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Fatty Acid Composition of Phospholipids from Subcellular Particles of Rat Liver

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The object of this work was to determine the fatty acid composition of the different phospholipids present in the mitochondria and microsomes of rat liver. Clément, Haimovici & Le Breton (1955) obtained data for the polyenoic acids of the neutral fat and of the total phospholipids, by spectrophotometry after alkali-isomerization, and Clément, Clément & Le Breton (1956) gave further data on the nature of the lipids. It appeared of interest to obtain more detailed information by preliminary fractionation of the lipids, and analysis of their fatty acids by gas chromatography. Part of this work has been briefly reported (Macfarlane, Gray & Wheeldon, 1960).

METHODS

Analytical methods. Methods used for P, N, amino N, inositol, fatty acid ester (colorimetric), iodine values, cholesterol, phosphomonoester and ester phosphatide were those used by Gray & Macfarlane (1958). Paper chromatography of water-soluble phosphoric esters obtained by mild alkaline hydrolysis (Dawson, 1954) was done on acid-washed Whatman no. 1 papers by the ascending method, with phenol saturated with 1% ammonia in water, or *tert.*-butyl alcohol–water 62:38 (v/v), trichloroacetic acid 10% (w/v). The P content of spots was estimated according to Bartlett (1959). The spots were identified with authentic markers. Chromatography on silicic acid paper was done according to Marinetti, Erbland & Kochen (1957).

Chromatography of lipid extracts on silicic acid. This was done as in Gray & Macfarlane (1958), by elution with increasing concentrations of methanol in chloroform.

Preparation of methyl esters of fatty acids. Phospholipid fractions (4–5 mg. of P) were saponified in aqueous N-NaOH at room temp. under N_2 for 24 hr. in the dark. Neutral-lipid samples were saponified by refluxing for 3 hr. in 0·5 N-NaOH in 95% ethanol; the hydrolysate was neutralized with HCl, the ethanol removed *in vacuo* and the aqueous mixture extracted with light petroleum (60–80°) before acidification and extraction of fatty acids. The acids were converted into methyl esters by refluxing in 2–3 ml. of methanolic 0·8 N-HCl for 3–4 hr.

Gas chromatography of the methyl esters. This was done as described by Gray (1960). The information necessary to identify the unsaturated acids was obtained by chromatographing the mixed fatty acid methyl esters of ox-liver kephalin and lecithin on Apiezon L and Reoplex 400 (James, 1959). It was therefore possible to identify the rat-liver acids by chromatography on the Reoplex 400 phase only. A chromatogram was also run after bromination as a check on the positions of unsaturated acids.

EXPERIMENTAL AND RESULTS

Preparation of sub-cellular particles. Rats of the Chester Beatty strain (350–400 g.) were used; these were kept on a stock diet (Rank no. 41 b), with small regular supplements of milk, cod-liver oil and Marmite. Three rats at a time were bled, and the liver was homogenized in a Craigie (1949) grinder in 0·25 M-sucrose (10 ml./g. of tissue). The homogenate was centrifuged successively at (*a*) 600 *g* for 10 min. to deposit nuclei, red cells and unruptured liver cells; (*b*) 12 000 *g* for 10 min., the grey–brown deposit being designated as mitochondria; (*c*) 78 000 (mean) *g* for 90 min., the red pellet being designated as microsomes.

Extraction of lipid. Samples of whole liver were minced finely with scissors and extracted with 19 ml. of chloroform–methanol (2:1, v/v)/g. of tissue. The mitochondria and microsomes were suspended in a small volume of water and stirred with 19 vol. of chloroform–methanol (2:1, v/v) at room temp. for a few minutes. The extract after filtration was washed twice with 0·2 vol. of 2 mM-$CaCl_2$: the chloroform layer was taken to dryness on a rotary evaporator at 30° *in vacuo* and the residue was dissolved in chloroform and stored in N_2 at −10°. The final supernatant after centrifuging of the homogenate at 78 000 *g* was evaporated to a small volume and extracted similarly. Extracts from a total of 120 g. of liver were pooled for fractionation.

