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Effect of Ascorbate on Phospholipids During Mitochondrial Swelling and Lysis *

by

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The large amplitude swelling or lysis induced by low concentrations of ascorbate on isolated rat liver mitochondria is distinct from most types of mitochondrial swelling (6, 7, 9). This effect is related to lipid peroxidation as shown by HUNTER *et al.* (7). It has also been reported that 75 % of the initial mitochondrial protein is found in the 100,000 × g supernatant after ascorbate treatment (5).

Since phospolipids are known to be necessary constituents in order to maintain mitochondrial structure, we decided to study the possible effect of ascorbate on the different phospholipids during the swelling and lysis process.

It is the purpose of this paper to report the decrease in mitochondrial phosphatidyl ethanolamine and phosphatidyl choline related to the aforementioned lysis, as well as the increase in water soluble organic phosphorus. These results were obtained by paper chromotagraphic studies and the use of autoradiograms of P^{32} labeled mitochondrial phospholipids.

The possible mechanism of the reported phospholipid effect and its relationship to mitochondrial lysis as affecting the membranous structure will be reported in the subsequent paper.

Materials and Methods

Male Wistar rats were fasted for 15 hours and killed. Livers were immediately removed and homogenized at 0° C in 8 volumes of 0.25 M sucrose in an all glass Elvehjem-Potter homogenizer. Mitochondria were isolated following the method of HOGEBOOM (4), slightly modified. The mitochondrial fraction was thoroughly washed by resuspension in 0.25 M

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sucrose and recentrifugation at 8,500 × g three times.

Mitochondria were then incubated at 30° C in a medium containing 0.02 M Tris buffer, *p*H 7.4, 0.001 M ascorbate and 0.25 M sucrose. Controls without ascorbate were incubated in the same way. Mitochondrial swelling was followed by the change in optical density in a 1 cm cuvette with a Zeiss PMQII Spectrophotometer at 520 mμ.

At different times aliquots were taken and concentrated $HClO_4$ added to give a final concentration of 0.3 N. Phospholipids were extracted from the precipitates with 5 ml of the solvent mixture described by FOLCH (3) consisting of chloroform, methanol and concentrated HCl (200: 100:1 by volume) and the extracts washed as described in Ref. 12. Chromatography of the phospholipids was carried out by the method of MARINETTI *et al.* (11) and after the spots were developed with Rhodamin B, they were cut out, wet ashed in concentrated $HClO_4$ at 180° C for 20 minutes and phosphorus determined by the method of BARTLETT (1).

In a different set of experiments mitochondria were labeled with radioactive phosphorus prior to incubation with ascorbate. Rat liver slices weighing up to 1.5 grams were incubated in bicarbonate-saline Krebs-Henseleit medium (8) containing 1 mc of radioactive orthophosphate at 37° C and homogenized in 0.25 M sucrose; mitochondria were then isolated, washed and incubated as indicated above. After paper chromatography, autoradiograms were prepared and the radioactivity present in the different phospholipids was counted with a thin window Geiger counter.

Protein was determined by the method of LOWRY *et al.* (10). Water soluble organic phosphorus in the perchloric acid supernatants was estimated as the difference between total phosphorus and inorganic phosphorus determined by the method of FISKE and SUBBAROW (2).

Results *

Figure 1 shows a marked decrease in optical density of the mitochondrial suspension during the incubation time. Change

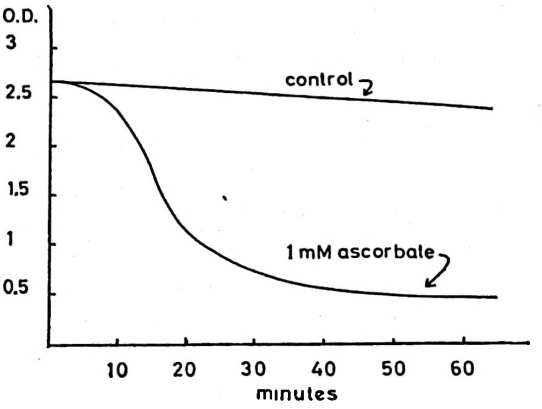

FIG. 1. Ascorbate-induced mitochondrial swelling. Incubation medium: 1 mM ascorbate, 20 mM Tris-HCl buffer, *p*H 7.4, 0.25 M sucrose. Mitochondrial protein 52,5 mg. Incubation volume: 70 ml. Temperature 30° C.

