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Rapid Development of Mitochondria in Pea Cotyledons during the Early Stage of Germination

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ABSTRACT

Rapid increases in activities and components of mitochondrial particles isolated from cotyledons of *Pisum sativum* var. Alaska during the early stage of germination are described. Respiratory rate of the cotyledons increased rapidly as hydration proceeded. A similar but slightly delayed increase in respiratory activity of the isolated mitochondrial fraction was observed. The respiratory control ratio and adenosine 5'-pyrophosphate/oxygen ratio rose during imbibition. Cytochrome oxidase and malate dehydrogenase activities in the mitochondrial fraction increased during the initial phase of imbibition. The increase seemed to precede that in respiratory activity. A significant activity of cytochrome oxidase and most of the malate dehydrogenase activity in the cotyledons were present in the postmitochondrial fraction in the case of the dry seeds. Mitochondrial protein and phospholipid also increased during imbibition, and the rise in the components seemed to concur with that in respiratory activity. The mechanism of mitochondrial development during imbibition is discussed.

It is well known that seed germination is accompanied by a marked increase in respiratory activity of the seeds, which seems dependent on water content of seeds (14). Electron microscopic studies have revealed that the respiration rate is linked with the organization of mitochondrial structure during germination. Bain and Mercer (1-3) demonstrated that poorly differentiated mitochondria could be resolved in pea cotyledons after the seeds passed into dormancy. The mitochondrial fraction isolated from cotyledons of dry peanut seeds contains few typical mitochondria in addition to many vesicular mitochondrial particles (9). Thus, only poor mitochondrial structure was detected in dormant cotyledon cells. However, more differentiated mitochondrial structure becomes evident as germination proceeds (1-3, 9, 13).

Synthesis of mitochondrial components occurs during germination. For instance, Breidenbach *et al.* (5, 6) showed increases in cytochrome, protein, and DNA contents of purified mitochondrial fractions prepared from peanut cotyledons by sucrose density gradient centrifugation during germination. They concluded that biogenesis of mitochondria occurs in the germinating cotyledons.

Although considerable information on the development of mitochondria during germination has been accumulated as described above, only a few reports are available as to changes in mitochondrial components or activities during the earliest stage of germination. A report of Young *et al.* (16) suggested that respiratory activity of mitochondria isolated from pea cotyledons increased rapidly to reach a plateau during the first 24 hr of germination with a lag time lasting about 12 hr.

The present paper describes marked increases in mitochondrial activities and components of pea cotyledons during the imbibing stage of germination.

MATERIALS AND METHODS

Plant Material. Pea seeds (*Pisum sativum* var. Alaska) were purchased from Watanabe Seed Co., Kogota, Miyagi, Japan. The seeds were soaked in a 1% (v/v) sodium hypochlorite solution for 10 min, washed thoroughly with tap water, and then rinsed once with deionized water. The surface-sterilized seeds were germinated in Petri dishes containing deionized water in the dark at 28 C. The cotyledons were taken at the required age, washed with water, and used as the experimental material.

Preparation of Mitochondrial Fraction. Mitochondrial fractions were prepared by a modification of the method of Bonner (4). The prechilled cotyledons obtained from 5 g of the dry seeds were ground with a pestle and mortar with 5 g of sea sand and 5 ml of grinding medium. The grinding medium was composed of 10 mM potassium phosphate buffer, pH 7.2; 0.7 M mannitol; 1.0 mM EDTA; 0.1% (w/v) BSA;[1] and 0.05% (w/v) cysteine. The resulting slurry was diluted with 25 ml of the grinding medium. The pH was adjusted to 7.2 with KOH. The resultant homogenate was squeezed through a double layer of gauze and centrifuged at 300*g* for 5 min. The precipitate was suspended in washing medium, of which the ingredients were the same as those of the grinding medium except that cysteine was omitted. The suspension was centrifuged at 300*g* for 5 min. The supernatant fractions were combined and centrifuged at 25,000*g* for 30 min. The precipitate was washed once with the washing medium and suspended in a small volume of the washing medium (mitochondrial fraction). The supernatant fractions from the centrifugations at 25,000*g* were combined and designated the postmitochondrial fraction.

For preparation of the mitochondrial fraction from dry seeds, the seeds were powdered with a motor pulverizer prior to grinding.

