Fatty liver induction: inverse relationship between hepatic neutral lipid accumulation and dietary polyunsaturated fatty acids in orotic acid-fed rats

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Abstract The levels of hepatic neutral lipids in the orotic acid-fed rat were inversely related to the dietary levels of polyunsaturated fatty acids, as in the choline-deficient rat. Hepatic microsomal protein and phospholipid and total hepatic phospholipid were increased in orotic acid-induced fatty livers. The increase in phospholipid was largely restricted to the phosphatidylethanolamines.

Supplementary key words choline · polyunsaturated fatty acids · linoleate · linolenate · phosphatidylethanolamine

It has been stated (1) that abnormal accumulation of fat, largely if not exclusively triglyceride, in the parenchymal cells is perhaps the most common response of the liver to injury. Farber (2) has stated: "It now appears that the genesis of fatty livers induced by ethionine, CCl_4, puromycin, white phosphorus, orotic acid, choline-deficiency, and possibly cerium may have a similar type of overall mechanism, even though the metabolic pathway with each agent is probably different." Care must, therefore, be taken to differentiate between observations related to the specific agent utilized and observations common to the group of agents.

Channon and Wilkinson (3) in 1936 reported that the amount of fat deposited in the livers of choline-deficient rats varied inversely with the iodine value of the dietary fat. Iwamoto, Hellerstein, and Hegsted (4) subsequently reported that saturated fatty acids increased the deposition of liver lipids, polyunsaturated fatty acids (PUFA) lowered the deposition, and monounsaturated fatty acids had relatively little effect.

Orotic acid was arbitrarily selected from the group of agents described above. Experiments, as reported herein, were undertaken to determine if hepatic neutral lipid accumulation was inversely related to dietary PUFA as in the choline-deficient rat (3, 4).

Abbreviations: PUFA, polyunsaturated fatty acids; PL, phospholipids.

MATERIALS AND METHODS

In the initial experiments weanling male and female rats of the Sprague-Dawley strain were fed a semisynthetic diet containing soy assay protein (18%), dextrose (67%), fat (10%), salt mixture 446 (5), and a complete vitamin mixture (5). This salt mixture supplies 0.13 ppm of selenium as sodium selenite. All animals received 7.5 mg of D-α-tocopheryl acetate three times per week in a fat-soluble vitamin mixture administered orally by dropper. The rats were maintained on these diets for 12, 13, or 14 days, after which 1.5% orotic acid was added in place of an equal weight of dextrose during the next 8 days. Control rats not fed orotic acid were restricted, by controlling food intake, to the same weight as the comparable orotic acid-fed rats, usually 85–100 g at the time of killing. The soy assay protein is low in methionine; other rats, not a part of the present experiment, fed these control diets ad lib. had an average weight of 90–110 g. For subsequent experiments involving a series of specially prepared fats, the soy assay protein was replaced by casein,

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TABLE 1. Composition of dietary fats

Fat No.	Double Bonds per Fatty Acid						
	0	1	2	3	4	5	6
	%						
1	9.3	89.0	1.7				
2	17.4	72.2	10.3				
3	25.7	55.1	18.8				
4	34.1	38.2	27.4				
5	42.5	21.2	36.0				
6	21.4	69.3	3.4	5.8			
7	33.8	49.5	5.0	11.6			
8	46.1	29.8	6.7	17.4			
9	58.5	10.0	8.4	23.1			
10	22.4	71.9	1.8	0.4	0.6	1.4	1.3
11	35.7	54.8	1.9	0.8	1.2	2.8	2.6
12	49.1	37.7	2.0	1.2	1.8	4.2	3.9
13	62.4	20.6	2.2	1.7	2.3	5.6	5.2
14	49.8	15.3	10.0	24.9			
15	42.5	15.0	10.0	32.5			
16	65.3	14.9	9.9	1.1	1.5	3.7	3.5
17	59.8	15.0	10.1	1.7	2.3	5.7	5.3
18	51.0	19.2	9.9	2.3	3.1	7.5	7.0

and only male rats were used. These rats and their controls were somewhat heavier, weighing approximately 110–125 g at the time of killing; this is to be compared with other rats, not a part of the present experiment, with average weights of 120–140 g when fed these control diets ad lib.

