ION-INDUCED ULTRASTRUCTURAL TRANSFORMATIONS IN ISOLATED MITOCHONDRIA

The Energized Uptake of Calcium

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ABSTRACT

The energized uptake of low levels of Ca^{2+} in the presence and absence of phosphate by isolated rat liver mitochondria, and the perturbation effected by this activity on ultrastructural and metabolic parameters of mitochondria have been investigated. In the presence of phosphate, low levels of Ca^{2+} are taken up by mitochondria and result in various degrees of ultrastructural expansion of the inner mitochondrial compartment. This indicates that low levels of Ca^{2+} in the presence of phosphate, are *accumulated* in an osmotically active form into the water phase of the inner compartment. The first clearly observable quantitative increase in the volume of the inner compartment occurs after the accumulation of 100 nmoles Ca^{2+}/mg protein. An accumulation of 150–200 nmoles Ca^{2+}/mg protein, which is equivalent to the osmolar concentration of endogenous K^+, is required to effect a doubling of the volume of the inner compartment. This degree of osmotic perturbation occurs as mitochondria transform from a condensed to an orthodox conformation. The osmotically induced orthodox conformation differs from the mechanochemically induced orthodox conformation previously described, in that its development is concomitant with a marked decrease in acceptor control and oxidative phosphorylation efficiency and it fails to transform to a condensed conformation in response to addition of ADP. In the absence of added phosphate, a maximum of 190 nmoles Ca^{2+}/mg protein was found to be taken up by mitochondria (state 6). Ca^{2+} is apparently *bound* under state 6 conditions since the uptake does not effect an ultrastructural expansion of the inner compartment. Phosphate added after state 6 Ca^{2+} binding, however, results in an immediate ultrastructural expansion of the inner compartment. The addition of phosphate to mitochondria in the absence of exogenous Ca^{2+} fails to effect an osmotic ultrastructural transformation. Under state 6 conditions, the binding of between 40 and 190 nmoles Ca^{2+}/mg protein results in the formation of dense matrix inclusions which appear to be composed of tightly packed, concentrically oriented membranes. Under conditions in which the bound Ca^{2+} is subsequently released, there is a concomitant loss in the density of these matrix inclusions, leaving behind morphologically distinct membrane whorls in the mitochondrial matrix.

It has been reported in several communications from this laboratory that isolated liver mitochondria undergo characteristic ultrastructural transformations in their electron-transport membrane formations in their electron-transport membrane as a function of respiratory state (1–4). Major transformations were found to occur under conditions which support steady-state electron transport independent of energized ion movements and were

identified as *mechanochemical ultrastructural transformations* (3). The results suggested that these transformations are manifestations of the conversion of the chemical energy of electron transport directly into conformational energy. Based on these results, the postulate was developed that conformational energy generated in the electron-transport membrane is the direct source of energy for the synthesis of ATP[1] in mitochondria (3).

Ultrastructural transformations of a different nature have recently been shown to occur in mitochondria under specialized conditions which support oscillatory electron-transport activity (5, 6). These transformations are secondary to osmotic perturbations in mitochondria brought about by an energized net accumulation of ions into the water phase of the inner compartment and may be referred to as *osmotic ultrastructural transformations* (3).

In the present communication, we focus attention on osmotic ultrastructural transformations induced by ion uptake which occur in mitochondria under conditions which support steady-state electron transport. We have measured the electron transport–dependent uptake of radiocalcium by mitochondria and have studied the perturbation of this activity on mitochondrial ultrastructure and metabolism.

The major purpose of this communication is to present results which establish the quantity of energized ion accumulation that is required to: (a) effect the first clear signs of increased volume in the osmotically active inner mitochondrial compartment; and (b) double the volume of the inner compartment. In addition, various degrees of osmotic perturbation of mitochondrial ultrastructure induced by ion uptake are assessed with respect to various metabolic parameters of mitochondria.

The results of this study bring to light some of the distinguishing characteristics between ion-induced osmotic ultrastructural transformation and mechanochemical ultrastructural transformation in isolated mitochondria.

MATERIALS AND METHODS

Mitochondria were isolated from the livers of male Sprague-Dawley rats in 0.25 M sucrose at 0°C accord-

ing to the method of Schneider (7). Protein was determined by a Biuret method (8).

Oxygen consumption was monitored with the Clark oxygen electrode (9). The accumulation of radiocalcium by mitochondria was determined by the Millipore filtration method described by Rassmussen et al. (10).

Microsamples of mitochondria in specific metabolic states were fixed for electron microscopy as described earlier (2). The fixative used was iso-osmotic, Na-phosphate buffered, 2% osmium tetroxide (pH 7.4) (2). Sections of Epon-embedded microsamples (2) were stained for 20 min at 60°C in 1.0% sodium borate solution saturated with uranyl acetate followed by lead hydroxide (11).

Electron micrographs were taken on Kodak 3¼ X 4 inch contrast plates at initial magnifications of 7,900 and 39,000 with an RCA 3 G electron microscope operated at 50 kv and equipped with an anti-contamination cold trap and a double condenser.

RESULTS

Ca²⁺ Uptake in the Presence of Phosphate

Table I and Figs. 1–4 contain results which contrast the affects of varying levels of Ca^{2+} on phosphate-succinate–supported electron transport (state 4P), on acceptor control, on phosphorylation efficiency, and on the ultrastructure of mitochondria. Ca²⁺ was present at zero time in these experiments and, as shown by the degree of the initial burst of oxygen consumption, was accumulated during the first 30 sec of electron transport (Fig. 1). Under the conditions of these experiments, it is well known that the Ca^{2+} accumulated is energy-dependent and is accompanied by phosphate (12, 13).

After the uptake of 40 nmoles of Ca^{2+}/mg of protein, state 4P mitochondria show no significant change in ultrastructure, in respiratory rate (Fig. 1), in subsequent ADP-induced acceptor control, or in phosphorylation efficiency (Table I), when compared to control state 4P mitochondria. These mitochondria show a condensed conformation (2) comparable to controls (Fig. 2).

After mitochondria accumulate 100 nmoles of Ca^{2+}/mg protein, there are signs of an osmotic perturbation as revealed by an increase (ca. 50%) in the volume of the inner mitochondrial compartment (Fig. 3) concomitant with a slight increase in the state 4P respiratory rate which follows the accumulation (Fig. 1) and some loss in acceptor control and phosphorylation efficiency (Table I). Mitochondria showing this degree of increased

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[1] Abbreviations used: ATP = adenosine triphosphate. ADP = adenosine diphosphate. ADP:O = adenosine diphosphate to oxygen ratio. DPN = diphosphopyridine nucleotide.

TABLE I

Ca^{2+} Accumulation by Rat Liver Mitochondria in the Presence of Phosphate at Various Concentrations of Ca^{2+}

Note that acceptor control and ADP:O ratios decrease in proportion to the amount of Ca^{2+} accumulated. Reaction system as in the legend for Fig. 1.

$^{45}Ca^{2+}$ Available nMoles/mg Protein	$^{45}Ca^{2+}$ Accumulated nMoles/mg Protein		Acceptor control @ 6 min	ADP/O Ratio
	@ 1.5 min	@ 4 min		
40	40	40	5.5	1.62
100	98	98	3.8	1.55
200	196	196	<2.0	<1.00
500	<25	<25	1.0	0

volume of the inner compartment have previously been designated "mitochondria of intermediate conformation" (2).

