REVISTA ESPAÑOLA DE FISIOLOGIA. 31, 215-222. 1975

Phospholipid Peroxidation and Release of Cytochromes from Inner Mitochondrial Membranes *

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(Received on 10 April, 1975)

N. LOPEZ-MORATALLA, J. L. SEGOVIA, J. EUGUI and E. SANTIAGO. *Phospholipid Perodixation and Release of Cytochromes from Inner Mitochondrial Membranes.* Rev. esp. Fisiol., 31, 215-222. 1975.

Phospholipid peroxidation of isolated rat-liver inner mitochondrial membranes induced by either ascorbate or cysteine was accompanied by a release of the different cytochromes. A straight correlation between this release and the alteration of molecular species of phosphatidylcholine and phosphatidylethanolamine containing one saturated and one unsaturated fatty acid has been found. These results suggest the existence of specific associations of these phospholipids with the cytochromes within the inner mitochondrial membrane.

It has already been shown that upon incubation of mitochondria or of isolated inner mitochondrial membranes in the presence of peroxidizing agents, such as ascorbate, cysteine, or glutathione a great loss of phospholipids takes place (14-17) due to alterations of their unsaturated fatty acids (18). It has also been reported that peroxidation leads to losses in activity of certain enzymes (2, 6, 10, 19, 22, 23) as well as to structural disaggregation of the membranes (7, 14, 16, 17). McKNIGHT and HUNTER (7) made the observation that a release of cytochromes to the suspending medium from incubated mitochondria took place in Fe^{++} induced peroxidations. Similar results were obtained by PÉREZ *et al.* (12) and SEGOVIA *et al.* (22) incubating inner mitochondrial membranes in the presence of ascorbate or cysteine; approximately 50 % of the cytochromes appeared in the supernatant of 100,000 × *g* centrifugations. In previous work from this laboratory the existence of areas within the inner mitochondrial membrane with different degrees of lipid unsaturation (12) has been proposed. One of these areas, containing predomi-

* This work was supported by a research grant from the «Ministerio de Educación y Ciencia», Spain. N. L.-M. was recipient of a Fellowship from the «Consejo Superior de Investigaciones Científicas», Spain.

nantly saturated phospholipids, was completely devoid of cytochromes; a second area possessed molecular species of phospholipids with one saturated and one unsaturated fatty acid; and a third area, where, molecular species of phospholipids with only unsaturated fatty acid were present together with other species containing one saturated and one unsaturated fatty acid. The cytochromes were found to be located exclusively in these two latter areas.

These findings prompted us to study the possible associations within the membrane between cytochromes and certain molecular species of the different phospholipids. Several authors (3, 5), have shown that *in vitro* cytochrome c has some preference in associating with certain purified phospholipids through electrostatic bonds. However, this does not necessarily reflect actual association in the membrane itself.

In the work here reported we have followed the alterations of the different molecular species of the phospholipids and the release of cytochromes from the inner mitochondrial membranes during peroxidation reactions induced by ascorbate or by cysteine. The results obtained permit an interpretation regarding the lipid environment of the cytochromes; they seem to be preferentially associated with molecular species of phosphatidylcholine and phosphatidylethanolamine possessing one saturated and one unsaturated fatty acid.

Materials and Methods

Male Wistar rats weighing approximately 200 g were used in all the experiments. Liver mitochondria were prepared according to the method of HOGEBOOM (4). Isolated mitochondria were subjected to osmotic rupture following the method of PARSONS *et al.* (11). 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, the pellet was thoroughly washed three times by resuspending it in 20 mM phosphate buffer (pH 7.4), centrifuging at 1,900 $\times$ *g* for 15 minutes, and once more resuspending it in 0.25 M sucrose, and centrifuging at 8,500 $\times$ *g* for 10 minutes.