Synthetic fats (Table 1) were prepared by the alkali (sodium methoxide)-catalyzed ester interchange of appropriate molecularly distilled methyl esters and glyceryl triacetin (5). Essentially complete triglyceride formation is achieved by continuous removal of methyl acetate under vacuum. 13 of these fats had an iodine value of 82 (6). This was achieved by starting with a sample of practical triolein, fat no. 1 in Table 1, replacing oleate by an appropriate quantity of linoleate, linolenate, or fish oil polyenes, and making up the difference with a mixture of palmitic and stearic acids. The remaining fats (fats 14–18, Table 1) were based on the direct one-for-one replacement of saturated fatty acids by PUFA in a fat originally containing 85% saturated and 15% monoenoic acids.

Animals were killed by decapitation, and the livers were rapidly excised and homogenized in methylal–methanol 4:1 (v/v) containing 0.01% DL-α-tocopherol (7). Lipid phosphorus was determined by the method of Zilversmit and Davis (8). Total lipids were determined gravimetrically. In one experiment involving female rats fed orotic acid and fat 1 (Table 1) in the soy assay protein diet, the hepatic neutral lipids and phospholipids were separated on a short silicic acid column (9), and individual phospholipid classes were then separated according to the method of Skipski, Peterson, and Barclay (10).

Small portions of the livers of two groups fed orotic acid and fats 1 and 6 in the diet containing soy assay were homogenized in suitable media, and the methods of Kato and Gillette (11) were used to determine the activity of the microsomal enzymes in metabolizing hexobarbital and aniline. Microsomal protein was determined by the biuret method of Layne (12).

RESULTS

Typical data on hepatic lipid composition of male and female rats fed orotic acid and fat 1 or fat 9 in the soy assay protein diet are listed in Table 2. In addition to the expected accumulation of neutral lipid, a significant accumulation of phospholipid was noted. Analysis of hepatic phospholipid classes in the female rats fed orotic acid (Table 3) indicated that the increase occurred

TABLE 2. Effect of orotic acid on hepatic lipids

Sex	Fat No.	Orotic Acid	n	Wt[a]	Liver	Neutral Lipid	Phospholipid
				g	*g/100 g* rat	*mg/liver/100 g rat*	*μmoles/liver/100 g rat*
F	1	−	20	98	4.34 ± 0.07[b]	111 ± 5	163 ± 3
F	1	+	20	96	6.05 ± 0.11	871 ± 31	219 ± 4
F	9	−	15	85	3.95 ± 0.14	87 ± 10	142 ± 4
F	9	+	10	82	5.12 ± 0.15	424 ± 29	194 ± 6
M	1	−	20	99	4.51 ± 0.12	117 ± 4	161 ± 3
M	1	+	20	102	5.62 ± 0.15	746 ± 24	220 ± 4
M	9	−	20	85	3.91 ± 0.10	90 ± 7	138 ± 4
M	9	+	10	84	5.14 ± 0.19	531 ± 8	196 ± 8

[a] Food intake of the control groups was restricted to maintain weight gains comparable to the groups fed orotic acid. The diet contained fats 1 or 9, Table 1, and the protein source was soy assay protein.
[b] Average ± SEM. In each case the value for the orotic acid-fed animals differed significantly ($P < 0.001$) from the corresponding value for the appropriate control group.

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TABLE 3. Effect of orotic acid on hepatic phospholipid classes

Orotic Acid	n		Phosphatidyl-choline		Phosphatidyl-ethanolamine		
			% of PL-P				
–	12		55.2 ± 0.8		26.4 ± 1.1		
+	9		51.5 ± 1.6		34.0 ± 1.8		
			$P < 0.05$		$P < 0.05$		
		PC		PE	PS + PI	SPM + LPC	TPL
				µmoles PL-P/liver/100 g rat			
–	12	89.3 ± 3.1		42.8 ± 2.4	19.9	9.4	164
+	9	111.9 ± 7.0		73.8 ± 4.9	20.8	10.3	217

Animals described in Table 2 were used for these analyses. Only female rats were used. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS + PI, phosphatidylserine plus phosphatidylinositol; SPM + LPC, sphingomyelin plus lysophosphatidylcholine; TPL, total phospholipid; PL-P, phospholipid phosphorus.

TABLE 4. Effect of orotic acid on metabolism of hexobarbital and aniline by hepatic microsomes

Orotic acid	–	+	–	+
Fat no.[a]	1	1	9	9
n	8	7	8	7
Liver (g/100 g rat)	4.24 ± 0.08[b]	5.67 ± 0.20	3.88 ± 0.10	4.90 ± 0.11
Microsomal protein (mg/liver/100 g rat)	148 ± 3	203 ± 20[c]	154 ± 8	165 ± 5
Microsomal phospholipid (µmoles/liver/100 g rat)	73 ± 3	98 ± 13	73 ± 3	73 ± 10
Hexabarbital oxidation (µmoles/hr/liver/100 g rat)	17.5 ± 1.0	15.2 ± 1.6	14.4 ± 2.2	20.4 ± 3.2
Aniline oxidation (µmoles/hr/liver/100 g rat)	3.90 ± 0.26	3.46 ± 0.32	3.29 ± 0.24	3.91 ± 0.36

[a] Fat composition is described in Table 1. Soy assay protein was fed in this experiment. Only male rats were used.
[b] Average ± SEM.
[c] $P < 0.05$.

largely in the phosphatidylethanolamine fraction and to a lesser extent in the phosphatidylcholine fraction.

