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THE RELATION BETWEEN STRUCTURE AND FUNCTION IN ELECTRON-TRANSPORT SYSTEMS

II. EFFECT OF SOLVENT TREATMENT ON MEMBRANE STRUCTURE

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SUMMARY

Changes in the structure of membrane fragments from beef heart mitochondrial cristae have been observed by negative staining technique following solvent treatment of the membranes. The changes in structure have been related to previously observed changes in enzyme activity. Isooctane treatment results in very little change in outer knob or inner bead structure except to cause an apparent spreading of the inner beads. Both succinoxidase and NADH-oxidase activity can be recovered. Ether treatment causes loss of outer knobs and a breakdown of inner beads to 30-Å particles. Succinoxidase activity is unaffected by this treatment but NADH-oxidase activity is lost. Finally acetone extraction causes both loss of knobs and further disintegration of inner beads to less than 30-Å units. Succinoxidase activity can still be restored to these membranes by addition of coenzyme Q, phospholipid and cytochrome *c*, whereas NADH-oxidase activity cannot be restored. In contrast to the changes observed by negative staining, membranes fixed with osmium tetroxide and sectioned still show normal membranous structure.

INTRODUCTION

The use of solvents to extract lipids from the membranes of cellular organelles has made possible the demonstration of a functional role for compounds such as coenzyme Q, vitamin K, and plastoquinones[1]. Extensive extraction has also been used to show the importance of phospholipids in mitochondrial electron transport[2]. Previous studies[3] on the ultrastructure of solvent-extracted membranes using classical electron-microscope techniques of fixation, embedding and sectioning have failed to reveal changes which correlate with lipid removal or enzyme inactivation.

In the present study using negative staining techniques, we have shown profound changes in membrane structure following solvent extraction. Extractions

using both polar and non-polar solvents are compared here, since it has previously been shown that very different patterns of enzyme inactivation result from these treatments[4,5]. A preliminary report of these results has been presented[6].

METHODS

Mitochondria were isolated by the method of LÖW AND VALLIN[7] and were stored in concentrated suspensions at −20° for periods up to one month before use. The following procedure was followed for all extractions whether with ether, isooctane or acetone. An aliquot of frozen mitochondria was thawed and added to a separatory funnel containing 5 volumes of solvent. The funnel was shaken for 1 h at room temperature (22°) in a mechanical shaker or for 1 min by hand in a cold room at 4° (in the latter case the solvent was also 4°). The residue was allowed to settle for 1 min, drained out of the funnel, and diluted with an equal volume of cold 0.25 M sucrose solution. It was centrifuged at 100 000 × *g* for 30–45 min to remove excess solvent and the pellet was resuspended by homogenization in 0.25 M sucrose solution.

NADH- and succinate-oxidase activities were measured polarographically as previously described[4]. Cytochrome- and indophenol-reductase activities were measured spectrophotometrically as previously described[4].

Small aliquots of the control or extracted mitochondria were prepared for electron microscopy by fixation for 2–4 hours at 0° in 1% osmium tetroxide buffered at pH 7.3 with 0.1 M phosphate. They were dehydrated in acetone and embedded in the Epon–Araldite mixture of MOLLENHAUER[8]. The sections were poststained with 1% barium permanganate. Other samples were diluted with distilled water to a protein concentration of about 1 mg/ml. A small amount of bovine serum albumin was added to the extracted membranes to facilitate spreading. A drop of the membrane suspension was spread on a carbon-stabilized, collodion-coated grid. A drop of 2% phosphotungstate solution neutralized to pH 6.8 with KOH was added, and as much liquid as possible was drawn off with filter paper. The grids were air-dried and observed and photographed in a Philips EM 200.

