Effect of essential fatty acid deficiency on release of triglycerides by the perfused rat liver

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ABSTRACT Studies are reported on release of triglycerides during perfusion of livers of male Sprague-Dawley rats fed a fat-free diet or diets containing hydrogenated coconut oil or corn oil. Perfusions were carried out with Krebs-Ringer bicarbonate buffer containing albumin with and without infusion of oleate or linoleate.

Infusion with sodium oleate or linoleate caused an accumulation of triglycerides in the livers of the corn oil-fed animals and stimulated the release of triglycerides into the perfusing medium. In similar experiments with essential fatty acid-deficient animals, which were fed fat-free diets or diets containing hydrogenated coconut oil, there was no increase in secretion of triglycerides into the perfusate, and the amount of triglyceride which accumulated in the liver was greater than in the livers of the control (corn oil-fed) animals. Tracer experiments with oleate-1-^{14}C or linoleate-1-^{14}C also showed that with livers of essential fatty acid-deficient animals, secretion of triglyceride into the perfusate was not stimulated by infusion of fatty acids into the perfusing medium. It is concluded that impairment of the secretion of triglycerides is a factor in the accumulation of fat in the livers of essential fatty acid-deficient animals.

SUPPLEMENTARY KEY WORDS fatty liver · liver triglycerides

Accumulation of fat in the liver of EFA-deficient animals has been well demonstrated (1) and is associated with low levels of plasma TG or VLDL (2–4). Similar observations have been made in animals with experimentally induced fatty livers, and it has been suggested that an important factor in the development of fatty liver under these conditions is an impairment in the secretion of TG (5, 6). Similar observations in the EFA-deficient rat indicate that impairment in the secretion of TG is responsible for accumulation of fat in the livers of these animals. However, Sinclair and Collins (2) reported that there was no impairment of TG secretion from the livers of EFA-deficient animals on the basis of the experiments with Triton injection, and they concluded that the etiology of the fatty liver in EFA deficiency was different from that in experimentally induced fatty liver. However, results of the Triton experiments upon which their conclusions were based, appeared to be inconsistent with observations of low levels of plasma VLDL and TG observed in animals with an EFA deficiency. Thus, because this point is basic to the mechanism of the accumulation of fat in the liver of EFA-deficient animals, it was decided to investigate further the release of triglyceride via isolated liver perfusion experiments. These studies are reported here.

MATERIALS AND METHODS

Animals

Weanling male rats of the Sprague-Dawley strain (obtained from Dan Rolfsmeyer Co., Madison, Wis.) were placed in individual cages and fed ad lib. for 3–4

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; TG, triglycerides; PL, phospholipids; FFA, free fatty acid(s); EFA, essential fatty acid(s); VLDL, very low density lipoprotein.

months, a basic fat-free diet (group I), or this diet supplemented with 10% hydrogenated coconut oil in place of an equal weight of sucrose (group II). The fat-free diet consisted of, by weight, 29% vitamin-test casein, 61% sucrose, 4% salt mixture (Wesson modified, Obsourne-Mendel salt mix; General Biochemicals, Chagrin Falls, Ohio), 4% cellulose (Nonnutritive cellulose Alphacel; Nutritional Biochemicals Corporation, Cleveland, Ohio), 1% casein containing vitamins in the required amounts (1), and 1% choline mixture (22% choline dihydrogen citrate, 78% casein).

As a control group (III), male Sprague-Dawley rats, 10 wk of age, were fed the basic fat-free diet supplemented with 10% corn oil in place of an equal weight of sucrose for approximately 1 month. Linoleic-1-^{14}C acid (SA 52.9 mCi/mmole) and oleic-1-^{14}C acid (SA 52 mCi/mmole) of 99% radiochemical purity was obtained from Nuclear-Chicago Corporation, Des Plaines, Ill. Oleic and linoleic acid, 99% purity, were purchased from the Lipids Preparation Laboratory of The Hormel Institute, Austin, Minn.