When mitochondria accumulate from 150 to 200 nmoles Ca^{2+}/mg protein, a dramatic osmotic perturbation occurs. The volume of the inner compartment doubles while the structurally intact mitochondria assume an orthodox (2) conformation (Fig. 4). Concomitant with the ultrastructural changes are a marked increase in the rate of electron transport (Fig. 1) and a considerable loss in subsequent acceptor control and phosphorylation efficiency (Table I).

In the above experiments, all the accumulated Ca^{2+} was retained by the mitochondria during subsequent state 4P respiration (Table I). In the presence of 500 nmoles of Ca^{2+}/mg protein, the volume of the inner compartment doubles while uncoupled rates of respiration (Fig. 1) and total loss of subsequent acceptor control (Table I) occur (state 3U). For the most part, these mitochondria are indistinguishable in structure from those which accumulated 200 nmoles Ca^{2+}/mg protein. Radiocalcium, however, is not found in these mitochondria after 1.5 min and, most likely, the marked uncoupling results in the release of the accumulated Ca^{2+} and phosphate before the 1.5-min sample can be taken for Ca^{2+} analysis.

This series of results shows that when mitochondria accumulate less than 100 nmoles of Ca^{2+}/mg protein in the presence of phosphate, a clearly defined osmotic ultrastructural transformation cannot be observed. Accumulations of 100 nmoles
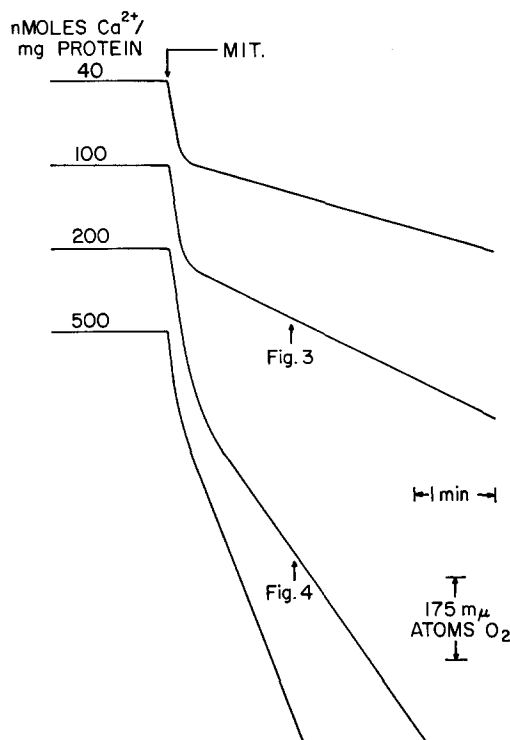

FIGURE 1 Rate of oxygen consumption by rat liver mitochondria in the presence of phosphate and at various concentrations of Ca^{2+}. Microsamples of mitochondria were fixed consecutively for electron microscopy at 1.5 and 4 min (see Figs. 3 and 4). The reaction system contained sucrose (0.113 M), PO_4 buffer (10 mM, pH 7.4), $MgCl_2$ (5.0 mM), succinate (5 mM), $CaCl_2$ (as indicated), and rat liver mitochondria (2 mg protein). Total volume 2.0 ml.

or more of Ca^{2+}/mg protein in the presence of phosphate result in various degrees of osmotic perturbation of mitochondrial ultrastructure. A doubling of the volume of the inner mitochondrial compartment, during which mitochondria transform from a condensed to an intact orthodox conformation, occurs with the accumulation of between 150 and 200 nmoles Ca^{2+}/mg protein.

It should be clear that the ultrastructural perturbations presented in this report represent the after effects of ion accumulation. Change in mitochondrial conformation, which may take place *during* ion accumulation, were not investigated.

Ca^{2+} Uptake in the Absence of Phosphate

Table II and Figs. 5–9 show the affects of Ca^{2+} present at zero time on phosphate-free, succinate-
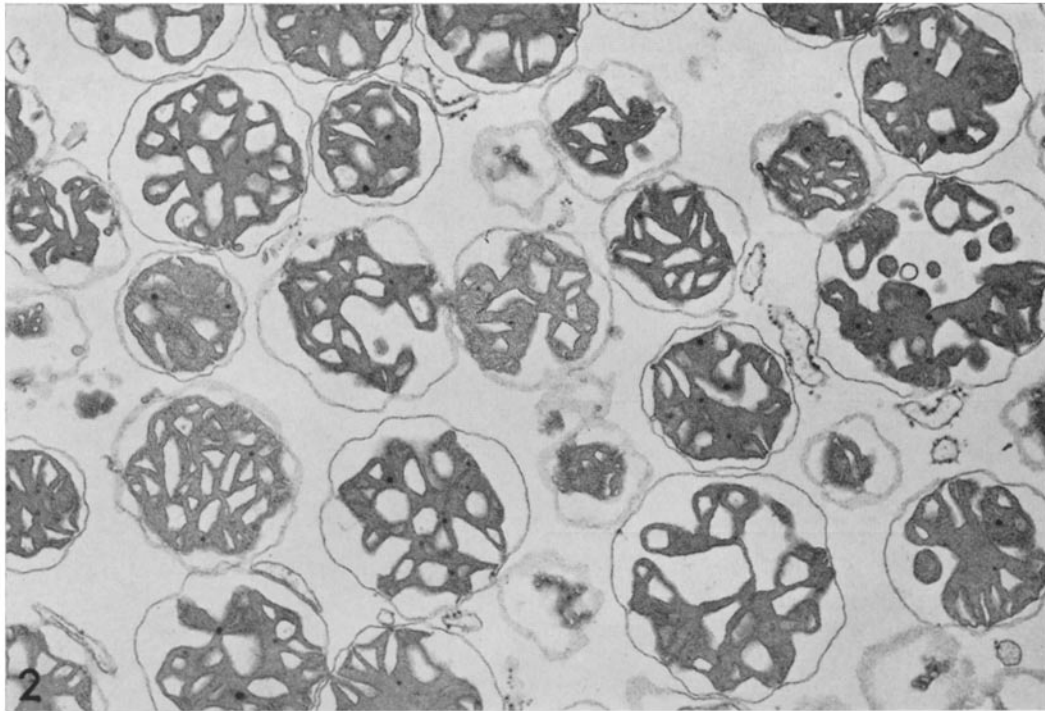
FIGURE 2   Control. Rat liver mitochondria isolated in 0.25 M sucrose showing the typical condensed conformation. × 23,700.
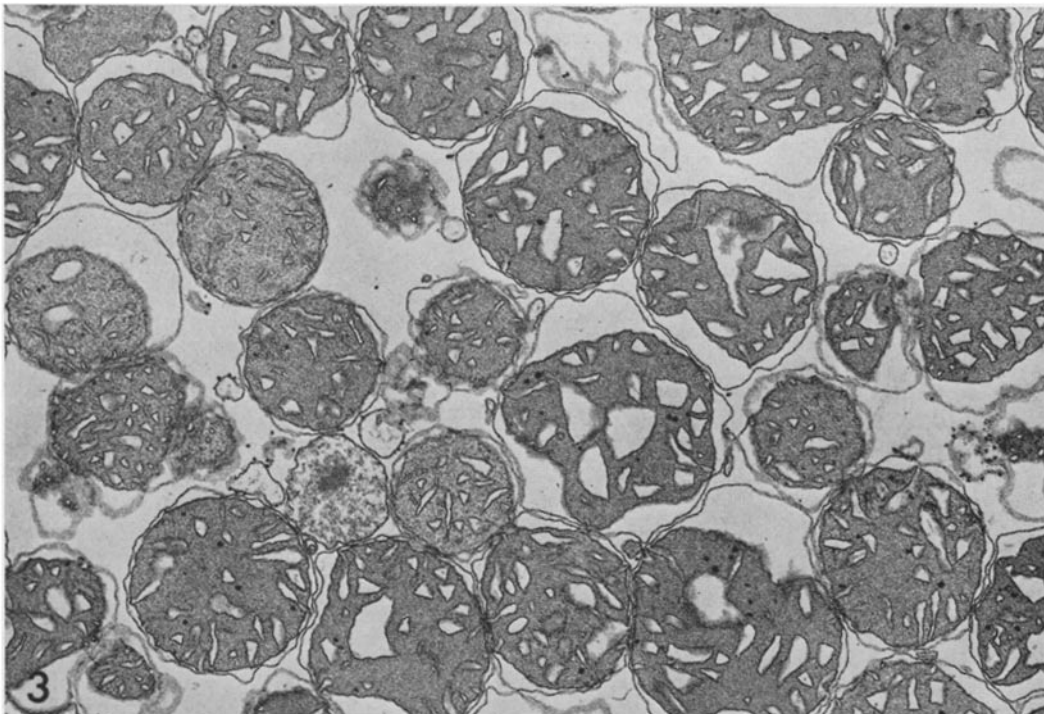

