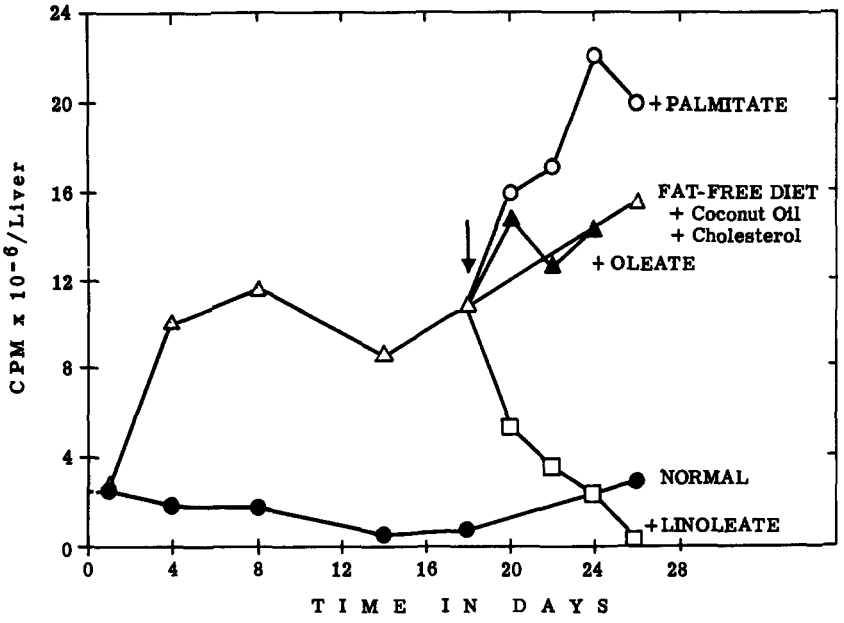

FIG. 4. Acetate incorporation into fatty acids by the liver supernatant fraction of mice receiving a linoleate-deficient diet supplemented with methyl linoleate, methyl palmitate, or methyl oleate.

Mice were the same as were used for Fig. 3. The yield of enzyme activity per liver is expressed in terms of acetate-1-C^{14} incorporation into fatty acids (as counts per minute) per hour. In the ordinate 10^6 cpm is equivalent to 0.91 μmole of acetate-1-C^{14} incorporated into fatty acids. The limiting enzyme in this assay is acetyl CoA carboxylase (31).

The assay system included potassium phosphate buffer (pH 6.5), 60 mM; isocitrate, 10 mM; $MnCl_2$, 0.8 mM; ATP, 4 mM; cysteine (pH 6.5), 8mM; TPN^+, 0.20 mM; CoASH 0.1 mM; acetate-1-C^{14} (2.4 $\times$ 10^6 cpm per assay), 2.2 mM; $KHCO_3$, 20 mM; and 0.5–3.0 mg of liver supernatant protein in a total volume of 1.0 ml. The incubation was carried out in glass stoppered tubes with a CO_2 atmosphere for 1 hr at 37°. Fatty acids were isolated and counted as described in Methods.

