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NADH-Cytochrome c Reductase, Succinate Cytochrome c Reductase and Phospholipids

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E. SANTIAGO, N. LOPEZ-MORATALLA, J. L. SEGOVIA and J. EUGUI. *NADH-Cytochrome c Reductase, Succinate Cytochrome c Reductase and Phospholipids.* Rev. esp. Fisiol., 33, 197-204. 1977.

Phospholipid peroxidation of isolated rat liver inner mitochondrial membranes induced by either ascorbate or cysteine was accompanied by a release of flavins and coenzyme Q. 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.

Peroxidation induced on molecular species of phosphatidylcholine and phosphatidylethanolamine containing only unsaturated fatty acids were accompanied by losses in enzyme activities of NADH-cytochrome c reductase and succinate cytochrome c reductase.

It is now well established that inner mitochondrial membranes when incubated in the presence of ascorbate or cysteine (20-23) undergo peroxidation of their phospholipid constituents, mainly phosphatidylcholine, phosphatidylethanolamine and cardiolipin; these alterations provoke a lysis of the membrane. The study of the fragments originated suggests the existence of three types of areas which differ in composition with regard to lipids and components of the respiratory chain (16).

It has also been observed (9, 10, 24) that together with the peroxidation of phospholipids an inactivation of membrane enzymes and release of cytochromes takes place. Moreover, a close correlation exists between the alteration of types of phospholipids, or of certain molecular species of them, and inactivation of certain enzymes. This parallelism might very well reflect the existence of specific associations of lipids and proteins within the membrane.

Several authors have also found that lipid peroxidation induced by Fe^{++} in mitochondrial (11) or sonic treatment of the membrane (15) gives raise to a release of flavins, cytochromes and coenzyme Q.

It is also known that delipidation of mitochondrial with organic solvents causes a loss of activity of succinate cytochrome c reductase and also that addi-

tion of certain phospholipids leads to a partial reactivation of the enzyme (3-5, 28).

In the present paper we report the findings with regard to the progressive release of flavins and coenzyme Q and the rate of inactivation of NADH cytochrome c reductase and succinate cytochrome c reductase which takes place simultaneously which the alterations of the different molecular species of the phospholipids during the peroxidation process induced on rat liver inner mitochondrial inner membranes. The results obtained may give some insight in the microscopic organization of the membrane.

Materials and Methods

Wistar rats weighing 210 ± 20 *g* were used in all the experiments. Livers were rapidly removed and homogenized in 0.25 M sucrose and mitochondria isolated according to the method of HOGEBOOM (7). Isolated mitochondria were subjected to osmotic rupture following the method of PARSONS *et al.* (14). 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 thoroughly washed three times resuspending it in 0.02 M phosphate buffer, pH 7.4, centrifugings 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 (19). Incubation of inner membranes was carried out in 20 mM Tris-HCl buffer, pH 7.4 1 mM ascorbate, 0.25 M sucrose, at 30° C, during one hour. In other experiments cysteine replaced ascorbate with a final concentration of 8×10^{-4} M. In every experiment controls without ascorbate or cysteine were incubated. The lysis of the membranes was followed by the changes of turbidity of the suspension in a 1 cm cuvet and setting the wavelength of the spectrophotometer at 520 nm.

Phospholipids were extracted as previously described (18) from 10 ml aliquots of the membrane suspensions after precipitation with enough concentrated $HClO_4$ to give a final concentration of 0.3 N.

Phospholipids were separated by thin layer chromatography according to the technique of NESKOVIC *et al.* (13). 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 (12) and analyzed by gas chromatography as described (16). Methylation of fatty acids still bound to proteins after lipid extraction 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 during 30 minutes, checking that the medium remained alkaline. The hydrolyzate was acidified with HCl and fatty acids extracted with 3 ml of petroleum ether; methyl esters were prepared as described above Flavin and coenzyme Q were determined by the method of CHANCE (2) and REDFEARN (17) respectively. Enzymes NADH cytochrome c reductase and succinate cytochrome c reductase were determined according to HATEFI and RIESKE (6) and TISDALE (26) respectively.

