The Lipid Composition of Rat-Liver Mitochondria, Fluffy Layer and Microsomes

By G. S. GETZ,* W. BARTLEY, F. STIRPE†, BRENDA M. NOTTON AND A. RENSHAW

Department of Biochemistry and Medical Research Council, University of Oxford

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The fatty acid compositions of the intracellular organelles of rat liver, separated by differential centrifuging, and the composition of the rat-liver lipids, have been described by Getz & Bartley (1961) and Getz, Bartley, Stirpe, Notton & Renshaw (1961). This paper describes the lipid composition of the mitochondria, fluffy layer and microsomes isolated from rat liver. A similar study has recently been published by Macfarlane, Gray & Wheeldon (1960).

EXPERIMENTAL

The animals and the cell-fractionation procedures were the same as those used by Getz & Bartley (1961). Two fractionations of liver were made: in the first (Expt. 1) 92 g. of rat-liver pulp was fractionated and in the second (Expt. 2) 130 g.

Lipid extraction. The lipids were extracted with either chloroform-methanol (2:1, v/v) (Expt. 1), or ethanol and ether (Expt. 2) as described by Getz & Bartley (1961). In Expt. 2 the light-petroleum extract containing the total lipids was evaporated to dryness, redissolved in chloroform-methanol (2:1, v/v) and washed according to Folch, Lees & Sloane-Stanley (1957).

Fractionation of lipids on silicic acid. In Expt. 1 the lipids were fractionated by chromatography on silicic acid (Mallinckrodt mesh 100) prepared according to Hirsch & Ahrens (1958) as described by Getz, Bartley, Stirpe, Notton, Renshaw & Robinson (1961), but the triglycerides were not separated from the lower neutral glycerides in the microsomal extract.

In Expt. 2 the method of Barron & Hanahan (1958) was used for the fractionation of neutral lipids. The phospho-

lipids were eluted from the same column with the solvent sequence used in Expt. 1. The least polar phospholipid was eluted with chloroform-methanol (9:1, v/v) instead of 7:1 (v/v). A small portion of each 10 ml. fraction collected was tested for acyl ester groups by the method of Rapport & Alonzo (1955). The colours developed were assessed visually and the tubes were combined on the basis of this assessment.

Solvents. All solvents were AnalaR grade, except for ethanol and methanol, which were used without further purification, and hexane which was the fraction distilled at 67-69° from crude laboratory hexane.

Analytical methods. These were described by Getz, Bartley, Stirpe, Notton & Renshaw (1961).

Paper chromatography. Chromatography on silicic acid-impregnated paper (Marinetti, Erbland & Kochen, 1957; Marinetti, Erbland & Stotz, 1958) was used to confirm the identity of the phospholipid fractions. In addition, an assessment of the major fractions was made with the quantitative paper-chromatographic examination of the water-soluble phosphate esters formed on mild alkaline hydrolysis of the phospholipids (Dawson, 1960).

Esterification of fatty acids and gas chromatography. The techniques were those described by Getz & Bartley (1961).

RESULTS

The dry weights, protein content, total lipid content and acyl-ester content of the liver pulp, mitochondria, fluffy layer and microsomes are given in Table 1. In Expt. 1 almost 60% of the lipid esters and 70% of the lipid phosphorus was recovered in the three cell fractions and in Expt. 2 the values were about 60% in both cases.

Identification of the major phospholipid fractions. The composition of the major phospholipid fractions of mitochondria, microsomes and fluffy layer was examined by the chromatographic method of Dawson (1960). The results (Table 2) show that the material eluted from the silicic acid

Table 1. *Analyses of cell fractions separated from rat liver*

Results are given as the mean of two experiments except for protein. Values are given/100 g. dry wt. of original liver.

Fraction	Dry wt. (g.)	Protein content (g.)	Fat content (g.)	Acyl-ester content (m-equiv.)
Pulp	100	55.6	21.0	36.9
Mitochondria	9.2	5.8	2.4	3.4
Fluffy layer	6.3	—	1.9	3.2
Microsomes	23.7	9.3	13.0	15.1

* Present address: Department of Chemical Pathology, Witwatersrand University, Medical School, Johannesburg, South Africa.

† Present address: Istituto di Patologia Generale dell'Università di Siena.

Table 2. *Phospholipids of rat-liver subcellular particles*

Values are percentages of the phospholipids recovered from the chromatogram (Dawson, 1960).

No. of expt.	Tissue fraction	Silicic acid fraction no.	Elution mixture (chloro- form- methanol, v/v)	Glyceryl- phosphoryl- choline derived from lecithin	Glyceryl- ethanol- amine derived from phosphatidylethanol- amine	Glyceryl- phosphoryl- serine derived from phosphatidylserine	Glycero- phosphate derived from phosphatidic acid	Cyclic glycero- phosphate derived from inositide	Polyglycerol phosphate derived from cardiolipin	Inorganic P	Alkali- stable lipid P
1	Mitochondria	4	4:1	—	79	—	2.4	—	9.7	27.2	—
1	Fluffy layer	4	4:1	—	72.8	—	—	—	—	12.0	—
1	Fluffy layer	6	3:2	80	—	—	—	7.0	—	—	1.4
1	Microsomes	4	4:1	—	85	—	—	—	5.9	—	—
1	Microsomes	6	3:2	93	—	—	2.7	4.0	—	—	1.8
1	Microsomes	4	4:1	—	74	—	18.0	17.2	—	—	—
2	Mitochondria	6	3:2	84	—	—	2.7	13.5	—	—	—
2	Fluffy layer	4	4:1	—	78	2.7	4.8	33	—	—	—
2	Fluffy layer	5	3:2	24	43*	—	—	—	—	—	—
2	Fluffy layer	6	3:2	98	—	—	—	—	—	—	2.9
2	Microsomes	4	4:1	—	79	9.6	—	9.5	—	—	—
2	Microsomes	6	3:2	102	—	—	—	—	—	—	3.1

* This figure is the sum of glycerylphosphorylethanolamine and the glycerylphosphorylserine.

column with a chloroform-methanol (4:1, v/v) mixture contained between 72.8 and 85 % of phosphatidylethanolamine, and the lecithin fraction gave more than 90 % of glycerylphosphorylcholine.

