ABSTRACT

Osmotically lysed rat liver mitochondria have been utilized for a study of the biochemical and ultrastructural properties in relation to divalent ion accumulation. Osmotic lysis of mitochondria by suspension and washing in cold, distilled water results in the extraction of about 50% of the mitochondrial protein, the loss of the outer mitochondrial membrane, an increase in respiration, and a marked decrease in the ability to catalyze oxidative phosphorylation. Nevertheless, except for a decrease in the ability to accumulate Sr²⁺ by an ATP-supported process, these lysed mitochondria retain full capacity to accumulate massive amounts of divalent cations by respiration-dependent and ATP-supported mechanisms. The decreased ability of osmotically lysed mitochondria to accumulate Sr²⁺ by an ATP-energized process does not appear to be due to a loss or inactivation of a specific Sr²⁺-activated ATPase. The energy-dependent accumulation processes in lysed mitochondria show an increased sensitivity to inhibition by monovalent cations. Extraction of cytochrome c from osmotically lysed mitochondria results in a complete loss of phosphorylation and the respiration-dependent accumulation of Ca²⁺; a lesser, but significant, decrease in the ATP-supported accumulation of Ca²⁺ also was observed. The addition of cytochrome c fully restores the respiration-dependent accumulation of Ca²⁺ to the level present in unextracted, osmotically lysed mitochondria. The ATP-supported process is not affected by the addition of cytochrome c to extracted mitochondria, indicating that cytochrome c is not involved in ion transport energized by ATP. The osmotically lysed mitochondria are devoid of outer membranes and contain relatively little matrix substance. The accumulation of Ca²⁺ and P₄ by lysed mitochondria under massive loading conditions is accompanied by the formation of electron-opaque deposits within the lysed mitochondria associated with the inner membranes. This finding suggests that the inner membrane plays a role in the deposition of divalent ions within intact rat liver mitochondria. The relevance of these observations to those of other investigators is discussed.

It has been clearly established by numerous investigations that, depending upon the conditions of incubation, isolated mitochondria can accumulate varying amounts of cations by a respiration-dependent or an ATP-supported mechanism (1–10). It also has been shown that,
concomitant with the in vivo accumulation of vast amounts of Ca\(^{2+}\) or Sr\(^{2+}\) and inorganic phosphate (Pi) by mitochondria, large electron-opaque deposits are formed within the inner mitochondrial compartment; these deposits are formed within the inner mitochondrial membrane and cristae (11–15). It appears that in the presence of excess Ca\(^{2+}\) (massive-loading conditions) respiratory energy is not conserved via the phosphorylation of ADP to ATP but rather is utilized in reactions supporting ion transport and accumulation (1, 2, 4, 8, 10). It also has been demonstrated that limited amounts of Ca\(^{2+}\) can be taken up by intact isolated rat liver mitochondria in the absence of added Pi (10). These latter conditions have been used extensively for probing into problems concerning the possible relationships of ion transport to oxidative phosphorylation and to the movement of other ions which accompanies the uptake of Ca\(^{2+}\) (8–10, 16).

In this regard, the study of factors affecting massive ion uptake may be less informative since conditions are admittedly unphysiological and Ca\(^{2+}\) is a strong uncoupling agent; however, mitochondria in tissues in certain pathological states do accumulate large amounts of calcium in the form of electron-opaque deposits (17–19). This fact provides adequate reason for investigating further the mechanism(s) controlling massive ion accumulation by mitochondria.

Osmotically lysed rat liver mitochondria, stripped of their outer membranes, are deficient in phosphorylating ability and contain only about 50% of their total protein (20–23). Nevertheless, these lysed mitochondria retain the capacity to accumulate massive amounts of divalent cations by an energy-dependent mechanism(s). The accumulated ions are deposited inside the lysed mitochondria and can be observed as large electron-opaque granules (21). The present paper describes results of correlated biochemical and ultrastructural studies on the accumulation of divalent cations by osmotically lysed rat liver mitochondria. Preliminary reports of some of these findings have been presented (20–22, 24).

METHODS AND MATERIALS

**BIOCHEMISTRY:** Mitochondria were isolated from the livers of adult male albino rats (Wistar strain, Carworth Farms, New City, N.Y.) by the procedure of Schneider (25) in 0.25 M sucrose. The isolated mitochondria were washed three times with 0.25 M sucrose. All steps in the mitochondrial isolation procedure were carried out at 0–4°.