Results and Discussion

The release of flavins and coenzyme Q with simultaneous alteration of phospholipids was determined during the peroxidation process induced by ascorbate or cysteine on rat liver inner mitochondrial membranes. Figure 1 shows that upon incubation of membranes both in the presence of ascorbate (fig. 1 *A*), or in the presence of cysteine (fig. 1 *B*), the rate of release of flavins and coenzyme Q was rather high at the beginning of the disaggregation of the membranes, when the changes in turbidity of the suspension are still low as compared to later

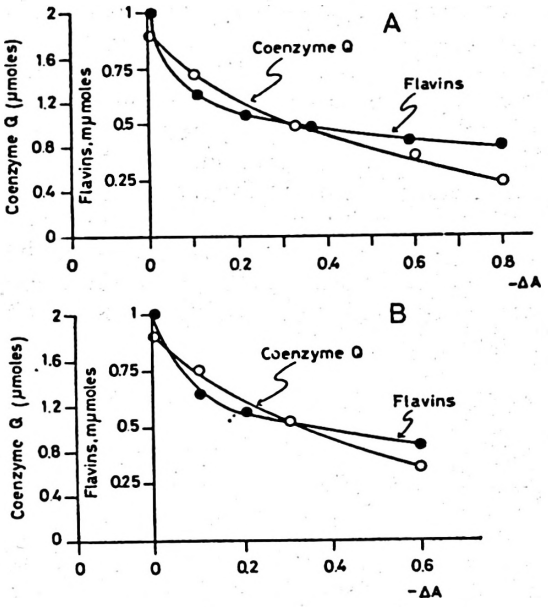

Fig. 1. *Release of flavins, coenzyme Q and changes in turbidity during the incubation of inner membranes in the presence of 1 mM ascorbate (A) or 0.8 mM cysteine (B).* Incubation was carried out at 30° C in 0.25 M Sucrose, Tris-HCl buffer, pH 7.4. Protein concentration was 0.8 mg/ml corresponding to an initial turbidity of 1.3 units at 520 nm, 1 cm light path. Results are expressed as mμ moles of flainvs and μ moles of coenzyme Q per mg protein in the initial suspension of membranes.

stages. Table I shows that these components were not altered during the incubation since they were completely recovered and distributed in the supernatant and in the pellet after centrifugation of the incubated membrane suspension at 100,000 × *g*. These results are in agreement mith those of Mc KNIGHT and HUNTER (11) after treatment of liver mitochondria with Fe^{++}.

Figure 2 shows that the amounts of phosphatidylcholine and phosphatidylethanolamine decreased progressively during the peroxidation process induced by either ascorbate or cysteine; however, cardiolipin remained unaltered in the early stages

Table I. *Distribution of flavins and Coenzyme Q in sediments and supernatants, after centrifugation at 100,000 × g of inner mitochondrial membranes incubated in the presence of ascorbate (60 minutes) or cysteine (120 minutes).*
Results are referred to 200 mg of protein in the initial suspension of inner membranes.

	Coenzyme Q (mμ moles)	Flavins (mμ moles)
Inner membranes	36.0	20.0
Inner membranes (ascorbate)	28.0	20.0
Sediment (ascorbate)	9.6	7.8
Supernatant (ascorbate)	17.6	12.0
Inner membranes (cysteine)	29.0	20.0
Sediment (cysteine)	13.0	8.0
Supernatant (cysteine)	15.8	11.0

after the onset of the peroxidation induced by cysteine.

The release of flavins and coenzyme Q was very noticeable in the early stages of the peroxidation (fig. 1). These results may suggest that the alterations of phosphatidylcholine and phosphatidylethanolamine could be related with the release of flavins and coenzyme Q, whereas no relationship would exist with cardiolipin.

In incubated controls, the change in turbidity, phospholipid losses and release of flavins and coenzyme Q were negligible.

The disappearance of molecular species of phosphatidylcholine and phosphatidylethanolamine was also studied in order to find out whether the alterations of some them would be in a closer parallelism with the release of flavins and coenzyme Q. The results obtained are shown in figures 3 and 4; it may be observed that a correlation exists between disappearance of saturated and unsaturated fatty acids and the release of flavins and coenzyme Q. The higher rate observed in the degradation of unsaturated with respect to saturated fatty acids in the early stages of the peroxidation and membrane

