Biogenesis of Mitochondria in Germinating Peanut Cotyledons II. Changes in Cytochromes and Mitochondrial DNA[1]

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Summary. Biogenesis of mitochondria occurs in the germinating cotyledons of peanuts. This process was demonstrated by measuring both constitutive and enzymatic properties of mitochondria as a function of germination time. Direct counting by phase contrast microscopy of sucrose density gradient preparations showed that the number of mitochondria increased markedly during germination. DNA with a buoyant density distinct from the major cellular DNA was associated with these mitochondrial preparations. During germination the amount of this DNA in mitochondrial pellets increased. This increase closely paralleled the increase in number of mitochondria.

Succinoxidase and succinic dehydrogenase increased during germination. Both activities were confined to the mitochondrial fraction. The rate of increase of succinoxidase activity was significantly greater than the rate of increase of succinic dehydrogenase and both increased at least initially at a greater rate than the amount of mitochondrial DNA or numbers of mitochondria.

The amounts of cytochromes present in mitochondrial preparations were measured spectrophotometrically. All of the cytochromes increased in amount during germination. The rate of increase of cytochrome a $-$ a_3 was very close to the rate of increase in succinoxidase activity.

The mobilization of food reserves in seed storage tissues is accompanied by a marked increase in respiratory activity (1, 2, 4, 8, 11).

In some seeds the respiratory increase is so immediate after imbibition that induction and synthesis of new enzymatic protein is improbable (15, 25). However, evidence from a number of studies suggests that there is an increase in the amount of mitochondrial protein or numbers of mitochondria, or both, during germination of at least some seeds (1, 2, 8, 10). These studies, as well as those of Howell (11) and Cherry (4) also indicate that there is an increase in the respiratory efficiency of the mitochondrial population during germination.

In view of this evidence, we undertook to estimate the contribution of mitochondrial biogenesis to the increased respiratory activity of peanut cotyledons during germination.

In a previous communication, we reported that the succinoxidase activity, the protein and the number of particles associated with the mitochondrial band in the sucrose density gradient increase for 9 days during seed germination (2). In this study, similar changes in the cytochromes and the mitochondrial DNA are described.

Materials and Methods

Materials. Reagents and materials were obtained as follows: Thiamine pyrophosphate, cytochrome c and Aquacide II from the California Corporation for Biochemical Research; ADP from Sigma Chemical Corporation; DPN from Boehringer and Sons; phenazine methosulfate from Nutritional Biochemical Corporation; sodium dichlorophenolindophenol from Eastman Chemical Company; electrophoretically purified bovine pancreas deoxyribonuclease and crystalline bovine pancreas ribonuclease from Worthington Biochemical Corporation; CsCl from Penn Rare Metals, Revere Penn, and Virginia jumbo peanuts from the Suffolk Peanut Company.

^{15}N-DNA from *Pseudomonas aeruginosa* (ρ = 1.745 g cm^{-3}) was the kind gift of Dr. R. H. Doi.

Methods. Peanut seeds were grown at 22 to 23° in moist perilite-vermiculite as described by Rebeiz and Castelfranco (16). Mitochondrial pellets were prepared from 25 or 50 g samples of tissue according to the method of Breidenbach, et al. (2). These pellets were either assayed directly or further purified by density gradient cen-

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trifugation. Density gradient centrifugation was performed as described previously. The density gradients were fractionated into thirteen 3 ml fractions. The fractions were designated by number from 1 to 13 beginning at the top of the gradient. The mitochondria were contained in fractions 4 to 7, but were mostly concentrated in fractions 5 and 6.

Succinoxidase was assayed with an oxygen cathode as described previously (2). Succinate dependent O_2 uptake by crude mitochondrial pellets was stimulated 30 % by ADP. However, mitochondria purified by density gradient centrifugation showed an 80 % stimulation by ADP. Succinate oxidation is coupled to ATP synthesis. With limiting amounts of ADP the oxygen uptake assumes a state 4 rate approximately equal to the state 2 rate. The values used to estimate succinoxidase activity were those obtained by measuring the ADP stimulated or state 3 rate.

Succinic Dehydrogenase Assay. Succinic dehydrogenase was assayed according to the method described by Hiatt (9). The reaction mixture contained in a total volume of 3 ml: 150 μmoles potassium phosphate (pH 7.4); 120 μmoles of neutralized sodium succinate; 25 μmoles KCN; 0.18 μmole sodium dichlorophenol-indophenol; 1.0 mg phenazine methosulfate and an amount of a suspension of crude mitochondrial pellet equivalent to the recovery from 100 mg fresh weight of cotyledon tissue. The rate of succinate oxidation in μmoles per minute was calculated using an extinction coefficient of 15.7 cm^2 $\mu mole^{-1}$ for dichlorophenol-indophenol (19).

Protein Assay. One-tenth to 0.2 ml aliquots of enzyme suspension to be assayed for protein were allowed to stand overnight at room temperature after addition of 0.45 ml of 1.0 N NaOH. The samples were then diluted to 1.0 ml with deionized water and assayed by the method of Lowry, et al. (13), but with the NaOH omitted from the reagents described in their procedure.

