Plant Physiol. (1991) 95, 390–398
0032-0889/91/95/0390/09/$01.00/0

Received for publication August 13, 1990
Accepted October 4, 1990

Oxidative Phosphorylation by Mitochondria Extracted from Dry Sunflower Seeds

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ABSTRACT

The role of mitochondria in the phosphorylation of ADP to ATP in the early steps of seed germination has been studied. Mitochondria were extracted from dry sunflower (*Helianthus annuus*) seeds. Adenylate kinase-dependent ATP synthesis was inhibited by p^1,p^5-di(adenosine-5′)pentaphosphate. Synthesis of ATP was observed with the different substrates: citrate, α-ketoglutarate, succinate, malate, pyruvate or NADH. This synthesis was activated by cytochrome *c*, and inhibited by cyanide, oligomycin, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone, and carboxyatractyloside. The ATP/O values with succinate were 0.85 and 1.2 in the absence or presence, respectively, of cytochrome *c*. Electron micrographs showed that mitochondria of dry tissues have different structures when observed *in situ* or *in vitro* after aqueous extraction, suggesting that profound changes occurred after the contact with the aqueous medium. These results confirm previous data obtained *in vivo* showing that mitochondria present in dry seeds are able to synthesize ATP as soon as the seeds are rehydrated.

The mechanisms by which ATP is produced upon imbibition of seeds and during the early phases of their germination has been a matter of debate for about three decades (for reviews see refs. 1, 17, 19). Although seed respiration has been established for a long time, its link with the phosphorylation of ADP to ATP is in doubt mainly for two reasons. Many authors observed no inhibition of germination by cyanide (19). Also, in spite of many attempts in various laboratories, phosphorylating mitochondria of germinating seeds have been obtained in only two cases, from peanut cotyledons imbibed for 40 min (27), and from cucumber cotyledons imbibed for 6 h (14). Moreover, the leakage of solutes during seed imbibition suggested some degree of membrane disorganization either in the dry state or following rehydration (5, 23, 26). It was believed, in particular, that mitochondria were damaged and had to be repaired during the germination period to become functional at the time of radicle emergence (13). Because fermentation has a low activity in most germinating seeds under aerated conditions (20), new pathways for ATP synthesis have been sought (17).

However, *in vivo* studies on lettuce seeds showed that the ATP synthesis which occurs upon imbibition of the seeds is O_2-dependent and KCN-sensitive, suggesting that the mechanism involved is oxidative phosphorylation (8). The same results were obtained in similar experiments with mung bean and cucumber seeds (14). It was concluded from all these results that, as soon as water enters into the cells, preexisting mitochondria synthesize ATP by oxidative phosphorylation. More recently, active ATPase and Cyt oxidase could be extracted from quiescent maize embryos (6). Thus protective mechanisms must occur in the dry seed which allow the membranes to withstand dryness or rehydration without disorganization (2, 9, 25). In the present paper, we show that mitochondria extracted from dry sunflower seeds (*Helianthus annuus*) are able to produce ATP by the oxidation of various organic acids or NADH, and this production is abolished by inhibitors of the partial reactions involved in the process of oxidative phosphorylation. The morphology of these mitochondria is also described and discussed in relation to their function.

MATERIALS AND METHODS

Plant Material

Sunflower seeds (*Helianthus annuus* cv Rodeo) obtained from CETIOM (France) were stored at 4°C in closed vessels containing silica gel. The seeds were dehusked by hand 1 or 2 d before utilization and stored at 4°C in flasks containing silica gel. Their water content was 4.5% (w/w) as determined after drying 24 h at 95°C.

Chemicals

All organic compounds were from Sigma and Merck, except Percoll (Pharmacia), Triton X-100 (Packard), β-mercaptoethanol (Serva), and glycerol (Prolabo).

Isolation of mitochondria

All procedures were carried out between 0 and 4°C. Twenty grams of dehusked seeds were ground by hand with a pestle and mortar in 100 mL of grinding medium containing 0.7 M sorbitol, 60 mM $Na_4P_2O_7$ (pH 7.5), 1 mM EDTA, 3% (w/v) defatted BSA, and 10 mM cysteine. The brei was squeezed through a 100 μm mesh nylon cloth. The crude mitochondrial pellet was prepared according to the method of Douce *et al.* (3) modified as follows. The filtrate was centrifuged at 120*g* for 15 min. The supernatant was recovered and centrifuged at 12,000*g* for 20 min. The resultant pellet was resuspended in a small volume of washing medium containing 0.7 M

sorbitol, 1 mM EDTA, 10 mM K_2HPO_4 (pH 7.2), and 3% (w/v) defatted BSA; the suspension was homogenized with a Potter-Elvehjem; the volume was adjusted to 35 mL; and the homogenate was centrifuged at 12,000*g* for 20 min. The pellet of washed mitochondria was suspended in approximately 1 mL of washing medium and purified by the procedure of Neuburger *et al.* (15) modified as follows. The suspension of mitochondria was layered on 35 mL of Percoll medium: 10% Percoll, 0.7 M sorbitol, 10 mM K_2HPO_4 (pH 7.2), 3% (w/v) defatted BSA, and centrifuged at 48,000*g* for 40 min. Two layers of mitochondria differing in density were obtained, as previously found in peanut seeds (27). Since electron microscopy showed that the heavy band was contaminated by peroxisomes and plastids (results not shown), the experiments were performed with the upper layer corresponding to the light mitochondria. The layer was recovered and diluted with 10 volumes of the washing medium described above and the organelles were sedimented by centrifugation at 12,000*g* for 20 min. The volume of the recovered pellet was 300 to 600 μL.

