Dietary Alterations of the Mitochondrial Lipid Pattern

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1. Changes in the lipid composition of rat-liver mitochondria from both sexes have been studied in response to normal, fat and fat-cholesterol diets. The cholesterol added to the diet was in low concentrations (0.2%). 2. In the non-phospholipid fraction, normal females had higher mitochondrial cholesterol concentrations than males, and the concentration of the free sterol was decreased in fat-cholesterol-fed females, but not in males. 3. In the phospholipid fraction, normal rats of both sexes had a predominance of mitochondrial lecithin over other phosphatides, but females had slightly higher lecithin concentrations than males. Fat-cholesterol-fed females had equal concentrations of lecithin and cephalin. 4. In the minor phosphatides, normal males had higher concentrations of phosphoinositides than females. The phosphatidic acid plus polyglycerophosphatide concentration was increased above normal in fat-fed females. Fat-cholesterol-fed females had higher concentrations of phosphoinositides than normal. 5. In general, changes in the mitochondrial lipid fractions occurred in female but not in male rats.

The living cell is generally regarded as a highly organized entity in which membranes constitute an important factor in metabolic regulation. The properties of multiple repeating units, of double structure and of high lipid (phospholipid and cholesterol) content have been repeatedly demonstrated in these membranes (Sjöstrand, 1960). On to these are attached specialized molecules and functional apparatus.

Most suggestions for the organization of lipids in these membranes were made for those containing cholesterol and phospholipid in ratios between 1:3 and 3:1 (Willmer, 1961). Different considerations might hold for mitochondria in which these lipids exist in ratios very different from those mentioned above. Recent evidence indicates the importance of lipids in mitochondrial function (Green & Fleischer, 1963). One possible way of studying the role of lipid classes in the mitochondrial function is to alter their proportions within physiological limits. The resultant change in the structure of the mitochondria and in the physiology of the cell may throw some light on the nature of this role.

The present paper reports alterations in the pattern of rat-liver mitochondrial lipids due to changes in the diet. It became apparent during this study that, under similar conditions of diet and methods of analysis, sex differences were exhibited both in the normal composition of the mitochondrial lipids and in their response to dietary changes.

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EXPERIMENTAL

Animals and diet. Piebald Lister rats (150–160 g.) were supplied by the Rowett Research Institute, Aberdeen. Litter mates were sometimes bred from these animals and used for the feeding experiment. One-week-old rats were subjected to the feeding experiment and designated 'immature animals'.

The diets consisted of ground animal cake (North-Eastern Agricultural Co-operative Society Ltd.) for normal animals, plus 17% of olive oil for fat-fed animals, and ground cake containing 0.2% of cholesterol dissolved in olive oil (17% of diet) for fat-cholesterol-fed animals. Olive oil (Boots Pure Drug Co. Ltd., Nottingham) was added to ensure complete absorption of cholesterol (Friedman, Byers & Shibata, 1953). This concentration of cholesterol in the diet does not produce pathological deposition of cholesterol in rat liver (Cook, 1953).

Table 1 summarizes the dietary habits of the six feeding groups (four rats per group) and the changes in their body weight. Normal animals consumed more food and water than fat-fed and fat-cholesterol-fed ones, which resulted in an almost isocaloric diet for females of the three dietary groups, but did not do so for males. The fat-cholesterol-fed animals gained more body weight than the fat-fed ones, although both received isocaloric diets. The difference was more marked in males than in females.

Isolation of the mitochondria. The liver isolated from the animal was immediately cooled on ice for 5 min., and homogenized (in a top-driven blender at top speed for 45 sec.) in 9 vol. of cold 0.25 M-sucrose containing EDTA (2 mM) (Hübscher, Dils & Pover, 1959). Cell debris and nuclei were removed by centrifugation of the homogenate at 1000 g_{av}. for 10 min. The mitochondria were sedimented by centrifugation of the supernatant at 8000 g_{av}. for 20 min. The pellet was resuspended in 10 vol. of 0.25 M-sucrose and again

Table 1. *Dietary habits and changes in the body weight of rats from the six feeding groups*

The feeding period extended for 28 days. Values for body wt. are the mean of those of four rats.