All procedures were carried out at approximately 4 C.

Sucrose Density Gradient Centrifugation. Sucrose density gradient centrifugation was based on the method of Sakano and Asahi (15). The mitochondrial fraction prepared from germinating pea cotyledons equivalent to 5 g of dry seeds was suspended in about 1.5 ml of the washing medium. The suspension was layered on 16 ml of a linear sucrose density gradient (32.5-65.0%, w/v) and centrifuged at 25,000 rpm for 3 hr in a Hitachi RPS 25-3A rotor at approximately 4 C.

[1] Abbreviation: BSA: bovine serum albumin.

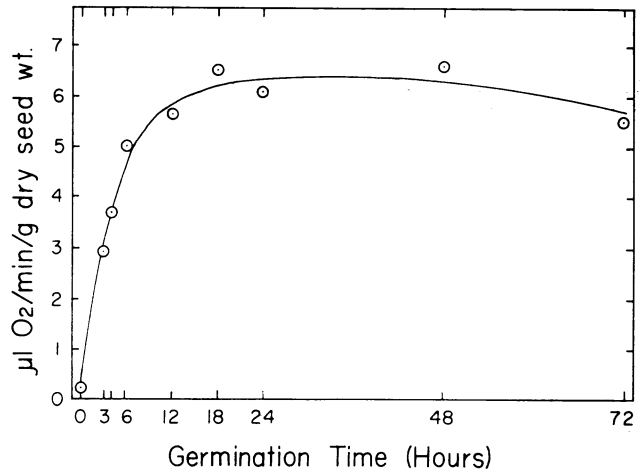

FIG. 1. Increase in respiratory rate of mitochondrial fraction of pea cotyledons during germination. Each value is the mean state 3 rate of four mitochondrial preparations. The rate in a preparation was calculated by averaging the rates obtained with three cycles of ADP addition.

Table I. *Changes in Respiratory Control Ratio and ADP/O of the Mitochondrial Fraction from Pea Cotyledons during Germination*

Each value is the mean ratio of four mitochondrial preparations. ADP was added to produce three cycles. The ratio in each cycle was calculated and is shown as the first, second, or third ratio.

Germination Time	RC Ratio			ADP/O		
	1st	2nd	3rd	1st	2nd	3rd
hr						
3	1.45	2.04	1.97	1.28	0.85	0.88
4	1.70	2.30	2.65	1.08	0.88	1.10
6	1.90	2.95	3.90	1.29	1.12	0.94
12	1.98	2.50	2.90	1.51	1.07	1.25
18	1.98	2.73	3.05	1.64	1.02	0.95
24	1.90	2.80	3.45	1.18	0.92	0.95
48	1.76	2.20	3.30	1.62	0.94	0.92
72	1.20	...	...	...	...	...

After centrifugation, the gradient was separated into 0.5-ml fractions.

Measurement of Respiratory Rate. Oxygen uptake by intact cotyledons was determined manometrically in a Warburg manometer apparatus at 30 C. The cotyledons were kept in a moist atmosphere; that is, water was added into the sidearm of flask but not into the main chamber containing the cotyledons.

Respiratory activity of the mitochondrial fraction was determined at 30 C by an oxygen electrode apparatus designed by Hagihara (11). The standard sequence of additions to the cuvette was as follows: 2.8 ml of reaction medium, 0.2 ml of mitochondrial fraction, 6 μmoles of succinate (2 min after mitochondria), and repeated additions of 0.25 μmole each of ADP. The reaction medium consisted of 10 mM potassium phosphate buffer, pH 7.2; 0.7 M mannitol; 10 mM KCl; 1 mM $MgCl_2$; and 0.1% (w/v) BSA. Respiratory control ratios were calculated according to the method of Chance and Williams (7, 8). ADP/O is the ratio between the known amount of ADP added to the reaction mixture and the amount of oxygen utilized during esterification of ADP to ATP.

Assay of Enzyme Activity. Malate dehydrogenase was assayed in a total volume of 3.0 ml that contained 50 mM potassium phosphate buffer, pH 7.5; 10 mM NADH; 12 mM oxalacetate; 0.2% (v/v) Triton X-100; and enzyme preparation. The reaction rate was recorded at 340 nm at 25 C by a Cary model 14 spectrophotometer.

