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Biochemical Studies on Development of Mitochondria in Pea Cotyledons during the Early Stage of Germination

EFFECTS OF ANTIBIOTICS ON THE DEVELOPMENT

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ABSTRACT

L-Leucine-U-^{14}C was incorporated into mitochondrial protein in pea (*Pisum sativum* var. Alaska) cotyledons during the imbibing stages. Incorporation was almost completely inhibited by cycloheximide but not by chloramphenicol. Both antibiotics did not affect increases in mitochondrial activities and components of the cotyledons during imbibition. Therefore, mitochondrial development seems to be achieved by a transfer of protein pre-existing in the cytoplasm into the mitochondria rather than by *de novo* synthesis of mitochondrial protein. Cycloheximide stimulated an increase in bile salt-soluble protein of mitochondria in imbibing pea cotyledons. The recovery of cytochrome oxidase activity after sucrose density gradient centrifugation was enhanced, and the morphological properties of mitochondria were altered by cycloheximide.

When water is supplied to dry seeds, a marked increase occurs in their respiratory activity. A number of studies have demonstrated that mitochondrial development takes place in the cotyledons or endosperms during the later stages of germination (1, 3-5, 8, 9, 13, 22). A rapid increase in mitochondrial activity was also observed during the early stages of germination of pea seeds (34). In a previous paper (20), we reported a rapid development of mitochondria including increases in their components and activities in the cotyledons of pea seeds during the imbibing stages. The structure of mitochondria seems to be stabilized as hydration proceeds. Recently, similar results were reported by Solomos *et al.* (31).

Our studies have been expanded in an attempt to elucidate the mechanism of development of pea cotyledon mitochondria during the imbibing stages. The purpose of the study described in this paper was to determine if the development involves the *de novo* synthesis of mitochondrial protein. The synthesis of mitochondrial protein is the result of two systems controlled by different genes; one is the cytoplasmic protein-synthesizing system controlled by nuclear genes, and the other is a mitochondrial protein-synthesizing system. The former is inhibited by CHI[1] and the latter by CAP (2, 25, 30, 32, 33). This paper deals with the effects of the antibiotics on the development of mitochondria in pea cotyledons during the imbibing stages. Our study would suggest that development does not require a *de novo* synthesis of mitochondrial protein but seems to be achieved by a transfer of pre-existing cytoplasmic protein to immature mitochondria.

[1] Abbreviations: CHI: cycloheximide; CAP: D-threochloramphenicol; SDGC: sucrose density gradient centrifugation; RCR: respiratory control ratios.

MATERIALS AND METHODS

Plant Material. Surface-sterilized pea seeds (*Pisum sativum* var. Alaska) were germinated in the dark at 28 C in Petri dishes containing deionized water or antibiotic solution (1 or 0.5 mM CHI, 5 or 1 mM CAP) 2.5 times that of the dry seed weight. The cotyledons were taken at the appropriate age, washed with water, and used as the experimental material. L-Leucine-U-^{14}C was supplied to the seeds by germinating them in the isotopic solution (484×10^4 dpm/2 g of dry seeds). After germination for the required time, the seeds were washed twice with 50 ml each of 2 mM unlabeled leucine solution (pH 7.0) for 30 min at 2 C with continuous stirring, and then the cotyledons were taken for the experimental material.

Preparation of Purified Mitochondrial Particles. Crude mitochondrial fraction was prepared from the cotyledons by differential centrifugation as described previously (20). The crude mitochondrial fraction was further fractionated to prepare purified mitochondrial particles by two methods.

Method I. Crude mitochondrial fraction in 1.5 ml of a medium, which was composed of 10 mM potassium phosphate buffer (pH 7.2), 0.7 M mannitol, 1 mM EDTA, and 0.1 % (w/v) bovine serum albumin, was layered on a linear sucrose density gradient (32.5-65.0 %, w/v), and the whole was centrifuged and fractionated as described previously (20).

