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The Liver-Lipid Constituents of Male and Female Rats

1. EFFECTS OF THE FAT-DEFICIENCY SYNDROME

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Green, Horner, Lowe & Morton (1957), studying the effect of feeding cholesterol to rats, noted a sex difference in that the treatment decreased liver vitamin A reserves in males but not in females.

It is well established that male rats have a much higher requirement for linoleic acid than females (see, for example, Greenberg, Calbert, Savage & Deuel, 1950). Linoleate is the predominant ester of cholesterol in the plasma of healthy rats (Klein, 1957). Peifer & Holman (1955) and Holman & Peifer (1960) have shown that dietary cholesterol accelerates the onset of fat deficiency.

On such evidence, Green *et al.* (1957) suggested that the sex difference which they had observed might be no less closely linked with the intake of polyunsaturated fatty acids than with that of cholesterol.

The experiments to be described were planned to reveal any sex difference in respect of liver vitamin A stored by fat-deficient rats. The paper immediately following (Horner & Morton, 1961) is concerned with similar experiments in which fat-deficient rats were fed with cholesterol.

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Liver lipids other than vitamin A were also determined. The cholesteryl esters, triglycerides and phospholipids were separated on silicic acid columns, and the polyunsaturated acids from these fractions were analysed by alkali-isomerization. These procedures were first applied by Mead & Fillerup (1954) to the study of rat plasma lipids. They have since been used by several workers to observe the incorporation into the esters of liver and plasma of the polyunsaturated fatty acids supplied by various edible fats.

In the present work the emphasis is on differences between the sexes and between fat-deficient animals and controls given the minimum amount of linoleic acid believed to be essential for good growth.

EXPERIMENTAL

Animals and diets. Hooded rats were fed on the following basal fat-free diet from weaning: sucrose, 73%; casein (Genatosan), 21%; salts (Hubbell, Mendel & Wakeman, 1937), 4%; Monotype paper punchings, 2%. The vitamin mixture (Table 1) was added (3 g./kg. of diet).

Procedure. Nine male rats and nine females selected from four litters born over a period of 5 days were given the fat-free diet from weaning. Three days after the youngest

animals had been weaned four experimental groups were formed. In group B, five males received the basal diet, and in group A, four males received the basal diet plus 100 mg. of linoleic acid per day. In group D, four females received the basal diet, and in group C, five females received the basal diet plus 25 mg. of linoleic acid per day.

The diets were fed *ad lib.* and water was freely provided. Linoleic acid was given in the form of the calculated volume of a tested cottonseed oil containing 54% of linoleic acid. The oil was added daily by pipette to a portion of the food of each individual animal. This was eaten promptly.

The male rats were killed after 190 days and the females after 200 days.

Vitamin A dosage. Every rat received eleven equal doses each of 800 i.u. of vitamin A given at weekly intervals from the start of the experiment. A known weight of synthetic vitamin A acetate (F. Hoffmann-La Roche and Co. Ltd.) was dissolved in a mixture of 95% ethanol (1 vol.) and glycerol (9 vol.). The standard dose was given orally by pipette in a volume of 0.2 ml. The dose also contained 2 mg. of DL- α -tocopheryl acetate.

Analysis of livers. The rats were killed with CHCl_3 vapour. Livers were washed, roughly dried on filter paper and weighed. Each liver was ground with anhydrous Na_2SO_4 and silver sand. The mixture was exhaustively extracted by boiling briefly with five 100 ml. portions of ether, and the bulked extracts were dried under N_2 on a water bath and dried under suction to constant weight. One-half of each sample of lipid was saponified for spectrophotometric estimation of vitamin A on the unsaponifiable fraction (Cama, Collins & Morton, 1951). The remaining portions of lipid were bulked within each experimental group and chromatographed.

