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Structural Development during Germination of Different Populations of Mitochondria from Pea Cotyledons[1]

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ABSTRACT

The crude mitochondrial fraction from pea cotyledons can, from days 1 to 7 of germination, be separated into three fractions by sucrose density gradient centrifugation. When seeds were grown in water (control) or cycloheximide (120 micrograms per milliliter of medium) for 4 days, the originally different populations of mitochondria acquired a uniform density and separated together in band 1 (density, 1.205 grams per milliliter). The oxidative and phosphorylative activities of mitochondria obtained from 4-day-old control and 4-day-old cycloheximide-treated pea seeds were the same. However, mitochondria from pea seeds that were grown in D-*threo*-chloramphenicol (1.5 milligrams per milliliter of medium) or erythromycin (0.5 milligram per milliliter of medium) for 4 days separate into three bands (fully developed mitochondria in the top band [band 1] and partially developed mitochondria in the lower two bands [bands 2 and 3]). Separation patterns and oxidative and phosphorylative activities were the same for mitochondria separated from 4-day-old cotyledons treated with D-*threo*-chloramphenicol or erythromycin and from 1-day-old cotyledons grown in water. This indicated that these inhibitors prevented the partially developed mitochondria originally in bands 2 and 3 from developing further. In contrast, cycloheximide did not seem to interfere with the mitochondrial structural development. These results along with those obtained from the experiments on the effects of D-*threo*-chloramphenicol, erythromycin, and cycloheximide on ^{14}C-leucine incorporation into mitochondrial membrane proteins suggest that the increase in mitochondrial activity during germination may be a result of structural development (membrane synthesis) in pre-existing mitochondria.

The respiratory activity of cotyledons from a variety of seeds has been shown to increase during the early stages of germination (1, 2, 4, 5, 20). Many investigators have attributed this increase to the *de novo* synthesis of mitochondria in the cotyledons of germinating seeds (4, 5, 20).

On the basis of cytological evidence, Bain and Mercer suggested that pea cotyledon mitochondria not only increase in number but also undergo further structural development during the first 5 days of germination (2). The present work was undertaken to study the effects of protein synthesis inhibitors on the structural development and biochemical properties of mitochondria.

Pea seeds were grown with and without cycloheximide, D-*threo*-chloramphenicol, or erythromycin. By means of sucrose step density gradient centrifugation, different populations of mitochondria were separated on the basis of their structural development. Parameters of mitochondrial activity were determined on days 1 and 4 of germination. Also the effects of D-*threo*-chloramphenicol, erythromycin, and cycloheximide on ^{14}C-leucine incorporation into mitochondrial membrane proteins were determined in order to find out whether the increase in mitochondrial activity during germination was mainly a result of structural development (membrane synthesis) in pre-existing mitochondria.

MATERIALS AND METHODS

Pea seeds (*Pisum sativum* L. var. Homesteader) were surface-sterilized with 0.5% (w/v) sodium hypochlorite for 15 min, then thoroughly washed and soaked in sterilized distilled water for 4 hr.

Inhibitor Treatment and Preparation of Different Fractions of Subcellular Particles. One hundred seeds of uniform appearance after they were soaked in water for 3 hr were placed between two filter papers that had been soaked with 50 ml of either water or the inhibitor solution (cycloheximide, 120 μg/ml of medium; D-*threo*-chloramphenicol, 1.5 mg/ml of medium; or erythromycin, 0.5 mg/ml of medium). The solutions were almost all taken up by the seeds at the end of day 1. On the 2nd and 3rd days after planting, the seeds were irrigated with another 25 and 15 ml, respectively, of the above mentioned solutions. On either day 1 or day 4 after planting, the seeds were peeled, and the crude mitochondrial pellet was isolated from the cotyledons and fractionated into different populations by sucrose step density gradient centrifugation (18). (The crude mitochondrial pellet has been shown to separate into three bands when subjected to these procedures.) The different bands of subcellular particles thus obtained (band 1, density 1.2050 g/ml; band 2, density 1.2331 g/ml; and band 3, density 1.3017 g/ml) were tested for mitochondrial respiratory activity as reported earlier (18). Mitochondrial protein was determined according to the method of Lowry *et al.* (11).