Method II. Crude mitochondrial fraction in 1.5 ml of the medium described above was layered on a discontinuous sucrose density gradient, which was composed of 5.5 ml of 60.0 %, 5.5 ml of 45.0 %, and 5.0 ml of 32.5 % (w/v) sucrose solutions. The whole was centrifuged at 24,000 rpm for 3 hr in a Hitachi RPS 25-3A rotor at approximately 4 C. Mitochondrial fraction at the interface, between 45.0 and 60.0 % sucrose solutions, was collected (3.0 ml), and its sucrose concentration was adjusted to about 32.5 % with the medium described above. The mitochondrial fraction was layered on 13 ml of a linear sucrose density gradient (35.0-60.0 or 65.0 %, w/v), and the whole was centrifuged at 24,000 rpm for 5 hr in a Hitachi RPS 25-3A rotor at approximately 4 C. Then, the gradient was separated into 0.5-ml fractions.

Fractionation of Mitochondrial Protein. The mitochondrial protein was fractionated into soluble, bile salt-soluble, and bile salt-insoluble protein fractions by the method of Sakano and

Asahi (28) with some modifications. A suspension of purified mitochondrial particles in 0.1 M NaCl, of which the sucrose concentration was adjusted to about 0.25 M, was sonicated with a sonicator device (Ohtake Works, Tokyo) at 20 kc for 60 sec. The sonicate was centrifuged at about 80,000*g* for 2 hr, and the supernatant was designated as soluble protein. The resulting precipitate was suspended in an aliquot of 0.1 M NaCl containing sodium cholate and sodium deoxycholate (3 and 6 mg/mg protein, respectively), and then the suspension was allowed to stand overnight at room temperature with occasional stirring. The insoluble material was separated by centrifuging the suspension at 80,000*g* and designated as the bile solt-insoluble protein. The supernatant was termed the bile salt-soluble protein.

Assays. Respiratory activity of intact cotyledons or crude mitochondrial fraction and enzymic activities were determined as described previously (20). RCR were calculated according to the method of Chance and Williams (11, 12). Protein was determined by the method of Lowry *et al.* (17). The amount of phospholipid was estimated from the phosphorus content in the lipid fraction extracted by the method of Folch *et al.* (15).

Radioactivity in the protein was determined by the method described by Sakano and Asahi (27) except that the protein solution in 0.3 ml of Hyamine 10X was mixed with 10 ml of Bray's scintillator solution (7). In case of components other than protein, aliquots of the sample solutions were mixed with 10 ml of Bray's scintillator solution, and the radioactivities were measured.

Electron Microscopy. Particles of the mitochondrial fraction purified by method I were collected by centrifugation and then transferred to 0.2 M phosphate buffer, pH 7.2, containing 5 % (v/v) glutaraldehyde and 0.5 M sucrose, and then the suspension was left for 1 hr at 0 C. The fixed material was washed by gentle decantation in three changes of the same buffer, after which it was transferred to 0.2 M phosphate buffer, pH 7.2, containing 1 % (w/v) OsO_4 and 4.6 % (w/v) sucrose, and left for 1 hr at 0 C. The fixed material was dehydrated in graded concentrations of ethanol and acetone. The sample was submerged in a solution of Epon mixture in absolute acetone for 3 hr at room temperature, then buried in the Epon mixture. A thin section was cut with a glass knife using a Japan Electron Optics Laboratory JUM 5A microtome. The section was stained in lead citrate (24) and observed with a Japan Electron Optics Laboratory EM-TS-7 microscope.

RESULTS

Incorporation of L-Leucine-U-^{14}C into the Mitochondrial Protein. When pea seeds were germinated in a solution of L-leucine-U-^{14}C, the cotyledons absorbed the radioisotope in parallel with water uptake (Fig. 1). Labeling of postmitochondrial protein set in after a lag phase and thereafter proceeded linearly (Fig. 1). Figure 2 shows the distribution of labeled protein after a linear SDGC of crude mitochondrial fraction. A peak of label at the middle part of the gradient corresponded in position to that of cytochrome oxidase activity. The onset of labeling of mitochondrial protein also showed a lag phase, and extensive labeling of the protein began after imbibing for 3 hr.

