Effect of Dietary Fat and Cholesterol on the Polyunsaturated Fatty Acids of Cholesterol Esters, Phospholipids and Triglycerides in the Liver of the Rat

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Garton & Duncan (1957) found that the cholesterol in the plasma of animals was combined mainly as the linoleate ester, so the relation of dietary fats with or without cholesterol to the component lipids of liver and plasma and to the levels of polyunsaturated fatty acids of liver- and plasma-lipid fractions has become an important subject of investigation. Alfin-Slater, Aftergood, Wells & Deuel (1954) reported that there was an accumulation of cholesterol esters in the livers of polyunsaturated-fatty acid-deficient rats. Klein (1958) observed that the liver cholesterol esters were increased in fat deficiency, were low in normal ranges of dietary fat and increased again with high levels of linoleic acid feeding. Klein (1958) and Okey, Lyman, Harris, Einset & Hain (1959) observed that esterified linoleic acid in liver was directly related to dietary linoleic acid. Thus it is reasonable to expect that an analysis of the fatty acid composition of each lipid class will yield information about the transport and metabolism of fatty acids.

In this paper results of the investigation of the effect of dietary fats with and without cholesterol on the polyunsaturated fatty acids of liver cholesterol esters, phospholipids and neutral glycerides are reported.

EXPERIMENTAL

Animals and diet. These results were obtained as part of a study of the relation of dietary fat and cholesterol to tissue and serum cholesterol and polyunsaturated fatty acids in rats (Patil & Magar, 1959). Estimations of polyunsaturated fatty acids were carried out on the cholesterol esters, phospholipids and neutral glycerides found in the livers of male albino rats maintained on diets containing 5% of coconut oil, dalda (hydrogenated fat), ghee (butter fat) or groundnut oil. The other ingredients of the diet were 22% of casein, 4% of salt mixture and 69% of sucrose. The rats were given all the required vitamins in sufficient quantities (Cheng, Alfin-Slater & Deuel, 1954). After 6 weeks on the above-mentioned diets the six rats of each group received 0.5 g. of cholesterol/kg. body wt. The cholesterol was mixed in the respective oils, which were then mixed with the diets. After 10 months of feeding on cholesterol, all rats were killed.

Extraction of lipids and analysis. The fresh liver tissue

was homogenized in 10% (w/v) trichloroacetic acid solution containing 0.4 M-MgCl₂ and the lipids were then extracted and purified by the method of Johnson & Dutch (1951). The phospholipids were precipitated with acetone from a known quantity of the lipid, and the acetone-soluble fraction and acetone-insoluble fractions were purified and weighed accurately. About 600–700 mg. of acetone-soluble lipids were chromatographed on silicic acid (Merck) by the method of Fillerup & Mead (1953) as modified by Mead & Fillerup (1954), 6.4 g. of activated silicic acid being used for 50 mg. of acetone-soluble lipids. Cholesterol esters were eluted first, followed by glycerides and free sterols. These fractions were evaporated *in vacuo*, dried and weighed. The weighed quantities of phospholipids, cholesterol esters and glycerides were saponified for the isolation of fatty acids according to the procedure of Wiese & Hansen (1953). The fatty acids were isomerized and analysed spectrophotometrically by the method of Herb & Riemenschneider (1953). Calculations of the percentage of the individual polyunsaturated fatty acids were made according to Herb & Riemenschneider (1953), as modified by Hammond & Lundberg (1953), for the inclusion of hexaenoic acid. In the formulae used in the present work, it was assumed that the pentaenoic acids consisted of 50% of docosapentaenoic acid and 50% of eicosapentaenoic acid.

RESULTS AND DISCUSSION

Liver lipids. The composition of component lipids of the liver in rats fed with diets containing 5% of fats with and without cholesterol is given in Table 1. The percentage of lipids accumulated in the liver of rats fed with diets containing 5% of various fats plus cholesterol was significantly higher than those of animals fed with fats alone. Table 1 shows that the amount of phospholipid in liver lipids from animals fed with diets containing 5% of various fats with cholesterol was largely reduced as compared with those values for animals fed only with fats. On the other hand, cholesterol esters and triglycerides were high in cholesterol-fed groups as compared with those of groups not fed with cholesterol. Liver phospholipids were slightly higher, whereas liver cholesterol esters and triglycerides were slightly lower in rats fed on groundnut oil with and without cholesterol as compared with those fed on the other three fats with and without cholesterol.