Washing the pellet of purified mitochondria in a medium without BSA caused a loss of 90% of the initial activities. Therefore BSA could not be omitted, with the result that the low amount of mitochondrial proteins could not be determined. Therefore, all activities are reported as a function of the weight of dehusked seeds.

Crude Extract Preparation

One gram of dry seeds was ground with an Ultra Turrax in 3 mL of a medium containing 100 mM K_2HPO_4 (pH 7.5), 14 mM β-mercaptoethanol, 2% (w/v) insoluble PVP, and 1% (v/v) Triton X-100. The homogenate was filtered through a 10 μm mesh nylon cloth and centrifuged at 20,000*g* for 15 min. The supernatant was recovered and desalted on an Econopac 10 DG column (Bio-Rad) (10 mL bed volume), equilibrated with 10 mM K_2HPO_4 (pH 7.5), 10% (w/v) glycerol, and 1 mM $MgCl_2$. The fraction containing the proteins was clarified by centrifugation at 20,000*g* for 15 min. The supernatant was recovered and stored at 4°C until use.

Enzyme Activities

SDH[1] (EC 1.3.5.1) activity was measured according to Singer *et al.* (22). Mitochondrial proteins were added to 1 mL of the standard reaction medium containing 40 mM Hepes (pH 7.5), 1 mM KCN, 20 mM succinate, 1% (v/v) Triton X-100. The reaction was started by the addition of 10 μL of a solution of dichlorophenolindophenol and phenazine methosulfate (0.5% [w/v] each). The reaction was followed at 25°C in a double beam spectrophotometer at 600 nm. Fumarase (EC 4.2.1.2) was measured as described by Hill and Bradshaw (7). Fumarate appearance was followed at 250 nm in 1 mL of 50 mM tricine buffer (pH 7.5) at 25°C. The reaction was started by the addition of 50 mM malate.

[1] Abbreviations: SDH, succinate dehydrogenase (EC 1.3.5.1); Ap5A, p^1,p^5-di(adenosine-5′)pentaphosphate; CAT, carboxyatractyloside; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone; SHAM, salicylhydroxamic acid.

Respiratory Measurements

Oxygen uptake by mitochondria was measured at 25°C using a Clark electrode system purchased from Hansatech Ltd. The reaction medium contained 10 mM K_2HPO_4 (pH 7.2), 0.1% (w/v) defatted BSA, 5 mM $MgCl_2$, 10 mM KCl, 0.7 M sorbitol, and 20 to 60 μL of the mitochondrial fraction corresponding to about 1 g of seeds, in a total volume of 1 mL. The O_2 concentration in air saturated medium was taken as 240 μM. The different substrates and cofactors used are described in the legends of the figures for each experiment. Respiration rate was corrected for the oxygen consumption by the electrode.

The oxygen uptake by seeds was measured at 25°C by the standard manometric method of Warburg using four dehusked seeds (about 0.2–0.3 g) with 1 mL of water in 15 mL vial.

Oxidative Phosphorylation Assay

The mitochondria from about 1 g of seeds were incubated in 400 μL of the medium used in the oxygen uptake assay. Reaction was started by the addition of either 50 μM ADP, after 10 mM substrate, or substrate after ADP. At various times, 20 μL of the incubation medium was taken and diluted into 2 mL of ice-cold medium containing 0.33 mM Tris (pH 7.4), 1.26 mM K_2SO_4, 0.33 mM $MgSO_4$, 0.045 mM EDTA. This medium is the same as that used for the enzymatic bioluminescence assay. Dilution stopped the reaction of ATP synthesis: we verified that the level of ATP in this medium remained constant for 1 h.

ATP Measurement

ATP was assayed by bioluminescence within 15 min after dilution. Fifty microliters of the diluted solution were mixed with 200 μL of the same medium. The tube was placed in a pico ATP luminometer (Jobin Yvon) and 50 μL of luciferin-luciferase solution were injected. This solution contained 0.14 mM luciferin, 5 $\mu g \cdot mL^{-1}$ purified luciferase (EC 1.13.12.7), 10 mM magnesium acetate, 0.1% BSA, 0.07 M Tris acetate (pH 7.75), and 2 mM DTT. ATP concentration was calculated from peak height, using internal ATP standards for each assay.

Electron Microscopy

The pellet of mitochondria was resuspended in a medium containing 0.7 M sorbitol, 100 mM Na phosphate buffer (pH 7.2), and 2.5% glutaraldehyde (Agar Aids), for 3 h at 4°C. The mitochondrial suspension was then centrifuged at 10,000*g* for 30 min, and the pellet carefully rinsed three times with the same medium minus glutaraldehyde. The pellet was then postfixed with 1% osmium tetroxide in the same buffer plus sorbitol, for 4 h at 4°C. After rinsing with the buffer minus sorbitol, the pellet was resuspended in 1% tannic acid (Mallinckrodt) in 100 mM Na phosphate buffer, for 30 min at 20°C, then pelleted, rinsed, and covered with 5% agar. After dehydration with ethanol and propylene oxide, the material was embedded in Epon 812. The ultrathin sections were mounted on 600 mesh copper grids, stained with uranyl and lead, and observed with a Philips CM10 electron microscope.

