THE EFFECTS OF OSMOTIC LYSIS
ON THE OXIDATIVE PHOSPHORYLATION
AND COMPARTMENTATION OF
RAT LIVER MITOCHONDRIA

ARNOLD I. CAPLAN and JOHN W. GREENAWALT

From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205. Dr. Caplan's present address is the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154

ABSTRACT

Rat liver mitochondria isolated in 0.25 M sucrose were osmotically lysed with distilled water. The effect of osmotic lysis on mitochondrial compartmentation was monitored by following the changes in the specific Mg$^{2+}$-ATPase and the stimulation of this activity by DNP. Each reuspension in distilled water caused a progressive increase in the specific Mg$^{2+}$-ATPase and a decrease in DNP-stimulation. Lysed mitochondria yielded P:O ratios of slightly less than 1.0 when each of the “site-specific” substrates, NADH, D-β-hydroxybutyrate, succinate, and ascorbate, were oxidized. These data indicate that only site 3 phosphorylation remained undiminished. The crude, lysed mitochondria were subfractionated by centrifugation on linear sucrose density gradients. Assays for protein, malate dehydrogenase, D-β-hydroxybutyrate dehydrogenase, and succinate dehydrogenase indicated that the inner compartment could be clearly separated from the outer membrane vesicles. The results also suggested that the small vesicle fraction contained a small proportion of vesiculated inner membranes. Inner mitochondrial compartments, “contracted” by preincubation in the presence of ATP, sedimented to a markedly lower density on the gradients than did the unincubated preparations and about 50% of the ghosts showed a highly condensed morphology. In the contracted preparations, relatively low malate dehydrogenase and D-β-hydroxybutyrate dehydrogenase activities were found in the fractions comprised of the inner compartments. The specific activity and distribution of succinate dehydrogenase were about the same as were found with the unincubated, lysed mitochondria.

INTRODUCTION

Previous studies have shown that osmotic lysis of isolated rat liver mitochondria with cold distilled water spatially separates the inner and outer membranes of these organelles; however, a number of biochemical activities found in intact mitochondria are unaffected by this treatment although others are modified considerably (1–5). Two populations of vesicles different in size and morphology but each enclosed by a single membrane, were sedimented as a pellet after water treatment, and it was suggested that the larger ghosts were comprised of the inner membrane which retained some matrix and that the smaller profiles represented vesiculated outer membranes (4). About 50% of the total mitochondrial protein including 1/5 of the soluble proteins of the matrix (as estimated by malate dehydrogenase) sedimented with the membranes of the lysed mitochondrial preparation. D-β-Hydroxybutyrate dehydrogenase, succinate dehydrogenase, phospholipid, and respiratory activity were completely recovered with the sedimented membranes. The
efficiency of massive calcium accumulation by respiration- or ATP-supported pathways was essentially the same as that found in intact mitochondria (1, 2, 5), but Vasington (3) has reported that lysed mitochondria are deficient in the ability to accumulate Sr++ by an ATP-supported mechanism. In addition, the rate of oxidative phosphorylation of ADP to ATP by osmotically lysed mitochondria was decreased to about 25% of that normally found in intact mitochondria, and no respiratory control (stimulation of oxygen uptake by ADP) was observed.

Studies on the phosphorylative capacity of lysed mitochondria have been extended in the present paper to determine whether specific phosphorylation sites are affected by the lytic treatment or whether all three phosphorylating sites are reduced in phosphorylating efficiency. The data indicate that phosphorylation at sites I and II is absent in the lysed preparations. The possibility of using the change in specific activity and the DNP-stimulation of Mg++-ATPase activity as criteria for monitoring the disruption of intact mitochondria during the lytic process was also investigated. Evidence is presented which suggests that the lytic procedure is a mild procedure as reflected by the gradual shift in mitochondrial ATPase activities.

Partial separation of the two membranous populations was achieved in the present study by centrifugation on sucrose density gradients. Results of enzymatic assays suggest that the ghosts (inner membranes plus some matrix) can be separated from the small vesicles; however, the smaller vesicle fraction obtained from the gradient contained activities attributable to both the inner and outer membranes. It appears that the inner membrane may also vesiculate to a lesser extent into small elements which have a density similar to that of the vesicles derived from the outer membrane.

METHODS

Mitochondria were isolated from the livers of well-fed rats (Carworth Farms, Sprague-Dawley strain) and osmotically lysed by washing three times in distilled water, as previously described (4). d-β-Hydroxybutyrate dehydrogenase activity (BDH), succinate dehydrogenase (SDH), malate dehydrogenase (MDH), phosphorylation, oxygen consumption, protein, and inorganic orthophosphate (Pi) were assayed by methods previously described (4).