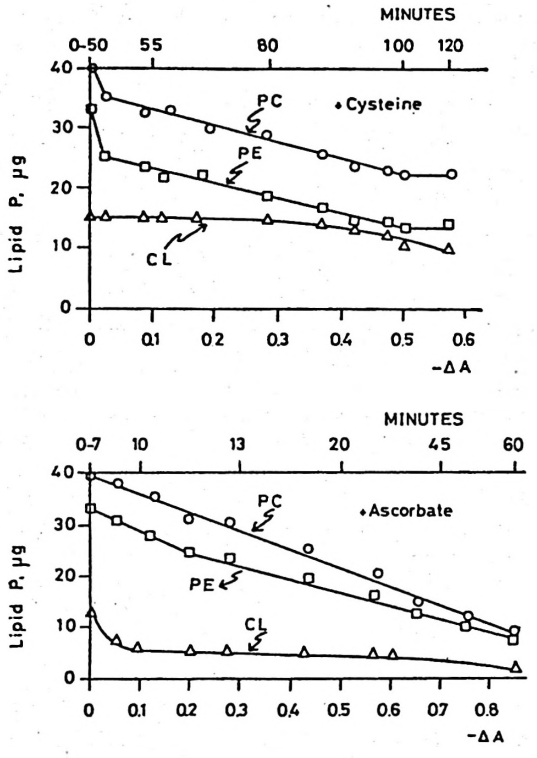

Fig. 2. *Changes in turbidity and changes in phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cardiolipin (CL) during the incubation of inner membranes in the presence of ascorbate or cysteine.*
The values of lipid P corresponding to each phospholipid are referred to 20 mg of protein in the initial suspension of inner membranes.

disaggregation in the presence of ascorbate (figures 3 *A* and 3 *B*) may indicate that during that interval the molecular species of phosphatidylcholine and phosphatidylethanolamine predominantly affected by peroxidation would be those possesing two unsaturated fatty acids and to a lesser exent those with one saturated and one unsaturated fatty acid. However, in the presence of cysteine the alteration of both kinds of species took place progressively after the onset of the reaction (figures 3 *B* and 4 *B*). This different peroxidation rate of membrane phospholipids when incubations are carried out with ascorbate or with cysteine had already been noticed in previous work from our laboratory (16). It had also been previously observed (16) that altered phospholipids bind neighboring proteins through the peroxidized fatty acids; those phospholipids can no longer be extracted with the usual procedures, and the corresponding lipid P could be found in the protein precipitate together with saturated or unsaturated fatty acids, which had

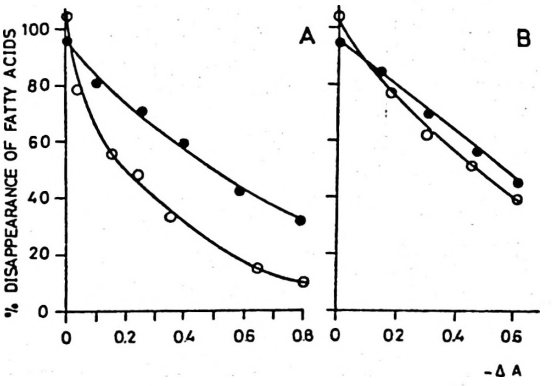

Fig. 3. *Changes in turbidity and percent losses of saturated (●) and unsaturated (○) fatty acids in phosphatidylethanolamine during the incubation of inner membranes in the presence of ascorbate (A) or cysteine (B).*

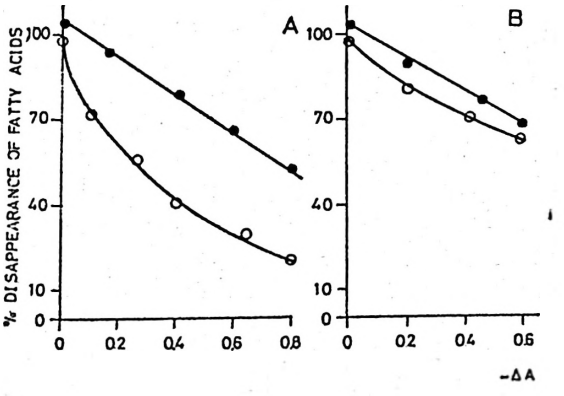

Fig. 4. *Changes in turbidity and percent losses of saturated (●) and unsaturated (○) fatty acids in phosphatidylcholine during the incubation of inner membranes in the presence of ascorbate (A) or cysteine (B).*

Table II. *Distribution of saturated and unsaturated fatty acids present in inner membranes after incubation with ascorbate or cysteine, and in the corresponding sediments and supernatants after centrifugation at 100,000 × g.*
Results are refered to 20 mg of protein in the initial suspension of inner membranes. The fatty acid values are expressed as areas, taking as 1.000 the total area of the fatty acids present in the initial suspension of inner membranes.