Cytochrome oxidase was assayed in a total volume of 3.0 ml that contained 0.5 ml of 0.5 M potassium phosphate buffer, pH 7.2; 1 mg of reduced cytochrome c; 0.065% (v/v) Triton X-100; and enzyme preparation. The reaction rate was recorded as for malate dehydrogenase, except at 550 instead of 340 nm.

Determination of Protein and Phospholipid. Protein was determinated by the method of Lowry *et al.* (12). The amount of phospholipid was estimated from the phosphorus content in the lipid fraction extracted by the method of Folch *et al.* (10).

RESULTS

The respiratory rate of intact pea cotyledons increased rapidly during a 6-hr imbibition and reached a plateau after 12 hr. There was practically no oxygen uptake by dry cotyledons. An apparent correlation between water content and oxygen uptake was observed for at least 12 hr.

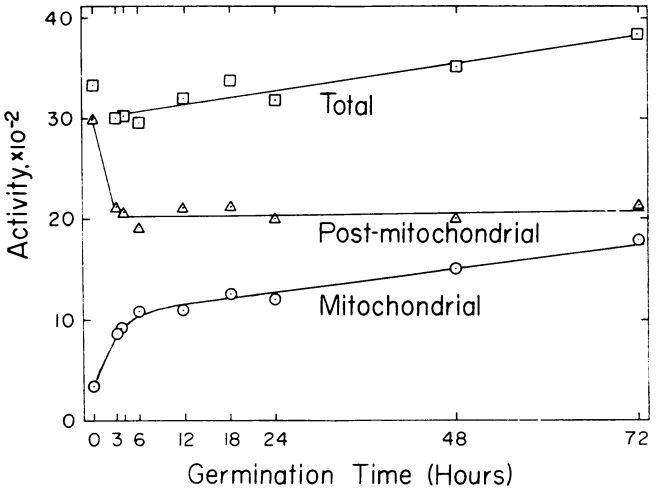

FIG. 2. Changes in malate dehydrogenase activity of pea cotyledons during germination. The activity was expressed as decrease in absorbance at 340 nm per min per cotyledons equivalent to 5 g of dry seeds.

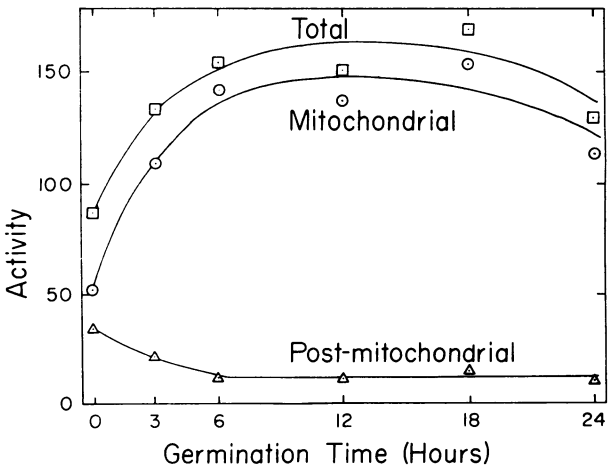

FIG. 3. Changes in cytochrome oxidase activity of pea cotyledons during germination. The activity was expressed as decrease in absorbance at 550 nm per min per cotyledons equivalent to 5 g of dry seeds.

The state 3 respiration rate of isolated mitochondria also increased rapidly during the first 6 hr of imbibition and reached a plateau after an additional imbibition for 12 hr (Fig. 1). The activities of mitochondrial fractions from the cotyledons at 3- and 6-hr ages were 48 and 80% of the value at the plateau, respectively. The activity of mitochondrial fraction from dry seeds was extremely low. The respiratory control ratio and ADP/O rose during imbibition for 6 to 12 hr (Table I).

Total activity of malate dehydrogenase in the cotyledons increased gradually during germination for 72 hr (Fig. 2). The activity of the postmitochondrial fraction remained unchanged during germination except for dry seeds. Most of the activity in dry seeds was present in the postmitochondrial fraction. A gradual increase in the activity of the particulate fraction was observed during germination after a rapid increment for the first 6 hr. Cytochrome oxidase activity increased rapidly during the early stage of imbibition and reached a plateau after 6 hr (Fig. 3). Most of the activity in the imbibing seeds was recovered in the mitochondrial fraction but a significant activity was present in the postmitochondrial fraction in the case of the dry seeds.

