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Biogenesis of Mitochondria in Imbibed Peanut Cotyledons[1]

II. DEVELOPMENT OF LIGHT AND HEAVY MITOCHONDRIA

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ABSTRACT

There are two types of mitochondria present in imbibed peanut cotyledons: a light type (density 1.182 grams per cubic centimeter) and a heavy type (density 1.205 grams per cubic centimeter). The membrane fractions from these two types can be distinguished using sucrose density gradient analysis, and differences in membrane density between the light and heavy types are reflected in differences in their protein N and phospholipid P composition. With increasing time after imbibition, there is a substantial increase in the amount and activity of the light type of mitochondria due to their *de novo* synthesis. The membrane density of the light mitochondrial fraction declines over 5 days after the start of imbibition as the phospholipid P to protein N ratio increases. The heavy mitochondrial fraction declines during the first 3 days after the start of imbibition, and then it remains at a low, but constant, level thereafter. Even during the decline, however, there is synthesis of proteins comparable to that into light mitochondria. The mitochondrial biogenesis that has been observed in peanut cotyledons is of the light type, the function and physiological importance of the minor heavy type is not known.

Many studies have been carried out on the development of mitochondria during and following imbibition of pea cotyledons (8, 10, 13, 14, 18), and it is accepted that the repair and activation of organelles already present in dry cotyledons are primarily responsible for mitochondrial development in this species. In contrast, there is evidence that, in peanut cotyledons, the increase in respiratory activity during and following germination results from the biogenesis of mitochondria (2, 11). It is of interest to compare the mechanism of mitochondrial development between pea and peanut cotyledons. But comparisons are difficult, because details of peanut cotyledon development are not available in the literature. This report provides some of these details.

MATERIALS AND METHODS

Plant Material. The two cotyledons of dry peanut seeds (*Arachis hypogaea* L.) cv. Early Spanish (Stokes Seeds Ltd., St. Catharines, Ontario) were separated, the embryonic axis remaining attached to one of them. The cotyledons with (attached) or without (detached) the embryonic axis were soaked for 6 h in water and then imbibed on wet filter paper at 25 C in the dark.

Preparation of Mitochondria. Cotyledons were homogenized in a mortar with a pestle with sand in 0.4 M mannitol, 0.05% cysteine (w/v), 1 mM EDTA, 0.5% BSA (w/v) in 30 mM phosphate buffer (pH 7.2) (2 ml per cotyledon). The homogenate was squeezed through four layers of cheesecloth after adjusting to pH 7.2 with KOH and centrifuged at 1,500*g* for 10 min. The supernatant was centrifuged at 20,000*g* for 15 min, and the resulting pellet was washed with 0.3 M mannitol, 1 mM EDTA, 0.1% BSA (w/v) in 10 mM K-phosphate (pH 7.2). The washed pellet was suspended in a small volume of the wash solution. To purify further the mitochondria, the washed mitochondrial fraction was loaded on a SDG[3] prepared by layering sucrose solutions in the following order: 8 ml 60% sucrose (w/v), 10 ml 50% sucrose, 10 ml 40% sucrose, and 8 ml 32.5% sucrose. The gradient was allowed to stand at 4 C for 1 day before use. The mitochondrial fraction was centrifuged at 25,000 rpm (85,000*g*) for 2.5 h in a Beckman SW 27 rotor (Beckman Instruments, Inc., Irvine, CA), and the gradient was collected as 30-drop fractions by puncturing the bottom of the tube.

Preparation of Mitochondrial Membrane Fraction. A mitochondrial membrane fraction (which might alternatively be called a submitochondrial particle fraction) was prepared according to the method of Sato and Asahi (17). After centrifugation and fractionation of the SDG (as above), fractions showing Cyt oxidase and/or succinate dehydrogenase activity were pooled. Sucrose in the mitochondrial suspension was diluted slowly to about 20% with 5% sucrose. The mitochondria were pelleted at 20,000*g* for 30 min, suspended in 0.1 M KCl, and then sonicated for 60 s (20 s × 3) on ice at the No. 8 position of a Branson Sonifer (Branson Ultrasonics Corp. Canada, Scarborough, Ontario, Canada). After adding 2 times the volume of 0.5 M sucrose in 10 mM Tris-HCl (pH 7.2), the sonicate was centrifuged at 35,000 rpm (150,000*g*) for 60 min in a Beckman Ti 60 rotor. The resultant precipitate was suspended in 0.25 M sucrose in 10 mM Tris-acetate buffer (pH 7.8), layered on a SDG, and centrifuged at 35,000 rpm (150,000*g*) for 2.5 h in a Beckman SW 41 rotor (Beckman Instruments, Inc.). The SDG was prepared by layering the following in order: 2 ml 60% sucrose (w/v), 2 ml 55% sucrose, 2 ml 50% sucrose, 2 ml 45% sucrose, 2 ml 40% sucrose, and 1.3 ml 35% sucrose. The gradient was allowed to stand at 4 C for 1 day before use. After centrifugation, the gradient was collected as 9-drop fractions by puncturing the bottom of the tube.

