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Ascorbate Induced Lysis of Inner Mitochondrial Membranes. Isolation and Electron Microscopic Studies of Three Subfractions

by

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As already shown in previous reports (5, 6), ascorbate induced swelling followed by lysis is accompanied by changes in mitochondrial phospholipid content. In order to understand which part of mitochondrial structure is affected by the ascorbate treatment we have incubated inner membranes from rat liver mitochondria isolated by the method of PARSONS *et al.* (4). Ascorbate treatment of isolated inner mitochondrial membranes produces a similar effect to that already described for whole mitochondria. A decrease of the optical density takes place similar to that observed in large amplitude swelling. These optical density changes seem to be associated with lysis of the inner mitochondrial membrane structure, which in turn depends on alterations of its phospholipid composition, consisting mainly of a decrease in phosphatidyl choline and phosphatidyl ethanolamine.

Electron microscope studies confirm a clear disruption of the inner membrane structure due to ascorbate treatment.

Three subfractions obtained by differential centrifugation were completely different both from the morphological and chemical point of view. We think therefore that ascorbate can be considered as a highly useful tool, as compared to other mitochondrial lytic agents, for the study of submitochondrial structures.

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Materials and Methods

Phospolipids were chromatographed by the method of MARINETTI *et al.* (3) and determined as described in previous work (6). Protein was determined by the method of LOWRY (2). Rat liver mitochondria were obtained by the method of HOGEBOOM (1) in 0.25 M sucrose. Inner mitochondrial membranes were prepared by the method of PARSONS *et al.* (4) and thoroughly washed three times by resuspension in 0.02 M phosphate buffer and recentrifugation at 1,900 × g and once more by resuspension in 0.25 M sucrose and centrifugation at 8,500 × g.

Incubation of the inner mitochondrial membranes was carried out at 30° C during one hour in the same way as indicated in the preceding communication (6). The change in optical density was followed in a 1 cm cuvette in a Zeiss PMQII Spectrophotometer.

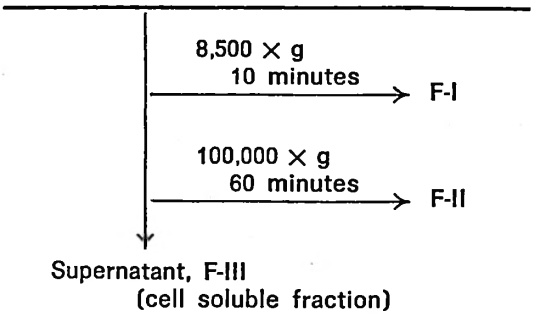
After the incubation, the suspension of inner mitochondrial membranes was chilled and centrifuged at 8,500 × g, during 10 minutes. The pellet obtained was called Fraction F-I. The supernatant was recentrifuged at 100,000 × g for one hour and another sediment was obtained; we will refer to this as Fraction F-II. The supernatant of this centrifugation will be referred to as Fraction F-III. This procedure is outlined in Table I.

Each fraction was examined by electron microscopy with negative staining after osmium tetroxide fixation, following a technique similar to that of PARSONS *et al* (4). The pellets obtained after centrifugation were resuspended in just enough unbuffered 0.25 M sucrose to give a slightly cloudy suspension. An equal volume of veronal buffered 2 % osmium tetroxide, *p*H 7.2, was added and the mixture kept at 2° C for 30 minutes. Preparations for electron microscopy were obtained by placing a small drop of the suspension on Formvar coated grids; excess liquid was removed with filter paper leaving a thin film; and then a small drop of 2 % phosphotungstate, *p*H 7.0, was applied to the grid. Supernatant fractions were studied by taking a small aliquot and mixing it with an equal volume of buffered osmium tetroxide and from this suspension grids were prepared as above. Electron micrographs were taken on a Siemens Elmiskop IA at 20,000 to 80,000 magnifications.

TABLE I

Procedure for Isolation of Subfractions of Inner Mitochondrial Membranes.

Differential Centrifugation
Suspension of inner mitochondrial membranes treated with ascorbate for 1 hour. Medium: 1 mM ascorbate, 20 mM Tris-HCl buffer, *p*H 7.4, 0.25 M sucrose:



Results *

Optical density changes during the incubation of the inner mitochondrial membranes in the presence of ascorbate are shown in Fig. 1. After a 5 minute lag a sharp decrease of the optical density took place reaching its lowest value around 30 to 40 minutes. The optical density of the controls remained completely unchanged.

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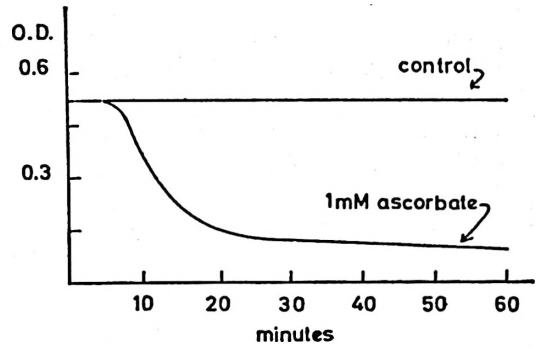

FIG. 1. Optical density changes of inner mitochondrial membrane suspension during ascorbate incubation. The decrease in optical density is related to structural disaggregation. Incubation medium: 1 mM ascorbate, 20 mM Tris-HCl buffer, 0.25 M sucrose. Mitochondrial membrane protein, 13.5 mg. Temp. 30° C. Incubation volume 30 ml.

