Fatty acid synthesis during fat-free refeeding of starved rats

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SUMMARY Refeeding starved rats a fat-free diet over a 48 hr period brings about a marked elevation in the activity of the enzymes in liver cytoplasm which catalyze the synthesis of saturated fatty acids from acetyl CoA and malonyl CoA. Acetate incorporation into palmitoleic and oleic acid is also accelerated during this period. Enhanced capability for the synthesis of these fatty acids is reflected in the net accumulation of saturated and monounsaturated fatty acids, as well as the triglyceride fraction of the liver lipids. Coincident with these events the relative amount of linoleic acid among liver fatty acids rapidly falls. These changes are substantially the same as those observed in early linoleic acid deficiency.

KEY WORDS rat · liver · linoleic acid · fatty acid composition · fatty acid biosynthesis · acetyl CoA carboxylase · fatty acid synthetase · fatty liver · fat-free refeeding · glucose-6-phosphate dehydrogenase · adaptive enzyme formation

In the preceding paper (1) it was found that linoleic acid deprivation in young mice led to relative depletion of linoleic acid in the liver within a week, long before the onset of any signs of the classical essential fatty acid deficiency syndrome. Coincident with the fall in linoleic acid there was a marked rise in the activity of the liver enzymes catalyzing the synthesis of long-chain, saturated fatty acids (predominantly palmitic) from acetate and from malonyl CoA, as assayed in isolated, soluble liver fractions in which these enzymes are quantitatively recovered. At the same time certain long-chain fatty acids accumulated, which resulted in striking changes in the composition of the fatty acid fraction. A fatty liver ensued which was due primarily to rigly ceride deposition in the tissue.

In the present study an experimental system is examined in which the events just described can be brought about within a 48 hr period in adult rats. If rats are starved for 2 days and then are refed a fat-free diet, the apparent adaptive increase in the liver enzymes leading to the net formation of fatty acids was detectable as early as 6 hr after the onset of refeeding (2–7). Coincident with the enzyme changes there is a precipitous fall in the relative linoleate content of liver, and a profound perturbation in the relative amounts of other long-chain fatty acids.

The above system is an extension of the observation of Tepperman and Tepperman (8, 9), who found that feeding a high-carbohydrate, low-fat diet to fasted rats greatly enhanced the capacity of liver slices to incorporate acetate into fatty acids. These authors also described greatly elevated levels of glucose-6-phosphate dehydrogenase activity in liver during the refeeding period. The present investigation was initially undertaken to determine whether or not an elevation in the activity of the enzymes directly catalyzing the synthesis of saturated fatty acids could explain the observed accelerated incorporation of acetate by the intact liver cell (2, 4, 10).

METHODS

Most of the methods employed in this study were described in the preceding communication (1).

Animals

Male, albino rats of the Holtzman strain, weighing between 100 and 200 g, were fed a balanced stock diet on arrival for not less than 3 days. Before an experiment was undertaken the variation in the weight of the individual normal rats selected for experimentation was less

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TABLE 1 FATTY ACID SYNTHESIS IN LIVER FRACTIONS FROM STARVED RATS REFED A FAT-FREE DIET OR A BALANCED STOCK DIET

Nutritional State	Acetate-1-C^{14} Incorporation (Supernatant)	Acetyl-1-C^{14} CoA Incorporation (P0–40)	Malonyl-1-C^{14} CoA Incorporation (P0–40)
N	1.00	1.00	1.00
S	0.11	0.08	0.19
R 10	0.18	0.24	0.39
R 23	0.51	1.01	1.28
R 48	1.44	1.79	2.31
F 10	0.35	0.15	1.09
F 23	1.47	2.09	3.58
F 48	11.0	6.19	12.8

Fatty acid synthesis was compared in normal rats (N) with rats starved for 2 days (S), with rats starved 2 days and refed a chow diet (R), or with rats starved for 2 days and refed a fat-free diet (F) for the number of hours indicated.

Fatty acid synthesis was assayed by acetate-1-C^{14}, acetyl-1-C^{14} CoA, or malonyl-2-C^{14} CoA incorporation into long-chain fatty acids. The acetate-1-C^{14} incorporation was assayed in the following system: potassium phosphate buffer (pH 6.5), 60 mM; potassium isocitrate, 10 mM; $MnCl_2$, 0.80 mM; ATP, 4.0 mM; cysteine (pH 6.5), 8.0 mM; TPN^+, 0.20 mM; CoASH, 0.09 mM; $KHCO_3$, 20 mM; acetate-1-C^{14} (2.4×10^6 cpm total), 2.2 mM; and 0.10–0.50 mg of liver supernatant protein in a total volume of 1.0 ml. The acetyl-1-C^{14} CoA incorporation was determined in a system similar to the one used for acetate-1-C^{14} except that the CoASH and acetate-1-C^{14} were replaced by acetyl-1-C^{14} CoA (1×10^6 cpm total), 30 μM. The malonyl-2-C^{14} CoA incorporation was assayed by the system described in Fig. 1. All assays were carried out at 37°. The fatty acids were isolated and counted as described in the preceding publication (1).

The results of the assays are expressed in terms of relative yield of activity per liver with the normal value set equal to 1.00. The observed normal values for each assay were: 0.325 μmole of acetate per 30 min per liver; 0.133 μmole of acetyl CoA per 10 min per liver; and 1.43 μmoles of malonyl CoA per 3 min per liver.

Each experimental value of N and S is the average of six (pooled) livers; each of the other values from three (pooled) livers.

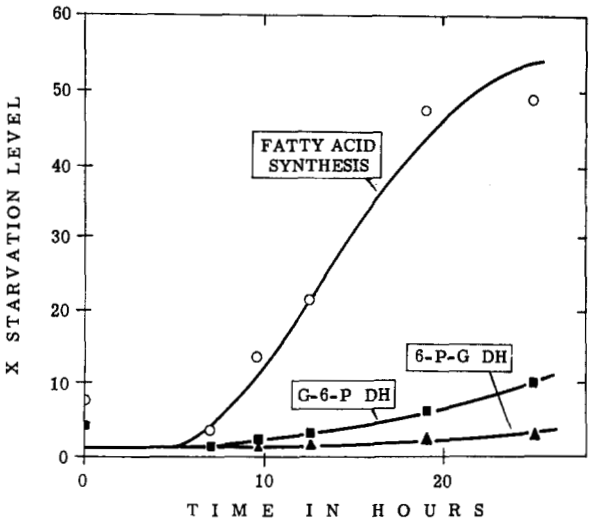

FIG. 1. Restoration of fatty acid synthesis in liver on refeeding starved rats a fat-free diet. Male rats were starved for 2 days, then refed a fat-free diet over a 24 hr period. Enzyme fractions were prepared from liver at the intervals indicated. The ordinate represents the total yield of enzyme activity in terms of malonyl CoA-dependent TPNH oxidation per liver, relative to the activity level at the end of the starvation period (time zero) set equal to 1.0. Similarly, the relative yields of glucose-6-phosphate dehydrogenase and of 6-phosphogluconate dehydrogenase are also given. The open circle and solid square at time zero represent the level of fatty acid synthesis and the hexose dehydrogenases, respectively, in normal animals. Each point is the result of three pooled rat livers.

