Liver Lipids of Choline-Deficient Rats

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1. Four-week-old male and female rats were given choline-deficient diets for 2 weeks. Deficient animals gained nearly as much weight as normal controls of the same sex. 2. The amounts of triglyceride and esterified cholesterol in liver lipids were increased threefold or more by the deficiency. The amounts of the major phosphatides and of unesterified cholesterol were unaffected. 3. In males, deficiency significantly increased the proportion of stearic acid in triglycerides, and, in females, the proportion of arachidonic acid was significantly decreased. 4. In the phospholipids of male rats, choline deficiency produced decreases in the amounts of linoleic acid and arachidonic acid and increased the amount of stearic acid. In the phospholipids of female rats, choline deficiency decreased the amount of arachidonic acid and increased that of linoleic acid. 5. The liver phospholipids of normal male rats had higher proportions of palmitic acid and lower proportions of stearic acid than were found in normal females. These sex differences became statistically insignificant in deficient animals.

Choline deficiency disturbs lipid metabolism severely in most animal species. In rat liver, there is an enormous deposition of triglyceride and a smaller increase in esterified cholesterol (Baxter & Goodman, 1955); in the circulation, concentrations of phospholipids, cholesterol esters, triglycerides and unesterified cholesterol all decrease. The concentrations of fatty acids of serum triglycerides decrease almost uniformly, but arachidonic acid and stearic acid concentrations undergo large decreases in serum phospholipids of both sexes (Tinoco, Shannon & Lyman, 1964). These effects on lipid composition resemble those found in ethionine-treated female rats (Arvidson & Olivecrona, 1962; Olivecrona, 1962), and these similarities indicate that the ultimate causes of both types of disturbance may be biochemically the same.

The liver lipids of choline-deficient rats were investigated to explore further the parallelism between the reported effects of choline deficiency and ethionine on serum lipid composition. We also wished to study the possibility that liver kephalins might accumulate in rats given a diet low in methyl groups, which was suggested by Stetten (1942) to explain the observation that choline-deficient rats given [¹⁵N]ethanolamine incorporate less label into liver kephalins than do normal rats. This finding could result from a diminished degree of incorporation, or through dilution of the incorporated label by accumulated kephalin. We were also interested in the change in liver lipid composition produced

by choline deficiency, and its possible relation to effects observed by Tinoco *et al.* (1964) in serum lipids of choline-deficient rats.

EXPERIMENTAL

The procedure for producing choline deficiency in rats was as described by Tinoco *et al.* (1964). Male and female weanling Long-Evans rats (Simonsen Laboratories, Gilroy, Calif., U.S.A.), averaging 48 g. and 47 g. respectively, were housed individually in wire-bottomed galvanized cages. All animals were given stock diet (Diablo Labration from Diablo Laboratories, Berkeley, Calif., U.S.A.) for the first week, and experimental diets for the next 2 weeks, *ad libitum* in both cases. Experimental diets contained 10% of safflower oil, 18% of soya-bean protein, sucrose, vitamins and minerals; they were low in methionine, contained no added vitamin B₁₂ and differed only in choline content. The control diet contained choline hydrogen tartrate (0.25% by wt. of the diets as choline), added at the expense of sucrose to the choline-deficient diet. Half of each sex group received the choline-deficient diet, and the other half were given the control diet. After the rats had been given the experimental diets for 2 weeks, they were starved for 18 hr. before being anaesthetized with Nembutal (Abbot sodium pentobarbital) and killed by heart-puncture exsanguination. Male rats gained an average of 125 and 123 g. for control and deficient groups respectively. Control and deficient females gained 88 and 86 g. respectively. These gains are based on body weights measured after the 18 hr. starvation period.

Livers were removed, weighed immediately and frozen on solid carbon dioxide. They were freeze-dried in a

modified vacuum oven and the lipids were extracted as described by Tinoco, Miljanich & Lyman (1963).

All solvents used in these experiments were freshly distilled in glass, except methanol (Merck reagent) and chloroform (B & A reagent).

The lipid extracts were analysed for total cholesterol (Sperry & Webb, 1950), total phosphorus (Sumner, 1944) and total lipid (Bloor, 1928). Phospholipid was calculated as phosphorus $\times$ 23.9.