Liver Perfusion

Livers were perfused essentially by the method described by Ruderman, Richards, Valles de Bourges, and Jones (7), using a modified Miller apparatus (model 81-68; Metaloglass Inc., Boston, Mass.) The basic perfusion medium consisted of 100 ml of Krebs-Ringer bicarbonate buffer containing 4 g of bovine serum albumin (0.8 moles of free fatty acid per mole of albumin; fraction V powder, Eastman Organic Chemicals, Rochester, N.Y.), 5 mg of potassium penicillin, 5 mg of streptomycin, and 200 mg of glucose. Prior to placement of the isolated liver in the apparatus, the perfusion medium was oxygenated with 5% CO_2 in oxygen.

The livers were prepared in the following manner: non-fasted rats were anesthetized with ether, and the bile duct and portal vein were cannulated *in situ*. Livers were placed in the perfusion apparatus, and residual blood was washed out of the liver with 50 ml of oxygenated Krebs-Ringer bicarbonate buffer which was then discarded. Flow rate of the medium through the liver was maintained at 40 ml/min by means of 15 cm of water pressure. Liver viability was assessed by gross appearance, bile production, flow rate of the medium, and time course of triglyceride release. Results were discarded when less than 1.0 ml of bile was secreted during the 3 hr perfusion period, when flow rate of medium dropped below 35 ml/min, or when triglyceride release decreased.

In experiments on the effect of fatty acids in the perfusate, 120 μmoles of sodium oleate or linoleate in 1 ml of water was infused first within 5 min. Then 360 μmoles of sodium oleate dissolved in 15 ml of water was infused over a 3 hr period by means of an infusion pump (model 1100; Harvard Apparatus Co. Inc., Millis, Mass.). Immediately upon completion of the perfusion, the livers were removed, perfused manually with 30 ml of cold (0°C), physiological saline, weighed, frozen on dry ice, and stored at −40°C until analyzed.

Analysis of Lipid

The lipid was extracted with chloroform–methanol 2:1 by the method of Folch, Lees, and Sloane Stanley (8) and washed with 0.2 volumes of 0.02 N HCl (9). TG was determined by the method of Van Handel and Zilversmit (10); PL was determined by phosphorus analysis using a modified Bartlett procedure (11), and FFA was analyzed by the method of Mahadevan, Dillard, and Tappel (12) after separation of PL using Florisil (13).

TG and total PL fractions were isolated by TLC, and fatty acid composition was determined by GLC of methyl esters prepared by interesterification (14). GLC was carried out with an F&M instrument model 1605 equipped with a hydrogen flame detector and a 6 ft × 1/4 in. column packed with 8% EGSS-X on Gas Chrom P (Applied Science Laboratories Inc., State College, Pa.) at a temperature of 185°C and nitrogen flow of 75 ml/min.

Perfusion experiments with 10 μCi of radioactive sodium oleate or linoleate diluted with 480 μmoles of unlabeled material were carried out as described above. In order to obtain complete recovery of radioactive CO_2 in these experiments, the gas (5% CO_2 in oxygen) was passed through the perfusate for 1 hr after the liver had been removed from the apparatus.

Distribution of radioactivity in TG, FFA, PL, and other fractions isolated by TLC, was determined by counting samples in a Packard Tri-Carb scintillation spectrometer. The scintillation solvent described by Snyder (15) was used. Radioactive CO_2 was trapped by passing the gas through two tubes in series, each containing 50 ml of 2-amino-ethanol–2-methoxy-ethanol 1:2 (v/v). Radioactive CO_2 was measured by counting aliquots dissolved in a dioxane scintillation solution (16). The radioactivity trapped by the second tube was approximately 1/25 of that of the first tube; tubes were replaced hourly to insure complete trapping of the radioactive CO_2.

RESULTS

The animals in groups I and II exhibited the high ratio of trienoic to tetraenoic acids in the liver PL (Table 1), and the dermal symptoms characteristic of an EFA deficiency. However, the color of livers from group II ranged from brown to slightly yellow indicating

variations in the onset of accumulation of fat in the liver. The output of bile indicated that liver function was not impaired throughout the course of the experiment. Liver viability was also indicated by CO_2 production and absorption of fatty acids from the perfusate. The increase of liver weight after perfusion was due largely to edema, but the increases were not significantly different among the three groups.