RESULTS

The most striking feature of negatively stained mitochondrial membranes are the rows of knob-like subunits seen in profile around the edges of the cristae fragments (see Fig. 1). These subunits have been named elementary particles by FERNANDEZ–MORAN *et al.*[9] who consider them to be part of a tripartite structure consisting of the 90-Å external knob or headpiece connected by a short stalk to a basepiece embedded in the membrane. When the knobs are clearly outlined by the phosphotungstate around the periphery of a cristae fragment, they usually seem to be attached to a continuous electron-translucent layer along the membrane edge which is not penetrated by the stain. This layer makes it difficult to ascertain the dimensions of the basepiece or its relation to the external units. Globular structures are seen in surface view on the membrane fragments, but it is difficult to distinguish which are embedded in the membrane and which protrude from it. The location of the mitochondrial enzyme complexes within the membranes has not been clearly

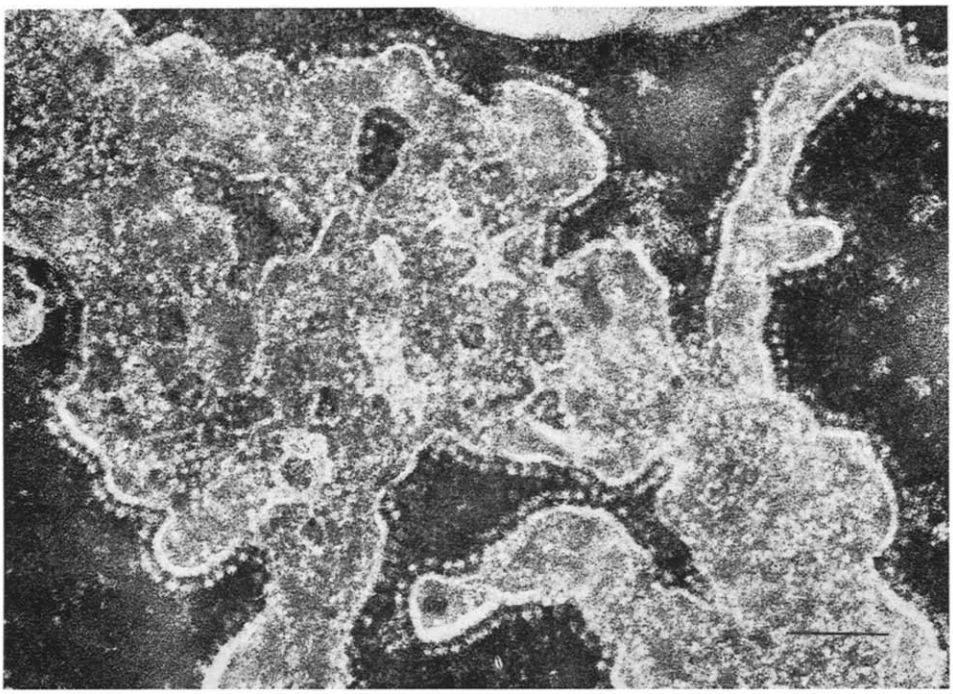


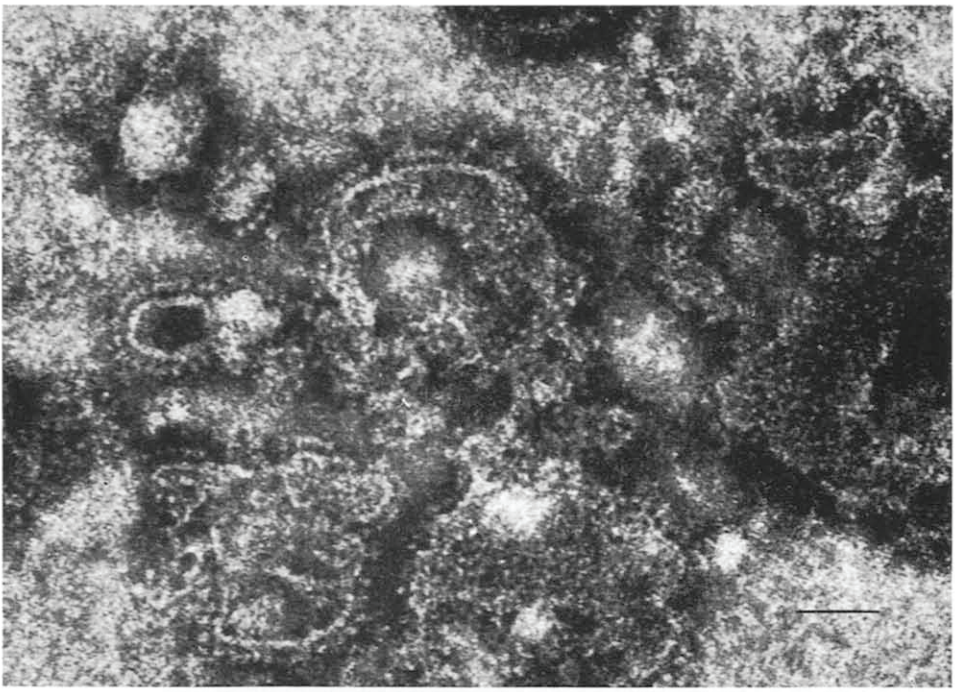
Fig. 1. Beef heart mitochondria membrane fragments negatively stained with phosphotungstate showing globular particles in profile and surface view. Marker represents 1000 Å.