Yields. Some analytical results for the extracts from whole liver, mitochondria and microsomes are given in Table 1. Considerable numbers of unruptured cells were present in the first deposit, so the recovery of particles was not quantitative; about 60% of the original lipid P was present in the mitochondria and microsomes recovered: the extract from the supernatant contained only 10 μg. of lipid P/g. of liver extracted, and was not investigated.

Table 1. *Analyses of lipid extracts from rat liver*

Lipid extract from	Whole liver	Mitochondria	Microsomes
Wt. of liver extracted (g.) ...	18	(from) 120	(from) 120
Lipid extract			
Dry wt. (g.)	1·04	1·62	1·13
P (mg.)	21	49	35
Amino N (mg.)	3·3	8·0	4·4
Total cholesterol (mg.)	21	31	21
P (% on dry wt.)	2·0	3·0	3·1

Table 2. *Phospholipid fractions from mitochondrial lipid*

Abbreviations: GPGPG, polyglycerophosphate from cardiolipin; GPS, glycerophosphorylserine; GPE, glycerophosphorylethanolamine; GPI, glycerophosphorylinositol; GPC, glycerophosphorylcholine.

Fraction	MA	MB	MC	MD	ME	MF
Eluate nos.	7–13	14–18	19–31	32–37	38–45	46–60
Eluent: $CHCl_3$–methanol (v/v)	19:1	4:1	4:1	3:2	3:2	1:4
Wt. (mg.)	123	408	83	167	191	161
P (mg.)	3·8	15·2	2·8	6·1	7·1	6·2
P (% on dry wt.)	3·1	3·7	3·4	3·7	3·7	3·8
Molar ratio (P = 100)						
Amino N	6	75	20	5	Nil	Nil
Fatty acid ester	197	190	177	195	193	188
Inositol	—	12	22	Nil	—	—
Double bonds/mol. of fatty acid	2·0	2·2	—	2·0	1·7	1·6
P in fraction (% of total)	9·2	37·0	6·8	14·8	17·2	15·0
Phosphoric esters*	GPGPG	GPS	GPS	GPC	GPC	GPC
	GPS	GPE	GPE	—	—	—
	—	GPI	GPI	—	—	—
	—	—	GPC	—	—	—

* By paper chromatography of water-soluble P esters formed by mild alkaline hydrolysis.

Fractionation of lipid extracts on silicic acid

Mitochondrial lipid. The material (46 mg. of P) in chloroform was placed on a column of 40 g. of silicic acid. Passage of chloroform eluted 230 mg. of lipid free from P (neutral-lipid fraction), containing 600 μequiv. of fatty acid ester and 2 mg. of cholesterol. Elution was continued with chloroform–methanol mixtures, 20 ml. fractions being collected, which were pooled on the basis of the P and amino-N content and further analysed (Table 2). The first fractions eluted after changing to chloroform–methanol (19:1, v/v) contained 46 mg. of lipid free from P, with about 15 μmoles of fatty acid ester and 19 mg. of cholesterol. This was followed by a phospholipid band low in amino N with 4 mg. of cholesterol (fraction MA); on silicic acid paper this gave a large cardiolipin spot and weak phosphatidylethanolamine and phosphatidylserine spots; the deacylated ester also gave a spot identical with that from cardiolipin. The main kephalin band (fraction MB) containing phosphatidylserine and phosphatidylethanolamine was eluted rapidly in chloroform–methanol (4:1, v/v), with the inositide overlapping. Owing presumably to the high degree of unsaturation the lecithin band, rather unusually, was eluted with this solvent, the front just overlapping into the tail (fraction MC) of the kephalin and inositide. The lecithin band was broad and successive eluates were pooled into three fractions MD, ME and MF with decreasing iodine values: the P in these fractions was all present as fatty acid ester phosphatide and was hydrolysed by *Clostridium welchii* lecithinase, and on paper chromatography the deacylated ester gave only a glycerylphosphorylcholine spot. About 97 % of the P was recovered from the column after passage of chloroform–methanol (1:4); sphingomyelin was not detected, but its presence in the original extract was not excluded. The kephalin and lecithin fractions were virtually free from plasmalogens.

