

BIOCHEMICAL AND ULTRASTRUCTURAL PROPERTIES OF OSMOTICALLY LYSED RAT-LIVER MITOCHONDRIA

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ABSTRACT

Isolated rat-liver mitochondria were osmotically lysed by suspension and washing 3 times in cold, distilled water. Pellets obtained by centrifugation at 105,000 *g* for 30 min were resuspended, fixed with glutaraldehyde and OsO₄, and embedded in Epon 812. Thin sections show the presence of two distinct membranous populations, each of which is relatively homogeneous in size and appearance. Swollen mitochondria (~1.5 μ in diameter), which have been stripped of their outer membranes, are largely devoid of matrix and normal matrix granules and are referred to as "ghosts." The smaller (0.2 to 0.4 μ in diameter), empty appearing, vesicular elements, derived primarily from the outer mitochondrial membrane, can be differentiated from the ghosts on the basis of their smaller size and complete absence of internal structures, especially cristae. Each membranous element is enclosed by a single, continuous membrane; the "double membrane" organization typical of intact mitochondria is not observed. These findings indicate that the outer membrane of rat-liver mitochondria is spatially dissociated from the inner mitochondrial membrane by osmotic lysis of the mitochondria in distilled water.

Three parameters of structural and functional significance in freshly isolated rat-liver mitochondria have been correlated with the structural alterations observed: (a) chemical composition (total protein, lipid phosphate and total phosphate), (b) specific and total activities of marker enzymes for mitochondrial matrix and membranes (malate dehydrogenase (MDH), D- β -hydroxybutyrate dehydrogenase (BDH) and cytochromes), and (c) integrated multienzyme functions (respiration, phosphorylation, and contraction). The data presented indicate that all mitochondrial membranes are completely conserved in the crude ghost preparation and that, in addition, about $\frac{1}{3}$ of the matrix proteins (estimated by assays for MDH activity and protein) are retained. The study of integrated mitochondrial functions shows that a number of physiologically important multienzyme activities also are preserved in the water-washed preparation. The respiratory rate of ghosts per milligram of protein is 1.5 to 2.0 times that of intact mitochondria, which shows that the respiratory chain in the ghosts is functionally intact. The rate of phosphorylation is reduced, however, to about 25% of that measured in freshly isolated mitochondria and accounts for lowered P:O ratios using succinate as substrate (P:O ranges from 0.4 to 0.9). The phosphorylation of ADP to ATP is the only biochemical function, so far investigated, that is greatly affected by osmotic lysis. In addition, two lines of evidence suggest that the ghosts undergo an energy-dependent transformation resulting in contraction: (a) suspensions of the crude ghost preparation in 0.02 M Tris-0.125 M KCl medium show a marked increase

in optical density upon the addition of ATP, and (b) ghost preparations incubated in ion-uptake medium in the absence of added calcium but in the presence of added ATP contain a large number of highly condensed ghosts (about 50% of the total profiles) when viewed as thin sections in the electron microscope. The correlated biochemical and morphological study presented here shows that the outer membrane of rat-liver mitochondria can be removed by controlled osmotic lysis without greatly impairing a number of integrated biochemical functions associated with the inner membrane.

INTRODUCTION

The identification of mitochondria as primary sites of electron transport and oxidative phosphorylation in aerobic cells has prompted many attempts to isolate and purify various enzymes and intermediates of these multienzyme systems by classical methods. One objective of such studies has been to reconstitute integrated, functional supramolecular complexes from isolated components (1, 2, 3). The subfractionation of mitochondria and the studies of the soluble and membrane-bound components have produced an increased awareness of the complex interrelationship of ultrastructure and biochemical function in these organelles (3-7). Recent studies suggest that the physical compartmentation of enzymes in the mitochondrion plays an important role in the regulation of biochemical function, and many enzymic activities of mitochondria appear to be woven intimately into the molecular fabric of the mitochondrial membranes (3, 8-10). Numerous investigators have used membrane subfractions of mitochondria, but few have utilized membrane preparations derived primarily from the inner or outer mitochondrial membrane.

Consideration of these facts suggested that the subfractionation of mitochondria by a method which emphasized the isolation of intact mitochondrial structures, i.e. inner and outer membranes, rather than specific enzymic or biochemical activities, might be helpful in elucidating the bases for the interdependence of structure and function in these organelles. The successful isolation of these membranes could provide valuable starting material for further extraction or fractionation procedures and, in addition, might provide some knowledge of the activities of the outer mitochondrial membrane about which little is now known with certainty (11).

The physical disruption of cells by osmotic lysis is a relatively gentle procedure and has been used to obtain fairly intact membranes ("ghosts") of red blood cells (12, 13) and bacterial protoplasts (14, 15). Isolated rat-liver mitochondria undergo spontaneous swelling in hypotonic media (16),

and phase-contrast microscopy shows that in distilled water they, too, are converted into ghostlike spheres which can be recovered by high-speed centrifugation.

The present paper describes the electron microscope appearance of rat-liver mitochondria lysed osmotically by washing with distilled water. Evidence is presented which indicates that the mitochondria are stripped of their outer membranes and that a large proportion of the dense-staining matrix is liberated by this procedure. Some general chemical and biochemical properties of the crude water-washed mitochondrial preparation recovered upon high-speed centrifugation are also presented. The data indicate that both mitochondrial membranes are recovered in the pelleted fraction and that, despite the physical separation of inner and outer membranes and loss of matrix proteins, the pelleted fraction retains many biochemical activities of intact rat-liver mitochondria.

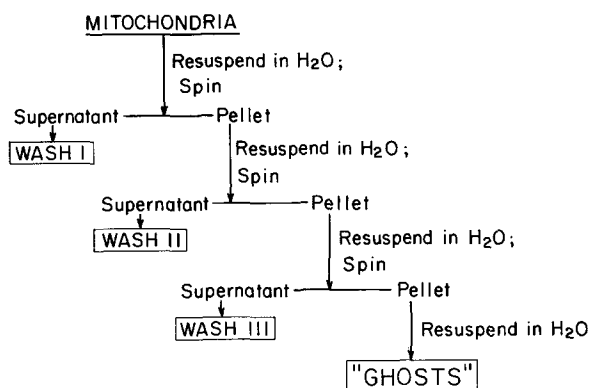
MATERIALS AND METHODS

Preparation of Water-Lysed Mitochondria (Ghosts)

Mitochondria were isolated from the livers of well fed albino rats (Carworth Farms, Sprague-Dawley strain) by the procedure of Schneider (17) and washed twice with 0.25 M sucrose. The mitochondrial pellet obtained from 10 g of rat liver was dislodged with the end of a cold test tube and diluted to a total volume of 10 ml with ice cold distilled water; the mitochondria were drawn into and expelled from a 10 ml pipette several times until a uniform suspension was obtained. Small aliquots were removed from the resulting suspension for protein and total phosphate determinations. The suspension was then centrifuged in a Spinco No. 40 head at 40,000 RPM (105,000 g) for a total time of 30 min.

The supernatant fraction from the first centrifugation was decanted and the pellet was dispersed with a cold stirring rod, diluted to 10 ml with cold water, resuspended with a pipette, sampled, and re-centrifuged as above. This procedure was repeated

twice; the supernatant fluid from the final centrifugation was decanted and the pellet was dispersed and resuspended to a volume which was varied from 3 to 10 ml to give the desired protein concentration. This final suspension is called the crude ghost fraction. One gram of rat liver normally yields 10 to 15 mg of ghost protein. A diagram outlining the preparative procedure is given in Fig. 1. All manipulations were performed at 0–4°C. When more than 10 g of rat liver are used, the volume of water must be varied so that the volume of the suspension (ml) equals the number of grams of rat liver from which the mitochondria were isolated. This 1:1 ratio of volume of the final suspension to the number of grams of liver from which the mitochondria are isolated must be carefully maintained to obtain the preparation described here.



and stained with lead by "Method B" of Karnovsky (19) or double-stained with uranyl acetate followed by lead citrate (20). Specimens were observed with a Siemens Elmiskop 1 double-condenser electron microscope at magnifications up to 80,000, using objective apertures of 20 or 50 μ in diameter.

Chemical and Biochemical Assays

Three parameters were used to determine the chemical and biochemical properties of the crude ghost preparation: (a) chemical composition, (b) selected enzymic activities, and (c) integrated multi-enzyme functions. Analyses of protein, phospholipid, and total phosphate were used to compare the relative chemical composition of the lysed mitochondria with that of intact mitochondria. Malate dehydrogenase (MDH) and D- β -hydroxybutyrate dehydrogenase

FIGURE 1 Diagram outlining the procedure used to prepare ghosts of rat-liver mitochondria. The mitochondria from 10 g (wet wt) of liver are resuspended in cold distilled water to give a total volume of 10 ml; the suspension is then centrifuged at 105,000 g for 30 min. This procedure is repeated twice and each pellet is resuspended to a total of 10 ml; each supernatant fluid (washes I, II, and III) is decanted and saved for analyses. The protein concentration (mg protein per milliliter) of each resuspension is critical in obtaining the preparation described here.