	Females			Males		
	Normal	Fat-fed	Fat-cholesterol-fed	Normal	Fat-fed	Fat-cholesterol-fed
Water consumption (ml./rat/day)	30	15	15	25	20	20
Food consumption (g./rat/day)	15	13	13	16	15	15
Caloric value (kcal./rat/day)	59	63	63	65	74	74
Total amount of food ingested for 28 days:	420	364	364	448	420	420
Olive oil (g.)	—	62	62	—	72	72
Cholesterol (g.)	—	—	0.73	—	—	0.84
Gain in body wt. (g.)	44	58	68	62	88	98

Table 2. *Sequence of solvents used in column chromatography and the various fractions eluted*

Sources of adsorbents were: Florisil, Floridine Co., Tallahassee, Fla., U.S.A.; silicic acid, Mallinckrodt, St Louis, Mo., U.S.A. (agents, Savory and Moore, London); Hyflo Super-Cel, Johns-Manville Co., London, S.E.11.

	Column	Solvent mixture (v/v)	Volume used (ml.)	Eluted fraction
Lipids of whole liver	Florisil (12g.)	Hexane	20	Hydrocarbons
		Ether-hexane (1:19)	30	Cholesterol esters
		Ether-hexane (3:17)	50	Triglycerides
		Ether-hexane (1:3)	70	Free cholesterol and diglycerides
		Methanol-ether (1:49)	70	Monoglycerides
		Acetic acid-ether (1:24)	70	Free fatty acids
Lipids of mitochondria	Silicic acid-Hyflo Super-Cel (2g.+1g.)	Chloroform	15	Non-phospholipids
		Methanol-chloroform (1:4)	50	Amino group containing phosphatides and their lyso- derivatives; also inositol phosphatides
		Methanol-chloroform (1:2)	40	Choline-containing phosphatides (including sphingomyelin)
		Methanol	20	Lysolecithin

centrifuged at 8000 g_{av} for 20 min., and the separated mitochondria were extracted immediately with organic solvents. All centrifugations were made at 4°. Since material was limited the total wet weight and dry weight of the mitochondria were obtained with one normal rat only. The values for this one sample were 150 mg. wet wt. and 23 mg. dry wt./g. fresh wt. of liver.

Extraction procedures. A portion of the whole liver (about 1g.) was extracted by homogenization first with 10 ml. of chloroform-methanol (1:1, v/v), then with 5 ml. of chloroform-methanol (2:1, v/v), and finally by warming under a condenser in 5 ml. of the latter solvent mixture at 40°. The extracts were pooled and washed (Folch, Lees & Sloane-Stanley, 1957). A milder extraction procedure was used for the mitochondrial fraction to preserve the integrity of its phospholipids. The mitochondria separated from one liver were extracted with 6 ml. of chloroform-methanol (1:1, v/v) for 1 hr. at room temperature, and the extraction was repeated with 5 ml. of chloroform-methanol (2:1, v/v). The extracts were combined and dried *in vacuo*, and the residue was washed by standing with 25 ml. of chloroform overnight in an atmosphere of nitrogen. Extraction with chloroform-

methanol mixtures alone was found by Garbus, De Luca Loomans & Strong (1963) to give as complete an extraction of the mitochondria as the procedure of Bligh & Dyer (1959). The lipid extract was dried *in vacuo* and stored at -16° under nitrogen.

Fractionation procedures. The sequence of solvents used for column chromatography on Florisil (Carroll, 1961) and on silicic acid columns (Marinetti, Erbland & Kochen, 1957), as well as the various fractions eluted with those solvents, are described in Table 2. A preliminary column was run for the total lipids of different dietary groups, and the positions of peaks containing esterified and free cholesterol were located by their Liebermann-Burchard reaction. In subsequent runs only these fractions were collected and the solvents evaporated in preparation for analysis.

Further fractionation of neutral lipids and of phospholipids was achieved by chromatography on silicic acid paper according to the techniques described by Marinetti (1962). The R_F values of neutral lipids were found to be slightly different from those given by Marinetti (1962), because his chromatograms did not contain the free fatty acid spot, and probably the R_F values of the fractions are affected by its

Table 3. *Total lipid, free cholesterol and esterified cholesterol of the whole liver from various dietary groups*

Results are presented as mg./g. fresh wt. of liver, $\pm$ S.E.M. The numbers of animals used for the determinations are presented in parentheses.

