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In vitro Effect of Ascorbate on the Phospholipid Composition of Various Subcellular Fractions

by

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In previous reports we have shown that rat liver mitochondria (6, 7) and inner mitochondrial membranes (8) incubated in the presence of ascorbate undergo profound changes in their total phospholipid content and composition; both phosphatidyl choline and phosphatidyl ethanolamine decreased to a great extent. The effect of ascorbate on the phospholipids is accompanied by lysis of the mitochondrial structure. We decided to investigate whether the action of ascorbate on phospholipids was limited to mitochondria and submitochondrial particles or, on the contrary, acted as well on other subcellular fractions.

In answering this question our approach has been to label rat liver slices by incubation in the presence of radioactive orthophosphate, to separate the various subcellular fractions, and after incubation in the presence of ascorbate to observe on autoradiograms of the extracted phospholipids the effect of ascorbate attack.

Materials and Methods

Male Wistar rats were fasted for 15 hours and killed. Two grams of liver slices were incubated for 3 hours at 37° C in 10 ml of bicarbonate-saline Krebs-Henseleit medium (3) containing 500 microcuries of radioactive orthophosphate. The slices were then removed from the radioactive medium, washed in cold 0.25 M sucrose and homogenized in 16 ml of 0.25 M sucrose with an all glass Elvehjem-Potter homogenizer. Nuclei and cell debris were sedimented at 800 × g.

Mitochondria were obtained by centrifuging 10 minutes at 8,500 × g. In order to thoroughly wash the mitochondrial fraction the pellet was succesively resuspended in 0.25 M sucrose and recentrifu-

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ged at 8,500 × g for 10 minutes, three times. To assure a pure microsomal fraction the supernatant was centrifuged at 15,000 × g for 10 minutes and the pellet discarded; the supernatant from this centrifugation was sedimented at 100,000 × g for one hour giving a microsomal pellet. The remaining supernatant was the cell soluble fraction. From a portion of the washed mitochondria, inner membranes were prepared by the method of PARSONS *et al.* (5) and thoroughly washed by resuspending them in 0.02 M phosphate buffer, *p*H 7.4, centrifuging at 1,900 × g for 15 minutes, and once more resuspending in 0.25 M sucrose and centrifuging at 8,500× g for 10 minutes.

From each of the inner mitochondrial membrane, microsomal, and cell soluble fractions, three aliquots were taken. One aliquot was incubated for 60 minutes at 30° C in 0.020 M Tris buffer, *p*H 7.4, 0.001 M ascorbate and 0.25 M sucrose. Controls without ascorbate were prepared by incubating another aliquot under the same conditions. The remaining aliquot served as a nonincubated control. After precipitation of proteins with $HClO_4$, phospholipids were extracted and phospholipid P determined as previously described (7). They were chromatographed on silicic acid impregnated paper by the method of MARINETTI *et al.* (4) and autoradiograms prepared. Protein was determined by the method of LOWRY *et al.* (2)

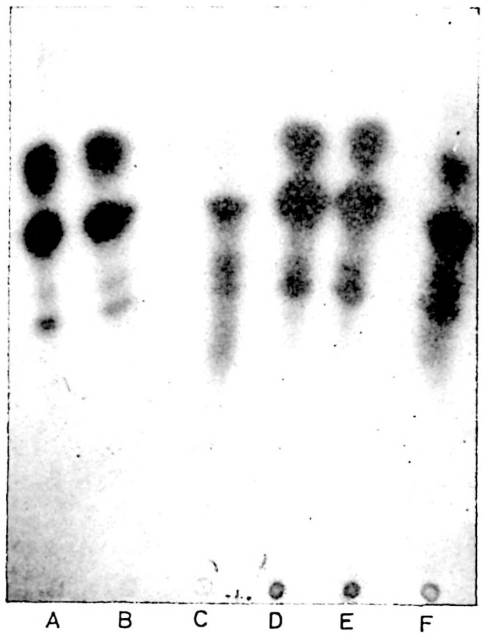

FIG. 1. Autoradiogram of P^{32} labeled phospholipids from inner mitochondrial membranes and microsomes. Effect of ascorbate on phospholipids of mitochondria and microsomes labeled prior to incubation with ascorbate. Samples: A: Inner mitochondrial membranes, non incubated control; B: Inner mitochondrial membranes, incubated control; C: Inner mitochondrial membranes, ascorbate added; D: Microsomes, nonincubated control; E: Microsomes, incubated control; F: Microsomes, ascorbate added. Spots: 1, Phosphatidyl inositol; 2, Sphingomyelin; 3, Phosphatidyl choline; 4, Phosphatidyl ethanolamine; 5, Phosphatadic acid. Developing solvent: Diisobutylketone, acetic acid and water (40:25:5) (4).

