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Short Communication

Ultrastructural Evidence for Calcium Phosphate Deposition by Isolated Corn Shoot Mitochondria[1]

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ABSTRACT

This study demonstrates that isolated corn (*Zea mays* L. Wf9 xM14) shoot mitochondria will accumulate calcium phosphate crystals *in vitro* that are comparable to those observed in both *in vivo* and *in vitro* animal mitochondria. The intensity of deposition, as observed by electron microscopy, increased with incubation time, substrate concentration, and concentration of calcium and inorganic phosphate. Calcium phosphate deposits were initially observed as amorphous, nearly spherical areas that lacked sharp or clear edges, but which were crystalline in nature under heavier loading conditions and longer incubation times.

Both isolated (3, 9, 16) and *in situ* animal mitochondria (7, 9) have been reported to accumulate Pi in the presence of Ca^{2+}, Sr^{2+}, and Ba^{2+}, but not Mg^{2+}. The solubility products of the salts can be exceeded within mitochondria, and the resulting precipitate has been identified as deposits of metal phosphate (21). Two recent studies (6, 17) have dealt primarily with strontium uptake in plant mitochondria. Hanson and Hodges (4) showed electron dense deposits in isolated corn mitochondria and suggested that these deposits were calcium phosphate, but detailed studies of calcium phosphate deposition have never been reported in plant mitochondria.

The Pi transport properties of isolated corn mitochondria, which are similar to *in vivo* properties (1, 7), have been reported by Hanson and Miller (5). They indicated that both Ca^{2+} and Pi were required for accumulation of either ion. Miller *et al.* (15) showed that little Pi was accumulated by isolated corn mitochondria in the presence of Mg^{2+}. It is believed that the transport of Ca^{2+} and Pi is at the expense of a phosphorylated high energy intermediate of oxidative phosphorylation. Concurrent with this transport is the acceleration of electron flow (13, 14).

MATERIALS AND METHODS

Mitochondria were isolated from 3-day-old etiolated corn seedlings (*Zea mays* L. Wf9xM14) according to the procedure of Miller *et al.* (13). Mitochondria were incubated in 4 ml of reaction media as noted in the figure legends. For observing calcium phosphate deposits, the following abbreviated fixation and dehydration procedures were used. After incubation, the suspension was centrifuged at 12,000 rpm for 10 min to pellet the mitochondria. The resulting pellet was fixed for 20 min in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4), was given three 10-min washes in buffer, and was quickly passed through a graded series of ethyl alcohols. Propylene oxide was used as a solvent for epon embedment (11). Control preparations and some preparations containing calcium phosphate deposits were fixed for 1 hr in 2% glutaraldehyde in 0.1 M phosphate buffer followed by secondary osmium fixation as above. Dehydration and embedment were also carried out as above. Thin sections were viewed unstained or doubly stained with uranyl acetate (19) and lead citrate (18). The technique of microincineration, as described by Thomas and Greenawalt (21), was performed by heating the sections, on grids, to 600 C for 15 min. Calcium uptake was monitored using murexide (ammonium purpurate) as a cation sensitive dye (12) on an Aminco, DW2 dual beam UV/Vis spectrophotometer.

RESULTS

Isolated corn mitochondria treated with only Pi in addition to the basic reaction media showed a uniformly low content of