Effect of Antibiotic Treatment on Incorporation of L-Leucine-U-^{14}C into Mitochondrial Protein. No detectable change

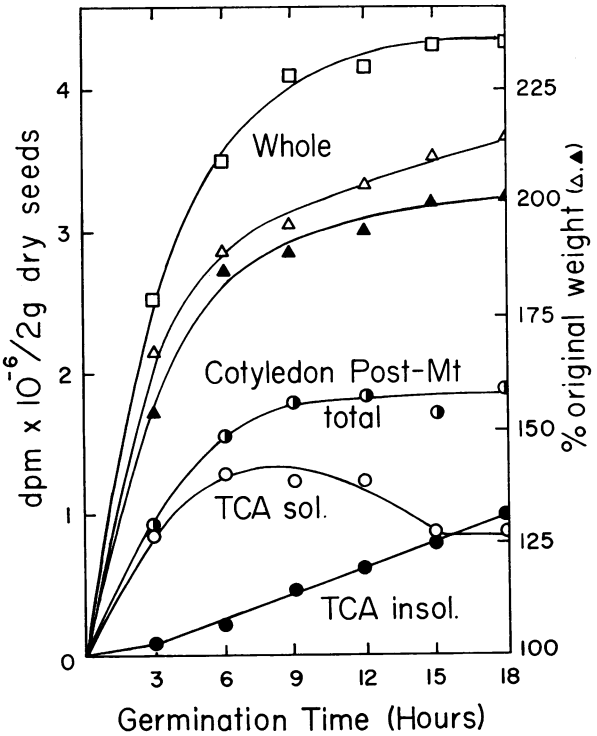

FIG. 1. Changes in L-leucine-U-^{14}C distribution in pea cotyledons during 18 hr of imbibition. The amounts of radioisotope taken up by whole seeds (□: whole) were calculated from differences between administrated and unabsorbed radioactivities. Postmitochondrial fraction (Post-Mt) is the supernatant obtained after centrifugation at 25,000*g* for 30 min. ◑: Radioactivity in total Post-Mt fraction; ○: radioactivity in trichloroacetic acid (TCA)-soluble materials in Post-Mt fraction; ●: radioactivity in TCA-insoluble materials in Post-Mt fraction; △: increase in fresh weight of whole seeds; ▲: increase in fresh weight of cotyledons.

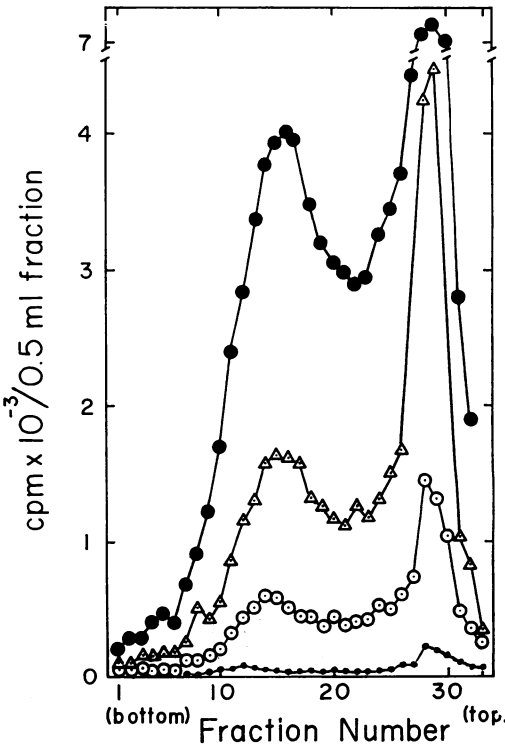

FIG. 2. Distribution of protein labeled with L-leucine-U-^{14}C after a linear SDGC of crude mitochondrial fraction prepared from germinating pea cotyledons. Crude mitochondrial fractions were prepared from cotyledons obtained from 2 g of dry seeds after imbibing for the hours indicated and subjected to SDGC as described in method I. ·: Imbibed for 3 hr; ⊙: imbibed for 6 hr; △: imbibed for 9 hr; ●: imbibed for 18 hr.