TABLE II

Total Phospholipid, Phosphatidyl Choline and Phosphatidyl Ethanolamine in Inner Mitochondrial Membranes.

Incubation medium: 1 mM ascorbate, 20 mM Tris-HCl buffer, pH 7.4, 0.25 M sucrose. Inner mitochondrial membrane protein, 13.5 mg. Final incubation volume, 30 ml. Temp. 30° C. Phospholipids were extracted after 60 minutes of incubation.

Fraction	Nonincubated control μg P	Incubated control μg P	+ Ascorbate μg P
Total phospholipid	68	67	40.5
Phosphatidyl choline	14	13	7
Phosphatidyl ethanolamine	8.7	8.5	2

Table II shows the values of total phospholipid P, phosphatidyl choline and phosphatidyl ethanolamine in extracts of inner mitochondrial membrane before and after incubation with and without ascorbate. Table III gives the values of phospholipid phosphorus, protein, and lipid P to protein ratios for the inner membrane and the submembranous structures of F-I, F-II and F-III, as well as the percent protein recovery. Protein recovered in the material sedimented at 8,500 × g was as high as 95 % for the incubated control, whereas it was only 4.5 % for the membranes incubated with ascorbate.

Our inner membrane preparations showed the typical ultrastructure with the projecting subunits. The material sedimented at 8,500 × g from incubated controls had a preserved inner membrane ultrastructure and only very occasionally, slight structural alterations were found after incubation. An electron micrograph of this light disaggregation is shown in

TABLE III

Total Phospholipid, Protein, Lipid to Protein Ratios, and Percent Protein Recovery in Different Subfractions from Inner Mitochondrial Membranes.

Fraction	Lipid P μg	Protein mg	Lipid/Protein μg/mg	Protein Recovery %
Inner membrane	68	13.5	5.03	—
Incubated control 8,500 × g fraction	67	12.8	5.22	95
Fraction I	6.3	0.61	10.3	4.6
Fraction II	6.45	2.05	3.12	15.2
Fraction III	14	11	1.27	81.5

Fig. 2-A, which has proved to be of great value for the interpretation of the material found in Fraction F-III.

Fraction F-I (Fig. 2-B) is composed of smooth membranous structures, completely lacking projecting subunits. Fraction F-II (Fig. 2-C) is similar to Fraction F-I but its membranous structure is much thinner and contains small aggregates of projecting subunits. Fraction F-III (Fig. 2-D) is very homogeneous and contains only granular structures around 80 Å in diameter with some material which tends to aggregate them.

Discussion

The large decrease in optical density of the inner membrane suspension is due to structural disaggregation caused probably by the attack of ascorbate on the phospholipids.

Figure 2-A appears to show areas of disruption of the inner membrane structure into particles of aggregates of projecting subunits along with their basal material. The obvious similarities of the disrupted area in Fig. 2-A and the photograph, Fig. 2-D, of Fraction F-III lead us to interpret it as consisting of projecting subunits along with their basal material. We also have observed that Fraction F-III contains more than 80 % of the total inner membrane protein and furthermore the lipid P to protein ratio is much smaller than that of the whole inner mitochondrial membrane.

The smooth membranous structures of Fraction F-I, Fig. 2-B, have a higher lipid P to protein ratio than that of whole inner mitochondrial membrane. This observation suggests to us that in this fraction we are dealing with a special structure of the inner membrane which has separated as such under the disruptive action of ascorbate and which in turn resisted any further lytic effect. Separately we will publish more definite evidence showing that these smooth structures are probably present as such in the inner membrane.

Fraction F-II, constituted by small fragments of submembranous structures, probably has a different nature than those of Fraction F-I, since its morphological appearance and lipid P to protein ratio are also different; however a definite answer must wait.

The fact that phosphatidyl ethanolamine and phosphatidyl choline decrease during ascorbate attack and the fact that different fractions do separate lead us to suggest that these phospholipids are in some way responsible for holding together different special structures of the inner mitochondrial membrane.

We are confident that this approach will prove to be a highly valuable tool in the study of mitochondrial structure.

Summary

Ascorbate treatment of isolated inner membranes from rat liver mitochondria produces a similar effect to that already described for whole mitochondria. A decrease of the optical density takes place similar to that observed in large amplitude swelling. These optical density changes seem to be associated with lysis of the inner mitochondrial membrane structure, which in turn depends on alteratations of its phospholipid composition, consisting mainly of a decrease in phosphatidyl choline and phosphatidyl ethanolamine.

FIG. 2. Electron micrographs of inner mitochondrial membrane subfractions. A: Tubular structure with projecting subunits. One of the tubules shows an area of disaggregation which constituted a very occasional finding in controls incubated without ascorbate. B: Smooth membranous structures corresponding to Fraction F-I. C: Smooth membranous structures corresponding to Fraction F-II. D: Granular structures corresponding to Fraction F-III, identical in appearance to the area of disaggregation shown in A.

Electron microscope studies confirm a clear disruption of the inner membrane structure due to ascorbate treatment.

Three subfractions obtained by differential centrifugation were completely different both from the morphological and chemical point of view.

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