Incubation of inner membranes was carried out in a medium, 1 mM ascorbate, or 0.8 mM cysteine, 20 mM Tris-HCl buffer (pH 7.4) and 0.25 M sucrose, at 30° C. Controls without ascorbate or cysteine were incubated simultaneously. The amount of inner membranes present in the medium was adjusted to give a final concentration of 0.8 mg protein/ml. The lysis of the membranes was followed by determining the turbidity changes at the conventional wavelength of 520 nm. To carry out the determinations of lipids 10 ml aliquots were taken at different intervals. Aliquots of 9 ml were also taken at the same intervals and discontinuous gradient centrifugation carried out as follows: the samples were rapidly cooled in an ice acetone bath during three minutes and a volume of 9 ml carefully layered on 2.5 ml of 0.5 M sucrose; the volume of the tube was completed with 0.25 M sucrose and centrifuged at 100,000 $\times$ *g* during 1 hour at 0° C; two fractions were separated, a sediment and a supernatant.

Lipids were extracted after precipitating the protein with $HClO_4$ to give a final concentration of 0.3 N, as previously described (18). Phospholipids were separated by thin layer chromatography according to the technique of NESKOVIC *et al.* (9). Lipid phosphorus was determined by the method of BARTLETT (1). Methyl esters of the fatty acids present in each phospholipid were prepared by direct transmethylation catalyzed by BF_3 as described by MORRISON and SMITH (8) and analyzed by gas chromatography as described (18) Methylation of fatty acids still bound to proteins after lipid extrac-

tion was carried out as follows: 6 ml of 96 % ethanol and 0.4 ml of 50 % NaOH were added to the protein precipitate amounting up to 20 mg, and heated in a water bath for 30 minutes, checking that the medium remained alkaline. The hydrolyzate was acidified with HCl and fatty acids were extracted with 3 ml of petroleum ether; methyl esters were prepared as described above. Cytochromes a, b, c_1 and c were determined by the method of WILLIAMS (25).

Results and Discussion

Figure 1 reflects the release of cytochromes from the membrane and the alteration of the major phospholipids during the peroxidation reaction induced with either ascorbate or cysteine. It may be observed that the cytochromes were liberated at a faster rate in the early stages of membrane disaggregation, as indicated by changes in turbidity, both in incubations with ascorbate (fig. 1 A) or with cysteine (fig. 1 C); however, the total amount of cytochromes liberated at the end of the incubation with ascorbate was higher than in the case of cysteine. Table I shows that the recovery of the different cytochromes was practically complete. The amounts of unaltered phosphatidylethanolamine and phosphatidylcholine decreased progressively coinciding with the membrane lysis as reflected by turbidity changes both in incubations with ascorbate (fig. 1 B) or with cysteine (fig. 1 D). Cardiolipins remained unchanged during the incubation with cysteine until a decrease in turbidity of approximately 0.4 absorbance units had taken place; and even after that moment the disappearance of this phospholipid was very limited. Cardiolipin disappeared at a fast rate during the early stages of the peroxidation induced by ascorbate and the total alteration of this phospholipid was considerably larger than in incubations carried out with cysteine (fig. 1 and table I). In incubated controls changes in turbidity, as well as phospholipid levels and release of cytochromes were negligible. From these observations it may be concluded that the release of cytochromes was unrelated to the alteration of cardiolipins, whereas this possibility cannot