Table I. *Effect of Antibiotic Treatment on Incorporation of L-Leucine-U-^{14}C into Protein of Pea Cotyledons during Imbibition for 18 hr*

Postmitochondrial fraction is the supernatant obtained after centrifugation at 25,000*g* for 30 min.

Treatments	Radioactivity			
	Uptake by whole seeds	Postmitochondrial fraction		Crude mitochondrial fraction
		Trichloroacetic acid		
		Soluble	Insoluble	
	dpm × 10^{-3}/2 *g dry seeds*			
Water	4307	1338	733	64
CHI, 0.5 mM	4313	3029	40	5
CAP, 1 mM	4284	1276	936	56

in water uptake by pea cotyledons was detected, when either CHI or CAP was supplied to the seeds. Neither CHI nor CAP affected the uptake of L-leucine-U-^{14}C by pea seeds during germination for 18 hr (Table I). Labeling of protein in either the postmitochondrial or crude mitochondrial fraction of the cotyledons was almost completely inhibited by CHI but not by CAP. CHI but not CAP treatment caused an accumulation of labeled compounds in the trichloroacetic acid-soluble fraction. Figure 3 shows the effect of CHI on the distribution of labeled protein after a second SDGC in method II. Labeling of mitochondrial protein, even at the 3-hr imbibition stage, was almost completely inhibited by CHI.

Effect of Antibiotic Treatment on Increases in Mitochondrial Activities and Components during Imbibition. These results indicate that *de novo* synthesis of mitochondrial protein occurs in pea cotyledons during the imbibing stages. Therefore, experiments were designed to determine whether or not the newly synthesized protein contributes to increases in mitochondrial components and activities during imbibiton. CHI did not affect a marked increase in respiratory activity of intact cotyledons and crude mitochondrial fraction during imbibition. Increases in RCR and malate dehydrogenase and cytochrome oxidase activities of crude mitochondrial fraction during imbibition were also insensitive to CHI. Similar results were obtained with CAP.

Figure 4 shows the distributions of protein and of malate dehydrogenase and cytochrome oxidase activities after a linear SDGC of mitochondrial fraction purified partially by a discontinuous SDGC from pea cotyledons germinated for 18 hr (refer to method II in "Materials and Methods"). The mitochondrial fraction was contaminated with other particles even after two successive SDGC. However, it is apparent that CHI enhanced the increase in mitochondrial protein during imbibition. Fractions containing cytochrome oxidase and malate dehydrogenase were collected and analyzed for protein and phospholipid. CHI stimulated increases in mitochondrial phospholipid as well as in mitochondrial protein during imbibition (Table II). CAP did not influence the increases. Of mitochondrial protein, soluble, bile salt-soluble, and bile salt-insoluble proteins were increased by about 130, 40, and 70 %, respectively, during imbibition for 15 hr (from 3-hr imbibition stage to 18-hr imbibition stage) (Fig. 5A). CHI stimulated only the increase in bile salt-soluble protein (Fig. 5B).

Effect of CHI Treatment on Other Properties of Mitochondria. Mitochondria of pea cotyledons germinated in a CHI solution were located at a distinctly higher position in the sucrose gradient than those germinated in water after a linear SDGC

