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Molecular Species of Phosphatidylcholine and Phosphatidylethanolamine in Subcellular Membranes from Rat Liver

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Four subfractions of phosphatidylcholine and phosphatidylethanolamine according to the degree of unsaturation of their fatty acids have been separated from lipid extracts of microsomes, and inner and outer mitochondrial membranes. The predominant species found in the three membranes contained one saturated and one unsaturated fatty acid. In microsomes completely saturated species of both phosphatidylcholine and phosphatideylethanolamine were practically nonexistent. In outer mitochondrial membranes species with two unsaturated fatty acids were absent. In the inner mitochondrial membranes, however, disaturated species and those with two unsaturated fatty acids were found.

The separation of different subfractions from each phospholipid type according to the degree of unsaturation of its fatty acids has been made possible after the introduction of thin layer chromatography with silica gel plates impregnated with silver nitrate (1).

The proportion of each subfraction for the different phospholipids has been determined in total rat liver (1-3, 8, 10, 12, 13, 17, 31). The results so far reported support the view that the proportion of fatty acids in each phospholipid type is more or less constant for a tissue regardless the membrane from which they have been extracted (7). It was thought to be of interest to know whether this constancy is also reflected in the proportions of the different molecular species within each particular phospholipid extracted from different cell membranes obtained from rat liver. Thus we have studied the distribution of molecular species of phosphatidylcholine and phosphatidylethanolamine according to their different degree of unsaturation in microsomes, and in inner and outer mitochondrial membranes. The results obtained show that in microsomes the completely saturated species were practically non-

existent. In outer mitochondrial membranes species with two unsaturated fatty acids were absent. However, both disaturated and diunsaturated species were found in inner mitochondrial membranes. In the three membranes studied the predominant species were those containing one saturated and one unsaturated fatty acids.

Materials and Methods

Male Wistar rats weighing approximately 200 g were used in all the experiments. Livers were homogenized in 0.25 M sucrose and mitochondria isolated according to the method if HOGEBOOM (9). Microsomes were isolated after removing mitochondria by centrifugation the mitochondrial supernatant at 105,000 × *g* for 60 minutes at 0° C. Isolated mitochondria were subjected to osmotic rupture following the method of PARSONS *et al.* (22). Inner mitochondrial membranes were obtained using Parsons «low speed pellet» as starting material. In order to remove the outer membranes still present in this fraction, it was throughly washed three times by resuspending it in 0.02 M phosphate buffer, pH 7.4, by centrifuging at 1,900 × *g* for 15 minutes and once more resuspending it in 0.25 M sucrose and centrifuging at 8,500 × *g* for minutes (26).

Outer mitochondrial membranes were prepared as described by PARSONS *et al.* (22).

Proteins were determined by the method of LOWRY *et al.* (15).

Lipids were extracted from the sediment obtained after precipitating the proteins with the addition of enough 70 % $HClO_4$ to give a final concentration of 0.3 N, as previously described (24). Phospholipids were separated by thin layer chromatography according to the technique of NESKOVIC *et al.* (19). Lipid P was determined by the method of BARTLETT (5).

Methyl esters of the fatty acids present in each phospholipid were prepared by direct transmethylation as described by MORRISON and SMITH (18), and analyzed in a Beckman Gas Chromatograph, Model GC-4, fitted with two 6 ft columns, 1/8 inch in diameter; solid phase was chromosorb W, particle size 42/60 μ, and liquid phase 20 % DEGS; hydrogen and air flow were respectively 55 cc/min and 250 cc/min; column temperature 160° C and that of the detectors, 280° C.

Among the different procedures described in the literature (3, 14, 20, 23, 31) for the separation of molecular species of phosphatidylcholine and phosphatidylethanolamine the method of ARVIDSON (3) was followed in spite of its more limited resolution. This latter method was found to be advantageous in our studies since much shorter times were needed, preventing thus the destruction of the species more highly unsaturated. On the other hand, its more limited resolution can be obviated by an easy calculation after knowing the composition in fatty acids of each group of species.