Mitochondrial lipids

Table 3 shows the analyses of the mitochondrial lipids separated by silicic acid chromatography in Expt. 1. Only 83 % of the total mitochondrial lipid phosphorus was recovered from the silicic acid column. The mitochondria of Expt. 2 gave analyses almost identical with those in Expt. 1. The main aminophosphatide fraction (phosphatidylethanolamine) was present in the mitochondria in amount almost equal to that of the lecithin. A substantial proportion of the lipid phosphorus in the mitochondria was a nitrogen-poor, cardiolipin-like compound (Getz & Bartley, 1959). The fatty acid composition of each of the lipid classes of the mitochondria is given in Table 4. Unless otherwise stated the results are the means of the two experiments. In general the values in the two experiments agreed but in the lecithin fraction the two values for palmitic acid were 23.5 and 15.8 % and for linoleic acid were 16.5 and 23.5 %. Only small differences between the fatty acid pattern of the mitochondrial lipids and the pulp lipids were found.

A comparison of the total methyl esters of the mitochondria and pulp showed that palmitic acid and oleic acid were less in the mitochondria (17.8 and 25 % for palmitic acid and 11.9 and 20.5 % for oleic acid). Conversely, the total mitochondrial esters contained a higher proportion of arachidonic and docosahexaenoic acids (17.0 and 9.8 % for arachidonic acid and 6.2 and 3.7 % for docosahexaenoic acid). The neutral-lipid fractions contained more stearic acid and arachidonic acid and less palmitic acid and C₁₈ unsaturated acids than the corresponding pulp fractions. The sterol ester fractions had a substantially higher palmitoleic acid content than the pulp.

All the mitochondrial phospholipids had less palmitic acid than the corresponding pulp fractions. The mitochondrial phospholipids with the exception of the inositide-containing fraction had a higher proportion of arachidonic acid than the corresponding pulp phospholipids.

The mitochondria have a high content of phosphatidylethanolamine, and all the fatty acids which predominate in the phosphatidylethanolamine are somewhat enriched in the mitochondria.

The fatty acid esters of the neutral-lipid fractions contained two substances which behaved in an anomalous manner on the gas chromatograph. On the Apiezon L columns these substances chromatographed with myristic acid and palmitic acid.

However, on a column of polyethylene glycol adipate the retention times (relative to stearate) were 1.64 and 2.94. The triglyceride fraction had the highest concentration of these two substances. When the triglyceride fraction was subjected to the micro-sublimation procedure of Stoffel, Chu & Ahrens (1959) a substance could be collected which on the gas chromatograph behaved as palmitic acid when an Apiezon column was used but had a retention time (relative to stearate) of 2.89 on adipate. The sublimed material was treated with dry methanolic HCl [as described for the preparation of methyl esters by Getz & Bartley (1961)] and again subjected to gas chromatography. Two peaks appeared on the gas chromatograph corresponding to the positions of palmitic acid and myristic acid when an Apiezon column was used but with retention times (relative to stearate) of 1.64 and 2.89 when an adipate column was used. The methanolic-HCl treatment thus had converted some of the material into the 'myristic acid-like' substance. These compounds showed no absorption of ultraviolet light.

A comparison of the areas of the chromatogram peaks showed that the original material separated by sublimation was present in greater quantity than the sum of the fatty acids isolated from the triglyceride fraction. It would appear that the material isolated by sublimation from the triglycerides was a volatile polar compound that was only partially methylated on its polar group by the treatment with methanol and HCl. Similar compounds have been found in the neutral-lipid fraction of the brain by Biran & Bartley (1961).

Microsomal lipids

The microsomes contained 558 μ equiv. of fatty acid ester/g. dry wt. compared with the 340 μ equiv. of fatty acid ester/g. dry wt. found in the mitochondria (cf. Tables 5 and 3). More than twice the quantity of lecithin was found in the microsomes than in the mitochondria. Less cardiolipin-like material was found in the microsomes compared

with the mitochondria (5.7 and 23.8 μ equiv. of fatty acid ester/g. dry wt. respectively). The microsomes contained up to twice the quantity of the other phospholipids in the mitochondria. In contrast with the mitochondria, where lecithin and aminophosphatide were present in equal amounts, the microsomes contained more than twice as much lecithin as aminophosphatide. In the microsomes the least-polar phospholipid (low-nitrogen-containing) constituted only 1% of the total phospholipids. The microsomal pellet would have to be contaminated with 10% of its weight of mitochondria if it were assumed that this phospholipid had its origin in the mitochondria. With the isolation procedures used for the preparation of the microsomes a contamination by mitochondria of less than 1% would be expected. In Expt. 2 the analyses were similar to those of Expt. 1, but lecithin constituted 70% of the phospholipids instead of the 60% in Expt. 1.

Table 6 gives the fatty acid composition of the various phospholipids of the microsomes. Special separations were made to demonstrate: (a) the free fatty acids; (b) the fatty acids of the lower neutral glycerides; (c) the fatty acids of the cardiolipin-like fraction; (d) the changes in fatty acid composition in the first third and later portion of the eluted lecithin peak (Harris, Robinson & Getz, 1960; Macfarlane *et al.* 1960).