The amounts of TG and PL in nonperfused and perfused livers of the three groups are shown in Table 2. Comparison of the nonperfused livers of the three groups showed that the levels of TG were higher in the EFA-deficient animals (groups I and II). The PL levels were not significantly altered by an EFA deficiency. Perfusion with oleate increased the TG content in the livers of the animals in all groups. The TG level in livers after perfusion was significantly greater in the animals of the fat-free group (I) than in those of the corn oil group (III). The amount of PL was also greater in the perfused livers than in nonperfused livers of group I, but the increases were small in comparison with TG.

Analyses of the lipids of the perfusate are shown in Table 3. There was a large uptake of FFA from the perfusate with all groups. The amount of FFA taken up by groups I and II was higher than or almost the same as that of group III, and the difference between groups II and III was statistically significant. On the other hand, the levels of TG in the perfusate during perfusion of the livers of groups I and II were significantly lower than those of group III in contradistinction to the TG content of the perfused livers (Table 2). The progressive release of triglyceride from the livers into the perfusate was also measured at selected intervals during the perfusion. These results (Fig. 1) showed that the average values for the release of TG from the liver was always greater in the animals of group III (corn oil-fed) than in those of groups I and II. The differences became significant at the 3 hr period as illustrated in Table 3.

Although infusion of oleate into the perfusates in the experiments with the livers of group I failed to increase TG secretion from the liver, it almost doubled the secretion of TG from the livers of the animals of group III. When livers were perfused without infusion of oleate, no significant difference was observed in the amount of TG secretion between groups I and III.

In order to determine differences in effects of essential and nonessential fatty acids on the secretion of triglycerides from livers of EFA-deficient animals, linoleate was used in place of oleate in several experiments. After 3 hr of perfusion, the amounts of TG secreted into the perfusate for animals of groups I, II, and III were 1.45 ± 0.30 [3], 1.76 [1], and 2.72 ± 0.78 [5] μmoles per g of liver, respectively (numbers in the brackets

TABLE 1 ANALYTICAL DATA ON ANIMALS

Group	Diet Supplement	Weight of Animals	Fatty Acid Analysis Liver Phospholipid		Liver Weights		Bile Production
			20:3 ($n-9$)	20:4 ($n-6$)	Nonperfused	Perfused	
		g	*% of total fatty acid*		*g*		*ml/g of liver per 3 hr*
I	Fat-free	339 ± 31 (10)	22.0 ± 1.3* (4)	5.5 ± 3.4* (4)	8.1 ± 0.7 (5)	10.6 ± 0.9 (5)	0.15 ± 0.04 (5)
II	Hydrogenated coconut oil	323 + 29* (12)	19.4 ± 3.7* (4)	7.5 ± 2.2* (4)	10.2 ± 21* (7)	11.8 + 2.9 (5)	0.14 ± 0.04 (5)
III	Corn oil	349 ± 46 (13)	nil (3)	32.7 ± 4.3 (3)	7.9 ± 1.3 (7)	10.6 ± 0.9 (6)	0.12 ± 0.01 (6)

Rats fed semisynthetic diet. In groups II or III, 10% of each oil was substituted for sucrose. Values are mean ±SD. Number in parentheses indicates number of animals. Liver PL column shows percentage of each fatty acid in control (nonperfused) rat.
* $P < 0.05$ when compared with group III.

TABLE 2 TRIGLYCERIDE AND PHOSPHOLIPID CONTENT OF NONPERFUSED AND PERFUSED LIVERS*

Group	Triglyceride		Phospholipid	
	Nonperfused	Perfused	Nonperfused	Perfused
	μmoles per liver†			
I	142 ± 48‡ (5)	323 ± 84§‖ (5)	354 ± 68 (4)	476 ± 56‖ (5)
II	256 ± 132§ (7)	468 ± 353 (5)	409 ± 74 (4)	463 ± 96 (5)
III	103 ± 25 (7)	202 ± 53 ‖(6)	359 ± 92 (4)	391 ± 60 (6)

* Perfusing medium contained 480 μmoles sodium oleate.
† Values are means ±SD. Number in parentheses indicates number of animals.
‡ $P < 0.10$ compared with group III.
§ $P < 0.05$ compared with group III.
‖ $P < 0.05$ compared with nonperfused liver of each group.

