# Mitochondrial Biogenesis during Germination in Maize Embryos[1]

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Mitochondrial biogenesis and metabolism were investigated during maize (*Zea mays*) seed germination. Mitochondria from dry and imbibed seed exhibited NADH-dependent $O_2$ uptake that was completely inhibited by KCN and antimycin A. Mitochondria in the dry seed had a lower rate of succinate-dependent $O_2$ uptake relative to that measured in imbibed and germinated seed. The activities of the tricarboxylic acid (TCA) cycle enzymes, pyruvate dehydrogenase complex, 2-oxoglutarate dehydrogenase complex, NAD-malic enzyme, and citrate synthase, are similarly low in mitochondria from dry seed and this correlates with a lower relative abundance of the mitochondrial matrix-located citrate synthase and pyruvate dehydrogenase complex E1α-subunit polypeptides. Electron microscopy revealed that mitochondria in the dry seed have a poorly developed internal membrane structure with few cristae; following 24 h of germination the mitochondria developed a more normal structure with more developed cristae. The mitochondria from maize embryos could be fractionated into two subpopulations by Suc density gradient centrifugation: one subpopulation of buoyant density equivalent to 22% to 28% (w/w) Suc; the other equivalent to 37% to 42% (w/w) Suc. These two subpopulations had different activities of specific mitochondrial enzymes and contained different amounts of specific mitochondrial proteins as revealed by western-blot analysis. Both subpopulations from the dry embryo were comprised of poorly developed mitochondria. However, during imbibition mitochondria in the heavy fraction (37%–42% [w/w] Suc) progressively acquired characteristics of fully functional mitochondria found in the germinated seedling in terms of structure, enzymic activity, and protein complement. In contrast, mitochondria in the light fraction (22% to 28% [w/w] Suc) show no significant structural change during imbibition and the amounts of specific mitochondrial proteins decreased significantly during germination.

Germination involves the mobilization of storage reserves and the initiation of growth and metabolic activity within the embryo. The generation of ATP underpins this process and it is not surprising that rapid increases in respiration rate accompany the earliest stages of germination following imbibition of the seed (for review, see Bewley and Black, 1994). The metabolic source of ATP in germinating embryos is not known, but it has been suggested that in some plant species fully functional mitochondria are present in the dry seed (Attucci et al., 1991), whereas in others, ATP synthesis is a product of fermentative pathways during the earliest stages of imbibition (Raymond et al., 1985). In the latter case it was argued that mitochondrial biogenesis (assembly) occurs during imbibition and is dependent upon ATP synthesized in the cytosol. The aim of this study was to distinguish between these possibilities through an examination of mitochondrial biogenesis and aspects of function during germination of maize (*Zea mays*) embryos.

Mitochondrial assembly requires the coordinated synthesis of mitochondrial proteins encoded in the nuclear and mitochondrial genomes, coupled to the import of the cytosolically synthesized polypeptides into the mitochondria. These processes require ATP for RNA and protein synthesis, and ATP and a potential difference across the inner mitochondrial membrane for protein import (for review, see Glaser et al., 1998) so that ATP synthesis is a prerequisite for de novo synthesis and assembly of functional mitochondria. Previous studies on mitochondrial biogenesis during germination have focussed on seed storage tissues in the post-germination period (i.e. after radicle emergence). In general, in storage tissues the number of functional mitochondria increases in parallel with increases in the rate of respiration. In pea cotyledons this has been suggested to result from the activation of preformed mitochondria through import of existing polypeptides in the cytoplasm (Nawa and Asahi, 1971; Satoh and Asahi, 1975). However, in peanut cotyledons de novo synthesis of mitochondrial proteins is apparently required (Morohashi et al., 1981). Functional mitochondria capable of ATP synthesis have been isolated from dry sunflower seeds (Attucci et al., 1991), suggesting that the energy for mitochondrial assembly may be supplied by oxidative phosphorylation. As well as a general increase in mitochondrial protein in the post-germination phase, there is also differential regulation of mitochon-

drial metabolism in lipid storing seeds, to support conversion of storage lipid to sugars for export to the growing embryo (Hill et al., 1992; Falk et al., 1998).

However, little is known of mitochondrial biogenesis and function in embryos. Ehrenshaft and Brambl (1990) studied mitochondrial assembly in maize embryo tissue immediately following imbibition and showed that the mitochondrial enzymes, cytochrome oxidase and ATP synthase, were present in an active form in mitochondria from dry embryos, but the extent to which functional electron transport occurs was not determined. They also showed that embryo respiration was cyanide-sensitive, suggesting the involvement of cytochrome oxidase; that there is de novo synthesis of mitochondrial proteins from 6 h after imbibition onwards; and that this depends on de novo transcription rather than pre-existing mRNA. In the work described in this paper we sought to improve our understanding of the fundamental events underlying initiation of mitochondrial function during germination and early seedling growth in maize. In particular we investigated whether a functional electron transport chain is present in mitochondria from dry embryos and the extent to which assembly of mitochondrial matrix components coincides with that of the electron transport chain.

## RESULTS AND DISCUSSION

### The Respiration Rate in Embryos Increases during Imbibition

The water content of excised embryos increased rapidly during the first 10 h of imbibition and then at a slower rate over the next 38 h (Fig. 1A). Radicle growth first became apparent between 36 to 48 h after the initiation of imbibition. After a lag of 4 h the rate of oxygen uptake increased over the first 36 h and plateaued thereafter (Fig. 1A). The initial lag in oxygen uptake contrasts with the data of Ehrenshaft and Brambl (1990) who found a significant increase in the rate of oxygen uptake by maize embryos in the 1st h after imbibition. This difference could be due to differences in imbibition conditions, which may affect the rate of water uptake, since Ehrenshaft and Brambl (1990) imbibed embryos in air-saturated water, whereas in this study seeds were imbibed between moist filter paper, and embryos were subsequently excised (see "Materials and Methods").