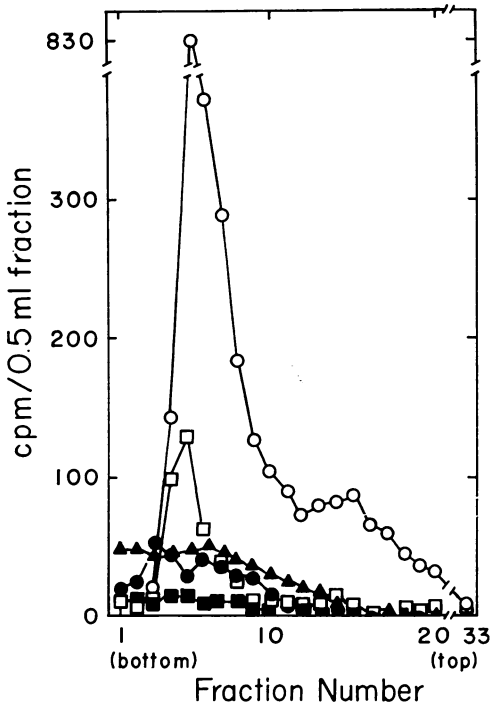

FIG. 3. Distribution of protein labeled with L-leucine-U-^{14}C after two successive SDGC of mitochondrial fractions prepared from pea cotyledons imbibed in water and in a CHI solution (0.5 mM). Mitochondrial fractions (equivalent to 2 g of dry seeds) purified by a discontinuous SDGC were subjected to a linear SDGC for 7 hr as described in method II. □: 3 hr in water; ■: 3 hr in a CHI solution; ○: 9 hr in water; ●: 9 hr in a CHI solution; ▲: 18 hr in a CHI solution.

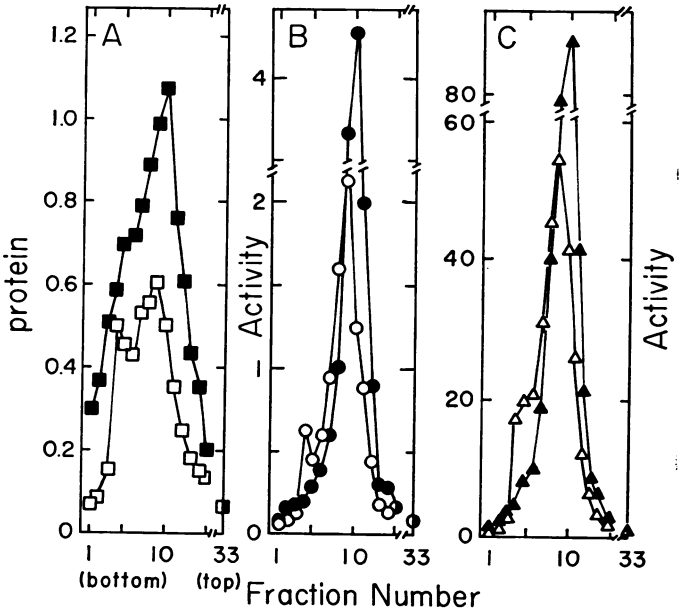

FIG. 4. Distribution of protein and cytochrome oxidase and malate dehydrogenase activities after two successive SDGC of mitochondrial fractions prepared from pea cotyledons germinated in water and in a 0.5 mM CHI solution for 18 hr. Mitochondrial fractions (equivalent to 2.5 g of dry seeds) purified by a discontinuous SDGC were subjected to a linear SDGC as for Figure 3. A: Protein (□: water; ■: CHI) in mg/0.5 ml; B: cytochrome oxidase (○: water; ●: CHI) expressed as decrease in absorbance at 550 nm/min·0.5 ml; C: malate dehydrogenase (△: water; ▲: CHI) expressed as decrease in absorbance at 340 nm/min·0.5 ml.

Table II. *Effect of Antibiotic Treatment on Increase in Mitochondrial Phospholipid and Protein*

Fractions containing cytochrome oxidase and malate dehydrogenase activities after the second SDGC of two successive SDGC (method II) were collected and analyzed for phospholipid and protein.

Treatments	Lipid Phosphorus	Protein
	μg/g dry seeds	*mg/g dry seeds*
3 hr water	6.6	1.21
18 hr water	10.7	1.88
18 hr CHI, 0.5 mM	16.3	3.40
18 hr CAP, 5 mM	11.2	1.77