TABLE 3 ANALYSES OF PERFUSATE LIPID*

Group	Free Fatty Acid		Triglyceride	Phospholipid	Triglyceride Without Infused Oleate
	Remaining	Removed†			
	μmoles			*μmoles/g of liver*	
I	69.4 ± 15.6 (4)	460 ± 16 (4)	1.46 ± 0.81‡ (5)	1.46 ± 0.22 (4)	1.71 ± 0.18 (3)
II	58.2 ± 10.6‡ (5)	472 ± 11‡ (5)	1.26 ± 0.59‡ (5)	1.07 ± 0.37‡ (4)	
III	84.2 ± 9.0 (5)	446 ± 9 (5)	2.71 ± 0.61 (6)	1.69 ± 0.13 (5)	1.40 ± 0.33 (4)

* Liver perfusion was performed with infusion of 480 μmoles of sodium oleate during 3 hr, except the groups in last column. Values of last column show the results of perfusion without infusion of oleate.

† Removed FFA includes the amount of FFA bound to albumin. Values are mean ± SD. Number in parentheses shows number of animals.

‡ $P < 0.05$ compared with group III.

designate the number of animals used). These results showed that the effect of linoleate was essentially the same as that of oleate and that impairment in the secretion of TG in groups I and II, in which oleate was infused into the perfusate, was not due to suppression of the utilization of oleate. Table 3 also shows that the amounts of PL secreted into the perfusates were smaller in groups I and II than in group III. The difference between groups II and III is statistically significant.

An estimation of the amounts of TG and PL fatty acids in the liver before and after perfusion with oleate is shown in Table 4. The results in Table 4 were calculated on the basis of fatty acid composition and the amounts of lipid in Table 2. The increase in fatty acid in the liver of

TABLE 4 AMOUNTS OF FATTY ACIDS IN NONPERFUSED AND PERFUSED LIVERS WITH SODIUM OLEATE*

Group		Total	16:0	16:1	18:0	18:1	18:2	20:3	20:4
Triglyceride									
I	Nonperfused	426	115	61		233			
	Perfused	969	239	107		594			
III	Nonperfused	309	79	22		79	112		
	Perfused	606	127	36		306	108		
Phospholipids									
I	Nonperfused	708	132	58	138	170	14	156	38
	Perfused	952	174	78	174	290	28	164	42
III	Nonperfused	718	146	20	176	56	82		234
	Perfused	782	154	26	166	104	100		232

* Values are the amounts of fatty acids (μmoles per liver) calculated on the basis of fatty acid composition, and the amounts of lipids (Table 2).

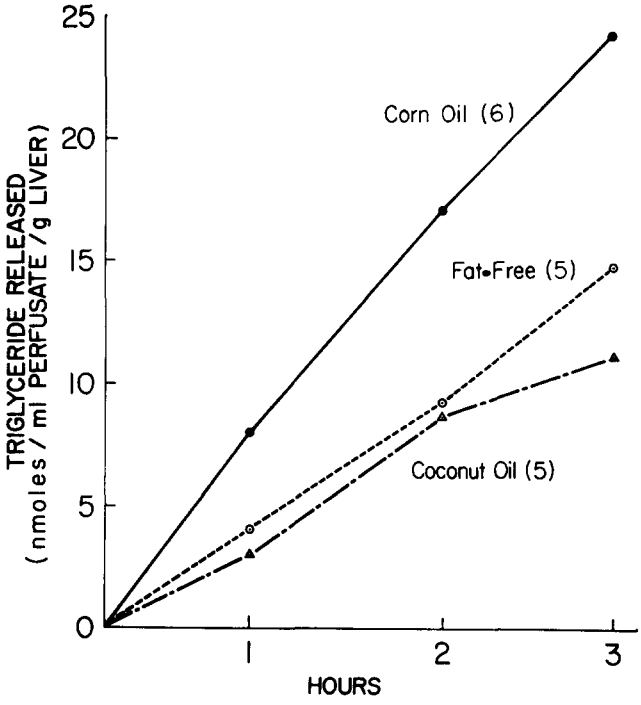

FIG. 1. Release of triglyceride from isolated liver during perfusion with sodium oleate. Each point represents the mean value for six animals in the corn oil group (III) and five animals in each of the fat-free (I) and hydrogenated coconut oil (II) groups.

