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ADENOSINE TRIPHOSPHATASE ACTIVITY IN GERMINATING LETTUCE SEEDS

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Mitochondria isolated from lettuce seeds and seedlings are capable of hydrolyzing ATP. On basis of pH optima, there are at least two and probably three enzymes possessing ATPase activity. One of them is inhibited by EDTA and all of them are inhibited by NaF.

The ATP ase activity of the mitochondria is very slight in imbibed seeds but increases considerably with seedling growth. Other components of the cell also contain ATP hydrolyzing enzymes; however, most of these phosphatases in the seedling seem to be soluble enzymes.

In previous papers from this laboratory various investigations on the metabolism of germinating lettuce seeds were reported (*1*, *2*). Among other things, it has been shown that the tricarboxylic acid cycle enzymes are active in mitochondria isolated from such seeds (*3*, *4*), but attempts to demonstrate oxidative phosphorylation failed; the P/O ratios obtained were always <1 and sometimes instead of phosphorylation, release of inorganic phosphorus occurred. It appeared therefore that the isolated mitochondria possesed comparatively strong adenosine triphosphatase (ATPase) activity. This activity was investigated and the results are reported in the present paper.

MATERIAL AND METHODS

Lettuce seeds (*Lactuca sativa* L.), variety Grand Rapids, were used throughout the experiments. The seeds were germinated in PETRI dishes at 26°. Two hours after the beginning of imbibition a light stimulus was given to the seeds to ensure full germination.

For isolation of mitochondria, the seeds or seedlings were ground in Tris buffer 0.025 *M* at pH 8.4~8.6, containing 0.5 *M* sucrose, 0.01 *M*

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate. ATPase, adenosine triphosphatase; EDTA, ethylenediamine tetraacetic acid; Pi, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane.

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EDTA and 0.001 M $MgSO_4 \cdot 7H_2O$. The resulting brei was squeezed through cheese-cloth and centrifuged for 5 *min* at 500× *g*. The supernatant was centrifuged, again, for 20 *min* at 20,000× *g*. The particulate precipitate was washed twice with the same buffer, but of pH 7.0~7.2, using an all-glass POTTER-ELVEHJEM homogenizer. Finally the particulate fraction was resuspended in the Tris buffer of pH 7.0~7.2, using one *ml* buffer for every gram of initial weight of seeds.

The ATPase activity was assayed by measuring the inorganic phosphorus (Pi) after incubation of the mitochondrial suspension, or the supernatant, for 30 *min* with ATP, at 26°. The reaction mixture contained: sucrose, 0.166 M; Tris buffer, 0.006 M; EDTA, 0.003 M; $MgSO_4 \cdot 7H_2O$, 0.0016 M; ATP 0.003 M; 0.5 *ml* enzyme preparation, the total volume being 1.5 *ml*. The reaction was stopped by adding 0.5 *ml* trichloroacetic acid (16.5%). Phosphorus was determined by the BERENBLUM and CHAIN method (*5*) as modified by BARTLEY (*6*). The ATP used was disodium ATP-Sigma. All other chemicals were analytically pure. Total nitrogen was estimated by Nesslerization.

When more detailed fractionation of the homogenate was required, the strained homogenate (prepared as outlined above) was broken down into 5 fractions: First the whole cells, nuclei, cell walls, etc. were sedimented by centrifugation at 500× *g* for 5 *min*, the sediment being fraction 1. The supernatant was then centrifuged at 5,000× *g* for 20 *min*. After this treatment a pellet of fat floated on the supernatant (fraction 2) and sediment formed on the bottom (fraction 3). The remaining fluid was centrifuged at 20,000 ×*g* for 20 *min*, the sediment being fraction 4 and the supernatant fraction 5. Fractions 1, 2, 3 and 4 were each resuspended in Tris-sucrose buffer using 1 *ml* of the buffer for each gram of initial weight of seeds, so that all fractions were brought to equal volume. Fraction 5 was left as it was, or occasionally diluted twice.

RESULTS AND DISCUSSION

The ATPase activity of mitochondria from lettuce seedlings was investigated at different substrate concentrations (Fig. 1) and after varying lengths of incubation period (Fig. 2). As it is seen from Fig. 1, the optimal substrate concentration is about $1.7 \times 10^{-3} M$, no inhibition being noted at higher substrate concentrations as reported by FORTI (*7*) for pea mitochondria.