FIGURE 3   Mitochondria fixed 1 min after the accumulation of 100 nmoles of Ca^{2+}/mg protein in the presence of phosphate. The volume of the inner compartment is noticeably increased in comparison to the control in Fig. 2. Reaction system as in the legend for Fig. 1. 100 nmoles of Ca^{2+}/mg protein were available (see Table I). × 23,700.
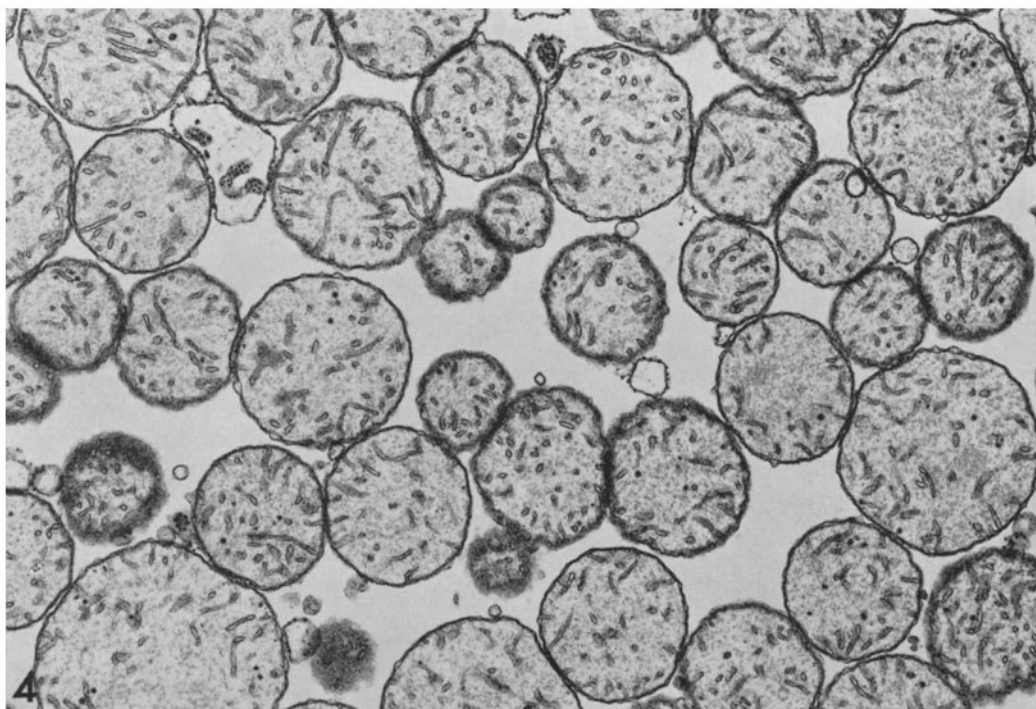
FIGURE 4 Mitochondria fixed 1 min after the accumulation of 200 nmoles of Ca^{2+}/mg protein in the presence of phosphate. The volume of the inner compartment is doubled in comparison to the control in Fig. 2, and the mitochondria show an orthodox conformation. Reaction system as in the legend for Fig. 1. 200 nmoles of Ca^{2+}/mg protein were available (see Table I). × 23,700.

TABLE II

Ca^{2+} Binding by State 6 Rat Liver Mitochondria at Various Concentrations of Ca^{2+}

Reaction system as in the legend for Fig. 5.

$^{45}Ca^{2+}$ Available nMoles/mg Protein	$^{45}Ca^{2+}$-Bound nMoles/mg Protein		
	@ 1.5 min	@ 4 min	@ 8 min
40	40	40	40
100	96	99	99
200	190	70	5
500	190	60	0
1000	160	0	0

supported, electron transport (state 4), and on mitochondrial ultrastructure. Tris-HCl was used as a buffer in place of phosphate in these experiments.

It was determined that 100–150 nmoles of Ca^{2+}/ mg protein can be taken up by mitochondria in the absence of phosphate (Table II) without effecting an appreciable osmotic ultrastructural transforma-tion (Fig. 6) or altering the subsequent state 4 respiratory rate (Fig. 5). The accumulated Ca^{2+} is retained (Table II) and structure remains stable (Fig. 7) during subsequent state 4 respiration. The *availability* of Ca^{2+} at levels of 200–1000 nmoles/mg protein, however, results in various levels of inhibi-tion of electron transport indicative of state 6 (14) as observed in Fig. 5, but does not initiate osmotic perturbation (Fig. 8). In no case, however, did the measurable accumulation of Ca^{2+} exceed 190 nmoles/mg protein (Table II). Once state 6 res-piratory inhibition is initiated in the presence of Ca^{2+}, retention of the accumulated Ca^{2+} over the next few minutes fails. As the accumulated Ca^{2+} is released (Table II), respiratory inhibition is re-leased (Fig. 5) and a gradual, presumably non-osmotic increase in the volume of the inner com-partment takes place (Fig. 9). As the volume of the inner compartment increases, and the accumulated Ca^{2+} is released, the mitochondrial membranes assume a characteristically wrinkled appearance.
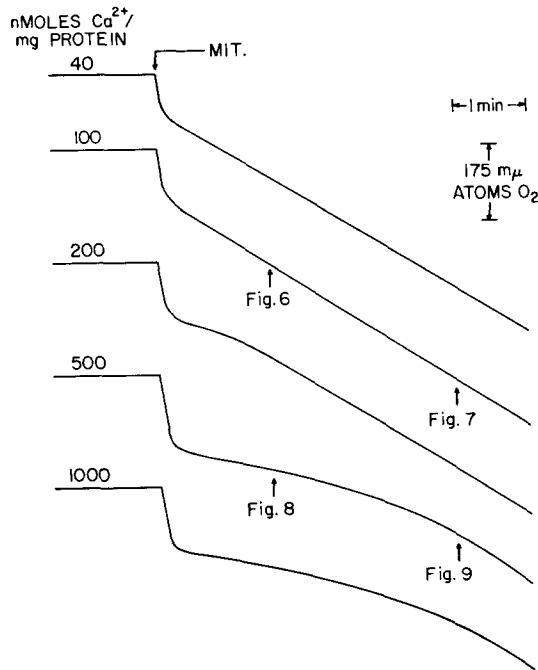
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nMOLES Ca²⁺/
mg PROTEIN

FIGURE 5 Rate of oxygen consumption by state 6 rat liver mitochondria at various concentrations of Ca²⁺. Microsamples of mitochondria were fixed consecutively for electron microscopy at 1.5 and 4 min (see Figs. 6–9). The reaction system contained Tris buffer (5 mM, pH 7.4), NaCl (80 mM), succinate (5 mM), CaCl₂ (as indicated), and mitochondria (2 mg protein). Total volume 2.0 ml.

