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Subfractions Obtained From Inner Mitochondrial Membranes Incubated With Cysteine. I. Fatty Acid Distribution in Phospholipids

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Three subfractions from inner membranes from rat liver mitochondria were separated by differential centrifugation after incubation in the presence of cysteine; these three subfractions were different in what respects to the fatty acid composition of their phospholipids. One of these subfractions (Subfraction I) which sedimented at 8,500 × *g* was further treated with ascorbate and fractionated into a new sediment of 8,500 × *g* and a supernatant. This new lysis would be due to the fact that the peroxidation induced by cysteine on the phospholipids destroyed almost completely the total content of arachidonic acid and affected linoleic acid in a small proportion; on the contrary, ascorbate destroyed almost completely both fatty acids. The data presented are consistent with the hypothesis of a heterogeneous distribution of the different molecular species of phospholipids and that the subfractions obtained would have their origin in areas of the inner membrane with a different degree of unsaturation of their phospholipids.

In previous work (10, 11) from this laboratory it has been found that upon incubation of inner mitochondrial membranes in the presence of cysteine a decrease of the lipid P extractable with organic solvents takes place, in a process caused by the peroxidation of the polyunsaturated fatty acids present in the phospholipids, and binding of the altered phospholipid molecules to the neighbouring proteins. The greatest diminution of this lipid P corresponded to phosphatidiylethanolamine, phosphatidylcholine, and cardiolipin; with respect to the fatty acids the greatest alteration was also found in these phospholipids, with a 70 %, 60 % and 30 % disappearance of their total fatty acids, respectively.

It has been already shown (10) that because of the peroxidation of the unsaturated fatty acids —a process known to cause disruption of biological membranes

(4, 7, 12) — the inner mitochondrial membrane was disaggregated into fragments of different size which could be separated by differential centrifugation in three subfractions very dissimilar in their morphology and lipid to protein ratio: a heavy fraction, constituted by particles that sedimented at 8,500 × *g* and had the greatest amount, about 50 %, of lipid P, and a very high lipid to protein ratio; a light fraction, the sediment of 100,000 × *g*, with a lower percent of phospholipids and a lower lipid to protein ratio; and the supernatant of 100,000 × *g* that presented the lowest amount of unaltered phospholipids and had also a very low phospholipid to protein ratio.

In the present paper we have studied in detail the fatty acid composition of these three submitochondrial fractions from inner membranes incubated with cysteine; the results here reported confirm previous data from similar experiments performed with ascorbate (9), another inducer of lipid peroxidation. These results are consistent with the existence of a heterogeneity of the inner mitochondrial membrane, which would be thus composed of zones with different degrees of unsaturation in their lipids.

Materials and Methods

Rat liver mitochondria were isolated by the method of HOGEBOOM (2) and the inner mitochondrial membranes were prepared by the method of PARSONS *et al.* (8) with the modifications described by SANTIAGO *et al.* (10).

Incubation of the inner mitochondrial membranes was carried out at 30° C during 2 hours, in a medium containing 0.02 M Tris-HCl buffer *p*H 7.4, 8 × 10^{-4} moles cysteine and 0.25 M sucrose. Controls without cysteine were incubated in the same way. After the incubation the suspension of inner mitochondrial membranes was centrifuged at 8,500 × *g* during 10 minutes: the sediment was called Subfraction I. The supernatant was centrifuged at 100,000 × *g* for one hour, and the sediment obtained will be referred to as Subfraction II and the supernatant as Subfraction III.

In other experiments 1 mM ascorbate was used in the incubations of Subfraction I during 1 hour. Two new subfractions were obtained centrifuging at 8,500 × *g*. The sediment will be referred to as Subfraction I A_{sed} and the supernatant as Subfraction I A_{sup}.

Proteins were determined by the method of LOWRY *et al.* (3). Phospholipids were extracter as previously described (9) and separated by thin layer chromatography as described by NESKOVIC *et al.* (6). Methyl esters of the fatty acids of the phospholipids were prepared by the method o MORRISON and SMITH (5) and analyzed by gas chomatography as previously described (9). Methylation of the fatty acids bound to proteins was carried out as described by PÉREZ *et al.* (9).

Results and Discussion

Three subfractions from inner mitochondrial membranes were separated after treatment with cysteine by centrifugation as described in Materials and Methods and the fatty acid distribution of the phospholipids present in each subfraction was studied.

Subfraction I (table I), which amounted to 30 % of the total protein of the inner membrane, contained a 42 % of fatty acids belonging to unaltered phospholipids. CL * was less affected by the peroxidation than PE and PC; and besides, it had in this fraction a remarkable amount of linoleic acid (18:2). The ratio of unsatured fatty acids to saturated plus monounsaturated fatty acids was smaller than that of the controls, because of the

* Abbreviations: CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

Table I. *Disappearance of fatty acids in phospholipids and recovery of fatty acids and lipid P bound to protein in subfraction I.*
The results are referred to the sum (taken as 100) of the areas of the different fatty acids present in each phospholipid in the incubated control. The values of lipid P are expressed in μg and corresponded respectively to 20 mg of protein in the inner membrane incubated control and to 6 mg of recovered protein in Subfraction I. See text for experimental details.