Samples containing about 20 mg. of lipid were fractionated on silicic acid into cholesterol esters, triglycerides, phospholipids and a small fraction containing mono- and di-glycerides, unesterified fatty acids and unesterified cholesterol (Lis, Tinoco & Okey, 1961). All fractions except triglycerides were saponified for 30 min. at 37° with 0.30 ml. of aq. 33% (w/w) KOH in 3 ml. of ethanol. This saponification procedure has been found to saponify quantitatively samples containing 1 mg. of cholesterol or less. Then 3 ml. of water and 10 ml. of light petroleum were added, and after 12 hr. the upper layer was decanted and analysed for cholesterol (Abell, Levy, Brodie & Kendall, 1952) in those fractions expected to contain it. Cholesterol is quantitatively extracted into the light-petroleum layer under these conditions. The aqueous phase was acidified with 9N-H₂SO₄ and 10 ml. of light petroleum was added. After 12 hr. the upper layer, containing all the extractable free fatty acids, was decanted. The remaining aqueous phase of the phospholipid fraction was analysed for phosphorus (Sumner, 1944). The phospholipid fraction was acidified immediately after saponification, and extraction of non-saponifiable matter was omitted. We have found that the same fatty acid pattern is obtained for liver phospholipids when this fraction is transmethylated without previous saponification.

Methyl esters from liver lipids were prepared by adding 10 ml. of 1% (v/v) H₂SO₄ in anhydrous methanol to 1–10 mg. of either free fatty acid or triglyceride, and evaporating this mixture by boiling to a volume of about 1 ml. Then 4 ml. of water and 10 ml. of light petroleum were added, and the mixture was allowed to stand, corked, overnight. The methyl esters migrated into the light-petroleum phase, which was decanted, concentrated to a volume of about 1 ml. and stored at below 0° until analysis. At the time of analysis, the remaining solvent was removed and the methyl esters were injected into the gas-liquid chromatography apparatus, in iso-octane solution.

An Aerograph A-90-C instrument (Wilkens Instrument Co., Walnut Creek, Calif. U.S.A.) was used. The liquid phase, poly(diethylene glycol succinate) was supported by silicone-treated Chromosorb W. The apparatus was calibrated daily by separation of a standard mixture. Relative areas of peaks were calculated from the product of peak

height and the retention time (measured as a distance on the recorder chart) (Carroll, 1961). The amount of fatty acid in the triglyceride fraction was determined by use of an internal standard (Tinoco, Shannon, Miljanich, Lyman & Okey, 1962).

An accurate analysis of the cholesterol ester fatty acids was not possible, because this very small fraction apparently became contaminated with extraneous fatty acid (Nelson, Bear, Dotson & Krause, 1963). The contamination was too small to produce any perceptible loss from either the triglyceride or phospholipid fractions.

Phospholipids were fractionated into cephalins, lecithins, sphingomyelins and lysolecithins by the method of Phillips (1958). The identities of the components and their chromatographic behaviour in this system have been confirmed by Lyman, Shannon, Ostwald & Miljanich (1964). Ten fractions of phospholipids were collected, and the amount of phosphorus in each was measured (Sumner, 1944).

RESULTS

Table 1 shows the amounts of major lipid classes found in normal and choline-deficient rat livers. In deficient animals, triglycerides accounted for most of the lipid accumulation; a smaller increase in cholesterol ester also occurred. These differences between control and deficient animals were statistically significant ($P < 0.01$) as determined by the *t* test (Snedecor, 1956). Males responded to the deficiency more strongly than did females, with sixfold and 20-fold increases in cholesterol ester and triglyceride contents respectively. Females showed approximately threefold increases in these fractions. Choline deficiency did not alter the amounts of phospholipid or unesterified cholesterol in either sex.

Subfractionation of phospholipids from livers of two normal and three deficient animals of each sex demonstrated that deficiency did not change the relative amounts of liver phospholipids, within experimental uncertainty. We found, as percentages of the total phosphorus recovered: neutral lipids, 0.2; cephalins, 34.7; lecithins, 55.1; sphingomyelins, 4.7; lysolecithins, 5.5. Other workers have obtained similar values (Getz, Bartley, Stirpe, Notton & Renshaw, 1961; Hanahan, Dittmer & Warashina, 1957; Figard & Greenberg, 1962).

Table 1. *Liver lipids in normal and choline-deficient rats*

Experimental details are given in the text. The results are given as means $\pm$ S.E.M.