About 5% of the mitochondria are ruptured during this time (Fig. 9).

It should be mentioned here that under conditions of state 6 the accumulation of low levels of Ca²⁺ (40–190 nmoles/mg protein) invariably results in the formation of electron-opaque inclusions in the matrix of mitochondria (Figs. 6–8). These dense inclusions appear to be tightly packed, concentrically oriented "membranes" reminiscent of myelin figures and show a periodicity of 45 A (Fig. 10). The inclusions remain stable with subsequent uninhibited respiration (Fig. 7). With state 6 inhibited respiration, however, the density of the matrix inclusions is lost as the accumulated Ca²⁺ is released, leaving behind clear evidence of membrane whorls, 60 A thick, in the mitochondrial matrix (Figs. 9, 11). The nature of these Ca²⁺-induced membranes in the matrix of mitochondria is presently being investigated.

These results show that mitochondria, in a phosphate-free medium, can sequester up to 190 nmoles

of Ca²⁺/mg protein without effecting an osmotic ultrastructural transformation or an appreciable change in the rate of subsequent state 4 respiration. The *availability* of 200 nmoles or more of Ca²⁺/mg protein results in various degrees of state 6 respiratory inhibition after Ca²⁺ accumulation but, again, no osmotic perturbation. However, state 6–related respiratory inhibition is unstable and soon leads to an ejection of the accumulated Ca²⁺ and an apparently nonosmotic ultrastructural transformation.

The Role of Phosphate

In all the experiments sited above, Ca²⁺ was present at zero time. When 150 nmoles of Ca²⁺/mg protein are added to mitochondria after a few minutes of respiration and in the presence of phosphate, an immediate osmotic ultrastructural transformation occurs. This perturbation is manifest as a doubling of the volume of the inner compartment while the mitochondria assume an orthodox conformation (Fig. 12).

Under all conditions studied, in which the order of addition of Ca²⁺ was varied with phosphate and ADP, once Ca²⁺ is accumulated and an osmotic perturbation established, subsequent oxidative phosphorylation occurs at low efficiency while acceptor control decreases to near unity (Fig. 12).

In Fig. 12, Ca²⁺ accumulation is identified by the short bursts of respiratory stimulation shown in the polarographic traces after Ca²⁺ addition. The availability of ADP has no influence on Ca²⁺-induced, phosphate-dependent, osmotic ultrastructural transformations (curves a and b). Although the presence of nucleotide and phosphate establishes "massive loading" conditions (15–17), it is to be reiterated that only a limited concentration of Ca²⁺ was used in these experiments.

Curves a–c of Fig. 12 show the nonosmotic affect of Ca²⁺ uptake by mitochondria in a phosphate-free system. The subsequent addition of phosphate in these three experiments, however, causes an osmotic perturbation in which the inner compartment doubles in volume. The dependency on phosphate for a Ca²⁺-effected osmotic perturbation is clearly established by these three results and agrees with light-scattering results reported from other laboratories (14, 21). Note that osmotic perturbation can occur independent of respiratory stimulation (curve c).

Curve d is especially interesting in that it demonstrates that the late addition of 500 nmoles of phosphate/mg protein to state 4 mitochondria fails
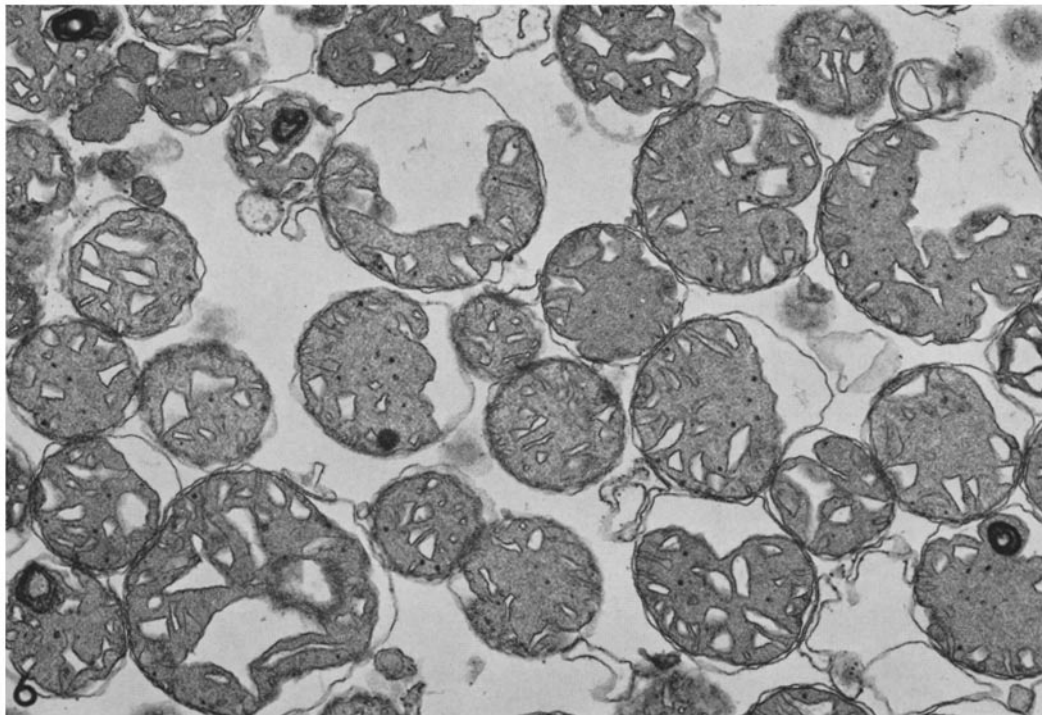
FIGURE 6 State 6 mitochondria fixed 1 min after the binding of 100 nmoles Ca^{2+}/mg protein. The volume of the inner compartment is not appreciably increased in comparison to the control in Fig. 2. Electron-opaque "membrane" inclusions are seen in the matrix of five mitochondria. Reaction system as in the legend for Fig. 5. 100 nmoles Ca^{2+}/mg protein were available (see Table II). × 23,700.

to cause an osmotic ultrastructural transformation. It is noteworthy that this quantity of inorganic phosphate is 15 to 20 times in excess of the endogenous inorganic phosphate in these mitochondria.[2] Curve d shows further that the subsequent addition of 150 nmoles of Ca^{2+}/mg protein to state 4P mitochondria, i.e. mitochondria to which exogenous phosphate is already available, effects an immediate osmotic ultrastructural transformation, apparently owing to Ca^{2+}-stimulated phosphate uptake.