Assays. Respiratory activities of mitochondrial fractions and activities of enzymes (except isocitrate lyase) were determined as described previously (12). Isocitrate lyase activity was assayed according to Hock and Beevers (4). Preparation of lipid and protein from membrane fractions of disrupted mitochondria utilized the method of Uritani and Yamaki (19). Phosphorus content of the lipid fraction and nitrogen content of the protein fraction were determined as by Bartlett (1) and Leung *et al.* (5), respectively. Protein in SDG fractions was measured by the Lowry *et al.*

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[3] Abbreviations: SDG, sucrose density gradient; CHX, cycloheximide; CP, chloramphenicol.

(6) method.

Amino Acid Incorporation into Protein of Isolated Mitochondria. Incorporation of amino acid into protein of isolated mitochondria was determined according to Forde *et al.* (3), with small modifications. Purified mitochondria (200 to 300 μg of protein) were incubated in 0.7 ml containing 0.35 M mannitol, 100 mM KCl, 12 mM Tricine buffer (pH 7.2), 6 mM K-phosphate (pH 7.2), 1 mM ethyleneglycol-bis(β-aminoethylether)*N*,*N*-tetraacetic acid, 17 mM succinate, 2 mM ADP, 2 mM GTP, 35 mM $MgCl_2$, 150 μM amino acid mixture (minus leucine), and 1.25 to 5.0 μCi L-[U-^{14}C]leucine (351 mCi/nmol; The Radiochemical Centre, Amersham) in tubes maintained at 27 C, shaken at about 120 cycles min^{-1}. After incubation for 1 h, TCA was added to a final concentration of 10% (w/v). The pellet obtained by centrifugation was suspended in 5% TCA, heated at 95 C for 15 min, and centrifuged. The pellet was collected on glass-fiber paper (Whatman GF83), washed three times with 5% TCA and, dried, and the radioactivity was monitored by liquid scintillation spectrometry.

Feeding Experiments. L-[U-^{14}C]Leucine (10 μCi) in 5 ml H_2O or antibiotic solution (1 mM CHX or 5 mM CP) was vacuum-infiltrated into twenty-five 2-day-old cotyledons. The cotyledons were bathed in the same solution for 3 h at 23 C. Mitochondria were fractionated by SDG centrifugation as above. Preparation of protein in each fraction from the gradient and measurement of radioactivity were carried out as described in the previous section.

Electron Microscopy. Mitochondrial pellets were fixed at 4 C in 4% glutaraldehyde buffered with 0.4 M sodium cacodylate (pH 6.8) containing 1 mM $CaCl_2$ for 4 h and then postfixed in buffered 2% osmium tetroxide for 4 h at 4 C. The fixed materials were then dehydrated in a graded acetone series and embedded in an Epon-Araldite (Polysciences, Inc., Warrington, PA) mixture (mixture 2 of Mollenhauer [9]). Ultrathin sections were cut with glass knives on the Reichert Ultracut ultramicrotome (Polysciences, Inc.) and double stained with uranyl acetate and lead citrate. The sections were examined and photographed with the JEOL 100S electron microscope (Polysciences, Inc.) at 60kv.

SDS Polyacrylamide Gel Electrophoresis. The total proteins of the mitochondrial pellets were extracted with 0.03 M Tris-HCl (pH 6.8) containing 2% mercaptoethanol, 10% glycerol, and 2% SDS. The homogenates were boiled at once for 2 min. The boiled extracts were centrifuged using an Eppendorf microfuge (Polysciences, Inc.) at 12,800*g* for 15 min. The supernatants were then applied to a 10% SDS polyacrylamide gel for protein analysis. SDS polyacrylamide gel electrophoresis was carried out by the procedure of Ogita and Markert (15).