The free fatty acid pattern was clearly different from that of the triglycerides but was nearer to that of the diglycerides. The cardiolipin fraction, although high in linoleic acid (57.8% of the total fatty acids), did not contain as much linoleic acid (74%) or have the same fatty acid pattern as the mitochondrial cardiolipin. The lecithin eluted initially had lower proportions of palmitic acid and linoleic acid and higher proportions of stearic acid, arachidonic acid and docosahexaenoic acid than the last lecithin eluted (Table 6).

The total fatty acid esters of the microsomes had higher proportions of stearic acid and arachidonic acid and lower proportions of oleic acid and linoleic acid than the pulp.

Table 3. *Analysis of lipids of rat-liver mitochondria*

The amounts are μ equiv./g. dry wt. of mitochondria. The results are those of Expt. 1.

Nominal lipid class	Acyl ester	Lipid P	Amino N	Choline	Free acids	Acyl ester:lipid P ratio	Amino N:lipid P ratio	Choline:lipid P ratio
Sterol esters	1.9	—	—	—	—	—	—	—
Triglycerides	29.5	—	—	—	16.5	—	—	—
Diglycerides	5.1	0.6	—	—	—	—	—	—
Cardiolipin	23.8	15.7	0.1	—	—	1.51	—	—
Aminophosphatides	118	60.4	61.7	0.15	—	1.95	1.02	—
Inositide	22.2	14.9	2.7	0.05	—	1.53	0.18	—
Lecithin	137	58.5	1.5	59.2	—	2.34	0.03	1.01
Polar lipid	2.9	6.5	0.3	0.2	—	0.45	0.05	0.03

Table 4. *Distribution of fatty acids in lipids from rat-liver mitochondria*

Results are the mean of two experiments except for triglycerides and free fatty acids. Values are percentages of the total area of the chromatogram peaks that was occupied by each individual peak. br., Branched-chain; uns., unsaturated.

Fatty acid. No. of C atoms and double bonds	Total fatty acid esters	Sterol esters	Trigly- cerides	Free fatty acids	Digly- cerides	Cardiolipin	Amino- phosphatide	Inositide- rich fraction	Lecithin	Polar lipid
≤10-0	—	—	—	—	—	0.1	—	—	0.1	—
11-0	0.1	—	1.9	—	—	0.1	—	—	0.1	—
*12-0 br.	0.1	—	0.4	—	0.1	—	—	—	0.1	—
12-0	—	0.9	—	—	0.1	—	—	0.3	—	1.8
*14-0 br.	—	1.3	0.2	—	0.2	—	—	0.1	—	0.7
*14-0 br.	—	3.7	2.2	—	0.7	0.5	0.1	0.9	0.5	1.2
14-0	0.3	0.4	—	—	0.6	0.1	—	—	—	—
14-1	—	0.2	—	—	0.6	—	—	—	—	—
*15-0 br.	0.1	0.6	0.3	—	0.2	0.1	—	—	—	0.6
*15-0 br.	0.4	0.6	0.7	—	0.4	0.2	0.2	1.5	0.5	0.6
15-0	—	2.2	—	—	0.3	0.1	—	1.1	0.1	0.6
*16-0 br.	—	21.8	22.6	18.8	18.8	3.9	20.2	12.1	19.7	33.6
16-0	1.6	10.6	2.5	—	2.0	2.5	0.8	0.8	1.5	2.5
16-1	—	0.2	—	—	—	0.1	—	—	—	—
*17-uns. br.	—	0.4	—	—	—	—	—	—	—	—
*17-0 br.	—	0.8	1.6	—	0.5	0.1	0.3	1.7	0.3	—
17-0	0.7	0.5	0.5	—	0.6	0.1	0.9	1.4	0.8	1.2
*18-0 br.	—	1.9	—	—	0.5	0.1	—	—	0.3	0.4
18-0	14.9	12.6	9.6	20.2	8.0	1.4	19.5	32.0	18.7	25.2
18-1	11.9	17.1	21.8	24.6	19.6	12.8	10.4	6.8	12.2	10.9
18-2	23.4	13.0	17.9	18.3	25.9	74.0	16.3	16.2	20.0	14.0
18-3	—	—	—	—	—	0.1	—	—	—	—
*19-0 br.	—	0.5	—	—	—	—	—	—	—	—
*19-0 br.	0.5	0.1	—	—	—	—	0.2	—	0.2	—
20-0	—	2.7	—	—	—	—	—	0.4	—	—
20-1	0.1	1.5	—	—	1.8	0.2	—	—	0.5	—
20-2	0.1	—	—	—	1.7	0.8	—	1.1	0.3	—
20-3	1.7	0.9	1.0	—	0.6	1.2	0.7	1.8	0.8	0.3
20-4	17.0	4.9	7.2	14.6	4.2	1.6	21.0	21.5	19.5	7.1
20-5	0.3	—	0.7	—	—	—	—	—	0.6	—
*21-0 br.	—	0.6	—	—	—	—	—	—	—	—
22-0	—	—	0.6	—	—	—	—	—	—	—
*22-3	—	—	3.2	—	—	—	—	—	—	—
22-5	0.9	—	1.6	—	—	—	—	—	—	—
22-6	6.2	0.3	3.5	—	—	0.5	1.0	0.9	0.4	—
23-0	—	—	—	—	1.5	0.4	8.7	3.4	3.4	—
*24-1	—	—	—	—	4.7	—	—	—	—	—

* Indicates tentative identification.