Freshly isolated mitochondria were osmotically lysed by resuspension and washing in cold, distilled water by the procedure described in previous communications (20, 23). About 50% of the total mitochondrial protein was extracted by the water-washing procedure, most of which was removed in the first and second water washes. Osmotically lysed, salt-extracted mitochondria were prepared by extracting freshly isolated mitochondria once or twice with distilled water, followed by two extractions with either 0.15 M KCl, 0.15 M NaCl, or 0.02 M MgCl\(_2\). The milliliters of water and salt solution used in the extractions were equivalent to the grams of tissue from which the mitochondria were prepared. The extracted mitochondria were isolated by centrifugation at 105,000 g for 20 min. After the final salt extraction, the mitochondria were finally washed once again with water and resuspended in water just before use.

Ca\(^{2+}\), Sr\(^{2+}\), and Pi uptake were measured as described in previous reports (2, 24). Protein was determined by the biuret method (26).

\(^{44}\)Ca\(^{2+}\) and \(^{85}\)Sr\(^{2+}\) were purchased from Oak Ridge National Laboratories, Oak Ridge, Tenn.; anti-

![Figure 1](image-url)
TABLE I
Effect of Inhibitors on Ca\(^{2+}\) Uptake by Osmotically Lysed Mitochondria

<table>
<thead>
<tr>
<th>System</th>
<th>Inhibitor added</th>
<th>Amount Ca(^{2+}) taken up ((\mu)atoms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiration-dependent</td>
<td>None</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>Ethanol (0.05 ml)</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>Antimycin A (0.95 (\mu)g per mg protein)</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>Antimycin A (2.4 (\mu)g per mg protein)</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>Oligomycin (1.6 (\mu)g per mg protein)</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>Oligomycin (4.0 (\mu)g per mg protein)</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>2,4-Dinitrophenol (0.5 mM)</td>
<td>1.4</td>
</tr>
<tr>
<td>ATP-supported</td>
<td>None</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>Ethanol (0.05 ml)</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>Antimycin A (2.4 (\mu)g per mg protein)</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>Oligomycin (1.6 (\mu)g per mg protein)</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Oligomycin (4.0 (\mu)g per mg protein)</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>2,4-Dinitrophenol (0.5 mM)</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*The reaction mixture for the respiration-dependent uptake contained 4 mM sodium phosphate (pH 7.0), 10 mM Tris-maleate (pH 7.0), 10 mM succinate, 10 mM MgCl\(_2\), 4 mM CaCl\(_2\) (labeled with Ca\(^{45}\)), 3 mM ATP and osmotically lysed mitochondria (5.1 mg protein) in a final volume of 3.0 ml. The reaction mixture for the ATP-supported uptake was the same as the one used in the respiration-dependent uptake experiment except that succinate was omitted and 15 mM ATP was added instead of 3 mM ATP. Antimycin A, oligomycin, and 2,4-dinitrophenol were added as indicated. Because antimycin A and oligomycin were dissolved in ethanol, control tubes receiving the same amount of ethanol were run. The final volume was 3.0 ml and the incubations were carried out for 20 min at 30°.

Effect of Osmotic Lysis on Respiration and Phosphorylation

As previously reported (20) and later confirmed (23), two of the most prominent biochemical changes observed in osmotically lysed mitochondria are an increased respiration rate and a marked loss in the ability to catalyze oxidative phosphorylation. The amount of residual phosphorylation present in osmotically lysed mitochondria was found to vary somewhat from preparation to preparation. These differences may be related to variation in the relative stability of different preparations of lysed mitochondria to prolonged incubations. In any case, the residual phosphorylation present in osmotically lysed mitochondria is quite labile (Fig. 1). As seen in Fig. 1, untreated mitochondria maintain their...
TABLE II
Effect of Cytochrome c on Ion Uptake by Osmotically Lysed and Osmotically Lysed, Salt-Extracted Mitochondria*