Data from preliminary experiments indicated that in contrast to other tissue lipids hepatic phospholipid fatty acids attained equilibrium compositions related to dietary fat composition within approximately 1 wk in the case of weanling rats.

Male rats fed orotic acid and fat 1 in the diet containing soy assay protein showed a significant ($P < 0.05$) increase in microsomal protein (Table 4), but the metabolism of hexobarbital or aniline was not altered significantly by ingestion of orotic acid. The groups fed fat 9 accumulated less neutral lipid and phospholipid when orotic acid was administered, and microsomal protein did not increase significantly (Table 4). Significant differences in hexobarbital and aniline metabolism were not observed.

Data for hepatic neutral lipid and hepatic phospholipid from male rats, in groups of 10, fed orotic acid and the synthetic fats in the casein-containing diet suggested an inverse relationship between the magnitude of the values and the level of dietary PUFA. The inverse relationship became particularly evident when the data were grouped in terms of the principal source of PUFA, linoleate, linolenate, or higher polyenes, in the dietary fat. Fat 16 was grouped with the fats containing higher polyenes as the principal source of PUFA, although it contained an equal amount of linoleate. Regression lines were fitted to the data by the method of least squares, and correlation coefficients (r) were calculated to determine the statistical significance of the fit of the data to these lines (13, 14).

The level of hepatic neutral lipid (N) in mg/liver/100 g rat was inversely related to the levels of the various PUFA (x) in g PUFA/kg diet (Fig. 1). For the first 12 fats with an iodine number of 82 (fats 2–13, Table 1), the relationships are described by the equations: $N = 489 - (3.40x)$, $r = -0.908$; $N = 637 - (9.52x)$, $r = -0.971$; and $N = 579 - (14.53x)$, $r = -0.994$, for linoleate, linolenate, and the higher polyenes, respectively. Each gram of linoleate added to the ration had significantly less effect in decreasing hepatic neutral lipid accumulation than did each gram of linolenate ($P < 0.05$) or each gram of higher polyenes ($P < 0.02$).

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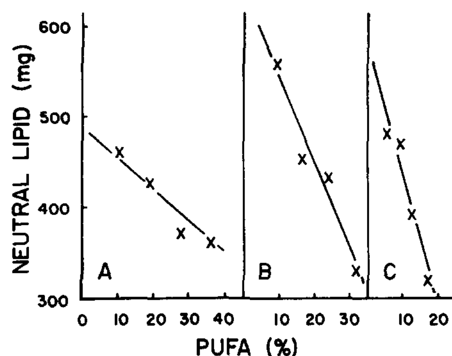

Fig. 1. Effect of dietary levels of polyunsaturated fatty acids on hepatic neutral lipid levels (mg/liver/100 g rat) of rats fed orotic acid. *A*, fats containing linoleate as the principal PUFA; *B*, fats containing linolenate as the principal PUFA; *C*, fats containing higher polyenoic acids as the principal PUFA. Each point is the average value for a group of 10 male rats. Crosses represent data from fats 2–13 (Table 1).

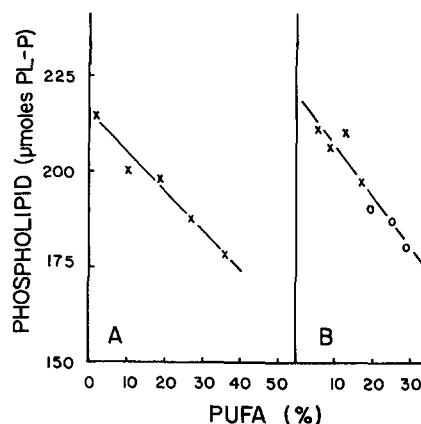

Fig. 2. Effect of dietary levels of polyunsaturated fatty acids on hepatic phospholipid levels (µmoles PL-P/liver/100 g rat) of rats fed orotic acid. *A*, fats containing linoleate as the principal PUFA; *B*, fats containing higher polyenoic acids as the principal PUFA. Each point is the average value for a group of 10 male rats. Crosses represent data from fats 1–5 and 10–13, and circles represent data from fats 16–18 (Table 1).