Table 5. *Analysis of lipids of rat-liver microsomes*

The amounts are μ equiv./g. dry wt. of microsomes. The results are those of Expt. 1.

Nominal lipid class	Acyl ester	Lipid P	Amino N	Choline	Free acids	Acyl ester:lipid P ratio	Amino N:lipid P ratio	Choline:lipid P ratio
Sterol esters	13.5	—	—	—	—	—	—	—
Neutral glycerides	49.5	—	—	—	20.5	—	—	—
Cardiolipin	5.6	3.2	0.25	—	1.8	1.7	0.05	—
Aminophosphatides	145	79.7	61.3	—	—	1.8	0.88	—
Inositides	47.0	29.1	8.6	0.5	—	1.6	0.32	0.02
Lecithin	290	183	4.0	116	—	1.6	0.02	0.63
Polar lipids	7.7	11.6	0.8	1.5	—	0.66	0.07	0.13

Compared with the total mitochondrial fatty acid esters, the microsomal fatty acid esters had higher percentage contents of palmitic acid and stearic acid and lower percentage contents of linoleic acid and docosahexaenoic acid.

Lipids of the fluffy layer

Analyses of these are in Table 7. The aminophosphatide:lecithin ratio in this fraction was intermediate between those for the mitochondrial and microsomal lipids. The fatty acid pattern of the fluffy layer lipids (Table 8) was also intermediate in composition between those for the microsomal and mitochondrial lipids.

Comparison of the lipid composition of mitochondria, microsomes and fluffy layer with that of the whole liver

The lipids of rat-liver pulp, mitochondria, fluffy layer and microsomes are summarized in Table 9. The results for lecithin and for aminophospholipids of mitochondria and microsomes agree well with the data of Kretschmer & Barnum (1951) on mouse liver and of Spiro & McKibbin (1956) on liver from young rats. Levine & Chargaff (1952) obtained higher values for the lecithin and cephalins of mitochondria, although they obtained 1.26 times as much nitrogen base as phosphorus in the phospholipids of their mitochondria. Pig-heart mitochondria (Marinetti *et al.* 1958) had a lower aminophospholipid and higher cardiolipin and phosphatidylinositol values than those for rat liver given in Table 9; pig-heart microsomes had a lower lecithin and higher cephalin and sphingomyelin contents than those of rat liver (Table 9). The analyses by Strickland & Benson (1960) on rat liver show slightly higher contents of lecithin and cardiolipin in their preparations of rat-liver mitochondria, and Macfarlane *et al.* (1960) found a higher lecithin and a lower cephalin content in their mitochondria than we found in our preparations.

Table 10 shows the distribution of the lipid classes in the cell fractions isolated by differential

centrifuging; 8.8% of the liver lipid phosphorus was recovered in the mitochondrial fraction. The mitochondrial phospholipid contained 12.4% of the liver phosphatidylethanolamine but only 7.5% of the liver lecithin. The microsomes contained 44.3% of the liver lipid phosphorus and 58.9% of the liver lecithin.

In general the particulate fractions of the cell contained a smaller portion of the neutral lipids of the liver than they did of the total lipids. Divergences from this were found in the neutral glycerides of the fluffy layer in Expt. 1 and the sterol esters of the microsomes in Expt. 2. Table 10 shows that the fluffy layer resembled the mitochondria more closely than the microsomes. The concentrations (expressed as amount of lipid/unit dry wt. of cell fraction) of each lipid class in the isolated fractions are compared with the concentration of the same lipid class in the whole liver. The ratio of the concentration of any lipid in a cell fraction to that in the whole liver is a measure of the degree of enrichment of that lipid in the cell fraction.

The mitochondria are enriched in cardiolipin, in aminophosphatides and, on the basis of Expt. 1, in the polar phospholipid (Table 10). The mitochondria are poor in lecithin and neutral lipids. On the other hand, the microsomes are poor in cardiolipin and enriched in aminophosphatides, lecithin and polar phospholipids.

DISCUSSION

In spite of the different function and spatial separation of the subcellular organelles within the cell, each lipid class has much the same fatty acid composition in each organelle.

The composition of the dietary fatty acids may affect the pattern of fatty acids found in the subcellular fractions [see, for example, Howitt, Century, Harvey & Navazio (1960) and Marco, Machlin & Gordon (1960)]. The small differences (higher content of unsaturated fatty acids in neutral-lipid, cephalin and lecithin fractions)

Table 6. *Distribution of fatty acids in lipids from rat-liver microsomes*

Results are the mean of two experiments except for free fatty acids and diglycerides. Values are percentages of the total area of the chromatogram peaks that was occupied by each individual peak. br., Branched-chain; hbr., highly branched-chain; uns., unsaturated.