established. It has been suggested that the elementary particles are the site of either the electron-transport chain[9] or the coupling factors for oxidative phosphorylation[10].

Mitochondrial membranes which have been fixed with osmium, embedded in plastic and sectioned thinly appear as typical unit membranes[11] as previously reported[12,13]. No surface structures are visible after this preparative technique, and no differences are distinguishable between the inner and outer mitochondrial membranes.

Extraction of mitochondria with non-polar solvents, such as isooctane results in the removal of coenzyme Q and a decrease in the succinate-oxidase activity. Restoration of the enzyme activity can be achieved by the readdition of coenzyme Q or cytochrome *c*. NADH-oxidase activity may even be increased by isooctane extraction[14,15]. We have not been able to find any change in the appearance of the mitochondrial membranes either by negative or positive staining after isooctane extraction. The membranes become more difficult to stain with phosphotungstate, but fairly regular arrays of subunits remain attached to the membranes (Fig. 2). The globular pattern within the membrane interpreted as representing basepieces of the elementary particles is often clearer after isooctane extraction. Due to the difficulty in staining extracted material, the spacing of these globules is not accurately measurable, but it may be that lipid has been removed from between the membrane-bound globules increasing the penetration of the stain.

In contrast to the results obtained with non-polar solvents, polar solvents produce very drastic changes in both the enzymatic activity and structure of mito-



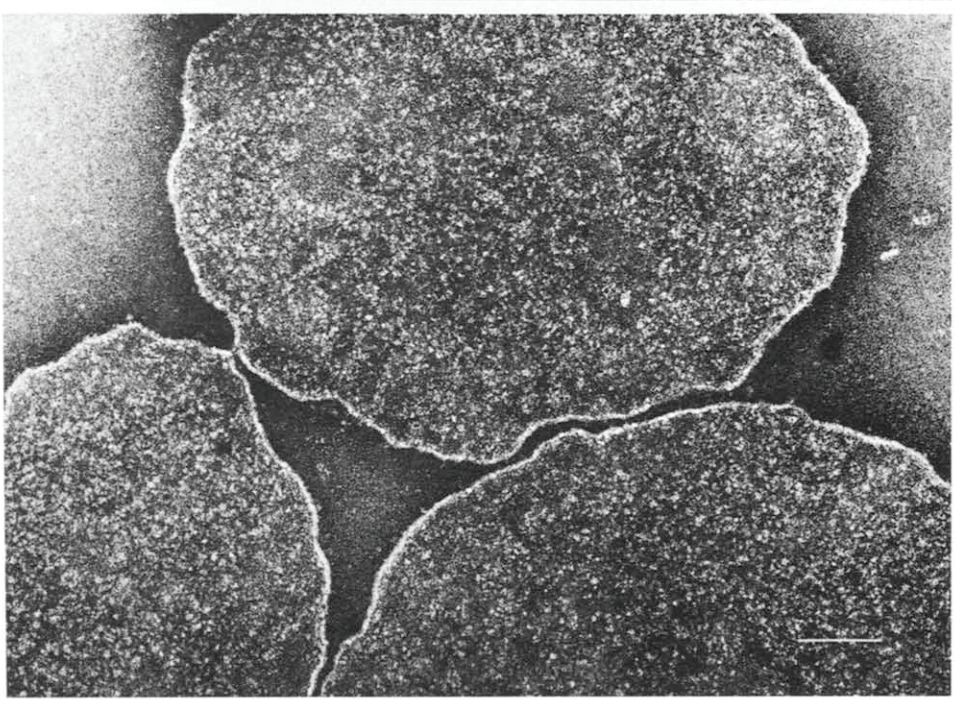


Fig. 2. Isooctane-extracted membrane residue. Note rows of elementary particles protruding from the membrane edges. Marker represents 1000 Å.