RESULTS

Distribution of Mitochondria after SDG Centrifugation. Figure 1 shows the distribution of mitochondria after SDG centrifugation of crude mitochondrial fractions prepared from cotyledons at different times from the start of imbibition. Succinate dehydrogenase and Cyt oxidase activities were assayed as the marker enzymes. Two peaks of mitochondrial enzyme activity were obtained. A major peak (designated as peak 2) was located in the middle part of the gradient at a density of 1.182 g/cubic centimeter, and a minor peak (peak 1) was in lower region of the gradient at a density of 1.205 g/cubic centimeter. Under the experimental conditions, glyoxysomes (isocitrate lyase as marker enzyme) were recovered in a pellet, and there was insignificant contamination of glyoxysomes with peak 1 or peak 2 (data not presented). Peak 2 increased markedly during and following germination, while peak 1 decreased slightly during the first 3 days after the beginning of imbibition and remained at a low level thereafter. Mitochondrial activities (respiration rate, ADP/O, and RC ratios) in these two peaks were assayed using succinate as substrate with the samples from 9-h-old cotyledons. Mitochondria from both peaks showed RC and oxidative phosphorylation,

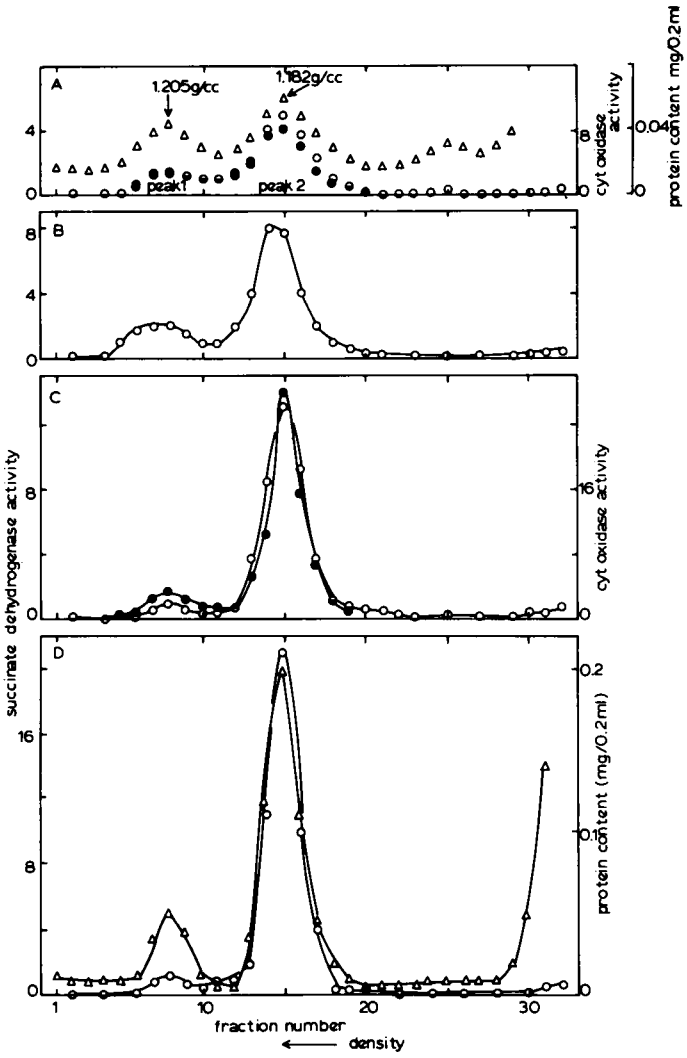

FIG. 1. Distribution of protein, Cyt oxidase, and succinate dehydrogenase after SDG centrifugation of mitochondrial fractions isolated from 20 attached cotyledons. A, 6-h-Imbibed cotyledons; B, 1-day-old cotyledon; C, 3-day-old cotyledons; D, 5-day-old cotyledon. (△), Protein content in mg/0.2 ml; (○), succinate dehydrogenase in $\Delta A_{600} \times 10^2$/min·0.1 ml; (●), Cyt oxidase in $\Delta A_{550} \times 10^2$/0.5 min·0.05 ml.