Fatty acid synthesis was determined from the initial rate of the malonyl CoA-dependent TPNH oxidation using the P0–40 fraction of the liver supernatant fraction (1). 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. Samples were incubated in the spectrophotometer at 37°. The baseline value of the zero time (48 hr starved) sample (set equal to 1.0) was 0.54 μmole of TPNH oxidized per min per liver.

The glucose-6-phosphate (G-6-P DH) and 6-phosphogluconate (6-P-G DH) dehydrogenases were assayed according to the procedure of Glock and McLean (18). The enzyme system consisted of glycylglycine buffer (pH 7.5), 5.0 mM; $MgCl_2$, 2.0 mM; TPN^+, 0.40 mM; and 0.1–0.5 mg of the whole, undialyzed, liver supernatant fraction in a total volume of 0.50 ml. Glucose-6-phosphate dehydrogenase activity was determined by the difference between the initial velocities of TPN^+ reduction with both glucose-6-phosphate (2.0 mM) and 6-phosphogluconate (2.0 mM) present in the incubation mixture, and the system with 6-phosphogluconate alone (ie., 6-phosphogluconate dehydrogenase activity). The baseline values of the zero time (48 hr starved) samples (set equal to 1.0) for glucose-6-phosphate dehydrogenase was 6.0 μmoles of TPN reduced per min per liver, and for 6-phosphogluconate dehydrogenase was 5.5 μmoles/min per liver.

than 15 g. Rats were starved for 48 hr and then refed for 48 hr ad lib. with either a balanced stock diet or a fat-free diet (see preceding paper for analyses of diets employed). Supplements were added to the latter diet, as indicated in the text. At various time intervals the rats were killed and the liver (and other tissues) removed for enzyme or tissue lipid analysis. Livers from three or more rats in each experimental category were pooled by homogenization and aliquots removed for the various analyses (1).

Enzyme Preparations and Assay

The soluble supernatant fraction of liver and the derived fraction obtained at 40% saturation with ammonium sulfate (P0–40) were prepared as previously described (1). With these fractions, fatty acid synthesis was determined by one or more of the following assays in which the enzyme is limiting (3, 11–17): acetate-1-C^{14} incorporation into fatty acids, using the whole, undialyzed supernatant fraction (11, 12) (Table 1); acetyl-1-C^{14} CoA incorporation into fatty acids using the supernatant fraction or the P0–40 fraction (11) (Table 1); malonyl-2-C^{14} CoA incorporation into fatty acids; and malonyl CoA-dependent TPNH oxidation, using the P0–40 fraction (3, 13) (Fig. 1). Pyruvate-2-C^{14} incorporation into fatty acids by adipose tissue was also

studied (19) (see Fig. 2). As previously indicated (1), the enzyme activity was determined from the rate of incorporation of label into the fatty acid fraction, and the results were finally expressed in terms of yield of enzyme activity per liver, rather than specific enzyme activity. In contrast with intact tissue or homogenates, only saturated fatty acids are synthesized by these supernatant enzymes (over 80% palmitic acid) (11–13, 15). Each experimental determination is the mean value of three or more animals. Since livers were pooled at the time of homogenization variation among individual animals was not examined.

Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were determined by the method of Glock and McLean (18) (see Fig. 1).

Lipid Analysis

With the exception of the special study described in Table 7, fatty acid analysis by gas–liquid chromatography was carried out as described in the preceding paper (1).

RESULTS

In the initial experiments it was of interest to determine if the yield of the enzymes catalyzing fatty acid synthesis, as assayed in soluble enzyme preparations, was elevated in starved rats refed a fat-free diet (2–4, 6, 7). In Fig. 1 are presented the results of liver enzyme assays of a series of rats starved for 48 hr then refed a fat-free diet for 24 hr. The data are given in terms of relative yield of fatty acid synthetase activity (malonyl CoA-dependent TPNH oxidation) in liver in reference to the activity level of the starved rats set equal to 1.0. As shown in Fig. 1, fatty acid synthesis began to be restored as early as 6 hr after refeeding and attained a level of 40 to 50 times the starved level at the end of 24 hr. In this experiment TPNH generation by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase was also determined. In starvation glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities are reduced to low levels and refeeding a fat-free diet results in a great increase of these activities (8, 9). However, in this experiment the