The distribution of P in the various phospholipids of the mitochondria was computed from the analyses in Table 2 as follows: cardiolipin 9; phosphatidylserine and phosphatidylethanolamine 31; phosphatidylinositol 6; lecithin 51 % of the total P.

Microsomal lipid. The material (34 mg. of P) was chromatographed in a similar way on 28 g. of silicic acid. The chloroform eluate (neutral-lipid fraction) contained 72 mg. of lipid with about 160 μequiv. of fatty acid ester and 1 mg. of cholesterol. With chloroform–methanol (19:1, v/v) a small band (0·36 mg. of P) with an amino N:P ratio of 0·06:1·0 was first eluted. This band contained 71 mg. of material, including about 20 mg. of total cholesterol; the deacylated product gave on paper-

chromatography spots corresponding to glycerophosphate and the polyglycerophosphate derived from cardiolipin. This band was immediately followed by the front of the kephalin band eluted with the same solvent. The remainder of the fractionation was very similar to that of the mitochondrial extract. Plasmalogens were virtually absent. The analyses of the fractions are given in Table 3. The distribution of P in the microsomal phospholipid was computed as follows: cardiolipin and phosphatidic acids, not more than 1; phosphatidylserine and phosphatidylethanolamine, 26; phosphatidylinositol, 10; lecithin 62% of the total P.

Fatty acid composition of fractions

Table 4 shows the fatty acid composition of: (*a*) the neutral lipid, mainly glycerides, in the chloroform eluates; (*b*) the main kephalin fraction; (*c*) the lecithin, as the weighted mean of the three fractions analysed; (*d*) the separate fractions of the mitochondrial lecithin. Fig. 1 shows the gas chromatograms of the fatty acids of the three lecithin fractions from the microsomes.

There is a striking similarity between the corresponding fractions of the mitochondria and microsomes. The neutral lipid contained more than 50%

Table 3. *Phospholipid fractions from microsomal lipids*

For explanation of abbreviations see Table 2.

Fraction	XA	XB	XC	XD	XE	XF	XG
Eluate nos.	1–5	6–11	12–14	15–26	27–30	31–36	37–55
Eluent: $CHCl_3$–methanol (v/v)	19:1	19:1	4:1	4:1	3:2	3:2	3:2, 1:4
Wt. (mg.)	71	65	211	75	192	179	192
P (mg.)	0·36	2·1	8·1	2·7	7·0	6·6	7·5
P (% on dry wt.)	0·5	3·2	3·8	3·6	3·6	3·7	3·9
Molar ratio: (P = 100)							
Amino N	6	77	76	30	5	Nil	Nil
Fatty acid ester	—	200	190	208	190	182	190
Inositol	—	24	22	34	—	—	—
Double bonds/mol. of fatty acid	—	2·1	2·0	0·8	2·2	1·5	1·0
P in fraction (% of total)	1·1	6·2	23·8	7·8	20·6	19·4	21·9
Phosphoric esters*	GP	GPE	—	GPE	—	—	—
	GPGPG	GPS	GPE	GPI	GPC	GPC	GPC
	—	GPI	GPI	GPC	—	—	—

* By paper chromatography of water-soluble P esters formed by mild alkaline hydrolysis.

