THE EXTRACTION OF STRUCTURAL PROTEIN FROM SUBMITOCHONDRIAL VESICLES

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Structural protein can be isolated from mitochondria by extraction with acid leaving most, if not all, of the phospholipid in the membranous residue (Zahler, Saito and Fleischer, 1968). On the other hand, the results of Richardson, Hultin and Fleischer (1964) show that isolated structural protein combines with mitochondrial phospholipid. The question remains whether structural protein is associated with phospholipid in the mitochondrion. It is possible that acidification of the mitochondria causes alteration and/or redistribution of the lipid and therefore such studies may not be relevant to the association of lipid with protein within the mitochondrion. We wish to report an independent procedure using urea for the extraction of structural protein. Membranous residues from urea and acid extraction of heart, kidney and liver submitochondrial vesicles have the same lipid content. These studies are compatible with the view that structural protein is not associated with lipid in the mitochondrion.

Materials and Methods

All operations unless otherwise specified were carried out at 0 - 4° C. Mitochondria were prepared from bovine tissues as described by Fleischer et al. (1967) except that 0.01 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.55, in 0.25 M sucrose was used as buffer. A Potter-Elveh-

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jem homogenizer was used in place of a Waring Blender in the preparation of liver and kidney mitochondria*.

Submitochondrial vesicles were prepared from mitochondria (2-10 mg protein/ml 0.25 M sucrose) with the use of a French Pressure Cell at a pressure of 2000 pounds per square inch (American Instrument Co., Inc., Silver Spring, Md.) (Fleischer, Fleischer and Stoeckenius, 1967). They were sedimented for one hour at 134,000 x g (average) and resuspended in 0.25 M sucrose.

Extraction of vesicles with urea: Vesicles, (5-20 mg protein in 0.75 ml 0.25 M sucrose) were mixed with 4.75 ml 8 M urea at room temperature. The mixture was placed in the cold for 30 minutes, then sedimented at 45,000 rpm for 150 minutes in the SW50 rotor in the Spinco Preparative Ultracentrifuge. The pellet was resuspended in 0.25 M sucrose.

Extraction of vesicles with acid: Vesicles (5-15 mg protein) in 4.4 ml 0.25 M sucrose) were mixed together with 1.1 ml of 7% acetic acid. After 30 minutes, the mixture was sedimented in the SW50 rotor at 45,000 rpm for 60 minutes. The residue was resuspended in 0.25 M sucrose.

The supernatant from urea or acid extraction was dialyzed vs 100 volumes of 5 mM Tris.HCl-0.25 mM EDTA, pH 8.0. The dialyzate from urea extraction was sedimented at 45,000 rpm for 60 minutes in a #50 rotor. The brown button was removed and resuspended for analysis. The resulting supernatant was acidified to pH 6.5. After 30 minutes, the white precipitate was collected by centrifuging in the clinical centrifuge and then resuspended for analysis.

The fractions were characterized by polyacrylamide gel electrophoresis and electron microscopy as previously described (Zahler, Saito and Fleischer, 1968) and analyzed for phosphorus (Chen et al., 1965) and protein (Lowry, et al., 1951).

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*We wish to thank Mr. Niels Nielsen for providing the liver and kidney mitochondria.

TABLE I

Comparison of Mitochondria, Vesicles and Extracted Vesicles

Source	Mitochondria	Vesicles	Urea Residue*		Acid Residue*	
	µg P/mg†	µg P/mg†	% Protein	µg P/mg†	% Protein	µg P/mg†
Heart	16.6	17.8	40	31	59	31
Kidney	12.2	16.4	33	35	57	35
Liver	8.6	17.2	25	35	52	36

*The extraction with acid or urea was carried out as described in "Materials and Methods". The amount of heart, kidney and liver mitochondria for extraction was 6, 6, and 10 mg respectively.

†The phosphorus is presented as µg phosphorus per mg protein.

Results

Mitochondria can be disrupted into small vesicles with the use of the French Pressure Cell. Submitochondrial vesicles from heart, kidney and liver mitochondria have higher phosphorus to protein ratios than the original mitochondria, and the ratios approach the same values (cf Table I). Further, a four-fold range of concentration (2.5 to 10 mg/ml) during vesiculation gives essentially the same phosphorus to protein ratio. When these vesicles are extracted, the phosphorus to protein ratio of the residue (pellet) is nearly two-fold higher than in the starting material (Table I). Similar ratios are obtained for the residues from heart, kidney and liver vesicles. It is significant that both the acid and urea methods give the same ratios.