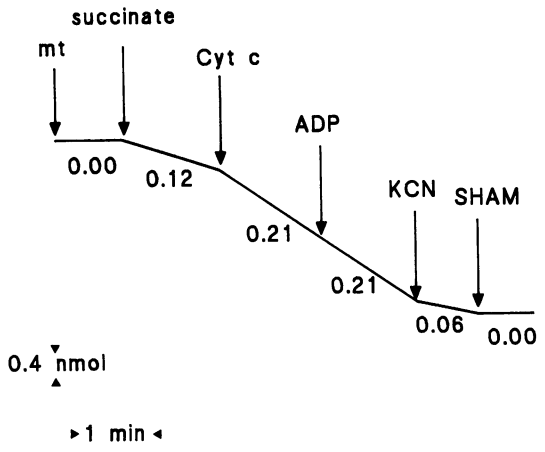

Figure 1. Succinate oxidation by purified mitochondria isolated from dry sunflower seeds. To 1 mL of reaction medium were added successively: mitochondria from 1 g of seeds (mt), 10 mM succinate, 32 μM Cyt *c*, 150 μM ADP, 2 mM KCN, 2 mM SHAM. Numbers are O_2 uptake rate in nmol·min^{-1}·(g seed dry wt)$^{-1}$.

Dry sunflower seeds were also fixed under aqueous conditions according to the same experimental procedure, without addition of sorbitol to the fixatives.

RESULTS

Mitochondria Extraction Method

Two major modifications to the classical method (3) allowed us to obtain mitochondria from dry tissues. We used a high concentration of sorbitol (0.7 M), necessary for the separation of the mitochondria from the spherosomes, which are very abundant in fatty seeds. The second modification to the usual method was the use of a high concentration (3% [w/v]) of defatted BSA. This is about 30 times the concentration in usual media. This modification was first used in (27). When lower concentrations of BSA were used during extraction, an increasing rate of O_2 uptake occurred during the assays: this was attributed to the uncoupling of the mitochondria by fatty acids present in the preparation. This high concentration of BSA could also be useful in preventing the interference by the phenolic compounds of seeds (11).

The usual assay of external membranes integrity (5) showed that respiration rate was sensitive to exogenous Cyt *c* in the presence of ascorbate (Fig. 1). This indicates some discontinuity of the external membrane (for further discussion, see below). However, this assay did not allow the determination of the percentage of intactness because oxygen uptake was lower after the osmotic disruption of the mitochondria (not shown); this effect is not understood.

Oxygen Uptake

The rate of oxygen uptake by mitochondria of dry sunflower seeds in the presence of 10 mM succinate was 0.12 nmol·min^{-1}·(g dry wt)$^{-1}$ (Fig. 1). It was doubled by the addition of 32 μM Cyt *c*.

The addition of ADP gave irreproducible results. In most cases we did not observe any significant increase of respiration after ADP addition (Fig. 1). However, a clear ADP effect was observed with mitochondria from seeds imbibed for 4 h or more (results not shown).

The succinate-dependent oxygen uptake was inhibited by 70% by 2 mM KCN and by 100% by a further addition of 2 mM SHAM.

Oxygen uptake with 10 mM citrate could not be detected. Other substrates were not tested.

Inhibition of the Adenylate Kinase-Dependent ATP Synthesis

A high ATP synthesis was observed when 50 μM ADP was added to a medium containing dry seed mitochondria in the absence of respiratory substrate (Fig. 2, curve a). It also occurred in the presence of cyanide. This ATP production was attributed to the activity of adenylate kinase, an enzyme located in the intermembrane space (19).

Adenylate kinase can be inhibited by the competitive inhibitor Ap5A. The concentration of Ap5A giving 50% inhibition is close to 3×10^{-7} M (10). A concentration of 10^{-4} M entirely inhibited the succinate-independent, cyanide-insensitive ATP production (Fig. 2, curve d). For each of the following experiments, the absence of adenylate kinase-dependent ATP synthesis was tested in the absence of respiratory substrate and in the presence of ADP, Ap5A, and KCN to eliminate any possible interference by residual endogenous substrates. The use of Ap5A allowed us to study specifically the ATP production by oxidative phosphorylation. However, Ap5A is also substrate for luciferase and gave a bioluminesence equivalent to about 2 pmol of ATP. This amount is relatively high since if ADP was quantitatively converted to ATP, 25 pmol of ATP should be determined in this assay.

ATP Synthesis by Oxidative Phosphorylation

The addition of succinate after ADP, or the addition of ADP after succinate, was followed by a linear increase of the

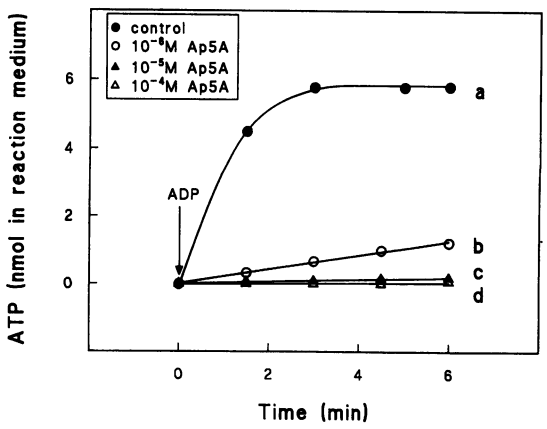

Figure 2. Effect of Ap5A on adenylate kinase dependent-ATP synthesis. The mitochondria from 2 g of seeds were incubated in 400 μL of reaction medium containing 2 mM KCN. The final ADP concentration was 50 μM.