Assay of ATPase

ATPase activity was measured by following the release of Pi in the presence of an ATP-regenerating system; a modification of the conditions described by Pullman et al. (7) was used. The incubation medium contained 10 mM Tris-HCl, pH 7.5; 3 mM ATP, pH 7.5; 2 mM MgCl2; 5 mM K-phospho(enol)pyruvate (PEP), pH 7.5; and 2 μl of pyruvate kinase (PK) (Sigma Rabbit Muscle Type 1, 47 mg/ml; 0.1 mg converts 0.38 μmole of PEP per min) and, when present, 5 × 10⁻⁴ M 2,4 dinitrophenol (DNP). The total volume equaled 0.5 ml. The reaction was started by addition of protein (enzyme) to the reaction medium. Incubation was carried out with shaking at 30°C for 5 min. The reaction was stopped by adding 0.5 ml of 5% TCA and placing the tubes in an ice bath. The TCA-insoluble material was sedimented by low-speed centrifugation, and 0.5 ml of the TCA-soluble supernatant was analyzed for inorganic phosphate.

The ATPase activity was measured of six different preparations including intact mitochondria, each resuspension of mitochondria in distilled water prior to centrifugation in the lytic procedure, the crude ghost preparation and sonicated ghost preparation. It should be emphasized that all fractions were stored at 0°C until used; the mitochondrial preparation, being the first isolated, was stored longest (in this case, in 0.25 M sucrose). Assays of ATPase in all samples were made simultaneously and immediately upon the preparation of the sonicated ghosts. Thus, no sample was stored much longer than 2 hr prior to assay.

Succinate Dehydrogenase (SDH)

Succinate dehydrogenase (SDH) was assayed spectrophotometrically at 400 nm with a Zeiss PMQII spectrophotometer equipped with a Sargent Model SRL Recorder. The 2-ml system contained 2 mM phenazine methosulfate (PMS), 0.05 mM dichlorophenol-indophenol (DCPIP), 1 mM KCN, 20 mM each of Na-succinate and Pi, all at pH 7.6. The amounts of PMS and DCPIP used were shown to give maximal SDH activities in the system described. The cuvettes with enzyme were preincubated in the presence of Pi and KCN at 37°C for 10 min before the addition of PMS and DCPIP. Preincubation of enzyme for 7-13 min was necessary for us to obtain maximal activities. Succinate was added to start the reaction. As reported previously (4), there is a substantial increase in activity upon sonication, and thus, all samples were sonicated prior to assay.

Succrose Density Gradients

Linear sucrose gradients were prepared with a Buchler density gradient apparatus (Buchler Instruments, Fort Lee, N. J.) and stored at 4°C for
at least 12 hr before use. Samples were layered on gradients of 25 ml of 35-50% or 40-50% sucrose (w/w) and contained about 15 mg of protein in a volume not greater than 3.0 ml. These gradients were centrifuged for 10 min at 10,000 rpm and then for 140 min at 25,000 rpm in a Spinco Model L centrifuge in the cold in a SW 25.1 rotor. Samples were collected dropwise by puncturing a hole in the bottom of the tubes and controlling the drop flow by the slow injection of extra heavy mineral oil "Nujol" through a very tightly fitted one-hole stopper. Sucrose concentrations, expressed in units % w/w, were read directly from a calibrated scale on a Bausch and Lomb Model Abbe-3L refractometer. Similar gradient patterns were obtained after 150 and 1500 min of high-speed centrifugation, which suggests that after 150 min the various membranous elements of the water-lysed mitochondrial preparation had migrated to a density very close to that which would be obtained at density equilibrium.

**Electron Microscopy**

Pellets were fixed with 6.25% glutaraldehyde in 0.1 m phosphate buffer, pH 7.4, for 1-2 hr followed by postfixation with 1% OsO4 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 Instrument Co.) 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 (8). The embedded pellets were sectioned on Porter-Blum or LKB microtomes with glass or diamond knives. The sections were collected on grids containing no supporting films and stained with lead by "Method B" of Karnovsky (9) or double-stained with uranyl acetate followed by lead citrate (10). Specimens were observed with a Siemens-Elmiskop 1 double-condenser electron microscope, at magnifications up to 80,000, with objective apertures of 20 or 30 μm diameter.