These results establish that the presence of phosphate is required for an osmotic ultrastructural transformation to occur in mitochondria during the energized accumulation of Ca^{2+} under steady-state conditions. The accumulation of Ca^{2+} in phosphate-free systems occurs without effecting an osmotic ultrastructural transformation. Finally, the addition of phosphate to Ca^{2+}-free state 4 mito-

chondria fails to initiate an osmotic ultrastructural transformation.

DISCUSSION

Osmotic Perturbation vs. Mechanochemical Activity in the Transformation of Mitochondrial Ultrastructure

Light-scattering methods have been used in several laboratories to ascertain that the energized accumulation of Ca^{2+} by mitochondria leads to mitochondrial "swelling" (18–21). Decrease in the light-scattering properties of mitochondria under prescribed conditions of Ca^{2+} accumulation is consistent with the idea that ion movements can initiate water movements in mitochondria (22, 23). The nonspecificity of light-scattering, however, leaves doubt as to what per cent of the mitochondria of a suspension "swells" and which compartment in the mitochondria undergoes "swelling"

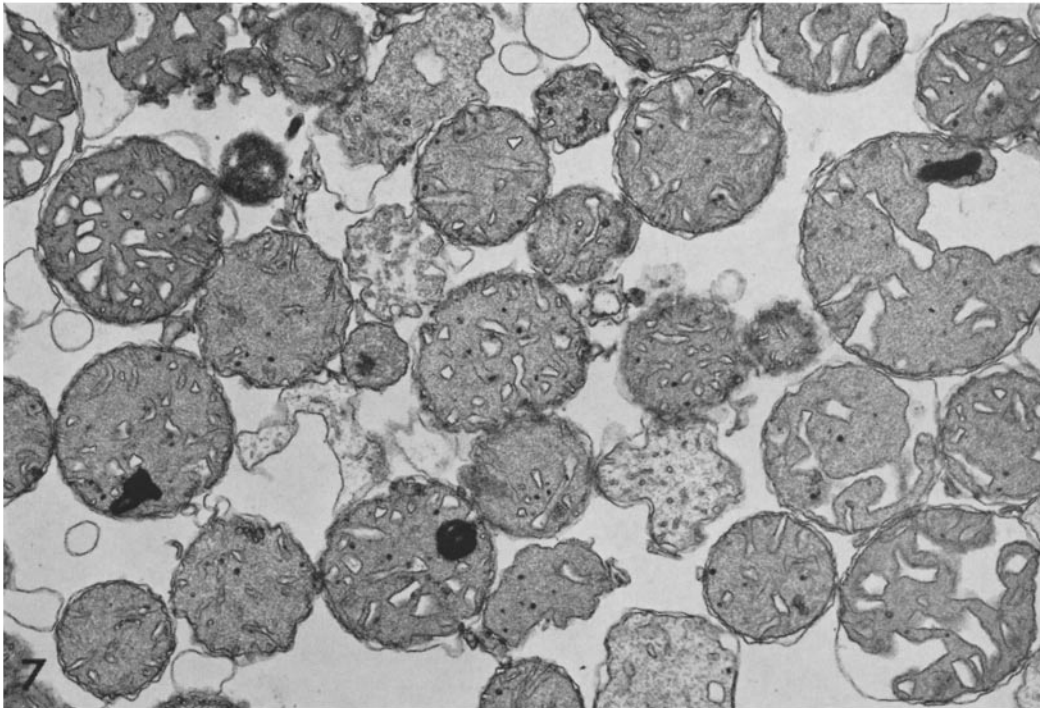[2] C. R. Hackenbrock and J. L. Gamble, Jr. In preparation.

FIGURE 7 State 6 mitochondria fixed 4 min after the binding to 100 nmoles Ca^{2+}/mg protein. The Ca^{2+} is retained (see Table II). The volume of the inner compartment is not appreciably changed in comparison to mitochondria fixed 1 min after state 6 Ca^{2+} binding (Fig. 6). Electron-opaque "membrane" inclusions are seen in the matrix of three mitochondria. Reaction system as in the legend for Fig. 5. 100 nmoles Ca^{2+}/mg protein were available (see Table II). × 23,700.

during ion-accumulation. In some cases, the "swelling" indicated by decreases in light-scattering signals is quantitatively greater than that which is predicted on the basis of the actual quantity of ion accumulation (24–26).

It is generally agreed that "swelling" measured by light-scattering changes is not quantitative (27). In addition, light-scattering signals fail to discriminate between ion-induced volume increases, mechanochemical expansion, structural disruption, and agglutination of mitochondria. Thus, light-scattering changes in many cases may be neither quantitative nor qualitative.

Tarr and Gamble (28) have pointed out that the sucrose-inaccessible water of mitochondria is osmotically active and is appropriate in volume to contain the endogenous electrolytes at iso-osmotic concentrations. It is generally agreed that the sucrose-inaccessible water of mitochondria is the water phase of the inner compartment. Using electron microscopy as a more direct approach,

Deamer et al. (5) and Packer et al. (6) were able to establish that, under conditions which support oscillatory electron transport, energized ion accumulation induces an oscillatory volume increase of the inner mitochondrial compartment. The maintenance of ion accumulation and the increased volume of the inner compartment is characteristically of short duration under oscillatory state conditions (ca. 1 min). Consequently, the quantitative aspects of ion accumulation related to the degree of volume increase, as shown by electron microscopy, were not investigated in these interesting studies.

In the present communication, we identify energized ion-induced osmotic ultrastructural transformations in mitochondria under steady-state conditions by ascertaining by direct means that: (a) ion was, in fact, accumulated by the mitochondria during increased electron transport; (b) the osmotically active inner mitochondrial com-
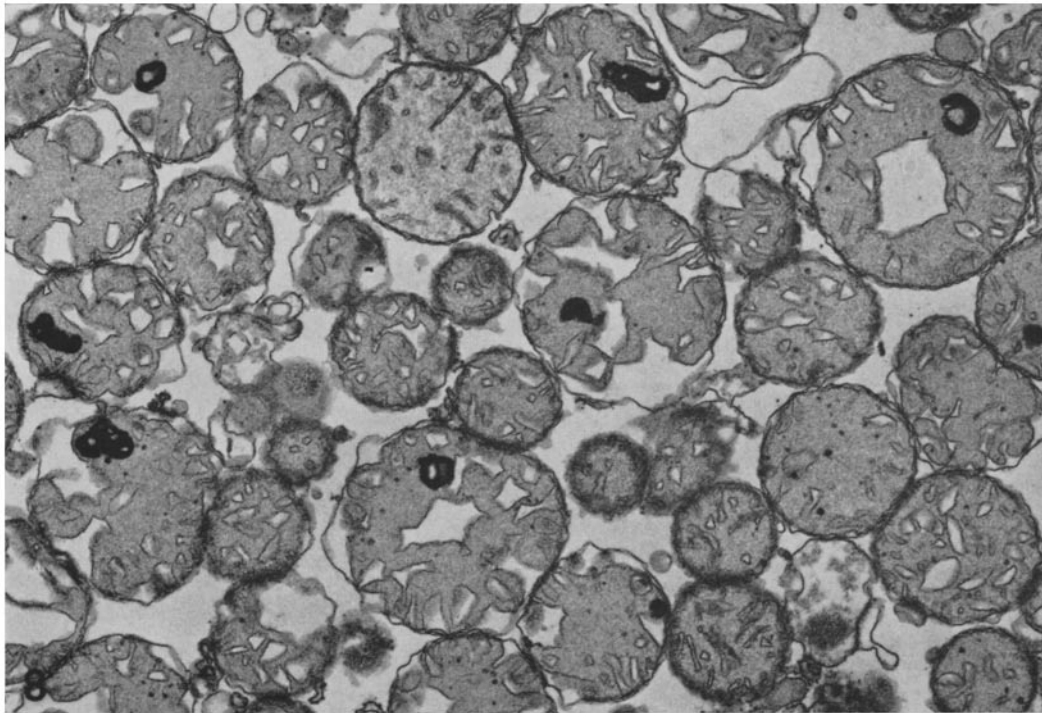
Figure 8   State 6 mitochondria fixed 1 min after the binding of 190 nmoles Ca^{2+}/mg protein. The volume of the inner compartment is not appreciably increased in comparison to the control in Fig. 2. Electron-opaque "membrane" inclusions (see Fig. 10) are seen in the matrix of eight mitochondria. Reaction system as in the legend for Fig. 5. 500 nmoles of Ca^{2+}/mg protein were available (see Table II). × 23,700.

partment increases in volume in proportion to the quantity of ion accumulated.