	Females			Males		
	Normal	Fat-fed	Fat-cholesterol-fed	Normal	Fat-fed	Fat-cholesterol-fed
Total lipids (mg./g.)	38.6 $\pm$ 0.44 (3)	45 $\pm$ 2.4 (3)	50 $\pm$ 2.5 (3)	41 $\pm$ 0.7 (2)	45 $\pm$ 2.9 (2)	47 $\pm$ 3.2 (2)
Free cholesterol (mg./g.)	3.2 $\pm$ 0.07 (3)	4.3 $\pm$ 0.43 (3)	3.4 $\pm$ 0.07 (3)	2.6 $\pm$ 0.34 (2)	3.2 $\pm$ 0.07 (3)	3.0 $\pm$ 0.09 (3)
Esterified cholesterol (mg./g.)	1.1 $\pm$ 0.14 (3)	1.5 $\pm$ 0.14 (3)	4.7 $\pm$ 0.28 (3)	0.9 $\pm$ 0.06 (2)	0.8 $\pm$ 0.06 (3)	2.0 $\pm$ 0.06 (3)
Free cholesterol (% of total cholesterol)	75 $\pm$ 2.8	69 $\pm$ 3.5	45.5 $\pm$ 3.9	74 $\pm$ 1.4	79 $\pm$ 1.1	60 $\pm$ 0.7

Table 4. *Total lipid, total cholesterol and free cholesterol of rat-liver mitochondria from various dietary groups*

Results are expressed as the mean weight/g. fresh wt. of liver $\pm$ S.E.M. Mitochondrial total cholesterol (MTC) is also expressed as percentages of mitochondrial total lipid (MTL), and mitochondrial free cholesterol as percentages of both MTC and MTL.

	Females			Males		
	Normal	Fat-fed	Fat-cholesterol-fed	Normal	Fat-fed	Fat-cholesterol-fed
Total lipids (mg./g.)	8.4 $\pm$ 0.7 (4)	6.8 $\pm$ 0.6 (3)	12.7 $\pm$ 0.5 (4)	7.6 $\pm$ 1.1 (3)	6.6 $\pm$ 0.4 (3)	6.0 $\pm$ 1.4 (4)
Total cholesterol (μ g./g.)	215 $\pm$ 7 (4)	143 $\pm$ 9 (3)	235 $\pm$ 15 (4)	127 $\pm$ 6 (3)	147 $\pm$ 12 (3)	180 $\pm$ 18 (4)
(% of MTL)	2.6	2.1	1.9	1.9	2.3	3.0
Free cholesterol (μ g./g.)	188 $\pm$ 11 (4)	110 $\pm$ 6 (3)	83 $\pm$ 8 (4)	67 $\pm$ 3 (3)	102 $\pm$ 11 (3)	94 $\pm$ 7 (4)
(% of MTC)	93 $\pm$ 2.6 (4)	77 $\pm$ 1.2 (3)	35 $\pm$ 1.7 (4)	85 $\pm$ 1.5 (3)	74 $\pm$ 0.7 (3)	66 $\pm$ 1.6 (4)
(% of MTL)	2.4	1.6	0.7	1.6	1.7	1.9

presence (e.g. Day & Fidge, 1962). Chromatograms were recorded photostatically (Sheltawy, 1963).

Pure phosphatides were prepared from hen's-egg yolk (Rhodes & Lea, 1957) and from ox brain (Folch, 1948).

Analytical procedures. Phosphatide P was determined (Bartlett, 1959) on the methanolic-HCl extracts of individual spots according to the procedure of Marinetti (1962).

Phosphatidylcholine and phosphatidylethanolamine were assayed (Hartree & Mann, 1961) only on chromatograms developed at 0°, the solvents having been driven off after development by a current of cold air from a hair-drier.