Fig. 3. Mitochondrial membranes after 1-min ether extraction. Phosphotungstate staining. Marker represents 1000 Å.

chondria. As has been previously reported[4] treatment with diethyl ether for as short a time as 1 min causes a complete loss of NADH-oxidase activity. The rate of succinate oxidation by the membrane residue is increased, however, by such treatments (Table I). No measurable removal of lipid material occurs under these conditions but an enzyme protein is released which we have identified as an amytal-sensitive NADH dehydrogenase[4].

TABLE I

EFFECT OF ETHER TREATMENT ON ENZYME ACTIVITY IN MITOCHONDRIA

Reaction conditions for: (i) NADH oxidase and succinoxidase: phosphate buffer, 100 μmoles (pH 7.2); mitochondrial protein, 0.5–2.0 mg; cytochrome *c*, 0.09 μmole, where indicated; NADH 3 μmoles or succinate 10 μmoles. (ii) NADH–cytochrome *c* reductase and NADH–DCIP reductase: phosphate buffer, 60 μmoles (pH 7.5); mitochondrial protein, 0.1–0.6 mg; NADH, 0.6 μmole; dichlorophenolindophenol (DCIP), 0.09 μmole or cytochrome *c*, 0.09 μmole, *plus* KCN, 10 μmoles. Values are expressed in μmole substrate oxidized per min per mg enzyme protein.

Treatment	*NADH oxidase (i)*		*Succinoxidase (i)*	*NADH–cyt. c reductase (ii)*	*NADH–DCIP reductase (ii)*
	−cyt. c	*+cyt. c*			
Control	0.044	0.144	0.042	0.142	0.078
Ether (1 min)	0.000	0.004	0.175	0.018	0.053
Ether (1 h)	0.003	0.004	0.075	0.022	0.080
Isooctane (1 h)	0.070	—	0.020	—	—

A negatively stained image of short-term ether-treated mitochondrial membranes is presented in Fig. 3. The filamentous cristae fragments outlined by rows of knobs seen in the control preparations are no longer visible. Instead large, rounded membrane fragments are seen which have a finely granular surface. The tightly packed granules are considerably smaller than those seen on the surface of normal mitochondrial membranes, being about 30–40 Å in diameter. Knob-like particles have not been found either in the membrane fraction or in the supernatant solution.

Longer extraction with ethyl ether, up to several hours of shaking at room temperature, results in the removal of lipid from the mitochondria but causes little further change in the enzyme activities (Table I). The main structural effects of this treatment are a reduction in the size of the membrane fragments and a change in the surface structures. Instead of the fine granules seen in Fig. 3 membrane surface has a fuzzy appearance (Fig. 4).

When the ether-extracted membranes are fixed, embedded, and sectioned, the original triple-layered structure appears unaltered. Long membrane profiles are visible, supporting the conclusion suggested by negative staining, that the mitochondrial membranes may have been broken and reformed into large, flattened vesicles.

Acetone extraction has an effect similar to that observed after extensive ether extraction. When examined by negative staining, the membrane residue consists of small vesicular fragments which have a fuzzy surface, lacking the typical protruding knob structures (Fig. 5). This effect is observed either after extraction