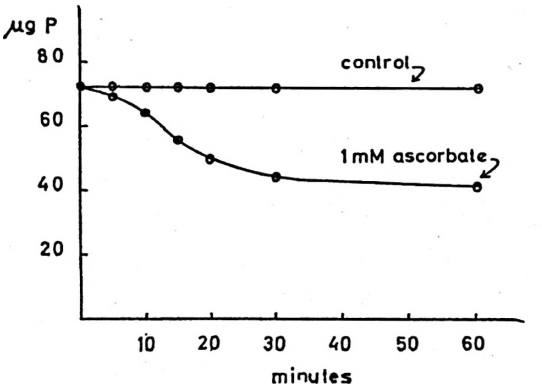

FIG. 2. Total mitochondrial phospholipid changes during ascorbate swelling. Total phospholipid phosphorus was determined on 10 ml aliquots containing 7.5 mg mitochondrial protein, after 5, 10, 15, 20, 30 and 60 minutes of incubation.

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of total phospholipid content determined at different intervals is shown in Fig. 2. It is evident that a parallelism exists between the decrease in optical density and the change in phospholipid content. The amounts of phosphatidyl choline and phosphatidyl ethanolamine contained in the aliquots at the indicated times are presented in Figs. 3 and 4. A progressive decrease took place in both lipids during the incubation time.

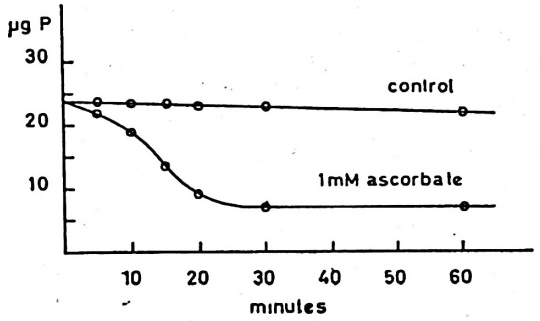

FIG. 3. Phosphatidyl choline changes during ascorbate swelling. Phospholipids were determined after chromatographic separation of total lipid extracts taken at the same incubation times as indicated in Fig. 2.

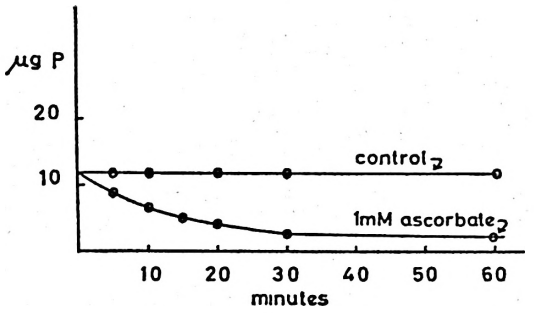

FIG. 4. Phosphatidyl ethanolamine changes during ascorbate swelling. See Fig. 3.

Figure 5 represents an autoradiogram of the chromatographed lipids after incubation of the P^{32} labeled mitochondria. A clear decrease in the radioactivity of the spots corresponding to phosphatidyl ethanolamine and phosphatidyl choline as compared to those of the incubated control can be easily appreciated. The autoradiogram also shows, together with this decrease in radioactivity, a streak due to some unidentified material produced by the effect of ascorbate on the phospholipids. It should also be noticed that no appreciable difference exists between the spots of the lipids extracted from incubated and nonincubated controls.

TABLE I

Effect of Ascorbate on P^{32} Labeled Mitochondria

Mitochondria were labeled with P^{32} prior to incubation with ascorbate (see details in text). Incubation medium: 1 mM ascorbate, 20 mM Tris-HCl buffer, pH 7.4, 0.25 M sucrose. Mitochondrial protein, 3.7 mg. Final incubation volume, 5 ml. Temp. 30° C. Phospholipids were extracted after 60 minutes and chromatographed.

Fraction	Nonincubated control		Incubated control		+ Ascorbate	
	Radioactivity	P	Radioactivity	P	Radioactivity	P
	c.p.m.	µg	c.p.m.	µg	c.p.m.	µg
Total phospholipid	50,000	37	50,000	37	35,800	25
Phosphatidyl choline	11,200	10.3	11,500	10.5	6,450	5.1
Phosphatidyl ethanolamine	10,180	3.6	10,200	3.6	1,030	0.3
Water soluble organic P	—	6	—	6	—	18

Table I summarizes the results obtained with P^{32} labeled mitochondria. In those mitochondria incubated with ascorbate the amount of phosphorus and radioactivity of the total phospholipid extract diminished 30 %. Most of this is accounted for by the loss of phosphatidyl choline and phosphatidyl ethanolamine; phosphorus and radioactivity of phosphatidyl choline decreased 50 % while that of phosphatidyl ethanolamine 90 %. It should also be noticed that the highest specific activity corresponds to phosphatidyl ethanolamine.