Fatty acids	PE		PC		CL	
	Control	Subfraction I	Control	Subfraction I	Control	Subfraction I
16 : 0	23.0	6.9	30.0	10.2	7	4.5
18 : 0	31.0	8.7	21.5	8.7	8	5.6
18 : 1	8.5	4.0	10.0	5.7	18	14.5
18 : 2	10.0	3.4	13.0	5.7	40	22.4
20 : 4	23.0	5.1	20.0	6.1	18	3.1
Total saturated + monounsaturated	65.1	20.7	64.9	25.5	37	27.4
Total polyunsaturated	35.5	8.9	34.5	12.3	64	28.6
Lipid P, μg	33.0	12.0	40.0	19.0	15	6.0

	Control	Subfraction I
Recovery of fatty acids		
Lipid extract	100	42.5
Bound to protein	0	13.2
Recovery of lipid P		
Lipid extract	100	51.0
Bound to protein	0	20.0

diminution of arachidonic acid (20 : 4). There was also a significant amount of fatty acids bound to protein, a 13 % versus a 42 % in the lipid extract corresponding to non attacked phospholipids; and the recovery of phosphate as lipid P bound to protein was 20 %. This would mean that this phosphate would correspond to molecules of phospholipids with at least one fatty acid not affected by the peroxidation reaction. The bond with the protein would be established through the fatty acids having undergone the peroxidation.

In similar experiments carried out with ascorbate (9), results were obtained which indicated the existence of zones of different lipid composition in the inner mitochondrial membrane: a zone, the most altered, rich in molecular species of phospholipids with two polyunsaturated fatty acids, a second one, constituted mainly by molecular species of phospholipids composed of one polyunsaturated and a saturated or monounsaturated fatty acids. And a thrid zone which presented a great proportion of molecular species of phospholipids with two saturated fatty acid. It is interesting to compare these data with those obtained from inner mitochondrial membranes incubated with ascorbate: Subfraction I from inner mitochondrial membranes incubated with cysteine had a larger amount of proteins, fatty acids from unaltered phospholipids, and fatty acids and lipid P bound to protein, than the sediment of 8,500 × *g* obtained from inner membranes incubated with ascorbate; and as it has been shown (9), this fraction would be originated in a zone of the inner membrane very rich in molecular species of highly saturated phospholipids and a small amount of molecular species of phospholipids with one polyunsaturated fatty acid; Subfraction I from inner mitochondrial membranes incubated with cysteine had a large amount of highly saturated phospholipids; besides, it had also molecular species of phospholipids with one polyunsaturated and

Table II. *Disaggregation of subfraction I re-incubated in the presence of ascorbate.*
Subfraction I was resuspended in a medium containing sucrose 0.25 M, 1 mM ascorbate, Tris-HCl buffer, *p*H 7.4 at 30° C during 1 hour. Protein concentration, 0.8 mg per ml. See text for other experimental details.

	Protein distribution (%)		Decrease in 18:2 %
	Subfraction I A_{sed}	Subfraction I A_{sup}	
Subfraction I Control	48	52	10
Subfraction I + Ascorbate	18	82	85

one saturated or monounsaturated fatty acid which could be recovered bound to protein after the peroxidation. These data indicate that Subfraction I contained parts of the inner mitochondrial membrane that have not been disaggregated by the action of cysteine, but that in the case of inner mitochondrial membranes incubated with ascorbate have been released and are found in the light fraction and in the supernatant.

In another set of experiments Subfraction I from inner mitochondrial membranes treated with cysteine were reincubated with 1 mM ascorbate during 1 hour, and two new subfractions obtained by centrifugation at 8,500 × *g*, a sediment (Subfraction I A_{sed}) and a supernatant (Subfraction I A_{sup}). The results are given in table II and indicate that the structural disaggregation initiated by cysteine was continued by ascorbate and only 18 % of the protein was recovered in the sediment of 8,500 × *g* (Subfraction I A_{sed}). There was also a great decrease in linoleic acid (18:2), mainly from CL. This can be easily explained by the fact already reported (11) that ascorbate produces a much deeper alteration in the phospholipids of the inner mitochondrial membrane than cysteine by causing a greater destruction of unsaturated fatty acids; this difference was especially remarkable in

Table III. *Disappearance of fatty acids in phospholipids and recovery of fatty acids and lipid P bound to protein in subfraction II.*
The results are referred to the sum (taken as 100) of the areas of the different fatty acids present in each phospholipid in the incubated control. The values of lipid P are expressed in μg and corresponded respectively to 20 mg of protein in the inner membrane incubated control and to 1.2 mg of recovered protein in Subfraction II. See text for experimental details.