Results *

Figure 1 shows the autoradiograms of the chromatographed phospholipids extracted from the inner mitochondrial membranes and the microsomes. In the case of inner membranes it is readily apparent that of all the phospholipids extracted after incubation in ascorbate, phosphatidyl ethanolamine has practically disappeared and phosphatidyl choline greatly decreased. This ascorbate effect was much less marked in the microsomal fraction.

The autoradiogram of the phospholipids extracted from the cell soluble fraction is

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shown in Fig. 2. The spot mainly affected by ascorbate treatment is that of phosphatidyl ethanolamine.

Table I gives the amount of radiactivity and lipid P present in total phospholipid extracts before and after incubation and with and without ascorbate for the different subcellular fractions. Incubation alone does not induce appreciable changes. But incubation in the presence of ascorbate affected the total phospholipid content of all the subcellular fractions. This effect was most marked in the case of the inner mitochondrial membranes and the cell soluble fraction where total phospholipid content decreased 50 % and 70 % respectively, whereas the total phospholipid extract from the microsomes decreased only 14 %.

Table II shows the amounts of radioactivity and lipid P present in the total phospholipid extract as phosphatidyl choline and phosphatidyl ethanolamine after the various treatments of the three subcellular fractions. Ascorbate treatment mainly affected phosphatidyl ethanolamine; in the case of the inner mitochondrial membranes and the cell soluble fraction only negligible amounts of radioactivity were found in the corresponding spots for this phospholipid and lipid P was not detected, Likewise phosphatidyl choline decreased,

TABLE I

Effect of Ascorbate on Total Phospholipids of Different P^{32} Labeled Subcellular Fractions.

Two grams of rat liver slices were incubated for 3 hours at 37° C in 10 ml of bicarbonate-saline Krebs-Henseleit medium (3) containing 500 microcuries of radioactive orthophosphate. The various cellular fractions were obtained after homogenization by differential centrifugation (see details in text). Inner mitochondrial membranes, microsomes and cell soluble fraction were incubated in a medium containing 0.001 M ascorbate, 0.020 M Tris-HCl buffer, *p*H 7.4, and 0.25 M sucrose. Temp. 30° C. Incubation time, 60 minutes. Final volumes: inner mitochondrial membranes, 3 ml (12 mg of protein); microsomes, 10 ml (60 mg of protein); cell soluble fraction, 10 ml (13 mg of protein). Phospholipids were extracted after precipitation with $HClO_4$.

Fraction	Total phospholipids	
	Radioactivity c.p.m.	P μg
Inner mitochondrial membranes:		
Nonincubated control	5,635	65.3
Incubated control	5,510	63.2
Incubated in ascorbate	2,908	33.5
Microsomes:		
Nonincubated control	55,975	305
Incubated control	55,800	300
Incubated in ascorbate	48,190	260
Cell soluble fraction:		
Nonincubated control	3,350	20
Incubated control	3,100	19
Incubated in ascorbate	905	6.3