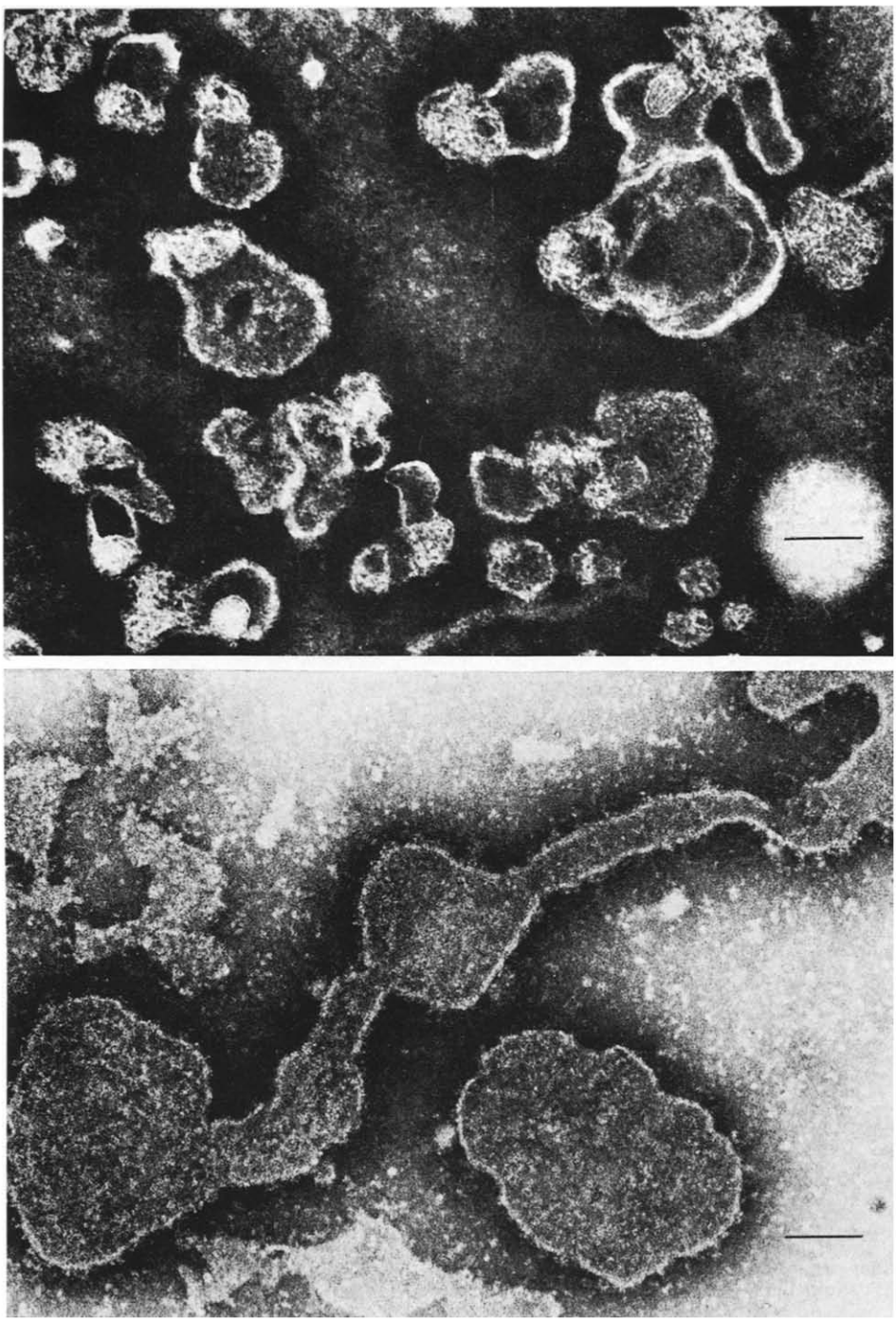


Fig. 4. Mitochondrial membrane after long-term ether extraction (1 h). Marker represents 1000 Å.

Fig. 5. Mitochondrial membrane after extraction with 96% acetone for 1 h. Similar structure is observed after extraction with 90% acetone for 10 min at 0°. Magnification 108 000 ×. Phosphotungstate staining.