Chromatography on silicic acid. The procedure was essentially that of Fillerup & Mead (1953); hydrocarbons, cholesteryl esters, triglycerides and free cholesterol were eluted with light petroleum (b.p. 40–60°) and diethyl ether in light-petroleum mixtures containing 2, 4 and 10% of ether (v/v) respectively. Phospholipids were eluted with methanol-ether (3:1, v/v) followed by absolute methanol.

Moist silicic acid (British Drug Houses Ltd.) was heated at 120° for 24 hr. and then ground mechanically to a fine powder. This was stirred three times with successive portions of warm methanol, the first of which removed a yellow contaminant. Particles of silicic acid which did not settle in methanol were removed by decantation. After three further washings with warm redistilled ether the material was spread on filter paper to dry and later was

again heated at 120° for 24 hr., cooled in a desiccator and stored in a tightly stoppered bottle. Most of the material was of particle size in the range 40–160 standard mesh.

Samples of lipid, each weighing 500–700 mg., were chromatographed on 30 g. of prepared silicic acid in columns of internal diameter 2.5 cm. with glass centre-rods of diameter 0.5 cm. About 200 ml. of solvent above the top of the adsorbent gave a head sufficient to maintain a good rate of flow.

The final fraction eluted with methanol usually contained silicic acid. The methanol was distilled off under reduced pressure, and the phospholipids were redissolved in ether and decanted from the insoluble residue.

Preparation of fatty acids. Free fatty acids for alkali-isomerization were obtained by the following methods from the various fractions.

(a) Cholesteryl esters were saponified on a boiling-water bath with N-KOH in ethanol for 1 hr. with frequent vigorous shaking. The mixture was acidified with conc. HCl and extracted with ether. The extract was freed from solvent, dried and dissolved in light petroleum and then chromatographed on a short column of MgCO_3 . A failure by light petroleum to elute any material was accepted as evidence of complete hydrolysis. Cholesterol was eluted with ether. The column was then dissolved in warm acidified ethanol, and the fatty acids were extracted with ether.

(b) Triglycerides and phospholipids were saponified with N-KOH in ethanol for 30 min. and 1 hr. respectively. The mixtures were acidified and extracted with ether. The product from phospholipids was expected to contain a little unsaponified material (see Lovern, 1955).

Alkali-isomerization. Samples of fatty acids weighing 1–10 mg. were alkali-isomerized by the micro-method of Holman & Hayes (1957). Analyses were made in triplicate and usually agreed well. (In a few cases turbidity raised extinction values considerably so that the results had to be rejected.)

RESULTS

Body weights and liver weights together with the liver-lipid analyses are given in Table 2.

Growth and deficiency symptoms. Deficient males and females ceased to increase in weight after 160 and 170 days respectively. Weights remained steady until the animals were killed after 190 and 200 days. All the rats showed the classical external signs of fat-deficiency (e.g. Burr & Burr, 1929) as well as the brown pigmentation of originally black hair reported by Basnayake & Sinclair (1955). Various symptoms and, in particular, scaly tails were more pronounced in the males than in the females. The control groups (both sexes) were still increasing in weight and seemed quite healthy at the end of the experimental periods.

Liver vitamin A. The mean total liver vitamin A value for deficient males was 5.25% higher than that of the control males but the difference lacked statistical significance ($t = 0.398$, $P > 0.5$). The value for deficient females was 9.9% lower than that of their controls, but again the difference was not significant ($t = 0.836$, $P > 0.4 < 0.5$).

Table 1. *Vitamin mixture*

	%	mg./100 g. of ration
Thiamine hydrochloride	0.167	0.5
Riboflavin	0.334	1.0
Pyridoxine hydrochloride	0.135	0.4
Calcium pantothenate	1.667	5.0
Nicotinic acid	0.334	1.0
Choline chloride	64.652	200.0
meso-Inositol	32.326	100.0
p-Aminobenzoic acid	0.334	1.0
Folic acid	0.033	0.1
Biotin	0.003	0.01
Menaphthone	0.015	0.05

Liver lipids. Data are presented for all the alkali-conjugatable polyunsaturated fatty acids, but, as knowledge of the metabolism of pentaenoic and hexaenoic acids is still fragmentary and no consistent changes were noted, the results for these acids will not be discussed.