the animals of group III was due mostly to oleic acid. In group I the amounts of palmitate and palmitoleate were also increased in the triglycerides. The increase in the phospholipids was due mostly to oleic acid in both groups I and III; the increase was much greater in the former group.

Amounts of individual fatty acids in the TG secreted from the liver with and without oleate in the perfusing medium are shown in Table 5. There was a much larger amount of fatty acid secreted from the livers of the corn oil group (III) when the perfusing medium contained oleate, and it is evident that the increase was due almost entirely to oleic acid. In contrast to the corn oil group, addition of oleate to the perfusing medium brought about little change in the total amount of TG fatty acid in the fat-free group (I).

In order to determine further the fate of infused acid, experiments were carried out with oleate-1-^{14}C or linoleate-1-^{14}C. In these experiments the radioactivity infused during first, second, and third hour period was 5.0, 2.5, and 2.5 μCi, respectively. Aliquots of the perfusate were taken at the end of each hour and counted as described above. Recovery of radioactivity was over 90%. Table 6 shows that the major part of radioactivity was in the liver TG. Perfusions with oleate

TABLE 5 FATTY ACID CONTENT OF PERFUSATE TRIGLYCERIDES AFTER PERFUSION*

Group		Total	14:0	16:0	16:1	18:0	18:1	18:2
		μmoles × 10⁻²/g of liver						
I	A†	513	8	156	87	11	251	
	B‡	438	6	95	52	7	278	
III	A	420	8	142	47	10	116	97
	B	813	9	136	50	11	513	94

* Values were determined from the total μmoles of TG (Table 3) on the basis of fatty acid composition.
† A, no sodium oleate in perfusing medium.
‡ B, 480 μmoles of sodium oleate infused into perfusing medium.

TABLE 6 RECOVERY OF RADIOACTIVITY IN CO_2, LIVER, AND PERFUSATE LIPIDS*

Group	Time	CO_2	Perfusate			Liver	
			FFA	TG	PL	TG	PL
	hr						
A. Sodium oleate-1-^{14}C†							
I (2)	1	1.9	16.3	1.3	0.3		
	2	3.1	9.2	3.7	0.6		
	3	4.2	6.8	3.1	0.8	53.0	22.7
III (2)	1	0.6	24.2	2.4	0.2		
	2	0.7	11.4	6.9	0.2		
	3	1.0	8.6	9.6	0.4	64.8	12.1
B. Sodium linoleate-1-^{14}C							
I (1)	1	2.3	12.3	1.1	2.0		
	2	3.3	9.0	1.8	2.0		
	3	4.7	4.0	2.4	1.4	52.1	20.8
III (1)	1	0.5	15.8	3.8	3.1		
	2	1.0	7.8	8.1	2.5		
	3	1.4	4.2	11.1	2.3	53.0	16.0

Results are expressed as percentage of added radioactivity.
* Number in parentheses indicates number of animals. A total of 10 μCi of radioactive fatty acid was infused into perfusate with 480 μmoles of the corresponding unlabeled fatty acid. Amounts of radioactivity infused during the first, second, and third hour periods were 5.0 μCi, 2.5 μCi, and 2.5 μCi, respectively.
† Values in A show the mean of two experiments.

and linoleate gave similar results. Analysis of radioactive CO_2 showed that the amount of oxidation during the experiment was small. However, the higher radioactivity in the CO_2 collected in experiments with the fat-free group suggested that there was a faster rate of oxidation of added fatty acids in the livers of the animals of this group than in those of the animals of the corn oil group. A high percentage of radioactivity was found in the perfusate TG in experiments with the livers of the corn oil group compared with those of the fat-free group, indicating a faster rate of TG secretion from the liver of the animals of group III.