Fatty acid. No. of C atoms and double bonds	Total fatty acid esters	Sterol esters	Trigly- cerides	Free fatty acids	Digly- cerides	Cardio- lipin	Amino phosphatide	Inositide- rich fraction	Initial lecithin eluted	Last lecithin eluted	Lecithin	Polar lipids
9-0	—	0.1	—	—	—	0.1	—	—	—	—	—	—
10-0	—	—	—	—	—	0.1	—	—	—	0.2	0.1	—
11-0	0.1	—	—	—	—	—	—	—	—	—	0.1	—
*12-0 br.	—	—	—	—	—	—	—	—	—	—	—	—
12-0	0.1	2.3	0.5	—	—	—	—	—	—	—	—	—
13-0 br.	—	—	—	0.1	—	0.1	—	0.2	0.2	0.1	0.1	—
*14-0 br.	—	0.1	0.1	—	—	0.1	—	—	—	0.2	0.2	0.7
14-0	0.3	3.0	1.0	1.2	0.4	0.5	0.1	0.3	0.2	0.5	0.4	1.9
14-1	—	0.5	—	0.1	—	0.1	—	0.1	0.1	—	—	—
15-0 hbr.	—	0.1	—	—	—	—	—	—	—	—	—	—
15-0 hbr.	—	0.1	—	—	—	0.1	—	—	—	—	—	—
*15-0 br.	0.1	0.5	—	0.1	—	0.2	—	0.2	—	—	0.1	0.5
15-0	0.4	0.9	0.4	0.6	0.7	0.6	0.2	0.2	0.2	0.4	0.5	1.3
16-0 hbr.	—	0.1	—	—	—	—	—	0.1	—	—	—	—
*16-0 br.	—	0.1	—	—	1.3	—	—	0.2	—	—	—	—
16-0	21.5	28.5	27.5	25.8	31.4	9.1	19.9	12.9	16.5	27.2	24.6	30.6
16-1	1.1	5.2	2.6	1.8	2.0	2.5	0.7	0.5	0.6	1.3	1.0	1.9
17-0 br.	0.2	0.4	0.8	0.2	—	0.4	0.4	0.2	0.1	0.2	0.3	0.4
17-0	0.6	0.3	0.3	0.4	1.1	0.2	0.7	0.9	0.6	0.5	0.7	0.8
18-0 hbr.	—	—	—	—	—	0.2	—	—	—	0.1	0.1	—
*18-0 br.	0.1	—	—	—	—	0.5	—	—	—	—	0.1	—
18-0	18.4	14.3	6.3	14.2	12.9	4.8	22.4	37.8	24.4	15.7	18.5	26.0
18-1	13.1	20.7	27.6	23.0	20.2	17.7	9.3	5.2	10.4	13.4	13.0	10.9
18-2	17.2	16.3	25.4	18.8	18.6	57.8	12.5	7.2	11.3	22.7	18.5	10.6
18-3	—	—	0.7	—	—	—	—	0.1	—	—	—	—
*19-0	—	0.4	—	—	—	—	—	—	—	—	—	—
*19-0 br.	0.5	0.3	0.3	—	—	0.2	0.2	—	—	0.1	0.2	—
20-1	—	—	—	0.6	—	—	0.4	0.2	—	0.2	0.3	—
20-2	0.5	—	0.1	0.4	—	0.5	0.4	0.2	—	0.2	0.3	—
20-3	1.8	0.4	0.4	0.7	2.2	1.5	1.1	1.6	1.1	1.1	1.4	0.9
20-4	17.0	6.1	4.0	9.2	5.0	3.1	20.0	26.3	26.4	14.2	17.2	9.8
20-5	—	—	1.1	—	—	—	—	0.3	—	—	—	1.0
22-5	0.9	—	0.3	0.6	2.0	—	2.0	0.6	0.6	—	0.1	—
22-6	4.3	—	0.7	2.3	2.3	0.4	8.6	5.3	7.0	1.8	3.2	2.0
24-0	—	—	—	—	—	—	—	—	—	—	—	1.3
24-0? uns.	—	—	—	—	—	—	0.2	—	—	—	—	—

* Indicates tentative identification.

Table 7. *Analysis of lipids from rat-liver fluffy layer*

The amounts are μ equiv./g. dry wt. of fluffy layer. Results are those of Expt. 2.

Nominal lipid class	Acyl ester	Lipid P	Amino N	Free acids	Acyl ester:lipid P ratio	Amino N:lipid P ratio
Sterol esters	4.9	—	—	—	—	—
Triglycerides	23.6	0.3	—	3.5	—	—
Diglycerides	8.7	0.1	—	—	—	—
Cardiolipin	15.3	7.9	0.5	—	1.9	0.07
Aminophosphatides	140	71.6	67.6	—	2.0	0.94
Inositides	40.3	21.1	12.6	—	1.9	0.60
Lecithin	180	90.6	2.3	—	2.0	0.03
Polar lipids	10.7	14.2	2.6	—	0.75	0.18

between our results and those of Macfarlane *et al.* (1960) could be due to the inclusion of cod-liver oil supplements in the diet of their rats. Rhodes (1958) showed that the inclusion of cod-liver oil in the diet altered the fatty acid composition of the phospholipid of hen's egg.

The technique of centrifuging used in the isolation of the cell fractions will influence their composition. This will be particularly so in determining the degree of contamination of mitochondria by fluffy layer and microsomes. Under any conditions of centrifuging the complete sedimentation of the mitochondria is accompanied by sedimentation of about 10% of the microsomes and a variable quantity of fluffy layer. Resuspension and centrifuging of the crude mitochondrial pellet reduces the microsomal content of the mitochondrial pellet to 1% and a repetition of the procedure reduces the amount of microsomes to about 0.1%. This double-washing procedure was used for all the mitochondrial preparations described in this paper.

The fluffy layer was poured from the surface of the mitochondrial pellet completely and a small portion of the least firmly packed mitochondria was allowed to follow. Thus the mitochondrial pellet is relatively uncontaminated with fluffy layer or microsomes but the composition of the fluffy layer will be somewhat variable. It would appear from a study of the centrifuging procedure used by Macfarlane *et al.* (1960) that at least some of the fluffy layer was included in their mitochondrial pellet, and since this pellet was unwashed some microsomes must also have been present. This difference in centrifuging procedure used by Macfarlane *et al.* (1960) could account for their higher ratios (compared with ours) for mitochondrial dry wt.:microsomal dry wt. and for lecithin:kephalin in the mitochondrial fraction.