### There Is No Change in Mitochondrial Genome Copy Number during Germination

To investigate whether germination is accompanied by changes in mitochondrial genome copy number DNA was isolated from embryos excised from the dry seed and at various times during imbibition. There was no significant change in the total DNA content of embryos during germination (Fig. 1B),
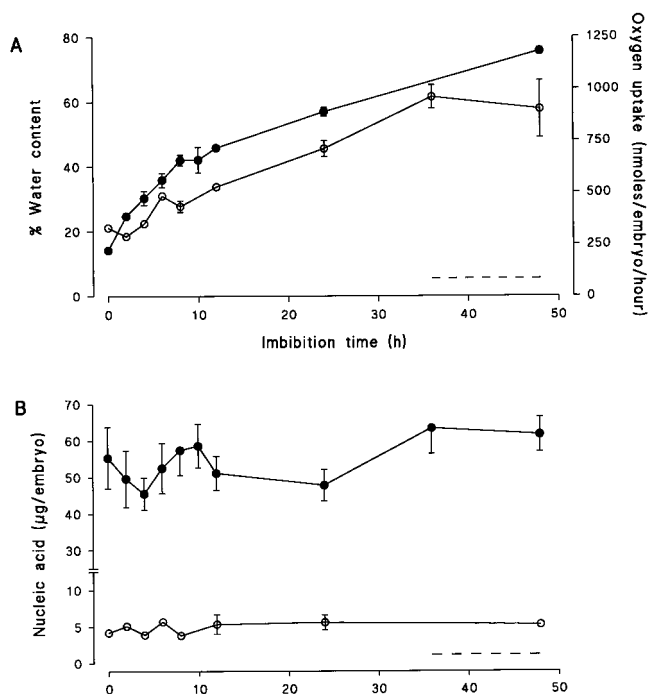


**Figure 1.** A, The water content (●) and rate of oxygen uptake (○) of maize embryos during imbibition. Water content was calculated from the change in weight following oven drying of the excised embryos relative to their fresh weight. Error bars indicate ±SE of three replicates of 10 (water content) or three (oxygen uptake) embryos. The dashed line indicates time of radicle emergence. B, The nucleic acid content of maize embryos during imbibition (●, RNA; ○, DNA). Values represent the average of three separate experiments, error bars indicate ±SE. The dashed line indicates time of radicle emergence.

suggesting that the number of new cells resulting from cell division is small compared with the total number of cells in the embryo. Mitochondrial genome copy number relative to nDNA content was analyzed by DNA-blot analysis. Total DNA blots were probed with a cDNA clone encoding mtHSP60 (a nuclear-encoded mitochondrial gene) and a genomic clone encoding cytochrome oxidase-I (COX-I) from the mitochondrial genome. The relative signal intensities give a measure of the number of mitochondrial genomes per nuclear genome. This ratio did not change significantly during the 48-h imbibition (data not shown), suggesting that there was no increase in mitochondrial genome copy number over this period.

### Abundance of Transcripts Encoding Mitochondrial Proteins Is Low until Radicle Emergence

To investigate changes in the steady-state transcript abundance for genes encoding mitochondrial proteins total RNA was extracted from embryos over the 48-h imbibition period. There was no significant change in the amount of RNA per embryo during imbibition (Fig. 1B). Total RNA at successive time
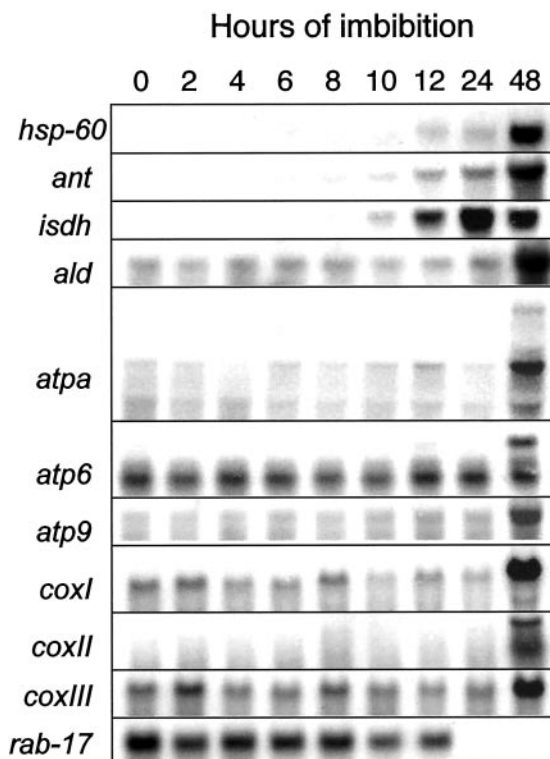
## Hours of imbibition



**Figure 2.** RNA gel-blot analysis of the steady-state abundance of various mitochondria-encoded (*atpa, atp6, atp9, coxI, coxII,* and *coxIII*) or nuclear-encoded (*isdh, ald, hsp60, ant,* and *rab-17*) gene transcripts in maize embryos during imbibition. Ten micrograms of total RNA was loaded per track.

points during imbibition was fractionated by gel electrophoresis, transferred to nitrocellulose membranes, and probed with the following cDNA and genomic DNA probes: *mthsp-60, ant, isdh, ald, atpa, atp6, atp9, coxI, coxII, coxIII,* and *rab-17* (Fig. 2). Previous studies had shown that RAB-17 (responsive to ABA) transcript and protein appears during the maturation phase of embryogenesis and have greatest steady-state abundance in the dry seed (Vilardell et al., 1990). As expression is progressively down-regulated during imbibition the transcript provides a useful internal control for RNA extraction from embryos during the early hours of imbibition. In general, the steady-state abundance of transcripts for nuclear- and mitochondrially encoded proteins was low until radicle emergence and then increased significantly between 24 and 48 h (Fig. 2). In general, these data strongly support the hypothesis that the de novo synthesis of mitochondrial proteins during imbibition is limited by transcript abundance until after 24 h of imbibition.