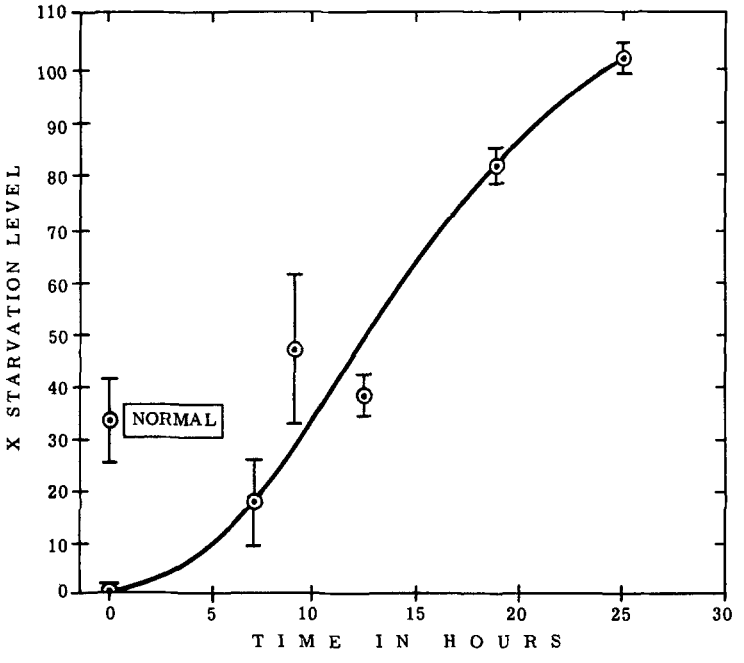

FIG. 2. Restoration of fatty acid synthesis in adipose tissue on refeeding starved rats a fat-free diet. Epididymal fat pads were extirpated from each of the rats in the series presented in Fig. 1. The ordinate represents the specific rate of pyruvate-2-C^{14} incorporation into fatty acids per g of adipose tissue, relative to the activity level at the end of the starvation period (time zero) set equal to 1.0 (observed baseline value: 0.36 μmole of pyruvate per g of tissue per 3 hr. Fat pads (400–500 mg) were incubated (15) with 5.0 ml of Krebs-Ringer bicarbonate buffer containing pyruvate-2-C^{14} (5000 cpm/μmole), 40 mM and glucose 10mM, in stoppered 50 ml flasks. The flasks were gassed with 95% O_2–5% CO_2 for 5 min and then were incubated at 37° for 180 min. Fatty acids were isolated and counted as described in the preceding publication (1). Each point represents the mean value for three animals. The limits of variance about the mean are indicated by the horizontal bars.

TABLE 2 FATTY ACID SYNTHESIS IN STARVED RATS REFED DIFFERENT DIETS AND THE LINOLEIC ACID CONTENT OF LIVER

Nutritional State	Acetate Incorporation	Linoleic Acid
		%
Normal	1.00	20.5
Starved	0.093	15.9
Refed R	1.50	12.6
Refed F	8.90	1.2
Refed FL	4.29	3.7
Fed F	6.13	3.8

Normal rats were starved for 2 days and refed a stock diet (Refed R); a fat-free diet (Refed F), or a fat-free diet containing 2% by weight of methyl linoleate (Refed FL) for 2 days. Another group of rats was fed a fat-free diet (Fed F) for 4 days (without previous starvation). In these animals fatty acid synthesis was assayed as acetate-1-C^{14} incorporation (see Table I). The extent of fatty acid synthesis was compared with the percentage of linoleic acid in the liver fatty acids (gas–liquid chromatography) (1). The observed normal value for acetate incorporation (0.983 μmole/hr per liver) was set equal to 1.00. Each experimental value is the average of three (pooled) livers.

restoration of the dehydrogenase activities appeared to occur after the increase in fatty acid synthesis (2–4), which suggested that the increase in fatty acid synthesis activity is not secondary to an enhanced capacity for TPNH formation (4, 10).

Adipose tissue also displayed the same general adaptation to the feeding of a fat-free diet to starved rats (4). In Fig. 2 the increase in fatty acid synthesis (pyruvate-2-C^{14} incubated with intact epididymal fat pads) is evident.

The rates of incorporation of acetate-1-C^{14}, acetyl-1-C^{14} CoA, and malonyl-2-C^{14} CoA into fatty acids by soluble enzyme preparations from liver were covariant in all studies in which this comparison was made. As presented in Table 1, these analyses were determined in a series of rats starved for 48 hr and then refed over a second 48 hr period, either with a fat-free diet or with a balanced stock diet. The three assays for fatty acid synthesis gave the same relative results: viz. synthetic

TABLE 3 FATTY ACID COMPOSITION OF RAT LIVER FROM STARVED RATS REFED VARIOUS DIETS

	Fatty Acids					
	16:0	16:1	18:0	18:1	18:2	20:4
				%		
Normal	30.6	0.3	24.5	13.7	20.5	10.6
Starved	40.5	0.9	28.7	10.1	15.9	5.6
Refed R	43.8	1.2	20.4	21.2	12.6	0.0
Refed F	42.9	13.9	9.1	32.2	1.2	0.0

The animals and feeding conditions were the same as described in Table 2.

The composition of the fatty acids of liver was determined by gas–liquid chromatography (1).

activity was depressed in starvation, elevated in refeeding, and especially elevated in refeeding with a fat-free diet (4, 10). It may be concluded that both fatty acid synthetase (malonyl CoA incorporation assay) and acetyl CoA carboxylase (acetate or acetyl CoA incorporation assay) vary in the same direction in these nutritional states provided that the assay systems are coupled directly to fatty acid synthesis (see Discussion).

Feeding a balanced stock diet, instead of a fat-free diet, to rats previously starved for 48 hr resulted in only a 2-fold increase in activity over normal levels (Table 2) (4). This is in contrast to the 9-fold increase over normal when a fat-free diet was fed. The addition of methyl linoleate to the fat-free diet tended to suppress the elevation of fatty acid synthesis seen when the fat-free diet alone was fed. As in the preceding study with linoleic acid-deficient mice (1), these results suggested that the elevated fatty acid synthesis was due in part to a lack of linoleic acid in the diet (2, 6, 7).

Since a relative decrease in liver linoleic acid accompanied the increase in fatty acid synthesis when mice were fed a linoleic acid-free diet (1), it was thought the linoleic acid content of the liver might also be decreased after feeding a fat-free diet to starved rats even though the refeeding period was 48 hr or less (2, 6, 7). In Table 2 the percentage of linoleic acid in the liver fatty acid fraction was compared with the level of fatty acid synthesis. As in the preceding study with linoleic acid deficiency in mice, fatty acid synthesis was inversely related to the relative linoleic acid concentration in liver. Prior starvation, however, accelerated these changes, and permitted the employment of adult rats in the present study. For comparison, analyses were also performed with a series of rats which were fed a fat-free diet, but without their being previously starved (Table 2) (see Discussion).

Accompanying the decrease in liver linoleic acid during the brief refeeding period there were profound changes in the composition of the total fatty acid fraction of liver (7) (Table 3): the relative concentration of palmitoleic (16:1) and oleic acids (18:1) increased, while that of stearic (18:0) and arachidonic acids (20:4) decreased. These changes have previously been observed in various tissues in established linoleic acid deficiency (20–25) and in liver of young mice fed linoleic acid-deficient diets for several days (1).