**RESULTS**

**ATPase Activity**

Fig. 1 shows that the rate of hydrolysis of ATP by the osmotically lysed mitochondrial preparation during the first 5 min of incubation was about three times greater in the presence than in the absence of an ATP-regenerating system. In the latter case, the ATPase activity reached an abrupt plateau after about 5 min, presumably caused by the production and accumulation of ADP which is known to inhibit the mitochondrial ATPase activity (7). In the presence of the regenerating system, on the other hand, linear rates of hydrolysis were observed for about 15 min. Because of these results, PEP and PK were included in the assay system in subsequent experiments. Lee and Ernster (11) have shown that the concentration of added MgCl₂ affects the ATPase activities and P:O ratios observed with submitochondrial particles. Also, pyruvate kinase has been shown to be activated by Mg⁺⁺ (36). Therefore, the concentration of MgCl₂ was varied from 0.01 to 9.0 mM so that we could determine the effect of Mg⁺⁺ concentration on the ATPase activity of the lysed mitochondrial preparation in the presence of the Mg⁺⁺-requiring regenerating system. As shown in Fig. 2, the ATPase activity increased sharply with the increasing concentrations of Mg⁺⁺ and reached a maximum at a concentration of 1-2 mM MgCl₂. At concentrations greater than 2 mM MgCl₂, the rate of ATP hydrolysis decreased (Fig. 2); half-maximal rates were observed at both 0.2 and at 8.0 mM MgCl₂. The ATPase activity, therefore, was assayed in media containing 2 mM MgCl₂. Under the assay conditions used, however,
the activation observed reflects the Mg++ requirements of both the ATPase and pyruvate kinase.

The Mg++-stimulated ATPase activity exhibited by the lysed mitochondria (crude ghost preparation) was completely inhibited by oligomycin at a concentration of 2 μg/mg of protein. Thus, the sensitivity of the Mg++-ATPase to oligomycin which is normally observed in intact mitochondria was not affected by the lytic procedures. The conditions described above, i.e., 2.0 mM MgCl₂ in the presence of an ATP-regenerating system, were utilized so that we could follow the sequential effects of the water-treatment on the ATPase activities of rat liver mitochondria as they were lysed (converted to ghosts and outer membrane vesicles).

Fig. 3 shows the specific activity of the Mg++-ATPase and the DNP stimulation of this Mg++-ATPase activity of each fraction obtained during the preparation of the crude ghost fraction (see legend Fig. 3). Since ATPase activities of mitochondria vary as a function of structural intactness, two preparations were used arbitrarily as bases for estimating the degree of structural alteration caused by each water-washing step. A sonicated, lysed mitochondrial preparation represented maximally disrupted mitochondria while intact mitochondria isolated in 0.25 M sucrose represented the most structurally intact sample assayed for ATPase activity.

Two changes occurred during the course of preparing the lysed mitochondria. A progressive increase in the specific activity of the Mg++-ATPase accompanied the sequential washing of the mitochondria; maximal specific activity was observed in the most disrupted sample (sonicated, lysed preparation). Also, the relative stimulation of the Mg++-ATPase by DNP was progressively reduced with increased washing of the mitochondria. In this case, minimal stimulation was observed in the most disrupted sample. These changes did not correlate quantitatively with the amount of total matrix protein lost during the preparative manipulations. If there were a simple direct correlation, the specific Mg++-ATPase activity would be expected to increase by a factor of about 2.0 since 50% of the total protein was removed by water-washing. In fact, the specific activity was increased by a factor of 8. Neither Mg++-ATPase nor DNP-stimulated Mg++-ATPase were detected in the water washes. All measurable Mg++-ATPase activity was retained by the membranes during the preparative procedures. An earlier report showed that other membrane-bound enzymes were also conserved in the crude ghost fraction (4).

The lytic procedure appears to be a relatively gentle treatment which produced a gradual increase in the observed Mg++-ATPase activity. By the criterion of relative Mg++-ATPase activity, the structural organization of the lysed mitochondria is intermediate between that of intact mitochondria and that of sonicated, lysed mitochondria.

**Oxidative Phosphorylation**

It has been shown that oxidative phosphorylation is significantly uncoupled in lysed mitochondria (1, 2, 4). Therefore, experiments were carried out to determine whether all three phosphorylation sites were partially uncoupled or whether one or more of these sites were completely uncoupled. Phosphorylation coupled to the oxidation of NADH, D-f-hydroxybutyrate (β-OH), succinate, and ascorbate was measured. The observed P:O ratios of the lysed mitochondria were below 1.0 for each of the site specific substrates tested (Table I). Intact mitochondria, under similar conditions, yielded P:O ratios of 2.6, 1.9, and 0.8 when β-OH, succinate, and ascorbate, respectively, served as substrates (12, 13). Since no increase was found in the P:O ratio in experiments with the lysed preparation when ascorbate was replaced by succinate, NADH, or β-OH, it is concluded that only the
third coupling site (between reduced cytochrome c and oxygen) is functional in these preparations.