The concentration of lipid in the livers of deficient males was 1.52 times that of the control males, whereas in the deficient females the corresponding value was very little higher (1.08 times) than the level shown by the control females. Cholesteryl ester concentrations were higher both in deficient males (3.36 times the control concentration) and deficient females (2.36 times the control level). Triglyceride concentrations also were higher in deficient animals, being 3.47 times the control group concentration in males, but only 1.15 times in females. The concentrations of other

constituents of the lipid material remained practically unchanged in fat deficiency.

A comparison of the two control groups shows that cholesteryl ester concentration in males was 1.31 times that of the females but the male triglyceride concentration was approximately half that of the female group.

There were marked changes in the conjugatable fatty acids in the livers of deficient animals. In males the total weights of dienoic and tetraenoic acids fell to 0.35 and 0.29 respectively of the control values, and trienoic acid rose by a factor of 2.47 times. In deficient females total dienoic and tetraenoic acids fell to 0.44 and 0.27 respectively of the control values, and trienoic acid rose by a factor of 1.71.

In control females the total weight of trienoic acid was higher (by a factor of 1.34) and the total

Table 2. *Body weights and liver weights, liver vitamin A and the weights of the various lipid fractions and their constituent fatty acids in the livers of fat-deficient male rats (group B) and their healthy controls (group A) and in fat-deficient female rats (group D) and their controls (group C)*

Control males were given 100 mg., and control females 25 mg., of linoleic acid per day.

Group	...	...	...	...	A	B	C	D
Mean final body wt. (g.)					238	167	196	133
Mean liver wt. (g.)					9.81	7.06	7.33	6.06
Mean total liver vitamin A (i.u.)					704 (± 56)*	741 (± 69)	996 (± 92)	897 (± 64)
Mean total liver lipid (mg.)					243	266	222	193
Mean wt. (mg.)/liver of:								
Phospholipids					134.0	96.0	106.2	84.0
Triglycerides					47.3	118.0	74.3	68.9
Cholesteryl esters					8.0	19.2	4.7	8.8
Free cholesterol					31.3	22.5	20.3	18.7
Hydrocarbons					3.6	3.1	1.7	1.7
Mean wt. (mg.)/liver of:								
					Phospholipid fatty acids			
Dienoic					4.40	1.11	3.06	0.86
Trienoic					2.20	5.63	3.07	5.38
Tetraenoic					6.16	1.67	4.91	1.33
Pentaenoic					2.18	0.32	2.22	0.25
Hexaenoic					0.95	0.03	1.26	0.36
Total polyunsaturated acids					15.89	8.76	14.52	8.18
Monoenoic and saturated					40.39	34.92	42.38	23.16
Mean wt. (mg.)/liver of:								
					Triglyceride fatty acids			
Dienoic					1.68	0.91	1.05	0.86
Trienoic					0.32	0.71	0.39	0.54
Tetraenoic					0.21	0.13	0.23	0.09
Pentaenoic					0.07	0.06	0.08	0.05
Hexaenoic					0.14	0.14	0.24	0.12
Total polyunsaturated acids					2.42	1.95	1.99	1.66
Monoenoic and saturated					39.39	104.25	63.25	58.17
Mean wt. (mg.)/liver of:								
					Cholesteryl ester fatty acids			
Dienoic					0.23	0.21	0.10	0.08
Trienoic					0.11	0.16	0.06	0.08
Tetraenoic					0.08	0.06	0.11	0.02
Pentaenoic					0.01	0.01	0.02	0.004
Hexaenoic					0.04	0.02	0.04	0.02
Total polyunsaturated acids					0.47	0.46	0.33	0.20
Monoenoic and saturated					4.01	7.87	1.76	3.06

* Standard deviation of the mean.

dienoic acid was lower (by a factor of 0.67) than in male controls. These values probably reflect the differences in cottonseed oil dosage; males received four times the dose given to females.