These initial studies indicated a very rapid onset of a relative linoleic acid deficiency state in the liver of adult, normal rats subjected to the starvation–refeeding experimental conditions. Consequently, the refeeding period was analyzed in some detail to determine the sequence of events. In Fig. 3 fatty acid synthesis and the relative linoleic acid content are compared in rats refed a fat-free diet. Fatty acid synthesis progressively in-

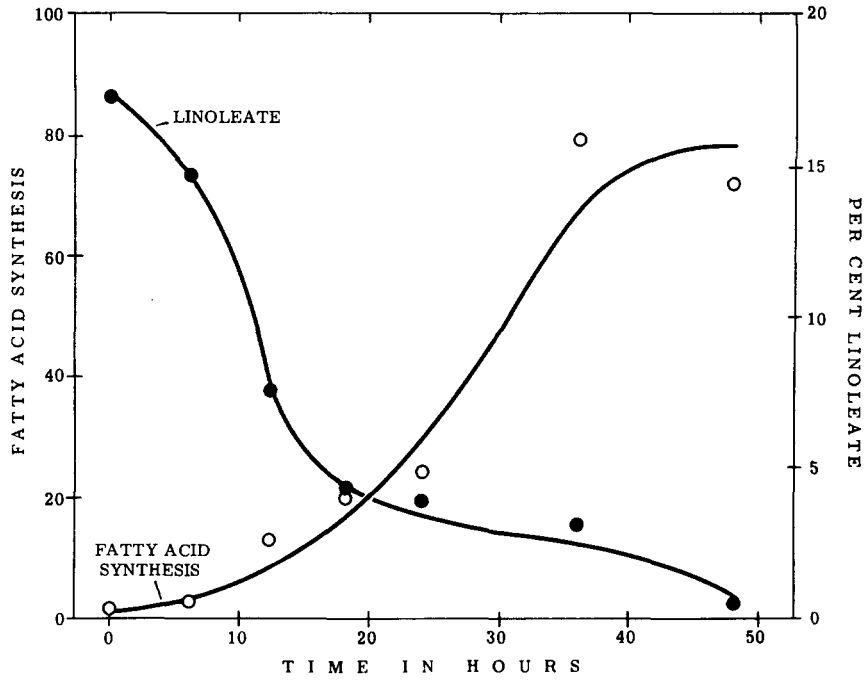

FIG. 3. Fatty acid synthesis and linoleic acid content of the liver during refeeding of fat-free diet. Male rats were starved for 48 hr, then refed a fat-free diet for 48 hr. The left ordinate represents the yield of malonyl CoA-dependent TPNH oxidation (fatty acid synthetase) relative to the activity level at the end of the starvation period (time zero) set equal to 1.0 (observed baseline value: 0.64 μmole of TPNH oxidized per min per average liver). The right-hand ordinate represents the percentage of linoleic acid in the fatty acid fraction. Each point is the result of three pooled rat livers. The linoleic acid content was determined by gas–liquid chromatography (see preceding paper) (1).

creased during the 48 hr refeeding period while the percentage of linoleic acid decreased correspondingly. Definite changes were observed as early as 6 hr after initiating refeeding.

Perturbations in the fatty acid composition as a function of time are plotted in Figs. 4 and 5. Note that during the brief (fat-free) refeeding period the relative concentration of palmitoleic and oleic acids progressively increased and stearic acid decreased (Fig. 4), whereas palmitic acid remained unchanged. As shown in Fig. 5, the relative quantity of linoleic acid decreased, but arachidonic acid first increased, then decreased nearly to zero. The transient rise in arachidonic acid may be the result of an accelerated conversion of the liver linoleic acid into arachidonic, the process coming to a halt as linoleic acid stores are depleted. No trienoic acids were detected in this early deficiency state.

The particulate fractions of liver seem to participate equally well in this rapid upheaval of the fatty acid composition. The results presented in Table 4 are indicative of rapid fatty acid turnover in the membranous components of liver. Linoleic acid does not seem to be preferentially preserved in any particular fraction, although the relative drop in mitochondria is slower.

The liver is not the only tissue that shows changes in fatty acid composition. It has been noted, however, that the extent of change is much more profound in liver during the limited 48 hr refeeding period. In Table 5 it is seen that adipose tissue (epididymal fat pad), heart tissue, and the remaining carcass also reflect the variations noted with liver, but the extent of change is less in the period studied.

During the refeeding period there is an accumulation of total fatty acids in the liver, principally as triglyceride. The rise in the quantity of total fatty acids per liver accompanies the enhanced capacity for fatty acid synthesis (enzyme assay) and the alteration in fatty acid composition (Fig. 6). A net increase in palmitic acid was observed even though the relative concentration of this acid does not change remarkably (see Fig. 4). Both oleic and palmitoleic acids accumulated (Fig. 7). The net quantity of stearic acid, however, does not change remarkably although the relative concentration is greatly decreased. The relative decrease in linoleic acid, as observed here, is primarily the result of the accumulation of the more saturated fatty acids. However, there was usually a significant net decrease of total linoleic acid per liver (Table 6). Periods beyond 48 hr were not examined.

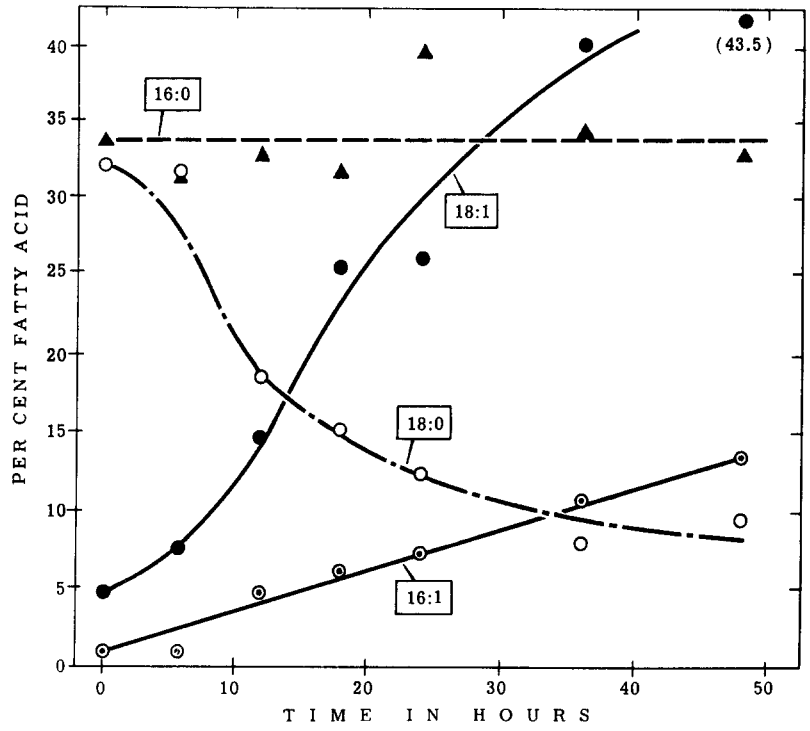

FIG. 4. Percentage of palmitic, palmitoleic, stearic, and oleic acids in the liver fatty acids during refeeding of fat-free diet. Analyses were performed with the same series of rats presented in Fig. 3.