### There Are Two Subpopulations of Mitochondria in Maize Embryos

To investigate possible changes in mitochondrial populations during germination, crude homogenates

were prepared from dry embryos or embryos dissected from seeds at intervals up to 48 h of imbibition. The homogenates were centrifuged at low speed (5,000*g* × 5 min) and the supernatant was fractionated on Suc density gradients. Total protein from each fraction was separated by SDS-PAGE and analyzed by immunoblotting using antibodies raised against a range of mitochondrial proteins (Fig. 3).
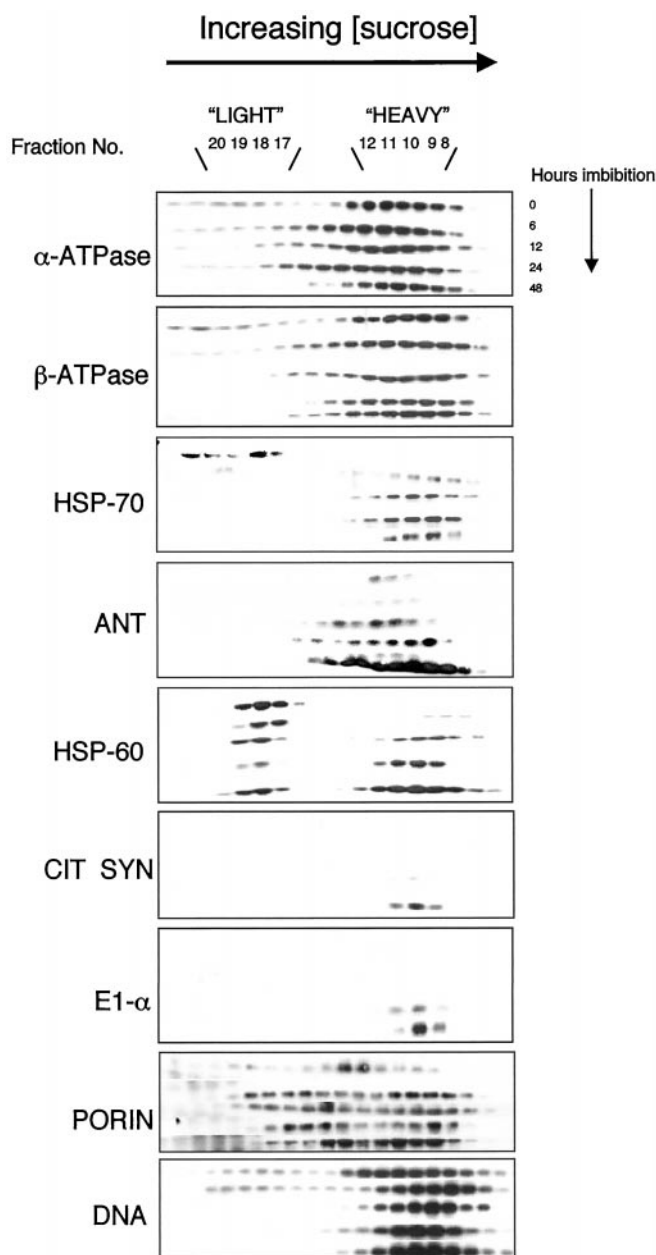


**Figure 3.** Distribution of mitochondrial proteins by immunoblotting with specific antibodies and mitochondrial DNA by Southern blotting with a mitochondria-specific gene probe (*cox I*). Subcellular homogenates were prepared from maize embryos after imbibition for 0 to 48 h and fractionated on Suc density gradients. The light and heavy subpopulations consist of fractions 17 to 20 (22%–28% [w/w] Suc) and 8 to 12 (37%–42% [w/w] Suc), respectively.

The profiles obtained suggested that two distinct subpopulations of mitochondria could be resolved over the time course under study. One subpopulation was found at an equilibrium density normally associated with mitochondria (37% to 42% [w/w] Suc), whereas the second was found at a lower density (22% to 28% [w/w] Suc). These populations can be conveniently referred to as heavy and light mitochondria, respectively. To confirm the identity of these fractions as mitochondria, cytochrome $c$ oxidase activity was measured and at all stages examined, both fractions contained substantial cytochrome $c$ oxidase activity (Table I). We also examined which of the Suc gradient fractions contained mtDNA by Southern-blot hybridization using $coxI$ as a probe (Fig. 3). This procedure showed that the distribution of mtDNA was coincident with that of mitochondrial proteins in the light and heavy fractions from the dry seed (Fig. 3). There was no detectable hybridization to nDNA on duplicate Southern blots using a probe derived from a nuclear-encoded mitochondrial gene ($mthsp60$, data not shown).

The heavy and light mitochondrial fractions were examined at the ultrastructural level by transmission electron microscopy (Fig. 4). The heavy fraction from 24 and 48 h-imbibed embryos contained double membrane-bound organelles with the expected appearance of mitochondria—there are numerous cristae, and the mitochondrial matrix is electron dense suggestive of a high protein concentration (Fig. 4, D and F). Mitochondria with this morphology are classified as type 1. In contrast the heavy mitochondrial fraction from dry embryos consisted of large double membrane-bound organelles with little internal membrane structure and no densely staining matrix (Fig. 4B). The light mitochondrial fraction at all three stages of germination contained double membrane-bound organelles, which have no or little internal membrane structure or densely staining matrix. Mitochondria composed of a double-unit membrane, but few cristae and a lightly staining matrix such as those isolated from dry embryos and light mitochondrial fractions at the other stages of germination are classified as type 2.

Dai and coworkers recently used differential centrifugation as a first step to separate mitochondria from mung bean seedlings into two subpopulations (Dai et al., 1998). One subpopulation was recovered by centrifugation at $14,000g_{max}$, whereas the other was produced by recentrifugation of the $14,000g_{max}$ supernatant at $26,000g_{max}$. The pellets obtained were then subjected to Suc density gradient fractionation on 20% to 50% (w/w) continuous or discontinuous Suc gradients, respectively. This procedure enabled these workers to isolate two fractions from each gradient, one banding at approximately 32% to 33% (w/w) and the other at 38% (w/w) Suc, which both contained cytochrome $c$ oxidase activity, mitochondrial protein, and mtDNA. Although there are differences between our subcellular fractionation procedure and that used by Dai and coworkers, it is clear that none of the subpopulations identified in mung bean seedlings is equivalent to the light (22%–28% [w/w] Suc) subpopulation we isolated from maize embryos following Suc density gradient centrifugation of the $5,000g \times 5$ min supernatant.