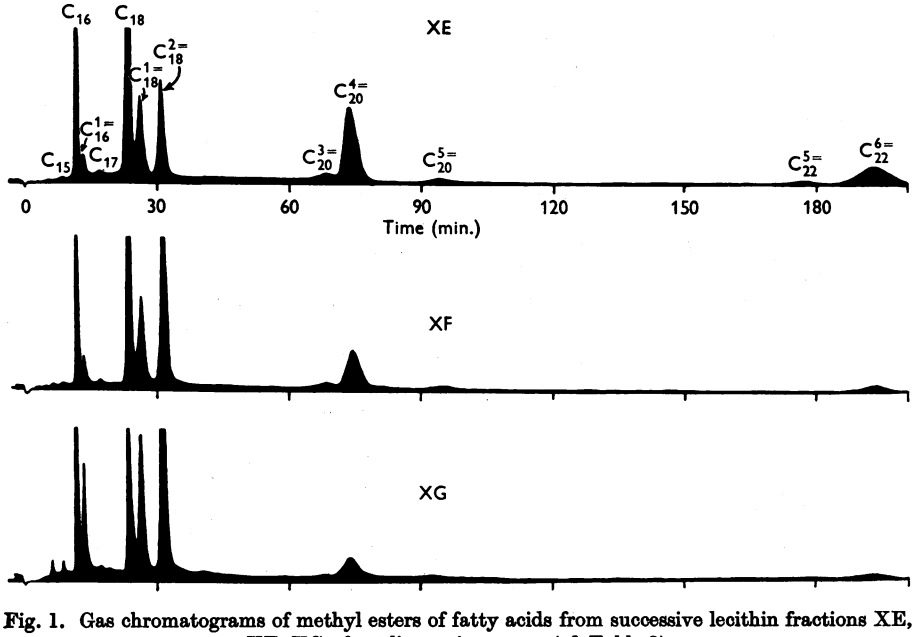

Fig. 1. Gas chromatograms of methyl esters of fatty acids from successive lecithin fractions XE, XF, XG of rat-liver microsomes (cf. Table 3).

Table 4. *Fatty acid composition of neutral-lipid, kephalin and lecithin fractions from rat-liver microsomes and mitochondria*

Values are expressed as percentages of total fatty acid methyl esters. Chromatography on Reoplex 400 at 190°.

		Neutral lipid		Main kephalin fraction		Lecithin*		Lecithin fractions		
								Mitochondria		
Fatty acid	Designation	Mitochondria $CHCl_3$ eluate	Microsomes $CHCl_3$ eluate	Mitochondria MC	Microsomes XB	Mitochondria MD–F	Microsomes XE–G	MD	ME	MF
Undecanoic	11:0	0·3	—	—	—	—	—	—	—	—
Lauric	12:0	—	—	0·4	—	—	—	—	—	—
Myristic	14:0	0·8	0·9	—	—	0·3	0·2	0·2	0·1	0·6
Pentadecanoic	15:0	0·2	0·5	0·2	—	0·2	0·2	0·2	0·1	0·3
Palmitic	16:0	23·9	22·5	17·5	17·5	13·2	13·2	11·0	13·3	15·2
Palmitoleic	16:1	10·1	11·6	1·0	0·7	3·5	3·7	1·4	3·2	6·2
Heptadecanoic	17:0	0·7	1·0	0·6	0·3	0·6	0·9	0·4	1·0	0·5
—	17:?	0·9	0·9	0·3	—	0·1	0·2	—	0·3	—
Stearic	18:0	2·1	2·4	30·4	34·5	21·9	24·9	22·2	21·6	21·9
Oleic	18:1	44·9	40·5	4·8	4·4	14·1	12·1	11·5	13·6	17·2
Linoleic	18:2	14·6	16·3	4·3	4·2	19·6	19·5	13·7	20·8	24·2
Linolenic	18:3	—	0·5	—	—	—	—	—	—	—
Nonadecanoic	19:0	—	0·8	—	—	0·5	—	1·1	—	0·5
Eicosatrienoic	20:3	—	—	0·8	1·3	1·5	1·2	2·0	1·4	1·3
Arachidonic	20:4	0·9	1·8	21·0	23·0	13·8	12·6	18·1	14·3	8·8
Eicosapentaenoic	20:5	0·5	—	2·4	0·6	1·9	1·4	1·1	3·6	0·9
Docosapentaenoic	22:5	—	—	2·0	2·0	1·2	0·4	2·9	0·9	—
Docosahexaenoic	22:6	—	—	14·0	11·7	7·9	7·7	14·3	7·3	2·2
Saturated acids	—	28·9	29·0	50·2	53·6	38·3	40·8	35·1	36·4	38·6
Monoenoic acids	—	55·0	52·1	5·8	5·1	14·7	15·8	12·9	16·8	23·4
C_{20}–C_{22} acids	—	1·4	1·8	40·2	38·6	26·3	23·3	38·4	27·5	13·2