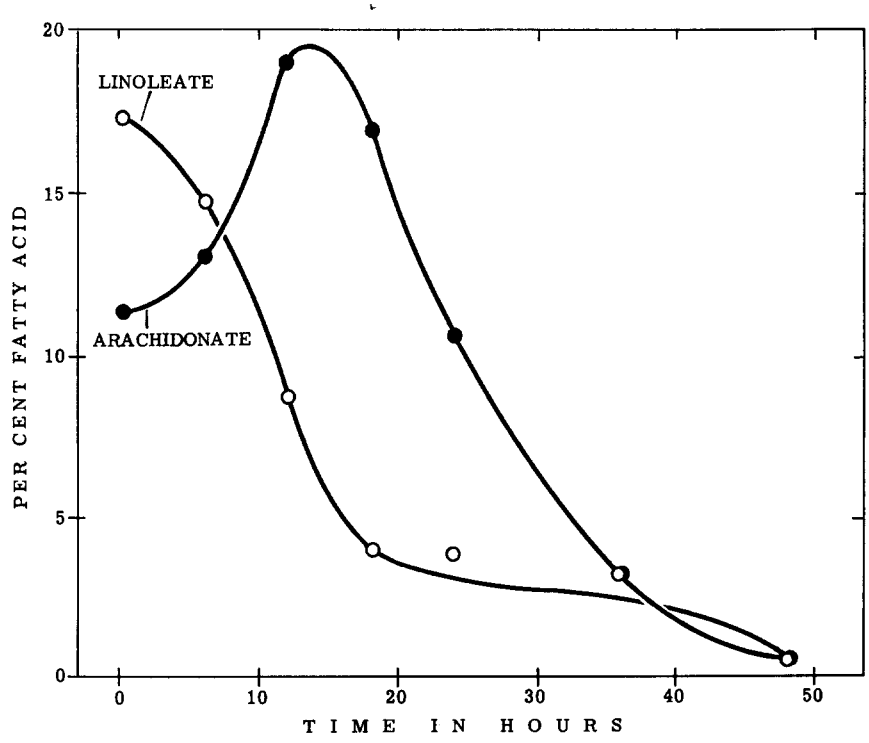

FIG. 5. Percentage of linoleic and arachidonic acids in the liver fatty acids during refeeding of fat-free diet. Analyses were performed with the same series of rats presented in Fig. 3.

TABLE 4 FATTY ACID COMPOSITION OF THE NUCLEI, MITOCHONDRIA, AND MICROSOMES IN STARVED RATS REFED A FAT-FREE DIET

Hr of Refeeding	Fatty Acid Composition of the Nuclei						Fatty Acid Composition of the Mitochondria						Fatty Acid Composition of the Microsomes					
	16:0	16:1	18:0	18:1	18:2	20:4	16:0	16:1	18:0	18:1	18:2	20:4	16:0	16:1	18:0	18:1	18:2	20:4
	%						%						%					
Normal	22.9	1.9	26.0	12.0	21.1	16.2	24.1	0.44	23.1	8.8	22.5	18.7	29.8	1.4	37.6	12.6	12.8	4.8
0	26.4	0.7	30.6	4.8	25.6	12.9	25.3	0.88	25.5	10.2	15.9	17.7	33.9	0.7	42.8	9.6	9.8	3.8
6	27.6	1.7	26.9	8.6	20.3	14.9	26.6	1.01	28.4	7.9	17.2	18.0	34.4	3.0	41.7	9.9	7.9	3.6
12	31.8	4.3	19.5	14.8	9.7	20.9	30.4	3.6	17.7	16.1	16.1	14.9	35.4	8.2	28.2	21.8	4.3	2.1
18	32.7	9.3	13.9	26.0	5.5	10.6	34.3	6.1	16.8	21.3	10.6	9.6	35.7	11.5	20.0	31.7	1.2	.25
36	40.0	12.5	7.6	35.0	1.4	1.0	33.8	9.3	9.9	33.3	6.0	6.0	37.1	10.8	12.2	39.6	.10	.10
48	38.6	14.9	7.1	38.6	0.0	0.0	33.2	9.4	9.9	31.3	5.25	8.25	37.9	10.8	12.3	38.8	.01	.01

The animals and feeding conditions were the same as in Fig. 3. The 0 hr sample is liver from rats previously starved for 48 hr. A pooled liver sample from three rats was homogenized in 0.25 M sucrose employing a glass–Teflon homogenizer for 1 min. The homogenate was centrifuged in a Spinco model L ultracentrifuge. The nuclei were sedimented by centrifuging at 300–500 × *g* for 10 min; the mitochondria at 10,000 × *g* for 10 min; and the microsomes at 104,000 × *g* for 60 min. The fatty acid composition was determined as previously described (1).

In the previous set of experiments there was a net accumulation of palmitoleic and oleic acids, in addition to palmitic acid, during the refeeding period. In view of this increase in the monounsaturated fatty acids an examination was made of the apparent rate of synthesis of fatty acids other than palmitic (the principal fatty acid synthesized in the soluble malonyl CoA system) by whole liver tissue. Liver slices were incubated in the presence of acetate-1-C^{14}. Fatty acids were isolated from the liver slices and separated by gas–liquid chromatography. The radioactivity in each isolated fatty acid was then determined. The data in Table 7 represent the net incorporation of acetate-1-C^{14} into each fatty acid. This information is also expressed in terms of the percentage of the total counts recovered in the entire fatty acid fraction for each fatty acid. Along with the expected increase in the incorporation of acetate into all fatty acids during refeeding, there was an increase in the fraction of the total fatty acid C^{14} in palmitoleic and oleic acids, relative to palmitic acid. In view of the net increase of acetate-1-C^{14} incorporated into each fatty acid, it would seem likely that there was accelerated synthesis by the intact liver cell, not only of palmitic acid, but also of palmitoleic and oleic acids during fat-free realimentation. Although there was no apparent net increase in the quantity of stearic acid, a turnover of this fatty acid is not excluded. (Acetate is efficiently incorporated.) Control experiments in the same series of rats showed, as before, that the rate of synthesis of saturated fatty acids from acetate and malonyl CoA was greatly increased in assays with the soluble cytoplasmic enzyme preparations (see Table 1).