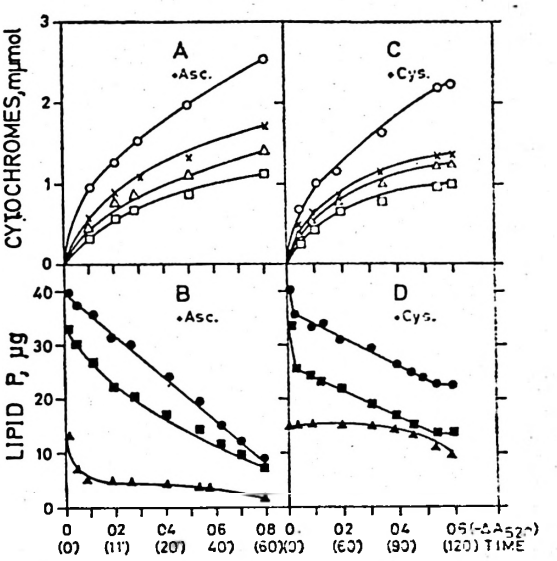

Fig. 1. *Release of cytochromes and phospholipids alteration during incubation of isolated rat liver inner mitochondrial membranes in the presence of ascorbate or cysteine.*
(A) Release of cytochromes to the suspending medium in the presence of 1 mM ascorbate. (B) Phospholipid alteration during the incubation in the presence of 1 mM ascorbate. (C) Release of cytochromes to the suspending medium in the presence of 0.8 mM cysteine. (D) Phospholipid alteration during the incubation in the presence of 0.8 mM cysteine. (○) cytochrome a; (×) cytochrome b; (△) cytochrome c; (□) cytochrome c_1; (●) phosphatidylcholine; (■) phosphatidylethanolamine; (▲) cardiolipin. Cytochromes were determined in the supernatants after centrifugation of 20 ml of membrane suspension at 100,000 × *g* as described in the text. The determination of the different phospholipids was carried out in 20 ml of the suspension. Under the scale corresponding to turbidity changes the approximate times in minutes, at which they take place, have been indicated in parenthesis.

be excluded in what respects to phosphatidylethanolamine or phosphatidylcholine. With the purpose of finding out whether the peroxidation of any molecular species of phosphatidylethanolamine or phosphatidylcholine might be involved in the liberation of cytochromes, the amounts of each cytochrome released during the incubation were plotted against the disappearance of the different fatty acids from

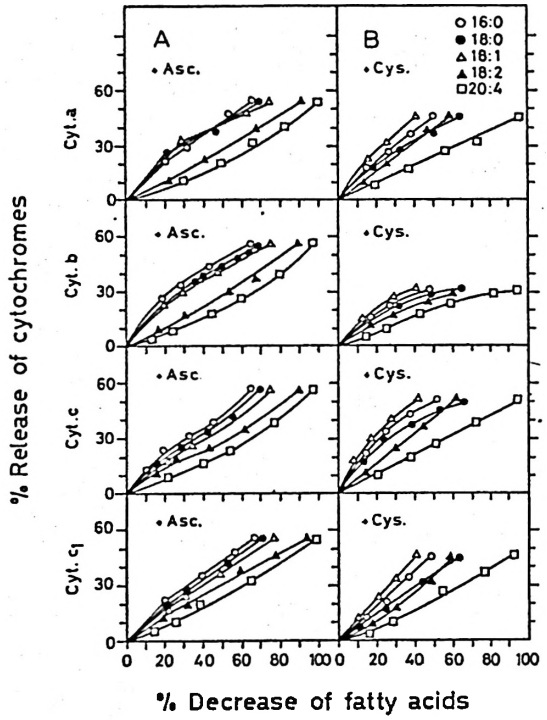

Fig. 2. *Correlation between percent release of cytochromes and disappearance of fatty acids in phosphatidylethanolamine during the incubation of inner mitochondrial membranes in the presence of either ascorbate (A) or cysteine (B).*
Cytochromes were determined in the supernatants obtained after centrifugation of the suspension at 100,000 × *g*. Fatty acids were determined in the isolated phospholipid from 30 ml aliquots after separation of the different lipids by thin layer chromatography as described in Materials and Methods. See figure 1 for other experimental details.