<table>
<thead>
<tr>
<th>Additions</th>
<th>Amount Ca++ taken up</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Respiration-dependent</td>
<td>ATP-supported</td>
<td></td>
</tr>
<tr>
<td>Untreated mitochondria</td>
<td>None</td>
<td>6.56</td>
<td>9.25</td>
</tr>
<tr>
<td>Lysed mitochondria</td>
<td>None</td>
<td>6.63</td>
<td>8.90</td>
</tr>
<tr>
<td></td>
<td>Cytochrome c</td>
<td>5.81</td>
<td>8.70</td>
</tr>
<tr>
<td>Lysed, MgCl₂-extracted mitochondria</td>
<td>None</td>
<td>1.52</td>
<td>4.40</td>
</tr>
<tr>
<td></td>
<td>Cytochrome c</td>
<td>6.64</td>
<td>4.26</td>
</tr>
</tbody>
</table>

* The reaction mixture for respiration-dependent Ca²⁺ uptake contained 4 mM sodium phosphate (pH 7.0), 10 mM Tris-maleate (pH 7.0), 10 mM MgCl₂, 10 mM succinate, 4 mM CaCl₂ (labeled with Ca⁴⁺), and 3 mM ATP in a final volume of 3.0 ml. The reaction mixture for the ATP-supported uptake was the same as the one described for the respiration-dependent uptake except that succinate was omitted and 15 mM ATP was added instead of 3 mM ATP. Cytochrome c (0.05 mM) was added where indicated. The reaction mixtures contained 7.9 mg of either untreated, osmotically lysed, or osmotically lysed, salt-extracted mitochondrial protein, where indicated. The incubations were carried out for 15 min at 30°C.

The ability to carry out net phosphorylation for 45–60 min under the incubation conditions used in these experiments, whereas lysed mitochondrial preparations phosphorylate ADP to ATP at a much lower initial rate and essentially stop phosphorylating after incubation for 10–15 min. In many experiments, it was observed that the amount of Pi esterified actually diminished after 15–20 min (Fig. 1); this may be due to glucose-6-phosphatase which could come from contaminating microsomes in the mitochondrial preparations. The lowered phosphorylative capacity of osmotically lysed mitochondria also has been shown in experiments measuring the formation of ATPπ from ADP and ATP during short periods of incubation (23). Phosphorylation is not restored by the addition of cytochrome c and/or NAD (20).

Ca²⁺ and Pi Accumulation and Effect of Metabolic Inhibitors

Osmotically lysed mitochondria show little or no respiratory control; i.e. the addition of ADP does not stimulate respiration, nor is respiration significantly stimulated by the addition of large amounts of Ca²⁺ (23). Therefore, it is of considerable interest that osmotically lysed mitochondria accumulate as much Ca²⁺ and Pi as do untreated mitochondria by both a respiration-dependent and an ATP-supported process (20). The uptake of Ca²⁺ and Pi seems to occur by the same processes in both intact and lysed mitochondria since the requirements for Ca²⁺ and Pi uptake by osmotically lysed mitochondria (20) are essentially the same as those reported for intact mitochondria. The pattern of inhibition by 2,4-dinitrophenol, antimycin A, and oligomycin also is the same as that observed with intact mitochondria (2, 3, 5, 6); i.e. 2,4-dinitrophenol inhibits both the respiration- and ATP-driven processes, antimycin A inhibits only respiration-supported ion accumulation, and oligomycin inhibits only ATP-supported ion accumulation (Table I).

Effect of Salt Extraction and Cytochrome c on Ion Accumulation

Extraction of osmotically lysed mitochondria with salts, such as MgCl₂, NaCl, or KCl, causes a marked reduction in the capacity of these preparations to accumulate Ca²⁺ (Table II); the respiration-independent Ca²⁺ accumulation process is affected to a much greater extent than is the

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ATP-driven process. MgCl₂ extraction of osmotically lysed rat liver mitochondria extracts essentially all of the mitochondrial cytochrome c, but little or none of the other cytochromes (Caplan and Greenawalt, unpublished observations). The addition of cytochrome c to water-lysed, salt-extracted mitochondria completely restores the respiration-dependent uptake of Ca²⁺ but has no effect on the ATP-supported ion accumulation process (Table II). Cytochrome c extraction also results in a complete loss of the residual phosphorylating activity found in the osmotically lysed mitochondria, and the addition of cytochrome c to these depleted preparations restores this process to that level initially observed in the lysed mitochondria prior to salt extraction (Chen and Vasington, unpublished data).