Fig. 2 shows clearly that during very short incubation periods, less than 5 *min*, no apparent ATPase activity of the mitochondria could be shown. On the contrary, apparently phosphorylation takes place and inorganic phosphorus disappears from the incubation medium. ATPase activity in these mitochondria could be clearly demonstrated after 15 *min*

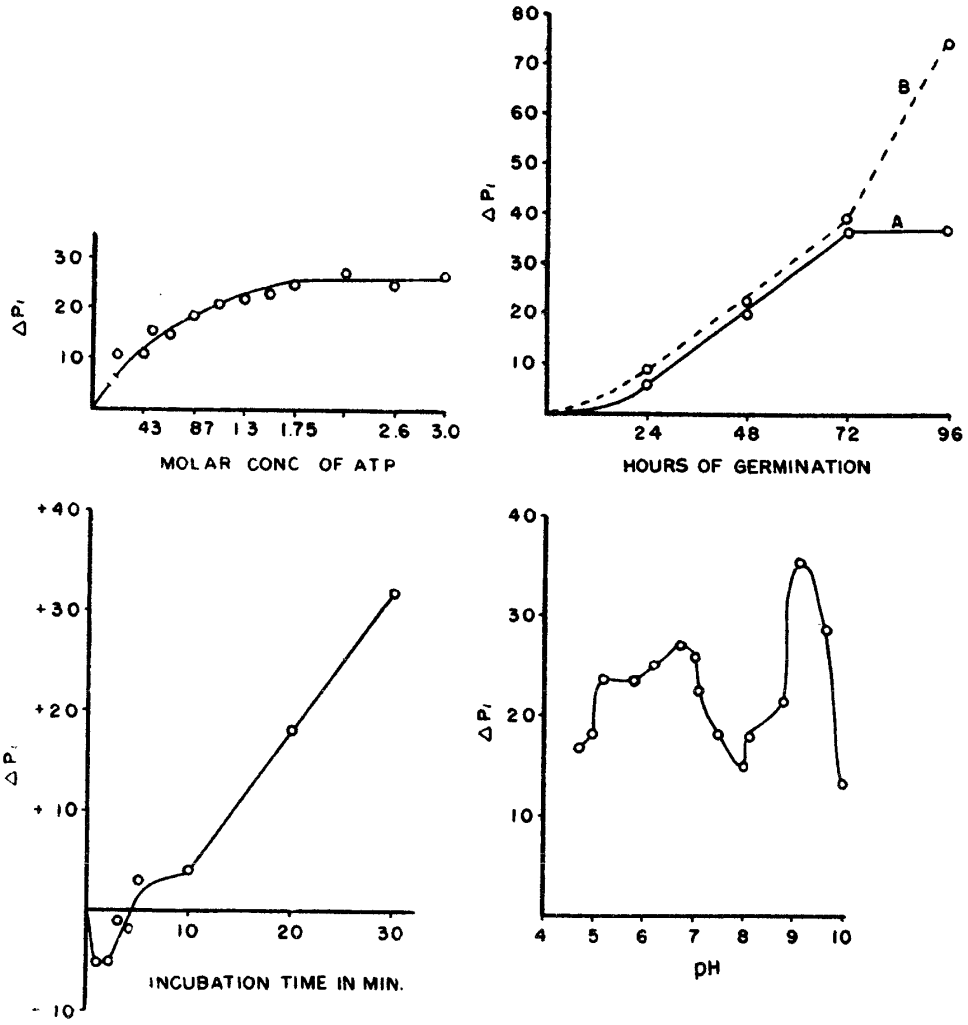

Fig. 1. (*Upper left*) Effect of substrate concentration on ATPase activity of lettuce mitochondria. Activity expressed as μg inorganic phosphorus ($\triangle$Pi) liberated by 0.5 *ml* of mitochondrial suspension (equivalent to 500 *mg* initial weight of seeds). Seeds germinated for 60–65 hours.

Fig. 2. (*Lower left*) Effect of length of incubation period on ATPase activity of lettuce mitochondria. Results expressed as in Fig. 1. Seeds germinated for 72 hours. The points drawn are the mean of 3 expts.

Fig. 3. (*Lower right*) Effect of pH on ATPase activity of lettuce mitochondria. Results expressed as in Fig. 1. Seeds germinated for 72 hours. The points drawn are the mean of 4 expts.

Fig. 4. (*Upper right*) Effect of seedling age on ATPase activity of lettuce mitochondria. A : results expressed as in Fig. 1. B : results expressed as $\triangle$Pi/*mg* nitrogen in the mitochondrial preparation.

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incubation and no special ageing procedure was necessary. The phosphorylation was possible, probably due to oxidation of some endogenous substrates still present in the mitochondria and to the presence of traces of ADP and Pi in the ATP preparation which was not chromatographically pure.