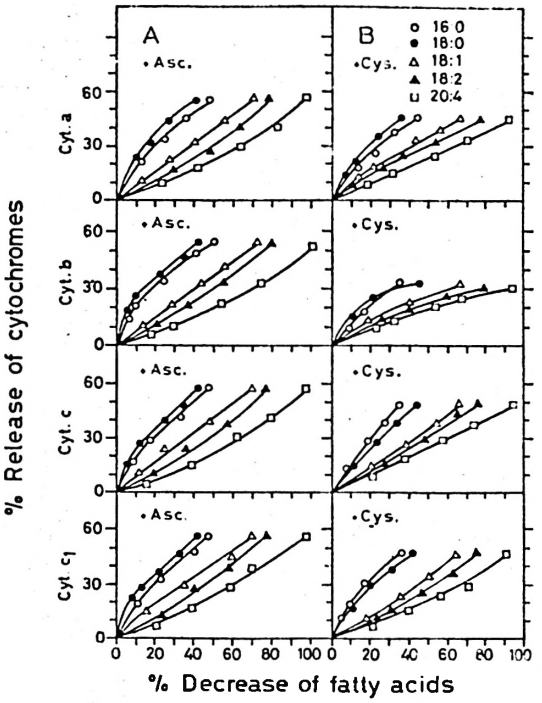

Fig. 3. *Correlation between percent release of cytochromes and percent disappearance of fatty acids in phosphatidylcholine during the incubation of inner mitochondrial membranes in the presence of either ascorbate (A) or cysteine (B).*
For experimental details, see figure 2.

both phospholipids separately. Figures 2 and 3 show the relationship between the release of the different cytochromes and fatty acids disappeared in phosphatidylethanolamine and phosphatidylcholine respectively during the incubation with ascorbate or with cysteine. It may be observed that straight correlations existed between the release of each one of the cytochromes and the disappearance of unsaturated fatty acids. With palmitic and stearic acids a similar relationship was also observed. In the case of cytochrome b there was a slight deviation which will be discussed below. These results are consistent with the hypothesis that the liberation of cytochromes would be related

to the peroxidation of molecular species of phosphatidylethanolamine and phosphatidylcholine with one saturated and one unsaturated fatty acid.

It has been demonstrated in previous work (18) that the phospholipids altered by peroxidation reactions become bound to neighboring proteins, through the peroxidized unsaturated fatty acid, and cannot be extracted with the usual organic solvents. The corresponding lipid P is found in the precipitated protein together with the saturated fatty acids and unsaturated fatty acids which might have escaped the peroxidation reaction. Table II shows the values of lipid P and fatty acids present in phosphatidylcholine, phosphatidylethanolamine and cardiolipin both in the sediments and in the supernatants of the centrifugations at 100,000 × *g* after incubating the membranes with ascorbate or cysteine. A small amount of phos-

Table I. *Recovery of cytochromes, lipid P and of different phospholipids after the Incubation of inner mitochondrial membranes in the presence of ascorbate or cysteine* [1].

	I. M. Control	I. M. + Ascorbate	I. M. + Cysteine	% Recovery	
				Ascorbate	Cysteine
Cyt. a (mμmol/20 mg protein) [2]	7	6.8	7	97	100
» b	6	5.64	5.7	94	95
» c_1	4.2	4.2	4.2	100	100
» c	5	4.58	4.6	92	92
Total P (μg/20 mg protein) [3]	100	28	60	28	60
Phosphatidylethanolamine [4]	33	8	11	24	33
Phosphatidylcholine [4]	40	11	15	27.5	37.5
Cardiolipin [4]	15	1.5	7	10	46.5

1. After incubating the membranes in the presence of 1 mM ascorbate or 0.8 mM cysteine.
2. Cytochromes were determined following the technique of Williams (22).
3. Total lipid P was determined in the total lipid extract according to Bartlett (20).
4. The different phospholipids were determined after separation by TLC (19).

Table II. *Distribution of saturated and unsaturated fatty acids in the different phospholipids present in inner membranes after incubation with ascorbate or cysteine and in the corresponding sediments and supernatants obtained by discontinuous gradient centrifugation* *.