Electron Microscopy

Pellets were fixed with 1% OsO_4 buffered with Veronal-acetate, pH 7.4, or with 6.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 to 2 hr followed by postfixation with 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4. In addition, small aliquots of well suspended pellets were mixed with an equal volume of 12.5% glutaraldehyde, and a sample was transferred to a Microfuge tube (Beckman Instruments, Inc., Fullerton, California) and centrifuged immediately for 4 min at maximum speed in the cold. The supernatant fluid was decanted and replaced with fresh glutaraldehyde. The volume of the sample was adjusted to give small pellets (about 0.5 mm thick) which could be sampled throughout their depth. Dehydration was accomplished by rapid passage through a cold alcohol series (-10°C), and embedding in Epon 812 was performed according to Luft's procedure (18). The embedded pellets were sectioned on Porter-Blum or LKB microtomes using glass or diamond knives. The sections were collected on grids containing no supporting films

(BDH) were used as specific enzyme markers for matrix and mitochondrial membranes respectively. The functional intactness of the mitochondria after water-washing was monitored by measuring respiration, phosphorylation, and contraction of the crude ghost preparation.

Protein was determined by the biuret method (21) or the method of Lowry et al. (22) using bovine serum albumin (fraction V, Sigma Chemical Co., St. Louis, Missouri) as a primary standard. A small, dark-brown, tightly packed "button" was present at the bottom of each pellet but was never included in the resuspensions. For this reason, total recovery of the protein present in the initial suspension was not achieved. Total phosphate was determined by the method of Gomori (23) after digestion with H_2SO_4 in the presence of H_2O_2 .

Oxygen uptake was measured with a Clark electrode by using the medium described by Chance and Williams (24); to avoid the complication of possible back-diffusion of O_2 into the polarographic chamber, all measurements were made using a closed system. Phosphorylation was measured by using the

medium of Gregg (25) and the method of Nielsen and Lehninger (26) as modified by Gregg (25). To determine the amount of P_i^{32} incorporated into ATP, parallel reactions were run in the presence and absence of 0.1 mM dinitrophenol (DNP); the radioactivity not extracted by isobutanol-benzene in the cuvette containing DNP was subtracted from the value obtained in the cuvette which did not contain DNP. Thus, the estimation of oxidative phosphorylation was corrected for substrate level phosphorylation which is DNP-insensitive.

To discover if similar or different mitochondrial proteins are extracted into washes I, II, and III, the proteins in the three washes were separated electrophoretically by three different procedures. Duplicate samples of each wash fluid were spotted in equal amounts, and the number of protein bands and the electrophoretic mobilities of the bands in each wash were compared. Disc electrophoresis was employed according to the methods of Ornstein and Davis (27). The electrophoresis of aliquots spotted on Sepharose III (cellulose polyacetate) strips (Gilman Instruments, Ann Arbor, Michigan) was conducted in 0.05 M Tris-HCl buffer (pH 8.5) containing 0.001 M ethylenediaminetetraacetate (EDTA) at 200 v and 2.3 ma for 1 hr. The proteins were fixed in the strips with trichloroacetic acid (TCA), stained with amidoblack dye, and washed with acetic acid. "Oxoid cellulose acetate strips" were used to separate the extracted proteins at pH 6.8 in 0.05 M imidazole buffer. Electrophoresis was carried out at 200 v and 4.3 ma for 1 to 2 hr. The bands were fixed with 20% sulfosalicylic acid, stained with comassie brilliant blue R-250, and washed with water.

Changes in absorbancy at 520 m μ were used, as previously described (28), to indicate high amplitude contractions.

Cytochromes *a*, *b*, *c*₁, and *c* in the various fractions were estimated by the spectral method of Williams (29), with a Carey Model 15 recording spectrophotometer adjusted so that the full range of the recorder measured 0.0 to 0.1 optical density units.

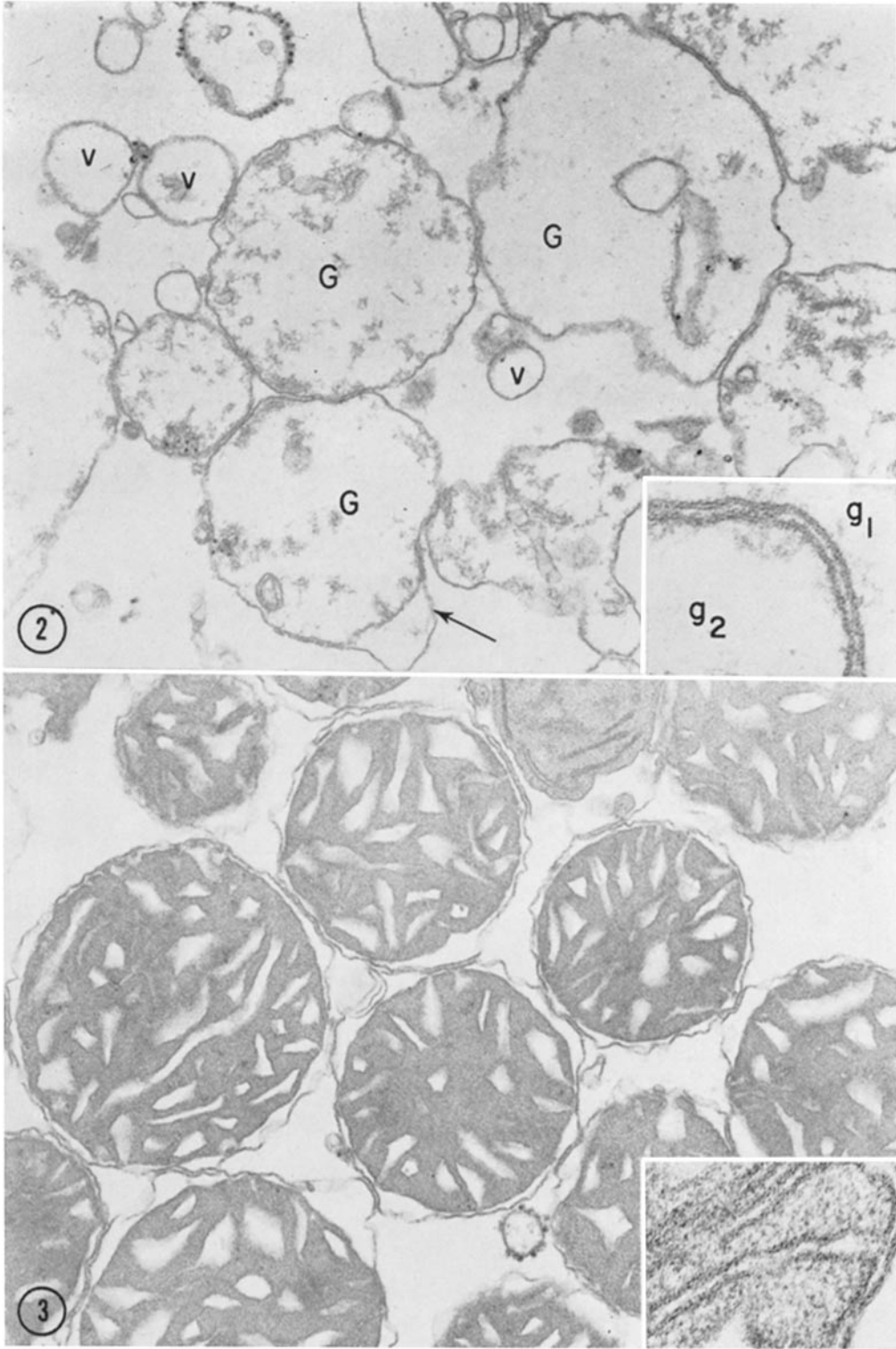
D- β -hydroxybutyrate dehydrogenase (BDH) was