The molecular species of each phospholipid type were separated according to the technique of ARVIDSON (3) with the following modifications: Silica gel G (Merck) was used instead of silica gel H (Merck). The plates were prepared by mixing 22.5 g or silica gel in 50 ml of an aqueous solution of 6 % $AgNO_3$ (w/v). Chromatography was performed at 4° C.

To protect lipids from peroxidation, ter-buthylhydroxytoluene was added to the lipid extracts to give an approximate concentration of 5×10^{-5} M.

The purity of each subcellular fraction was followed by enzyme markers; the cross contamination of microsomal and mitochondrial fractions was established after determining activities of cytochrome oxidase (28) and glucose-6-phosphatase (25); cross contamination of inner and outer mitochondrial membranes was established after determining cytochrome oxidase (28) and monoamine oxidase

(32). In no cases the contamination was greater than 3 %.

Results and Discussion

Four subfractions with different degree of unsaturation corresponding to phosphatidylcholine and phosphatidylethanolamine were obtained from different cell membranes from rat liver through the use of thin layer chromatography with silica gel impregnated with silver nitrate. In each subfraction lipid P and the distribution of each fatty acid determined. Table I shows the percent of lipid P as well as their fatty acid distribution in the four subfractions of phosphatidylcholine and phosphatidylethanolamine obtained from the microsomal fraction of rat liver. The values corresponding to whole mitochondria, outer and inner mitochondrial membranes have been collected in tables II, III and IV respectively.

The amount of PC, PE expressed in μg of P/mg protein was a follows: in

Table I. *Fatty acids distribution in the different subfractions of PC and PE from rat liver microsomes.*

PC_t and PE_t represent respectively total phosphatidylcholine and phosphatidylethanolamine. PC_6, PC_4, PC_2, PC_1 and PE_6, PE_4, PE_2, PE_1, indicate respectively the hexaenoic, tetraenoic, dienoic and monoenoic subraction of phosphatidylcholine and phosphatidylethanolamine separated by thin layer chromatography. The fatty acid distribution in each subfraction has been expressed as mole percent. Lipid P in PC_t represented 54.6 %, and 22.3 % in PE_t in the corresponding total lipid extract from microsomes. Number of experiments, 10. Mean values ± S.E. Materials and Methods for experimental details.

Fatty acids	PC subfractions					
	Lipid P (%) / PC_t 100	PC_6 13.3 ± 0.4	PC_4 48.5 ± 0.3	PC_2 25.6 ± 0.6	PC_1 12.6 ± 0.4	Recovery
16:0	26.00±0.94	27.70	18.68	32.30	35.80	25.50
16:1	0.75±0.35	—	0.14	1.38	2.64	0.74
18:0	20.07±0.87	21.16	24.20	14.60	17.80	20.51
18:1	7.02±0.68	3.95	2.50	3.34	32.81	6.58
18:2	15.00±0.59	1.57	1.27	48.35	10.92	14.55
20:4	26.10±0.81	6.19	53.18	—	—	26.61
22:6	5.03±0.44	39.40	—	—	—	5.24
Sat. Unsat.	0.85	0.95	0.75	0.88	1.15	

Fatty acids	PE subfractions					
	Lipid P (%) / PE_t 100	PE_6 25.0 ± 0.4	PE_4 45.0 ± 0.5	PE_2 23.0 ± 0.5	PE_1 7.0 ± 0.6	Recovery
16:0	21.90±1.04	28.80	12.41	29.39	30.23	21.64
16:1	0.83±0.27	—	0.45	1.90	3.20	0.85
18:0	24.77±0.99	19.29	31.25	18.85	20.80	24.66
18:1	5.10±0.75	3.79	2.79	3.25	34.81	5.36
18:2	12.81±0.59	3.80	2.11	46.58	10.93	13.36
20:4	24.33±0.98	4.49	50.96	—	—	24.05
22:6	10.23±0.64	39.80	—	—	—	9.95
Sat. Unsat.	0.87	0.92	0.77	0.93	1.04	