Two levels of osmotic perturbation were studied. The first clearly defined level is that which occurs after the accumulation of 100 nmoles Ca^{2+}/mg protein in the presence of phosphate. The volume of the inner compartment increases by approximately 50%, and subsequent respiratory rate, acceptor control, and phosphorylation efficiency are slightly altered. Lower levels of osmotic perturbation were found to be difficult to quantitate with the electron microscope.

The second level of osmotic perturbation studied and the one to which we will devote most of this discussion, results in a doubling of the volume of the inner compartment while the mitochondria transform from a condensed to a structurally intact orthodox conformation. The accumulation of 150–200 nmoles of Ca^{2+}/mg of protein in the presence of phosphate is required for effecting this degree of osmotic perturbation. Although this quantity of accumulated ion results in a loose coupling rate of

respiration and a major loss of acceptor control, the accumulated Ca^{2+} is retained in the osmotically perturbed mitochondria.

It is known that the inner compartment of mitochondria can expand to twice its volume during the state 4 mechanochemically induced condensed-to-orthodox ultrastructural transformation (3). During mechanochemical ultrastructural transformation, however, an increase of total ion does not occur, while high oxidative phosphorylation efficiency and acceptor control ratios are maintained by the mitochondria (3). It is most significant that the mechanochemically induced orthodox conformation is immediately reversed to a condensed conformation during ADP-initiated oxidative phosphorylation (3).

In contrast with these characteristics of mechanochemical ultrastructural transformations, the present study reveals that, when the orthodox conformation is osmotically induced in mitochondria by ion accumulation, ADP cannot reverse the orthodox conformation to a condensed conforma-
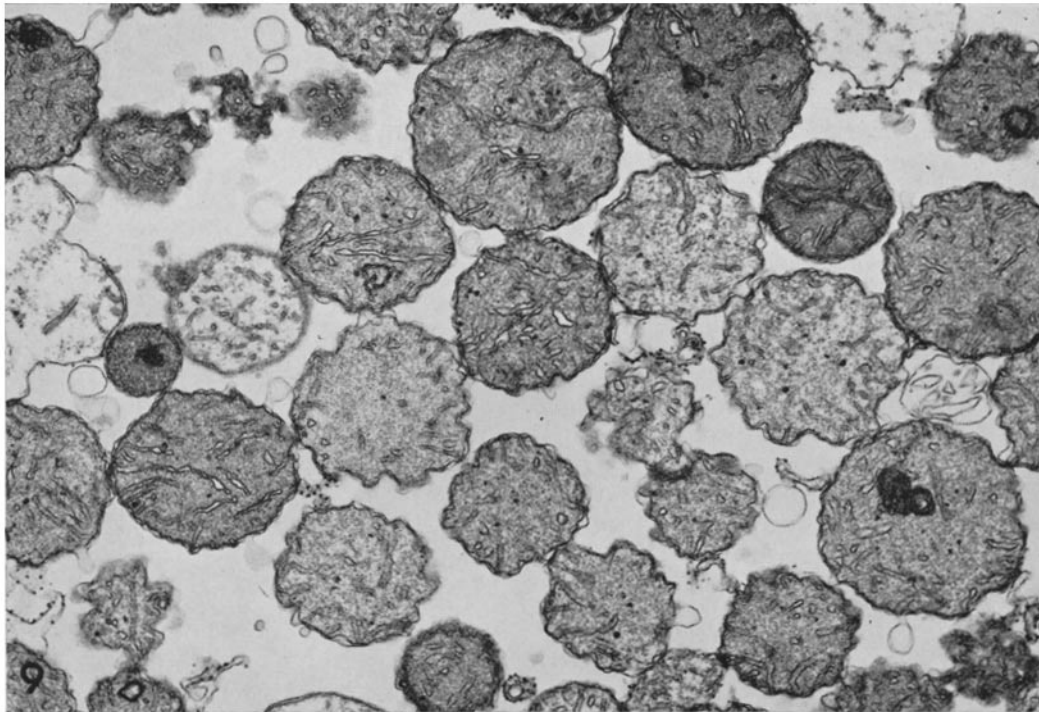
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FIGURE 9 State 6 mitochondria fixed 4 min after the binding, followed by the spontaneous release, of 190 nmoles of Ca^{2+}/mg protein (see Table II). The volume of the inner compartment is approximately doubled in comparison to mitochondria fixed 1 min after state 6 Ca^{2+} binding (Fig. 8), and the mitochonrial membranes show a wrinkled appearance. Approximately 5% of the mitochondria are ruptured. Membrane whorls (see Fig. 11) are observed in the matrix of six mitochondria. Reaction system as in the legend for Fig. 5. 500 nmoles Ca^{2+}/mg protein were available (see Table II). × 23,700.

tion and that, in addition, these mitochondria are invariably found to be loosely coupled.

Quantitative Ion-Induced Osmotic Ultrastructural Transformation in Mitochondria

Liver mitochondria isolated in this laboratory show the condensed conformation (Fig. 2) and contain a water content of 2.4 μl/mg protein, of which 1.2 μl is sucrose-inaccessible.[2] These findings are consistent with the view that the sucrose-inaccessible water resides in the inner mitochondrial compartment. A doubling of the volume of the inner compartment (Fig. 4) requires an accumulation of 150 nmoles of Ca^{2+}/mg protein and presumably an uptake of 1.2 μl of osmotic water/mg protein into the inner compartment. With this osmotically induced doubling of the water content of the inner compartment, the accumulated Ca^{2+}

amounts to a final concentration of 60 milliosmolar in the inner compartment. This is approximately equivalent to the resulting osmolar concentration of the endogenous K^+ of the inner compartment. The concentration of K^+ in the condensed mitochondria isolated in our laboratory is 115–150 milliosmolar.[2] K^+ is the major osmotically active endogenous cation of the inner compartment and, along with its counteranion, effectively maintains osmotic equilibrium in the condensed mitochondria after isolation in 250 milliosmolar sucrose. Thus, when the accumulated Ca^{2+} and its anion reach the osmolar concentration of the endogenous K^+ and its anion, a doubling of the volume of the inner compartment occurs.