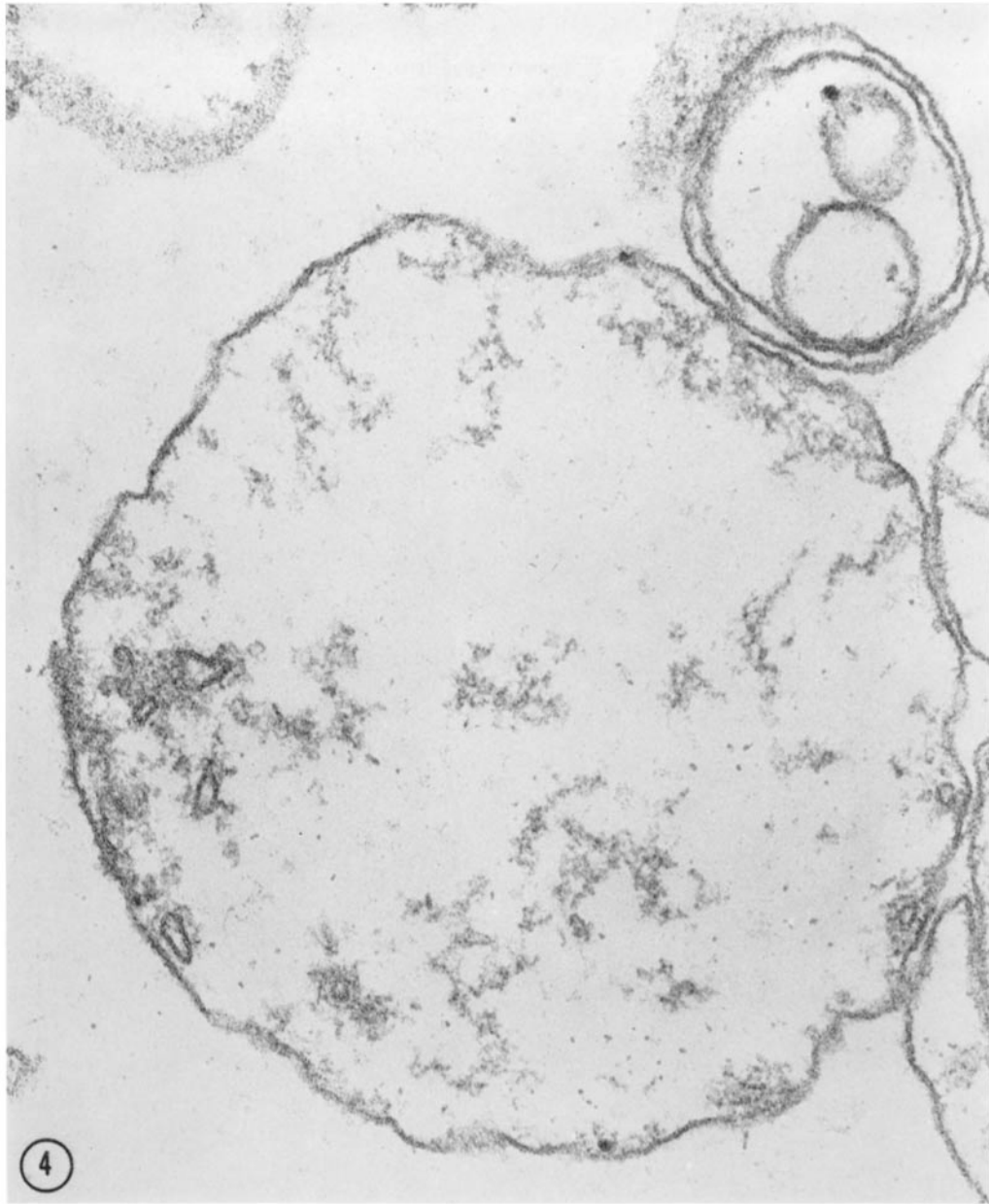
measured by following the rate of reduction of NAD⁺ at 340 m μ with a Zeiss PMQ II Spectrophotometer equipped with a Sargent Model SRL Recorder. The extinction value used for NADH was 6.22 mm⁻¹ cm⁻¹ (30). The medium contained 1 mM NAD⁺, 75 mM Tris-HCl (pH = 8.1), 1 mM Amytal, and 2 to 50 μ l of ghost suspension (20 to 50 μ g protein); the final volume of the system was 2.0 ml. The reaction was started by the addition of 15 μ l of 1.5 M D- β -hydroxybutyrate. Amytal was used to block the reoxidation of NADH by the respiratory chain; its presence accounts for a 10 to 15% increase in the observed rate of reduction of NAD⁺. Malate dehydrogenase (MDH) was assayed according to the method of Ochoa (31) by following the oxidation of NADH at 340 m μ . Amytal (1 mM) was also present in the assay medium to block the oxidation of NADH by the respiratory chain. Samples were sonically disrupted with an MSE sonicator (MSE Instrument Associates, New York) or by a Sonifier model LS75 equipped with an exponential tip (Branson Instruments, Inc., Stamford, Connecticut). Maximal BDH and MDH activities were recorded after samples (total volume equal to 0.5 to 2.0 ml) were sonicated for 15 to 45 sec. All enzymic assays were run at room temperature.

Lipids were extracted into chloroform-methanol by a modification of the procedure of Folch et al. (32). The sample was diluted to 20 times its volume with chloroform-methanol (2:1, v/v). The insoluble material was suspended by a brief homogenization in a Waring blender and then warmed to near 50°C, cooled, and stirred at room temperature for 1 to 2 hr. The remaining insoluble material was collected by filtration on a Büchner funnel, and the residue was rinsed with a volume of chloroform-methanol which represented 5% of the total volume of the filtrate. A volume of sodium chloride (0.9%, w/v), equalling 20% of the total volume, was added to the filtrate and shaken vigorously for several minutes. The resulting emulsion was broken by a short, high-speed centrifugation; the resulting two phases were separated and assayed for total phosphate.

FIGURE 2. Thin sections of crude ghost preparation. Fixed with glutaraldehyde and OsO₄; stained with uranyl acetate and lead. Ghosts (*G*) and small vesicles (*v*) are enclosed by single, continuous membranes. An extremely rare profile, possibly with a remnant of the outer membrane attached to a ghost, is shown (arrow). Inset, "unit membranes" of two adjacent ghosts (*g*₁ and *g*₂). Fig. 2, \times 40,000; inset, \times 140,000.

FIGURE 3. Control. Unlysed mitochondria fixed with glutaraldehyde-OsO₄ and double-stained with uranyl acetate and lead. The double membranes and condensed matrices contrast with the ghosts (cf. Fig. 2). Inset, "unit membranes" are revealed at higher magnification and are similar in appearance to those observed in the ghosts (cf. Fig. 2, inset). Fig. 3, \times 40,000; inset, \times 140,000.





FIGURES 4 and 5 The effects of osmotic lysis on mitochondrial ultrastructure. The structural features of ghosts and freshly isolated, unlysed mitochondria are compared. Fixed with glutaraldehyde- OsO_4 and stained with uranyl acetate followed by lead. $\times 100,000$.

FIGURE 4 A section of an individual ghost showing the single, trilamellar membrane, the diminished matrix contents, the relatively few cristae, and absence of normal matrix granules.

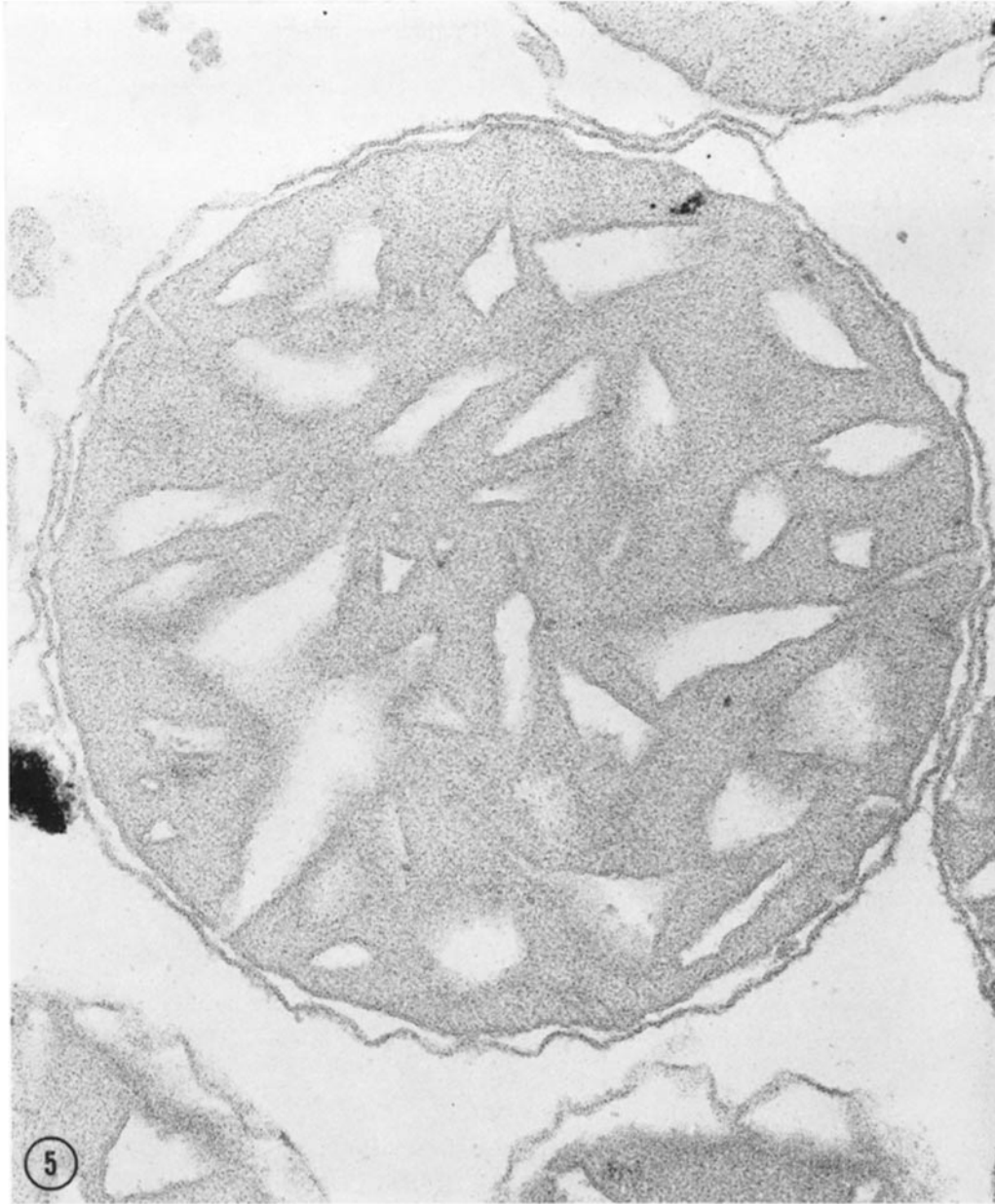


FIGURE 5 Section of a freshly isolated mitochondrion. The double-membrane organization and densely staining matrix contrast with the single-membrane-limited ghosts (cf. Fig. 4). Both inner and outer surface membranes and the cristae appear trilamellar.