Incorporation of ^{14}C-Leucine into Mitochondrial Structural Proteins. One hundred partially imbibed seeds (imbibed in water for 3 hr) were transferred to 1-liter sterile conical flasks containing either 25 ml of ^{14}C-leucine solution (control) or 25 ml of ^{14}C-leucine solution and one of the following inhibitors: D-*threo*-chloramphenicol (1.5 mg/ml of medium), erythromycin (0.5 mg/ml of medium), cycloheximide (120 μg/ml). (The activity of the solution varied from 250 $\times$ 10^3 to 250 $\times$ 10^4 cpm/25 ml.) After 5 hr, 25 ml more of water or inhibitor solu-

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tion were added. After a further 19 hr, the seeds were placed between filter papers that had been soaked with the corresponding inhibitor solutions and allowed to germinate for a further 72 hr in the dark in the conical flasks. On day 4 after planting, the seeds were peeled, and the mitochondria were isolated by sucrose step density gradient centrifugation as described previously (18). The structural proteins of mitochondria were prepared by the method of Criddle, Bock, Green, and Tisdale (6). Mitochondria were treated at 0 C with deoxycholate (2 mg/mg of protein), cholate (1 mg/mg of protein), and 0.75 mg of SDS per mg of protein. After centrifugation at 40,000*g* to remove the residue (protein-free), enough solid $Na_2S_2O_4$ was added to reduce the cytochromes: then solution was brought to 12% saturation with respect to ammonium sulfate (pH kept at 7.0, and temperature 0–5 C). The resulting white precipitate was then treated with butanol (20% by volume) in the presence of 20% saturated ammonium sulfate and deoxycholate (1 mg/mg of protein) to remove lipids. The extracted precipitate was washed in 0.25 M sucrose and was then extracted with 10 volumes of 75% methanol at 50 C 10 to 20 times to remove deoxycholate.

The mitochondrial pellet or the structural proteins were then treated with cold trichloroacetic acid solution to give a final trichloroacetic acid concentration of 10% (w/v). The pellet obtained by centrifugation was dissolved in 1 to 2 ml of 1 N NaOH solution and again precipitated with 10% trichloroacetic acid and centrifuged. The pellet was washed once with 5% cold trichloroacetic acid and finally taken up in 1 to 2 ml of 1 N NaOH solution. In order to secure complete solution of the pellet, the mixture was warmed in a water bath (75–80 C) for 15 min. An aliquot of 0.2 ml of this solution was transferred to a vial containing 10 ml of liquid scintillation mixture (384 ml of dioxane, 384 ml of xylene, 231 ml of absolute ethanol, 80 g of naphthalene, 5 g of PPO, and 100 mg of POPOP) and counted by use of a Nuclear-Chicago liquid scintillation counter.

Chemicals. Cyclohemixide and erythromycin were from Sigma and D-*threo*-chloramphenicol was from Calbiochem Laboratories. PPO and POPOP were obtained from Amersham/Searle. Other chemicals were of analytical grade, from Fisher Scientific Company.

RESULTS AND DISCUSSION

The number of bands of different populations of mitochondria and other subcellular particles obtained from pea cotyledons after sucrose density gradient centrifugation depends upon the stage of germination (18). At the early stages of germination (days 1–7), the pea cotyledon crude mitochondrial fraction separates into three bands. On day 1, the top band (band 1, density 1.205 g/ml) contains mainly mitochondria. The middle band (band 2, density 1.2331 g/ml) contains mitochondria, peroxisome-like structures and protein bodies. The bottom band (band 3, density 1.3017 g/ml contains mainly peroxisome-like structures, protein bodies, and some mitochondria. On day 4 of germination, pea cotyledon mitochondria acquire a uniform density of 1.205 g/ml. Bands 2 and 3 consist of only peroxisome-like structures and protein bodies. Regardless of age of the tissue, band 1 (density, 1.205 g/ml) always contains fully developed mitochondria without any contamination (18).

Effects of Inhibitors on Respiratory Activities and Protein Content of Mitochondria. In the control experiment, on day 1, all three bands showed O_2 utilization (Table I). However, only bands 1 and 2 showed ADP control. On day 4 (control experiment), all three bands showed O_2 utilization (although that of bands 2 and 3 was low), and only band 1 showed ADP control (Table I). Our results (in preparation) based on ^{14}C-leucine

Table I. *Effects of Cycloheximide,* D-*threo-Chloramphenicol, and Erythromycin on the Respiratory Activities (Ability to Oxidize Succinate) of Three Different Populations of Particles Obtained from the Crude Mitochondrial Preparation from Germinating Pea Seeds*

One hundred partially imbibed seeds (imbibed in water for 3 hr) were allowed to germinate in conical flasks containing 25 ml of either water (control) or solutions of cycloheximide (120 μg/ml), D-*threo*-chloramphenicol (1.5 mg/ml), erythromycin (0.5 mg/ml), or cycloheximide and D-*threo*-chloramphenicol together (above concentrations). After 5 hr, 25 ml more of the respective solution were added. After a further 19 hr, the seeds were placed between the filter papers that had been previously soaked with the respective solutions, and allowed to grow for another 3 days. After either 1 day or 4 days a crude mitochondrial pellet was prepared and fractionated by use of sucrose density gradient centrifugation as described in "Materials and Methods." For measurement of succinate oxidation, the concentrations, in a final volume of 3.2 ml, were: 0.3 M mannitol, 4 mM $MgCl_2$, 0.75 mg/ml bovine serum albumin, 0.05 M TES, and 8 mM succinate (pH 7.2) at 25 C. The reaction was run at 25 C. The data in this table are representative of several runs, and the variation between the runs was minimal.

