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 control (corn oil-fed) animals. Tracer experiments with oleate-1-14C or linoleate-1-14C 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
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-1-14C acid (SA 52.9 mCi/m mole) and oleic-1-14C acid (SA 52 mCi/m mole) 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% CO2 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 X 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 CO2 in these experiments, the gas (5% CO2 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 CO2 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 CO2 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 CO2.

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 CO₂ 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 perfused livers of the EFA-deficient animals (groups I and II). The increases were not significantly different among the comparison groups.

Perfusing medium contained 1.45 ± 0.30 [3], 1.76 [1], and 2.72 ± 0.78 [5] μmoles per g of liver, respectively (numbers in the brackets). TG and PL in nonperfused and perfused livers of the three groups are shown in Table 2. The levels of TG were higher in the perfused livers of the animals than in the nonperfused livers of the same group.

Table 2: Triglyceride and Phospholipid Content of Nonperfused and Perfused Livers

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet Supplement</th>
<th>Weight of Animals</th>
<th>20:3 (n = 9)</th>
<th>20:4 (n = 6)</th>
<th>Nonperfused</th>
<th>Perfused</th>
<th>Bile Production ml/g of liver per 3 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Fat-free</td>
<td>339 ± 31 (10)</td>
<td>22.0 ± 1.3* (4)</td>
<td>5.5 ± 3.4* (4)</td>
<td>8.1 ± 0.7 (5)</td>
<td>10.6 ± 0.9 (5)</td>
<td>0.15 ± 0.04 (5)</td>
</tr>
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<td>II</td>
<td>Hydrogenated coconut oil</td>
<td>323 ± 29* (12)</td>
<td>19.4 ± 3.7* (4)</td>
<td>7.5 ± 2.2* (4)</td>
<td>10.2 ± 21* (7)</td>
<td>11.8 ± 2.9 (5)</td>
<td>0.14 ± 0.04 (5)</td>
</tr>
<tr>
<td>III</td>
<td>Corn oil</td>
<td>349 ± 46 (13)</td>
<td>nil (3)</td>
<td>32.7 ± 4.3 (3)</td>
<td>7.9 ± 1.3 (7)</td>
<td>10.6 ± 0.9 (6)</td>
<td>0.12 ± 0.01 (6)</td>
</tr>
</tbody>
</table>

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 3: Analytical Data on Animals

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<tr>
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* P < 0.05 when compared with group III.
TABLE 3 ANALYSES OF PERFUSATE LIPID*

<table>
<thead>
<tr>
<th>Group</th>
<th>Free Fatty Acid</th>
<th>Triglyceride</th>
<th>Phospholipid</th>
<th>Triglyceride Without Infused Oleate</th>
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<tr>
<td></td>
<td>Remaining μmoles</td>
<td>Removed μmoles</td>
<td>μmoles/g liver</td>
<td>μmoles/g liver</td>
</tr>
<tr>
<td>I</td>
<td>69.4 ± 15.6 (4)</td>
<td>460 ± 16 (4)</td>
<td>1.46 ± 0.81† (5)</td>
<td>1.46 ± 0.22 (4)</td>
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<tr>
<td>II</td>
<td>58.2 ± 10.6† (5)</td>
<td>472 ± 11 (5)</td>
<td>1.26 ± 0.59† (5)</td>
<td>1.07 ± 0.37† (4)</td>
</tr>
<tr>
<td>III</td>
<td>84.2 ± 9.0 (3)</td>
<td>446 ± 9 (5)</td>
<td>2.71 ± 0.61 (6)</td>
<td>1.69 ± 0.13 (5)</td>
</tr>
</tbody>
</table>

* 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 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-1-14C or linoleate-1-14C. 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. Perusions with oleate...
and linoleate gave similar results. Analysis of radioactive CO₂ showed that the amount of oxidation during the experiment was small. However, the higher radioactivity in the CO₂ 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 oil group. A high percentage of radioactivity was 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.

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

<table>
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<th>TABLE 5</th>
<th>Fatty Acid Content of Perfusate Triglycerides After Perfusion*</th>
</tr>
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<tbody>
<tr>
<td>Group</td>
<td>CO₂ 10⁻¹⁴CO₂</td>
</tr>
<tr>
<td>I (1)</td>
<td>1</td>
</tr>
<tr>
<td>II (2)</td>
<td>2</td>
</tr>
<tr>
<td>III (3)</td>
<td>3</td>
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<tr>
<td>B (4)</td>
<td>1</td>
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<tr>
<td></td>
<td>2</td>
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<td></td>
<td>3</td>
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</table>

Results are expressed as percentage of added radioactivity.

* Values were determined from the total moles 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.

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.
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 accumulation 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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