Free cholesterol from the Florisil column or the silicic acid paper was determined by a mild bromination procedure (A. Sheltawy, unpublished work). The sample in chloroform-methanol (1:1, v/v) was allowed to react with a dilute solution of bromine and the excess of reagent was detected with *p*-ethoxychrysoidine. The results agree with those obtained by the colorimetric procedure of Zlatkis, Zak & Boyle (1953), which was also used for the determination of cholesterol esters. The analytical values presented in the Results section both for free and esterified cholesterol are corrected for chromatographic recovery (70%).

RESULTS

Lipids of whole liver. Changes in the total lipid and cholesterol content of whole liver are described in Table 3. Males and females feeding on fat and fat-cholesterol diets had slightly higher concentra-

tions of liver lipids than had normal animals. Of these, the fat-cholesterol-fed females had the highest values (50 mg./g.).

The concentrations of free cholesterol were not substantially increased above normal when the animals were fed on the fat and fat-cholesterol diets ($P \approx 0.1$). The esterified cholesterol concentrations increased above normal in fat-cholesterol-fed males and females ($P < 0.001$). Accordingly, the ratio of free to total cholesterol decreased in fat-cholesterol-fed males and females ($P = 0.1$ and < 0.001 respectively).

Mitochondrial lipids. Changes in the mitochondrial lipid fractions are shown in Tables 4, 5 and 6. In Table 4, the mitochondrial total lipids (mg./g. fresh wt. of liver) showed no difference in their distribution between normal male and female animals ($P = 0.6$). The fat diet had no effect on these concentrations in both sexes, whereas the fat-cholesterol diet caused a significant increase in the mitochondrial total lipids of females over normal ($P < 0.01$). These values for the fat-cholesterol-fed females were also higher than the corresponding concentrations in males ($P < 0.01$).

Male and female rats exhibited a different mitochondrial cholesterol pattern either in their normal

[illegible]

This phospholipid was not detected in the present mitochondrial preparations whether chromatography was carried out at 15° or 0°.

Sphingomyelin fraction. In general, mitochondrial sphingomyelin (Table 5) did not respond to dietary alterations ($P > 0.4$). The only exception was the increase after 8 weeks of feeding (Expt. 2) in both the sphingomyelin concentration ($P < 0.01$) and its molar ratio to mitochondrial total phospholipids ($P \approx 0.05$) of the fat-cholesterol-fed over that of the fat-fed males.

Kephalin fraction. This fraction (phosphatidylethanolamine and -serine, and their lyso-derivatives) could only be further fractionated on paper chromatograms into the mono- and di-acyl derivatives. However, because of the small amounts obtained from each single liver, no fractionation of the acid hydrolysis products to estimate serine was attempted.

In contrast with the diacylphosphatides, which showed a negative test for unsaturation as performed by potassium permanganate solution on paper chromatograms, the monoacyl derivatives reacted positively, showing a high degree of unsaturation. The latter fraction, however, varied considerably from 0 to 60% of the total amino group containing phosphatides, and the variation was unrelated to diet. The simultaneous appearance of variable concentrations of free fatty acids in the non-phospholipid fraction suggested that the monoacyl derivatives might have been formed from the diacylphosphatides by hydrolysis during the separation procedure. On chromatographic fractionation at 0°, these lyso-kephalins were absent and the unsaturation, characteristic of them, was detected in the diacyl fraction. The hydrolysis was not therefore produced by phospholipases during the separation of the mitochondria but occurred during the chromatographic fractionation.

After 4 weeks of feeding (Expt. 1 in Table 5) the similarities between various dietary groups were again more striking than the differences (average $P \approx 0.4$). However, a difference in sex pattern was exhibited where the females had higher concentrations of kephalin P ($\mu\text{g./g.}$) than normal when fed on the fat-cholesterol diet ($P \approx 0.05$), but the ratio of this fraction to the mitochondrial total lipids remained constant.

Extension of the feeding period to 8 weeks (Expt. 2 in Table 5) showed that the fat-cholesterol-fed females had higher concentrations of kephalin P ($\mu\text{g./g.}$) than the fat-fed females ($P < 0.001$). When expressed as a ratio to the mitochondrial total phospholipid, the values reflected the same increase, although in this case they were slightly masked owing to the small number of samples and the wide scatter of mitochondrial total phospholipid values ($0.2 > P > 0.1$).