Table II. *Fatty acids distribution in the different subfractions of PC and PE from rat liver mitochondrias.*

PC_t and PE_t represent respectively total phosphatidylcholine and phosphatidylethanolamine. PC_6, PC_4, PC_2, PC_1 and PE_6, PE_4, PE_2, PE_1, indicate respectively the hexaenoic, tetraenoic, dienoic and monoenoic subfrantion of phosphatidylcholine and phosphatidylethanolamine separated by thin layer chromatography. The fatty acid distribution in each subfraction has been expressed as mole percent. Lipid P in PC_t represented 38.2 %, and 35.5 % in PE_t in the corresponding total lipid extract from mitochondrias. Number of experiment, 10. Mean values ± S.E. See Materials and Methods for experimental details.

	PC subfractions					
Fatty acids	Lipid P (%) PE_t 100	PC_6 7.5 ± 0.5	PC_4 51.0 ± 0.4	PC_2 31.0 ± 0.7	PC_1 10.5 ± 0.4	Recovery
16:0	25.89±0.73	27.48	18.37	31.62	37.21	25.12
16:1	1.05±0.34	—	0.15	1.50	3.00	0.84
18:0	23.04±0.85	21.40	27.40	17.00	20.78	23.02
18:1	5.56±0.46	1.34	1.10	5.85	30.74	5.69
18:2	17.58±0.98	2.66	6.45	44.00	8.24	17.97
20:4	23.85±1.03	6.24	46.50	—	—	24.17
22:6	3.00±0.82	40.85	—	—	—	3.06
Sat. Unsat.	0.95	0.95	0.84	0.94	1.38	
	PE subfractions					
Fatty acids	Lipid P (%) PC_t 100	PE_6 7.0 ± 0.6	PE_4 62.0 ± 0.5	PE_2 18.0 ± 0.3	PE_1 13.0 ± 0.7	Recovery
16:0	22.41±0.96	26.15	14.16	28.24	49.15	21.98
16:1	0.69±0.30	—	—	1.25	2.45	0.54
18:0	27.38±0.84	22.90	30.96	19.12	21.18	26.98
18:1	7.63±0.66	1.00	4.79	8.23	27.19	8.04
18:2	10.20±0.71	1.12	5.11	43.13	—	10.99
20:4	28.17±1.12	3.30	44.95	—	—	28.09
22:6	3.49±0.79	45.50	—	—	—	3.18
Sat. Unsat.	0.99	0.96	0.82	0.90	2.73	

microsomes 10.8 and 4.0; in mitochondria 1.9 and 1.8; in inner membranes 2.4 and 2.0; in outer membranes 7.2 and 5.7 respectively.

It can be observed that in each one of these subfractions of phosphatidylcholine and phosphatidylethanolamine of the different membranes an unsaturated fatty acid predominates: docosahexaenoic for hexaenoic subfraction, arachidonic for tretraenoic subfraction, linoleic for dienoic subfraction, and oleic for monoenoic subfraction. Stearic was the predominant fatty acid in the tetraenoic subfraction, and palmitic acid in the other three subfractions. The preferential combination of stearic and arachidonic acid agrees with the findings already reported by other authors (3, 31) in total rat liver.

For all the membranes studied the tetraenoic subfraction was the predominant one for both phosphatidylcholine and phosphatidylethanolamine.

It may be observed that in the different membranes studied the ratio of saturated to unsaturated fatty acid was very close to

one for all the subfractions of each phospholipid with the exception of the monoenoic subfraction. This would indicate that the most abundant molecular species would be those constituted by a saturated and an unsaturated fatty acid. This is in agreement with previous studies on the composition of molecular species of phosphatidylcholine and phosphatidylethanolamine in total rat liver (2, 3). In microsomes and inner mitochondrial membranes some of the molecular species should necessarily contain two unsaturated fatty acids; however, in outer mitochondrial membranes some of the molecular species should contain two saturated fatty acids. The existence of molecular species with two saturated fatty acids cannot be excluded in microsomes and in inner mitochondrial membranes, nor the existence of molecules with two unsaturated fatty acids in the outer mitochondrial membranes if the data of the composition in fatty acids of these two phospholipids is taken into consideration. Since molecules with two saturated fatty acids migrate in thin layer chro-