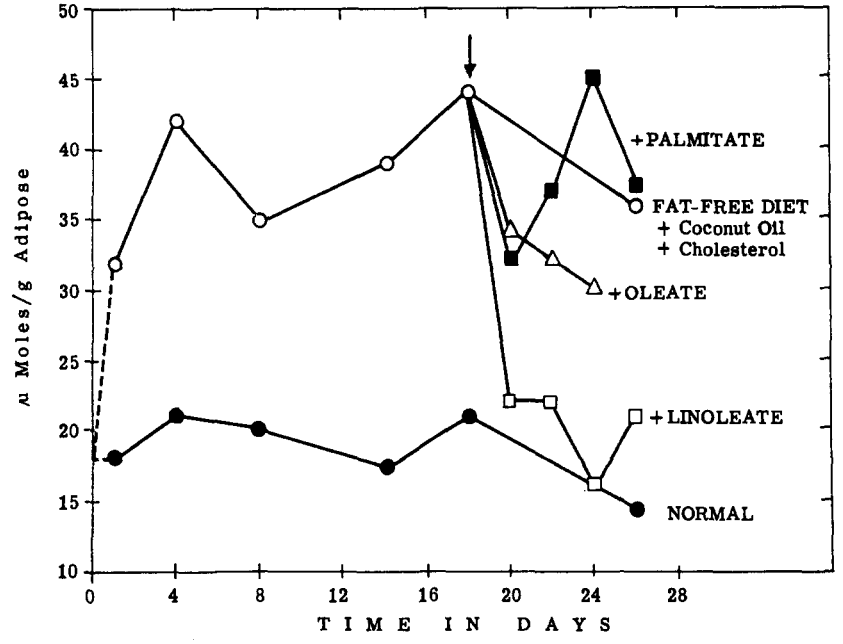

FIG. 5. Incorporation of pyruvate-2-C^{14} into fatty acids by adipose tissue of mice receiving a linoleate-deficient diet supplemented with methyl linoleate, methyl palmitate, or methyl oleate.

Epididymal fat pads were extirpated from each of the mice in the experiment presented in Fig. 3. The fat pads (0.4 to 0.5 g) were incubated (43) with 5.0 ml of Krebs-Ringer bicarbonate buffer containing pyruvate-2-C^{14} (5000 cpm/μmole), 40 mM; and glucose, 10 mM. The tissue was incubated for 180 min at 37° in stoppered 50 ml flasks. The systems were gassed with 95% O_2–5% CO_2 for 5 min before incubation. The fatty acids were isolated and counted as described in Methods. The incorporation of pyruvate-2-C^{14} into fatty acids is expressed in terms of μmoles of pyruvate incorporated per 180 min per g of adipose tissue.

It was surprising that only a few days were needed to bring about a dramatic fall in the relative linoleate concentration in liver (see Table 5) since 10 or more weeks are ordinarily required to establish the classical manifestations of essential fatty acid deficiency (32, 33). As indicated in the following paper (34), refeeding a starved animal with a fat-free diet initiates depletion of liver linoleate within 8 hr.

The enzyme changes were coincident with the shifts in fatty acid composition of liver. In Table 5 the yield of enzyme activity (both acetate incorporation into fatty acids and malonyl CoA-dependent TPNH oxidation) is compared with the percentage of linoleate in liver fatty acids. As in previous experiments, mice were fed a linoleate-deficient diet over a 19 day period. Methyl linoleate was then added to the diet in place of hydrogenated coconut oil for an additional 6 days. As early as 5 days after initiating the deficient diet elevated enzyme levels were observed, coincident with a sharp drop in the percentage of linoleate in liver fatty acids. Returning linoleate to the diet tended to restore both the enzyme values and the fatty acid composition toward normal.

TABLE 4 GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY IN LIVERS OF MICE FED A FAT-FREE DIET

Diet	Days	Yield of Dehydrogenase Activity
N	8	2.4
N	24	1.6
F	1	1.2
F	4	4.2
F	8	8.8
F	17	17.6
F	31	26.4
F	45	36.3
F + CO	28 + 3	13.7
F + CO	28 + 6	10.7
F + CO	28 + 13	5.3
F + CO	28 + 17	4.9

The experimental animals were taken from the series presented in Fig. 1. Mice were fed a fat-free diet over a total 45 day period (F). Some of these, beginning on the 28th day, were given a fat-free diet supplemented with corn oil (10% by weight) for an additional 17 day period (F + CO).