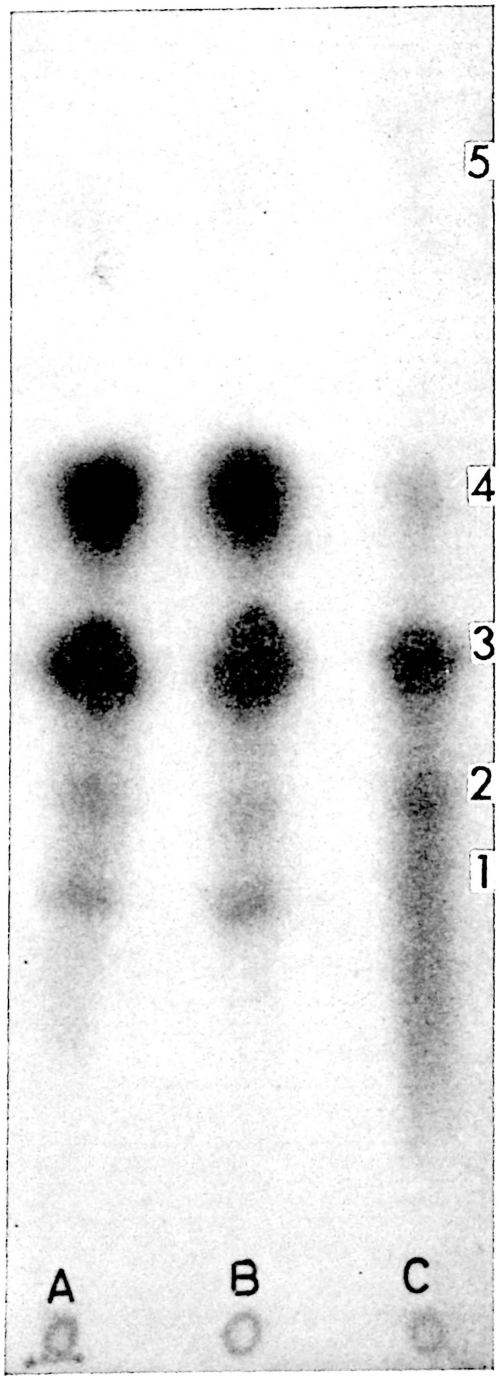

FIG. 5. Autoradiogram of radioactive phospholipids from mitochondria. Effect of ascorbate on the different phospholipids. Mitochondria were labeled with P^{32} prior to incubation with ascorbate. Medium: as in Fig. 1. Final incubation volume, 5 ml. Mitochondrial protein, 3.7 mg. Phospholipids were extracted after 60 minutes of incubation. A, Nonincubated control; B, Incubated control; C, Ascorbate added. Spots: 1, phosphatidyl inositol; 2, sphingomyelin; 3, phosphatidyl choline; 4, phosphatidyl ethanolamine; 5, cardiolipin.

Discussion

HUNTER *et al.* (7) have suggested that optical density changes during incubation in ascorbate could be explained by an increase in permeability followed by a disintegration of mitochondrial membranes. The same authors stated that ascorbate-induced swelling may be due to lipid peroxide formation.

Our results clearly establish the fact that ascorbate has a marked effect on mitochondrial phospholipids. The formation of peroxides alone would not explain the decrease of the phosphorus content in the total phospholipid extract since peroxides or the phosphorus containing compounds originated from breaking the double bonds of fatty acids would be expected to remain

in the organic phase during the extraction procedure; on the other hand water soluble organic phosphorus increased during incubation with ascorbate. Therefore it would seem reasonable that changes other than, or in addition to, peroxide formation have taken place in the phospholipid molecule to give a water soluble phosphorus containing moiety. For instance, ascorbate might render the mitochondrial lipids more accessible to the action of lipases.

The phosphorus containing material which streaks during chromatography (Fig. 5) could be the result of ascorbate-induced lipid breakdown. A knowledge of its chemical nature would be of great value for the understanding of ascorbate action. At present this point is receiving attention in our laboratory.

It is interesting to note that after the labeling of mitochondria with P^{32}, the specific activity in phosphatidly ethanolamine was higher than the average in the total phospholipid mixture. This higher phosphorus turnover may be an indication of some important role of phosphatidyl ethanolamine as a dynamic constituent of mitochondrial structure. It is also striking that phosphatidyl ethanolamine is the phospholipid mainly affected by the ascorbate incubation.

Knowledge of the chemical changes associated with mitochondrial swelling will lead to a better understanding of the process of permeability at the molecular level. In this respect the phospholipid composition may be of capital importance in regulating the permeability of mitochondrial membranes.

Summary

Ascorbate induced swelling and lysis of rat liver mitochondria causes a progressive decrease in phosphatidyl ethanolamine and phosphatidyl choline. The decrease of both these phospholipids is parallel to the usual optical density curve obtained during swelling experiments. Experiments were carried out with mitochondria labeled with P^{32} prior to incubation with ascorbate. Phospholipids were extracted and after chromatography autoradiograms were prepared. These data confirmed the breakdown effect of ascorbate on phospholipids.

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