To investigate whether the mitochondrial subpopulations visualized by electron microscopy of the gradient fractions could be identified in situ, dry and imbibed maize embryos were sectioned and examined by transmission electron microscopy (Fig. 5). The structure of mitochondria within dry embryo tissue resembles the type 2 mitochondria: the outer and inner membrane is visible, but there is little internal structure (Fig. 5A). After 48 h imbibition two distinct populations of mitochondria could be identified in the embryo tissue (Fig. 5B). One population consists of apparently mature mitochondria containing many cristae and an electron-dense matrix (type 1) whereas the other resembles the type 2 organelles present in dry tissue: double membrane-bound organelles with poorly developed cristae and matrix. Electron microscopy of scutellum tissue revealed that in the dry seed, mitochondria were type 2 (Fig. 5C), whereas only type 1 mitochondria were found within the scutellum excised from germinated seed (i.e. after a 48-h imbibition; Fig. 5D).

**Table I.** *Enzyme activities in mitochondrial fractions from germinating maize embryos*

Values represent the means of three separate experiments ± SE.

| Enzyme | Activity in Fraction | | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | Light mitochondria | | | Heavy mitochondria | | |
| | 0 h of imbibition | 6 h of imbibition | 48 h of imbibition | 0 h of imbibition | 6 h of imbibition | 48 h of imbibition |
| | *nmol min$^{-1}$ mg$^{-1}$ protein* | | | | | |
| Cytochrome $c$ oxidase | 72 ± 13 | 144 ± 6 | 87 ± 9 | 200 ± 7 | 188 ± 5 | 186 ± 8 |
| Citrate synthase | 4 ± 1 | 4 ± 1 | 3 ± 1 | 15 ± 4 | 56 ± 6 | 139 ± 13 |
| NAD malic enzyme | 2 ± 2 | 6 ± 1 | 2 ± 0 | 5 ± 3 | 12 ± 1 | 28 ± 7 |
| Pyruvate dehydrogenase complex | ND[a] | ND | 1 ± 1 | 2 ± 1 | 12 ± 1 | 19 ± 4 |
| 2-Oxoglutarate dehydrogenase complex | ND | ND | 1 ± 1 | 2 ± 1 | 8 ± 1 | 14 ± 3 |

[a] ND, Not detected.

**Figure 4.** Transmission electron micrographs of the light (A, C, and E) or heavy (B, D, and F) mitochondrial subpopulations obtained after subcellular fractionation of embryos excised from dry maize seed (A and B) or seed imbibed for 24 h (C and D) or 48 h (E and F). Plain arrows indicate type 2 mitochondria; arrows ending with a circle indicate their poorly developed cristae. Scale bar = 500 nm.
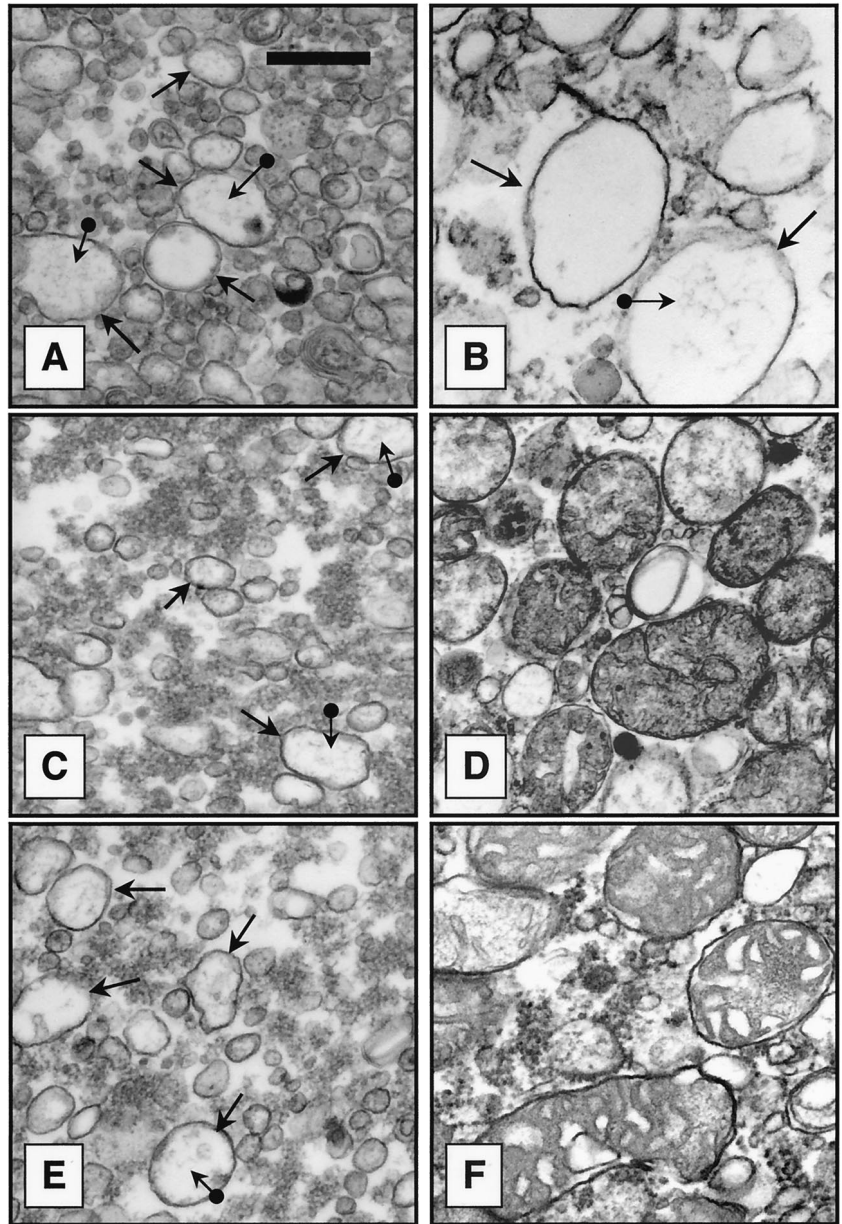


## Heavy and Light Mitochondrial Subpopulations Have Different Protein Compositions

To investigate the protein composition of the mitochondrial fractions mitochondrial proteins were fractionated by SDS-PAGE and probed with a range of antibodies against selected mitochondrial proteins (Fig. 3). Both fractions contained the outer mitochondrial-membrane protein porin at all stages examined (Fig. 3). The light fraction (type 2) from dry embryos contains subunits of the $F_1$-$F_0$ ATPase and the chaperones, mtHSP70 and mtHSP60. However, the corresponding fraction isolated from embryos at later stages of imbibition contains only trace amounts of $F_1$-$F_0$ ATPase subunits and no detectable mtHSP70. The heavy mitochondrial frac-

tion (type 2) from dry seeds contains porin, mtHSP60, the adenine nucleotide translocator (ANT), and subunits of the $F_1$-$F_0$ ATPase. Following imbibition, mtHSP70, citrate synthase, and the PDC E1$\alpha$-subunit are also detectable in the heavy fraction. A summary of the changes in specific mitochondrial proteins in the light and heavy fractions from dry and 48-h imbibed embryos is shown in Figure 6.