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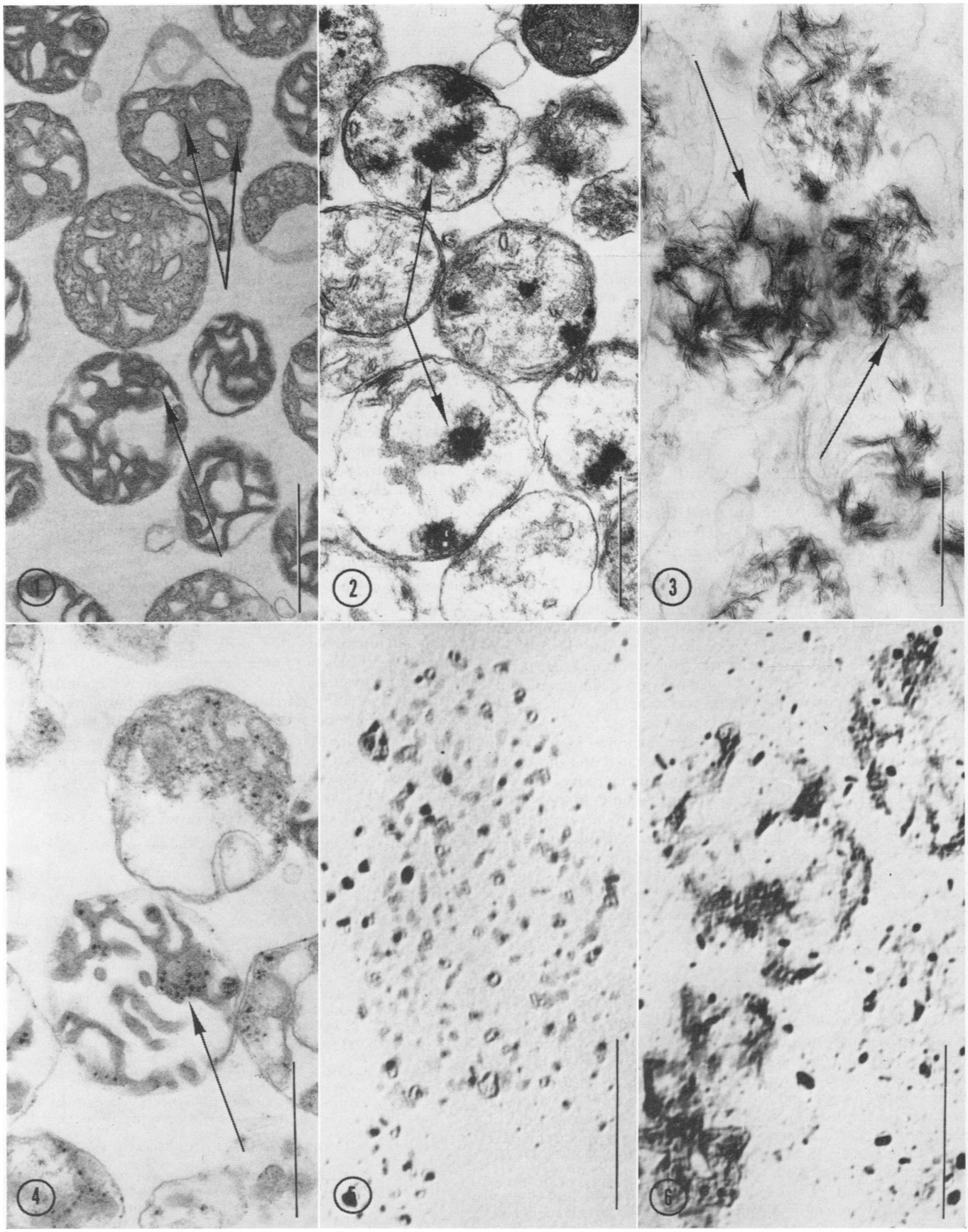
FIGS. 1-6. Line scale in Fig. 1 represents 1 μm. Figs. 2 to 6 are at same magnification as Fig. 1. Fig. 1: mitochondria incubated for 3 min in 4 ml of reaction media containing 200 mM KCl, 20 mM tris-HCl (pH 7.4), 1 mg/ml bovine serum albumin, and 4 mM Pi. Arrows point to normal granules (N). Fig. 2: incubated for 3 min in reaction medium as in Fig. 1 before addition of 40 μmoles of malate + 40 of μmoles pyruvate and 20 μmoles of $CaCl_2$. Three μmoles of NADH were also added. Mitochondria were fixed 15 min after initial substrate addition. Fig. 3: same as Fig. 2 but with three additions of 40 μmoles of malate + 40 of μmoles pyruvate, 3 μmoles of NADH, 64 μmoles of Pi, and 80 μmoles $CaCl_2$ spaced 5 min apart. Mitochondria were fixed 25 min after the initial substrate addition. Large amounts of substrate and longer loading time are essential for the accumulation or massive crystalline deposits. Arrows show crystalline deposits. Fig. 4: corn mitochondria held overnight in 2% glutaraldehyde. No exogenous substrate or Ca^{2+} was added before fixation. Note granules similar to those shown by Hanson and Hodges (4). Fig. 5: incinerated preparation similar to Fig. 2 but incubated with $MgCl_2$ instead of $CaCl_2$. Fig. 6: same as Fig. 5 but incubated with Ca^{2+} instead of Mg^{2+}.



FIGS. 1–6.

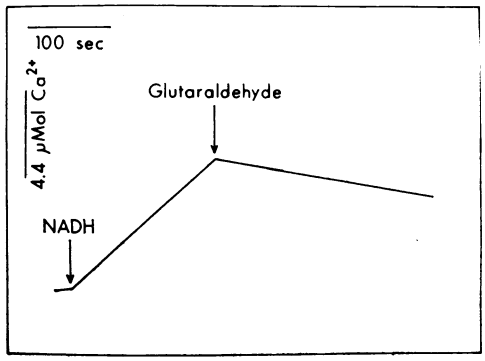


FIG. 7. Uptake of Ca^{2+} by isolated mitochondria and its release by glutaraldehyde. The 3-ml reaction medium contained 0.3 M sucrose, 20 mM tris-HCl (pH 7.4), 1 mg/ml bovine serum albumin, 1 mM Pi, 1 mM Ca^{2+}, and 40 μmoles of murexide (ammonium purpurate). The reference wavelength used was 510 nm, and the measuring wavelength was 540 nm. Glutaraldehyde (final concentration 2% v/v) and 3 μmoles of NADH were added as indicated. The upward deflection of the trace represents Ca^{2+} accumulation by the mitochondria.