	PC			PE			CL		
	Lipid P μg	Fatty acids		μg Lipid P	Fatty acids		Lipid P μg	Fatty acids	
		Sat.	Unsat.		Sat.	Unsat.		Sat.	Unsat.
Inner membranes	40.0	190.0	200.0	33.0	160.0	155.0	15.0	28.0	103.0
Inner membranes (ascorbate)	11.0	62.0	26.0	8.0	45.0	15.0	1.5	8.0	5.5
Sediment (ascorbate)	8.0	46.0	19.0	6.5	35.0	9.0	1.2	4.5	2.0
Supernatant (ascorbate)	2.5	13.0	7.0	1.5	10.0	3.0	0.3	3.0	—
Innner membranes (cysteine)	22.0	81.0	74.8	14.0	55.4	44.7	6.6	21.0	69.6
Sediment (cysteine)	21.0	80.0	74.5	13.3	55.2	44.4	6.6	21.0	69.6
Supernatant (cysteine)	1.0	0.5	0.2	0.7	0.2	0.1	—	—	—

escaped the peroxidation reaction, but still forming part of altered phospholipids. Table II shows the values of lipid P and of fatty acids present in phosphatidylethanolamine, phosphatidylcholine and cardiolipin in sediments and supernatants after centrifuging at 100,000 × *g* membranes previously incubated with ascorbate or with cysteine. In the sediments obtained after incubating with ascorbate, small amounts of phosphatidylcholine and phosphatidylethanolamine were found, together with negligible amounts of cardiolipin; comparing the values of the different fatty acids presen in them, with those of the corresponding fatty acids in the inner membranes incubated as controls, it may be seen that the phospholipids in the sediments were more saturated. In the case of the incubations carried out in the presence of cysteine, the sediments contained higher amounts of unsaturated fatty acids in the unaltered phosphatidylcholine and phosphatidylethanolamine. Only negligible amounts of phospholipids were present in the supernatants after the incubation with either ascorbate or cysteine. The molecular species of phosphatidylcholine and of phosphatidylethanolamine predominantly altered would be those having two unsaturated fatty acids. In the case of the incubation of the membranes in the presence of cysteine, the sediments obtained after centrifugation at 100,000 × *g* still contained species of unaltered phosphatidylcholine and phosphatidylethanolamine with unsaturated fatty acids. The amounts of the different phospholipids in the supernatants, after incubation with either ascorbate or cysteine, were negligible.

Lipid P and fatty acids remaining bound to protein after extraction with solvent was also determined in membranes incubated with ascorbate or with cysteine. Figure 5 shows that the ratio of bound lipid P to bound fatty acids was much higher in the supernatants than in the sediments. These results may be interpreted in the sense that the material present in the supernatants came from areas of the membrane with a high degree of unsaturation of their phospholipids; these phospholipids would belong mainly to species containing only unsaturated fatty acids, together with some others containing one unsaturated fatty acid and another saturated; phospholipids belonging to the former species are liable to peroxidation of all their fatty acids and the phosphorus

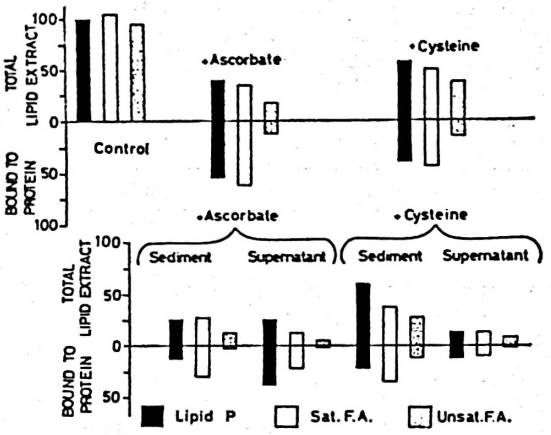

Fig. 5. *Lipid P, saturated and unsaturated fatty acids in inner mitochondrial membranes incubated in the presence of ascorbate or cysteine.*

Incubations were carried as described in figure 1. After incubation the membrane suspension was centrifuged at 100,000 × g and sediment and supernatant were obtained. The values of lipid P, saturated and unsaturated fatty acids present in the total lipid extract, as well as those bound to protein after precipitation and solvent extraction are referred to the total lipid extract of non incubated inner membranes taking the value of 100 for the total lipid P and 200 for the sum of all the saturated and unsaturated fatty acids.

containing moiety would bind to the protein. Fatty acids bound to protein would belong to the latter species. Approximately 40 % of flavins and coenzyme Q (Table I) was recovered in this supernatants fraction.