RESULTS

Electron Microscopy

Electron microscope examination of thin sections of the water-washed mitochondria shows that

the crude ghost preparation consists of two morphologically and structurally distinct elements: (a) relatively small (0.2 to 0.4 μ in diameter), empty appearing vesicles, and (b) larger particles (about 1.5 μ in diameter) which appear as swollen

mitochondria and contain some matrix, a few cristae, and few, if any, normal matrix granules (see Fig. 2). Each of the populations is relatively homogeneous in size and appearance, and each is enclosed by a single, intact membrane. By analogy, with respect to membranes obtained from other sources by osmotic lysis (12-15), the larger elements, which are structurally altered mitochondria, are called ghosts. The smaller, vesiculated elements bear little resemblance to either intact mitochondria or ghosts. Examination of sections from the top, middle, and bottom of the pellets or of sections taken from throughout the small Microfuge pellets indicates that the images shown in Fig. 2 are representative of the entire population. Hundreds of sections from more than

The integrity and continuity of the single membrane enclosing the ghosts is further illustrated in Fig. 4. Comparison of a ghost (Fig. 4) with an intact, control mitochondrion (Fig. 5) at this magnification clearly shows the relative absence of matrix and cristae in the ghosts. Some internal membranes, probably cristae, and some matrix material are present, however. The small vesicle in Fig. 4 (upper right corner) shows a profile of membranes enclosed by other membranes. In some preparations of water-washed mitochondria, these profiles are quite numerous. The outer membrane of the small vesicle in Fig. 4 may be continuous with the adjacent ghost membrane and suggests that some vesiculation of the inner mitochondrial membranes also occurs. The enwrap-

TABLE I
Comparison of the Protein and Total Phosphate Contents of Submitochondrial Fractions Obtained by Osmotic Lysis

See text for details of fractionation and isolation procedures.

Sample	Experiment 26		Means of 20 experiments		
	Total protein	Total P _i	Total P _i /total protein	Total protein	Total P _i
	mg	μmoles	μmoles/mg	%	%
Mitochondria	280	80	0.29	100	100
Wash I	48	8	0.15	16 ± 5*	9 ± 2*
Wash II	42	12	0.19	22 ± 3	17 ± 3
Wash III	7	3	0.20	7 ± 1	6 ± 2
Crude ghosts	133	47	0.35	46 ± 2	65 ± 8

* ± represents deviation from the means.

a hundred different preparations of water-washed mitochondria have been carefully examined in the electron microscope. Only rarely has a ghost been seen which appeared to have fragments of the outer membrane retained (Fig. 2, arrow). This is taken as evidence that osmotic lysis effectively disrupts the outer mitochondrial membranes and spatially separates them from the ghost. Fig. 3 shows the typical appearance of the mitochondrial control preparation prior to osmotic lysis.

Despite the over-all alterations in the mitochondria as a result of the water treatment, the membranes surrounding the ghosts show a trilamellar appearance similar to that observed in membranes of intact mitochondria (cf. insets to Figs. 2 and 3). No alteration in the ultrastructure of the inner mitochondrial membrane enclosing the ghosts is revealed by the electron microscope.

ment of inner and outer membranes during vesicle formation could explain why the inner and outer mitochondrial membranes in water-washed preparations are not completely separated by centrifugation on density gradients, although considerable enrichment is obtained (unpublished observations).

Protein

The distribution of protein in the various fractions obtained during the preparation of the crude ghosts is shown in Table I; the results from a single experiment, and the means and deviation from the means calculated from the results of 20 experiments, are presented. Washes I and II each contain about 20% of the protein which was present in the initial mitochondrial suspension, whereas the third wash contains less than 10% of the total protein. The amount of material extracted from

TABLE II

Effect of Sonic Disruption on Malate (MDH) and D-β-hydroxybutyrate (BDH) Dehydrogenase Activities of Ghosts and Freshly Isolated Mitochondria

See text for methods of assay.

Sample	Sonication	MDH*	BDH‡
Mitochondria§	+	1.56	0.276
Ghosts	—	0.46	0.179
	+	1.13	0.565

* μ mole of NADH disappearing per minute per milligram of protein.

‡ μ mole of NADH appearing per minute per milligram of protein.

§ Mitochondria undergo high amplitude swelling under the hypotonic assay conditions; therefore, no measurements of NADH appearance or disappearance were made using the unsonicated mitochondrial samples.

different mitochondrial preparations by each water washing varies considerably; this is especially true of the amount of protein liberated by the first water washing. The protein content of the final crude ghost fraction, however, is remarkably reproducible and does not differ significantly from preparation to preparation.

Total Phosphate

The amount of orthophosphate (P_i) in each fraction analyzed shows no direct correlation with the amount of protein extracted by each water washing. This is indicated by the ratios of P_i to protein present in the various fractions (Table I). About 9% of the total phosphate is found in wash I, about 17% in wash II, and about 6% in wash III; the final ghost fraction contains about 65% of the total P_i initially present in the mitochondrial preparation. The phosphate (μ moles) to protein (mg) ratio of the ghost fraction is 0.35 ± 0.04 . This value is close to the amount of lipid phosphate present in the ghost fraction (see Phospholipid Assay).

Electrophoresis

Experiments were conducted to determine whether the proteins in the three washes appeared qualitatively similar or different in electrophoretic mobility. Washes I, II, and III appear to contain the same number of bands of protein which, under

the three conditions of electrophoresis used, were identical in mobility though some bands stained more heavily than the corresponding bands in another wash. It is tentatively concluded that each water wash extracts similar, if not identical, groups of proteins.

Enzyme Assays

Several methods for obtaining maximal enzymic activities were tested; sonication proved the most useful in providing maximal activities of the two enzymic reactions studied. The need for disruption to obtain maximal BDH activity was noted earlier by Lehninger et al. (33). Also, sonication has been used by Klingenberg and Pfaff (8) to obtain the maximal release of MDH activity from the matrix space of the mitochondria. It can be seen from Table II that large increases in the BDH and MDH activities of the crude ghost fraction are obtained by sonication. The increase in MDH activity in the ghost preparation upon sonication suggests that a fraction of MDH activity is not liberated by the water-washing procedure and, thus, remains compartmented within the crude ghost preparation. Subsequent determinations of BDH and MDH activities were made on samples which were sonicated.

MDH Activity

Mitochondrial malate dehydrogenase has been characterized as a soluble enzyme localized in the matrix of these organelles (8). Therefore, the MDH activity in each water wash and in the final crude ghost fraction was used as a marker by which the liberation of matrix protein could be monitored. Klingenberg and Pfaff (8) have shown that about 90% of this enzyme is released upon sonication and is not sedimented when the sonicate is centrifuged at high speed. Table III shows that about two-thirds of the MDH activity is released by the water-washing procedure and that this activity is distributed almost equally between wash I and wash II. About one-third of the original MDH activity is retained in the crude ghost fraction.

These observations suggested that the proteins found in the supernatant fluids are extracted from the matrix. To determine if mitochondrial components which are exclusively bound to the mitochondrial membranes could be detected, the washes and ghost fraction were assayed for BDH, phospholipids, and cytochromes.

TABLE III

Comparison of the Total and Specific Malate and D- β -hydroxybutyrate Dehydrogenase Activities of the Submitochondrial Fractions Obtained by Osmotic Lysis

See text for assay methods and fractionation procedures.

Sample*	Specific activity		Total mitochondrial activity	
	MDH \dagger	BDH \S	MDH	BDH
			%	
Mitochondria	1.0	0.276	100	100
Wash I	1.8	0	30	0
Wash II	1.6	0	24	0
Wash III	0.6	0	2	0
Ghosts	0.7	0.565	33	100

* All samples sonicated.