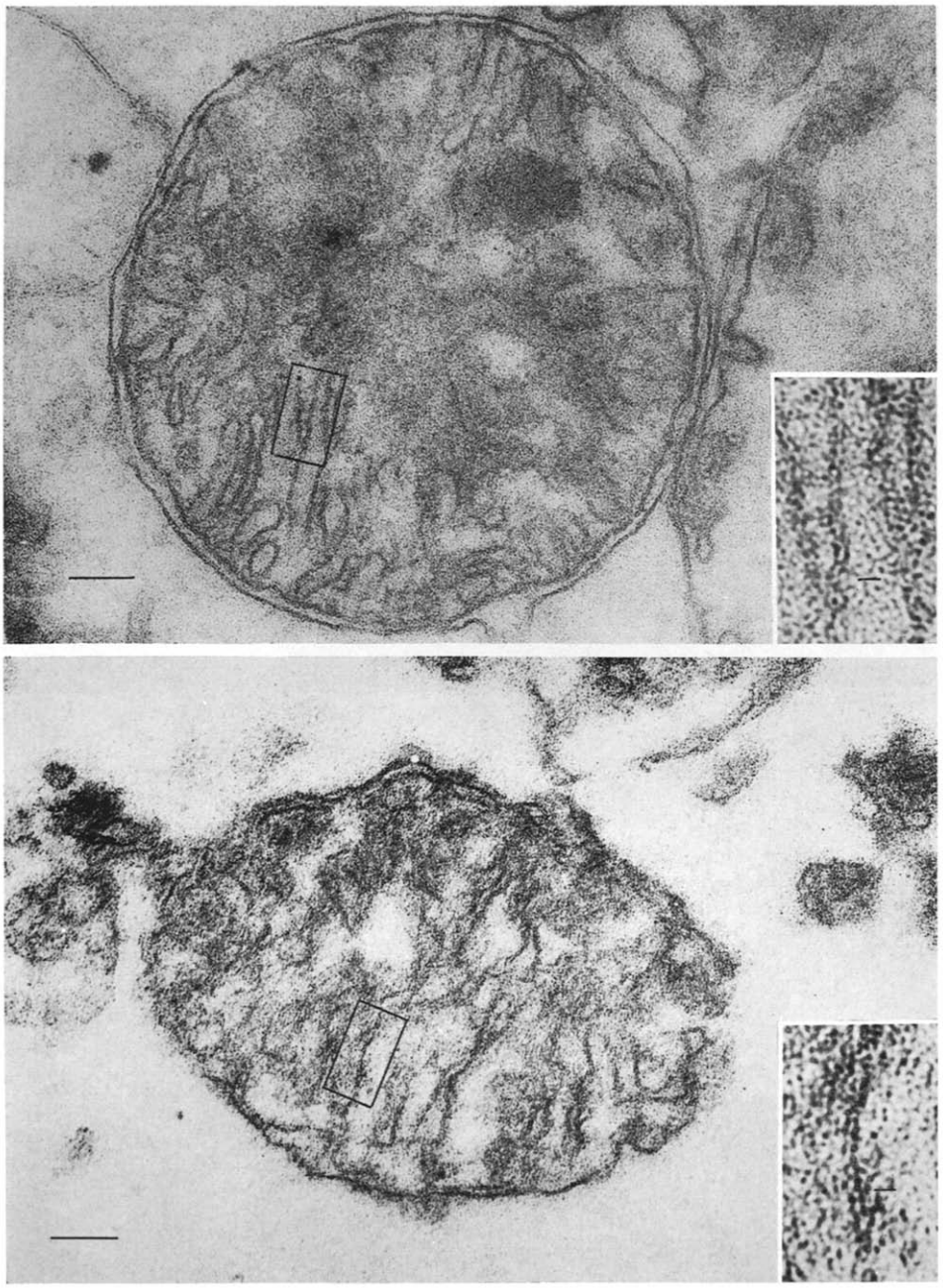


Fig. 6. Section of untreated mitochondria fixed with osmium tetroxide and poststained with barium permanganate after sectioning. Magnification 88 000 ×. The marker represents 1000 Å. Insert shows detail of membrane sections. Marker represents 100 Å.