Day after Planting	Treatment	Band No.	RCR	ADP:O	O_2 Utilization of State 3	Total Protein
					nmoles O_2/min·mg protein	*mg*
1	Control	1	2.1	1.0	34	16.9
		2	1.9	1.0	26	5.3
		3	0	0	8	5.1
4	Control	1	3.4	1.3	38	21.2
		2	0	0	5	4.2
		3	0	0	6	3.9
4	Cycloheximide	1	3.2	1.1	32	20.9
		2	0	0	6	4.8
		3	0	0	6	5.7
4	D-*threo*-Chloramphenicol	1	2.0	1.2	30	17.8
		2	1.7	1.2	18	4.9
		3	0	0	6	5.1
4	Cycloheximide + D-*threo*-chloramphenicol	1	2.3	1.2	35	15.5
		2	2.0	1.1	23	5.1
		3	0	0	0	5.6
4	Erythromycin	1	2.3	1.1	25	18.4
		2	2.0	1.1	11	5.2
		3	0	0	6	5.5

incorporation studies in the presence of protein synthesis inhibitors suggest that the mitochondria that are present originally in bands 2 and 3 go through structural changes and then move up to band 1 during a later stage of germination (day 4). Table I also shows that the total mitochondrial protein on days 1 and 4 remained about the same. On day 4, there was a decrease in the total protein content of bands 2 and 3 whereas the protein content of band 1 increased, suggesting disappearance of mitochondria from band 2 and their movement to band 1. For both days 1 and 4, membranes of mitochondria in bands 2 and 3 are probably not fully developed. Sucrose enters these mitochondria and makes them heavier. They will thus settle at a higher density than the fully developed mitochondria in band 1.

The concentration of cycloheximide (120 μg/ml) that was used in these studies was such that it inhibited germination completely during treatment but permitted normal germination

if removed after 4 days of treatment. D-*threo*-Chloramphenicol at a concentration of 1.5 mg/ml and erythromycin at a concentration of 0.5 mg/ml only slightly retarded germination. These are concentrations that had no effect on cytoplasmic protein synthesis but partially inhibited mitochondrial protein synthesis (unpublished data).

Effects of Inhibitors on Oxidative and Phosphorylative Activities of Mitochondria. In higher plants, in addition to their effects on protein synthesis, antibiotics such as cycloheximide and chloramphenicol can, at certain concentrations, affect energy transfer and processes (such as ion uptake) dependent upon them (8, 13). In order to obtain a meaningful interpretation of the results, one must establish whether the effects of antibiotics on a particular tissue are mediated *via* inhibition of protein synthesis or by interference with energy transfer. To do this, the effects of the inhibitors on the oxidative and phosphorylative abilities of the mitochondira were determined. The seeds were treated with inhibitors in the same way as described above. It was found that none of these inhibitors had any appreciable effect on oxidative and phosphorylative activities of mitochondria (Table II).

Ellis (8) has reported that only if the effect (inhibition of protein synthesis) is produced specifically by the D-*threo* isomer of chloramphenicol should an interpretation directly involving protein synthesis be invoked. In the present studies, only D-*threo* isomer of chloramphenicol was used.

Effects of Inhibitors on Mitochondrial Development. In *in vitro* experiments, cycloheximide has been shown to inhibit cytoplasmic protein synthesis in peanut cotyledons (7) and peas (10) and to have no effect on the protein synthesis of uncontaminated soybean hypocotyl mitochondria (3). However, in *in vivo* experiments, cycloheximide inhibited yeast mitochondrial protein synthesis by about 90% (19). (The remaining protein synthesized in yeast mitochondria was considered to be directly under the control of mitochondrial protein-synthesizing machinery.) D-*threo*-Chloramphenicol, on the other hand, inhibited the protein synthesis only within the mitochondria (from sterile beet discs [9] and sterile soybean hypocotyl [3]) and had no effect on the cytoplasmic protein synthesis of peanut cotyledons (14) and castor bean embryos (15).