The ratio lecithin:kephalin is always higher in the isolated microsomal fraction than in the isolated mitochondria. A survey of published work giving the relative proportions of lecithin and kephalin in mitochondria and microsomes is given in Table 11. This suggests that the relative distri-

bution of lecithin and kephalin between mitochondria and microsomes is common to many different tissues.

Special characteristics of mitochondrial lipids. Mitochondria contain most of the cell cardiolipin and a large proportion of the cell linoleic acid. The phosphatidylethanolamine of mitochondria also contains a higher proportion of linoleic acid than other cell fractions together with a higher concentration of arachidonic acid and of docosahexaenoic acid than is found in other lipids. Although rat-liver mitochondria contain large amounts of linoleic acid, those from other species or organs may contain only small quantities. For example, Tappel & Richardson (1961) showed that fish-liver mitochondria were low in linoleic and arachidonic acids but had large amounts of docosapentaenoic acid and docosahexaenoic acid. Also rat-brain mitochondria (Biran & Bartley, 1961) have a low content of linoleic acid. In rat-liver mitochondria most of the linoleic acid is associated with a polyglycerolphosphatide (cardiolipin). Many other species have been shown to contain polyglycerolphosphatide, particularly in the mitochondrial fraction (Strickland & Benson, 1960) and in other organelles (e.g. the chromatophores of *Rhodospirillum rubrum*) concerned in electron transport (Benson & Strickland, 1960). Although linoleic acid constitutes some 80% of the fatty acids of the rat-liver cardiolipin it seems that other fatty acids may take the place of linoleic acid in cardiolipins from fish liver or plant sources. This is supported by the findings of Strickland & Benson (1960) and of Renkonen & Renkonen (1959) who have separated polyglycerolphosphatide into different fractions. The cardiolipin fraction of brain mitochondria (Biran & Bartley, 1961) has a different fatty acid composition (with only 10.3% of linoleic acid) from that of rat liver. Although this concentration is low, it is still 5–10 times higher than in any other brain phospholipid.

The connexion between cardiolipin and electron-transport mechanisms is supported by the finding that cytochrome oxidase, cytochrome *b* and

Table 8. *Distribution of fatty acids in lipids from rat-liver fluffy layer*

Results are the mean of two experiments except for free fatty acids. Cardiolipin is given separately for each experiment. In Expt. 1 it was eluted from the silicic acid column with a chloroform-methanol (7:1, v/v) mixture; in Expt. 2 the eluting solvent was chloroform-methanol (9:1, v/v). The values are percentages of the total area of the chromatogram peaks that was occupied by each individual peak. br., Branched-chain; hbr., highly branched-chain; uns., unsaturated.

Fatty acid. No. of C atoms and double bonds	Total fatty acid esters	Sterol esters	Trigly- cerides	Free fatty acids	Digly- cerides	Cardio- lipin [Expt. 1]	Cardio- lipin [Expt. 2]	Amino phosphatides	Inositide- rich fraction	Lecithin	Polar lipids
8-0	—	—	0.1	—	—	—	—	—	—	—	—
9-0	0.1	—	0.2	—	0.1	—	—	—	—	—	—
10-0	—	—	—	—	0.1	—	—	—	—	—	—
11-0	0.1	—	0.3	—	—	—	0.3	0.1	—	—	—
*12-0 br.	—	—	0.2	—	—	—	0.2	0.1	—	—	—
12-0	—	—	0.1	—	—	—	—	—	0.1	—	0.2
13-0	—	—	—	—	0.1	—	—	—	—	—	0.1
*14-0 br.	—	0.2	0.1	—	0.1	—	—	—	—	—	0.2
14-0	0.4	0.5	1.1	0.8	0.8	0.3	0.6	0.2	—	0.5	0.4
14-1	—	0.2	—	—	—	—	—	—	—	—	0.1
*15-0 br.	—	0.4	0.1	—	0.1	—	—	0.1	—	—	—
15-0	0.5	0.3	0.6	0.6	0.5	0.2	0.2	0.3	0.2	0.6	0.3
16-0 br.	—	1.5	0.5	0.1	0.6	—	0.4	0.4	0.1	0.2	0.5
16-0	20.4	20.3	25.6	25.9	22.5	12.8	5.1	21.8	17.8	27.7	24.6
16-1	1.2	5.6	2.6	2.0	1.5	0.8	2.7	0.9	0.6	1.5	0.9
*17-0 hbr.	—	0.3	0.1	—	—	—	—	—	—	—	—
17-0 br.	0.2	0.5	0.8	0.4	0.3	0.3	0.7	—	0.4	0.4	—
17-0	0.7	0.3	0.5	0.6	0.2	0.6	0.2	0.7	0.9	0.8	0.8
*18-0 hbr.	—	0.4	0.3	—	—	—	—	0.2	0.1	0.3	—
18-0 br.	—	1.5	0.6	0.1	—	0.3	—	0.4	0.2	—	0.4
18-0	18.5	14.5	5.4	13.3	9.3	19.1	1.9	23.2	33.3	16.7	24.4
18-1	13.4	23.8	26.9	23.4	16.7	10.8	13.8	10.1	7.9	12.6	8.2
18-2	17.2	16.8	24.8	18.3	19.7	25.8	68.5	15.4	10.8	21.1	10.1
18-3	—	—	0.3	—	0.3	—	0.3	—	—	—	—
*19-0 br.	0.3	—	0.2	0.2	0.2	0.3	—	0.3	0.1	0.2	—
20-0	—	—	0.1	—	0.2	—	—	0.1	—	—	1.0
20-1	0.1	—	0.5	0.6	0.2	—	—	0.2	0.5	0.3	0.3
*20-2	—	—	0.2	—	—	—	—	—	—	—	—
20-2	0.3	0.3	0.3	0.4	0.3	0.5	0.9	0.1	0.5	0.2	—
20-3	1.1	1.5	0.2	0.9	0.4	0.8	1.5	0.9	1.3	0.9	—
20-4	16.2	8.6	3.5	8.1	6.8	16.8	1.6	18.0	22.2	13.8	11.3
20-5	0.2	—	1.2	—	0.6	—	—	0.5	—	0.4	2.3
*21-?	—	0.7	—	—	—	—	—	—	—	—	—
22-0	—	—	—	—	1.3	—	—	—	—	—	—
*22-2	—	—	—	—	1.6	—	—	—	—	—	0.7
*22-3	—	—	0.1	—	1.8	—	—	—	—	—	1.6
*22-4	—	—	—	—	—	—	—	—	—	—	4.9
22-5	—	—	—	—	—	—	—	—	—	—	0.6
22-6	0.9	—	3.0	1.5	—	1.3	—	0.9	0.5	0.4	2.2
*23-2	4.9	1.1	1.3	2.7	4.8	9.5	0.9	5.7	2.9	2.1	—
24-0	—	—	—	—	3.9	—	—	—	—	0.2	—
*24-1	—	—	—	—	1.1	—	—	—	—	—	—
*24-uns.	—	—	—	—	1.4	—	—	—	—	—	4.7