Table I. *Activities of Mitochondrial Fractions Isolated from Attached 9-h-old Cotyledons*

Succinate was used as substrate.

Mitochondrial Peak	O_2 Uptake		RC	ADP/O
	nmol/min·cotyledon	*nmol/min·mg protein*	*ratio*	
1	1.0	22.3	1.54	0.84
2	1.8	28.3	1.65	0.84

although the activities of peak 1 mitochondria were a little less than those of peak 2 (Table I).

Previously, we observed a marked development of mitochondria in peanut cotyledons after the beginning of imbibition (11). There can be no doubt that this development is due to an increase in peak 2 mitochondria. In the previous report (11), we also showed that the development of mitochondria was retarded in detached cotyledons. Little difference in the peak 1 and peak 2 distribution of succinate dehydrogenase was observed between attached and

detached cotyledons, although activity of this enzyme was higher overall in mitochondria from attached cotyledons than in those from detached ones (Fig. 2). This suggests that mitochondria in detached cotyledons do not differ qualitatively from those in attached cotyledons.

Attached cotyledons were imbibed in 50 μM CHX for 6 h and then incubated for 3 days on filter paper wetted with the same solution. CHX reduced the development of succinate dehydrogenase (Fig. 2) and Cyt oxidase (data not shown) activities in both peaks 1 and 2. Protein content in peak 2 mitochondria from CHX-treated cotyledons (12 μg protein N per cotyledon) was lower than that in mitochondria from untreated ones (22 μg N per cotyledon).

Properties of Mitochondrial Membranes. Peak 1 and peak 2 mitochondria from 6-h-old cotyledons were broken by sonication and fractionated by SDG centrifugation. Cyt oxidase in peak 1 mitochondria was recovered in a lower region of the gradient at density 1.213 g/cubic centimeter, while Cyt oxidase in peak 2 mitochondria was in a light region at density 1.182 g/cubic centimeter (Fig. 3). When the membranes of peak 2 mitochondria from 5-day-old cotyledons were subjected to SDG centrifugation, Cyt oxidase activity was found in a lighter region of the gradient at density 1.166 g/cubic centimeter (Fig. 3). Cyt oxidase in the membrane of peak 2 mitochondria from 3-day-old cotyledons was located between the two regions of density, 1.182 g/cubic centimeter and 1.166 g/cubic centimeter (Fig. 3). Thus, the membranes of peak 2 mitochondria became lighter with increasing time after the start of imbibition. No difference was observed in the distribution of Cyt oxidase in membrane fractions of peak 2 mitochondria between attached and detached cotyledons (Fig. 3). This again suggests that mitochondria in attached cotyledons are qualitatively similar to those in detached ones.

The content of protein N and lipid P (on a per-cotyledon basis) in membrane fractions increased markedly over the 5 days from imbibition (Table II). The relative content of phospholipid to protein in peak 2 mitochondria was higher than in peak 1 mitochondria and increased appreciably with time (Table II).

Electrophoresis. Polypeptides of mitochondria obtained by the SDG centrifugation were analyzed by SDS polyacrylamide gel electrophoresis (15). No qualitative or quantitative differences in the pattern of major polypeptides were observed between peak 1 and peak 2 mitochondria (data not presented). Furthermore, the pattern did not change substantially during germination, nor did CHX treatment bring about any alteration of the pattern.

***In Vivo* Incorporation of Amino Acid into Mitochondrial Proteins.** Figure 4 shows the distribution of labeled proteins after SDG centrifugation of the mitochondrial fraction from 2-day-old cotyledons incubated with [U-^{14}C]leucine. A peak of label in the heavy region and another in the lighter region corresponded in position to peak 1 and peak 2, respectively (Fig. 1). Since the peak 1 fraction was contaminated with particles other than mitochondria (see below), it could be argued that the radioactivity in peak 1 is a result of incorporation of leucine into nonmitochondrial particles. But this is unlikely, because the distribution of radioactivity after SDG centrifugation of membranes prepared from the peak 1 fraction corresponds very well with the distribution of Cyt oxidase (Fig. 5A). Similarly, there is good correspondence between radioactivity and enzyme activity in the peak 2 fraction (Fig. 5B). Therefore, the radioactivity in peak 1 and peak 2 fractions is justifiably presumed to be largely due to incorporation into mitochondrial proteins. Incorporation into peak 1 and peak 2 fractions was roughly equivalent to the amount of Cyt oxidase activity in these peaks (Fig. 4), and it seems that, on an activity basis, leucine incorporation into protein in peak 1 mitochondria is comparable with that into peak 2 mitochondria.