The yield of dehydrogenase activity is expressed in terms of the initial reaction rate of TPNH formation (in μmoles/min per liver). Aliquots of the whole liver supernatant fraction were employed in this spectrophotometric assay according to the procedure of Glock and McLean (18). The incubation media contained the following: $MgCl_2$, 2.0 mM; glycylglycine buffer (pH 7.5), 5.0 mM; TPN^+, 0.4 mM; and 0.10–0.50 mg of whole undialyzed supernatant protein in a total volume of 0.5 ml. The glucose-6-phosphate dehydrogenase activity was determined by the difference in the initial velocity of TPN^+ reduction when both glucose-6-phosphate (2 mM) and 6-phospho-gluconate (2 mM) were present and when 6-phosphogluconate alone was present. The enzyme activities were determined from the initial velocities at 38° (usually between 30 and 60 sec).

In this study it was noted that the changes in fatty acid composition of tissues, previously described as occurring in long-standing essential fatty acid deficiency (35–37), were also observed in liver in a period of less than 5 days. These changes in composition included a large relative increase in palmitoleate (16:1) and oleate (18:1), and a drop in stearate (18:0), linoleate, and arachidonate (Table 6). The relative amount of palmitate ordinarily remains about the same. Fatty acids of the same chromatographic characteristics as eicosatrienoic acid (20:3) appeared only in small relative amounts during short-term linoleate deficiency (32, 38). The addition of linoleate to the diet (in place of hydrogenated coconut oil) initiated a slight trend toward a more normal composition in the limited period studied. The return was slight, except in the case of linoleate itself.

The effect of the restriction of linoleate in the diet does not seem to depend on the presence of other lipids in the diet. As shown in Table 7, a simple fat-free diet will rapidly bring about a relative depletion in liver linoleate, as well as the other changes in fatty acid composition noted previously.

In early linoleic acid deficiency tissues other than liver show fluctuations in fatty acid composition of the same sort as observed in liver, but the changes are much less striking. For example, the change in composition of heart fatty acids in response to linoleate deficiency is presented in Table 8. Under the same conditions of linoleic acid deficiency adipose tissue reflects the changes

TABLE 5 FATTY ACID SYNTHESIS AND THE RELATIVE LINOLEIC ACID CONTENT OF THE LIVER FATTY ACIDS

Diet	Days	Acetate Incorporation	TPNH Oxidation	Per Cent of Linoleic Acid in Liver Fatty Acids
N	10	0.0208	0.297	18.1
N	24	0.0143	0.162	20.5
F	5	0.0500	3.06	4.7
F	19	0.0603	5.83	1.2
F + L	19 + 2	0.0528	1.70	2.1
F + L	19 + 6	0.0192	0.780	8.4

Male mice were fed either a linoleate-free diet (F) (fat-free diet plus 1% cholesterol and 2% hydrogenated coconut oil) or a normal stock diet (N). After 19 days the coconut oil of the deficient diet was replaced by methyl linoleate (F + L).

Livers were removed for analysis at 2 days (19 + 2) and 6 days (19 + 6) after initiating methyl linoleate replacement.

Acetate incorporation into fatty acids is expressed as μmoles/30 min per average liver. The conditions of this assay are given in Fig. 4. Malonyl CoA-dependent TPNH oxidation is expressed as μmoles of TPNH per min per average liver. TPNH oxidation was assayed as indicated in Fig. 1.

The percentage of linoleic acid in liver fatty acids was determined by gas–liquid chromatography.

Each experimental value is the average of 15–20 (pooled) livers.

TABLE 6 FATTY ACID COMPOSITION OF MOUSE LIVER

Diet	Days	Fatty Acid Composition							
		16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4
						%			
N	10	32.6	2.2	13.5	18.4	18.1	0.0	0.0	14.7
N	24	30.9	4.3	14.9	19.0	20.5	0.0	0.0	9.8
F	5	21.1	7.3	5.7	56.4	4.7	2.2	0.0	1.0
F	19	20.4	8.1	5.1	59.5	1.2	1.5	1.0	2.1
F + L	19 + 2	20.5	10.1	3.8	56.4	2.1	1.9	2.4	2.2
F + L	19 + 6	21.1	6.3	6.1	50.2	8.4	0.6	5.3	1.4

The animals and diets are the same as those indicated in Table 5.