\dagger μ moles of NADH disappearing per minute per milligram of protein.

\S μ moles of NADH formed per minute per milligram of protein.

BDH Activity

As shown in Table III, BDH activity was not detected in the water washes, whereas the specific activity of BDH in the ghost fraction is about twice that of the original mitochondria. Table I shows that the crude ghost fraction retains about 50% of the mitochondrial protein. The water washes show no BDH activity; therefore, the approximate doubling of the specific BDH activity in the ghost fraction indicates complete conservation of this membrane-bound enzyme.

Phospholipid Assay

Each of the fractions obtained during preparation of ghosts was analyzed for phospholipids. Since about 90% of the lipids of mitochondria is phospholipids (3, 34), the total phosphate in the lower phase of the chloroform-methanol-NaCl extract was used as an estimation of lipid content of each sample. Table IV lists the amount of phospholipid in the various samples, and shows that washes I, II, and III contain no phospholipid. The ratio of lipid phosphate to milligram of protein in the crude ghost fraction is about twice that found in the unlysed mitochondria. This doubling of lipid phosphate content agrees with the increase in the specific activity of BDH and shows a complete conservation of phospholipids and, therefore,

TABLE IV

Comparison of the Lipid and Nontlipid Phosphate in the Submitochondrial Fractions Obtained by Osmotic Lysis

See Methods and Materials for procedures.

Sample	μ mole P ₁ per mg of protein	
	Lipid phase	Aqueous phase
Mitochondria	0.178	0.028
Wash I	<0.004	0.088
Wash II	<0.004	0.083
Wash III	<0.002	0.093
Crude ghost fraction	0.326	0.075

of mitochondrial membranes in the crude ghost fraction. Also, the lipid phosphate to protein ratio are nearly equal in the crude ghost fraction (cf. Tables I and IV). This suggests that 90% or more of the phosphate in the ghost fraction is present as phospholipids.

Cytochromes

Spectral analysis of the mitochondria, the water washes, and the crude ghost fraction for cytochromes *a*, *b*, *c*₁, and *c*, which are membrane-bound, showed that there was a complete recovery of these respiratory carriers in the ghost fraction. As shown in Fig. 6, the difference spectrum of the crude ghost fraction is the same as that found with intact mitochondria (29). No cytochromes were detected in washes I, II, or III. Thus, as in the cases of phospholipid and BDH activity, these components of the respiratory chain associated with the mitochondrial membranes are recovered in the ghost fraction.

The complete conservation of membranes in the crude ghost preparation indicates that this fraction contains elements from *both* inner and outer mitochondrial membranes. This interpretation is suggested also by the electron microscope study presented here.

Oxygen Uptake and Oxidative Phosphorylation

One of the important integrated functions of intact mitochondria is respiration. Ghost preparations have the same general respiratory properties as do freshly isolated rat liver mitochondria; they oxidize succinate, β -hydroxybutyrate, and ascorbate. Respiration is blocked by amytal, antimycin A, and CN⁻. However, the ghost fraction, in

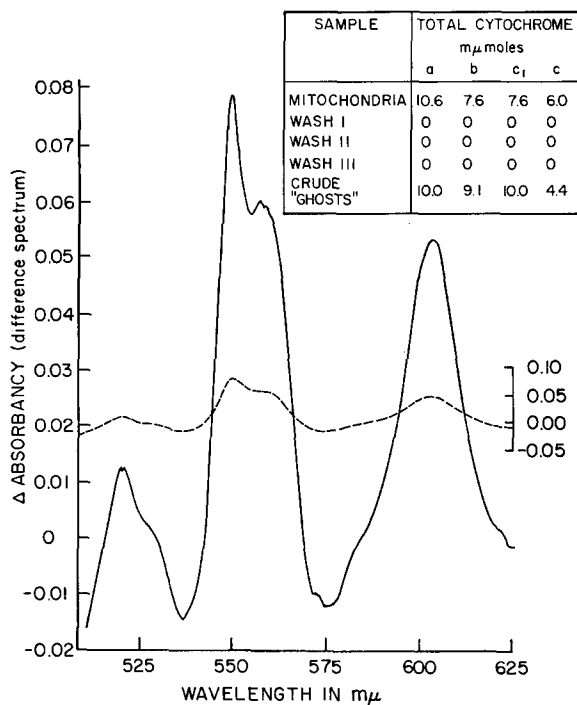


FIGURE 6 Cytochrome content of the crude ghost fraction. Typical difference spectra for reduced-oxidized cytochromes are given, showing peaks for cytochromes *a*, *b*, *c*₁, and *c* at 605, 563, 554, and 550 $m\mu$, respectively. The continuous and broken lines compare the traces obtained using an expanded and unexpanded scale, respectively. The data in the table have been calculated by the method of Williams (29) and show that no cytochromes are detectable in washes I, II, or III. The values shown for the cytochrome content of the mitochondria and the ghosts were obtained from analysis of 30 mg of mitochondria and from the ghost material which was derived from this amount of mitochondria.

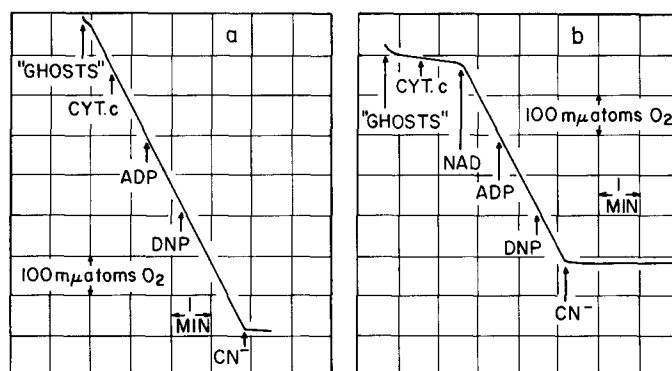


FIGURE 7 Polarographic traces of oxygen uptake of crude ghosts in the medium described by Chance and Williams (24). Arrows indicate the sequence of additions of: ghost suspension (4.6 mg of protein), cytochrome *c* (25 μ l of 0.068 M), ADP (10 μ l of 0.050 M), DNP (10 μ l of 0.01 M), KCN (10 μ l of 0.1 M) and NAD^+ (25 μ l of 0.02 M). The pH of all reagents was adjusted to pH 7.2-7.4. Fig. 7 *a*, substrate is 0.010 M succinate. Fig. 7 *b*, substrate is 0.011 M *D*- β -hydroxybutyrate.

contrast to intact rat-liver mitochondria, does not show respiratory control upon the addition of phosphate acceptor. As seen in Fig. 7 *a*, the addition of cytochrome *c* and of ADP has no effect on the rate of oxygen uptake; the ghost fraction thus may be termed "loosely coupled." The absence of any detectable effect, when cytochrome *c* is added, agrees with the absence of detectable cytochromes

in the wash fluids and shows that any loss of cytochrome *c* during preparation does not limit the rate of oxygen consumption. This was previously shown by Vasington and Greenawalt (35). The earlier work of Lehninger (36) showed that the addition of exogenous NAD^+ is an absolute requirement for the oxidation of *D*- β -hydroxybutyrate by mitochondria suspended in hypotonic

TABLE V

Comparison of the Oxidative and Phosphorylative Capacities of Freshly Isolated Mitochondria and the Crude Ghost Fraction

See text for details.

Sample	O ₂ uptake*	P _i uptake†	P:O
Mitochondria	40.0	80.0	1.85
Ghost fraction	78.6	40.6	0.72

* mμ atoms per minute per milligram of protein.

† mμmoles of P³² into ATP per minute per milligram of protein.

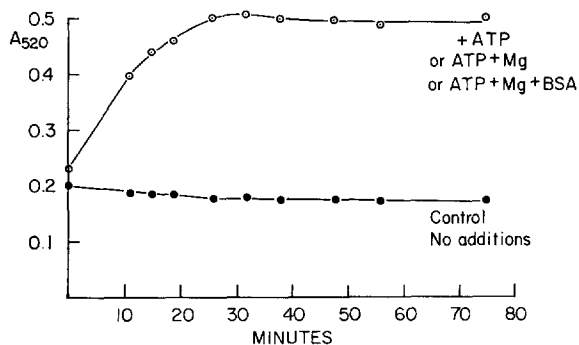


FIGURE 8 ATP-induced absorbancy changes in ghost fractions. Contraction medium consisted of 0.02 M Tris-0.125 M KCl (pH 7.4) to which were added (where indicated) ATP (4.7 μmoles), MgCl₂ (15 μmoles) and/or BSA (10 mg). Total volume of reaction medium equalled 5.0 ml. The reaction was started by adding 40 μl (1.2 mg of protein) of ghost suspension to the test and control tubes.

media. These respiratory properties of water-lysed rat-liver mitochondria are illustrated by the polarographic trace shown in Fig. 7 b.