Labeling of mitochondrial protein was severely inhibited by

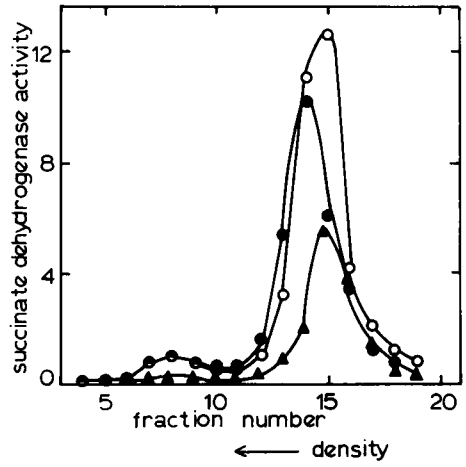

FIG. 2. Distribution of succinate dehydrogenase after SDG centrifugation of mitochondrial fractions isolated from 20 3-day-old cotyledons. (○), Attached cotyledons; (●), detached cotyledons; (▲), CHX-treated, attached cotyledons. Enzyme activity, $\Delta A_{600} \times 10^2$/min·0.1 ml.

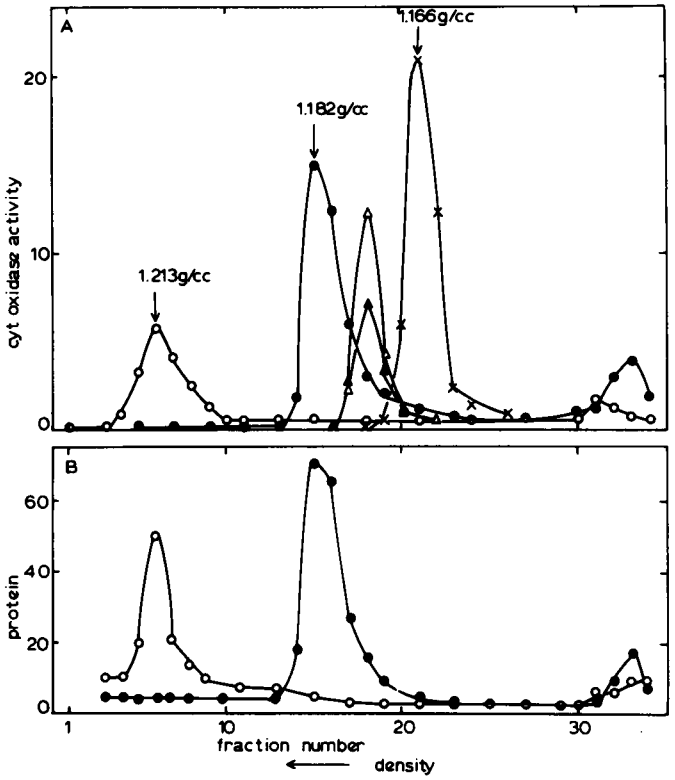

FIG. 3. Distribution of Cyt oxidase and protein after separation of mitochondrial membranes by SDG centrifugation. A, Cyt oxidase; B, protein. (○) and (●), Peak 1 and peak 2 mitochondria, respectively, from 100 6-h-old attached cotyledons. (△) and (▲), Peak 2 mitochondria from 13 3-day-old attached and detached cotyledons, respectively. (X), Peak 2 mitochondria from 25 5-day-old cotyledons. Enzyme activity, ΔA_{550}/0.5 min·0.025 ml; protein, μg/0.05 ml.