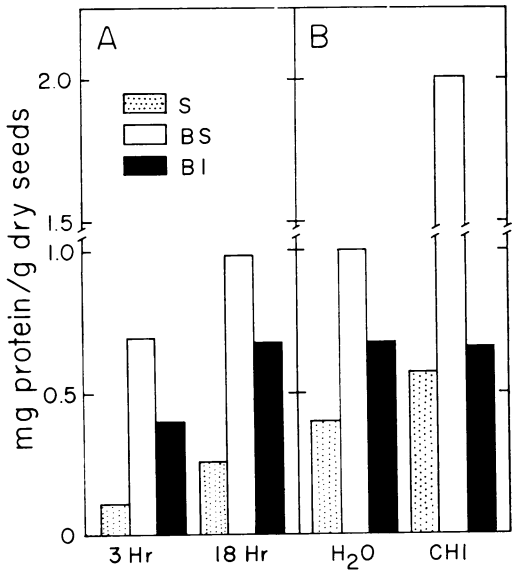

FIG. 5. Increases in soluble, bile salt-soluble, and bile salt-insoluble proteins of purified mitochondrial fraction during imbibition for 18 hr. A: Protein contents in pea cotyledons germinated in water for 3 and 18 hr; B: protein contents in pea cotyledons germinated in water and a CHI solution (0.5 mM) for 18 hr. Purified mitochondrial fractions were obtained as for Table II and subjected to the fractionation of mitochondrial protein as described in "Materials and Methods."

of crude mitochondrial fraction (Fig. 6). There was no significant difference in the position of mitochondria in the sucrose gradient between the cotyledons germinated in water and a CAP solution (Fig. 6). Both mitochondrial fractions from pea cotyledons germinated in water and a CHI solution were located at lower positions in sucrose gradient after second centrifugation of two successive SDGC as compared with the respective positions after a linear SDGC of crude mitochondrial fraction (compare Figs. 4 and 6). For instance, mitochondria of the cotyledons germinated in water were located at the region of about 52 % (w/v) sucrose in Figure 6, whereas they were at the region of about 55 % (w/v) sucrose in Figure 4. Even after two successive SDGC, however, mitochondria of pea cotyledons germinated in a CHI solution were located at a region of density lower than those of the cotyledons germinated in water (Fig. 4).

In addition, cytochrome oxidase of pea cotyledons germinated in water was greatly inactivated during SDGC, but that of CHI-treated cotyledons were affected only slightly. The recovery of cytochrome oxidase activity after SDGC in case of CHI-treated cotyledons was higher than that in other cases (Figs. 4 and 6).

Electron Microscopic Observation of Isolated Mitochondria. Figure 7 shows electron micrographs of the mitochondrial fractions purified from pea cotyledons germinated for 18 hr by method I. In members, the mitochondrion was the major organelle and existed in different conformational states. However, there were other membranous materials and particles. There was no detectable difference in the forms of mitochondria between mitochondrial fractions from pea cotyledons germinated in water and a CAP solution (Fig. 7, A and B, respectively). However, some of the mitochondria from CHI-treated cotyledons were in an unusual form with diffused electron-dense matrices. The mitochondria were not uniform in size, and some of them were very tiny (less than 0.3 μ in diameter). No significant difference in the extent of contamination with other particles among the mitochondrial fractions was detected.

DISCUSSION

The incorporation of labeled leucine into mitochondrial protein set in after a short lag phase in pea cotyledons during the imbibing stages. The failure of CAP to influence the incorporation indicates that the incorporation was not due to bacterial contamination. It is now evident from radioisotopic experiments in this study that *de novo* synthesis of mitochondrial protein occurs in the cotyledons during the imbibing stages. The fact that CHI inhibited almost completely the synthesis but CAP did not suggests that synthesis of protein does not occur on mitochondrial ribosomes in pea cotyledons during imbibition. Probably, only mitochondrial protein controlled by nuclear genes is synthesized in imbibing pea cotyledons.