The precise pathway by which external NADH is oxidized by intact mitochondria is still not known although a number of pathways have been proposed (14–22). As in intact mitochondria, external NADH is oxidized by the lysed mitochondria via a nonphosphorylating, antimycin A-insensitive pathway. As a result, the observed P:O ratios were very low with external NADH as a substrate (P:O \( \leq 0.1 \) to 0.2). A correction was made for the nonphosphorylating, NADH oxidation by subtracting the amount of O\(_2\) consumed, which was antimycin A-insensitive, from that obtained in the absence of the inhibitor. The calculated respiratory chain-linked P:O ratios ranged from 0.5 to 0.6 (see Fig. 4). The differences in the oxidative pathways of externally added NADH and \( \beta \)-OH are significant. It can be seen (Fig. 4) that the external, nonphosphorylating, antimycin A-insensitive oxidation of NADH is as efficient as the respiratory chain-linked, antimycin A-sensitive oxidation. The oxidation of \( \beta \)-OH seems to be much more efficient than the external NADH-linked pathways as evidenced by the relative rates of \( \beta \)-OH and NADH oxidation. These observations suggest that the NAD associated with membrane-bound \( \beta \)-DH is more effectively linked to the respiratory chain than is external NADH.

Subfractionation of Osmotically Lysed Mitochondria on Sucrose Density Gradients

Biochemical and ultrastructural evidence presented in earlier communications (1–5) indicated that lysed mitochondrial preparations contained small vesicular elements derived from the outer mitochondrial membranes as well as morphologically distinct "ghosts" comprised of components from the inner mitochondrial compartment. It was shown that about \( \frac{3}{4} \) of the total matrix proteins of freshly isolated mitochondria was retained, presumably, within the ghosts in the lysed preparations. To provide a basis for further investigations concerning the interrelationship of the function...
TABLE I
Oxidative Phosphorylation by Lysed Mitochondria

The P:O ratios obtained with succinate, β-OH, and ascorbate (all 10 mM) as substrates of respiratory chain-linked oxidations by the lysed mitochondria are compared with those of intact rat liver mitochondria. The medium is that described by Gregg (35). TMPD (N,N,N',N'-tetramethyl-p-phenylene-diamine) was present in a concentration of 0.1 mM and NAD+ in a concentration of 1 mM. Antimycin A was used to block the contributions of the respiratory chain-linked phosphorylation sites other than that of the third site (that from cytochrome c and oxygen).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Substrate</th>
<th>Phosphate-linked ADP*</th>
<th>Oxygen uptake†</th>
<th>P/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact mitochondria</td>
<td>β-OH</td>
<td>1561</td>
<td>598</td>
<td>2.61</td>
</tr>
<tr>
<td>Intact mitochondria</td>
<td>Succinate</td>
<td>1145</td>
<td>612</td>
<td>1.87</td>
</tr>
<tr>
<td>Intact mitochondria</td>
<td>Ascorbate (TMPD + antimycin A)</td>
<td>482</td>
<td>598</td>
<td>0.81</td>
</tr>
<tr>
<td>Ghost fraction</td>
<td>β-OH (+NAD+)</td>
<td>546</td>
<td>696</td>
<td>0.80</td>
</tr>
<tr>
<td>Ghost fraction</td>
<td>Succinate</td>
<td>462</td>
<td>710</td>
<td>0.65</td>
</tr>
<tr>
<td>Ghost fraction</td>
<td>Ascorbate (TMPD + antimycin A)</td>
<td>551</td>
<td>710</td>
<td>0.78</td>
</tr>
</tbody>
</table>

* m mol P\textsubscript{i} incorporated into ADP.
† m atoms Oxygen consumed.

Figure 4 Comparison of the amounts of oxygen consumed by the lysed mitochondria (4.5 mg per 3 ml system) with β-OH (β-hydroxybutyrate fortified with NAD\textsuperscript{+}) and with externally generated NADH (alcohol dehydrogenase (ADH) plus ethanol (EtOH)) as substrates. See Methods and Materials. Trichloroacetic acid (TCA) was used to terminate the reaction; the TCA-soluble fraction was analyzed for that \textsuperscript{32}P which had been incorporated into ATP. Oxidation in the presence and absence of antimycin A (anti A) demonstrates the existence of an external, antimycin A-insensitive, nonphosphorylating NADH oxidation pathway. The “corrected” P:O ratio is that calculated for the antimycin-sensitive oxidation of NADH.

and structure of the two mitochondrial membranes, we made attempts to isolate these two components by centrifugation on linear sucrose density gradients. It seemed likely that separation might be achieved if (a) the inherent phospholipid-to-protein ratios of the outer vs inner membranes were significantly different, and/or (b) the protein retained within the ghosts contributed sufficiently to the density of the ghosts so that their apparent density increased significantly. The possibility of utilizing the “contractibility” of the ghosts in the presence of ATP (4) as an additional aid to separation was also investigated.