Table III. *Fatty acids distribution in the different subfractions of PC and PE from rat liver outer membranes.*

PC_t and PE_t represent respectively total phosphatidylcholine and phosphatidylethanolamine. PC_6, PC_4, PC_2, PC_1 and PE_6, PE_4, PE_2, PE_1, indicate respectively the hexaenoic, tetraenoic, dienoic and monoenoic subfraction of phosphatidylcholine and phosphatidylethanolamine separated by thin layer chromatography. The fatty acid distribution in each subfraction has been expressed as mole percent. Lipid P in PC_t represented 40.4 %, and 31.6 % in PE_t in the corresponding total lipid extract from mitochondria. Number of experiment, 10. Mean values ± S.E. See Materials and Methods for experimental details.

Fatty acids	PC subfractions					
	Lipid P (%) PC_t 100	PC_6 4.0 ± 0.3	PC_4 48.0 ± 0.5	PC_2 31.0 ± 0.8	PC_1 17.0 ± 0.3	Recovery
16:0	31.35±1.04	27.30	23.45	34.82	46.12	30.91
16:1	0.85±0.38	—	—	1.50	2.36	0.86
18:0	23.42±0.84	22.36	29.10	15.10	20.89	23.08
18:1	5.95±0.59	1.78	1.31	2.34	28.34	6.22
18:2	15.27±0.69	2.73	1.39	46.21	2.26	15.46
20:4	21.16±0.96	2.94	44.72	—	—	21.57
20:6	1.97±0.41	42.86	—	—	—	1.71
Sat. Unsat.	1.21	0.98	1.10	0.99	2.03	

Fatty acids	PE subfractions					
	Lipid P (%) PE_t 100	PE_6 5.0 ± 0.5	PE_4 56.0 ± 0.5	PE_2 20 ± 0.7	PE_1 19.0 ± 0.4	Recovery
16:0	24.16±0.95	29.21	18.19	28.72	37.39	24.48
16:1	0.86±0.37	—	—	1.13	3.46	0.87
18:0	30.27±1.05	21.10	35.06	21.22	24.70	29.61
18:1	7.62±0.74	1.17	2.17	2.27	32.27	7.84
18:2	10.07±0.61	1.30	1.37	46.63	2.15	10.54
20:4	24.35±0.97	2.26	43.18	—	—	24.29
22:6	2.64±0.48	44.93	—	—	—	2.24
Sat. Unsat.	1.19	1.01	1.13	0.99	1.63	

Table IV. *Fatty acids distribution in the different subfractions of PC and PE from rat liver inner membranes.*

PC_t and PE_t represent respectively total phosphatidylcholine and phosphatidylethanolamine. PC_6, PC_4, PC_2, PC_1 and PE_6, PE_4, PE_2, PE_1, indicate respectively the hexaenoic, tetraenoic, dienoic and monoenoic subfraction of phosphatidylcholine and phosphatidylethanolamine separated by thin layer chromatography. The fatty acid distribution in each subfraction has been expressed as mole percent. Lipid P in PC_t represented 38.0 %, and 36.8 % in PE_t in the corresponding total lipid extract from mitochondrias. Number of experiment, 10. Mean values ± S.E. See Materials and Methods for experimental details.