To further characterize mitochondrial metabolism during the first 48 h of imbibition the activities of cytochrome $c$ oxidase, citrate synthase, NAD malic enzyme, PDC, and 2-oxoglutarate dehydrogenase complex were measured in the heavy and light mitochondrial fractions (Table I). As described earlier,
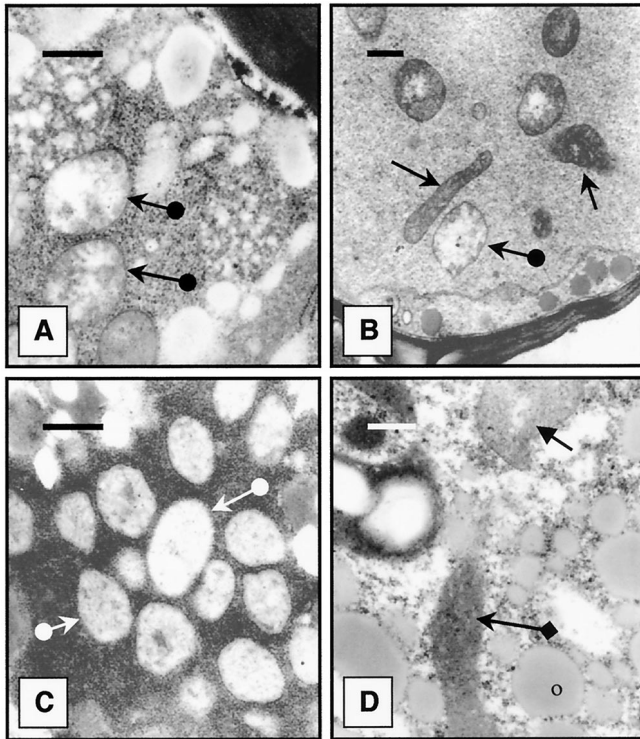
**Figure 5.** Transmission electron micrographs of ultra-thin sections of maize embryo (A and B) or scutellum (C and D) tissue isolated from dry (A and C) or 48-h imbibed and germinated (B and D) maize seeds. Type 1 mitochondria are indicated by plain arrows; type 2 by arrows ending with a circle. The arrow ending with a diamond in D indicates a glyoxysome. O, Oil body. Scale bar = 500 nm.

cytochrome $c$ oxidase activity was detected in both fractions at all stages. In the light fraction the cytochrome $c$ oxidase activity was higher after 6 h of imbibition than in the dry embryo or after 48 h, but remained essentially constant in the heavy fraction over the same period. The four tricarboxylic acid (TCA) cycle enzymes were present at low activities or undetectable in the light mitochondrial fraction at all stages (Table I). The results for citrate synthase and PDC are consistent with the immunological evidence (Fig. 3) as neither citrate synthase nor the E1$\alpha$-subunit were detectable in the light fraction from dry embryos or during imbibition. In contrast mitochondria in the heavy fraction contained low activities of TCA cycle enzymes in the dry seed, but all four enzymes increased in activity during imbibition (Table I).

**A Functional Electron Transport Chain Is Present in Both Mitochondrial Subpopulations, But TCA Cycle Enzyme Activities Are Only Measurable in the Heavy Mitochondrial Fraction**

Taken together, the data presented in Figure 3 and Table I suggest that the capacity for electron transport exists in both mitochondrial populations throughout imbibition, but that TCA cycle activity is only measurable in the heavy mitochondrial fraction after rehydra-

tion of the dry tissue. To investigate the extent to which mitochondrial electron transport operates in dry and imbibing embryos the rates of external NADH and succinate-dependent $O_2$ consumption by the mitochondrial fractions were measured (Table II). Rates of NADH-dependent respiration in both mitochondrial fractions were high and did not change during imbibition provided that additional cytochrome $c$ was provided. Outer membrane intactness was only 70% for mitochondria from dry embryos, and the degree to which cytochrome $c$ stimulated NADH-dependent $O_2$ consumption was correlated with the outer membrane integrity (Table II). This suggests that cytochrome $c$ was lost during extraction of mitochondria from dry seed. NADH-dependent $O_2$ consumption was insensitive to 50 $\mu$M rotenone and completely inhibited by 100 $\mu$M dicumerol (3, 3'-methylene-bis(4-hydroxycoumarin) (data not shown), confirming that NADH was oxidized via the externally facing NADH dehydrogenase rather than complex I. NADH-dependent $O_2$ consumption was completely inhibited by KCN and antimycin A (data not shown), suggesting that the alternative oxidase did not contribute to the measurable respiratory activity.

In contrast to the utilization of NADH, succinate-dependent $O_2$ consumption was low in both mitochondrial subpopulations from dry embryos (Table II) and increased significantly in the heavy mitochondrial fraction during imbibition, but not in the light mitochondrial fraction (Table II). The rate of succinate-dependent respiration was not stimulated by addition of cytochrome $c$ and was completely inhibited by KCN and antimycin A (data not shown). The low rates of succinate-dependent consumption relative to those of NADH-dependent $O_2$ consump-
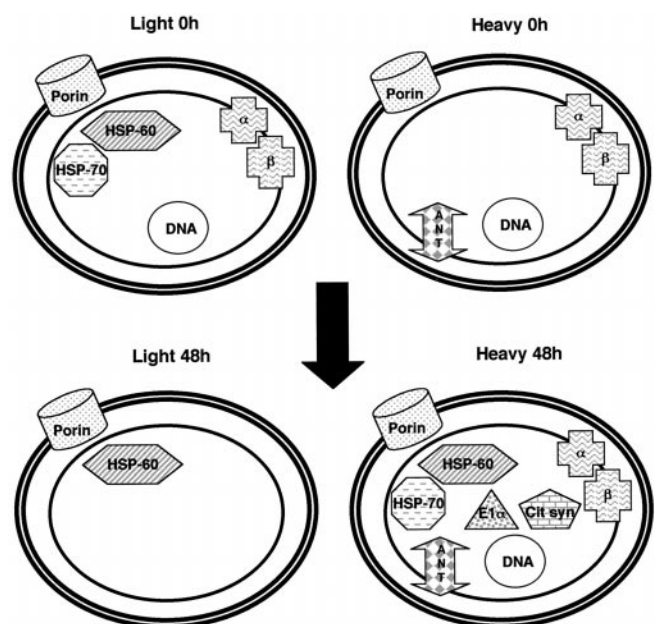


**Figure 6.** Schematic diagram summarizing the changes in distribution of mitochondrial proteins and DNA (data shown in Fig. 3).