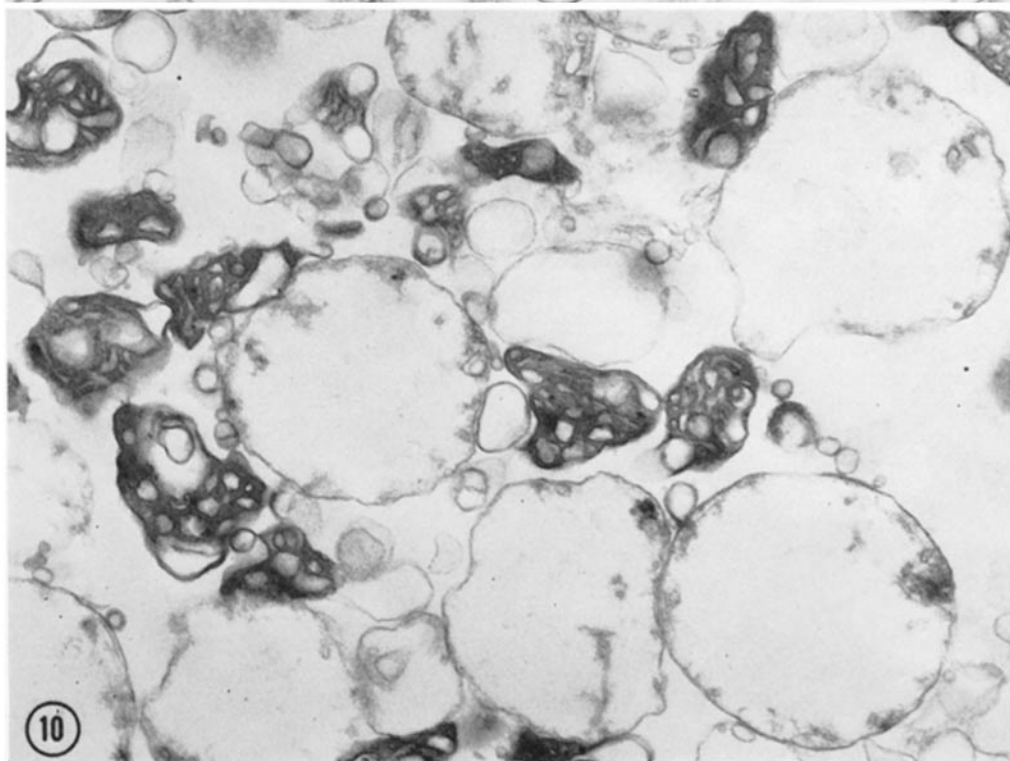
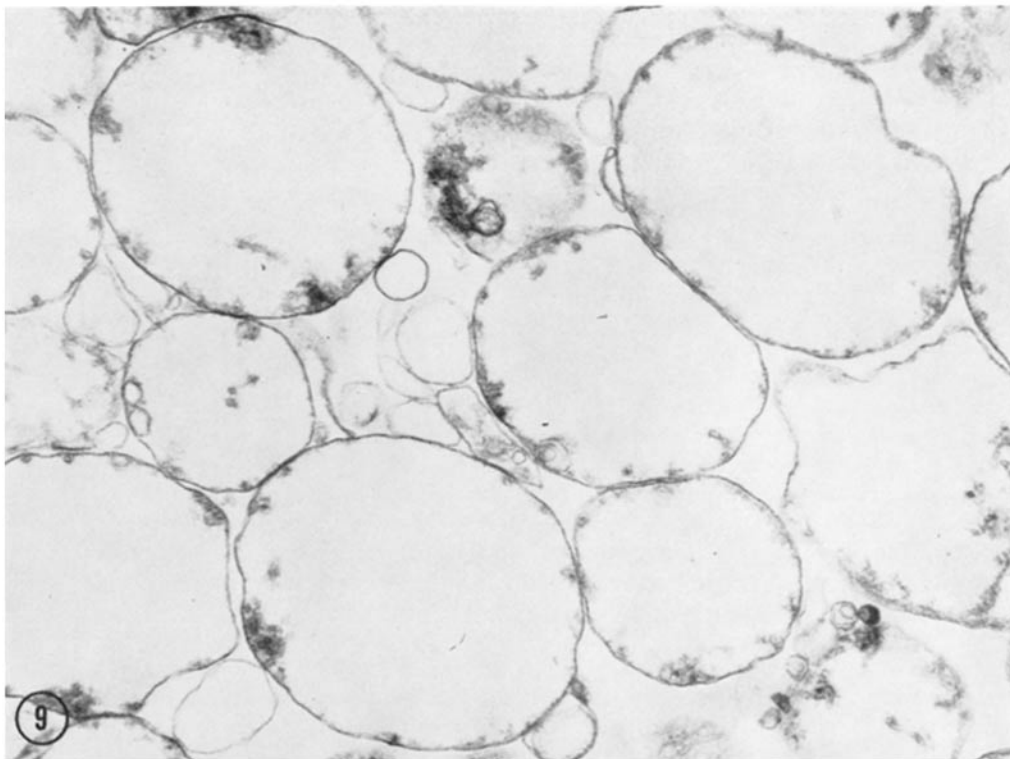
The P:O ratios of the crude ghost fraction vary

from 0.4 to 0.9 with succinate as substrate; these low ratios could arise from alteration in either the phosphorylating ability or the respiratory capacity of the ghost fraction. It can be seen in Table V that the rate of oxygen uptake per milligram of ghost protein is almost twice the rate per milligram of protein of intact rat-liver mitochondria, whereas the rate of phosphorylation per milligram of protein is decreased by about one-half. The ghost fraction usually takes up about 1.5 to 2.0 times as much O₂ per minute per milligram of protein as do intact mitochondria (State III). This suggests that

the respiratory chain is functionally intact in the ghost fraction. The data indicate that the respiratory capacity is unaltered whereas the phosphorylating ability has been decreased by the

FIGURE 9 ATP-induced contraction of ghost fraction. Control. Micrograph of a thin section of a pellet obtained after an aliquot of the crude ghost fraction was incubated under ion-uptake conditions in the absence of added ATP. The preparation shows the structural characteristics routinely observed with freshly prepared ghosts. Ghosts are enclosed by single membranes, and practically no internal structures are visible. Fixed with OsO₄ and stained with lead. An aliquot of crude ghost fraction (5 mg of protein) was incubated at 30°C for 20 min in a medium consisting of 4 mM sodium phosphate (pH 7.0), 10 mM Tris-maleate (pH 7.0), 10 mM MgCl₂, 10 mM succinate, and 4 mM CaCl₂; final volume of the reaction mixture was 3.0 ml. No ATP was added; therefore, no Ca⁺⁺ was accumulated (see reference 35). × 26,000.

FIGURE 10 ATP-induced contraction of ghost fraction. Micrograph through a thin section of pellet obtained after the crude ghost fraction was incubated under ion-uptake conditions in the presence of added ATP but no added CaCl₂ (see legend to Fig. 9). It can be seen that about 50% of the profiles consists of electron-opaque, highly condensed bodies, presumably contracted ghosts. Membranes, similar in general conformation to the cristae of intact mitochondria, can be seen, and "intracrystal" spaces are also apparent. The densely staining internal substance may correspond to the matrix materials still retained in the ghosts. The contracted forms are much smaller in diameter than freshly isolated mitochondria, indicating a high degree of contractility in these ghosts. Fixed with OsO₄ and stained with lead. × 26,000.



water-washing procedures. It should be mentioned that the ghost fraction exhibits an absolute requirement for Mg^{++} in the phosphorylating medium; 1 to 3 mM Mg^{++} does not seem to affect O_2 uptake greatly although no detailed investigation has been made to determine the requirements necessary for maximal O_2 uptake.

Contraction

The study of crude ghost preparations in the electron microscope following the accumulation of massive amounts of Ca^{++} showed that under these conditions a large number of ghosts assume a densely staining, highly condensed state (37). This finding suggested that, in at least some of the ghosts, the membranes underwent gross conformational changes resulting in the contracted profiles observed.

To investigate further the possibility that elements in the crude ghost preparation are capable of contracting, two types of experiments were conducted. Aliquots of the crude ghost preparation were incubated in KCl-Tris medium under conditions previously described for following the swelling-contraction cycle of intact rat-liver mitochondria (28). Changes in absorbancy at 520 $m\mu$ have been utilized in numerous studies to indicate "high amplitude" swelling or contraction of isolated mitochondria (16). Details of the incubation conditions are given in the legend to Fig. 8. This figure shows that ATP, as well as ATP + Mg^{++} or ATP + Mg^{++} + BSA, produces a marked increase in the absorbancy of a ghost suspension at 520 $m\mu$. The increase in absorbancy is maximal and levels off at about 30 min after the addition of ATP. Controls incubated in the absence of added ATP show no change in absorbancy during the time course of the experiment. The change of absorbancy with time follows a curve similar to that observed during contraction of swollen rat-liver mitochondria (16, 28).