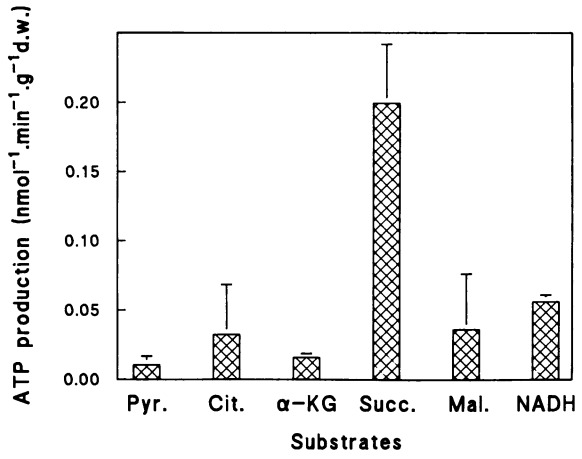

Figure 3. ATP synthesis from different respiratory substrates. The mitochondria from 1 g of seeds were incubated in 400 μL of reaction medium containing 10^{-4} M Ap5A, 50 μM ADP, and one of the following substrates: 10 mM succinate (Succ.), 20 mM Malate (Mal.), 10 mM citrate (Cit.), 2 mM α-ketoglutarate (α-KG), 10 mM pyruvate (Pyr.) plus 0.5 mM malate, 1 mM NADH. Mean ± SD; $n = 3$ mitochondrial preparations for all substrates except succinate, $n = 5$.

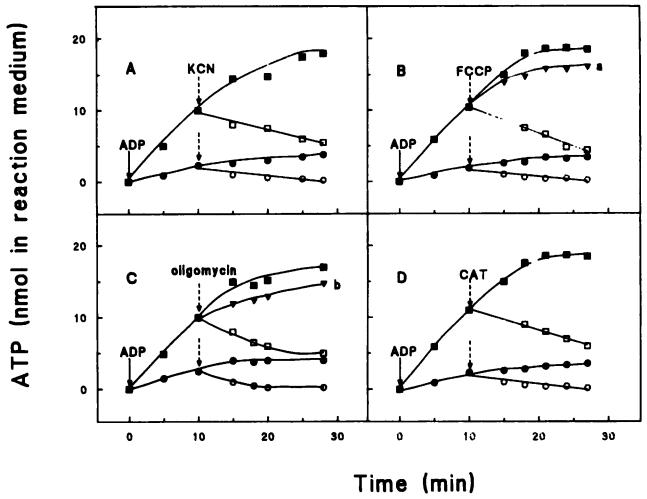

Figure 5. Effect of different inhibitors on ATP synthesis. The mitochondria from 2 g of dry sunflower seeds were added to 400 μL of the standard reaction medium containing 50 μM ADP, 10^{-4} M Ap5A, 10 mM succinate. The reactions were run with (■, □, ▼) or without Cyt *c* (●, ○). A, 2 mM KCN; B, 20 μM FCCP; C, 10 μg oligomycin; D, 100 μM carboxyatractyloside. FCCP and oligomycin were dissolved in 8 μL methanol and 4 μL ethanol, respectively. Controls with methanol (B, curve a), and ethanol (C, curve b).

level of ATP at a rate of 0.2 nmol·min^{-1}·(g dry wt)$^{-1}$. The bioluminescence signal of the synthesized ATP was twice the blank value after 2 min. The ATP production was linear for about 10 min: the rate then decreased, and a plateau was reached where only about one-third of the initial ADP had been transformed to ATP. After adding 0.1 mM phospho*enol*pyruvate and 200 units pyruvate kinase (EC 2.7.1.40), close to 100% of the ADP added at time 0 was recovered as ATP: this showed that the plateau was not due to the degradation of ADP. The incomplete conversion of ADP to ATP is explained below.

The ATP production was tested with other respiratory substrates. In all cases, a linear increase of ATP followed by a plateau was observed. The rates calculated from the linear part of the curves were about 3 to 20 times lower than with succinate (Fig. 3).

With all the substrates tested (results not shown), Cyt *c* increased the rate of ATP synthesis. With succinate as substrate the maximum rate was attained with 8 μM Cyt *c* which induced a 2.5-fold increase (Fig. 4). In the presence of Cyt *c*, 90 to 100% of ADP was transformed to ATP.

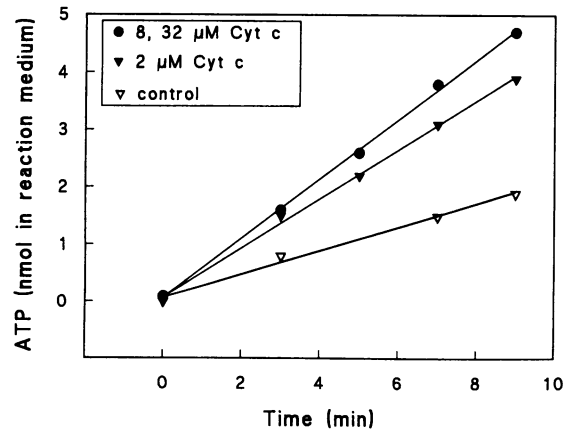

Figure 4. Effect of Cyt *c* on ATP synthesis. The mitochondria from 1 g of seeds were incubated in 400 μL of reaction medium containing 150 μM ADP, 10^{-4} M Ap5A, and 10 mM succinate, either in the absence or with different concentrations of Cyt *c*.