The effect of pH on the ATPase activity was studied; the results (Fig. 3) show certainly two and probably three pH optima: 9.0~9.5, 6.5~7.0 and possibly 5.0~5.5, suggesting that the mitochondria contain more than one system capable of hydrolyzing ATP. Calcium and Mg ions stimulated slightly ATPase activity. NaF at a concentration of $1.5\times10^{-2}\,M$ completely inhibited the ATPase activity. The activity at pH 9.0 was depressed by $10^{-3}\,M$ EDTA. These results, although varying in details of concentrations and degree of inhibition, agree in general lines with the results obtained for the ATPase of mitochondria from other plant and animal sources (*7—14*).

A comparison was made of the mitochondrial ATPase activity with the activity of this enzyme in the other fractions of the homogenate. Fractionation was achieved by differential centrifugation of the strained homogenate as described under METHODS. Five fractions were prepared. No attempt was made to wash the fractions; but for every fraction a control was run, in which the liberation of Pi (by the endogenous enzyme) from the endogenous substrates was measured. The ATPase activity, using exogenous ATP as substrate, was corrected for these results. The activity of each fraction was calculated as percentage of the total activity of all the fractions together. The results are summarized in Table I. As may be seen from these results, all the

TABLE I

ATPase activity of various fractions of the homogenate

The homogenate was prepared from lettuce seeds germinated for 72 hours. Details of preparation of the fractions are given in the text. Reaction mixture contained 0.166 *M* sucrose, 0.06 *M* Tris, 0.003 *M* EDTA, 0.0016 *M* Mg: Substrate (when present): $3\times10^{-3}\,M$ ATP. Incubation for 30 *min* at 26°. Results are given as percent of the total activity.

Fraction No.	Specification	Activity on exogenous ATP as % of activity of total homogenate	Activity on endogenous (unknown) substrate as % of activity of total homogenate
1	Sedimented at $500\times g$	10	2.3
2	Floating pellet after centrifugation at $5{,}000\times g$	15	2.3
3	Sedimented at $5{,}000\times g$	21	4.6
4	Sedimented at $20{,}000\times g$	12	3.1
5	Supernatant	42	87.7

fractions were capable of hydrolyzing ATP. The endogenous substrate was apparently concentrated in the supernatant; the endogenous activity of the other fractions may be due to the presence of traces of this or other substrates, as the fractions were not washed. No attempt was made to elucidate the nature of the endogenous substrate but a possibility exists that it may be either phytin or some intermediate breakdown products of phytin which are abundant in germinating lettuce seeds (MAYER, *15*; GESUNDHEIT, *16*).

Most of the ATPase activity was also concentrated in the supernatant, this being in agreement with the results for soluble ATPase in alaska peas (YOUNG *et al.*, *16*). The mitochondrial preparation used throughout this work consisted of fractions 3 and 4, and possessed approximately one third of the ATPase activity of the germinating seeds. However, most of this activity was concentrated in the heavier particles sedimenting at $5,000 \times g$.

The oxidative activity of lettuce mitochondria is extremely iow in dry seeds and during the first period of germination. It is only in later stages of seedling growth that this activity increases considerably (POLJAKOFF-MAYBER & EVENARI, *4*). These conclusions were based on calculations of activity per unit nitrogen. It is not yet clear whether this change in oxidative activity is due to increase in numbers of mitochondria in the sediment, or to the increase in the enzymatic activity. Lettuce seeds contain considerable amounts of storage proteins, some of which may be of a specific gravity that enables them to sediment together with the mitochondria, thus diluting the activity of the enzymatic protein. As growth of the seedlings proceeds, the amount of these enzymatically inactive proteins decreases and the activity per *mg* nitrogen approaches the specific activity of the enzyme. It was interesting, therefore, to follow up the change in another mitochondrial enzyme, ATPase activity of the mitochondrial fraction, during germination and initial growth of the seedling. The results are given in Fig. 4, calculated either on the basis of activity per *mg* nitrogen or per 500 *mg* initial seed weight. There was an extremely slight increase in the ATPase activity during the first 24 hours of incubation, although germination was almost complete in that period as the seeds were given a light stimulus. Then, almost linear increase in activity occurred during the following two days of incubation, which paralleled seedling growth. On the fourth day there was no further increase in activity if calculated per weight of seeds, but there was a very marked increase if calculated per *mg* nitrogen. No attempt has been made as yet to understand this difference, but tentatively it may support the assumption that the ratio between the active enzyme and non active storage protein increased during seedling growth, and that after three days the storage protein was all used up for the growth of the seedling. There may also have been a

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parallel increase in the number of mitochondria in the isolated fraction. To test this possibility morphological and protein fractionation studies should be carried out.

This paper is part of the M. Sc. Thesis of one of us (R.A.).

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