TABLE 7 FATTY ACID COMPOSITION OF LIVER FROM MICE FED A FAT-FREE DIET

Diet	Days	Fatty Acid Composition						
		16:0	16:1	18:0	18:1	18:2	20:3	20:4
					%			
N	8	40.5	1.3	15.3	13.6	16.4	0.0	10.7
N	21	35.0	2.2	15.2	23.4	13.6	0.0	10.2
F	1	22.3	5.3	10.7	39.1	11.0	0.0	8.5
F	5	25.4	7.0	13.1	41.3	4.4	0.0	7.7
F	13	23.2	7.4	9.2	51.8	4.5	0.0	5.5
F	18	23.6	8.4	9.2	50.0	4.6	0.0	4.0
F	26	22.5	8.2	10.0	51.0	1.6	4.5	2.7
F + L	18 + 3	29.3	9.0	11.7	43.8	4.7	0.0	1.4
F + L	18 + 5	22.3	5.9	6.8	33.4	14.6	0.0	6.2
F + L	18 + 8	24.0	5.6	8.9	41.5	11.5	0.0	7.6

In this experiment mice were fed a fat-free diet (F) over a total 26 day period. In a group of the F series, on the 18th experimental day, methyl linoleate (2% by weight) was added to the deficient diet for 8 more days (F + L).

Each experimental value is the average of 10 (pooled) livers.

TABLE 8 FATTY ACID COMPOSITION OF MOUSE HEART

Diet	Days	Fatty Acid Composition				
		16:0	16:1	18:0	18:1	18:2
				%		
N	10	28.7	0.5	26.0	22.1	21.3
N	24	27.0	1.4	26.5	17.2	24.4
F	5	23.0	3.6	20.0	33.0	18.0
F	19	25.0	7.4	14.0	36.8	12.0
F + L	19 + 2	21.2	5.9	16.8	31.2	22.1
F + L	19 + 6	20.0	5.0	15.5	32.0	27.0

The animals and diets are the same as indicated in Table 5.

TABLE 9 PER CENT LINOLEATE IN LIVER AND ADIPOSE TISSUE FATTY ACIDS

Diet	Days	Liver	Adipose Tissue
		% 18:2	
N	8	16.4	32.5
N	21	13.6	22.9
F	1	11.0	34.3
F	5	4.4	19.5
F	13	4.5	11.9
F	18	4.6	9.8
F	26	1.6	8.3
F + L	18 + 3	4.7	8.5
F + L	18 + 5	14.6	8.2
F + L	18 + 8	11.5	11.5

These data were obtained in the same series of mice described in Table 7.

seen in liver, although the degree of deficiency that developed in adipose tissue is not as severe (Table 9).

Mice maintained on a fat-free or a linoleate-free diet developed fatty livers (39). This was grossly apparent in the more yellowish color of the tissue and by the increase in liver weight. Intracellular deposits of lipid were made visible microscopically by staining frozen sections with Sudan IV. Control livers showed very few sudanophilic particles. In the deficient series the distribution of lipid deposits was predominantly in the portal (peripheral) region of the liver lobule. Addition of methyl linoleate or corn oil to the fat-free diet tended to restore the normal appearance of the liver.

Chemical analysis confirmed that total lipid is elevated in liver even during short-term linoleic acid deficiency (Fig. 6). The bulk of the accumulated lipid was triglyceride, although there was also an increase in