Effect of Salt and Sucrose on Ca²⁺ Accumulation by Osmotically Lysed Mitochondria: As reported previously (20), the requirements for Ca²⁺ and P₁ uptake by osmotically lysed mitochondria and intact mitochondria are essentially the same. One interesting difference, however, is the effect of salt concentration on the uptake process. In an earlier report it was shown that the uptake of Ca²⁺ by intact mitochondria is stimulated in the presence of 0.05-0.1 M NaCl (2). Higher concentrations of NaCl were found to be inhibitory. The data in Fig. 2 show that Ca²⁺ uptake by osmotically lysed mitochondria is not stimulated by NaCl. In fact, it was found that NaCl inhibited Ca²⁺ uptake by lysed mitochondria at all levels tested and that lysed mitochondria are much more sensitive to the inhibitory effect of NaCl than are untreated mitochondria (Fig. 2). In a typical experiment shown in Fig. 2, 0.1 M NaCl stimulates Ca²⁺ uptake by untreated mitochondria whereas the uptake process in lysed mitochondria is inhibited about 76%. The differential effect of NaCl is manifested in both the respiration- and ATP-dependent processes. The action of these salts is probably not simply an osmotic effect, since as much as 0.5 M sucrose has relatively little effect on Ca²⁺ uptake (Fig. 3).

Sr²⁺ Accumulation: Osmotically lysed mitochondria also accumulate Sr²⁺ by a respiration-dependent process (Table III) similar to that

![Figure 2](image)

**Figure 2** Effect of NaCl concentration on Ca²⁺ accumulation by intact and osmotically lysed mitochondria. The complete reaction mixtures for the respiration-dependent and ATP-supported processes were the same as described in Table I. NaCl concentrations are as indicated. The incubation periods for the respiration- and ATP-dependent Ca²⁺ uptake reactions were 25 and 15 min, respectively, at 30°. Mitochondria + succinate and mitochondria + ATP = respiration-dependent and ATP-supported accumulation, respectively, by intact mitochondria; lysed mitochondria + succinate and lysed mitochondria + ATP = respiration-dependent and ATP-supported accumulation, respectively, by osmotically lysed mitochondria.

![Figure 3](image)

**Figure 3** The relative effects of sucrose and NaCl on Ca²⁺ uptake by osmotically lysed mitochondria. The complete reaction mixtures for the respiration-dependent and ATP-supported processes were the same as those described in Table I except that 6 mg of lysed mitochondrial protein were added. Sucrose and NaCl concentrations are indicated. The incubations were carried out at 30° for 20 min for the respiration-dependent and for 15 min for the ATP-supported uptake reactions.
TABLE III
Requirements for Sr$^{2+}$ Uptake by Osmotically Lysed Mitochondria*

<table>
<thead>
<tr>
<th>System</th>
<th>Amount Sr$^{2+}$ accumulated (µatoms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiration-dependent</td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>5.21</td>
</tr>
<tr>
<td>Complete, minus succinate</td>
<td>1.61</td>
</tr>
<tr>
<td>Complete, minus Mg$^{2+}$</td>
<td>2.34</td>
</tr>
<tr>
<td>Complete, minus ATP</td>
<td>3.23</td>
</tr>
<tr>
<td>Complete, minus Pi</td>
<td>4.06</td>
</tr>
<tr>
<td>Complete, minus succinate &amp; ATP</td>
<td>0.59</td>
</tr>
<tr>
<td>ATP-supported</td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>2.38</td>
</tr>
<tr>
<td>Complete, minus ATP</td>
<td>1.33</td>
</tr>
<tr>
<td>Complete, minus Mg$^{2+}$</td>
<td>1.32</td>
</tr>
<tr>
<td>Complete, minus Pi</td>
<td>1.82</td>
</tr>
</tbody>
</table>

* The complete reaction mixture for the respiration-dependent process contained 4 mM sodium phosphate (pH 7.0), 10 mM Tris-maleate (pH 7.0), 10 mM succinate, 10 mM MgCl$_2$, 3 mM ATP, 2 mM SrCl$_2$ (labeled with $^{85}$Sr), and 4 mg lysed mitochondrial protein. The complete reaction mixture for the ATP-supported process was the same as that described for the respiration-dependent process except that the ATP concentration was reduced to 15 mM and succinate was omitted. Incubations were carried out at 30°C for 20 min.