Fig. 7. Section of acetone-extracted mitochondria fixed as for Fig. 6. Magnification 88 000 ×. The marker represents 1000 Å. Insert shows membrane detail. Note that the dimensions of the sectioned membrane are about the same as in the untreated material. Marker represents 100 Å.

with aqueous acetone or after lyophilization and extraction with dry acetone. Untreated mitochondria fixed with osmium tetroxide and sectioned are shown in Fig. 6. A similar section of mitochondria extracted according to FLEISCHER *et al.*[3] is shown in Fig. 7. As has been previously reported[5,16] the acetone-extracted membranes show the typical unit membrane structure without any change in spacing relatable to the removal of lipid. Like the ether-extracted membrane, the acetone residue very often does not show typical mitochondrial form but seems to be aggregated into long vesicular profiles.

Both NADH-oxidase and succinate-oxidase activity are lost after acetone extraction. NADH oxidase cannot be restored by any known additive but succinate oxidase or succinate cytochrome-*c* reductase activity can be restored by adding back coenzyme Q_{10} and phospholipid to the extracted membranes.

DISCUSSION

The relation between structures observed in dry films exposed to phosphotungstate and the structures present in aqueous suspension remains to be determined. It appears that there are two alternative explanations of the structural phenomena which we observe. One explanation is that the structures observed actually represent structures present in the hydrated membrane and the changes observed after solvent extraction represent rearrangement in the membrane components reflected by alterations in enzymatic activity. A second explanation is that the structures observed with phosphotungstate may be reconstructions of the components of the hydrated membrane. In this case solvent treatment may not cause actual change in shape in the hydrated membrane but may predispose the membrane to break down into a more dispersed structure during the staining procedure. In other words the solvent treatment would weaken some bonds in the membrane which would be disrupted on exposure to drying and phosphotungstate. The fact that acetone-extracted membranes show the same double-layered structure as untreated membranes when fixed with osmium tetroxide and sectioned can be taken as evidence that the solvent treatment is only weakening bonds and not causing an immediate change in membrane structure. An alternative explanation is that reduced osmium is deposited at the interfaces of membranes giving a unit membrane image regardless of the actual structure. The release of a NADH dehydrogenase (EC 1.6.99.3) by ether treatment[4] which is not released by sonication or freezing and thawing certainly indicates weakening of inner membrane bonding and perhaps actual structural change which permits the release of the enzyme. Other approaches such as low-angle X-ray diffraction studies of the membranes will be desirable before a clear decision concerning the sequence of events can be made.

It is clear from these studies that an intact electron-transport system does not require the type of membrane structure which appears to have 90-Å knobs either on or within the membrane after negative staining. By the simplest interpretation one can propose that electron transport is accomplished by interaction of 30–40-Å units. Size considerations eliminate the possibility of a complete electron-transport chain in one 30–40-Å unit so the parts of the chain must be variously disposed in different units[7]. If phosphotungstate staining truly represents membrane structure then electron transport can continue even when the 30–40-Å structures are no longer

associated in larger units within the membrane. If phosphotungstate staining merely indicates a predisposition to disruption on drying because of weaker association of components, then the larger 90-Å units may be necessary for electron transport. This weakening should be reflected in a greater fragility of the electron-transport system in extracted membranes than in normal mitochondria. So far the only evidence we see for this in the succinoxidase system is the requirement for cytochrome *c* after ether treatment. Since a cytochrome *c* requirement is also induced by isooctane treatment this requirement does not correlate well with the observed structural change.

Acetone treatment provides a further useful dimension to this study in that a requirement for coenzyme Q is induced in the succinoxidase system together with a requirement for cytochrome *c*. This treatment extracts lipids from the mitochondria and the average size of the residual units in the membrane is smaller than after any other treatment. The fuzzy surface appearance observed after this treatment may be the result of disruption of lipoprotein bonds. Extraction of lipids from the 30-Å units would be consistent with these observations and addition of coenzyme Q and cytochrome *c* may be necessary to allow the 30-Å units to interact to complete an electron-transport system. One approach to the effects which we see is to interpret the 30-Å units as units of the electron-transport system as envisioned by the fractionation studies of GREEN AND FLEISCHER[2]. They find fractions which include the succinic- and NADH dehydrogenases, a cytochrome *b* and c_1 complex and cytochrome–oxidase complex. Perhaps the 30-Å units which we see represent individual units. If so, separation of the individual units or complexes should be facilitated by solvent treatments.

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