	Phosphatidylcholine			Phosphatidylethanolamine			Cardiolipin		
	Lipid P, μg	Fatty acids		Lipid P, μg	Fatty acids		Lipid P, μg	Fatty acids	
		Sat.	Unsat.		Sat.	Unsat.		Sat.	Unsat.
Inner membranes	40	190	200	33	160	155	15	28	103
Inner membranes (Ascorbate)	11	62	26	8	45	15	1.5	8	5.5
Sediment (Ascorbate)	8	46	19	6.5	35	9	1.2	4.5	2
Supernatant (Ascorbate)	2.5	13	7	1.5	10	3	0.3	3	—
Inner membrane (Cysteine)	22	81	74.8	14	55.4	44.7	6.6	21	69.6
Sediment (Cysteine)	21	80	74.5	13.3	55.2	44.4	6.6	21	69.6
Supernatant (Cysteine)	1	0.5	0.2	0.7	0.2	0.1	—	—	—

* The results are referred to 20 mg of protein in the original inner membrane preparation. The values of fatty acids are given as areas and referred to 1,000 as the total area of all the fatty acids present in the original inner membrane preparation. See text for experimental details.

Table III. *Cytochrome content of inner mitochondrial membranes and of sediments and supernatant obtained by discontinuous gradient centrifugation* *.

	Cytochromes (mμmol)				Protein recovery %
	a	b	c_1	c	
Inner membranes	0.35	0.3	0.21	0.25	100
Inner membranes (Ascorbate)	0.34	0.28	0.21	0.23	100
Sediment (Ascorbate)	0.15	0.13	0.08	0.1	18.5
Supernatant (Ascorbate)	0.19	0.14	0.12	0.13	81.5
Inner membrane (Cysteine)	0.35	0.29	0.21	0.23	100
Sediment (Cysteine)	0.17	0.19	0.1	0.12	38
Supernatant (Cysteine)	0.17	0.09	0.1	0.1	62

* After incubating the membranes in the of 1 mM ascorbate or 0,8 mM cysteine. See text for experimental details.

phatidylcholine and phosphatidylethanolamine remained together with negligible amounts of cardiolipin in the sediments obtained after incubation with ascorbate; the amounts of saturated and unsaturated fatty acids present in phosphatidylcholine and phosphatidylethanolamine, as compared to the values in the control inner membranes, indicate that in this sediment the predominant molecular species of these phospholipids were saturated. In the sediments obtained after treatment with cysteine the molecular species of phosphatidylcholine and phosphatidylethanolamine remaining unaltered contained more unsaturated fatty acids. The amounts of the different phospholipids present in supernatants were very small.

Figure 4 shows the values of lipid P and of fatty acids present in the lipid extract and in the protein precipitate after the usual lipid extraction, both in membranes incubated with ascorbate or with cysteine and in the corresponding sediments and supernatants. It may be observed that the ratio of protein bound lipid P to protein bound fatty acids is much higher in supernatants than in sediments after incubation with ascorbate or with cysteine. These results may be taken as an indication that the material present in the supernatants originated in areas of the membrane predominantly unsaturated, possessing molecular species of phospholipids having only unsaturated fatty acids, plus other species with saturated and unsaturated fatty acid in the same molecule. The former species would have all their fatty acids peroxidized; their phosphate would become strongly bound to the extracted precipitated protein, and found together with the saturated fatty acids and phosphorus which were forming part of the latter species. Approximately 50 % of all the cytochromes were present in this supernatant fraction (fig. 1).

Following a similar reasoning it might be concluded that the sediments could have their origin in an area of the membrane where the molecular species of the phospholipids attacked by the peroxidation reaction would be those containing saturated and unsaturated fatty acids in the same molecule, since the ratio of bound lipid P to bound saturated fatty acid is approximately 1. The phospholipids which were not peroxidized could be easily extracted with the usual organic solvent mixture; it should be observed that these lipids had a very high proportion of saturated fatty acids. The remaining 50 % of all the cytochromes was present in these sediments. The disaggregation of this area was more limited, due perhaps to its lower degree of unsaturation with respect to that which gave origin to the supernatants.

These data may be interpreted in the

sense that all the cytochromes are accompanied by molecular species of monosaturated phosphatidylethanolamine and phosphatidylcholine within the areas which gave origin to the sediments as well as to the supernatants. The cytochromes surrounded by their specific phospholipids — monosaturated phosphatidylethanolamine and phosphatidylcholine — and located in areas of higher degrees of unsaturation would be more easily released from the membrane when this is peroxidized with ascorbate or cysteine, than those located in more saturated areas.