Patterns obtained by polyacrylamide gel electrophoresis of the residues from beef heart submitochondrial vesicles extracted with either acid or urea appear very similar. The patterns for beef heart submitochondrial vesicles and derived fractions are shown in Fig. 1. The major band previously designated as structural protein (Zahler, Fleischer, and Saito, 1968) is missing in the extracted residues. The structural protein fraction as isolated by

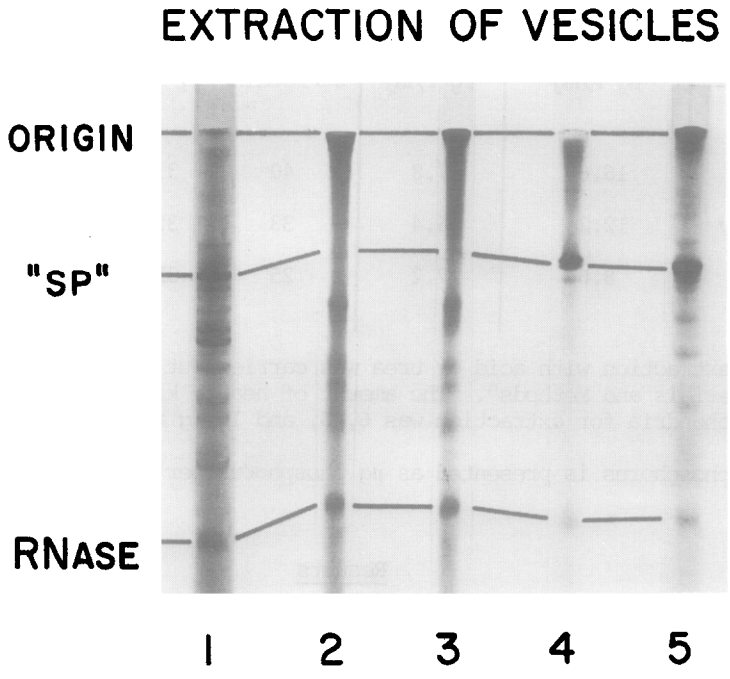

Fig. 1. Polyacrylamide gel electrophoresis of beef heart submitochondrial vesicles and fractions obtained by urea and acid extraction. They are: 1) submitochondrial vesicles; 2) the residue after urea extraction; 3) the residue after acid extraction; 4) structural protein isolated from the urea extract; and 5) structural protein isolated from the acid extract. Electrophoresis was carried out for 3-1/2 hours at 2.5 milliamps per tube. Ribonuclease has been added to each tube so that relative mobilities can be measured for characterization of the bands.

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both procedures is shown for comparison. Both procedures are therefore equally effective in removing structural protein associated with the membranes.

It may be noted in Table I that the percent protein remaining in the residue fraction is greater with acid extraction. The urea procedure gives lower values because of technical problems arising from the density of the 7 M urea. For this reason the acid extraction procedure gives a more reliable distribution of protein. The percent protein recovered in the residue frac-