Linear density gradients (35–50% sucrose, w/w) were layered with either (a) unincubated, lysed mitochondria or (b) lysed mitochondria which had been “contracted” in the presence of ATP; these tubes were centrifuged and fractions were collected dropwise from each. Correlated enzymatic and morphological studies of various subfractions from the gradients were made in order to estimate the degree of separation of the two populations. The subfractions were assayed for protein, BDH, SDH, and MDH; samples
(marked A, B, C, D, E, and F) were taken for examination in the electron microscope (see Fig. 5) as described in Methods.

**SOLUBLE FRACTIONS**

In both tubes, unincubated and contracted (I and II, Fig. 5), three subfractions were separated: a heavy somewhat diffuse subfraction having a density greater than that of 40% sucrose, a light subfraction which banded near the top of the gradient (density about that of 35% sucrose), and a "soluble" fraction (density less than that of 30% sucrose). The soluble fractions contained no detectable membranous elements or BDH or SDH activities; substantial quantities of MDH activity were present. In this respect, this fraction from the gradient resembled the water-washes (4) and probably represents soluble matrix proteins from the water-lysed mitochondria which did not penetrate into the gradient but remained on top as a solubile fraction.

**MEMBRANE FRACTIONS—UNINCUBATED, LYSED MITOCHONDRIA—TUBE I**

Electron micrographs of samples of the heavy and light subfractions from the unincubated preparation (Fig. 6 A, B, C) showed that the heavy subfraction (Fig. 6 A, B) was composed almost exclusively of large, single membrane-limited ghosts which had some stainable matrix material and internal membranes interpreted to be cristae. The light subfraction (Fig. 6 C) was composed of empty-appearing vesicles limited by single membranes and generally much smaller than the vesicles in the heavy subfraction. Occasionally seen in this fraction were some vesicles about the size of the larger, more dense vesicles, but these from the upper band were usually empty-appearing. Morphological examination of the bands indicated, therefore, that the two populations of vesicles in the lysed mitochondrial preparations were separated.

Assays for protein, MDH, BDH, and SDH on fractions collected from the gradients supported the morphological evidence, presented above (Fig. 5 a and b, Tube I). The distribution of major peaks of protein and BDH and SDH activities on the gradient corresponded to the location of the heavier subfraction (Fig. 6 A and B). The distribution profiles of BDH and SDH were essentially identical. These enzymes have been reported to be tightly bound to the inner mitochondrial membranes (23-26). Also, 80-90% of the total SDH or BDH activity was recovered with the heavier (ghost) subfraction. This heavier subfraction also contained most of the recovered MDH activity (Fig. 5 b, Tube I). From these observations, it is suggested that the heavier subfraction is derived exclusively from the inner membrane compartment of intact mitochondria.

The light membranous subfraction contained little of the total MDH activity (Fig. 5 b, Tube I) of the lysed preparation although some cross-contamination with the soluble fraction resulted from the technique of collecting the samples from the small orifice in the bottom of the tube. This interpretation was supported by experiments in which the soluble fraction was removed from the top of the gradient with a Pasteur pipet. When the small vesicle subfraction was then collected in this fashion, it contained no measurable MDH activity. It seems clear that MDH activity is not concentrated with the lighter small vesicle fraction but is distributed between the soluble and heavier, ghost fractions. Of importance, in this regard, are the observations (a) that MDH activity sharply increased in fractions from the gradient which corresponded to the marked decrease in sucrose concentrations at the interphase between the gradient and sample volume, (b) that the small vesicle subfraction did not have detectable MDH activity, and (c) that the vesicles of this lighter fraction appeared empty, i.e. devoid of stainable matrix (Fig. 6 C). The larger, empty-appearing vesicles which were sometimes seen in the lighter subfractions were probably inner membrane vesicles from which most, or all of the matrix protein had been extracted. Also, the distribution of BDH and SDH suggests that a portion of the smaller vesicles in the lighter subfraction was comprised of vesiculated inner membrane; this lighter subfraction may, therefore, contain empty vesicles derived from both inner and outer membranes. If so, then the inner membrane (devoid of matrix material) would appear to have a density very similar to that of the outer membrane, and the presence of matrix protein in the larger vesicles may contribute significantly to the distribution patterns observed on the density gradients.