Sex	Diet	No. of animals	Liver wt. (g.)	Total lipid (mg./liver)	Phospholipid (mg./liver)	Total cholesterol (mg./liver)	Cholesterol as ester* (mg./liver)	Unesterified cholesterol* (mg./liver)	Triglyceride (mg./liver)*
Male	Control	9	6.23 $\pm$ 0.25	288 $\pm$ 7.5	209 $\pm$ 6.5	20.4 $\pm$ 1.2	5.26 $\pm$ 0.98	13.4 $\pm$ 1.0	74 $\pm$ 5.4
Male	Deficient	5	7.25 $\pm$ 0.34	1710 $\pm$ 200	204 $\pm$ 5.2	48.7 $\pm$ 4.3	34.5 $\pm$ 4.6	14.6 $\pm$ 0.37	1500 $\pm$ 180
Female	Control	11	5.02 $\pm$ 0.12	243 $\pm$ 11.3	154 $\pm$ 4.4	15.5 $\pm$ 0.8	3.51 $\pm$ 0.28	9.13 $\pm$ 0.71	92 $\pm$ 25
Female	Deficient	5	4.94 $\pm$ 0.10	513 $\pm$ 70	154 $\pm$ 4.0	24.9 $\pm$ 2.1	12.4 $\pm$ 1.86	10.8 $\pm$ 0.70	316 $\pm$ 55

* Analyses performed on five animals only in each group.

Table 2. *Fatty acid patterns of liver triglycerides and phospholipids in normal and choline-deficient rats*

Liver lipids from five animals in each experimental group were individually fractionated and analysed. Experimental details are given in the text. The results are expressed as percentages by weight of the total fatty acids in each fraction, and are given as means $\pm$ S.E.M. The percentages of some minor components have been omitted.

		Fatty acid composition (% of total)						
Sex	Diet	C _{14:0} acid Triglyceride	C _{16:0} acid	C _{16:1} acid	C _{18:0} acid	C _{18:1} acid	C _{18:2} acid	C _{20:4} acid
Male	Control	0.58±0.05	21.1±0.45	2.46±0.16	2.60±0.10	15.0±0.92	48.6±1.1	8.64±1.2
Male	Deficient	0.74±0.09	23.6±1.6	2.60±1.2	3.76±0.21	14.1±1.8	45.1±3.1	7.52±1.1
Female	Control	0.40±0.10	21.5±0.59	2.02±0.23	2.88±0.24	14.1±0.52	49.3±0.84	8.72±0.77
Female	Deficient	0.58±0.07	21.8±0.81	1.98±0.18	2.74±0.22	12.9±0.28	52.8±1.3	6.54±0.32
Phospholipid								
Male	Control	—	18.2±1.7	0.48±0.12	25.1±0.85	3.46±0.13	13.3±0.49	36.4±0.90
Male	Deficient	0.12±0.04	20.2±0.96	0.32±0.16	28.4±0.61	3.06±0.21	11.5±0.10	33.3±0.91
Female	Control	—	15.4±0.41	0.20±0.08	28.9±0.83	2.86±0.04	12.3±0.21	37.8±0.50
Female	Deficient	0.14±0.02	17.7±1.1	0.26±0.07	26.5±1.1	2.82±0.06	13.5±0.41	33.5±1.3

Choline deficiency produced two statistically significant changes in the fatty acid patterns of triglycerides (Table 2). In males, the livers of deficient animals contained significantly larger proportions of stearic acid (C_{18:0}) than did normal livers ($P < 0.01$); in females, deficiency decreased the proportions of arachidonic acid (C_{20:4}) significantly ($P < 0.05$). Only deficient animals showed significant sex differences in triglyceride composition; males had higher proportions of stearic acid and lower proportions of linoleic acid (C_{18:2}) than did females ($P < 0.01$ and < 0.05 respectively). Choline deficiency affected the qualitative composition of liver triglyceride only slightly, although this fraction underwent the greatest quantitative change. In livers of both normal and deficient animals, linoleic acid and arachidonic acid together comprised more than half of the triglyceride fatty acids.