Fatty acids	PE		PC		CL	
	Control	Subfraction II	Control	Subfraction II	Control	Subfraction II
16 : 0	23.0	0.57	30.0	0.8	7	1.68
18 : 0	31.0	0.64	21.5	0.6	8	2.4
18 : 1	8.5	0.62	10.0	0.7	18	4.2
18 : 2	10.0	0.21	13.0	0.25	40	1.8
20 : 4	23.0	0.21	20.0	0.25	18	0.4
Total saturated + monounsaturated	65.1	2.00	64.9	2.35	37	9.17
Total polyunsaturated	35.5	0.57	34.5	0.64	64	2.7
Lipid P, μg	33.0	1.30	40.0	1.8	15	0.6

	Control	Subfraction II
Recovery of fatty acids		
Lipid extract	100	5.5
Bound to protein	0	4.0
Recovery of lipid P		
Lipid extract	100	6.6
Bound to protein	0	8.0

the case of linoleid acid, which, in turn, is the more abundant fatty acid of CL, a phospholipid, which, at difference with others, was much less affected by cysteine than by ascorbate. This dissimilar action of these who peroxidizing agents on CL could explain the greater disaggregation of the inner membrane caused by ascorbate. Other authors (1) have also emphasized the role of CL on the maintenance of the structure of the inner membrane, that is preserved even after complete hydrolysis of PC and PE by phospholipase A from *Naja naja* venom, but it becomes deeply damaged when about 60 % of CL has been hydrolyzed.

Subfraction II (table III) had a smaller amount of fatty acids than Subfraction I, especially of arachidonic acid (20:4), and of linoleic acid (18:2). This would indicate that it comes from an area of the membrane more altered by the peroxidation process. It may be seen that this subfraction, which amounted only to 6 % of the protein of the inner membrane, had a large percent of fatty acids bound to protein; the fatty acid to protein ratio was greater than that of Subfraction I, and the ratio of lipid P bound to protein to fatty acids bound to protein would mean that the molecular species of phospholipids mainly affected by the peroxidation were those having one saturated or monounsaturated fatty acid and one polynsaturated fatty acid. And even more, considering the very small amount of saturated fatty acids in the phospholipids extracted from this fraction it can be said that Subfraction II should have its origin in areas of the inner membrane predominantly constituted by those molecular species of phospholipids.

Subfraction III contained a very small content of fatty acids, which amounted only to a 2 % of the controls (table IV). CL was not detected in this fraction. The amount of fatty acids bound to protein was also very small; however, the amount of lipid P bound to protein was 4 or 5 times greater than in the other two sub-

Table IV. *Disappearance of fatty acids in phospholipids and recovery of fatty acids and lipid P bound to protein in subfraction III.*

The results are referred to the sum (taken as 100) of the areas of the different fatty acids present in each phospholipid in the incubated control. The values of lipid P are expressed in μg and corresponded respectively to 20 mg of protein in the inner membrane incubated control and to 12 mg of recovered protein in Subfraction III. See text for experimental details.

Fatty acids	PE		PC		CL	
	Control	Subfraction III	Control	Subfraction III	Control	Subfraction III
16 : 0	23.0	0.15	30.0	0.24	7	—
18 : 0	31.0	0.13	21.5	0.17	8	—
18 : 1	8.5	0.22	10.0	0.40	18	—
18 : 2	10.0	0.035	13.0	0.066	40	—
20 : 4	23.0	0.05	20.0	0.11	18	
Total saturated + monounsaturated	65.1	0.57	64.9	0.96	37	—
Total polyunsaturated	35.5	0.12	34.5	0.24	64	—
Lipid P, μg	33.0	0.80	40.0	0.90	15	—

	Control	Subfraction III
Recovery of fatty acids		
Lipid extract	100	2.0
Bound to protein	0	2.6
Recovery of lipid P		
Lipid extract	100	2.4
Bound to protein	0	11.6

fractions; this would imply that a large amount of phospholipids constituted by two polyunsaturated fatty acids have been destroyed and their P became bound to protein. Subfraction III would correspond then to a zone of the inner mitochondrial membrane with a high content of polyunsaturated phospholipids, and therefore very sensitive to the peroxidation process.

Thus it may be concluded that the inner mitochondrial membrane from rat liver is heterogeneous with respect to lipid distribution having zones with a different degree of unsaturation of the fatty acids present in its phospholipids.

Resumen

Mediante centrifugación diferencial de membranas internas mitocondriales de hígado de rata incubadas en presencia de cisteína se han separado tres subfracciones que han resultado ser diferentes en cuanto a composición en ácidos grasos de sus fosfolípidos. Una de estas subfracciones (Subfracción I) que sedimenta a $8.500 \times g$ se ha podido a su vez fraccionar en otras dos por incubación en ascorbato y nueva centrifugación a $8.500 \times g$ durante 10 minutos. La causa de esta nueva lisis se debe al hecho de que la peroxidación inducida por la cisteína sobre los fosfolípidos destruye casi por completo su contenido total de ácido araquidónico y afecta sólo en escasa proporción al ácido linoleico; por el contrario, el ascorbato destruye prácticamente por completo ambos ácidos grasos. Los datos presentados son coherentes con la hipótesis de una distribución heterogénea de las diferentes especies moleculares de fosfolípidos, de forma que las subfracciones obtenidas tendrían su origen en áreas de la membrana interna que difieren en el grado de insaturación de los ácidos grasos de sus fosfolípidos.

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