Morphological studies were made of the ghost preparation incubated in ion-uptake medium (from which calcium was omitted) in the absence and presence of added ATP. The legends to Figs. 9 and 10 give details of the incubation conditions. Figs. 9 and 10 are electron micrographs of a ghost preparation incubated for 20 min in the absence and presence of ATP, respectively. The ghosts incubated in the absence of ATP (Fig. 9) show the structural characteristics of unincubated ghosts prepared by osmotic lysis and fixed with OsO_4 .

However, about one-half of the ghosts incubated in the presence of ATP (Fig. 10) appear tightly condensed, presumably because of the contraction of the remaining inner mitochondrial membrane and possibly because of the condensation of the matrix contents retained within the ghosts. The small diameters of the contracted ghosts indicate definite changes in the conformation of the membranes.

It cannot be assumed from these studies that the changes in absorbancy shown in Fig. 8 result from the condensation of ghosts shown in Fig. 10, although this seems probable. Electron microscope studies correlated with the changes in absorbancy are required before further comments on this point can be made. However, these two independent lines of evidence, (a) absorbancy changes under conditions promoting the contraction of intact mitochondria and (b) structural changes visualized in the electron microscope, provide strong presumptive evidence that the mechanism regulating contraction in intact mitochondria is also functional in at least some of the ghosts obtained by the osmotic lysis of isolated mitochondria.

DISCUSSION

The most distinctive feature of the structural architecture of mitochondria is the unique spatial configuration of membranes which partitions these organelles into two aqueous phases or compartments: the inner compartment or "soluble" matrix and the outer compartment, which is bounded by the paired surrounding membranes and is continuous with the intracristal spaces. The normal relative volumes of these compartments may vary greatly in mitochondria of various cell types *in situ*, as a result of differences in the number of cristae, or, in isolated mitochondria of a given cell type, as a result of the osmolarity of the suspending medium (16) and possibly the metabolic state of the mitochondria (38).

The present study describes some ultrastructural and biochemical properties of the crude ghost preparations of rat-liver mitochondria obtained by osmotically lysing these organelles with distilled water. The objectives of this study were: (a) to determine whether or not chemical and biochemical analyses support the interpretations of morphological studies which indicate that water-washed preparations of rat-liver mitochondria contain spatially dissociated inner and outer mito-

chondrial membranes and that the protein liberated by the washing procedure is extracted primarily from the mitochondrial matrices; (b) to establish the degree of functional integrity of the phosphorylative, respiratory, and contractile mechanisms of the lysed mitochondria; and (c) to characterize the crude ghost preparation by biochemical activities which could be used in future studies designed to subfractionate the crude preparations into ghosts and outer membranes. Preliminary results of such fractionation studies have been briefly communicated (37); a detailed report of the separation of inner and outer membranes from rat-liver mitochondria is in preparation.

The matrices of lysed mitochondria are significantly less electron-opaque than the unlysed controls (cf. Figs. 2 and 3, 4 and 5), which, in a qualitative way, indicates the loss of matrix material. A great difference in the density of staining is observed even though glutaraldehyde-OsO₄-fixed ghosts are only slightly larger in diameter than the unlysed controls. Thus, this decrease in density appears to result primarily from the extraction of matrix constituents and not from pronounced swelling accompanied by dilution of the matrix. In addition, the matrices of the entire population of water-washed mitochondria appear to be of comparable densities; this fact suggests that protein is extracted rather uniformly from all the mitochondria during ghost preparation and is not due to the complete loss of matrix by some mitochondria and complete retention by others.

The present morphological data show that thin sections of ghosts prepared by the suspension and washing of mitochondria in distilled water can be readily distinguished from unlysed mitochondria on the basis of (a) decreased density of the matrix, (b) increased diameter, (c) the presence of a single surrounding membrane, (d) the decreased number of cristae, and (e) the absence of normal matrix granules.

The observation that the surface envelopes of both ghosts and the smaller vesicles consist of a single membrane is of considerable significance. In only an insignificant number of sections of water-washed mitochondria out of hundreds examined has the double-membrane organization typical of intact mitochondria been observed. The presence of two surrounding membranes clearly present in intact mitochondria (Figs. 3 and 5) would be detected in ghosts even if the spatial organization were altered so that the inner and

outer membranes were in close apposition. In such a case the surrounding membranes of ghosts would be noticeably thicker than a single membrane and would be marked by a dense mid-line where the membranes were apposed; this is not observed (Figs. 2 and 4). Furthermore, the resolution of a single "unit membrane" circumscribing the ghosts (Fig. 2, inset) shows that only one membrane is present on the surface of the ghosts and that the inner and outer mitochondrial membranes clearly are spatially dissociated in these preparations. The small vesicles present in the osmotically lysed mitochondrial preparations studied here resemble somewhat the water-treated mitochondria briefly studied by Watson and Siekevitz (39) who systematically disrupted rat-liver mitochondria by several methods. These workers observed small, vesiculated elements in their 105,000 *g* fraction of water-washed mitochondria. In the present study, similar vesicles are observed in the crude ghost pellets. It is suggested that these vesicles are fragments derived primarily from the disrupted, outer membrane. This interpretation is consistent with both the structural evidence and the chemical data which indicate that both membranes are present in the crude water-lysed mitochondrial preparation. It is possible that vesiculated inner membranes may also contribute to this population. The elements designated ghosts in the present paper are about the size of intact mitochondria when fixed with glutaraldehyde-OsO₄; they contain small amounts of lightly staining, flocculent material (residual matrix) and enclose some membranous structure interpreted to be cristae. If major reorganization of the ghost membranes has occurred, it is not detected by high resolution electron microscopy (Figs. 2, inset and 4).

The results presented here confirm earlier findings that 40 to 50% of the total mitochondrial protein is recovered in the crude ghost preparations (Table I). The total amount of protein retained by lysed mitochondria is very reproducible, although the amount liberated in a given wash varies from preparation to preparation. The total phosphate to total protein ratio in the crude ghost fraction (0.35 μ moles/mg of protein, Table I) is about equal to the lipid phosphate to protein ratio (0.326 μ moles/mg of protein, Table IV); this indicates that almost all (~90%) the phosphate in the crude ghost preparation is in the form of phospholipids. In addition, the phospholipid to protein ratio in the crude ghost fraction is about

twice that of intact mitochondria. Direct analysis of the washes shows that no phospholipids (membranes) are present in these soluble fractions. Thus, the protein liberated during ghost preparation contains essentially no phospholipid, and, therefore, few, if any, lipoprotein components of the membranes.

Crypticity in the activities of a number of enzymes associated with mitochondria has long been recognized and has been interpreted to reflect the presence of structural barriers preventing enzyme-substrate interactions (8). In the present study, maximal BDH and MDH activities in ghosts or intact mitochondria were obtained only after sonication. Assays for total and specific MDH activities indicate that large amounts of matrix proteins are liberated into the soluble washes. In fact, over 60% of the total MDH activity recovered in the various fractions is found in the supernatant fluids, and less than 40% remains associated with the membranes of the crude ghost fraction. These data agree with the morphological interpretation that the liberated protein is derived largely from the matrix. That considerable physical compartmentation is retained by the water-washed mitochondria is suggested by the increased MDH activity obtained upon sonication as well as by the integrity of the ghost membranes seen in thin sections.

The results indicate that the amount of matrix protein (based on MDH activity) still retained in the ghost fraction approximates 20% of the total mitochondrial protein. Thus, if the ghosts retain all the membrane-bound protein of intact mitochondria (30 to 40%) plus this protein from the matrix (20%), the crude ghost preparation should contain from 50 to 60% of the total mitochondria protein. Direct analyses indicate that about 46% is retained. These figures agree remarkably well, considering the fact that completely quantitative recoveries of total protein and MDH activities were not achieved. The data of Klingenberg and Pfaff (8) show that 30 and 70% of the total mitochondrial protein fall into "membrane-bound" and "nonmembrane-bound" protein, respectively. It has been shown that about $\frac{1}{3}$ of the mitochondrial MDH activity remains in the crude ghost fraction and that $\frac{2}{3}$ is released into the washes. Therefore, $\frac{2}{3}$ of the nonmembrane-bound protein (70%) or about 47% of the total mitochondrial protein should be recovered in the soluble fraction. This value is about equal to the

amount of protein (50%) which is extracted from the mitochondria by the water treatment. This type of calculation suggests that 80 to 90% of the protein found in the water washes is derived from the matrix. The significant increase in the specific MDH activities of washes I and II and the decrease in the ghost preparation also indicate that matrix proteins are concentrated in the soluble fractions. The similarity of the specific activity of MDH in the water washes, coupled with the electrophoretic patterns of the water washes, is interpreted to suggest that the same proteins are probably liberated in all three washes. However, additional experiments are required to establish this with certainty.

The estimations of the membrane-bound enzyme, BDH, indicate that this tightly bound protein is not present in the soluble fractions. The total BDH activity of the intact mitochondria is recovered in the crude ghost fraction, and the specific BDH activity of this fraction is twice that of the intact mitochondria. The complete retention of the mitochondrial cytochromes in the ghost fraction shows that none of the inner mitochondrial membranes, which are thought to contain these proteins as well as the BDH activity, are released into the water washes.