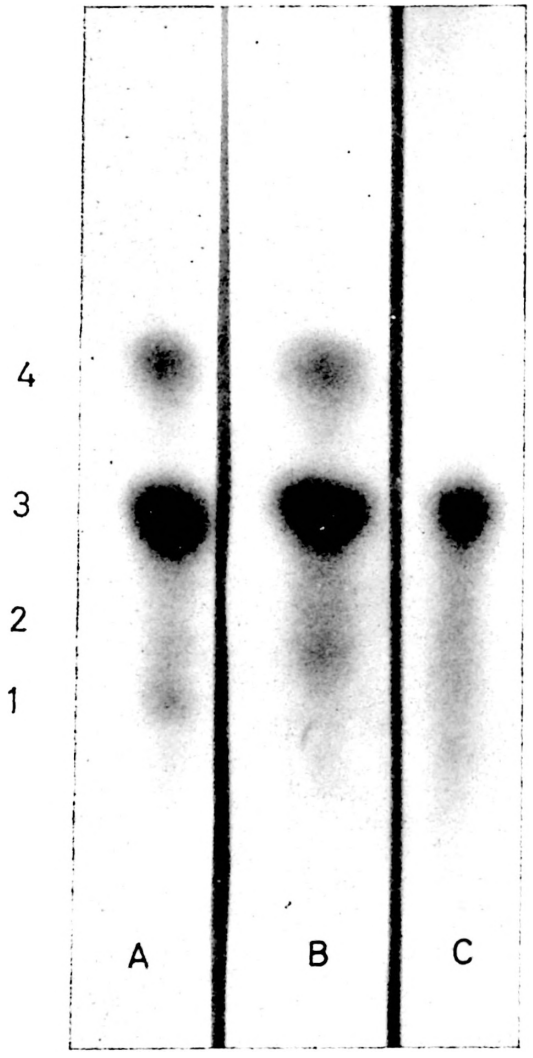

FIG. 2. Autoradiogram of P^{32} labeled phospholipids from cell soluble fraction. Effect of ascorbate on phospholipids of cell soluble fraction labeled prior to incubation with ascorbate. Samples: A: Nonincubated control; B: Incubated control; C: Ascorbate added. Spots: 1, Phosphatidyl inositol; 2, Sphingomyelin; 3, Phosphatidyl choline; 4, Phosphatidyl ethanolamine. Developing solvent: See Fig. 1.

but only 60 % in mitochondrial inner membranes and about 50 % in the cell soluble fraction. In the microsomal fraction, radioactivity decreased 46 % in phosphatidyl ethanolamine and 40 % in phosphatidyl choline.

Discussion

It is readily apparent from our results that ascorbate affects the total phospholipid content and composition in all the subcellular fractions studied. This effect was undoubtedly more pronounced in the inner mitochondrial membrane and cell soluble fractions and more restricted in the microsomal fraction. The phospholipid mainly affected in all cases was phosphatidyl ethanolamine.

The chemical nature of the ascorbate attack on the phospholipid molecule is not completely clear. One explanation could be that unsaturated fatty acids form peroxides, but doubts are raised that this gives a complete explanation; see discussion in Ref. 7. The difference observed in the various subcellular fractions could be due to specific fatty acid patterns in their phospholipids. However, this hypothesis would be difficult to reconcile with the data reported by BARTLEY (1) showing similar fatty acid patterns in the lipids from different subcellular fractions which tended to be characteristic of the lipid; on the other hand, the differences in fatty acid composition between the phosphatidyl choline and phosphatidyl ethanolamine reported by the same author would not be sufficient to justify the different effect of ascorbate on these two phospholipids in the various fractions studied. Therefore, relying on Bartley's data, the differences observed must be accounted for by other environmental conditions, such as protein make up and lipid-protein interactions.

TABLE II

Effect of Ascorbate on Phosphatidyl Choline and Phosphatidyl Ethanolamine of Different P^{32} Labeled Subcellular Fractions.

After incubation of different P^{32} labeled cellular fractions (see details in Table I) in ascorbate, phospholipids were extracted, chromatographed and autoradiograms prepared. Spots were cut out, radioactivity counted and lipid P determined.

Fraction	Phosphatidyl choline		Phosphatidyl ethanolamine	
	Radioactivity c.p.m.	Lipid P µg	Radioactivity c.p.m.	Lipid P µg
Mitochondrial inner membrane:				
Nonincubated control.	1,850	6.2	1,350	4
Incubated control	1,800	6	1,300	4
Incubated in ascorbate	650	2.5	20	n. d.
Microsomes:				
Nonincubated control.	26,700	79.3	11,375	36.5
Incubated control	26,200	75	11,180	35
Incubated in ascorbate	15,200	45.2	4,550	18.7
Cell soluble fraction:				
Nonincubated control.	1,340	4	588	2
Incubated control	1,350	4	560	2
Incubated in ascorbate	642	2	15	n. d.

Summary

The phospholipid composition of subcellular fractions of rat liver are affected by low concentrations of ascorbate when *in vitro*. Inner mitochondrial membranes, microsomes and cell soluble fraction were separated by differential centrifugation. Phosphatidyl choline and phosphatidyl ethanolamine decreased considerably in all fractions studied; phosphatidyl ethanolamine practically disappeared in the inner membrane and cell soluble fraction.

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