electron-dense granules (Fig. 1). Other micrographs (not shown) of mitochondria treated with only Ca^{2+} or with both Mg^{2+} and Pi were essentially the same. In all three situations, amorphous granules were observed that were similar in appearance to those described by Thomas and Greenawalt (21). These are termed normal granules and may possibly be fixation artifacts.

The presence of calcium phosphate deposits was easily discerned in samples prepared by the abbreviated procedure. Normal granules in corn mitochondria had a sharp boundary, *i.e.* a clear cut edge (Fig. 1). Calcium phosphate deposits initially formed as amorphous, nearly spherical areas that lacked sharp or clear edges (Fig. 2). Under heavier loading conditions the crystalline nature of the deposit was clear (Fig. 3).

Incineration at 600 C destroys organic matter and leaves a residue containing inorganic granules. Samples incubated in Mg^{2+} with Pi and Ca^{2+} with Pi were incinerated and are shown in Figures 5 and 6, respectively. After incineration the Ca^{2+} with Pi-treated mitochondria left considerably more residue than did the Mg^{2+} with Pi treatment.

Calcium phosphate deposits were exceedingly difficult to find in samples prepared with glutaraldehyde fixation. Figure 7 shows the accumulation of Ca^{2+} by mitochondria upon the addition of exogenous NADH. After addition of glutaraldehyde, Ca^{2+} began to leak out of the mitochondria back into solution. Electron microscopy would reveal such a preparation to appear similar to that in Figure 1.

DISCUSSION

The formation of electron-diffractive (metal phosphate) deposits in animal mitochondria (*in vitro*) depends on the presence of both Ca^{2+} and Pi (9). Strontium, Ba^{2+}, and Mn^{2+} will partially substitute for Ca^{2+}, but Mg^{2+} will not (10). Deposition requires energy, and the number and form of the deposits depends on the loading time (2). With short loading time, a scattering of particles was observed, but with longer loading time, the particles (Sr^{2+} with Pi) assumed a needle shape and appeared crystalline (2).

Since Ca^{2+} and Pi transport properties of plant and animal mitochondria are similar in most respects (8, 20), it would be expected that plant mitochondria would transport Ca^{2+} and Pi and would form noticeable internal deposits. The only study reporting such deposits in plant mitochondria is that of Hanson and Hodges (4). We have reason to believe that the granules those authors interpreted as calcium phosphate deposits were actually fixation artifacts. Mitochondria held overnight in 2% glutaraldehyde have granules similar to those shown by Hanson and Hodges (Fig. 4). These granules were present even when Ca^{2+} and Pi were completely lacking from the incubation medium. Because the calcium phosphate deposits are not stable, we shortened the fixation, wash, and dehydration time. These artifact granules appear to become more prominent with extended fixation, washing, and dehydration.

From Figures 2 and 3, it can be seen that, in the presence of both Ca^{2+} and Pi, corn shoot mitochondria transported sufficient Ca^{2+} and Pi to cause precipitation inside the mitochondrion. Both ions were necessary, the process required energy, and Mg^{2+} would not substitute for Ca^{2+}. It can also be seen in Figures 2 and 3 that both amorphous and crystalline deposits were formed. Incineration experiments confirmed that the deposits were inorganic. The results presented here indicate that *in vitro* corn shoot mitochondria are similar to animal mitochondria in terms of Ca^{2+} and Pi transport and deposition. Although deposition of phosphate compounds has been shown in animal mitochondria *in vivo*, we have as yet been unable to demonstrate similar calcium phosphate deposits in *in vivo* corn mitochondria.

A recent study (17) of strontium deposition remarked on the paucity of calcium deposits in plant mitochondria and indicated the reason for this was unclear. We feel that Figure 7 demonstrates the reason for this paucity of calcium phosphate deposits in normal preparations. The calcium phosphate deposits are apparently soluble enough to wash out under certain fixation procedures. This same study (17) also notes needle-like structures, but comments that these are probably not strontium phosphate crystals but rather membranes lined with amorphous strontium phosphate. We feel that Figure 3 demonstrates this latter interpretation, at least for calcium phosphate, to be incorrect. Due to the abundance and position of the needle-like structures, it is impossible to interpret them as segments of membrane lined with metal-phosphate. These needle-like structures are most likely calcium phosphate crystals.

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