**Table II.** *Substrate oxidation by mitochondrial fractions from germinating maize embryos*

Rates of $O_2$ consumption were measured in the presence of NADH (1 mM) or succinate (M) + ATP (0.1 mM) following the addition of a saturating amount of ADP. Where indicated, cytochrome *c* (50 μM) was added before addition of ADP. Outer membrane integrity was measured as the latency of cytochome *c* oxidase activity using Triton X-100 (0.05% [w/v]) to obtain maximal activity. Values represent the means of three separate experiments ± SE.

| | Rate of Oxygen Uptake in fraction | | | | | |
|---|---|---|---|---|---|---|
| Substrate | Light mitochondria | | | Heavy mitochondria | | |
| | 0 h of imbibition | 6 h of imbibition | 48 h of imbibition | 0 h of imbibition | 6 h of imbibition | 48 h of imbibition |
| | *nmol min$^{-1}$ mg$^{-1}$ protein* | | | | | |
| NADH | 9 ± 2 | 25 ± 3 | 25 ± 1 | 24 ± 3 | 54 ± 6 | 72 ± 5 |
| NADH + cytochrome *c* | 39 ± 4 | 45 ± 8 | 38 ± 2 | 123 ± 9 | 131 ± 16 | 78 ± 4 |
| Succinate | 3 ± 1 | 2 ± 1 | 5 ± 1 | 7 ± 3 | 19 ± 1 | 46 ± 3 |
| Outer membrane intactness (%) | 69 ± 5 | 62 ± 5 | 81 ± 3 | 66 ± 6 | 84 ± 3 | 93 ± 1 |

tion suggests that succinate oxidation is restricted by the low capacity of the dicarboxylate transporter and/or succinate dehydrogenase (complex II). A low rate of oxidation of succinate in the dry embryo relative to NADH contrasts with the result of Attucci et al. (1991) obtained using mitochondria isolated from dry sunflower seeds.

The data of Tables I and II suggests that there is a functional electron transport chain present in both mitochondrial subpopulations in the dry embryos and during the first 48 h of imbibition. Measurement of the maximal catalytic activities of the pyruvate dehydrogenase complex (PDC), the 2-oxoglutarate dehydrogenase complex, citrate synthase, and NAD-malic enzyme in mitochondrial fractions from maize embryos suggest that TCA cycle enzyme activities appear to be confined to the heavy mitochondrial subpopulation and increase during imbibition.

However, an increase in the TCA cycle enzyme activities does not necessarily mean that there will be an increased flux through the TCA cycle in vivo. We therefore investigated the in vivo TCA cycle flux by supplying $^{14}C$ Glc, labeled specifically at carbon-6, to excised maize embryos and determining the extent of labeling of released $CO_2$ (Fig. 7). The majority of the C-6 carbon of Glc will be released as $CO_2$ during oxidation of pyruvate by the TCA cycle, and the rate of this $CO_2$ release gives an estimate of the TCA cycle flux. However, the C-6 carbon can also be released as $CO_2$ by other processes, notably during pentose synthesis. Pentoses are primarily produced as components of cell walls, and we argue that since cell wall biosynthesis is likely to be low during the initial 48 h of embryo imbibition (during which cell expansion, rather than cell division occurs), the release of $CO_2$ during pentose synthesis will be minimal and will not interfere with our estimations of TCA cycle flux. It is not possible to estimate an absolute flux in this manner, since the specific activity of Glc-6-P that enters glycolysis is not known. This specific activity will be primarily affected by the extent of recycling of Glc-6-P through the oxidative pentose phosphate pathway. Estimations of the oxidative pentose phosphate pathway flux as the ratio of $^{14}CO_2$ release from [1-$^{14}C$]Glc and [6-$^{14}C$]Glc showed that this flux remained constant throughout the first 48 h of imbibition (data not shown). Thus, if no other factor affects Glc-6-P specific activity, then we can make accurate estimations of the TCA cycle flux during embryo imbibition. We found using this approach that TCA cycle activity was low in embryos from dry seeds, but increased rapidly during imbibition, reaching a plateau by 48 h (Fig. 7). Thus an increased capacity of the TCA cycle as evidenced by an increase in the

**Figure 7.** The rate of $^{14}CO_2$ release from [6-$^{14}C$]Glc supplied to maize embryos during imbibition. The dashed line indicates time of radicle emergence. The SEs of the means were smaller than the symbols (*n* = 3).
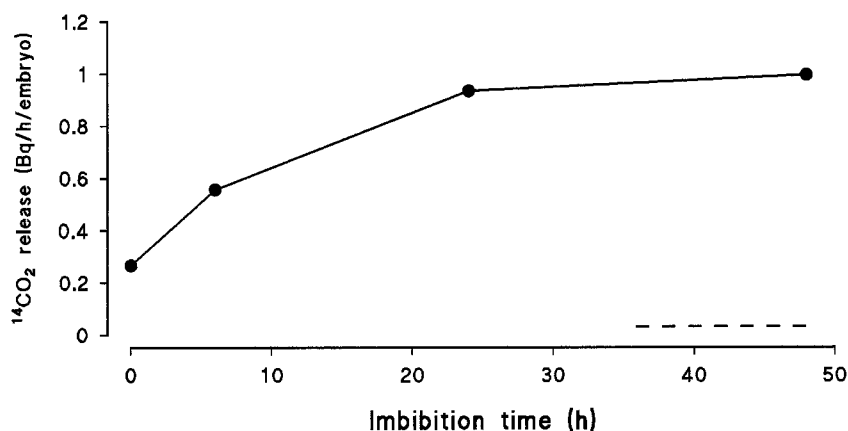
activity of TCA cycle enzymes is reflected by an increase in the in vivo flux.