Table II. *Content of Phospholipid P and Protein N in Mitochondrial Membrane Fractions*

Peak	Cotyledon Age	P/cotyledon	N/cotyledon	P/N × 10^2
		μg		
1	6 h	0.19	4.59	4.1
2	6 h	0.41	7.21	5.7
2	3 days	1.43	22.5	6.4
2	5 days	2.26	27.0	8.4

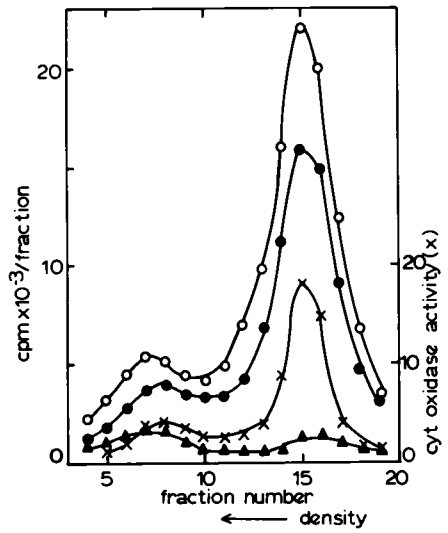

FIG. 4. Distribution of protein labeled with [U-^{14}C]leucine and of Cyt oxidase after SDG centrifugation of crude mitochondrial fractions from 2-day-old attached cotyledons. Radioactivity: (○), H_2O-treated cotyledons; (●), CP-treated cotyledons; (▲), CHX-treated cotyledons. Cyt oxidase in ΔA_{550}/0.5 min·0.05 ml·(x), H_2O-treated cotyledons.

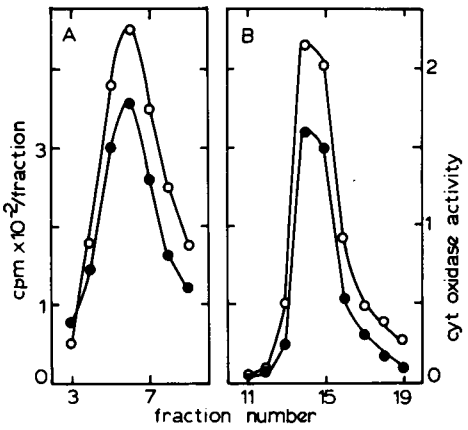

FIG. 5. Distribution of protein labeled with [U-^{14}C]leucine and of Cyt oxidase after separation of mitochondrial membranes on SDG. A, Peak 1 mitochondria; B, peak 2 mitochondria. (○), Radioactivity; (●), Cyt oxidase expressed as relative activity. Unlabeled membranes were added as carriers.

Table III. *In Vitro [U-^{14}C]Leucine Incorporation into Protein by Mitochondria Isolated from 1-day-old Cotyledons*

Mitochondrial Peak	Radioactivity Incorporated	
	cpm × 10^{-3} per 25 cotyledons	*cpm × 10^{-3} per mg protein*
1	15	11
2	47	22

CHX, but only slightly by CP (Fig. 4). These results are in good agreement with those reported by others (8, 14) for pea cotyledon mitochondria.

***In Vitro* Incorporation of Amino Acid into Mitochondrial Protein.** Peak 1 and peak 2 mitochondria were prepared from 1-day-old cotyledon, and their ability to incorporate amino acid into protein *in vitro* was determined. Both peaks of mitochondria incorporated [U-^{14}C]leucine into their protein (Table III). The incorporation rate per mg protein of peak 2 mitochondria was twice that of peak 1 mitochondria. But this does not necessarily reflect any real difference in incorporation between the two types of mitochondria, since their pool size of endogenous leucine and their relative membrane permeabilities are not known. When succinate was omitted from the incubation medium, incorporation of leucine was severely retarded (Table IV). Acetate, which cannot serve as a respiratory substrate, could not substitute for succinate (Table IV). The failure of acetate to support amino acid incorporation and the failure of streptomycin to reduce incorporation (Table V) suggest that bacterial contamination plays a negligible part in the incorporation reaction. CP severely inhibited *in vitro* amino acid incorporation into mitochondrial protein, while CHX stimulated the incorporation (Table V). These results are in good accord with the observation that CP inhibits the mitochondrial protein-synthesizing system and that CHX does not (16). Why stimulation of the incorporation occurs in the presence of CHX is not clear. Taking the results of Figure 4 and Table V together, we deduced that most mitochondrial proteins are synthesized in the cytoplasm and are imported to mitochondria.

It has been reported that antibiotics can affect mitochondrial activities as well as it affects protein synthesis (7, 21). But, we found that CP, CHX, and streptomycin had no significant affect on activities of our isolated mitochondria (data not presented).