* Weighted mean.

of monoenoic acids, very small amounts of C_{20}–C_{22} polyenoic acids and palmitic acid as the predominant saturated acid. The analyses of the kephalin bands, which contained 10–20% of phosphatidylinositol as well as phosphatidylserine and phosphatidylethanolamine, give a general picture of the components as compounds rich in C_{20}–C_{22} polyenoic acids (40%) and low in oleic acid and linoleic acid. This kephalin differs from that of ox-heart kephalin in three main respects: (1) the content of palmitic acid (20% against 4%); (2) the high content of arachidonic acid (21% against 12%); (3) the remarkable content (12–14%) of docosahexaenoic acid; C_{22} acids were not detected in ox-heart kephalin (Gray, 1960).

The lecithin fraction of the liver particles is even more remarkable in its high content (25%) of C_{20}–C_{22} polyenoic acids, including 8% of docosahexaenoic acid, for these acids are present in very small amounts, if at all, in heart-muscle lecithin and egg lecithin (Gray, 1960; Hawke, 1959); also, unusually, there is more stearic acid than palmitic acid. The successive lecithin fractions MD, ME and MF all had about the same proportion of saturated acids; the decrease in iodine values throughout the band is due to the substitution of oleic acid and linoleic acid for arachidonic acid and docosahexaenoic acid.

Linolenic acid was present (0·5%) in the microsomal neutral lipid, but was not detected in any of the phospholipid fractions. C_{15} and C_{17} acids were present in small amounts in all the fractions and C_{20} trienoic and pentaenoic acids and C_{22} pentaenoic acids in the kephalin and lecithin. In contrast with ox liver, C_{22} hexaenoic acid preponderates over the C_{22} pentaenoic acid in rat liver.

DISCUSSION

The only striking difference between the phospholipids of the mitochondria of rat liver and those of the microsomes is in the content of cardiolipin, which is about 9% of the total in mitochondria and not more than 1% in the microsomes. Marinetti, Erbland & Stotz (1958) found that mitochondria of pig-heart ventricle contained significantly more polyglycerophosphatide than did the microsomes. Getz & Bartley (1959) isolated the polyglycerophosphatide from rat-liver mitochondria and found the fatty acid composition was similar to that of cardiolipin from heart muscle.

The neutral-lipid fractions from mitochondria and microsomes are very similar in fatty acid composition, and so are the corresponding phospholipid fractions. The results are broadly in agreement with those of Clément *et al.* (1955, 1956) but the content of hexaenoic acids in the phospholipids is considerably higher.

The very high content of 'essential' fatty acids, reckoned as the sum of linoleic acid and C_{20}–C_{22} polyenoic acids, and particularly of the polyenoic acids, in the phospholipids is of interest in several respects. It is not clear what part phospholipids play in the metabolic activity of the subcellular particles, but it is known from the action of lecithinases that the integrity of the lecithin in mitochondria is necessary for the succinoxidase activity (Macfarlane, 1950; Nygaard & Sumner, 1953; Macfarlane & Datta, 1954) and doubtless the other phospholipids are also of functional significance. A dietary deficiency of essential fatty acids would presumably limit the rate of production of mitochondria and microsomes and hence the rate of growth, though it would not necessarily affect the metabolic activity of the particles already present.