* Indicates tentative identification.

Table 9. *Comparison of the lipid composition of mitochondria, microsomes and fluffy layer with that of the whole liver*

The values are given as equivalents of acyl glyceryl ester or g.atoms of P/100 g.atoms of phospholipid P calculated from the phospholipid P recovered from the silicic acid chromatograms. Phosphatidylserine and phosphatidylethanolamine are calculated from the total amino N of the lipids extracted from each cell fraction. Lecithin, polyglycerolphosphatides and inositolphosphatide were calculated similarly from the differences between the lipid P content and the amino N content in the respective fractions eluted from the silicic acid column, e.g. inositolphosphatide = lipid P - amino N in kephalin fractions. The mean values from both experiments are given.

Component	Liver pulp	Mitochondria	Fluffy layer	Microsomes
Acyl glyceryl ester	218	237	211	200
Phosphatidylserine and phosphatidylethanolamine	30.9	42.0	34.5	27.0
Lecithin	54.9	38.6	47.3	62.6
Polyglycerolphosphatides	2.3	8.4	4.5	0.8
Inositolphosphatides	9.1	9.9	9.9	9.3
Polar lipids	3.6	2.1	4.7	3.3
Lysophosphatides	23.7	3.0	26.9	23.7

Table 10. *Distribution and enrichment factor of lipids in isolated cell fractions from rat liver*

Calculations of quantities of lipids are as described in Table 9. The results are the mean of the two experiments except where indicated by *. The values for 'Lipid (%)' are the percentages of each lipid recovered in the cell fraction.

$$\text{Enrichment factor} = \frac{\mu\text{moles of lipid/g. dry wt. of cell fraction}}{\mu\text{moles of lipid/g. dry wt. of whole liver}}$$

Nominal lipid class	Mitochondria		Fluffy layer		Microsomes	
	Lipid (%)	Enrichment factor	Lipid (%)	Enrichment factor	Lipid (%)	Enrichment factor
Sterol esters	3.4	0.41	3.6	0.58	35.3	1.5
Neutral glycerides	5.6	0.62	8.7	1.5	26.7	1.1
Cardiolipin	33.2	3.5	11.5	2.9	12.8	0.53
Aminophosphatides	12.4	1.5	10.5	1.3	40.9	1.6
Inositol-rich phosphatide	9.9	1.1	10.1	1.6	29.0	1.2
Lecithin	7.5	0.67	7.8	1.3	58.9	2.1
Polar phospholipid	12.1*	1.6*	9.8	1.8	35.2	1.8
Total phospholipids	8.8	0.96	9.3	1.5	44.3	1.8

Table 11. *Comparison of lecithin and kephalin in mitochondria and microsomes from different tissues*

The values are percentages of the lipid P of each cell fraction that was found as lecithin or kephalin.

Reference	Animal and tissue	Mitochondria			Microsomes		
		Lecithin	Kephalin	Lecithin: kephalin ratio	Lecithin	Kephalin	Lecithin: kephalin ratio
*Levine & Chargaff (1952)	Rat liver	—	—	1.05	—	—	1.37
†Spiro & McKibbin (1956)	Rat liver	49.0	29.0	1.69	58.0	27.0	2.15
		37.0	37.0	1.00	57.0	26.0	2.20
Strickland & Benson (1960)	Rat liver	49.0	30.0	1.63	69.0	15.0	4.53
Macfarlane, Gray & Wheeldon (1960)	Rat liver	51.0	31.0	1.65	62.0	26.0	2.38
Collins & Shotlander (1961)	Rat liver	31.0	20.0	1.55	39.0	12.0	3.25
This paper	Rat liver	39.0	42.1	0.93	64.7	27.1	2.33
Petersen & Schou (1955)	Rat brain	17.8	68.3	0.26	26.2	64.6	0.41
Biran & Bartley (1961)	Rat brain	32.1	50.0	0.64	38.9	41.4	0.94
Marinetti, Erbland & Stotz (1958)	Pig heart	36.2	28.1	1.29	41.8	24.8	1.69

* Isolated from 0.88M-sucrose; all other fractions were isolated from 0.25M-sucrose.