found in intact mitochondria by Carafoli et al. (29). There is a marked dependence on added substrate, ATP and Mg$^{2+}$. Whereas osmotically lysed mitochondria accumulate Ca$^{2+}$ equally well by the ATP- and respiration-dependent processes, lysed mitochondria exhibit a markedly reduced capacity to accumulate Sr$^{2+}$ by the ATP-dependent process (Table III and Fig. 4).

Electron microscopic studies: The appearance of osmotically lysed mitochondria viewed in the electron microscope (Fig. 6) is compared to that of intact, isolated rat liver mitochondria (Fig. 5). It can be seen that the inner and outer mitochondrial membranes have been spatially separated by the lytic treatment and that relatively little matrix substance is retained within the inner mitochondrial compartment. Schnaitman et al. (30) recently subfractionated lysed mitochondrial preparations and showed that the small vesicles (v, Fig. 6) are largely derived from the outer mitochondrial membranes and that the larger vesicles (G, Fig. 6) consist of inner membrane plus retained matrix. Lysed mitochondria incubated in Ca$^{2+}$ have essentially the same ultrastructure as the unincubated, lysed mitochondria. Higher magnification (Fig. 7) of a lysed mitochondrion incubated in the Ca$^{2+}$ uptake medium without added Ca$^{2+}$ shows that only a single membrane encompasses the larger, inner compartments ("ghosts"). These results are consistent with the findings reported by Caplan and Greenawalt (23) who have further characterized osmotically lysed rat liver mitochondria.

Figs. 8 and 9 are micrographs of osmotically lysed mitochondria incubated in Ca$^{2+}$ uptake medium containing ATP but from which Ca$^{2+}$ has been omitted. Under these conditions no large opaque deposits are observed. However, in addition to inner compartments (G) and the small vesicles (v), many profiles, which appear to be highly condensed inner mitochondrial compartments, are seen (Fig. 8, CG). At higher magnification (Fig. 9), granules (g) reminiscent of "normal" matrix granules in size and electron-opacity are visible in the condensed forms. Such granules are not apparent in the uncondensed "ghosts" (Fig. 9). About 50% of the inner membrane "ghosts" appear to undergo this condensation, and it has been suggested that these marked changes in membrane conformation account for the “contraction” of osmotically lysed mitochondria as measured by optical density changes (23).

Figs. 10 and 11 show that the accumulation of Ca$^{2+}$ and Pi by osmotically lysed mitochondria under massive loading conditions is accompanied by the formation of huge electron-opaque deposits; such deposits are formed within intact mitochondria under similar conditions of incubation (11-13). It is also clear from Fig. 10 that both the condensed and uncondensed forms of the "ghosts" contain these deposits of calcium phosphate. The study of many sections of the loaded, lysed mitochondria suggests that the small vesicles derived from the outer membranes do not contain such deposits. That the deposits in the "ghosts" are frequently associated with the inner membranes and cristae is illustrated at higher magnification in Fig. 11. This interpretation is consistent with the suggestion that the inner membranes play a role in the deposition of Sr$^{2+}$ and Ca$^{2+}$ within intact liver mitochondria (15).
**DISCUSSION**

Treatment of mitochondria with water under conditions which cause osmotic lysis and extraction of approximately 40-50% of the mitochondrial protein has distinct effects on the biochemical and structural properties of mitochondria (20, 23). One of the effects observed is a marked decrease in the ability of lysed mitochondria to catalyze oxidative phosphorylation. Yet these lysed mitochondria retain full capacity to accumulate large quantities of Ca\(^{2+}\) and P\(_i\) by both a respiration-dependent and an ATP-supported process (20).