Electron Microscopic Observation. Figure 6 shows electron micrographs of mitochondria fractions from 6-h-, 2-day-, and 4-day-old cotyledons. In the peak 2 fraction from 6-h-old cotyledons, many membrane-bound structures can be seen, but it is difficult to recognize them as mitochondria. They were also difficult to distinguish in electron micrographs of intact cells (11). Mitochondria are more easily distinguishable in the peak 1 fraction, but this fraction is contaminated with many particles other than mitochondria. Mitochondria are the major organelle in the peak 2 fraction from 2-day-old cotyledons and, in this fraction, cristae are more developed. Only mitochondria are present in peak 2 fraction from 4-day-old cotyledons, and they are fully developed.

DISCUSSION

Two types of mitochondria are present in imbibed peanut cotyledons: heavy (more dense) mitochondria and light (less dense) ones, although the relative amounts of these two types changes with time after the beginning of imbibition. The nature of the minor peak (peak 1) is not known. It may contain aggregates

Table IV. *Effects of Energy Sources on [U-^{14}C]Leucine Incorporation into Protein by Isolated Mitochondria*

Mitochondrial Peak	Energy Source	Relative Radioactivity Incorporated
1	Succinate	100
	Acetate	9
	None	15
2	Succinate	100
	Acetate	4
	None	4

Table V. *Effects of Antibiotics on [U-^{14}C]Leucine Incorporation into Protein by Isolated Mitochondria from Peaks 1 and 2*

Treatment	Relative Radioactivity Incorporated	
	Peak 1	Peak 2
Control	100	100
CP, mM	4	1
CHX, 0.2 mg/ml	149	138
Streptomycin 0.2 mM	97	87