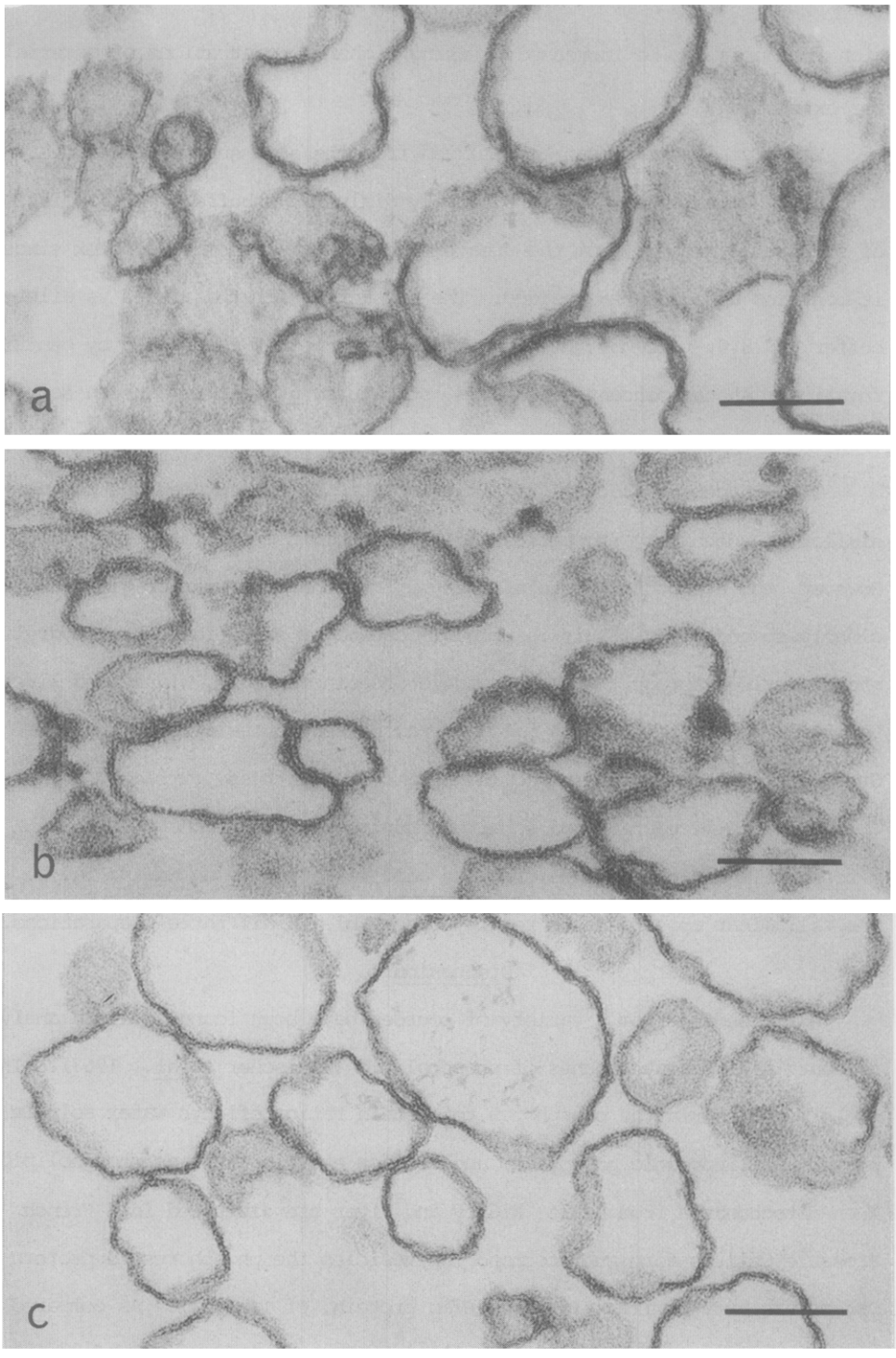

Fig. 2. Electron micrographs of: a) Beef heart submitochondrial vesicles; b) the urea-extracted residue; and c) the acid-extracted residue. The trilaminar arrangement is visible in each preparation. The optical magnification is 50,000; the bar represents 0.1 micron.

tion from urea can be increased by using higher concentrations of material for extraction.

Structural protein can be isolated from the acid supernatant either by direct neutralization to pH 6.5 or by dialysis vs buffer. The isolation of structural protein from the urea extract is somewhat more complex since it contains unsedimented residue. The extract is first dialyzed vs dilute buffer, pH 8.0, to remove the urea and a brown pellet is removed by centrifugation. At the concentration used, structural protein remains in solution and is precipitated by titration to pH 6.5.

The structural protein fractions isolated by both procedures are very similar in solubility properties and electrophoretic mobility (Fig. 1). However, the structural protein isolated from the urea extract gives two closely spaced bands. This is probably caused by oxidation of sulfhydryl groups during storage. The extractable protein of heart, kidney and liver vesicles amounts to 40 - 45% based on the percent protein in the residue. Structural protein is the predominant component in these extracts.

Electron micrographs of submitochondrial vesicles from beef heart mitochondria and the derived urea and acid residues are shown in Fig. 2. The trilaminar appearance is readily observable in all three preparations.

Discussion

Mitochondria from a variety of sources have been found by lipid analysis to contain different amounts of phospholipid (Fleischer et al., 1967). In whole mitochondria the phosphorus to protein ratio reflects water soluble phosphorus (inorganic phosphate, nucleotides etc.) as well as phospholipid. When mitochondria from heart, kidney and liver are disrupted in a French Pressure Cell to form submitochondrial vesicles the phosphorus to protein ratios are very similar (16-18 μg P/mg protein, cf Table I). As compared with the original mitochondrion the phosphorus to protein ratio of vesicles is a better index of phospholipid content, and is indicative of a similar lipid content in these preparations.

The extraction of submitochondrial vesicles with acid or urea removes structural protein leaving a membranous residue with a high phosphorus to protein ratio. On the other hand the structural protein fraction contains little phosphorus. These data are in good agreement with previous results (Zahler, Saito and Fleischer, 1968) for the acid extraction of whole mitochondria. The agreement of the phosphorus to protein ratios and the similarity of the electrophoresis patterns lead to the conclusion that the residue from urea extraction has the same composition as the residues from acid extraction of either vesicles or whole mitochondria. Since extraction with urea produces the same results as with acid, it is unlikely that redistribution of lipid occurs. Further, the complex of structural protein with mitochondrial phospholipid, prepared according to Richardson, Hultin and Fleischer (1964), is not dissociated by urea (S. Fleischer, unpublished studies). This evidence clearly indicates that structural protein is not associated with lipid in the mitochondrion.

Finally, the residues from acid and urea extraction retain the trilaminar arrangement characteristic of membranes. Thus, it is clear that structural protein is not required to maintain the structure of the membrane as observed by electron microscopy. In light of these results the suggestion that structural protein performs a structural role in the membrane will have to be reevaluated (Green et al., 1961; Criddle et al., 1962; Richardson, Hultin and Green, 1963).

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