**MEMBRANE FRACTIONS—LYSED MITOCHONDRIA CONTRACTED IN THE PRESENCE OF ATP—TUBE II**

From previous experiments (4), it was concluded that about 50% of the ghosts in the crude lysed
**Figure 5** Fractionation of osmotically lysed mitochondria (crude ghost preparation) on linear sucrose density gradients. The sedimentation profiles and the distribution of enzymic activities in subfractions of unincubated, lysed mitochondria (unincubated ghosts) vs subfractions of lysed mitochondria contracted by incubation in the presence of ATP (+ATP) were compared. Osmotically lysed mitochondria were contracted by incubation for 20 min at 30°C in medium consisting of NaCl (80 mM), Tris-chloride buffer pH 7.4 (5 mM), MgCl₂ (5 mM), orthophosphate (2 mM), Na-succinate (5 mM), and ATP (5 mM). Aliquots (15 mg of protein) of the unincubated “ghosts” and ATP-contracted ghosts were layered on the gradients and centrifuged. See Methods and Materials. The linearity of the sucrose gradients and the concentration of sucrose (% w/w) in each fraction is shown by the open circles (O—O). The gradients ranged in concentration from 35% (w/w) sucrose (density = 1.151) to 50% (w/w) sucrose (density = 1.229). Samples were taken at points A, B, and C and at D, E, and F for electron microscopic examination (See Fig. 6 A, B, and C and 7 D, E, and F). Enzyme assays are described in Methods and Materials.

**Figure 5 a** The distribution of succinate dehydrogenase (SDH, X--X), and D-3-hydroxybutyrate dehydrogenase (BDH, Δ-Δ-Δ), and protein (●—●—●) are shown. Sedimentation profiles of unincubated ghosts and ATP-contracted ghosts are schematically illustrated by the drawings on the left of the distribution curves. I. Fractionation of unincubated ghosts. II. Fractionation of ATP-contracted ghosts.

preparations undergoes high amplitude contraction in the presence of ATP. In the present study, experiments were conducted to determine whether this effect would aid in the separation of inner and outer mitochondrial membranes. Samples of the lyed preparation were incubated for 20 min under ion uptake conditions (see legend for Fig. 5) in the absence of Ca²⁺ but with ATP present in the
The distribution of proteins (---) and malate dehydrogenase (MDH, - - - -). Sedimentation profiles of unincubated ghosts and ATP-contracted ghosts are indicated (as in 5 a) by the drawings to the left of the distribution curves. I. Fractionation of unincubated ghosts. II. Fractionation of ATP-contracted ghosts.

FIGURE 5b The distribution of proteins (---) and malate dehydrogenase (MDH, - - - -). Sedimentation profiles of unincubated ghosts and ATP-contracted ghosts are indicated (as in 5 a) by the drawings to the left of the distribution curves. I. Fractionation of unincubated ghosts. II. Fractionation of ATP-contracted ghosts.

Aliquots were then layered on linear sucrose density gradients and centrifuged. The distribution patterns of protein, SDH, BDH, and MDH shown in Figs. 5 a and 6, Tube II were observed; samples were taken at points D, E, and F for examination in the electron microscope.

Fig. 5, Tube II, shows that, as with the unincubated preparation, two membranous subfractions were obtained from the density gradients in addition to the soluble fraction. The lighter subfraction sedimented to the same density as that of the lighter subfraction obtained with the unincubated sample (cf. Figs. 5, I), and in the electron microscope, the two lighter, small vesicle fractions appeared identical (cf. Figs. 7 F and 6 C). In addition the protein and enzymic contents of the small vesicle fraction in the unincubated preparations were very similar.

However, comparison of Fig. 5 I and II shows that the heavier components of the lysed preparation which had been incubated in the presence of ATP sedimented to a density considerably lower than that observed with the unincubated fraction. Also, the ATP-incubated preparation gave a less symmetrical elution pattern with a pronounced shoulder at the trailing edge of the band. Examination of the heavier fraction and shoulder in the electron microscope (Figs. 7 D and E) showed that both contained two types of profiles: (a) ghosts with the typical morphology, enclosed by a single membrane and containing a few cristae and some stainable matrix, and (b)}
Subfraction A taken from the leading edge of the denser band. Composed almost entirely of ghosts, i.e. the inner mitochondrial compartment. Some stainable matrix and recognizable cristae are seen.

Subfraction B is from about the middle of the more diffuse, tailing portion of the denser band. Also composed primarily of ghosts. This subfraction may contain slightly less flocculent appearing matrix than subfracion A but is otherwise very similar in appearance.

Subfraction C is comprised primarily of small single membrane-limited vesicles, about 0.4 μ in diameter, containing no stainable matrix. Numerous profiles of concentrically arranged membranes are present.
FIGURE 7  Subfractions of lysed mitochondria contracted with ATP. The lysed preparation was contracted by preincubation with ATP and contains ghosts and small vesicles much like those in the uninoculated sample. Profiles of highly condensed ghosts are also present. The denser subfraction of the ATP-contracted preparation has a much lower average density than does the dense subfraction of the “uninoculated” sample (see Fig. 5). X 28,000.

Subfractions D and E. About 50% of the ghost profiles in these subfractions are similar to those of the uninoculated subfractions A and B but about 50% are highly condensed. A few small vesicles may also be present. Subfraction D may contain slightly more flocculent matrix than subfraction E, but, in general, D and E appear very similar. It may be that D and A sediment to higher densities than E and B, respectively, because they contain a greater concentration of matrix protein within the single-limiting membrane.