Figures 4 and 5 show the distribution of protein after sucrose density gradient centrifugation of the mitochondrial fraction prepared from the cotyledons. Cytochrome oxidase was plotted as a marker enzyme to show the distribution of mitochondria. A major peak of the enzyme was located in the middle part of the gradient and a minor one was at the top after the centrifugation of the mitochondrial fraction of imbibing seeds (Fig. 4). The distribution of malate dehydrogenase coincided with that of cytochrome oxidase. However, there were three fractions of cytochrome oxidase in the case of the dry seeds (Fig. 5). One of them did not possess malate dehydrogenase. Protein was distributed broadly and no peak of protein content was detected in the middle part

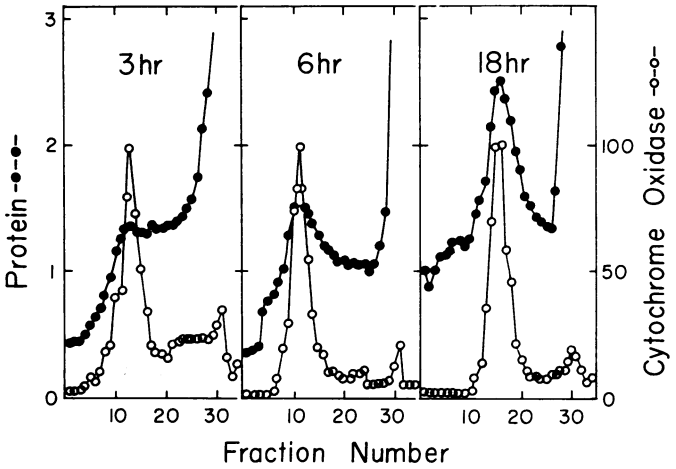

FIG. 4. Distribution of protein and cytochrome oxidase activity after sucrose density gradient centrifugation of mitochondrial fractions prepared from cotyledons of pea seeds germinated for 3, 6, and 18 hr. BSA was omitted from grinding and washing medium for the preparation of mitochondrial fraction from the cotyledons at 3 and 18 hr ages. ●: Protein content in mg per 0.5 ml; ○: cytochrome oxidase activity, expressed as the percentage of the activity of a given fraction relative to that in the peak.

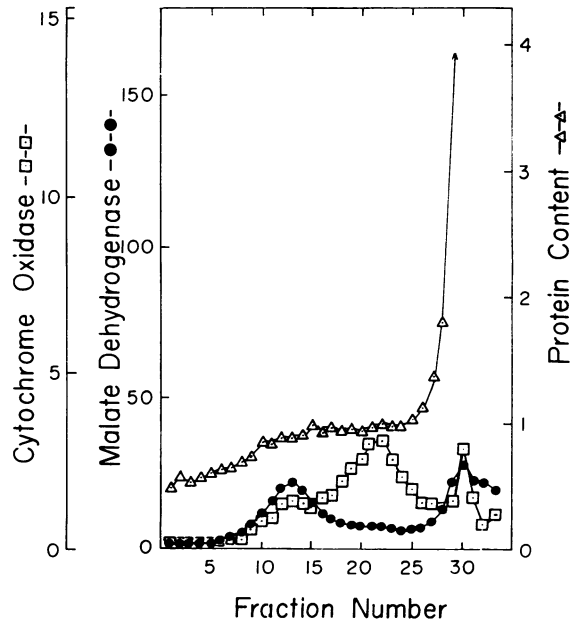

FIG. 5. Distribution of protein and cytochrome oxidase and malate dehydrogenase activities after sucrose density gradient centrifugation of mitochondrial fraction prepared from dry pea seeds. △: Protein content in mg per 0.5 ml; ⊡: cytochrome oxidase activity in decrease in absorbance at 550 nm per min per 0.5 ml; ●: malate dehydrogenase activity in decrease in absorbance at 340 nm per min per 0.5 ml.