	PC subfractions					
Fatty acids	Lipid P (%)	PC_t 100	PC_6 7.4 ± 0.7	PC_4 51.0 ±0.8	PC_2 25.2 ± 0.4	PC_1 16.4 ± 0.3	Recovery
16:0	25.55±0.91		57.52	19.60	31.57	38.63	26.24
16:1	1.25±0.44		—	0.25	1.65	2.15	0.87
18:0	22.35±0.81		20.90	26.35	16.13	19.33	22.11
18:1	8.60±0.53		1.85	2.67	6.77	32.48	8.37
18:2	14.74±0.68		2.21	4.50	43.85	7.38	14.58
20:4	24.28±0.99		5.85	46.60	—	—	24.17
22:6	3.20±0.57		41.64	—	—	—	2.91
Sat Unsat.	0.92		0.94	0.85	0.91	1.37	
	PE subfractions					
Fatty acids	Lipid P (%)	PE_t 100	PE_6 8.0 ± 0.4	PE_4 65.3 ± 0.8	PE_2 15.7 ± 0.3	PE_1 11.0 ± 0.8	Recovery
16:0	20.15±0.86		27.23	13.85	27.32	45.35	20.50
16:1	0.56±0.24		—	—	1.13	3.20	0.53
18:0	28.54±0.93		22.45	31.34	20.47	20.31	27.71
18:1	8.05±0.67		1.10	5.65	8.43	28.19	8.20
18:2	9.65±0.56		1.13	4.28	42.62	2.92	9.89
20:4	29.37±0.89		3.20	44.85	—	—	29.55
22:6	3.65±0.63		44.86	—	—	—	3.59
Sat. Unsat.	0.94		0.99	0.82	0.92	1.91	

matography together with the monoenoic subfraction, the proportion of the different subfractions of each phospholipid and the ratio of saturated to unsaturated fatty acids within each subfraction allows a calculation of the distribution of molecular species with two saturated fatty acids, with one saturated and one unsaturated, with two unsaturated fatty acids (table V).

The results here presented point out that in spite of the similarity in distribution of fatty acids in phosphatidylcholine and phosphatidylethanolamine a certain selectivity exists regarding the distribution of molceular species of each of these phospholipid according to the membrane from which they have been obtained. It seems reasonable to admit that all the molecular species of phosphatidylcholine and phosphaditylethanolamine of microsomes and mitochondrial membranes should be very similar since both phospholipids are synthesized in the endoplasmic reticulum (16, 27, 30) and transferred to outer mitochondrial membranes through protein

Table V. *Calculated percent distribution of molecular species of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in microsomes, and outer and inner mitochondrial membranes from rat liver.*

	Microsomes		Inner membrane		Outer membrane	
Fatty acids	PC	PE	PC	PE	PC	PE
Sat.-Sat.	0.9	0	2.5	3.4	5.7	4.5
Sat.-Unsat.	90.2	92.6	92.1	89.8	94.3	95.5
Unsat.-Unsat.	8.6	7.4	5.4	6.8	0	0

factors (4, 11, 16, 33-35, 37). The outer mitochondrial membrane participates in the exchange of phosphatidylcholine and phosphatidylethanolamine between the endoplasmic reticulum and the inner mitochondrial membrane (6, 16, 36). Cycles of deacylation and reacylation (29) could be responsible for the small differences in composition of the molecular species of phosphatidylcholine and phosphatidylethanolamine in microsomes and mitochondrial membranes. These small differences are in agreement with the observation that deacylation and reacylation cycles are of minor significance in mitochondria when compared with the bulk of phospholipids transferred from the endoplasmic reticulum to the mitochondria (21).

The disagreement of some of our results referring to the composition of phosphatidylcholine in mitochondria and microsomes with those found by PARKES and THOMPSON (20) might be due to the different source of the biological material, or to the use by them of a lengthier procedure.

Resumen

De acuerdo con el grado de insaturación de sus ácidos grasos constituyentes, se han separado cuatro subfracciones de fosfatidilcolina y cuatro de fosfatidiletanolamina obtenidas a partir de extractos lipídicos procedentes de microsomas y membranas internas y externas mitocondriales. Las especies moleculares que contenían un ácido graso saturado y un insaturado fueron las predominantes en las tres membranas estudiadas. Las membranas microsomales prácticamente no contienen especies completamente saturadas de ambos fosfolípidos. En las membranas externas mitocondriales no se han encontrado especies con dos ácidos grasos insaturados; sin embargo, en las membranas internas mitocondriales aparecen tanto especies con dos ácidos grasos insaturados como con dos saturados en los dos fosfolípidos estudiados.

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