Subfraction F of the ATP-contracted sample appears to be identical with that of the less dense, uninoculated sample (subfraction C) and sediments to about the same density on the gradient. Only small vesicles enclosed by single membranes are seen, although concentrically arranged vesicles are seen.

ARNOLD I. CAPLAN AND JOHN W. GREENAWALT  Osmotically Lysed Mitochondria 27
highly condensed or contracted ghosts. Results shown in Fig. 5 a, II indicate that the relative amount of measurable SDH was about equal to that found with the uninubcated samples and that this enzyme was distributed in both the denser subfraction and shoulder. The observed MDH activity (Fig. 5 b, II) was distributed almost exclusively in the shoulder and in the soluble fractions; the amount of activity in the shoulder compared to that found in the uninubcated sample was somewhat low. The BDH activity found in the lighter, small vesicle subfraction was of the magnitude expected from the results obtained with the uninubcated preparation; however, almost no BDH function in the intact mitochondrion. With this in mind, mitochondria were disrupted by means of osmotic shock and the resulting lysed preparations of mitochondria were characterized in order to determine the effects of water-washing on mitochondrial structure, on enzyme activities and localization, and on integrated mitochondrial functions such as oxidative phosphorylation and contraction (1-5). The present study supports earlier results, which were interpreted to indicate that the inner and outer membranes were spatially separated by osmotic lysis (4), and further characterizes the water-lysed preparations. The results presented here also show that separation of the small vesicle fraction (outer membrane) and ghosts (inner membrane plus matrix) can be achieved by centrifugation on sucrose density gradients, but that the small vesicles are derived to a minor extent from inner as well as outer membrane.

It has now become generally accepted that the in vitro response of the mitochondrial Mg++-ATPase of rat liver to stimulation by DNP reflects the structural integrity of the mitochondria (7, 26). That is, freshly isolated, intact mitochondria have a relatively low Mg++-ATPase activity which can be greatly stimulated by DNP, whereas aged or disrupted mitochondria exhibit a high level of Mg++-ATPase which is not stimulated by the addition of DNP (6). To what extent structural alterations or conformational changes in membrane components are involved in this phenomenon is not precisely known. Submitochondrial particles obtained by sonic oscillation (6) or by mechanical disruption (7) catalyze oxidative phosphorylation, retain no respiratory control, and show little or no dinitrophenol-(DNP)–stimulated Mg++-ATPase activity. The specific Mg++-ATPase activity of such preparations is 10- to 20-fold higher than the Mg++-ATPase activity of intact mitochondria which can be substantially stimulated by DNP. The present results indicate (a) that osmotic lysis is a relatively gentle procedure which produces a gradual shift toward a more "disorganized state" (high Mg++-ATPase activity, low DNP stimulation); (b) that with regard to structural integrity, as judged by this criterion (relative Mg++-ATPase activity), the lysed preparations are intermediate between intact mitochondria and sonicated lysed mitochondria; and (c) that the mitochondrial ATPase remains associated with the membranes after lysis.

Inhibitor studies indicate that the Mg++-ATPase of mitochondria is functionally linked to
oxidative phosphorylation (28, 29). In addition, this enzymic activity has been identified with the “inner membrane subunits” observed in negatively stained preparations of intact mitochondria (30). Similar appearing particles are readily visible in negatively stained samples of osmotically lysed preparations of rat liver mitochondria (J. W. Greenawalt, unpublished observations). Furthermore, it was shown by Caplan and Greenawalt (4) that the single membranes enclosing individual ghosts in the lysed mitochondrial preparations are continuous, appear intact, and have the basic trilamellar architecture of a “unit membrane.” Thus, the effects on mitochondrial membrane organization which cause the observed shifts in ATPase activity do not seem to result in observable structural alterations of the inner membrane per se. Rather, structural integrity seems to be required either at the molecular level, where changes are below the resolution of the electron microscope, or at the organelle level, i.e. related to the maintenance of a double-membrane construction and/or the retention of all, or specific components normally contained within the two compartments. In any case, it is clear from the present study that the inverse relationship between high Mg\(^{2+}\)-ATPase activity and DNP stimulation can be used to monitor the lytic procedure and to estimate the relative degree of “structural integrity” of the components in the lysed preparations.

Two observations indicate that phosphorylation sites 1 and 2 were not functional in lysed mitochondria: (a) P:O ratios, under a variety of conditions, were consistently below 1.0, a value expected if only a single phosphorylation site were functioning, and (b) \(\beta\)-OH + NAD\(^+\), NADH, and succinate (which should give P:O ratios of 3, 3, and 2, respectively, if all sites were functional) gave experimental P:O values approximately equal to that obtained with ascorbate + TMPD, a substrate specific for phosphorylation at site 3 (12, 13).