Table 1. *Composition of liver lipids of rats fed on diets containing 5% of various fats with and without cholesterol*Figures represent the average values for six rats $\pm$ S.D.

Diet	Lipids (% of liver wt.)	Component lipids as percentage of total lipids			
		Phospholipids	Sterol esters	Triglycerides	Free sterols
Coconut oil	8.0 $\pm$ 0.7	42.9 $\pm$ 2.5	1.5 $\pm$ 0.1	53.0 $\pm$ 2.0	2.6 $\pm$ 0.2
Coconut oil + cholesterol	11.1 $\pm$ 1.1	18.2 $\pm$ 1.8	13.5 $\pm$ 0.9	65.5 $\pm$ 2.7	2.8 $\pm$ 0.2
Dalda	7.8 $\pm$ 0.6	43.1 $\pm$ 2.8	1.4 $\pm$ 0.2	52.9 $\pm$ 2.1	2.6 $\pm$ 0.1
Dalda + cholesterol	11.2 $\pm$ 0.8	21.0 $\pm$ 1.5	14.1 $\pm$ 1.0	62.1 $\pm$ 1.8	2.8 $\pm$ 0.2
Ghee	7.5 $\pm$ 0.6	46.5 $\pm$ 2.2	1.4 $\pm$ 0.1	49.4 $\pm$ 2.5	2.7 $\pm$ 0.2
Ghee + cholesterol	10.4 $\pm$ 0.8	25.0 $\pm$ 1.5	11.8 $\pm$ 0.8	60.2 $\pm$ 2.3	3.0 $\pm$ 0.2
Groundnut oil	6.9 $\pm$ 0.5	54.3 $\pm$ 2.1	1.2 $\pm$ 0.1	41.7 $\pm$ 1.6	2.8 $\pm$ 0.2
Groundnut oil + cholesterol	10.1 $\pm$ 0.7	30.1 $\pm$ 2.0	10.0 $\pm$ 0.7	57.0 $\pm$ 2.2	2.6 $\pm$ 0.2

Table 2. *Polyunsaturated fatty acids in liver cholesterol esters of rats fed on diets containing 5% of various fats with and without cholesterol for 10 months*Figures represent the average values for six rats $\pm$ S.D.

Diet	Polyunsaturated fatty acids as percentage of total cholesterol ester fatty acids				
	Dienoic	Trienoic	Tetraenoic	Pentaenoic	Hexaenoic
Coconut oil	6.43 $\pm$ 0.35	1.15 $\pm$ 0.08	2.20 $\pm$ 0.32	0.12 $\pm$ 0.03	0.8 $\pm$ 0.07
Coconut oil + cholesterol	4.12 $\pm$ 0.28	2.30 $\pm$ 0.09	1.24 $\pm$ 0.28	0.00	0.58 $\pm$ 0.07
Dalda	6.34 $\pm$ 0.23	1.82 $\pm$ 0.12	2.34 $\pm$ 0.26	0.18 $\pm$ 0.02	0.92 $\pm$ 0.08
Dalda + cholesterol	3.87 $\pm$ 0.32	2.10 $\pm$ 0.06	1.13 $\pm$ 0.27	0.00	0.71 $\pm$ 0.06
Ghee	6.94 $\pm$ 0.25	1.92 $\pm$ 0.12	2.26 $\pm$ 0.30	0.20 $\pm$ 0.04	1.1 $\pm$ 0.05
Ghee + cholesterol	5.19 $\pm$ 0.32	2.51 $\pm$ 0.10	1.41 $\pm$ 0.30	0.00	0.8 $\pm$ 0.04
Groundnut oil	13.2 $\pm$ 0.34	1.00 $\pm$ 0.07	3.52 $\pm$ 0.31	0.23 $\pm$ 0.04	1.41 $\pm$ 0.04
Groundnut oil + cholesterol	10.4 $\pm$ 0.51	1.50 $\pm$ 0.08	2.00 $\pm$ 0.34	0.00	0.97 $\pm$ 0.03

Table 3. *Polyunsaturated fatty acids in liver phospholipids of rats fed on diets containing 5% of various fats with and without cholesterol for 10 months*Figures represent the average values for six rats $\pm$ S.D.