The increase of total fatty acids per liver during fat-free alimentation is also reflected in an accumulation of

TABLE 5 FATTY ACID COMPOSITION OF LIVER, HEART, ADIPOSE TISSUE, AND CARCASS FROM STARVED RATS REFED A FAT-FREE DIET

Tissue	Nutritional State	Fatty Acid					
		16:0	16:1	18:0	18:1	18:2	20:4
		%					
Liver	N	36.3	1.4	24.9	13.3	16.6	4.9
	F 48	38.8	12.1	6.0	36.4	0.1	0.1
Heart	N	19.5	1.0	27.0	9.65	29.8	10.4
	F 48	21.6	2.0	27.0	18.0	18.0	11.9
Adipose	N	27.5	5.1	3.6	37.6	23.3	0.0
	F 48	24.0	6.8	4.5	42.5	19.0	0.0
Carcass	N	24.8	4.1	7.0	36.4	23.0	0.0
	F 48	26.1	2.2	8.1	44.5	17.0	0.0

Comparison of the fatty acid composition of normal rats (N) and starved rats refed a fat-free diet (F 48) for 48 hr. The fatty acids were isolated, esterified, and analyzed as described earlier (1). The remaining carcass consisted of the whole animal minus the head, heart, liver, epididymal fat pad, and intestine.

TABLE 6 QUANTITY OF FATTY ACIDS AND LINOLEIC ACID IN RAT LIVER

	Total Fatty Acids		Linoleic Acid		
	mg/ total liver	*mg/g liver*	*mg/ total liver*	*mg/g liver*	*% of fatty acids*
N	200	21	33	3.5	16.6
S	51	11	6.2	1.4	12.2
R 10	62	8.6	11	1.5	18.0
R 23	97	12	11	1.3	11.3
R 48	169	21	18	2.2	10.7
F 10	72	9.1	7.5	0.9	10.4
F 23	155	17	7.1	0.7	4.6
F 48	321	33	0.32	0.03	0.1

The animals and feeding conditions are the same as those described in Table 1 (also see Tables 7 and 8). With the exception of the column on the right, results are expressed in terms of milligrams of methyl ester of total fatty acids or of linoleic acid. The absolute values for linoleic acid were calculated from the percentage of linoleic acid in the total fatty acid fraction (right column).

the triglyceride fraction. In Table 8 data are presented showing that the triglyceride content increased to about 3 times its normal level when a fat-free diet was fed to rats for 48 hr after a prior starvation. This fraction increased only to a normal level when a balanced diet was refed. Phospholipid returned to normal values during the realimentation phase in both series of rats. As in the case of mice maintained on a linoleic acid-deficient diet (1),

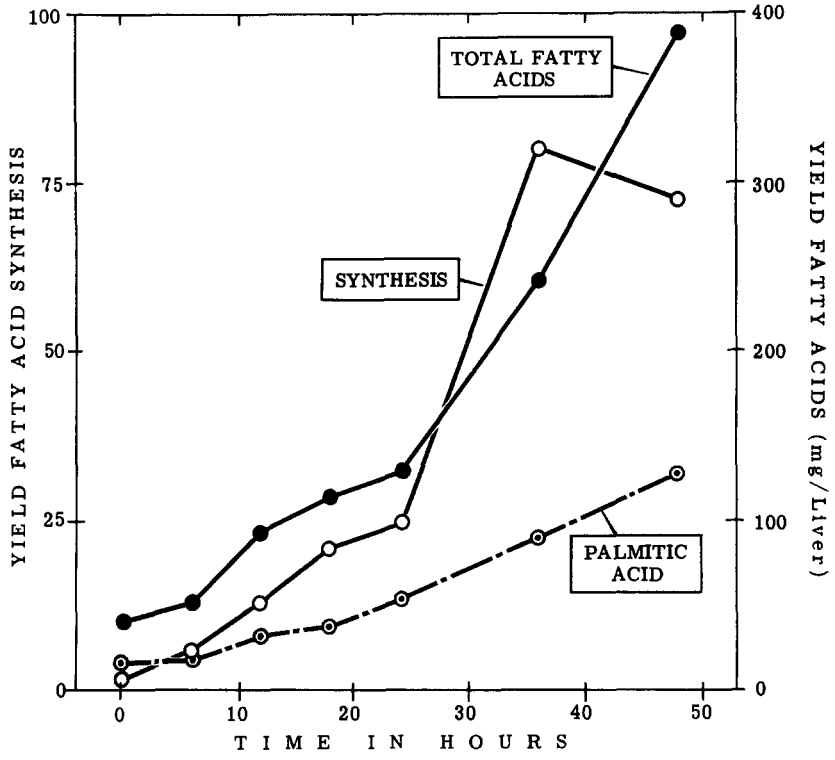

FIG. 6. Fatty acid synthesis, total fatty acids, and the yield of palmitic acid in liver during refeeding of fat-free diet. Analyses were performed with the same series of rats presented in Fig. 3. The left ordinate represents the yield of malonyl CoA-dependent TPNH oxidation (fatty acid synthetase) relative to the activity level at the end of the starvation period (time zero set equal to 1.0 (observed baseline value: 0.64 μmole of TPNH oxidized per min per average liver). The latter curve is also presented in Fig. 3. The total fatty acids (●) were measured as the hydroxamate (26) formed from the methyl esters of the fatty acid fraction (right ordinate). The palmitic acid content (⊙) was calculated from the percentage of palmitic acid in the total fatty acid fraction.