Table 6. *Plasmalogen fractions of rat-liver mitochondria from the fat-fed and fat-cholesterol-fed groups*

Results are presented as $\mu\text{equiv./g.}$ fresh wt. of liver, as a ratio to the phosphatidylcholine P (PC P) or phosphatidylethanolamine P (PEA P), and as a ratio to the mitochondrial total lipid P (MTL P). The fractions designated phosphatidyl- and phosphatidylethanolamine include the corresponding serine derivatives. Immature animals were 1 week old, and were fed for 4 weeks on their respective diets. Adults were similarly fed for 8 weeks. Chromatography of the mitochondrial lipid extract was carried out at 0°. The numbers of animals used are indicated in parentheses.

	Males					
	Females			Males		
	Immature		Adult	Immature		Adult
	Fat-fed	Fat-cholesterol-fed	Fat-fed	Fat-fed	Fat-cholesterol-fed	Fat-cholesterol-fed
Phosphatidylcholine ($\mu\text{equiv./g.}$)	0.47 (3)	0.23 (3)	0.36 (3)	0.17 (2)	0.20	0.10 (2)
($\mu\text{equiv./100 } \mu\text{g. of PC P}$)	0.30 (3)	0.10 (3)	0.29 (3)	0.15 (2)	0.17	0.09 (3)
($\mu\text{equiv./100 } \mu\text{g. of MTL P}$)	0.14 (3)	0.05 (3)	0.15 (3)	0.08 (2)	0.09	0.04 (2)
Phosphatidylethanolamine ($\mu\text{equiv./g.}$)	0.56 (3)	0.35 (2)	0.60 (3)	0.36 (2)	0.37	0.42 (2)
($\mu\text{equiv./100 } \mu\text{g. of PEA P}$)	0.60 (3)	0.24 (2)	0.83 (3)	0.51 (2)	0.51	0.52 (2)
($\mu\text{equiv./100 } \mu\text{g. of MTL P}$)	0.18 (3)	0.08 (2)	0.24 (3)	0.16 (2)	0.16	0.17 (2)
						0.34 (3)
						0.33 (3)
						0.10 (3)

Immature rats fed for 6 weeks did not exhibit similar changes in this fraction (about 30% of mitochondrial total lipid P) as did adult animals.

Phosphoinositide fraction. Two components of the fraction were always demonstrable, having R_F values on silicic acid paper chromatograms of 0.15 and 0.24. These, according to the tentative identification of Marinetti (1962), correspond to mono- and di-phosphoinositides respectively. However, owing to their small concentrations they were always combined and presented as one single value.

Ratios of phosphoinositide P to mitochondrial total lipid P were higher in the liver mitochondria of normal males than in those of females ($0.05 > P > 0.02$; Table 5). After 4 weeks (Expt. 1 in Table 5) the fat-cholesterol-fed females showed an increase in phosphoinositide P concentration ($P \approx 0.02$) and in its ratio to total mitochondrial lipid P ($P \approx 0.05$).

Results obtained from males after 4 weeks of feeding the fat and fat-cholesterol diets were too variable to allow coherent quotation. Values obtained from adult rats fed for 8 weeks and from immature rats fed for 6 weeks were similar and did not alter with dietary changes or sex.

Phosphatidic acid fraction. The compounds collectively designated as phosphatidic acid in the present study were not further fractionated on other chromatographic systems (Hübscher, 1961) because of the small concentrations dealt with. Also, the streaking of this fraction on silicic acid paper chromatograms did not always allow complete recovery.

In females fed on the fat diet for 4 weeks (Expt. 1 in Table 5) a significant increase above normal in this fraction was observed, both when expressed in absolute concentrations ($P = 0.001$) and as a fraction of mitochondrial total phospholipids ($P < 0.01$). The latter increase was also repeated, but to a less-pronounced degree, in animals fed for 8 weeks (Expt. 2; $P < 0.01$).

Plasmalogen fractions. Mitochondrial plasmalogens were determined in the livers of several fat-fed and fat-cholesterol-fed animals (Table 6). Despite the scatter of the values, two general tendencies were noticed: (i) phosphatidylethanolamine concentrations were higher than those of the corresponding choline compound (0.6 and 0.36 $\mu\text{equiv./g.}$ fresh wt. of liver respectively for females, and 0.42 and 0.1 $\mu\text{equiv./g.}$ fresh wt. of liver respectively for males); (ii) females had higher concentrations of plasmalogens than males (see above).