The intact mitochondrion is a composite of at least two membranes and the components of the two compartments enclosed by these membranes. Frisell et al. (31) have recently shown that heavy rat liver mitochondria can be separated from light mitochondria by centrifugation and are distinguished by their higher content of matrix protein, sarcosine dehydrogenase, and electron transfer flavoprotein per unit of membrane protein and lipid. Since in the lysed preparations the outer membrane is spatially removed from the inner mitochondrial compartment, the sedimentation characteristics of the “ghosts” on density gradients are determined primarily by the amount of matrix material remaining and by the density of the inner membrane. The results of the present study indicate that the presence of unextracted matrix protein in the inner compartment (as marked by MDH activity and electron microscopy) may be an important factor in the separation of the light and heavy subfractions from the lysed mitochondrial preparations.

The MDH, SDH, and BDH activities of the gradient fractions indicate that the lighter subfraction which has been shown to contain a high percentage of vesicles from the outer membrane (27) also contains some contaminating inner membrane vesicles. The fact that vesicles from both membranes may be present in the same subfraction suggests that the inner and outer membranes are of similar densities. However, the present study provides no data concerning the possible rules that different lipid-to-protein ratios and qualitative differences in chemical composition in the membrane subfractions may play in the separation of these components. Parsons et al. have reported that the outer membrane has a density of 1.12–1.14 and the inner membrane a density of 1.21 (32, 33). This interpretation must, however, be viewed with some reservation since the inner compartment appears in the electron microscope still to contain stainable matrix material and would thus be more dense when compared to the outer membrane. Our studies have shown that, after three water-washes, approximately one-third of the matrix is retained inside the inner membrane compartment which, including the matrix, has a density of 1.20. Apparently, slightly more matrix material has been removed by the water-washing procedures used by the present authors than was removed by the procedure of Parsons et al. Further studies clearly demonstrating that matrix material is not present within the inner compartment must be performed before meaningful comparisons of the relative densities of the two membranes can be made.

The relationship between the inner membrane and the matrix should be considered further. In the discussion presented above, it is assumed that most matrix material is not associated structurally with the inner membrane. If this assumption is valid, then it should be possible to further extract...
the ghosts (heavy subfraction) and characterize inner membranes essentially free of matrix. To date, no such experiments have been reported. However, it should be noted that during the preparation of ghosts by osmotic lysis no concomitant extraction of lipid occurs (4). Whether or not the remaining third of the matrix proteins can be extracted without removal of components inherent to the inner membrane remains to be seen.

The observation that the heavy subfraction separated after contraction with ATP is less dense than the corresponding unincubated heavy subfraction suggests that upon contraction the contents remaining within some ghosts are concentrated within a smaller volume. Electron micrographs support this interpretation. The density of a membrane-limited vesicle can be decreased by an increase in the lipid-to-protein ratio of the membranes, but also by a change in the relative concentration of contents contained within the membrane. Since the shift of "ghosts" to a lower density accompanies contraction by ATP which results in a highly condensed profile, it seems likely that the density change is due to the extrusion of water and/or proteins from the matrix. This possibility is being investigated further.

It seems certain that the heavy subfraction separated as a band on the sucrose density gradients represents the inner mitochondrial membrane plus matrix. This view is supported by electron microscopic evidence, by the presence of marker enzymes for matrix (MDH) and enzymes bound to the inner mitochondrial membrane (BDH and SDH), by the absence of marker enzymes for the outer mitochondrial membrane (27), and by the retention of functions linked with oxidative phosphorylation (ATP-driven contraction and ion accumulation) (1-5). The difficulty encountered in attempting to unmask the latent BDH activity in contracted ghosts argues that the ATP-supported contraction restores the vesicles of the heavy subfraction to a form more closely resembling the molecular features of intact mitochondria which also exhibit a high degree of latency of this enzyme. Hackenbrock (34) has recently demonstrated that the inner mitochondrial compartment undergoes large-scale structural alterations (in the form of relaxation and contraction) which are closely associated with the respiratory state of the mitochondria. Thus, the capacity to contract is an important integrated function which is retained by some elements in preparations of water-lysed mitochondria.

The authors wish to thank Dr. A. L. Lehninger for his helpful discussions and Mr. Glenn L. Decker for his expert technical assistance with the electron microscope. This work was supported in part by Training Grant GM184 and Research Grant 12125 From the National Institutes of Health, United States Public Health Service. It was based on studies submitted by Dr. Caplan to the Faculty of Philosophy at the Johns Hopkins University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Received for publication 17 July 1967; revision accepted 31 August 1967.

REFERENCES