On the basis of these findings, the conclusion can be made that an energized ion-induced osmotic perturbation of mitochondrial ultrastructure, in which the osmotically active inner compartment doubles in volume, requires a doubling of the os-
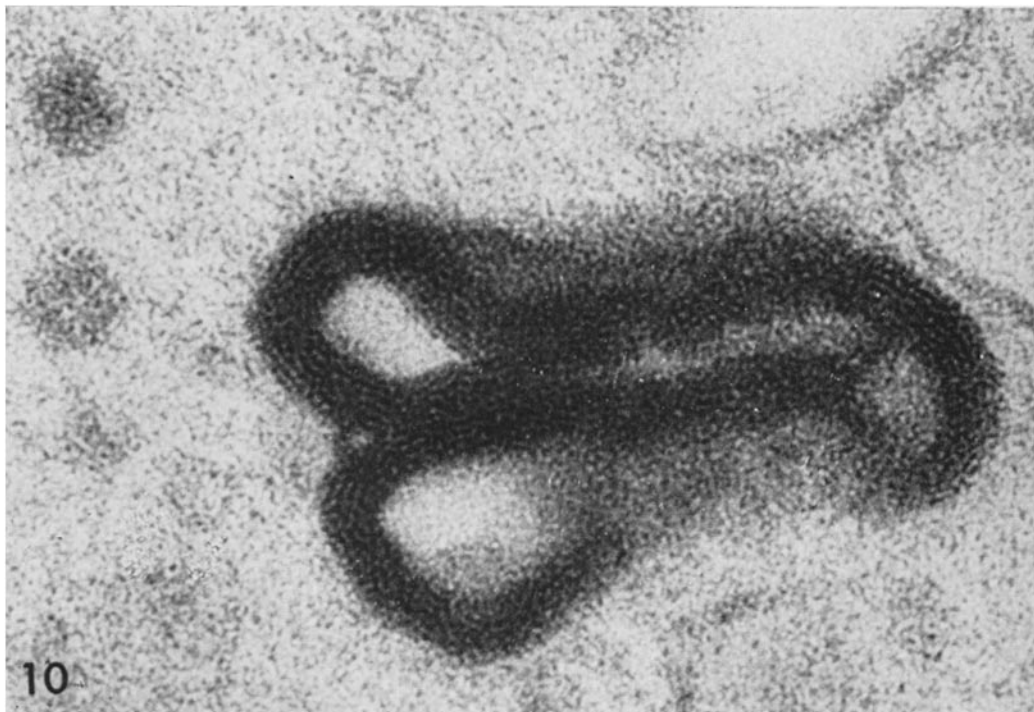
FIGURE 10 Ca^{2+}-induced formation of electron-opaque "membrane" inclusions in the matrix of state 6 mitochondria. The concentrically arranged "membranes" show a periodicity of 45 A. Two typical intramitochondrial granules are seen in the upper left. Conditions as in the legend for Fig. 8. $\times$ 278,000.

FIGURE 11 Membrane whorls in the matrix of state 6 mitochondria after the spontaneous release of previously bound Ca^{2+}. The membranes are 60 A thick. Conditions as in the legend for Fig. 9. $\times$ 278,000.
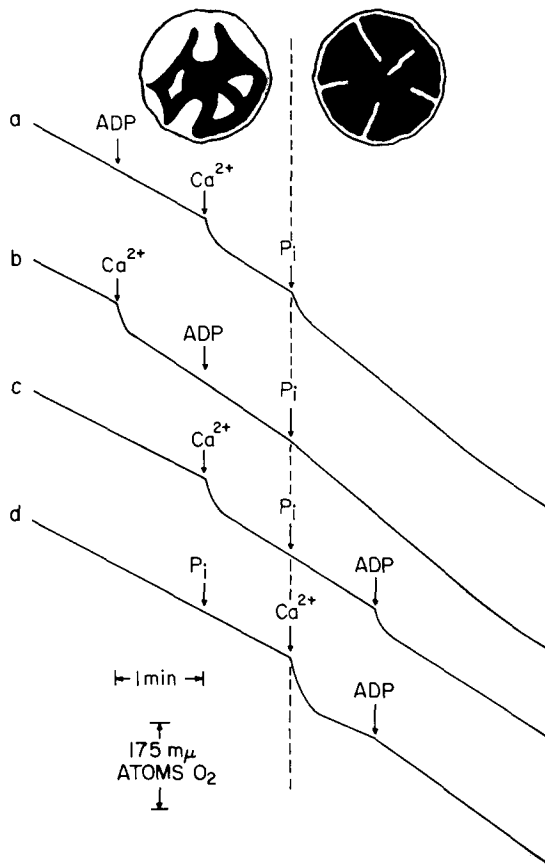
Figure 12 Rate of oxygen disappearance after additions of ADP, Ca^{2+}, and P_i to state 4 mitochondria. Ca^{2+} accumulation is identified by the short bursts of respiratory stimulation. Mitochondrial structure is shown diagrammatically and indicates the time (vertical dash) at which the inner compartment doubles in volume. Osmotic perturbation occurs only when Ca^{2+} and P_i are present and can be independent of respiratory stimulation (curve c). The state 4 reaction system contained Tris buffer (5 mM, pH 7.4), $NaCl_2$ (80 mM), succinate (5 mM), and mitochondria (2 mg protein); also, when added, ADP (0.5 μmoles), P_i (1.0 μmoles), and $CaCl_2$ (300 nmoles). Total volume 2.0 ml.

motic equivalents of the major endogenous cation, namely K^+, and its counteranion, in the water phase of the inner compartment of mitochondria. During such a perturbation induced by the accumulation of Ca^{2+} and phosphate, mitochondria transform from a condensed to an orthodox conformation as considerable uncoupling takes place. It is concluded that, under conditions in which the inner mitochondrial compartment doubles in volume during transformation from a

condensed to an orthodox conformation without an energized accumulation of ions, the volume expansion of this compartment is due to mechanochemical activity. Electron transport–dependent ultrastructural transformations, during which a doubling of the volume of the inner mitochondrial compartment occurs without any change in total ion content, has recently been observed in this laboratory.[2]

It should be mentioned here that Ca^{2+} accumulation supported by nucleotide and phosphate can be as high as 2600 nmoles/mg protein, which leads to observable electron-opaque precipitates in the inner compartment of mitochondria as well as ultrastructural disruption (29, 30). We have not observed electron-opaque precipitates in mitochondria when physiological levels of Ca^{2+} are accumulated in the presence of phosphate, i.e. levels of less than 200 nmoles/mg protein which do not cause ultrastructural disruption. Apparently, the solubility product of this complex precipitate is reached only after the accumulated free calcium and phosphate reaches an osmolar concentration which disrupts the ultrastructure of isolated mitochondria.

Ion Accumulation by Mitochondria without Osmotic Ultrastructural Transformation

The present study clearly establishes that added phosphate is required for Ca^{2+}-induced osmotic ultrastructural transformations in mitochondria. It is well known, however, that divalent cations can be accumulated independently of phosphate (31). The present study reveals that, in systems free of exogenous phosphate, up to 190 nmoles Ca^{2+}/mg protein is sequestered by mitochondria and does not effect any appreciable increase in the volume of the inner mitochondrial compartment. The availability of more than 200 nmoles Ca^{2+}/mg protein also does not effect osmotic perturbation, but does initiate respiratory inhibition indicative of state 6. State 6 respiratory inhibition results in a loss of the accumulated Ca^{2+} and a gradual, presumably nonosmotic, ultrastructural change. As Ca^{2+} is lost, the mitochondria tend to become "orthodox" but their membranes usually assume a wrinkled appearance. Our data show that the minimum concentration of available Ca^{2+} which causes these state 6–related structural alterations is in the range of 150–200 μM. This concentration of Ca^{2+} is also the minimum amount which stabilizes

both DPN and cytochrome b in a highly reduced state in phosphate-free systems (14).