In a preliminary paper (20), we reported that some of our lysed mitochondrial preparations were completely nonphosphorylating but still retained the full capacity to accumulate Ca\(^{2+}\) and P\(_i\). On the basis of these observations, we suggested that, if the respiration-dependent and ATP-supported ion accumulation processes involve the same high-energy intermediate, ATP must form this intermediate by a pathway other than the simple reversal of the oxidative phosphorylation reactions (20). It is now necessary to withdraw this earlier suggestion because it has not been possible to demonstrate the complete loss of phosphorylation in recent preparations obtained by the lysis technique. We cannot satisfactorily account for our inability to confirm this aspect of our initial findings, but our data do not indicate any correlation between the amount of residual phosphorylating capacity of lysed mitochondria and their ability to accumulate Ca\(^{2+}\).

The mechanism for the respiration-dependent accumulation of Sr\(^{2+}\) also is unaffected by water-treatment. However, lysed mitochondria do show a greatly reduced capacity to accumulate Sr\(^{2+}\) by the ATP-supported process (24). The decrease in the ATP-supported accumulation of Sr\(^{2+}\) by lysed mitochondria does not appear to be due to an effect on a specific Sr\(^{2+}\)-stimulated ATPase since neither intact nor lysed mitochondria exhibit a Sr\(^{2+}\)-stimulated ATPase activity (24, 31). In fact, the ATPase activity in the presence of Sr\(^{2+}\) is essentially the same in intact and osmotically lysed mitochondria (24). Furthermore, Sr\(^{2+}\) actually inhibits mitochondrial ATPase activity stimulated by Ca\(^{2+}\) and Mg\(^{2+}\) (24). The fact that osmotically lysed mitochondria do accumulate and retain Sr\(^{2+}\) as effectively as they do Ca\(^{2+}\) by the respiration-dependent process indicates that a selective change in membrane permeability cannot account for the reduced ATP-supported accumulation of Sr\(^{2+}\).

Since approximately 50% of the mitochondrial protein is extracted by the water lysis procedure, it is possible that some essential factor(s) necessary...
for the ATP-supported accumulation of Sr~+ has been damaged or extracted. Brierley et al. (32) were able to restore the ATP-supported uptake of Ca~+ in aged beef-heart mitochondria by preincubation with ATP or succinate in the presence of Mg~+. So far it has not been possible to restore Sr~+-uptake by our preparations by such treatment with these reagents. Furthermore, attempts to restore the ATP-supported accumulation of Sr~+ by the addition of water extracts of the mitochondria to the incubation medium have met with limited success to date. However, the addition of Ca~+ to the incubation medium results in a 70% or greater restoration of the ATP-supported accumulation of Sr~+ (Chen and Vasington, unpublished data). This effect appears to be specific for Ca~+; Zn~+, Ba~+, and Cd~+ are not effective.

Several investigators have found that concomitant with the salt-extraction of cytochrome c from mitochondria, there is a marked decrease in the ability of these mitochondria to catalyze oxidative phosphorylation (33–36). It also was observed that the addition of cytochrome c to these cytochrome c-deficient mitochondria resulted in a considerable restoration of the P:O ratio (35, 36). The extraction of rat liver mitochondria with water as described in this report also causes a marked decrease in the ability of mitochondria to catalyze oxidative phosphorylation. However, this decrease in oxidative phosphorylation is not due to the loss of cytochrome c, since no cytochrome c has been detected in the water extracts (23) and the addition of cytochrome c to water-extracted mitochondria does not restore phosphorylation (20). When water-lysed mitochondria are depleted of cytochrome c by extraction with salt solutions (Caplan and Greenawalt, unpublished data), they exhibit a complete loss of the ability to catalyze oxidative phosphorylation and respiration-dependent ion uptake; only a partial loss of the ATP-supported ion accumulation process occurs. Addition of cytochrome c to be depleted, lysed mitochondria restores oxidative phosphorylation (Chen and Vasington, unpublished data) and the respiration-dependent ion accumulation process only to the level present in lysed mitochondria; cytochrome c addition has no effect on the ATP-supported ion transport process. These results are somewhat similar to those obtained by MacLennan et al. (36), but different from those observed by Penniston et al. (37). The latter investigators found a complete loss of the ATP-supported ion uptake process in cytochrome c-deficient mitochondrial particles and a restoration of this process by the addition of cytochrome c. Furthermore, the addition of cytochrome c did not restore phosphorylation in the deficient particles (37). On the basis of their observations, Penniston et al. (37) stated that cytochrome c is involved in the formation of a high-energy intermediate at the third phosphorylation site and in the coupling of this intermediate to ion translocation. Our findings with cytochrome c-depleted osmotically lysed mitochondria, which, similar to the preparations studied by Penniston et al. (37), couple phosphorylation only at the third phosphorylation site, are not consistent with this view. It appears more likely that the observed decrease in the ATP-supported ion accumulation process following salt extraction is due to the removal or damage of some component, other than cytochrome c, essential to and specific for the ATP-energized mechanism and that cytochrome c plays no role in this process.