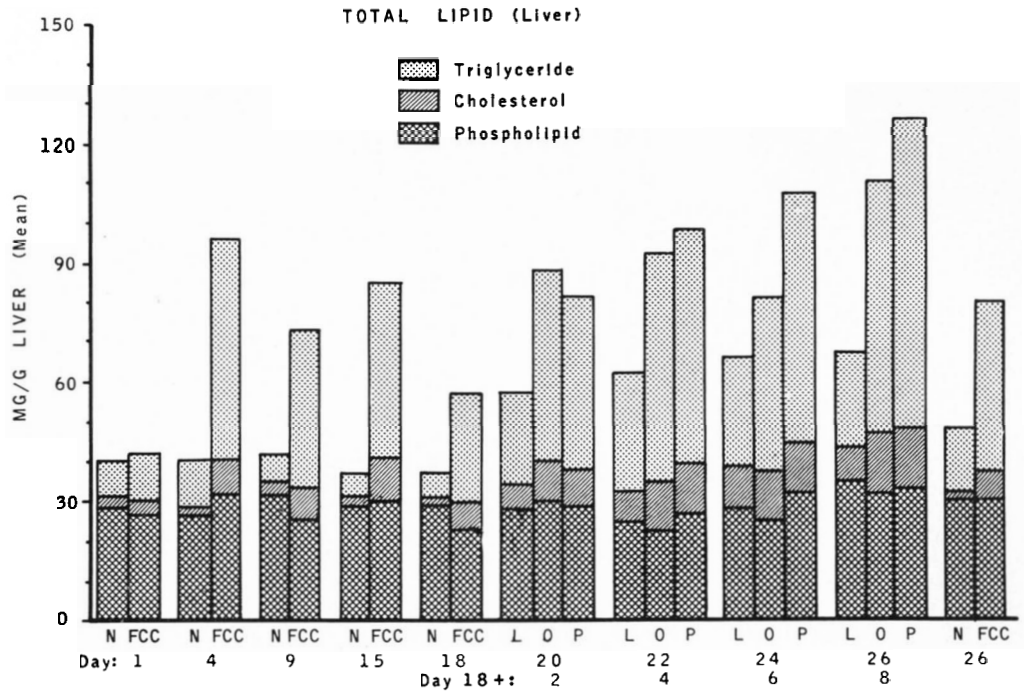

FIG. 6. The total liver triglyceride, phospholipid, and cholesterol content in mice receiving a linoleate-deficient diet supplemented with methyl linoleate, methyl palmitate, or methyl oleate.

The total lipid and the major lipid classes of liver were determined in control mice (N), and mice maintained on a linoleate-deficient diet (FCC) over a total period of 26 days. From the 18th experimental day groups of the deficient mice were fed the restricted diet supplemented (2% by weight) with methyl linoleate (L), methyl oleate (O), or methyl palmitate (P) over an 8 day period. The same experiment is cited in Fig. 3.

The height of each bar represents the weight of the lipid in milligrams/gram of liver tissue.

total cholesterol. Phospholipid levels did not change significantly.

In Fig. 6 the lipid fractions of liver from the control series (N) are compared with a series on the linoleic acid deficient diet (FCC) over a 26 day period. Triglyceride and cholesterol were much greater than normal after 4 days on the restricted diet. At 18 days the deficient diet was supplemented with methyl linoleate (L), methyl palmitate (P), or methyl oleate (O). At the dosage of linoleate employed (2% by weight) only partial restoration of the normal liver lipid pattern was achieved in 8 days. Nevertheless, the quantity of triglyceride and cholesterol was appreciably smaller in the livers of the L series than in the O or P series (the potential of these same livers for fatty acid synthesis was previously presented in Figs. 3–5).

Consistent with the picture of elevated liver triglyceride and cholesterol esters (35, 40) in linoleate deficiency was the rise in total liver fatty acids. After 20 days on a fat-free diet total liver fatty acid levels were three times greater than normal.