It has been suggested that yeast mitochondrial DNA contains information for the synthesis of at least 10 to 15% of the total mitochondrial membrane proteins (16, 19). Table I shows that on day 4 of germination of pea seeds in cycloheximide solution, the amounts of protein(s) in the three bands were very similar to those obtained from day 4 control. Moreover, RCR,[2] ADP:O, and O_2 utilization of three different populations of mitochondria obtained from 4-day-old germinating cotyledons in cycloheximide were the same as those from the day 4 control. These results suggest that cycloheximide had no inhibitory effect on the membrane protein synthesis in mitochondria. Under these conditions, mitochondria from bands 2 and 3 may undergo normal membrane protein synthesis and, therefore, move up to their newly acquired sucrose equilibrium density of "mitochondria proper" (fully developed mitochondria). This reasoning is supported by the protein data (Table I).

On the other hand, D-*threo*-chloramphenicol and erythromycin, potent inhibitors of mitochondrial membrane protein synthesis, kept the mitochondria in bands 2 and 3 from further development and thus prevented them from acquiring the sucrose equilibrium density of fully developed mitochondria (Table I). In the presence of D-*threo*-chloramphenicol and erythromycin, the protein content, RCR, and ADP:O values of mitochondria from 4- and 1-day-old cotyledons were about the same (Table I), suggesting that D-*threo*-chloramphenicol and erythromycin inhibit membrane protein synthesis of mitochondria. This prevention of further structural development of mitochondria from bands 2 and 3 in the presence of D-*threo*-chloramphenicol and erythromycin may be the main reason that they fail to acquire the uniform sucrose equilibrium density of 1.205 on day 4. The mitochondria will, therefore, have three different populations or densities as long as the cotyledons are grown in D-*threo*-chloramphenicol or erythromycin solutions. When cycloheximide and D-*threo*-chloramphenicol both were used together, results similar to those with D-*threo*-chloramphenicol alone were obtained. This behavior was noticed even on day 8, provided the seeds were grown continuously in the presence of D-*threo*-chloramphenicol.

To explore further the above results, the effects of D-*threo*-chloramphenicol, erythromycin (inhibitors of mitochondrial protein synthesis), and cycloheximide (inhibitor of cytoplasmic protein synthesis) on ^{14}C-leucine incorporation into mitochondrial membrane proteins were studied. D-*threo*-Chloramphenicol and cycloheximide were used at the same concentrations as discussed above. Table III shows that some of the mitochondrial proteins are synthesized *de novo* during the first 4 days of germination. About 60 to 63% of the total *de novo* synthesis of mitochondrial proteins occurred in the mitochondrial structural membranes (Table III). When the pea seeds were grown in D-*threo*-chloramphenicol (1.5 mg/ml medium) or erythromycin (0.1 mg/ml medium), there was about 16 to 20% inhibition of ^{14}C-leucine incorporation into mitochondrial membrane proteins (Table IV). On the other hand, when the seeds were grown in cycloheximide (120 μg/ml), there was only

Table II. *Effects of Cycloheximide, D-threo-Chloramphenicol, and Erythromycin on Oxidative Phosphorylation and Respiration of the Mitochondrial Fraction Obtained from Cotyledons of 4-day-old Pea Seeds*

Experimental conditions were the same as those described in Table I, except that succinate was replaced by 8 mM α-ketoglutarate, 5 mM malonate, and 70 mM TPP. Erythromycin was used at a concentration of 0.5 mg/ml medium. After the inhibitor treatment, the seeds were removed from the flask, washed with distilled water, and used for the preparation of the mitochondrial fraction (5,000*g* spin for 5 min followed by 20,000*g* spin for 10 min).

Experiment	Mitochondrial Preparation	Inhibitor	RCR	ADP:O	State 3 Oxygen Uptake
					nmoles O_2/min·mg protein
1	1	Control	4.05	2.6	20.7
	2	Cycloheximide	4.00	2.7	20.2
	3	D-*threo*-Chloramphenicol	4.15	2.8	19.2
2	4	Control	4.3	2.7	20.8
	5	Cycloheximide	4.25	2.7	21.4
	6	D-*threo*-Chloramphenicol	4.2	2.7	21.1
3	7	Control	4.65	2.6	26.5
	8	Erythromycin	4.8	2.6	27.0
4	9	Control	4.3	2.8	21.0
	10	Erythromycin	4.18	2.7	20.5

[2] Abbreviations: RCR: respiratory control ratio (rate of oxygen utilization when ADP is present/rate of oxygen utilization when ADP is limiting); ADP:O: adenosine diphosphate to oxygen ratio (μmoles of ADP esterfied/μatoms of oxygen consumed); TES: *N-tris*(hydroxymethyl)methyl-2-aminoethane sulfonic acid; SDS: sodium dodecylsulfate; TPP: thiamine pyrophosphate.