Linolenate and the higher polyenes did not differ significantly, however, in their effect on hepatic neutral lipid levels.

When the data for hepatic neutral lipid levels in the rats fed fats 6–9 were corrected for the effect of dietary linoleate, each gram of linolenate added per kilogram of diet appeared to lower hepatic neutral lipid levels by approximately 12.2 mg/liver/100 g rat. Similarly, correction of the data for the effects of linoleate and linolenate on rats fed fats 10–13 suggested that each gram of the higher polyenoic acids added per kilogram of diet decreased hepatic neutral lipid levels by approximately 18.2 mg/liver/100 g rat. Lower hepatic neutral lipid levels were noted in rats fed the casein diet, and the data in Fig. 1 differ markedly from data obtained when these fats were fed in the soy assay protein diet (Table 2). This difference presumably arose from the low methionine, and hence low lipotrophic activity, of the soy assay protein.

Restriction of the final iodine value of fats 1–13 determined the maximum level of PUFA which could be incorporated into these fats, particularly in the case of the higher polyenoic acids. Use of several additional fats (fats 14–18) was therefore necessary in the study of the relationship between hepatic phospholipid levels and dietary PUFA. The level of hepatic phospholipids (*PL*) in µmoles phospholipid P/liver/100 g rat was inversely related to the dietary level of PUFA (x) in g PUFA/kg diet according to the equations: $PL = 215 - (1.02x)$, $r = -0.982$; $PL = 225 - (1.36x)$, $r = -0.707$; and $PL = 220 - (1.36x)$, $r = -0.957$, for linoleate, linolenate, and higher polyenes, respectively (Fig. 2). Dietary linoleate, linolenate, and the higher polyenoates did not differ significantly in their inverse relationship to hepatic phospholipid levels. Combining the data on linoleate and higher polyenoates, $PL = 217 - (1.14x)$, still resulted in a high correlation coefficient, $r = -0.959$.

DISCUSSION

It should be emphasized that in the present investigation an effort was made to determine if observations on PUFA metabolism in other studies were related to the specific means of fatty liver induction or if these observations represent general phenomena characteristic of fatty livers induced by a group of agents acting via a similar overall mechanism (2). The use of orotic acid in this investigation represents an arbitrary selection of hepatotoxin from this group, and no effort was made to study the action of orotic acid per se.

Higher levels of hepatic neutral lipids were noted in rats fed soy assay protein than in rats fed casein, but this is presumably related to the relative methionine contents of the two proteins. Since the action of the hepatotoxin, rather than the lipotrophic activity of the protein, was under study, the use of soy assay protein appeared to add an unnecessary and undesirable complication.

In general agreement with the studies on choline deficiency-induced fatty livers (3, 15, 16), it was found that with orotic acid feeding, the level of hepatic neutral lipids was inversely related to the dietary level of PUFA. With groups of 10 rats per diet and 12 synthetic fats, this relationship was readily apparent. Furthermore, the more highly unsaturated the dietary PUFA, linoleate vs. linolenate vs. higher polyenes, the greater the effect noted. This effect is not due to lipid antioxidant contained in the dietary fat, since these specially prepared fats were essentially free of tocopherols, and all rats received the same level of α-tocopheryl acetate in the

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form of a direct oral administration by dropper three times per week.

It is possibly of interest to note that Kohout, Kohoutova, and Heimberg (17) found that the triglyceride output of the normal perfused liver was inversely related to the degree of unsaturation of the fatty acid in the perfusing medium. While the rise in hepatic lipid levels may signify establishment of the block in release of lipoproteins into the bloodstream, the extent of the increase may be related to the composition of the dietary fat and the animal's adipose tissue.

With the increase in liver size, increases in total hepatic phospholipid, microsomal protein, and microsomal phospholipid were also observed. The increase in phospholipid was largely restricted to the phosphatidylethanolamines. An increase in phosphatidylethanolamines in the choline-deficient rat has been described by Haines and Rose (18).

In the present experiment it could not be established that the increase in microsomal protein resulted from an enzyme induction related to drug detoxification; of the two activities assayed, aliphatic and aromatic hydroxylation, neither was significantly increased in these young rats. This, however, does not preclude possible increases in other mixed-function oxidases.

Since experiments demonstrating an inverse relationship between dietary PUFA and the extent of hepatic neutral lipid accumulation in choline-deficient rats have been duplicated in rats fed orotic acid, this phenomenon may be related to the general mode of action of a group of agents inducing fatty livers via the same overall mechanism.

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