Our data suggest that the accumulation of TCA cycle enzymes occurs after the initial phase of imbibition and requires the de novo synthesis of TCA cycle enzymes (Fig. 3; Table I), which in turn will require ATP and a mitochondrial membrane potential (for protein import into the mitochondria) both of which could be generated by the oxidation of external NADH.

## Mitochondrial Biogenesis during Germination of Maize Embryos

The data presented demonstrate that two subpopulations of mitochondria, identifiable by differences in buoyant density, enzyme activity, and protein complement are present in the germinating maize embryo. During imbibition there are significant changes in enzyme activities and protein complement between the two subpopulations, which can be interpreted to describe the process of mitochondrial development/biogenesis during germination. During imbibition the amount of specific mitochondrial proteins and DNA in the light mitochondrial fraction diminishes progressively. Mitochondria in the light fraction from germinated embryos (48 h of imbibition) only contain small amounts of two of the proteins measured and mtDNA is undetectable. The absence of detectable mtDNA together with the absence of mtHSP70, which is necessary for protein import (Scherer et al., 1990), in the light population after germination suggest that this subpopulation of mitochondria are the remains of mitochondria that were active in the developing embryo during the maturation phase of seed development prior to desiccation.

It is hypothesized that during desiccation mitochondria active during the maturation phase are partially degraded, loosing the bulk of the contents of the matrix, and as a result, a functional TCA cycle. We suggest that upon rehydration of the embryo this programmed degradation process continues. The light mitochondria in the dry embryo retain the components necessary for protein import: the chaperones mtHSP70 and mtHSP60 for import and correct folding of imported polypeptides, and the ability to generate a membrane potential (NADH oxidation) enabling import of nuclear-encoded degradative enzymes.

The process of mitochondrial biogenesis is clearly delineated by analysis of the development of more typical mitochondria present in the heavy fraction from the Suc density gradient. The heavy mitochondria in the dry embryo are deficient in certain enzyme activities and matrix components (notably the TCA cycle and the chaperones mtHSP60 and mtHSP70). However, within 6 h from the start of imbibition heavy mitochondria contain the components necessary for mitochondrial biogenesis: an active electron transport chain and ATP synthesis machinery for the production of ATP and a mitochondrial membrane potential, and mtHSP70 and mtHSP60. After 24 h of imbibition and prior to the culmination of germination as evidenced by protrusion of the radicle, the heavy mitochondria import further electron transport components and matrix enzymes, leading to the production of mature mitochondria capable of full TCA cycle and electron transport chain function. We suggest that the heavy mitochondria in the dry embryo and early stages of imbibition are pro-mitochondria. We hypothesize that these pro-mitochondria are generated prior to desiccation possibly as a division product of mature mitochondria that then become programmed for disassembly and degradation (see above). It is possible that during germination, commencing with the onset of imbibition, these pro-mitochondria develop rapidly as a result of import of precursor polypeptides coupled to synthesis of mitochondrially encoded polypeptides.

## External NADH Oxidation Plays a Fundamental Role in Mitochondrial Assembly

A key feature of the proposed pro-mitochondria is the presence of electron transport driven by oxidation of extra-mitochondrial NADH. Assigning a biochemical function to the externally facing NADH dehydrogenase of plant mitochondria has been elusive (Menz and Day, 1996). Its high $K_m$ for NADH suggests that it will only operate at a significant rate if cytosolic NADH concentrations are high (Kromer and Heldt, 1991; Hanning and Heldt, 1993), but the ubiquitous presence of the enzyme in plant mitochondria suggests an important role. We suggest that one role fulfilled by this enzyme is the provision of ATP in cells that have yet to develop fully functional, mature mitochondria, and in particular, lack the capacity to generate NADH within the mitochondria through the TCA cycle. In a cell with rapid glycolysis, but no TCA cycle, NADH generated from glycolysis will accumulate. In a similar manner, if there is rapid extra-mitochondrial $\beta$-oxidation of fatty acids (Beevers, 1978), there is also likely to be a highly reduced nucleotide pool. Engagement of externally facing NADH dehydrogenase would allow efficient generation of ATP from this NADH and, perhaps more importantly, generates a mitochondrial membrane potential, allowing import of new proteins into the mitochondria.

## CONCLUSIONS

We have provided evidence that there are two subpopulations of mitochondria in maize embryos, and we have characterized these populations in terms of their ultrastructure, protein composition, and metabolic function. Our observations lead us to

hypothesize that mitochondria are disassembled in the embryo during desiccation, and that pro-mitochondria formed at this time undergo re-differentiation and biogenesis during imbibition. In the reassembly phase there is de novo synthesis of some, but not all, mitochondrial proteins encoded by the nuclear and mitochondrial genomes. ATP synthesis during the early stages of imbibition and biogenesis of mitochondria appears to be dependent upon the supply of cytosolically synthesized NADH that is oxidized by the mitochondrial electron transport chain via the externally-facing NADH dehydrogenase.

## MATERIALS AND METHODS

### Plant Material

Seeds of the maize (*Zea mays*) hybrid 3394 were a kind gift of Pioneer Hybrid International (Des Moines, IA). Seeds were surface sterilized in a 10% (v/v) solution of sodium hypochlorite for 20 min followed by rinsing with sterile MilliQ (Millipore, Bedford, MA) water. Seeds were air dried and stored at room temperature. Seeds were imbibed in the dark at 25°C between five sheets of sterile filter paper (No. 1, Whatman, Clifton, NJ) in a sterile plastic Petri dish to which was added 15 mL of sterile MilliQ water. Embryos, consisting of the embryo axis and scutellum were excised from dry and imbibed seed by the use of a small electric saw and scalpel.