It appears that the presence of the outer mitochondrial membrane is not necessary for ion accumulation, since this membrane is separated from oxidative phosphorylation and respiration-dependent ion uptake; only a partial loss of the ATP-supported ion accumulation process occurs. Addition of cytochrome c to be depleted, lysed mitochondria restores oxidative phosphorylation (Chen and Vasington, unpublished data) and the respiration-dependent ion accumulation process only to the level present in lysed mitochondria; cytochrome c addition has no effect on the ATP-supported ion transport process. These results are somewhat similar to those obtained by MacLennan et al. (36), but different from those observed by Penniston et al. (37). The latter investigators found a complete loss of the ATP-supported ion uptake process in cytochrome c-deficient mitochondrial particles and a restoration of this process by the addition of cytochrome c. Furthermore, the addition of cytochrome c did not restore phosphorylation in the deficient particles (37). On the basis of their observations, Penniston et al. (37) stated that cytochrome c is involved in the formation of a high-energy intermediate at the third phosphorylation site and in the coupling of this intermediate to ion translocation. Our findings with cytochrome c-depleted osmotically lysed mitochondria, which, similar to the preparations studied by Penniston et al. (37), couple phosphorylation only at the third phosphorylation site, are not consistent with this view. It appears more likely that the observed decrease in the ATP-supported ion accumulation process following salt extraction is due to the removal or damage of some component, other than cytochrome c, essential to and specific for the ATP-energized mechanism and that cytochrome c plays no role in this process.

It appears that the presence of the outer mitochondrial membrane is not necessary for ion accumulation, since this membrane is separated from

**FIGURES 5 and 6** Comparison of freshly isolated rat liver mitochondria and osmotically lysed mitochondria. Fixed with OsO4; stained with lead.

**FIGURE 5** Control mitochondria, freshly isolated in 0.25 M sucrose. Double membranes, cristae, matrix, and “normal” matrix granules are visible. × 32,500.

**FIGURE 6** Osmotically lysed rat liver mitochondria. Two populations of vesicles are observed: small (0.2–0.5 μ in diameter), empty vesicles derived from the outer membrane (v); and larger (1.0–1.5 μ in diameter) “ghosts” (G) comprised of inner mitochondrial membrane plus some matrix. The double-membrane organization, typical of intact mitochondria, is absent. Little matrix, few cristae, and no “normal” granules are seen. This micrograph also illustrates the appearance of lysed mitochondria which were incubated in Ca~+ uptake medium minus ATP to serve as controls in ion accumulation studies (see text and Fig. 7). × 36,000.
the inner compartment in water-lysed mitochondria. This interpretation is consistent with the idea that the outer membrane is freely permeable to small solutes (38, 39). However, the Ca$^{2+}$ ion accumulation process in osmotically lysed mitochondria is much more sensitive to inhibition by monovalent ions, such as Na$^+$ and K$^+$, than it is in intact mitochondria. It is possible that the outer membrane does show some selective permeability to monovalent ions, accounting for the higher concentrations of Na$^+$ and K$^+$ needed to inhibit the accumulation process in intact mitochondria. However, damage to the structural integrity of the inner membrane could also account for this phenomenon. It is of interest to note that, whereas Na$^+$, K$^+$, and Li$^+$ stimulate Ca$^{2+}$ and P$_i$
uptake at relatively low concentrations in intact mitochondria, these ions do not stimulate Ca\(^{2+}\) and P\(_i\) uptake by lysed mitochondria at any concentrations tested in the present study. As stated previously, the actions of these salts are probably not simply an osmotic effect since similar concentrations of sucrose have relatively little effect on Ca\(^{2+}\) uptake by lysed mitochondria.