Diet	Polyunsaturated fatty acids as percentage of total phospholipid fatty acids				
	Dienoic	Trienoic	Tetraenoic	Pentaenoic	Hexaenoic
Coconut oil	8.72 $\pm$ 0.73	0.85 $\pm$ 0.12	13.6 $\pm$ 1.2	1.51 $\pm$ 0.36	2.10 $\pm$ 0.32
Coconut oil + cholesterol	6.25 $\pm$ 0.53	1.40 $\pm$ 0.16	9.42 $\pm$ 1.1	2.82 $\pm$ 0.31	1.28 $\pm$ 0.21
Dalda	8.93 $\pm$ 0.65	0.55 $\pm$ 0.08	13.2 $\pm$ 1.3	2.72 $\pm$ 0.30	2.52 $\pm$ 0.22
Dalda + cholesterol	6.11 $\pm$ 0.55	1.12 $\pm$ 0.10	10.4 $\pm$ 1.1	2.12 $\pm$ 0.25	1.82 $\pm$ 0.21
Ghee	8.56 $\pm$ 0.91	0.64 $\pm$ 0.06	12.8 $\pm$ 0.9	2.67 $\pm$ 0.32	2.22 $\pm$ 0.18
Ghee + cholesterol	6.21 $\pm$ 0.62	1.72 $\pm$ 0.13	10.1 $\pm$ 0.8	1.09 $\pm$ 0.24	1.81 $\pm$ 0.31
Groundnut oil	12.3 $\pm$ 0.71	0.31 $\pm$ 0.05	17.7 $\pm$ 1.1	3.20 $\pm$ 0.31	2.97 $\pm$ 0.20
Groundnut oil + cholesterol	10.1 $\pm$ 0.78	0.67 $\pm$ 0.08	14.6 $\pm$ 1.0	2.41 $\pm$ 0.33	2.32 $\pm$ 0.20

Table 4. *Polyunsaturated fatty acids in liver triglycerides of rats fed on diets containing 5% of various fats with and without cholesterol for 10 months*Figures represent the average values for six rats $\pm$ S.D.

Diet	Polyunsaturated fatty acids as percentage of total triglyceride fatty acids				
	Dienoic	Trienoic	Tetraenoic	Pentaenoic	Hexaenoic
Coconut oil	5.21 $\pm$ 0.65	4.23 $\pm$ 0.35	2.61 $\pm$ 0.28	0.24 $\pm$ 0.06	0.30 $\pm$ 0.05
Coconut oil + cholesterol	3.94 $\pm$ 0.51	6.40 $\pm$ 0.40	2.30 $\pm$ 0.31	0.21 $\pm$ 0.03	0.21 $\pm$ 0.03
Dalda	5.56 $\pm$ 0.50	3.95 $\pm$ 0.51	2.65 $\pm$ 0.32	0.75 $\pm$ 0.07	0.25 $\pm$ 0.04
Dalda + cholesterol	3.38 $\pm$ 0.45	6.93 $\pm$ 0.62	2.54 $\pm$ 0.32	0.71 $\pm$ 0.05	0.22 $\pm$ 0.03
Ghee	5.39 $\pm$ 0.62	3.75 $\pm$ 0.40	2.41 $\pm$ 0.25	0.61 $\pm$ 0.06	0.12 $\pm$ 0.03
Ghee + cholesterol	3.98 $\pm$ 0.45	7.65 $\pm$ 0.62	2.40 $\pm$ 0.21	0.60 $\pm$ 0.07	0.12 $\pm$ 0.04
Groundnut oil	7.90 $\pm$ 0.70	1.57 $\pm$ 0.32	2.91 $\pm$ 0.36	1.41 $\pm$ 0.05	0.82 $\pm$ 0.04
Groundnut oil + cholesterol	7.22 $\pm$ 0.65	2.85 $\pm$ 0.40	2.81 $\pm$ 0.31	1.28 $\pm$ 0.05	0.71 $\pm$ 0.04

Polyunsaturated fatty acids of cholesterol esters, phospholipids and triglycerides. The individual polyunsaturated fatty acids of cholesterol esters, phospholipids and triglycerides of liver lipids of animals fed with fats with and without cholesterol are represented in Tables 2, 3 and 4 respectively. In animals fed with fats only, dienoic acids appeared to be the major group in both the cholesterol esters and phospholipid fractions, occurring to the extent of 6.3–13.2% and 8.5–12.3% respectively. The level of this acid was low (5.2–7.9%) in the triglyceride fraction. Trienoic acid occurred in the largest quantities in the triglycerides (1.6–4.2%) and in the lowest amounts in the phospholipids (0.3–0.8%). Table 3 shows that the liver phospholipids contained tetraenoic acid, pentaenoic acid and hexaenoic acid in greater proportion than those present in the liver cholesterol esters (Table 2) and triglycerides (Table 4). It is clear from Tables 2 and 4 that the distribution of polyunsaturated fatty acids in the triglycerides was similar to that of cholesterol esters.