† In these experiments the rats were given choline as a control group for choline-deficiency experiments; the first values were obtained from rats on the diet for 2 weeks and the second values from rats on the diet for 4-6 weeks.

cytochrome *c* preparations from pig heart (Marinetti, Erbland, Kochen & Stotz, 1958) contain a larger proportion of cardiolipin than the parent mitochondria. Cardiolipin is also concentrated in the cytochrome oxidase isolated from ox-heart mitochondria by Fleischer, Brierley & Klouwen (1961).

Special characteristics of microsomal lipids. Most of the lipid of the cell is associated with the microsomal fraction. Of the phospholipids, cardiolipin is present in only small quantities but more than half the cells' complement of lecithin is found in the microsomes.

The microsomal fraction actively incorporates amino acids into its protein and suggestions have been made that the lipid portion of the microsomes is concerned in this process (Hendler, 1958, 1959; Westley, Wren & Mitchell, 1957; Gaby, Hadley & Kaminski, 1957; Haining, Fukui & Axelrod, 1960; Gaby, Wolin & Zajac, 1960; Wallach, Soderberg & Bricker, 1960; Gaby, Naughten & Logan, 1959), involving lipid-bound amino acids.

Hokin & Hokin (1956) and Redman & Hokin (1959) believe that phospholipids are concerned in transport mechanisms across membranes. Phosphoinositides and phosphatidic acids may be concerned in these mechanisms which have been demonstrated in the isolated microsomal fraction of pancreas.

Lipid composition of the fluffy layer. The lipid analyses of the fluffy layer are consistent with it being a mixture of mitochondria and microsomes. This has been suggested by Novikoff, Podber, Ryan & Noe (1953) and by Miller, Bagot & Greenberg (1955), but in contrast Laird, Nygaard, Ris & Barton (1953) believe that the fluffy layer contains a distinct kind of mitochondria. Jackson, Walker & Pace (1953) believe that the fluffy layer belongs to the microsomal fraction. The lecithin:kephalin ratio (Table 10) resembles that in the mitochondria rather than that in the microsomes, and the enrichment of cardiolipin (Table 10) is similar to that of the mitochondria. The enrichment of phosphatidylethanolamine is like that of the microsomes but the enrichment in lecithin is intermediate between those of the two fractions (Table 10).

SUMMARY

1. The lipids of rat-liver mitochondria, microsomes and fluffy layer have been separated by silicic acid chromatography and the fatty acid composition of each fraction has been measured.

2. On a weight basis 26% of the mitochondria was lipid, whereas the fluffy layer contained 33% and the microsomes 55%.

3. Lecithin and kephalin were present in the mitochondria in almost equal amounts (each about

38% of the total phospholipid), whereas there was twice as much lecithin as kephalin in the microsomes (lecithin 60% of total phospholipid, kephalin about 30%). The fluffy layer had quantities of lecithin and kephalin intermediate between those of the mitochondria and the microsomes.

4. Cardiolipin was concentrated in the mitochondria to some 3.5 times that found in the whole cell.

5. Kephals had a higher proportion of arachidonic acid and docosahexaenoic acid than had other lipids. Thus, as the proportion of kephals to lecithins in the mitochondria is higher than that in the microsomes, the mitochondria contain a higher proportion of these polyunsaturated fatty acids.

6. The proportions of fatty acids in the lipid classes of mitochondria, fluffy layer and microsomes are presented.

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Biochem. J. (1962) **83**, 191

An Enzymic Method for the Assay of Pyridine Nucleotides in Extracts of Animal Tissues

By CLAUDE A. VILLEE

Department of Biological Chemistry, Harvard Medical School, and the Research Laboratories of the Boston Lying-in Hospital, Boston, Mass., U.S.A.

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An enzymic assay of oxidized and reduced di- and tri-phosphopyridine nucleotides was described by Glock & McLean (1955). In their assay system the continuous reduction of DPN⁺ in the presence of an excess of ethanol and alcohol dehydrogenase was coupled with DPNH-cytochrome *c* reductase prepared from pig heart. In a similar fashion the continuous reduction of TPN⁺ in the presence of glucose 6-phosphate and glucose 6-phosphate dehydrogenase was coupled with TPNH-cytochrome *c* reductase prepared from brewer's yeast. The reduction of cytochrome *c* was followed spectrophotometrically at 550 m μ . The present report describes a modification of this assay.

Diaphorase and dichlorophenol-indophenol are used in place of the cytochrome *c* reductases and cytochrome *c*. This permits the assays to be carried out with enzyme preparations that are commercially available. The dehydrogenases are absolutely specific for their respective pyridine nucleotides; hence the additional specificity of the cytochrome *c*

reductases can be dispensed with. A preliminary account of this work has appeared previously (Vilee, 1961).

MATERIALS AND METHODS

Samples (about 200 mg.) of tissue are blotted free of blood and weighed rapidly on a torsion microbalance. Glass Potter-Elvehjem tissue homogenizers containing 5 ml. of 0.1N-HCl or 0.1N-NaOH are placed in a boiling-water bath. The tissues are added and 30 sec. later the homogenizers are removed from the boiling water. The tissues are homogenized for 90 sec. and then transferred to an ice bath. 0.2M-Tris buffer, pH 7.4 (1 ml.) is added to the content of each homogenizer and the solutions are neutralized by the dropwise addition, with constant stirring, of dilute acid or base. The neutralized extracts are then centrifuged at 18 800*g* at 0° for 30 min. The oxidized coenzymes are relatively stable in dilute alkali and the reduced coenzymes are relatively stable in dilute acid (Schlenk, 1951; Glock & McLean, 1955). Thus the acid extract contains DPNH and TPNH, whereas the alkaline extract contains DPN⁺ and TPN⁺. The usual volume of extract required for