Effect of Various Inhibitors of Oxidative Phosphorylation

To establish that ATP synthesis was due to oxidative phosphorylation, the effects of different inhibitors of this process were studied: 2 mM KCN, an inhibitor of Cyt oxidase; 20 μM FCCP, an uncoupler; 10 μg oligomycin, an inhibitor of Fo-ATPase; and 100 μM carboxyatractyloside, an inhibitor of the ATP/ADP translocator, were used. Succinate was used as substrate with or without 32 μM Cyt *c* (Fig. 5).

The addition of any of the four inhibitors of oxidative phosphorylation blocked the production of ATP. Since FCCP and oligomycin were dissolved in methanol and ethanol respectively, a control with the solvents alone was performed and showed that most of the inhibition was due to the inhibitors (Fig. 5, B and C).

The block of ATP synthesis was followed by a decrease of the ATP level (Fig. 5). A higher rate of ATP degradation in the presence of Cyt *c* was related to the higher concentration of ATP in the medium: incubation of the mitochondria with increasing levels of exogenous ATP showed that the rate of degradation was proportional to the concentration of ATP (results not shown). Therefore, the presence of a plateau in the absence of Cyt *c* represents a steady state concentration of ATP where synthesis balances degradation.

The ATP/O ratio was calculated from the rates of ATP

Table I. *Succinate Dependent ATP Synthesis and Oxygen Uptake by Mitochondria Extracted from Dry Sunflower Seeds*

V_{ATP} was the initial rate of ATP synthesis calculated from the curves of Figure 5. V_{O_2} was measured on the same batch of mitochondria, as described in Figure 1. Values are mean ± SD; $n = 5$.

	−Cyt *c*	+32 μM Cyt *c*
	nmol·min^{-1}*·(g dry wt)*$^{-1}$	
V_{ATP}	0.20 ± 0.03	0.49 ± 0.06
V_{O_2}	0.12 ± 0.02	0.21 ± 0.03
ATP/O	0.85	1.20

synthesis and oxygen uptake measured with succinate as substrate. The ATP/O ratios were 0.85 and 1.2 in the absence and presence of 32 μM Cyt *c*, respectively (Table I).

Yield of Mitochondria

The yield of mitochondria was calculated by two methods: first, by comparing the oxygen uptake of the mitochondria to that of fully imbibed seeds and second, by comparing the activity of marker enzymes in the mitochondrial fraction and in a crude extract.

Figure 6 shows that the level of hydration and the respiratory rate of the seeds increased gradually and in parallel during the first 2 h of imbibition (germination phase I), reaching a plateau corresponding to germination phase II (1). This suggests that the increase in respiration rate corresponds to an increase in the number of hydrated cells. The yield of mitochondria, calculated from the respiratory activities, was 0.075% or 0.14% without and with Cyt *c*, respectively.

The activities of SDH and fumarase in a crude extract from dry seeds were 540 and 661 nmol·min^{-1}·(g dry wt)$^{-1}$, respectively (Table II). In the mitochondrial fraction these activities were 0.6 and 0.8 nmol·min^{-1}·(g dry wt)$^{-1}$, respectively (Table II). The recovery of mitochondria calculated from these data is close to 0.11%. Similar results for the yield of mitochondria were obtained from either mitochondrial marker enzymes or the oxygen uptake of the seeds.

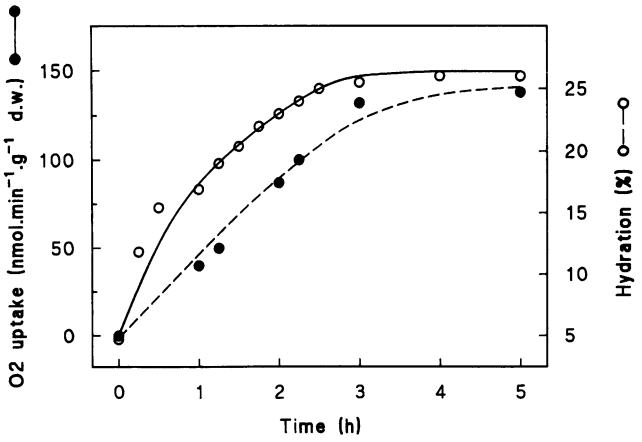

Figure 6. Oxygen uptake and hydration of sunflower seeds. Hydration is expressed in g H_2O·100·(g dry wt)$^{-1}$. Experimental procedures are described in "Materials and Methods."

Table II. *Fumarase and Succinate Dehydrogenase Activities in a Crude Extract and in the Mitochondrial Fraction from Dry Sunflower Seeds*

For the experimental procedures see "Materials and Methods." Values are mean ± SD for $n = 2$.