Effect of dietary fats on polyunsaturated fatty acids of liver cholesterol esters, phospholipids and triglycerides. The percentages of dienoic acid and tetraenoic acid in the liver phospholipids and the levels of dienoic acids in the cholesterol esters of animals fed with a diet containing 5% of coconut oil, hydrogenated fat or butter fat were observed to be lower (Tables 2 and 3) than those of animals fed with a diet containing 5% of groundnut oil. Klein (1958) found a high percentage of dienoic acid (45.5 mg./100 mg. of total fatty acids) in cholesterol ester fatty acids in the livers of rats fed with diets containing 81.2 mg. of linoleic acid/g. of diet. Also, Okey *et al.* (1959) obtained 16–27% and 13–21% of dienoic acid from liver cholesterol ester fatty acids in rats fed with diets containing 10% of corn oil and 10% of groundnut oil respectively. In the present experiments the comparatively low levels of dienoic acids in the cholesterol esters, phospholipids and triglycerides were due to the low percentage of dietary fat, and the small amounts of linoleic acid contained in coconut oil, hydrogenated fat, butter fat (2.0–2.5%) and groundnut oil (26.2%) as compared with the linoleic acid contained in corn oil (50–60%).

Table 2 suggests that the tetraenoic acids were low in the liver cholesterol esters of animals fed on coconut oil, hydrogenated fat, butter fat or groundnut oil. This is in conformity with the results of Klein (1958), which showed low levels of tetraenoic acid in liver cholesterol esters of animals fed on diets containing 30% of corn oil.

The levels of tetraenoic acid, pentaenoic acid and hexaenoic acid were very low in the triglycerides. Trienoic acid was high and dienoic acid was low in the liver triglycerides of animals fed on coconut

oil, hydrogenated fat or butter fat as compared with those of animals fed on groundnut oil. Mead & Slaton (1956) expressed the opinion that the trienoic acid in liver may be 5:8:11-eicosatrienoic acid and not linolenic acid. This trienoic acid was shown by Montag, Klenk, Hayes & Holman (1957) to be structurally related to oleic acid.

Effect of feeding with cholesterol on polyunsaturated fatty acids of liver cholesterol esters, phospholipids and triglycerides. The levels of dienoic acids were significantly decreased in the liver phospholipids (Table 3), cholesterol esters (Table 2) and triglycerides (Table 4) of animals fed on fats with cholesterol as compared with those fed with fats alone. The levels of dienoic acid were decreased in liver phospholipids, cholesterol esters and triglycerides and the levels of trienoic acid were significantly increased in the triglycerides of animals fed with coconut oil plus cholesterol, hydrogenated fat plus cholesterol, butter fat plus cholesterol as compared with those of animals fed on groundnut oil plus cholesterol (Tables 2–4).

Pentaenoic acid was completely absent and tetraenoic acid and hexaenoic acid were lowered in the liver cholesterol ester of animals fed with cholesterol (Table 2). The levels of tetraenoic acid, pentaenoic acid and hexaenoic acid were reduced in the liver phospholipids as a result of cholesterol feeding (Table 3). The changes in the levels of tetraenoic acid, pentaenoic acid and hexaenoic acid in the liver triglycerides of cholesterol-fed animals were, however, very small.

SUMMARY

1. Liver phospholipids were reduced and cholesterol esters and triglycerides were increased as a result of feeding cholesterol to rats.

2. In rats fed with diets containing 5% of various fats for 10 months, the dienoic acid level was high in liver cholesterol esters and phospholipids. Tetraenoic acid level was low in the cholesterol esters and triglycerides and high in phospholipids. The liver phospholipids contained more tetraenoic acid, pentaenoic acid and hexaenoic acid than the cholesterol esters and triglycerides.

3. The levels of polyunsaturated fatty acids of liver lipids decreased greatly on feeding on more saturated fats with and without cholesterol as compared with those obtained when less saturated fats, with and without cholesterol, were fed.

4. Trienoic acids were increased on cholesterol feeding much more in the liver triglycerides than the cholesterol esters and phospholipids.