Changes in polyunsaturated fatty acid composition due to fat deficiency were most marked in the phospholipids. In both deficient groups the weight of phospholipid-bound tetraenoic acid was only 0.27 that of the control value. In deficient animals phospholipid trienoate was 2.56 times the control value in males and 1.75 times in females. Phospholipid dienoate in deficient males was 0.25 times the control level and in deficient females 0.28 times the level for control females.

Falls in dienoic and tetraenoic acids and rises in trienoic acid levels were also evident in the triglycerides of deficient animals. In terms of absolute weights of acids involved the changes were much less marked than those of the phospholipid fatty acids, and on a concentration basis also they were less striking. Thus in deficient males the weights of triglyceride dienoate and tetraenoate fell to 0.54 and 0.62 of their control values, whereas triene rose by a factor of 2.22. In deficient females the corresponding factors were 0.82, 0.39 and 1.38.

Changes in polyunsaturated fatty acid composition caused by fat deficiency were least evident in the cholesteryl esters. Differences from normal followed the same pattern as in other lipid groups and were slight, with one exception; tetraenoic acid in females fell to 0.16 times the control value; in males the corresponding change was a fall to 0.75 times the control level.

DISCUSSION

Liver vitamin A stores were not significantly affected by fat deficiency in either male or female rats. The amount of vitamin A stored in the liver depends both on the efficiency of absorption and of storage and on the rate at which it is subsequently utilized. It is still possible that control rats may initially accumulate larger vitamin A reserves than the fat-deficient animals. The controls are, however, appreciably larger animals and they may draw more rapidly on the reserves and thus decrease their stores to levels similar to those of their deficient counterparts. Experiments to measure vitamin A utilization have been performed and will be reported separately.

Deficiency of fat causes a rise in liver-cholesterol concentrations. This effect has been reported previously by Deuel, Alfin-Slater, Wells, Kryder & Aftergood (1955). The present work shows that the increase is entirely in respect of esterified cholesterol and is more pronounced in males than in females.

Klein (1958) compared the fatty acids of the liver cholesteryl esters under various conditions of linoleate intake and concluded that the concentrations of cholesteryl esters reflected the amount of linoleate fed, being high with none, low with some and high again with more. His results cannot be closely compared with ours, for daily linoleic acid intakes of groups receiving fat would increase throughout the experiment as more food was eaten by the growing rats (fat was an integral part of the diet). However, Klein (1958) gave data for one group of male rats on a fat-free diet. If weights of cholesteryl ester fatty acids are expressed as mg./100 g. of liver, Klein's values are: dienoic, 3.6 (3.0); trienoic, 1.2 (2.3); tetraenoic, 0.1 (0.8); pentaenoic, 0.2 (0.1). Our own values for group B are given in parentheses; these rats were almost certainly more severely fat-deficient than Klein's corresponding group. The lower dienoic and higher trienoic levels support this, but the considerably higher tetraenoic value is anomalous.

Klein (1958) found a direct relationship between the dietary level of linoleic acid and its concentration in the liver cholesteryl esters. The data for our control groups A and C fit very well the straight line which correlates the logarithms of Klein's variables. (The mean individual food intakes of rats in groups A and C were assessed at 15 g. and 12 g. per day respectively.)

Table 2 brings to light many marked differences between liver-lipid levels in males and females. The rise in triglyceride concentration in deficient males was the most striking change of all, whereas the corresponding effect of fat deficiency in females was very small.

The falls in total liver tetraenoic acid were very similar in deficient males and females relative to their respective controls, but decreases in dienoic and increases in trienoic acids were more marked in males.