Thus, quantitative chemical analyses of membrane components (total phosphate, phospholipids, and protein) and specific enzymic markers for (a) matrix proteins (MDH) and (b) membrane-bound proteins (cytochromes and BDH) support the views that both membranes of the intact mitochondrion are completely conserved in the water-washed preparation and that the protein liberated by the osmotic lysis is extracted almost entirely from the matrices. In addition, it is concluded that the matrix proteins (using MDH as a marker) are not completely extracted by the water washing but that the ghosts retain about $\frac{1}{3}$ of the matrix material originally present in unlysed mitochondria. These data agree with the interpretation of the morphological findings, and lead to the conclusions that both mitochondrial membranes are present in the crude ghost fraction, although the "double membrane" organization of intact mitochondria no longer exists, and that only reduced amounts of matrix are retained.

Measurement of the oxidative capacity of the water-washed mitochondria shows that the multi-enzyme functions of the respiratory chain remain essentially intact. The complete conservation of

the cytochromes and the doubling of the rate of oxygen uptake per milligram of protein in the ghosts when succinate is oxidized indicate that the electron transfer system of mitochondria is probably completely functional. Electron micrographs suggest that the structural integrity of the membrane surrounding the ghosts is also preserved to a high degree. The capacity of a large proportion of the ghosts to assume a highly contracted state upon the addition of ATP provides additional evidence that the water-washed mitochondria retain a number of integrated, multienzyme functions. Of the three integrated functions measured, only phosphorylative activity seems reduced in the ghost preparation. The increased specific uptake of O₂ by the crude ghost fraction vs. intact mitochondria is readily explained by the loss of matrix protein during washing; therefore, a given amount of ghost protein contains about twice as much inner membrane protein (twice as many functional, respiratory chains) as does an equal amount of mitochondrial protein.

The drop in phosphorylating ability in the ghost accounts for the lowered P:O ratios observed; the ghost preparations phosphorylate about 25% as efficiently as intact mitochondria since twice as much inner membrane is added per milligram of ghost protein as is added per milligram of mitochondrial protein. Two possibilities may be offered to explain the decreased phosphorylative activity of the ghosts: (a) the water-washing procedure may partially extract a soluble factor(s) (no attempts to reconstitute phosphorylation by recombining soluble fractions have yet been made); or (b) the phosphorylating machinery may remain in the ghost fraction but may function with diminished efficiency as a result of some molecular reorganization caused by the osmotic lysis of the intact mitochondria. Each of these possibilities could involve population differences within the ghost preparation, i.e. some of the ghosts might retain full capacity to phosphorylate ADP to ATP whereas others might be completely devoid of phosphorylating activity, or, alternatively, all

the ghosts might retain a diminished capacity to couple phosphorylation to oxidation. Ghosts in thin sections show a high degree of uniformity in morphology and structural integrity when observed in the electron microscope; however, only a fraction of the ghosts appear to contract upon the addition of ATP. Thus, some multienzyme functions of mitochondria appear to be reduced in the ghost preparations as in the case of phosphorylation, whereas others, e.g. respiration, are completely functional. In addition, differential contraction of ghosts upon the addition of ATP suggests that other activities are retained completely by some ghosts and are completely lost by others.

Attempts to fractionate the crude ghost preparation into inner and outer membranes are in progress. Success in these attempts and analyses of the isolated fractions may aid in understanding the supramolecular complexes involved in the structural-functional interdependence of rat-liver mitochondria. The present data confirm and extend the results of Greenawalt, Vasington, and Caplan (37) and show that the crude preparation of water-washed mitochondria, enclosed by single unit membranes and deficient in matrix protein, are capable of carrying out major energy-dependent properties of intact mitochondria: (a) respiration, (b) ion accumulation, and (c) contraction. As far as has been determined now, the reduced capacity to phosphorylate ADP to ATP marks the only major biochemical alteration resulting from the osmotic lysis which spatially separates the inner and outer membranes.

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REFERENCES

1. KING, T. E., and TAKEMORI, S., *J. Biol. Chem.*, 1964, **239**, 3546.
2. GREEN, D. E., and HECHTER, O., *Proc. Nat. Acad. Sc.*, 1965, **53**, 318.
3. LEHNINGER, A. L., *The Mitochondrion*, New York, W. A. Benjamin, Inc., 1964.
4. CHAN, P. C., LEHNINGER, A. L., and ENNS, T., *J. Biol. Chem.*, 1960, **235**, 1769.

5. LEHNINGER, A. L., and GREGG, C. T., *Biochim. et Biophysica Acta*, 1963, **78**, 12.
6. GREGG, C. T., and LEHNINGER, A. L., *Biochim. et Biophysica Acta*, 1963, **78**, 27.
7. COOPER, C., and KULKA, R. G., *J. Biol. Chem.*, 1961, **236**, 2351.
8. KLINGENBERG, M., and PFAFF, E., in Symposium on Regulation of Metabolic Processes in Mitochondria, Bari, Italy, (J. M. Tager, S. Papa, E. Quagliariello, and E. Slater, editors), New York, Elsevier Publishing Co., 1965, 180.
9. RACKER, E., CHANCE, B., and PARSONS, D. F., *Fed. Proc.*, 1964, **23**, 431.
10. FERNANDEZ-MORAN, H., in Symposium on the Plasma Membrane, New York, American Heart Association and New York Heart Association, 1962, 1039.
11. ALLMANN, D. W., BACHMANN, E., and MCCAMAN, R. D., *Fed. Proc.*, 1966, **23**, 414.
12. PARPART, A. K., *J. Cellular and Comp. Physiol.*, 1942, **19**, 248.
13. HILLIER, J., and HOFFMAN, J. F., *J. Cellular and Comp. Physiol.*, 1953, **42**, 203.
14. WEIBULL, C., *J. Bact.*, 1953, **66**, 688.
15. WEIBULL, C., *Exp. Cell Research*, 1956, **10**, 214.
16. LEHNINGER, A. L., *Physiol. Rev.*, 1962, **42**, 467.
17. SCHNEIDER, N. C., in Manometric Techniques, (W. W. Umbreit, R. Burris, and J. E. Stauffer, editors), Minneapolis, Burgess Publishing Co., 1956, 188.
18. LUFT, J. H., *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 409.
19. KARNOVSKY, M. J., *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 729.
20. REYNOLDS, E. S., *J. Cell Biol.*, 1963, **17**, 208.
21. GORNALT, A. G., BARDAWALL, C. T., and DAVID, M. M., *J. Biol. Chem.*, 1963, **177**, 574.
22. LOWRY, O. H., ROSENEROUGH, N. F., FARN, A. L. and RANDELL, R. J., *J. Biol. Chem.*, 1951, **193**, 265.
23. GOMORI, G., *J. Lab. and Clin. Med.*, 1962, **27**, 467.
24. CHANCE, B., and WILLIAMS, G. R., *J. Biol. Chem.*, 1955, **217**, 295.
25. GREGG, C. T., *Biochim. et Biophysica Acta*, 1963, **74**, 573.
26. NIELSEN, S. O., and LEHNINGER, A. L., *J. Biol. Chem.*, 1955, **215**, 555.
27. ORNSTEIN, L., and DAVIS, B. J., Disc Electrophoresis, Distillation Products Industries, Rochester, New York, 1962.
28. CAPLAN, A. I., and CARAFOLI, E., *Biochim. et Biophysica Acta*, 1965, **104**, 317.
29. WILLIAMS, J. N., *Arch. Biochem. and Biophysics*, 1964, **107**, 537.
30. Pabst Laboratories, Circular OR-18.
31. OCHOA, S., in Methods in Enzymology, (S. Colowick and N. Kaplan, editors), New York, J. Wiley & Sons, Inc., 1955, **1**, 735.
32. FOLCH, J., LEES, M., and SLOANE-STANLEY, G. H., *J. Biol. Chem.*, 1957, **226**, 497.
33. LEHNINGER, A. L., SUDDUTH, H. C., and WISE, J. B., *J. Biol. Chem.*, 1960, **235**, 2450.
34. GETZ, G., BARTLEY, W., STRIPE, F., NOTTON, B., and RENSHAW, A., *Biochem. J.*, 1962, **83**, 181.
35. VASINGTON, F. D., and GREENAWALT, J. W., *Biochem. and Biophysic. Research, Commun.*, 1964, **15**, 133.
36. LEHNINGER, A. L., *J. Biol. Chem.*, 1951, **190**, 345.
37. GREENAWALT, J. W., VASINGTON, F. D., and CAPLAN, A. I., *J. Cell Biol.*, 1965, **27**, No. 2, 38A (abstract).
38. HACKENBROCK, C. R., and BRANDT, P. W., *J. Appl. Physics*, 1965, **36**, 2626.
39. WATSON, M. L., and SIEKEVITZ, P. J., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 639.