	Crude Extract	Mitochondria
	nmol·min^{-1}*·(g dry wt)*$^{-1}$	
V_{FU}	662 ± 86	0.80 ± 0.12
V_{SDH}	541 ± 78	0.60 ± 0.07

Electron Microscopy

In the highly dehydrated cells of the cotyledons, the cytoplasm is very dense and clumped (Fig. 7a), the membrane systems are hardly visible and appear negatively stained—except for the plasma membrane (Fig. 7b). The mitochondria cannot be clearly recognized and are probably present as very small structures—0.15 μm mean diameter—with a clear matrix and indiscernible membrane boundaries (Fig. 7c). In the axis, the mitochondria are larger than in the cotyledons—0.20 to 0.30 μm mean diameter—and both envelope membranes are visible. However, whereas the inner membrane is distinctly trilaminar (dark-light-dark construction), the structure of the outer membrane cannot be clearly resolved (Fig. 7d).

The pellet corresponding to the mitochondrial fraction (Fig. 7e) also contains spherosomes, large lipid bodies, small dense clusters originating in the protein bodies of the dry seed, and a few membrane vesicles, but it is substantially free of other organelles. The size of mitochondrial sections ranges from 0.4 to 1.8 μm mean diameter. Their shape is very heterogeneous, often roughly circular or somewhat branched.

Mitochondria extracted from dry seeds are characterized by the very low density of the mitochondrial matrix (Fig. 7f), where only small protein clusters are electron dense. DNA nucleoids are ribosomes are not clearly seen. In all mitochondria sections, the inner membrane is distinct and continuous, but only scarce cristae protrude within the matrix and appear as small membrane vesicles. In contrast, the outer envelope membrane is discontinuous or absent. Most often, the outer membrane covers only distinct parts of the organelle, giving a patchy appearance to the mitochondrial envelope. Many pictures suggest the existence of contact zones between both envelope membranes at the margins of the outer membrane fragments. In a few cases, both membranes are present and continuous, as in the envelope of mitochondria of hydrated cells. In addition, the trilaminar structure of both the outer and the inner membranes can be hardly seen, in contrast to the situation in hydrated maturing sunflower seeds processed according the same experimental procedure (Fig. 7g).

All these observations are suggestive of considerable modifications of the mitochondrial membranes following dehydration during maturation of the seeds (Fig. 7, *cf.* g and c, d) or rehydration during extraction (Fig. 7, *cf.* c, d, and e, f).

DISCUSSION

Mitochondria Extraction Method

Only a few reports can be found in the literature concerning the occurrence of oxidative phosphorylation in isolated mi-