It appears that in systems free of exogenous phosphate the Ca^{2+} taken up is for the most part *bound* to the electron-transport membrane of mitochondria rather than *accumulated* into the water phase of the inner mitochondrial compartment. This explains the osmotic stability of the inner compartment after the sequestering of 150–190 nmoles Ca^{2+}/mg protein in the absence of phosphate. It has been reported that the Ca^{2+}-binding capacity of mitochondria is 100–150 nmoles/mg protein (32). It is thought that the additional 40 nmoles of Ca^{2+} sequestered under state 6 conditions in the present investigation may enter the inner compartment, owing to the endogenous freely mobile phosphate present in this compartment, or may be complexed in the dense matrix inclusions formed under these conditions (Fig. 10). It will be recalled that in the presence of phosphate the accumulation of 150–200 nmoles Ca^{2+}/mg protein is required for a doubling of the volume of the inner mitochondrial compartment.

That phosphate is essential for Ca^{2+} accumulation to induce an osmotic ultrastructural transformation is further supported by the finding that after Ca^{2+} binding takes place in a system lacking added phosphate, the subsequent addition of phosphate effects an immediate osmotic perturbation in the inner mitochondrial compartment. Thus, it follows that the Ca^{2+}-binding sites of the electron-transport membrane release bound Ca^{2+} into the osmotically active water phase of the inner mitochondrial compartment in the presence of phosphate. Although the binding of Ca^{2+} is essentially respiratory-dependent, the phosphate-induced re-

lease of membrane-bound Ca^{2+} into the inner compartment can be independent of respiratory stimulation (Fig. 12, curve c). The release of mitochondrial-bound Ca^{2+} by permeant anion has previously been suggested by investigators in other laboratories (14, 33). We have not studied permeant anions other than phosphate.

The present results also show that the addition of phosphate to mitochondria at any time during state 4 respiration fails to effect an osmotic ultrastructural transformation. This finding is in agreement with the observation that phosphate is not appreciably accumulated by state 4 mitochondria (34, 35).

Under the conditions for steady-state electron transport, it is concluded that: (a) in the absence of phosphate, Ca^{2+} is bound by mitochondria without effecting an osmotic ultrastructural transformation; (b) in the presence of phosphate, Ca^{2+} is accumulated along with phosphate into the inner mitochondrial compartment to effect an osmotic ultrastructural transformation; (c) in the case in which Ca^{2+} is previously bound, the subsequent addition of phosphate initiates release of the bound Ca^{2+} into the inner mitochondrial compartment and, along with the phosphate, effects an osmotic ultrastructural transformation; and (d) in the absence of exogenous Ca^{2+}, phosphate is not accumulated by state 4 liver mitochondria and does not effect an osmotic ultrastructural transformation.

REFERENCES

1. HACKENBROCK, C. R. 1965. *Anat. Rec.* **151**:356.
2. HACKENBROCK, C. R. 1966. *J. Cell Biol.* **30**:269.
3. HACKENBROCK, C. R. 1968. *J. Cell Biol.* **37**:345.
4. HACKENBROCK, C. R. 1968. *Proc. Nat. Acad. Sci. U.S.A.* **61**:598.
5. DEAMER, D. W., K. UTSUMI, and L. PACKER. 1967. *Arch. Biochem. Biophys.* **121**:641.
6. PACKER, L., J. M. WRIGGLESWORTH, P. A. G. FORTES, and B. C. PRESSMAN. 1968. *J. Cell Biol.* **39**:382.
7. SCHNEIDER, W. C. 1948. *J. Biol. Chem.* **176**:259.
8. LAYNE, E. 1957. *In* Methods in Enzymology. S. P. Colowick and N. O. Kaplan, editors. Academic Press Inc., New York. **3**:447.

9. CLARK, L. E., JR., R. WOLF, D. GRANGER, and Z. TAYLOR. 1953. *J. Appl. Physiol.* **6**:189.
10. RASMUSSEN, H., A. WALDORF, D. D. DZIEWIATKOWSKI, and H. F. DELUCA. 1963. *Biochim. Biophys. Acta.* **75**:250.
11. KARNOVSKY, M. J. 1961. *J. Biophys. Biochem. Cytol.* **11**:729.
12. ROSSI, C. S., and A. L. LEHNINGER. 1963. *Biochem. Z.* **338**:698.
13. LEHNINGER, A. L., E. CARAFOLI, and C. S. ROSSI. 1967. *In* Advances in Enzymology. F. F. Nord, editor. J. Wiley and Sons, Inc., New York **29**:259.
14. CHANCE, B. 1965. *J. Biol. Chem.* **240**:2729.
15. VASINGTON, F. D., and J. V. MURPHY. 1961. *Fed. Proc.* **20**:146.

16. VASINGTON, F. D., and J. V. MURPHY. 1962. Biol. Chem. 237:2670.

17. DELUCA, H. F., and G. W. ENGSTROM. 1961. Proc. Nat. Acad. Sci. U.S.A. 47:1744.

18. RASMUSSEN, H., B. CHANCE, and E. OGATA. 1965. Proc.Nat. Acad. Sci. U.S.A. 53:1069.

19. CHAPPELL, J. B., and A. R. CROFTS. 1965. Biochem. J. 95:378.

20. CROFTS, A. R., and J. B. CHAPPELL. 1965. Biochem. J. 95:387.

21. AZZI, A., and G. F. AZZONE. 1966. Biochim. Biophys. Acta. 113:438.

22. PRICE, C. A., A. FONNESU, and R. E. DAVIES. 1956. Biochem. J. 64:754.

23. MACFARLANE, M. G., and A. G. SPENCER. 1953. Biochem. J. 54:569.

24. COCKRELL, R. S., E. J. HARRIS, and B. C. PRESSMAN. 1966. Biochemistry. 5:2326.

25. PRESSMAN, B. C., 1965. Proc. Nat. Acad. Sci. U.S.A. 53:1976.

26. HARRIS, E. J., R. COCKRELL, and B. C. PRESSMAN. 1966. Biochem. J. 99:200.

27. LEHNINGER, A. L. 1962. Physiol. Rev. 42:467.

28. TARR, J. S., and J. L. GAMBLE, JR. 1966. Amer. J. Physiol. 211:1187.

29. GREENAWALT, J. W., C. S. ROSSI, and A. L. LEHNINGER. 1964. J. Cell Biol. 23:21.

30. WEINBACH, E. C., and T. VON BRAND. 1967. Biochim. Biophys. Acta. 148:256.

31. CHAPPELL, J. B., M. COHN, and G. D. GREVILLE. 1963. In Energy-Linked Functions of Mitochondria. B. Chance, editor. Academic Press Inc., NewYork. 232.

32. ROSSI, C., and A. L. LEHNINGER. 1964. J. Biol. Chem. 239:3971.

33. GEAR, A., C. S. ROSSI, B. REYNAFARJE, and A. L. LEHNINGER. 1967. J. Biol. Chem. 242:3403.

34. CARAFOLI, E., C. S. ROSSI, and A. L. LEHNINGER. 1964. J. Biol. Chem. 239:3055.

35. PRESSMAN, B. C., and J. K. PARK. 1963. Biochem. Biophys. Res. Commun. 11:182.