DISCUSSION

In one of the first studies on linoleic acid deficiency in rats, Wesson and Burr concluded that fatty acid formation is enhanced in the deficient animal since they observed respiratory quotient values greater than unity (41). Later studies pointed out that the essential fatty acid deficiency syndrome was associated with lipid deposition in the liver (39, 40). The accumulation of eicosatrienoic acid (20:3) (44) was studied by Holman (32), who considered its presence diagnostic for the established deficiency syndrome. Fulco and Mead (45) proved that this acid is derived from oleic acid (18:1) through a series of enzymatic steps apparently identical with those responsible for converting linoleic acid (18:2) into arachidonic acid (20:4). Eicosatrienoic acid is in effect a polyunsaturated acid that can be synthesized de novo from glucose or acetate. In lieu of linoleic acid, the trienoic acid may step in to play a vital role in maintaining certain cellular functions (42). In addition to the relative decrease in linoleic and arachidonic acid and the rise in eicosatrienoic acid in the various tissues of rats during long-term deficiency, other changes in the composition of fatty acids have recently been described (35–37, 46–48). These include a marked relative rise in palmitoleic and oleic acids and a concomitant fall in stearic acid (18:0).

In the present studies it was found that the capacity of the liver to synthesize saturated long-chain fatty acids

is greatly enhanced in mice maintained on a linoleic acid-deficient diet or on a fat-free diet. The increased activity levels of these liver enzymes, assayed as soluble protein fractions of liver, was well established within 4 days after initiating the linoleic acid-deficient feeding schedule.

During this period of elevated enzyme activity there was a profound change in the composition of the liver fatty acids, the most striking being a fall in the relative amount of linoleic acid. This fall occurred more promptly in liver than in adipose or heart tissue. As with the increase in liver enzymes, the changes in the fatty acid composition of liver were underway in less than 4 days, long before the classical signs of the essential fatty acid deficiency syndrome appear (33, 41, 42). The observed metabolic perturbations may be the earliest changes following linoleic acid restriction.

Under the conditions of these experiments, supplementation of the deficient diets with pure methyl linoleate, or with fats containing linoleate, returned liver linoleic acid levels and the synthetic capacity for saturated fatty acids toward normal. The altered lipid composition of liver was not completely restored in the experimental period examined.

Accumulation of triglyceride in liver (and thus accumulation of total fatty acids) likewise was evident early in the deficiency state. It was not established in this study that the apparent net gain of certain fatty acids (palmitic, palmitoleic, and oleic) was the result of enhanced synthesis of saturated fatty acids and of enhanced conversion of the latter to monounsaturated fatty acids (52). This explanation does seem warranted, however, in view of subsequent studies with animals maintained on a fat-free diet (see following paper) (34). As indicated in Fig. 1 and Table 7 a fat-free diet brings about changes in enzyme content and fatty acid composition that are identical with those observed during linoleic acid restriction.

Linoleic acid requirements for an animal are increased if cholesterol and saturated triglycerides are provided in the diet. This has been interpreted to mean that linoleic acid is required in the metabolism and transport of these lipids (42, 49). Providing the diet with cholesterol and saturated triglycerides would tend to divert existing linoleic acid supplies to the task of processing these exogenous lipids. In the present study it was found that supplementation of the fat-free diet with cholesterol and saturated triglycerides (separately or combined) did intensify the consequences of the fat-free diet (see Figs. 1 and 2). The relative content of linoleic acid in liver may fall rapidly under these conditions since (*a*) the exogenous linoleic acid supply is terminated, (*b*) a flow from adipose tissue of free fatty acids (containing linoleic acid) is not encouraged by a high carbohydrate diet (50), and (*c*) endogenous liver linoleate is probably employed to transport exogenous and newly synthesized triglycerides out of the liver.

The means by which the enzymes catalyzing fatty acid synthesis become more active during linoleic acid deficiency may be explained by two possible mechanisms: (*a*) a diminution in liver tissue of "free" long-chain fatty acids (or acyl CoA), which nonspecifically inhibit the synthesizing enzymes (53), or (*b*) an increase in enzyme protein (adaptive enzyme formation). The apparent specific sensitivity of the system synthesizing fatty acids to the presence or absence of linoleic acid in the diet, under the conditions of these experiments, need not be attributed to a selective, direct inhibition of the enzymes by linoleic acid itself. Conceivably tissue linoleate could influence the concentration of (nonspecific) fatty acyl inhibitors or act by way of repressing enzyme formation.

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