TABLE 7 INCORPORATION OF ACETATE-1-C^{14} INTO FATTY ACIDS BY LIVER SLICES

	<16:0	16:0	16:1	I	18:0	18:1	>18:1
Normal	0.54 (7.4)	5.18 (70.0)	0.15 (2.1)	0.15 (2.1)	0.67 (9.1)	0.15 (2.1)	0.52 (7.0)
FF 10	0.22 (4.1)	3.52 (65.6)	0.26 (4.9)	0.11 (2.0)	0.60 (11.2)	0.46 (8.5)	0.18 (3.4)
FF 23	0.88 (4.8)	9.85 (53.7)	1.26 (6.9)	0.08 (0.5)	1.86 (10.2)	3.92 (21.4)	0.42 (2.3)

The incorporation of acetate-1-C^{14} into the various fatty acids of liver was determined by incubating liver slices from normal rats and from rats fed a fat-free diet for 10 hr (FF 10) and 23 hr (FF 23) after a starvation period of 48 hr. The animals employed in this study were selected from the series described in Table 1 (also see Tables 6 and 8). Net incorporation is expressed as μmoles of acetate per 90 min per average liver. The values in parentheses are percentage of the total incorporation. The column designated "I" contains the values obtained for the effluent emerging from the column after 16:1 and before 18:0. Liver slices (500 mg) were incubated in 5.0 ml of Krebs-Ringer bicarbonate buffer, containing 5 μmoles of acetate-1-C^{14} (8×10^5 cpm) for 90 min at 37°. The fatty acids were isolated and esterified as described earlier (1). Each fatty acid was collected in a U-shaped glass tube (immersed in ice water) connected to the outlet of the gas–liquid chromatograph. The fatty acid fraction was eluted from the U-tube with 15 ml of toluene containing 0.40% 2,5-diphenyloxazole and counted in a liquid scintillation counter (Packard Tri-Carb).

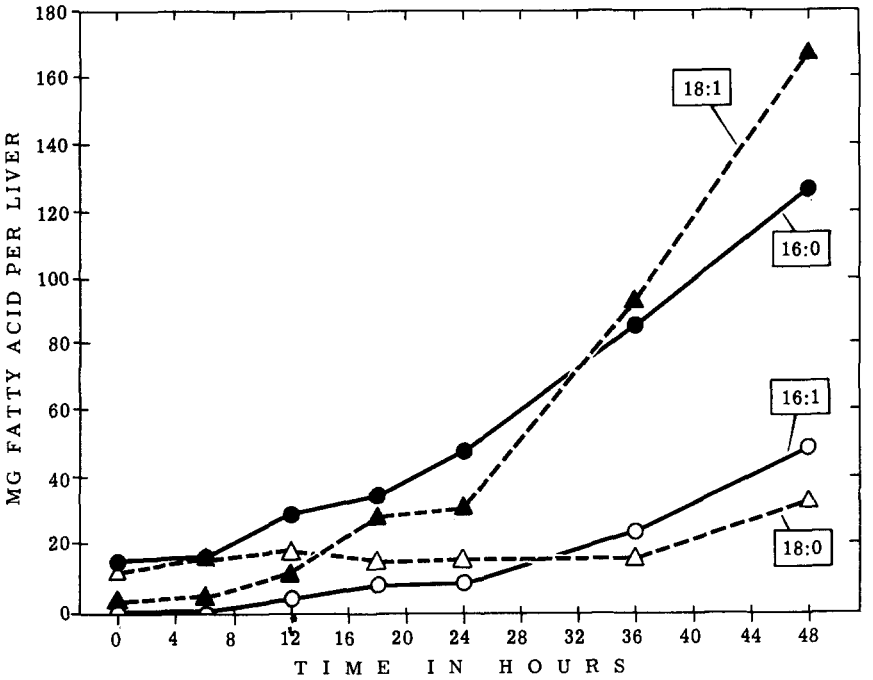

FIG. 7. Yield of palmitic, palmitoleic, stearic, and oleic acids in the liver during refeeding of fat-free diet. Analyses were performed with the same series of rats presented in Fig. 3. The individual fatty acids are represented in terms of milligrams of fatty acid methyl ester per liver.

lipid accumulation was also evident grossly and microscopically.

DISCUSSION

Refeeding a starved animal a balanced diet restores the normal status of the liver as reflected in the levels of the enzymes catalyzing fatty acid synthesis and in the composition of the liver fatty acids. The restoration of nearly normal levels from the starvation state is not accompanied by a linoleic acid deficiency in the liver tissue.

TABLE 8 CHANGES IN THE TRIGLYCERIDE AND PHOSPHOLIPID CONTENT OF LIVER OF STARVED RATS REFED A BALANCED STOCK DIET OR A FAT-FREE DIET

Nutritional State	Triglyceride	Phospholipid
	mg/liver	
Normal	57.4	282
Starved	24.2	81
R 10	57.6	174
R 23	90.0	195
R 48	80.2	195
F 10	54.4	165
F 23	129.0	183
F 48	164.0	174

The animals and feeding conditions were the same as described in Table 1. The liver triglyceride and phospholipid analyses were previously described (1). (Also see Tables 6 and 7.)

Refeeding with a fat-free diet, however, permits the levels of the synthetic enzymes to rise far above normal. Coincident with the enhanced enzyme activity there is net accumulation of saturated and monounsaturated fatty acids, principally in the triglyceride fraction of liver (2–9). Aside from the rapid onset, the latter events are the same as those attending the feeding of young mice (which were not starved) a fat-free or a linoleic acid-free diet (1). In both studies supranormal activity of the enzymes catalyzing fatty acid synthesis was observed only under those conditions that brought about a relative depletion of linoleic acid in the liver. A similar study by Reiser et al. (27) has recently been reported in which fatty acid synthesis was evaluated by the incorporation of acetate into fatty acids in intact rats fed purified lipids (30% of the diet).

The liver is the first tissue to register an upheaval in fatty acid composition in response to the fat-free diet. Epididymal fat pads, heart muscle, and the remaining carcass responded to a less significant degree in the brief time interval of the experiment. Although there is a net decrease in the total amount of linoleic acid in liver the precipitous fall in the relative linoleic acid content may be attributed to the great accumulation of saturated and monounsaturated fatty acids (1). All of the liver cellular subfractions promptly display linoleic acid depletion and an altered composition that indicates a rapid turnover of

all fatty acid moieties in the various lipid compartments of the cell.

Coincident with a fall in the linoleic acid content of liver is the dramatic rise in the enzymes catalyzing the incorporation of acetyl CoA or malonyl CoA into fatty acids as assayed in soluble, semipurified fractions of liver. These assays measure acetyl CoA carboxylase and fatty acid synthetase activities which are directly coupled to the synthesis of saturated fatty acids. The activities of these two enzymes, as determined in the assays employed, were covariant under all experimental conditions examined. The assay in which $C^{14}O_2$ incorporation into malonate is followed (a "direct" assay of acetyl CoA carboxylase) was not covariant (28), employing the standard conditions published for this determination (29). The apparent "uncoupled" activity of acetyl CoA carboxylase may, however, be influenced by the presence of malonyl CoA decarboxylase or other factors.