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REFERENCES

- Alfin-Slater, R. B., Aftergood, L., Wells, A. F. & Deuel, H. J., jun. (1954). *Arch. Biochem. Biophys.* **52**, 180.
 Cheng, A. L. S., Alfin-Slater, R. B. & Deuel, H. J., jun. (1954). *J. Nutr.* **54**, 201.
 Fillerup, D. L. & Mead, J. F. (1953). *Proc. Soc. exp. Biol., N.Y.* **83**, 574.
 Garton, G. A. & Duncan, W. R. H. (1957). *Biochem. J.* **67**, 340.
 Hammond, E. G. & Lundberg, W. O. (1953). *J. Amer. Oil Chem. Soc.* **30**, 433.
 Herb, S. F. & Riemenschneider, R. W. (1953). *Analyt. Chem.* **25**, 953.
 Johnson, R. M. & Dutch, P. H. (1951). *Proc. Soc. exp. Biol., N.Y.*, **78**, 662.
 Klein, P. D. (1958). *Arch. Biochem. Biophys.* **76**, 56.
 Mead, J. F. & Fillerup, D. L. (1954). *Proc. Soc. exp. Biol., N.Y.*, **86**, 449.
 Mead, J. F. & Slaton, W. H. (1956). *J. biol. Chem.* **219**, 705.
 Montag, W., Klenk, E., Hayes, H. & Holman, R. T. (1957). *J. biol. Chem.* **227**, 53.
 Okey, R., Lyman, M. M., Harris, A. G., Einset, B. & Hain, W. (1959). *Metabolism*, **8**, 241.
 Patil, V. S. & Magar, N. G. (1959). *Indian J. med. Res.* **47**, 446.
 Wiese, H. F. & Hansen, A. E. (1953). *J. biol. Chem.* **202**, 417.

Tissue-Fractionation Studies

14. THE ACTIVATION OF LATENT DEHYDROGENASES IN MITOCHONDRIA FROM RAT LIVER*

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Several mitochondrial enzymes are known to occur in a latent form, for example adenosine triphosphatase (Kielley & Kielley, 1951), glutamic dehydrogenase (Hogeboom & Schneider, 1953), aconitase (Dickman & Speyer, 1954), cytochrome *c* reductases (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955; Borgström, Sudduth & Lehninger, 1955), cytochrome oxidase (Simon, 1958), rhodanese (Greville & Chappell, 1959) and alkaline deoxyribonuclease (Baudhuin, 1959). The activities of the enzymes in intact mitochondria towards added substrates are relatively low and can be increased, sometimes tenfold or more, by damage to the particles. The acid hydrolases which are localized in the lysosomes of rat liver behave in a similar manner (de Duve *et al.* 1955). These enzymes can be liberated into solution and their activities revealed by various procedures such as incubation at 37°, freezing and thawing, treatment in a Waring Blender, osmotic shock and treatment with detergents and certain enzymes (Berthet & de Duve, 1951; Berthet, Berthet, Appelmans & de Duve, 1951; Appelmans & de Duve, 1955; Gianetto & de Duve, 1955; Wattiaux & de Duve, 1956; Beaufay & de Duve, 1959). In graded activation experiments the activities of the various hydrolases appear concurrently. There is good evidence that the latency of lysosomal enzymes is due to the inability of the

substrates to penetrate the membrane of the particle, and that the apparent activation is due to rupture of the membrane and release of the enzymes into solution.

This paper describes the results of a parallel study of the release of enzymes associated with the two types of particle. Acid phosphatase was taken as being a typical lysosomal enzyme and glutamic dehydrogenase was chosen to represent mitochondria. Some experiments were also done with malic and D- β -hydroxybutyric dehydrogenases. A brief account of the results has already been given (Bendall, 1958), and evidence that these dehydrogenases belong to the true mitochondria has been described (Beaufay, Bendall, Baudhuin & de Duve, 1959; Beaufay, Bendall, Baudhuin, Wattiaux & de Duve, 1959).

EXPERIMENTAL

Methods

All experiments were performed on a combined heavy and light mitochondrial fraction (which contains most of the lysosomes as well as the mitochondria) isolated in 0.25 M-sucrose from rat liver by the method described by Appelmans, Wattiaux & de Duve (1955). The particles were washed once, resuspended in 0.25 M-sucrose (at a concentration corresponding to 0.5–1.0 g. of original liver/ml.) and stored at 0° before use. In a few experiments the preparation and suspension medium contained mM-ethylene-diaminetetra-acetic acid (EDTA), pH 7.7.

Acid phosphatase. Free acid phosphatase was measured as described by Gianetto & de Duve (1955). Total activities were determined by the addition of 0.1% of Triton X-100

* Part 13: Beaufay, Bendall, Baudhuin, Wattiaux & de Duve, 1959.

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