DISCUSSION

The concentration of cholesterol used during this study (0.2%) is only double that actually present in the animal cake (Riddell & Cook, 1955), a condi-

tion that might pertain in the normal diet of the animal. On feeding this cholesterol-containing diet, the concentration of cholesterol in male rat liver was increased, but to a smaller extent than was observed by Ridout, Lucas, Patterson & Best (1954). The semi-synthetic diet used by these authors was essentially choline-deficient, thus helping excessive deposition of lipids in the liver. However, in agreement with these workers, it was found that dietary cholesterol increased the liver total cholesterol in females to a greater extent than in males.

The present analysis of cholesterol in the mitochondria from various groups of animals revealed some hitherto unnoticed sex differences (Table 4). Kritchevsky, Tepper, Staple & Whitehouse (1963) noticed that the mitochondria from female rat liver have higher rates of oxidation and biosynthesis of cholesterol than the corresponding male preparations. It is not surprising therefore that, during this study, sex differences in the metabolism of cholesterol were reflected on the cholesterol concentrations in the mitochondria, both of normal rats and of rats fed on the fat-cholesterol diet.

The observation of Schotz, Rice & Alfin-Slater (1953) on the increase in the proportion of esterified to total cholesterol in liver mitochondrial preparations from rats fed on a diet containing 1% of cholesterol has been confirmed in this work with a diet containing 0.2% of cholesterol (19 and 58% above normal values in males and females respectively; Table 4).

In agreement with most previous work (see Ball & Joel, 1962), the present study shows that lecithin predominates in the lipids of normal rat-liver mitochondria. Only two studies seem to deviate from this general observation (Collins & Schotlander, 1961; Getz, Bartley, Stirpe, Notton & Renshaw, 1962). In the first, the low proportions of phosphatidylcholine and -ethanolamine (31 and 21% of mitochondrial total lipid P respectively) are due, according to the authors, to the presence of the remainder of these fractions in the form of 'complex phosphatides'. These were separated by the mild procedure of countercurrent distribution with neutral solvent mixtures. In the second, it was found that the phosphatidylcholine fraction was present in an almost equal molecular proportion to phosphatidylethanolamine (39 and 42% of mitochondrial total phospholipids respectively). This was probably due to the slightly altered centrifugation conditions used by the authors to separate the mitochondria. Apart from these methodological causes, differences in the mitochondrial lipid pattern could be attributed to the age of the animal (Biezenski, Speat & Gordon, 1963), and, according to the present work, to its sex and the diet administered.

The proportion of phosphatidylcholine to total

phospholipids in the mitochondrial preparation from female rats was decreased when the animals were fed on the fat-cholesterol diet. The concentration ($\mu\text{g. of P/g. fresh wt. of liver}$) did not change, but the decrease was a reflexion of the increase in total mitochondrial lipids, mainly due to the increase in the kephalin fraction. Changes in the distribution of lecithin in the mitochondria have previously been induced in rats on a choline-deficient diet (Gallo & Cornatzer, 1954; Spiro & McKibbin, 1956). The decrease in the proportion of phosphatidylcholine in those studies was, however, also accompanied by a decrease in the concentration of mitochondrial total phospholipids.

Lysolecithin was not detected in the present normal liver mitochondrial preparation, nor was it detected in the mitochondria from other dietary groups. Getz *et al.* (1962) have reported lyso-phosphatides in small concentrations in the rat-liver mitochondrial fraction, although the possibility of its production during the cell fractionation or the silicic acid chromatographic procedures was not excluded by these authors. During the present work, the detection of varying concentrations of lysokephalins after chromatography at 15° and their absence at 0° indicated their production during the chromatographic procedure. Plasmalogens could be excluded as a possible source (see Hack & Ferrans, 1959), since they were not detected in normal rat-liver mitochondria (Macfarlane, Gray & Wheeldon, 1960) and the concentrations found during the present work (on chromatography of the lipids at 0°) in the fat-fed and fat-cholesterol-fed animals were not high enough to account for the lysokephalins found.

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