Evidence has been presented which indicates that acetate incorporation into palmitoleic, stearic, and oleic acids by liver slice preparations was enhanced during the fat-free refeeding period. Synthesis of these acids requires a set of particulate enzymes in order to lengthen the palmitate carbon chain and to introduce the $\Delta 9$ unsaturation (30–33). Eicosatrienoic acid formation via oleic acid (34, 35) was barely detectable during these very brief periods of linoleic acid restriction.

The net increase in saturated and monounsaturated fatty acids appears primarily in the triglyceride fraction of the liver lipids. There is also a definite increase in total liver cholesterol, probably in the form of cholesterol ester (36), and an initial jump in phospholipid. Since virtually no exogenous sources of lipid are available to the liver it seems reasonable to conclude that fatty acids and triglyceride accumulate in the liver as a result of the enhanced capacity for synthesizing these compounds.

Liver triglycerides are the precursors of the plasma triglycerides in the low density β-lipoproteins (37). The increased synthesis of liver triglycerides may thus be of critical importance in generating the lipemia observed in the course of feeding high-carbohydrate, low-fat diets (38).

The fall in linoleic and arachidonic acids, and the accumulation of palmitic, palmitoleic, and oleic acids in the liver lipids should greatly influence the physicochemical nature of the lipids that comprise membrane systems and of the circulating lipids originating in the liver (39). The swelling of liver mitochondria (40, 41), the sensitivity of adipose tissue to insulin (42) that is observed in animals receiving a fat-free diet, and the abnormal equilibration of circulating lipids with the vascular endothelium in atherosclerosis (43, 44), may be the direct result of altered tissue or plasma lipoprotein properties. A study of the composition of the fatty acid moiety of cholesterol esters found in the early "fatty streaks" of the arterial intima has shown that linoleic acid levels are well below normal (45).

The changes in the liver described in this study were brought about acutely, in adult rats, long before the possibility of general depletion of linoleic acid in other tissues, as seen in the classical essential fatty acid deficiency syndrome (34, 46, 47). Thus the consequences of early liver depletion of linoleic acid may have far-reaching effects on other tissues because of the central role of liver in the metabolism of the animal. The liver, however, is not the only tissue to respond to these nutritional states. For example, the rise in fatty acid biosynthesis in the epididymal fat pad was coincident with a significant change in the general fatty acid composition and in the relative linoleic acid concentration of the adipose lipids.

It is not unexpected that other enzymes, generally concerned with triglyceride formation from glucose, may also be increased during the refeeding period. For example, as originally described by Tepperman and Tepperman (8, 9), glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities (assayed in the soluble liver supernatant fraction) increased during the refeeding period, but well after the rise in fatty acid synthesis (see Fig. 1) (4). In the intact cell these ancillary enzymes provide a continuing supply of reduced TPN, a necessary component for the synthesis of both saturated and monounsaturated fatty acids. The activities are elevated although the rate of generation of TPNH is not necessarily limiting. The activity of the citrate cleavage enzyme (48, 49) responds to starvation and fat-free refeeding in a manner similar to the enzymes synthesizing fatty acids (50). This list may also include the malic enzyme (51) and glucokinase (52, 53).

The events accompanying the refeeding of a starved animal with a fat-free diet rest primarily on the fact that the enzymes catalyzing fatty acid synthesis are hyperactive. This rise in activity may be due to a paucity of long-chain fatty acyl inhibitors in the liver tissue. Following the preliminary observations that a diet high in fat depresses fatty acid formation in vivo, and in vitro by liver slices (14, 54–58), evidence has recently accumulated showing that fatty acid derivatives directly inhibit the enzymes catalyzing fatty acid synthesis (15, 16, 59–64). Whether or not linoleic acid is a more effective inhibitor than other long-chain acids has not been established. The tissue concentration of acyl CoA is elevated over the normal in starvation (65, 66) and during the feeding of fat; but the levels are lower than normal during high-carbohydrate alimentation. It has also been suggested (62) that fatty acids may interfere with the citrate-dependent association of inactive

acetyl CoA carboxylase subunits into the complete, competent enzyme (17). Palmityl CoA is also known to inhibit purified citrate synthetase enzyme (63).

The picture which emerges is that several long-chain fatty acids (as the acyl CoA) nonspecifically inhibit acetyl CoA carboxylase and possibly fatty acid synthetase. This provides a basis for the depressed fatty acid synthesis observed in alloxan diabetes and starvation (2,10, 28, 67), situations in which the release of free fatty acids from adipose tissue is accelerated (68). Studies with anti-insulin serum support this view (69). If diminished activity is found in a soluble fraction of liver from an animal in starvation, then it might be surmised that the enzyme protein was present but its activity was hampered by a bound fatty acid inhibitor. This being the case, then the inhibitor is not readily dissociated by the fractionation process used to isolate the enzyme fraction. By the same token, animals on a fat-free diet or a linoleic acid-free diet would possess enzyme free of the inhibitor (even though the enzyme is isolated from grossly fatty livers). Realimentation of the starved animal with a high-carbohydrate, low-fat diet would favor the generation of α-glycerophosphate and the esterification of fatty acids (65, 70), so that low levels of acyl CoA would be expected.

The elevated enzyme activities observed in the present experiment could also be the result of an adaptive synthesis of new enzyme. Puromycin and actinomycin injected in vivo at the onset of the refeeding period blocks the elevation in enzyme activity and the associated changes in fatty acid composition.[1] Such an effect is considered to be diagnostic of adaptive enzyme formation even in the intact animal (71, 72). Insulin may play a direct role in determining enzyme levels. The return of enzyme activity in the soluble fractions of liver following insulin treatment of the alloxan diabetic rat and following refeeding of the starved animal has been reported previously (2, 10, 28). A similar response has now been observed with the glucokinase enzyme system of liver (52, 53). The malic enzyme of liver rises to very high levels following the injection of insulin (73). The metabolism of glucose by epididymal fat pads from rats maintained on a fat-free diet responds more dramatically to insulin in vitro than control preparations (42).

The pattern of changes seen in early linoleic acid deficiency (1) appear to be the same as those accompanying fat-free refeeding. In both studies hyperlipogenesis (beyond the recovery of normal enzyme activity levels) was coincident with a fall in the relative linoleate concentration in liver lipids. It might be postulated that such a radical shift in the composition of the fatty acids making up the lipid-containing structures in all parts of the cell (Table 4) could influence the accessibility (compartmentalization) of nonspecific fatty acid inhibitors (including "free" linoleic acid itself), as well as the acceleration of new enzyme formation.

[1] S. E. Hicks, D. W. Allmann, and D. M. Gibson (unpublished data).

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