CHI failed to inhibit increases in respiratory and enzymic activities, protein, and phospholipid of mitochondria in pea

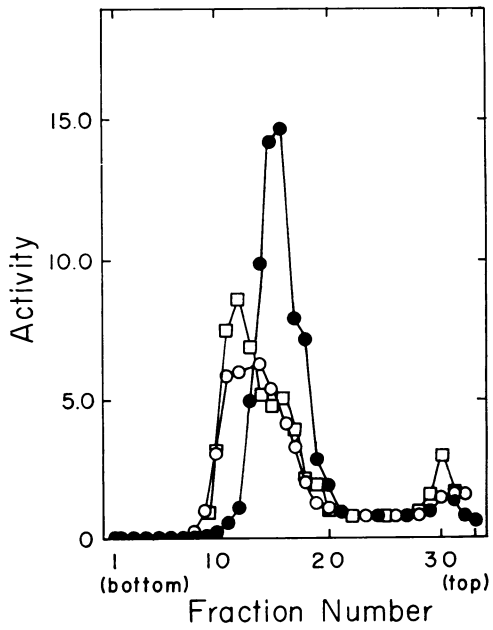

FIG. 6. Comparison of relative position of mitochondrial peaks after a linear SDGC among pea cotyledons germinated in water, a CHI solution, and a CAP solution for 18 hr. Crude mitochondrial fractions (equivalent to 3 g of dry seeds) were fractionated by a linear SDGC as for Figure 2, and the distribution of cytochrome oxidase activity was analyzed. The activity was expressed as decrease in absorbance at 550 nm/min·0.5 ml. ○: Water; ●: 1 mM CHI; □: 5 mM CAP.