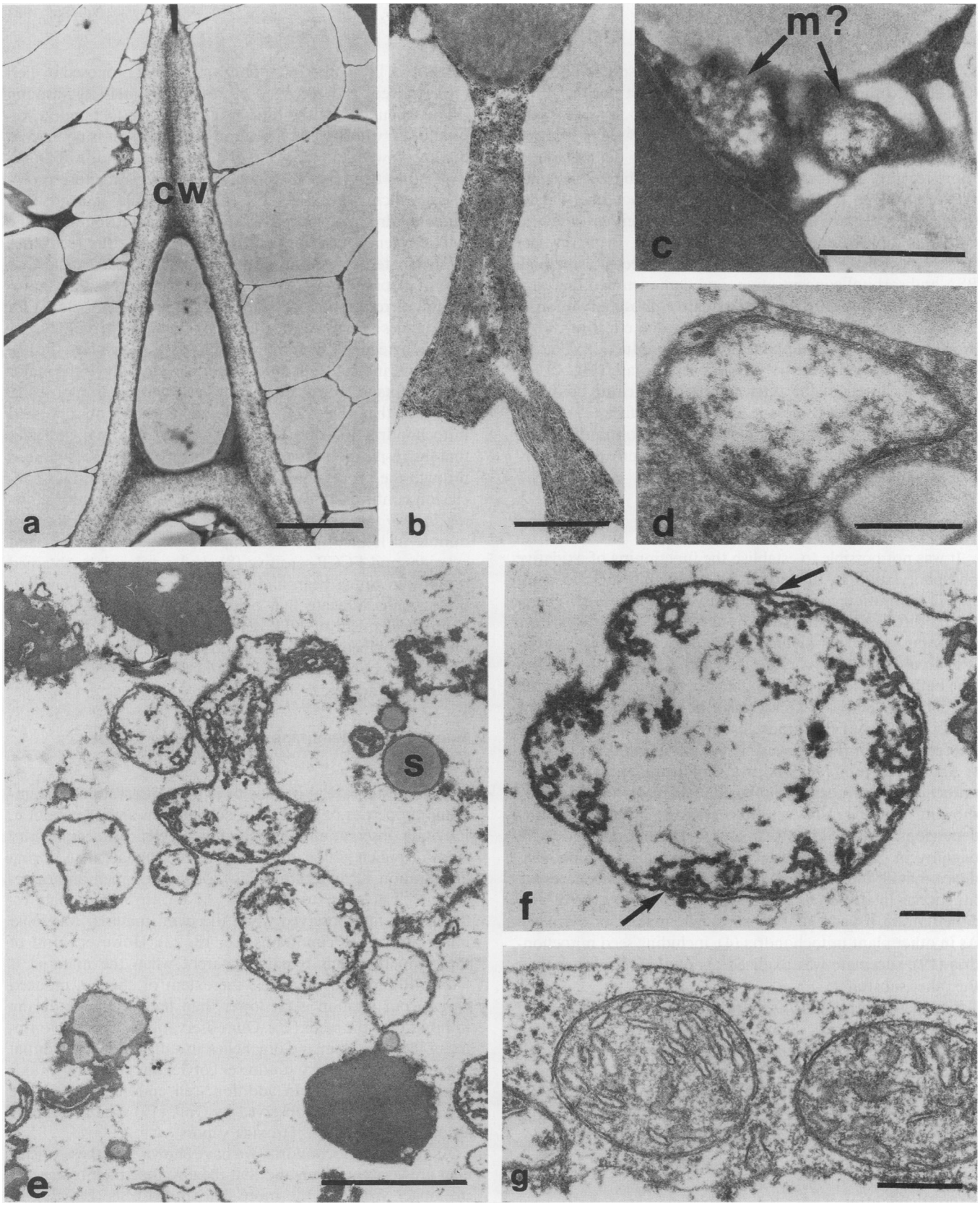

Figure 7. Electron micrographs of sunflower seed material. a, Low magnification view of dry cotyledons cells showing the reticulation of the dehydrated cytoplasm (CW = cell wall) (×15,500; bar, 1 μm). b, Detail of cytoplasmic trabecule showing negatively stained endomembranes (×58,000; bar, 0.3 μm). c, Putative mitochondria (m?) of dry cotyledons cells (×85,000; bar, 0.3 μm). d, Mitochondrion of dry axes; both membranes are present and the inner membrane is clearly trilaminar (×63,000; bar, 0.3 μm). e, Overall view of the mitochondrial pellet from dry seeds (s = spherosomes) (×25,500; bar, 1 μm). f, Detail of an isolated mitochondrion showing the intactness of the inner membrane; the outer membrane is present only in the right part of the section, close to the inner membrane at the margins (arrows) (×38,000; bar, 0.3 μm). g, Mitochondrion of a maturing sunflower seed: both membranes and inner cristae are well delineated (×49,500; bar, 0.3 μm).

tochondria at early stages of germination. Wilson and Bonner (27) studied this mechanism in mitochondria purified from peanut cotyledons after 40 min of imbibition. An ATP synthesis which was activated by Cyt *c* and was respiratory substrate-dependent and KCN-sensitive was demonstrated. More recently a mitochondrial fraction, capable of phosphorylating ADP to ATP, was obtained from cucumber cotyledons imbibed for 6 h (14). However, in neither case was contamination by adenylate kinase excluded. In the present work we extracted mitochondria from dry seeds, using high BSA and sorbitol concentration, and demonstrated that they were able to synthesize ATP by oxidative phosphorylation.

Two apparently conflicting results are the effect of exogenous Cyt *c* on oxygen uptake and ATP production (Figs. 1, 4, 5) and the high activity of adenylate kinase (Fig. 2), an enzyme localized in the intermembrane space or bound at the outer surface of the inner membrane surface (19). However, both results are consistent with the discontinuity of the external membrane shown by electronic microscopy in apparently intact mitochondria (Fig. 7f).

Oxidative Phosphorylation

It was not possible to establish the functioning of oxidative phosphorylation by the usual polarographic method since state 3 respiration could not be consistently observed. Therefore, a direct ATP estimation by the firefly bioluminescence assay was used. This method is specific and highly sensitive. It is an alternative to a radio enzymatic assay with ^{32}P (19). The bioluminescence method does not require any separation of ATP but adenylate kinase must be inhibited. Its activity, which was about 10 times higher than that of oxidative phosphorylation (Figs. 2 and 5), was fully inhibited by 10^{-4} M Ap5A. Using this method, we demonstrated that dry seed mitochondria are capable of oxidative phosphorylation by showing that the ATP synthesis was respiratory substrate-dependent, activated by Cyt *c*, and sensitive to KCN, FCCP, oligomycin, and carboxyatractyloside. We also obtained evidence that all the tricarboxylic acid cycle dehydrogenases are functional in dry seeds since ATP synthesis occurred with either citrate, α-ketoglutarate, succinate, malate, or pyruvate. As in other plant mitochondria (4), including seed mitochondria (19), succinate was oxidized about 10 times faster than the other substrates.

To calculate the ATP/O ratio, the initial velocity of ATP production was used. Since the rate of ATP degradation appeared to be proportional to the concentration of ATP and the increase of ATP approximated linearity, degradation of ATP (Fig. 5) was considered negligible. The values of 0.85 and 1.2, with and without Cyt *c*, respectively, are close to that of 0.9 found in mitochondria from imbibed peanut embryos (27).

Seed Physiology

The aim of the present work was to establish whether, as previously suggested (8), oxidative phosphorylation plays a major role in ATP regeneration during the imbibition and early germination of seeds. If not, it would be necessary to consider the operation of other oxygen-dependent mechanisms of ATP synthesis, as for example, that proposed by Perl (17). Indeed, the recovery of respiratory activity (around 0.1%) calculated as the ratio of extracted mitochondria respiration to whole seed respiration is very low: it is about 5 to 30 times lower than the yields of intact mitochondria obtained with other tissues such as potato tubers or sycamore protoplasts which are 0.5 to 3% (4). This could indicate that mitochondrial respiration is a marginal contributor to the total oxygen uptake of seeds. However, since the recoveries of succinate dehydrogenase and fumarase are very close to that of respiratory activity, it is clear that, rather than mitochondrial respiration, the yield of mitochondria is low (dry seed tissues are obviously an unfavourable material). Therefore, in agreement with many results obtained *in vivo* (8, 19), our results indicate that mitochondrial respiration is the major oxygen uptake process during the early germination of seeds. The fact that the ATP/O ratio, determined on dry seed mitochondria respiring succinate, is close to normal, indicates that the respiration of seeds is closely linked to ATP synthesis through the process of oxidative phosphorylation. This conclusion confirms an earlier report from our laboratory showing *in vivo* that the ATP synthesis which occurs in the first minute of lettuce seed imbibition is oxygen-dependent and cyanide- and FCCP-sensitive (8), and similar data obtained recently in mung bean and cucumber seeds (14). Together, these results indicate that oxidative phosphorylation is the main mechanism of ATP regeneration in seeds, as soon as they are imbibed. There is no need to consider other mechanisms to account for the ATP production in early germinating seeds under aerobic conditions.