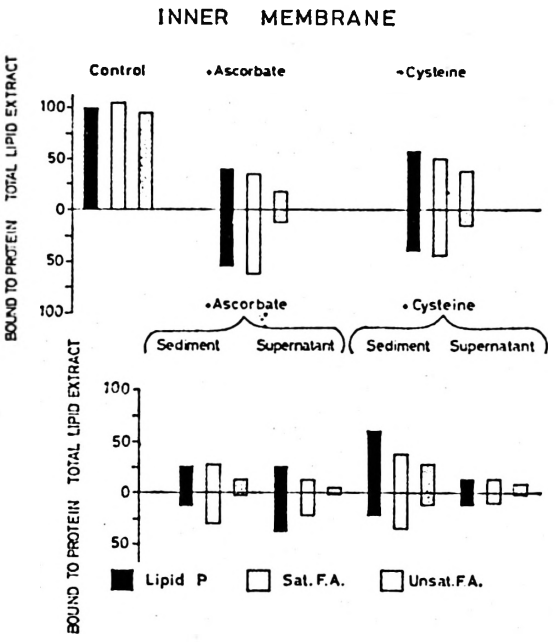

Fig. 4. *Lipid P, saturated and unsaturated fatty acids in inner mitochondrial membranes incubated in the presence of ascorbate or cysteine.*

Incubation conditions were as described in figure 1. After the incubation the inner membrane suspensions were centrifuged in a discontinuous gradient and sediments and supernatants obtained as described in Materials and Methods. The values of lipid P, saturated and unsaturated fatty acids present in the total lipid extract, as well as bound to protein have been referred to those of the total lipid extract of non-incubated inner membranes (100 for lipid P and 200 for the sum of saturated plus unsaturated fatty acids). See text for other experimental details. (F. A.) fatty acids.

In a previous work (21) in thas been demonstrated that the alteration of molecular species of phosphatidylethanolamine and phosphatidylcholine with one saturated and one unsaturated fatty acid takes place progressively after the peroxidation process has been initiated either by ascorbate or by cysteine; if the incubation is carried out with ascorbate the polyunsaturated species of the different phospholipids are altered almost completely in the early stages after the initiation of the peroxidation reaction. This would explain why at those early stages the percent release of the cytochromes was higher than the percent disappearance of saturated fatty acids, since the disintegration originated by the alteration of species having only unsaturated fatty acids would add up to that originated by the alterations of monosaturated species.

The lower liberation of cytochrome b in comparison with the other cytochromes, in the case of the incubation with cysteine, might be due to a preferential localization in areas of the membrane less unsaturated and therefore less disaggregated.

This hypothesis of a distribution of the cytochromes in two areas of the membrane, which differ in the degree of unsaturation of their phospholipids, but in every case with their specific environment of phosphatidylethanolamine and phosphatidylcholine with one saturated and one unsaturated fatty acids does not contradict the localization on different sides of the membrane proposed by several authors (20, 24), since the liberation of the cytochromes during the peroxidation process is due to alterations undergone by the phospholipids; the sidedness of the membrane migth also influence the cytochrome release, and perhaps for this reason cytochrome b, which TYLER *et al.* (24) have suggested to be located on the

inner side of the membrane, is released in a lower proportion than the other cytochromes.

Acknowledgements

We are grateful to Mrs. M. Preciado, and Miss J. Domezain for their technical asistance.

Resumen

La peroxidación de los fosfolípidos de las membranas internas mitocondriales de hígado de rata inducida por el ascorbato y la cisteína va acompañada de una liberación de los diferentes citocromos. Se ha encontrado la existencia de una estrecha correlación entre esta liberación y la alteración de especies moleculares de fosfatidilcolina y fosfatidiletanolamina que contienen un ácido graso saturado y otro insaturado. Estos resultados sugieren la existencia de una asociación específica de estos fosfolípidos con los citocromos en la membrana interna mitocondrial.

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