Table II. *Increase in Protein and Phospholipid Contents in the Purified Mitochondrial Fraction during Germination*

The purified mitochondrial fraction was obtained by collecting Fraction 5–20 (in the case of the cotyledons at the 3-hr age) or Fraction 10–25 (in the case of the cotyledons at the 18-hr age) of Figure 4. Each value is the average of two separate experiments.

Germination Time	Protein	Lipid Phosphorus
hr	*mg/g dry seed weight*	*μg/g dry seed weight*
3	3.20	17.9
18	5.70	32.7

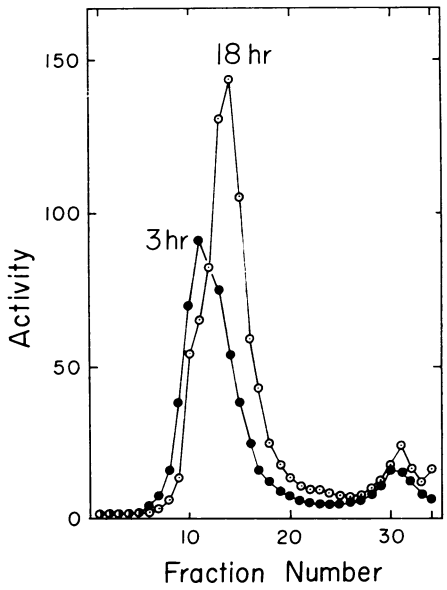

FIG. 6. Comparison of the position of the mitochondrial peaks after sucrose density gradient centrifugation between cotyledons of pea seeds germinated for 3 and 18 hr. The distribution of malate dehydrogenase activity after the centrifugation was shown. The activity was expressed as decrease in absorbance at 340 nm per min per 0.5 ml.

of the gradient in the cases of the dry seeds and the seeds imbibed for 3 hr (Figs. 4 and 5). A peak of the content appeared as germination proceeded (Fig. 4). The base line of protein distribution was considerably high in all cases. Omission of BSA from the washing medium did not reduce the base line. Therefore, real mitochondrial protein could not be determined. However, it was evident that the protein increased during germination. The increase in the protein seemed to follow that in cytochrome oxidase activity and to occur in parallel with that in the respiratory activity of the mitochondrial fraction. Fractions in the major peak of cytochrome oxidase were collected and assayed for protein and phospholipid content. About an 80% increase in the content was observed during germination for 15 hr after the first 3 hr (Table II).

There were slight differences in the position of the major peak of malate dehydrogenase or cytochrome oxidase among the mitochondrial fractions prepared from the different ages of seeds. Mitochondria seemed to become lighter as germination proceeded. As shown in Figure 6, the mitochondrial peak was at Fraction 11 in the case of the seeds imbibed for 3 hr but at Fraction 14 in the case of the seeds imbibed for 18 hr. Similar results were observed with germinating mung bean seeds.

DISCUSSION

The rate of oxygen uptake by cotyledons rises markedly during germination. The rise in bean cotyledons proceeds for 3 to 5 days in two phases: a rapid rise as the result of hydration in the early stage of germination and a slower one in the later stage (14). An apparent correlation between water content and oxygen uptake in the early stage of germination was also observed in the present study with pea cotyledons. The data presented here show that the hydration induces a rapid development of mitochondria including increases in their activities and components.

It has been demonstrated by electron microscopic studies that there are poorly differentiated mitochondria in dormant cotyledon cells (1–3, 9). The present study indicates the presence of peculiar mitochondria in dry seeds, which have little respiratory activity. Some of them did not contain malate dehydrogenase (Fig. 5). The fact that the particles contained a small amount of protein supports the electron microscopic observation. It is very possible that the structure of mitochondria in dry seeds is very unstable and thus malate dehydrogenase is released from the particles during isolation.

As imbibition proceeds, mitochondria become rich in protein and lipid and active in biological function. Moreover, their structure seems to become stable during imbibition. These results suggest a maturation of mitochondria, that is, a growth of vesicular particles to functional mitochondria during imbibition. A shift in the density of mitochondria (Fig. 6) may support the hypothesis. There seem to be two phases in the development of mitochondria during imbibition. In the first phase (the first 3 hr) enzymatic activities in mitochondria increase rapidly. In the second phase, there are increases in respiratory activity and protein and lipid contents. It remains unknown as to whether the development of mitochondria during imbibition involves an increase in the number of particles.

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