Phospholipid concentrations were not altered by fat deficiency, but the changes in the pattern of polyunsaturated fatty acids were very marked and followed the pattern already described for liver fatty acids as a whole. This was to be expected as the phospholipids were much richer in polyunsaturated acids than were the other lipids.

Reiser, Gibson, Carr & Lamp (1951) found that the rise in trienoic acid in egg-yolk fat from fat-deficient hens was five times as great in the phospholipids as in the neutral fat.

In triglycerides and cholesteryl esters also, rises in trienoic and falls in dienoic acid concentrations were more marked in males than in females. The qualitatively important difference from the overall pattern of changes in the phospholipids and liver lipids as a whole was that the falls in triglyceride and cholesteryl ester tetraenoic acid were very much greater in females than in males.

The extra triglycerides and cholesteryl esters which accumulated in fat-deficient animals were not esterified with polyunsaturated acids other than trienoic acid.

Values for the concentrations and ratios of dienoic, trienoic and tetraenoic acids in each lipid fraction are tabulated in the following paper (Horner & Morton, 1961), which compares the effects of fat deficiency itself with effects due to the addition of cholesterol to a fat-free diet.

Analysis by alkali-isomerization throws little light on the complex changes in trienoic acid levels which occurred in fat deficiency. The triene which accumulates in this condition is eicosa-5:8:11-trienoic acid (Mead & Slaton, 1956), which the animal forms from oleic acid (Fulco & Mead, 1959). In our experiments the picture is complicated by the fact that the cottonseed oil given to controls contained some linolenic acid (0.74%). Therefore male and female controls received 1.36 and 0.34 mg. of linolenic acid per day respectively.

In the present work most of the changes from normal in liver-lipid constitution, i.e. accumulation of cholesteryl esters and triglycerides, a decrease in dienoic acid and an increase in trienoic acid, were more marked in males than in females.

Thus, as with the effect of cholesterol on liver vitamin A (Green *et al.* 1957), the female rat is more resistant than the male to nutritional hazards which disturb lipid metabolism.

There are, however, instances in which the converse holds. The accumulation of liver lipid caused by feeding a choline-free diet is more marked in female rats than in males (Deuel & Hallman, 1941). Females also show the greater 'exogenous ketonuria' when fed ketogenic acids after a period of inanition (Butts & Deuel, 1933). They are also less resistant to the 'endogenous ketonuria' which results from feeding a high-fat, low-protein, choline-free diet (Deuel, Hallman & Murray, 1937). Artom (1959) has reported that large injected doses of DL- or L-ethionine given to female rats caused some increase in liver-triglyceride concentration and markedly impaired the oxidation of fatty acids by liver homogenates. These effects were not seen in males.

SUMMARY

1. The liver vitamin A stores of male and female fat-deficient rats did not differ significantly from those of their controls which received linoleic acid, administered as cottonseed oil.

2. Fat-deficient rats had higher liver-lipid concentrations than their controls, owing to increases in cholesteryl esters and triglycerides. Both effects were more marked in males than in females.

3. Control males, which received 100 mg. of

linoleic acid per day, had considerably higher cholesteryl ester concentrations and lower triglyceride concentrations than control females which received 25 mg. of linoleic acid per day.

4. The total weights of dienoic and tetraenoic fatty acids fell, and the weight of trienoic acid rose, in the livers of fat-deficient rats. Quantitatively these effects were mostly due to changes in the phospholipid fatty acids.

5. Falls in total liver and phospholipid-bound tetraenoic acids were almost equally marked in male and female fat-deficient rats. Losses of dienoic and increases in trienoic acids in the total liver lipid and phospholipids were more marked in males.

6. In liver triglycerides and cholesteryl esters, rises in trienoic and falls in dienoic acid levels due to fat deficiency were more marked in males, but tetraenoic acid levels fell to a greater extent in females.

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