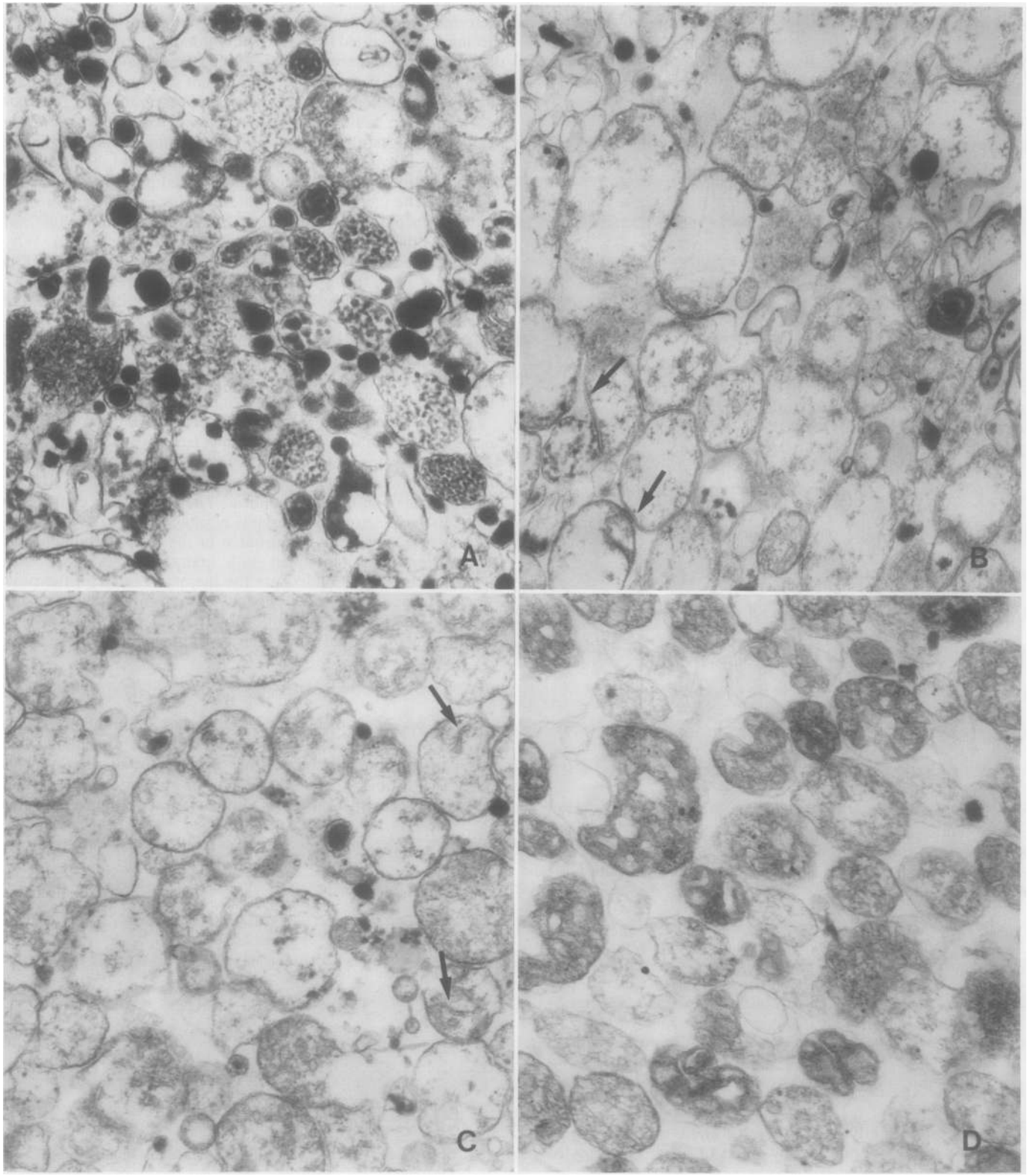

FIG. 6. Features of peanut cotyledon mitochondria isolated during the first 4 days from the start of imbibition. A, Thin section of the peak 1 mitochondrial pellet prepared 6 h after the start of imbibition. This fraction contains numerous membranous structures, but it is difficult to distinguish structures that can be identified unequivocally as mitochondria. B, Thin section of the peak 2 mitochondrial pellet prepared 6 h after the start of imbibition. The mitochondria have the typical double membrane structure (arrow), although the internal membrane system (arrow) has not developed. C, Thin section of the mitochondrial pellet prepared 2 days after the start of imbibition. The internal membrane system (arrow) is better developed at this time. D, Thin section of the mitochondrial pellet prepared 4 days after the start of imbibition showing mitochondria with well-developed cristae (× 34,000).

of mitochondria or an aggregation of mitochondria with other cellular membranes, *i.e.* heavy mitochondria might be an aggregate form of light ones and be produced as an artifact during extraction. However, there are three lines of evidence that do not support this possibility. First, we could not observe such aggregate forms in electron micrographs of the heavy mitochondrial fraction. Second, the membrane obtained from ruptured peak 1 mitochondria was recovered after SDG centrifugation as a well-defined band in a region quite different from that where the membrane from peak 2 mitochondria was recovered. Third, the membranes of the two types of mitochondria differ in relative composition of lipid and protein. This suggests that the mitochondrial membranes in peaks 1 and 2 are different. On the basis of these results, we speculate that there are two different types of mitochondria in peanut cotyledons and that one of them, the light mitochondria type, becomes predominant with increasing time after imbibition. Wilson and Bonner (20) have also reported two types of mitochondria in embryonic axes of imbibing peanut seeds. According to them, however, it is the heavy mitochondria which become the major type as germination proceeds. This suggests that there is an inherent difference between the pattern of mitochondrial development in peanut axes and cotyledons.

The density of the membranes from ruptured peak 2 mitochondria decreases with time after imbibition. This observation agreed with that of Breidenbach *et al.* (2), who reported that mitochondria in peanut cotyledons become less dense with time. The present study shows that at least one of the factors involved in the decrease in density is an increase in the ratio of lipid to protein content in the mitochondrial membranes. Also, the high density of the membrane of peak 1 mitochondria is explicable in terms of lipid and protein composition. Changes in the density of mitochondria from heavy to light during germination have been reported for pea cotyledons (14, 18).

It has been suggested that the development of mitochondria in imbibed peanut cotyledons is due to the biogenesis of these organelles rather than to repair or activation of mitochondria already existing in dry cotyledons (2, 11). There can be no doubt that, in peanut cotyledons, the biogenesis of light (peak 2) mitochondria is responsible for the increase in mitochondrial activities. On the other hand, the amount of activity in peak 1 decreases during the first 3 days from imbibition, which suggests that these heavy mitochondria degenerate. The amount of peak 1 activity does remain at a low, but constant, level between days 3 and 5. Moreover, the data shown in Figure 4 and Table III suggest that, even when peak 1 activity is declining, there is synthesis of protein by heavy mitochondria and the import of cytoplasmic proteins into them. It is difficult to conclude, therefore, that the heavy mitochondria are simply degraded during and following imbibition. It remains to be elucidated whether the heavy type of mitochondria have any physiological function or significance.

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