Another interesting aspect is the susceptibility of the highly unsaturated acids to peroxidation. Tappel & Zalkin (1959), for example, have shown that lipid peroxidation in isolated mitochondria is accompanied by deterioration in enzymic activity. Horgan, Philpot, Porter & Roodyn (1957) investigated the toxicity of auto-oxidized linoleic acid, and the formation of peroxides *in vivo* in relation to the toxicity of radiation, and suggested that chain auto-oxidation of essential fatty acids would produce lethal doses of peroxide in sites not protected by vitamin E. The toxicity may possibly be referred to the direct inhibition by peroxidation of the metabolic activity of the mitochondria or microsomes.

SUMMARY

1. The lipid extracted from the mitochondria of rat liver was similar to that from the microsomes except that the mitochondria contained significantly more cardiolipin than the microsomes.

2. The fatty acid composition of the neutral-lipid, kephalin and lecithin fractions of the mitochondria and microsomes of rat liver was determined by gas chromatography. The corresponding fractions from the two kinds of particles were very similar.

3. Monoenoic acids (53%), palmitic acid (23%) and linoleic acid (15%) were the main acids in the neutral lipid.

4. The lecithin and kephalin fractions contained respectively 25% and 40% of the fatty acids as C_{20} and C_{22} acids, with a high proportion of arachidonic acid and docosahexaenoic acid.

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Improved Fractionations of Arginine-Rich Histones from Calf Thymus

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It has been shown by a variety of methods that calf-thymus histones can be separated into fractions relatively rich in arginine (Grégoire & Limozin, 1954; Daly & Mirsky, 1955; Davison & Shooter, 1956) and in lysine (Stedman & Stedman, 1951; Davison & Butler, 1954; Crampton, Moore & Stein, 1955; Daly & Mirsky, 1955). Further chromatographic separation on Amberlite IRC-50 resin columns was also achieved by Luck, Rasmussen, Satake & Tsvetikov (1958) with a gradient of guanidinium chloride for elution. Davison (1957) first reported the use of carboxymethylcellulose columns with a salt gradient at pH 4·2 which gave two main peaks, in addition to an initial small fraction very rich in aspartic and glutamic acids. The first main peak was a lysine-rich histone and the second, larger, peak was heterogeneous, with a higher arginine content in the part eluting more slowly. Later it was possible to elute histone as two fractions from carboxymethylcellulose columns with dilute hydrochloric acid, the fractionation being followed by *N*-terminal amino acid analysis (Phillips & Johns, 1959). The two fractions had mainly proline and mainly alanine *N*-terminal groups respectively. These two systems of elution have now been combined to give an improved fractionation into three parts. A bulk fractionation process has also been worked out, which readily gives arginine-rich histones, with alanine as virtually the sole *N*-terminal group. This method is based on the extraction of the whole tissue with acid in the presence of a high concentration of ethanol. It was thought that under these conditions the action of tissue proteinases, such as that shown to be present in calf thymus (Phillips & Johns, 1959), would be prevented. It was realized that proteins other than histones would be dissolved from the tissue in this procedure, but judging by the analytical results on the product it is thought that these have been eliminated. This paper is mainly confined to the study of the arginine-rich histones obtained by the chromatographic and the extraction procedures.

EXPERIMENTAL AND RESULTS

Improved chromatographic separation on carboxymethylcellulose. The histone was prepared from calf-thymus glands by the methods previously described, with slightly acidified sodium chloride washes (Phillips & Johns, 1959). The histone (100 mg.) was dissolved in 20 ml. of buffer, pH 4·2, containing 0·1 M-acetic acid and 0·03 M-sodium hydroxide, and applied to carboxymethylcellulose in the form of a column, 14 cm. long and 2 cm. in diameter, which had been prepared by allowing a slurry of 5 g. of carboxymethylcellulose in the same buffer to settle in the chromatographic tube under gravity. Fractions of 3–5 ml. were collected automatically at a flow rate of about 0·5 ml./min. and the progress of the elution was followed by measuring the extinction of the fractions at 278 mμ. Elution was carried out in four steps as follows: (1) a buffer, pH 4·2, containing 0·1 M-acetic acid and 0·03 M-sodium hydroxide; (2) a buffer, pH 4·2, containing 0·17 M-acetic acid, 0·051 M-sodium hydroxide