It was earlier shown by Vasington (40) in studies with digitonin particles prepared from rat liver mitochondria that disruption of mitochondrial integrity results in a change in the sensitivity of the Ca\(^{2+}\) accumulation process to monovalent cations. Digitonin particles show the same response to monovalent cations as do osmotically lysed mitochondria; i.e. the addition of monovalent cations, in contrast to the effect on intact mitochondria, does not stimulate Ca\(^{2+}\) uptake; the only effect observed was an inhibition. However, sucrose concentrations up to 0.5 M have little effect on Ca\(^{2+}\) uptake by lysed mitochondria, whereas 0.5 M sucrose markedly inhibits Ca\(^{2+}\) accumulation by digitonin particles. The fact that sucrose inhibits several partial reactions of oxidative phosphorylation catalyzed by digitonin particles (41–43) suggests that inhibition of some essential enzymatic reaction accounts for the inhibition of Ca\(^{2+}\) uptake by these particles.

It is clear from this and other correlated biochemical and ultrastructural studies (21–23) that the inner and outer membranes of rat liver mitochondria are spatially dissociated by osmotic lysis. Ca\(^{2+}\) and P\(_i\) are massively accumulated by the lysed preparation under much the same conditions as are required by intact mitochondria, and large electron-opaque deposits are formed only when all these requirements are present in the reaction mixture. The close association of the large calcium phosphate granules with the “ghost” membranes suggests that deposition may involve specific binding sites on the membranes; such a possibility has been suggested for Ca\(^{2+}\) and Sr\(^{2+}\) accumulation in intact mitochondria (15). Since these deposits are formed within the “ghosts,” Ca\(^{2+}\) and P\(_i\) are clearly transported across the inner mitochondrial membrane, i.e. the delimiting membrane of the “ghosts.” Energy is thus apparently required for the transport of ions across this barrier and for retention of the deposits within the inner compartment.

The condensation of “ghosts” in the presence of ATP has been reported previously (23), but the mechanism of this process is as yet unknown. It is curious that only about 50% of the “ghosts” appear condensed, yet both condensed and uncondensed profiles contain the electron-opaque deposits of calcium phosphate. The presence of what appear, morphologically, to be “normal” matrix granules in the condensed forms is also an interesting observation. Further studies are required before any definite functional role can be related to these latter observations.

This study was supported by grants from The National Institutes of Health, United States Public Health Service (GM 12125 and 12717) and the University of Connecticut Research Foundation.

Received for publication 13 December 1957, and in revised form 9 August 1968.

REFERENCES


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**FIGURES 8 and 9**  
**Ca**²⁺ accumulation studies. Control. Fixed with OsO₄; lead stained.

**FIGURE 8** Osmotically lysed mitochondria incubated in **Ca**²⁺ uptake medium minus **Ca**²⁺ (see text). No large deposits of calcium phosphate are visible (see Figs. 10 and 11). However, about one-half of the mitochondrial compartments ("ghosts") are highly condensed (CG); in the other 50%, cristal profiles are frequently observed, but these "ghosts" are not tightly condensed. The small vesicles (v) appear unchanged. × 66,000.

**FIGURE 9** Condensed (CG) and uncondensed "ghosts" (G) at higher magnification. The condensed "ghost" (CG) is densely stained and contains electron-opaque granules (g) similar in appearance to "normal" matrix granules. No such granules are seen in the uncondensed "ghost"; however, numerous cristae are visible. No large, electron-opaque depositions of calcium phosphate are present (see Figs. 10 and 11). × 65,000.
**Figure 10 and 11**  Ca\(^{2+}\) accumulation by osmotically lysed mitochondria. Fixed with OsO\(_4\); stained with lead.

**Figure 10** Large electron-opaque deposits are seen in osmotically lysed rat liver mitochondria which have accumulated Ca\(^{2+}\) and P\(_i\). Such deposits are formed within the condensed and uncondensed "ghosts." No such deposits are seen clearly associated with the small vesicles. \(\times 26,000\).
Deposits of calcium phosphate are formed on the inside of the single membrane delimiting the "ghost." The close association of the large opaque granules with the inner mitochondrial membrane (IM), the membrane which delimits the "ghost," and with the cristae (c) is illustrated. The large deposits often cause the "ghost" membrane to "bleb" outward as indicated on the right side of this micrograph. × 130,000.