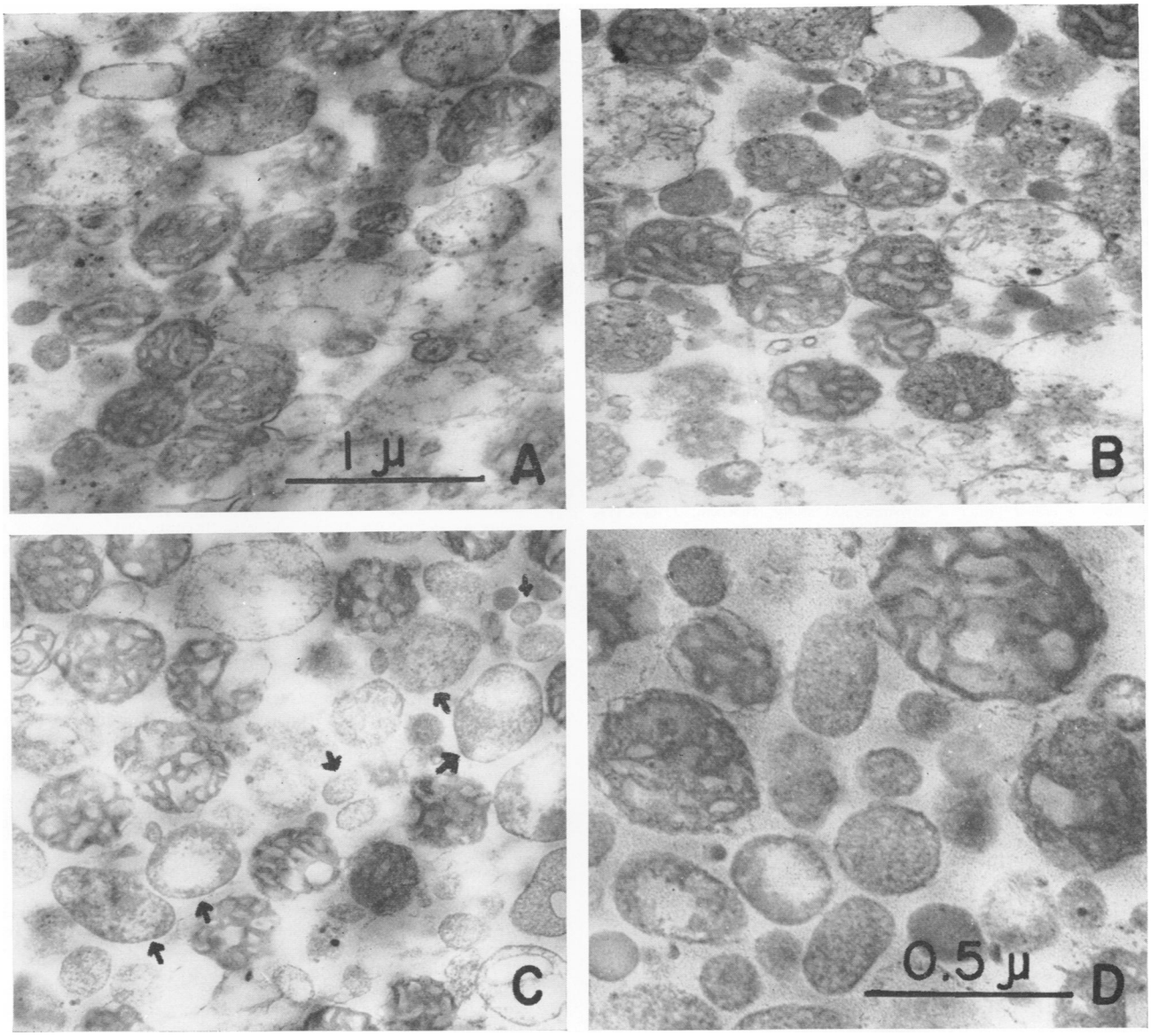

FIG. 7. Electron micrographs of mitochondrial fraction purified by a linear SDGC (method I) from pea cotyledons germinated in water, a CAP solution (1 mM), and a CHI (0.5 mM) for 18 hr. A: Water. B: CAP. C: CHI, × 30,000. Arrows indicate mitochondrial particles in an unusual form with diffused electron-dense matrices. D: CHI, × 60,000.

cotyledons during imbibition. These results indicate that the newly synthesized mitochondrial protein plays no important role in the development of mitochondria in pea cotyledons during imbibition. The failure of both CHI and CAP to inhibit the development of mitochondria suggests that the biogenesis of new mitochondria may not be involved in the increases in mitochondrial activities and components. We infer that the development of mitochondria is achieved by incorporation of preexisting cytoplasmic protein into the immature mitochondria. Fractionation of mitochondrial protein revealed that membrane-bound protein (bile salt-soluble and -insoluble proteins) as well as phospholipid increased during the imbibing stages. Therefore, cytoplasmic protein may be transferred into the immature mitochondria to form new mitochondrial membrane in imbibing pea cotyledons. Sakano and Asahi (28) reported the existence of bile salt-insoluble protein in sweet potato root mitochondria and its importance in the increase of mitochondrial function. Thus, the protein may also play an important role in the development of mitochondria in imbibing pea cotyledons.

CHI stimulated the accumulation of bile salt-soluble protein of mitochondria in imbibing pea cotyledons. Recently, some unexpected effects of CHI and CAP on cellular metabolism have been reported (6, 14, 19). Of special interest to us is the effect of CHI on the metabolism of orotidine-5′-P in germinating pea cotyledons, which was reported by Ross and Murray (26). They proposed that CHI might inhibit the accumulation of a protein capable of destroying the enzymes metabolizing orotidine-5′-P. Thus, it is possible that CHI inhibits the accumulation of a proteolytic enzyme and protects bile salt-soluble protein from destruction.

Although CHI stimulated the formation of mitochondrial membrane in imbibing pea cotyledons, it failed to affect the increases in mitochondrial activities during imbibition. CHI influenced the density or rate of sedimentation in sucrose gradient of mitochondria, the recovery of cytochrome oxidase activity after SDGC, and the conformation of mitochondria. Changes in the density of mitochondria have been observed in glucose derepressed yeast (10, 21, 29), in postnatal and perinatal rat

liver (16, 23), in neonatal swine hepatocyte (18), and in imbibing pea cotyledons (20, 31). The present study showed that mitochondria became denser during two successive SDGC. Pollak and Munn (23) showed that the permeability of inner mitochondrial membrane to sucrose influenced the density of mitochondria. Therefore, we propose that CHI caused an increase in the impermeability of the inner mitochondrial membrane to sucrose. Since CHI stimulated only the accumulation of bile salt-soluble protein in pea cotyledon mitochondria during imbibition, the bile salt-soluble protein may contribute to the changes in mitochondrial conformation, permeability, and stability.

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