A similar reasoning leads us to think that the fraction sedimenting at 100,000 × *g* would correspond to areas of the membrane where most of their phospholipids would contain one saturated fatty acid together with another unsaturated since molar ratio of bound lipid P to bound fatty acids in the precipitated and extracted protein was approximately equal to one. Approximately 60 % of flavins and coenzyme Q were recovered in this fraction (Table I).

The present results suggest that flavins and coenzyme Q would be associated to unsaturated species of phosphatidylcholine and phosphatidylethanolamine in two areas of the membrane with different degrees of unsaturation and giving rise to the supernatant and sediment fractions after peroxidation and centrifugation at 100,000 × gram. Components located in those areas would be more easily released than others located in areas with a higher proportion of saturated lipids.

There is also experimental evidence (9) that cytochromes would be also distributed in those two more unsaturated areas approximately 50 % in each one.

At the same time that the release of respiratory chain components during the process of peroxidation, the losses in enzymatic activity of rotenone sensitive NADH-cyt. c reductase and succinate-cyt c reductase was also investigated. Figure 6 shows that in the presence of ascorbate the losses in activity of both enzymes were very high and they took place very rapidly during the early stages after the onset of the peroxidation and when the changes in turbidity of the suspension were still very small. In the presence of cysteine the losses in activity were more limited and they took place slowly during the process of peroxidation.

Since the NADH-cyt. c reductase contains flavins, coenzyme Q and cytochromes (8), and the enzymes succinate cyt. c reductase contains cytochromes and coenzyme Q (27) it is reasonable to think that losses in enzyme activity of those complexes would depend on the alteration of phospholipids and the simultaneous release of their components. Figure 6 shows that a relationship exists between alterations of phosphatidylcholine and phosphatidylethanolamine and losses in enzyme activities; the lack of correlation with cardiolipin is also obvious. The fast rate of inactivation of these enzymes, when incubations were carried out in the presence of ascorbate, may indicate that they would

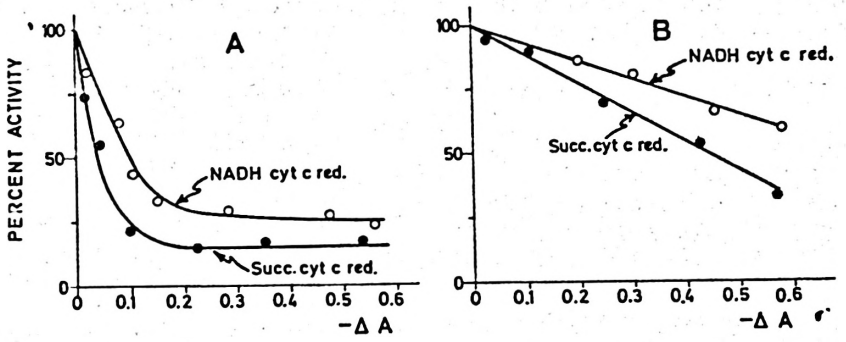

Fig. 6. *Changes in NADH-cyt c reductase and succinate-cyt c reductase activities during the incubation of innter membranes in the presence of ascorbate (A) or cysteine (B).*

be associated with highly unsaturated molecular species of phosphatidylethanolamine and phosphatidylcholine, since the species mainly affected in the early stages of peroxidation would be those constituted by two unsaturated fatty acids. This would also explain why the losses in enzyme activity would be larger than the extent of the release of flavins and coenzyme Q during the early stages of the peroxidation; they would still be associated to the membrane, but already inactivated through the alterations provoked by the peroxidation reaction.

Resumen

La peroxidación de fosfolípidos de membranas internas mitocondriales inducida por ascorbato o por cisteína está acompañada por una liberación de flavinas y coenzima Q. Se ha encontrado una estrecha correlación entre esta liberación y la alteración de las especies moleculares de fosfatidilcolina y fosfatidiletanolamina cvonstituidas por un ácido graso saturado y otro insaturado.

La peroxidación inducida sobre las especies moleculares de fosfatidilcolina y fosfatidiletanolamina que contienen solamente ácidos grasos insaturados se acompaña por una pérdida de las actividades enzimáticas NADH-citocromo c reductasa y succínico citocromo c reductasa.

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