Patterns of the phospholipid fatty acids were significantly altered by choline deficiency (Table 2). In this fraction, proportional changes in fatty acid pattern must represent absolute changes in the amounts of individual fatty acids, because deficiency did not alter the total amount of phospholipid. In males, choline deficiency increased the amount of stearic acid and decreased the amounts of linoleic and arachidonic acid ($P < 0.02$, < 0.01 and < 0.05 respectively). In deficient females, the amount of arachidonic acid was lower and that of linoleic acid was higher than in their controls ($P < 0.02$ and < 0.05 respectively). Significant sex differences appeared in control animals in that male rats had more palmitic acid (C_{16:0}) and less stearic acid than females ($P < 0.05$ and < 0.02 respectively). These differences decreased and

became statistically insignificant in deficient animals ($P > 0.1$ in both cases).

DISCUSSION

Liver triglyceride. Accumulation of liver lipid can come from increased absorption from the circulation, or from increased net synthesis within the liver itself. In these experiments, synthesis could account for only half of the liver triglyceride at most, because in both control and deficient animals 50–60% of the triglyceride was arachidonic acid and linoleic acid, which were derived only from the diet. If increased synthesis did occur, it must have been closely paralleled by a selective increased absorption (or decreased removal) of essential fatty acids, since proportions of all fatty acids remained nearly constant. Our results suggest therefore a defect in hepatic triglyceride transport as the major cause of accumulation, but cannot distinguish between abnormally high absorption or depressed removal. Clearly, even a small defect in removal of triglyceride will result in accumulation of this fraction in the liver.

Liver phospholipid. The amount of total phospholipid in rat liver did not change in choline deficiency, and, surprisingly, neither did the proportions of choline phosphatide. Since the liver contains more than 20 times the amount of phospholipid present in the circulation, a decrease in the amount of circulating phospholipid would be much more easily detectable than an equal loss from the liver. The decrease in the amount of circulating phospholipid that occurs very soon after a deficient diet is fed and before any changes in liver lipid are perceptible (Rosenfeld & Lang,

1957) suggests that formation of phospholipid by the liver is decreased.

In choline deficiency, we should expect impairment of choline phosphatide formation. Two processes that could be affected are: the reaction of diglyceride with CDP-choline, and the methylation of phosphatidylethanolamine to form lecithin. The first process probably remains normal in choline deficiency, because the amount of liver CDP-choline does not change as the deficiency progresses (Wilgram, Holoway & Kennedy, 1960). The decreased incorporation of labelled ethanolamine into both kephalins and choline phosphatides in livers of choline-deficient rats (Stetten, 1942) suggests that methylation of phosphatidylethanolamine may be impaired in choline deficiency. This decreased incorporation can no longer be attributed to dilution of the label by accumulated liver kephalins, because our measurements showed that kephalins did not accumulate.

Liver cholesterol. Esterified cholesterol accounted for the entire increase in liver cholesterol in choline deficiency. The change could be caused by increased synthesis within the liver or by absorption from the circulation, or by decreased removal from the liver. Impaired removal of cholesterol seems to be one possible reason for the accumulation, because choline-deficient rats have been observed to produce significantly less bile than normal controls, though the composition of the bile remains unchanged (Colwell, 1951).

Comparison of effects of choline deficiency and of ethionine. Some effects of choline deficiency on lipid composition are similar to those produced by ethionine treatment. In fatty livers produced by either treatment, the accumulated lipid is mainly triglyceride, and the phospholipid content does not change. Both treatments decrease the concentrations of circulating lipoproteins and all lipid classes in serum (Olson, Jablonski & Taylor, 1958; Harris & Robinson, 1961; Tinoco *et al.* 1964). The correspondence extends to the fatty acid patterns of liver and serum lipids (Arvidson & Olivecrona, 1962; Horning, Earle & Maling, 1962; Tinoco *et al.* 1964; Table 2). A particularly interesting example of the similarity is the decrease in the amount of arachidonic acid in all lipid classes analysed, except the liver triglyceride, in which the amount of arachidonic acid greatly increases under both conditions (Arvidson & Olivecrona, 1962; Olivecrona, 1962; Tables 1 and 2). In serum of rats given either treatment, the amounts of both arachidonic acid and linoleic acid decrease, producing a diminished concentration of circulating essential fatty acid.

Ethionine interferes with protein synthesis (Robinson & Harris, 1961) and transmethyations,

apparently by trapping ATP in the form of *S*-adenosylethionine (Villa-Trevino, Shull & Farber, 1963). The similarity of effects produced by ethionine and by choline deficiency suggests that transmethyations may also be impaired in choline deficiency.

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