Table III. *Incorporation of ^{14}C-Leucine into Mitochondria and Mitochondrial Membrane Proteins of Germinating Pea Seeds*

One hundred partially imbibed seeds (imbibed in water for 3 hr) were allowed to germinate in conical flasks containing 25 ml of ^{14}C-leucine solution. After 5 hr, 25 ml of water were added. After a further 19 hr the seeds were placed between filter papers that had been previously soaked with water and were allowed to grow for another 72 hr. After a total of 4 days, the cotyledons were peeled, mitochondria (band 1) were isolated, and the incorporation of ^{14}C-leucine into mitochondrial membrane proteins was determined as discussed in "Materials and Methods." Each experiment was run with different mitochondrial preparations.

Experiment	Radioactivity Recovered in Mitochondria	Radioactivity Recovered in Mitochondrial Membrane Proteins	Radioactivity Taken up by Mitochondria That Was Recovered in Mitochondrial Membrane Proteins
	cpm/100 seeds	*cpm*	%
1	1485	940	63.4
2	1259	755	60.0
3	815	515	63.2

Table IV. *Effects of D-threo-Chloramphenicol, Erythromycin, and Cycloheximide on the Incorporation of ^{14}C-Leucine into Mitochondrial Membrane Proteins from Cotyledons of Pea Seeds*

One hundred partially imbibed seeds (imbibed in water for 3 hr) were transferred to conical flasks containing either 25 ml of ^{14}C-leucine solution (control) or 25 ml of ^{14}C-leucine solution and one of the following inhibitors: D-*threo*-chloramphenicol (1.5 mg/ml), erythromycin (0.5 mg/ml), cycloheximide (120 μg/ml). After 5 hr, 25 ml more of water or respective inhibitor solution were added. After a further 19 hr, the seeds were placed between filter papers that had been previously soaked with the respective solutions and were allowed to grow for another 72 hr. After a total of 4 days the seeds were peeled, mitochondria were isolated, and the incorporation of ^{14}C-leucine into mitochondrial (band 1) membrane proteins was determined as described in "Materials and Methods."

Experiment	Mitochondrial Preparation	Treatment	Radioactivity Recovered in Mitochondrial Membrane Proteins	Inhibition of ^{14}C-Leucine Incorporation
			cpm/100 seeds	%
1	1	Control	340	
	2	D-*threo*-Chloramphenicol	285	16.2
2	3	Control	307	
	4	D-*threo*-Chloramphenicol	251	18.4
3	5	Control	415	
	6	D-*threo*-Chloramphenicol	335	19.0
4	7	Control	329	
	8	Erythromycin	271	17.6
5	9	Control	350	
	10	Erythromycin	281	19.7
6	11	Control	287	
	12	Erythromycin	233	19.8
7	13	Control	262	
	14	Cycloheximide	248	5.3

about 5% inhibition of ^{14}C-leucine incorporation into mitochondrial membrane proteins (Table IV). D-*threo*-Chloramphenicol and erythromycin had no effect on the incorporation of ^{14}C-leucine into soluble mitochondrial proteins, whereas cycloheximide inhibited this incorporation by about 90%. These results support our previous findings (18) that a certain amount of mitochondrial membrane proteins are newly synthesized during the first 4 days of germination. These proteins appear to be very important for the development of the full respiratory and phosphorylation capacities.

The mitochondria that are initially present in bands 2 and 3 move to band 1 during a later stage of germination (18). Our present work suggests that this behavior results from structural development of the mitochondria originally present in bands 2 and 3. This concept is supported by the electron microscopic studies of Lund, Vatter, and Hanson (12), who suggested that there are two types of mitochondria in the roots of *Zea mays*. As the cells enlarge, mitochondria regarded as immature could no longer be seen, but only mitochondria with well developed cristae (12). The development of these cristae was found to be associated with an increase in respiration of the tissue as well as with increased rates of oxidation and phosphorylation of isolated mitochondria. As the cells grow and mature, mitochondria make up an increasing percentage of the total cytoplasmic protein (12). Similar results were obtained by Simon and Chapman (17), who reported that at the earliest stage of *Arum* spadix development there was an average of 9 sections of cristae per mitochondrion. In later stages of development, the number of cristae rose to 22.

It is concluded from our results that the increase in mitochondrial activities during early stages of pea seed germination may be mainly a result of further structural development of pre-existing mitochondria in dormant seeds.

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