Mitochondrial Membranes in Dry Seeds: Electron Microscopy

These results are also relevant to the conservation of membrane properties of seeds after dehydration and rehydration. Previous observations on dehydrated cells, from either dry seeds or pollen grains, have emphasized the fact that, during dehydration, structural modifications of cell membranes occur which give rise to characteristic ultrastructural pictures: these were first observed when the plant material was fixed under anhydrous conditions (16, 18, 23). However, most of these modifications remain apparent when the material is fixed with aqueous fixatives: the extent of changes induced by aqueous fixation is far lower than that occurring during imbibition of the seeds (26). Our observations of dense clumping of the cytoplasm and organelles also suggest that the initial organization of the dry sunflower cotyledons was barely modified during fixation. In addition, cell endomembranes are negatively stained, as observed by Opik (16) after anhydrous fixation with osmium tetroxide vapors.

As regards mitochondria, we have shown that these organelles are very shrunken and that the envelope membranes are no longer visible in the more dehydrated cells (Fig. 7c), in contrast to the situation in hydrated tissues (Fig. 7g). These results are also in agreement with previous observations of distinct modifications of the outer mitochondrial membrane in dry tissues. When rice seeds are fixed with osmium tetroxide vapor, the mitochondria are wrinkled, the internal membrane systems are sparse and the outer membrane of the

mitochondrial envelope is hardly visible, or absent (16). In cowpea seeds fixed in anhydrous conditions with 4% paraformaldehyde dissolved in glycerol (23), the organelles are ill-defined and irregular in outline and the outer membrane is hardly seen. In dry cotyledons of soybean fixed with glutaraldehyde in phosphate buffer, the outer membrane of many mitochondria is distended so that it appears regular, thin, and tenuous whereas in the imbibed cells it appears intact (26). In dry pollen grains quench-frozen in liquid propane and freeze-substituted by 1% osmium tetroxide in acetone, tightly packed, multilamellate membrane profiles are found in association with all cell membranes and specially mitochondria, but they are no longer visible in hydrated pollen (2 min imbibition) (24).

All these observations suggest that drastic changes in membrane organization occur during seed or pollen dehydration, and that the mitochondrial outer membrane is specially affected by these changes. It is likely that these modifications, which may lead to an apparent absence of the outer membrane, are related to the hydration status of the cell. Recently, several physical parameters have been determined in seeds of two *Phaseolus vulgaris* cultivars (28): 0.11 and 0.15 g water·(g dry wt)$^{-1}$ are critical moisture levels for imbibitional injury, and intracellular sugars enter into solution above 0.14 g water·(g dry wt)$^{-1}$. Since at a given water potential, the lipid-rich sunflower seeds contain less water than starchy *Phaseolus*, the levels determined in *Phaseolus* may correspond to 0.05 to 0.09 g water·(g dry wt)$^{-1}$ for sunflower seeds (21). Therefore, it is likely that the seeds used in this study (0.045 g water·[g dry wt]$^{-1}$) were below the critical moisture levels. While some biological membranes are capable of maintaining their bilayer configuration at water levels as low as 2.3% (18), the molecular arrangement of the mitochondrial membranes of dry sunflower seeds did not produce the trilaminar structure after aqueous fixation with glutaraldehyde and osmium tetroxide (Fig. 7 c, d). It can be concluded that the mitochondrial outer membrane is present in a highly modified state which prevents resolution by the usual electron-microscopy processing.

All these morphological manifestations of membrane rearrangements are transient and disappear as soon as the seeds or pollen grains are hydrated to restore a normal mitochondrial membrane structure with a well defined outer membrane and an inner membrane with cristae (18, 23).

In the present study, the contact of the dry mitochondria with water was very rapid and occurred at a low temperature which is likely to increase the constraints on the membranes (25). Hydration induced a swelling of mitochondria and a partial restoration of the membrane structure. However, the outer membrane remained often incomplete and the trilaminar structure could not be resolved in both membranes, indicating clearly that the environmental conditions and the water supply were completely different from that occurring during normal imbibition of the tissues. In spite of this, the organization of the mitochondrial structure necessary for the functioning of oxidative phosphorylation was restored, or preserved.

The leakage of solutes which occurs upon imbibition of seeds was first interpreted as an indication that cellular membranes could be disrupted either by dehydration or by the entry of water into the seed. More recently, it was suggested (5) that leakage of electolytes is almost entirely due to passive diffusion through membranes, and not necessarily an indication of cellular rupture. In the past few years, different mechanisms have been proposed to explain the stabilization of membranes in the dry state: either the lipid composition of the membranes (12), bound residual water (25), the replacement of water by sucrose (4), or hydrophobic protein (9), could contribute to membrane stabilization in dehydrated seeds. Although the mechanisms responsible for the protection of the mitochondrial membranes are not yet identified, it is clear that they are very efficient.

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