### Subcellular Fractionation

Isolated embryos (10 embryos per 17 mL Suc gradient, 20 embryos per 38 mL gradient) were ground at 4°C in five volumes of grind buffer (0.3 M Suc, 50 mM MOPS [3-(N-morpholino)-propanesulfonic acid], pH 7.8, 5 mM MgCl$_2$, 2 mM EDTA, 1% [w/v] bovine serum albumin [BSA], 0.6% [w/v] polyvinylpyrrolidone-40, and 20 mM Cys) using a pestle and mortar. The homogenate was filtered through one layer of Miracloth (Calbiochem, La Jolla, CA) and centrifuged at 5,000$g_{av}$ for 5 min. The supernatant was layered onto a 17- or 38-mL continuous 30% to 50% (w/w) Suc gradient and centrifuged at 80,000$g_{av}$ for 16 h. This method produces a linear gradient from 14% to 50% (w/w) Suc. Gradients were fractionated into 0.5 mL (17-mL gradient) or 1.5 mL (38-mL gradient) fractions. Fractions were used immediately or frozen in liquid nitrogen and stored at −80°C. Purification of mitochondria for measurement in the oxygen electrode followed a similar procedure with the exception that mitochondria were purified on a discontinuous Suc gradient composed of 20%/28%/37%/42% (w/w) Suc and centrifuged for 2 h. The 20%/28% and 37%/42% interfaces were collected, diluted slowly with four volumes of wash buffer (0.3 M Suc, 10 mM TES [N-Tris(hydroxymethyl)-2-aminoethanesulfonic acid]-KOH, pH 7.2, and 0.1% w/v] BSA), and collected by centrifugation at 20,000$g_{av}$ for 20 min. The pellets were resuspended in 200 μL of wash buffer and assayed immediately.

### Enzyme Assays

O$_2$ consumption was measured in an O$_2$ electrode (Hansatech, King's Lynn, UK) in 1 mL of reaction medium containing 0.3 M Suc, 10 mM TES-KOH, pH 7.5, 5 mM KH$_2$PO$_4$, 10 mM NaCl, 2 mM MgSO$_4$, and 0.1% (w/v) BSA. Succinate (5 mM), ATP (0.1 mM), NADH (1 mM), and ADP (0.1–1 mM) were added as required. PDC, 2-oxoglutarate dehydrogenase complex, NAD-malic enzyme, citrate synthase, and cytochrome *c* oxidase activities were measured according to standard protocols (Millar et al., 1999). Protein concentrations were determined by Bradford bioassay (Bio-Rad, Hercules, CA) using BSA as a standard.

### SDS-PAGE and Immunoblotting

SDS-PAGE under denaturing conditions was performed according to Laemmli (1970). Gels were electroblotted onto reinforced nitrocellulose using Dunn buffer (Dunn, 1986). Immunodetections were performed using horseradish peroxidase-linked secondary antibodies (Amersham, Buckinghamshire, UK) and an chemiluminescence kit (NEN-Enhance, NEN, Boston). Blots were probed with monoclonal antibodies to the maize α- and β-subunits of the mitochondrial F$_1$F$_0$-ATPase (Luethy et al., 1993), maize outer mitochondrial membrane porin, and E1α-subunit of the PDC (Luethy et al., 1995) and maize mtHSP70 (Lund et al., 1998), and polyclonal antisera to *Tetrahymena thermophila* mtHSP60 (McMullin and Hallberg, 1988), maize adenine nucleotide translocator (A.D. Liddell and C.J. Leaver, unpublished data), and yeast citrate synthase (Suissa et al., 1984). All antibodies were monospecific.

### DNA Isolation and Southern-Blot Analysis

DNA was isolated from 10 excised maize embryos using a cetyl-trimethyl-ammonium bromide method and fractionated by electrophoresis in 0.9% (w/v) agarose gels. Quantification based on ethidium bromide fluorescence was performed using a Fluor-S MultiImager (Bio-Rad) using *Hin*dIII cut lambda DNA as a standard. For analysis of DNA on Suc gradients aliquots of the Suc gradients fractions were prepared for DNA gel electrophoresis according to Meijer et al. (1996). After electrophoresis through 0.9% (w/v) agarose gels the DNA was transferred to nitrocellulose membrane according to standard procedures. Hybridization was carried out in a formamide containing buffer and followed by washing at high stringency (0.2× SSC, 0.1% [w/v] SDS, 42°C). DNA probes for nuclear-encoded *mthsp60* and mitochondria-encoded *coxI* were labeled by random priming.

### RNA Isolation and Gel-Blot Analysis

Total RNA was extracted from 10 excised maize embryos using a urea-SDS method. Ten micrograms of RNA was fractionated in a denaturing 1% (w/v) agarose/formaldehyde gel, transferred to nitrocellulose, and hybridizations and washes were performed as above. Blots were hybridized with probes and prepared by random priming to the

following genes: *isdh, ald, mthsp60, ant, atpa, atp6, atp9, coxI, coxII, coxIII,* and *rab-17.* Probes for *ald* and *isdh* were prepared from rice expressed sequence tags (GenBank accession nos. D38826 and D21069, respectively); all others were from maize genomic or cDNA clones.

## Transmission Electron Microscopy

Subcellular fractionation was performed as above. Fractions corresponding to light (20%–28% [w/w] Suc) or heavy (37%–42% [w/w] Suc) mitochondrial subpopulations were combined, diluted, and centrifuged as detailed above. The pellets were resuspended in 0.3 M Suc, 100 mM sodium phosphate, pH 7.2, and 2.5% (v/v) glutaraldehyde, and incubated for 1 h on ice. The resuspended material was pelleted at $20,000g_{av} \times 20$ min and washed three times by resuspension in buffer minus glutaraldehyde followed by recentrifugation. The material was post-fixed in 1% (v/v) osmium tetroxide for 2 h in the same buffer after which it was rinsed in buffer minus Suc and resuspended in 1% (w/w) tannic acid. The material was pelleted as before, rinsed, resuspended in 5% (w/v) low melting point agarose, dehydrated, and embedded in Epon resin. Ultra-thin sections were cut and stained with uranyl acetate followed by lead citrate prior to observation. Embryonic axes and scutellum tissue material was fixed, embedded, and stained in the same way with the exception of treatment with tannic acid.

## Incubation of Embryos with [6-14C]Glc

Embryos were dissected from dry seeds or from seeds imbibed for between 6 and 48 h. Three embryos from each time point were incubated in 3 mL of 200 mM [6-$^{14}$C Glc] (0.16 MBq/mmol), 20 mM MES (pH 6.5) in a sealed conical flask for 2 h. Released $^{14}CO_2$ was trapped in an Eppendorf tube containing 0.5 mL of 20% (w/v) KOH, which was suspended inside the flask.

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