DISCUSSION

In accordance with previous reports (9, 17), release of triglycerides was stimulated from livers of the corn oil-fed (normal) animals (group III) in liver perfusion experiments by elevated levels of FFA in the perfusate (Table 3). Thus, failure of infused oleate or linoleate to stimulate the release of TG from livers of the animals in groups I and II (Table 3) indicates that secretion of liver TG is impaired by an EFA deficiency. The tracer experiments with radioactive fatty acids also demonstrated that the capacity of the liver to secrete triglycerides was impaired by an EFA deficiency (Table 6). Evidence of this is the higher ratio for the distribution of radioactivity between the triglycerides of the liver and perfusates of the fat-free (group I) animals compared with that of the corn oil (group III) animals. An interesting aspect of these experiments is the higher percentage of radioactivity in the phospholipids of the animals of the fat-free group compared with that of the corn oil group. It appears that this observation is due to the high rate of turnover of fatty acids in the phospholipids of EFA-deficient animals (18) and indicates that changes occur in other facets of the metabolism of lipids in the livers of EFA-deficient rats in addition to an influence on TG secretion.

In the present study the development of fatty liver as the result of an EFA deficiency was not marked in accordance with reports of a number of other investigators (19–21). The large amount of fat (9–10%) in the livers of the EFA-deficient animals in the experiments of Sinclair and Collins (2) is highly unusual, and we suspect that it may be due to a suboptimal amount of choline in the diet. Although other dietary ingredients can substitute to some extent for choline, the amount added to the diet was only approximately 40% of that recommended by Holman (1) for EFA-deficient rats. Moreover, the effect of choline deficiency in causing fatty livers is progressive and may be aggravated by the stress of an EFA deficiency as it is by high levels of cholesterol in the diet (22). Thus, the effect of Triton in the animals in the studies by Sinclair and Collins (2) may be explained on this basis because otherwise it is inconsistent with the observation of low levels of plasma VLDL and TG in EFA-deficient animals.

Failure of the development of overt fatty liver in EFA-deficient animals indicates that impairment of the secretory capacity of the EFA-deficient liver is not so severe as in poisoning by carbon tetrachloride and becomes manifest only as hepatic lipogenesis and (or) TG synthesis increases.

In an EFA deficiency, plasma FFA level is reported to be high (2, 23), and FFA uptake of the liver of the EFA-deficient animals is as efficient as that of normal or control animals as shown in Table 3. Moreover, fatty acid synthesis is reported to be increased by an EFA deficiency (24–27). The increase of 16:0 in the liver of group I is in accord with these observations. Thus, in an EFA deficiency a higher rate of TG formation in the

liver is highly probable, and this, in turn, should be a stimulus for the formation of VLDL (7, 28). If a sufficient amount of VLDL were formed, there would be no overt increase of TG content of the liver even though TG formation would be increased. However, if the liver can only produce a basal amount of VLDL (but not enough in response to the higher rate of TG formation), TG will accumulate accordingly.

Impairment of the secretion of TG from livers of EFA-deficient animals may be attributed to a number of factors. EFA may be required for the formation of the physicochemical structure of lipoprotein for transport of triglyceride into the perfusate. Evidence for this effect is disarrangement of membrane (lipoprotein) structure, such as in mitochondria or red blood cells, due to an EFA deficiency (29). Another possibility is that EFA may be required for the synthesis of protein moiety of lipoprotein. Ruderman et al. (7) demonstrated that liver released and possibly synthesized apoprotein of VLDL in response to high concentrations of linoleate in the perfusate. Since our conditions of perfusion were the same as theirs, new synthesis of apoprotein probably occurred in the livers of the corn oil group (III) concomitant with the secretion of triglyceride into the perfusate. Accordingly, secretion of triglyceride from livers of the EFA-deficient animals may not be stimulated because of impairment in the synthesis of apoprotein. This facet of fatty liver development is currently under investigation.

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