Cytochrome Assay. Amounts of cytochromes were determined from difference spectra obtained with mitochondrial samples purified by sucrose density gradient centrifugation. Aliquots (2.2 ml) of mitochondrial suspension were placed in each of 2 matched microcuvettes (pathlength 5 cm; nominal volume 2.2–2.6 ml) along with 50 mg of sodium deoxycholate and 50 μmoles of potassium phosphate buffer (pH 7.3). To 1 cuvette, 12.5 μmoles of potassium ferricyanide was added and to the other 12.5 μmoles of sodium ascorbate. The final volume after all additions was 2.5 ml. The cuvettes were mixed by repeated inversion for 1 minute, then a few sodium hydrosulfite crystals were added to the ascorbate containing cuvette and the contents again thoroughly mixed. Two minutes after mixing, the samples were scanned from 630 to 500 mμ with a Cary Model 14 recording spectrophotometer equipped with a 0 to 0.1 OD slidewire. Scans of partially and fully reduced samples were used to ascertain the existence of an isosbestic point at 630 mμ. Other isosbestic points were determined by noting all of the crossover points when scans were overlayed and aligned at 630 mμ. Sixteen scans were compared in all combinations by this procedure. Isosbestic points were found at 589 ± 4 mμ, 567 ± 2 mμ, and 545 ± 2 mμ. Since the extinction coefficients are not available for these cytochromes the data are presented as the absorbancy at maxima for the α bands of cytochromes of the a, b and c type measured relative to the nearest isosbestic point. To wit: cytochrome a − a_3 was measured relative to the isosbestic point at 630 mμ, cytochrome b was measured relative to the isosbestic point at 567 mμ and cytochrome c was measured relative to the isosbestic point at 545 mμ (table I, fig 1).

Extraction, Purification, and Estimation of DNA. Mitochondrial pellets to be assayed for DNA were suspended in 0.15 M NaCl, 0.1 M EDTA, 0.02 M tris, 2 % sodium dodecylsulfate (pH 7.9) (SEDT). The suspensions were frozen and thawed, then heated for 10 minutes at 60°. The lysed samples were shaken for 15 minutes with an equal volume of SEDT-saturated phenol. The aqueous phase was removed and extracted twice with one-half volume of diethyl ether and once with one-half volume of chloroform-isoamyl alcohol (24:1). The aqueous extracts were then dialyzed for 24 hours against two 6 liter changes of 0.1 M NaCl to 0.001 M EDTA. The samples were next treated with 50 μg/ml of ribonuclease for 20 minutes at room temperature. The ribonuclease used had been previously heated for 10 minutes at 100° and pH 5 to destroy any deoxyribonuclease. The extracts were again dialyzed for 24 hours against two 6 liter changes of NaCl-EDTA. The resulting dialysates were concentrated with Aquacide II and adjusted to a volume equivalent to the recovery from 12.5 g fresh weight of tissue per ml.

To obtain the total DNA, 10 g of cotyledons were extracted with 20 ml SEDT by thorough blending in a Virtis tissue homogenizer. The homogenates were frozen and thawed, heated to 60° for 10 minutes and centrifuged at 34,000 × *g* for 10 minutes. The residue was extracted with an additional 5 ml SEDT and the combined extracts were then treated as described above. The volume of the final dialysates was adjusted so that 1 ml contained the recovery from 2 g of tissue.

Extraction of DNA from mitochondria purified by density gradient sedimentation was achieved by pooling fractions 4, 5, 6 and 7 from several gradient tubes and dialyzing against 300 volumes of 0.15 M NaCl, 0.1 M EDTA, 0.02 M tris (pH 7.9) for 12 hours. After dialysis, sodium dodecylsulfate was added to a concentration of 2 % and the resulting SEDT preparations were treated as described above.

Suspensions of crude mitochondrial pellets were treated with deoxyribonuclease to test the resistance of the mitochondrial DNA to enzymatic digestion

(14). Pellets were suspended in 0.4 M sucrose, 0.1 M tris, 0.005 M $MgCl_2$, pH 7.2 at a concentration equivalent to the recovery from 12.5 g fresh weight of cotyledon tissue per ml. Deoxyribonuclease was added to a final concentration of 50 μg per ml and the suspension incubated for 1 hour at 0°. The reaction was stopped by adding EDTA to a concentration of 0.025 M, diluting to 40 ml with sucrose-tris-EDTA, and resedimenting the mitochondria. The resulting pellets were resuspended in SEDT and treated as described above for DNA extraction.

Total amounts of DNA in various samples were assayed colorimetrically by the method of Burton (3).

Mitochondrial DNA was determined using buoyant density centrifugation on CsCl gradients in a Beckman Model E analytical ultracentrifuge as described by Shildkraut, et al. (20). A known quantity of *P. aeruginosa* ^{15}N-labeled DNA was added with each unknown to the CsCl gradient. Ultraviolet photographs were scanned with a Beckman microdensitometer and the areas under the component peaks were measured. Absolute amounts of mitochondrial DNA were determined by reference to the *P. aeruginosa* ^{15}N-DNA standard.

Bacterial Count. In one experiment bacterial counts were made on the whole homogenate and crude mitochondrial pellet from 4.5 day old cotyledons. After suitable dilution with sterile water, aliquots were plated on 1 % agar, 1 % trypsin, 0.5 % NaCl medium containing 1 mg vit_{b_1} and 5 mg thymidine per liter. The resulting plates were incubated at 30° for 24 hours and counted.

Results

Cytochromes a − a_3, b and c are associated with the mitochondrial band on sucrose gradients. Difference spectra obtained with the mitochondria preparations purified by density gradient centrifugations exhibit absorption maxima at 603 mμ, 560 mμ and 552 mμ corresponding respectively to the α bands of cytochromes a − a_3, b and c. There is also a maximum at 527 mμ corresponding to the β bands of cytochromes b and c. A typical difference spectra is shown in figure 1.

Measurements obtained from difference spectra show that the amounts of all 3 cytochromes increased during germination (table I). It is impossible to calculate the molar concentration of each species because the respective extinction coefficients are not known. Nevertheless if one considers the level of each cytochrome present in the mitochondria of 2.5 day old cotyledons and the level in each subsequent increment (from 2.5–4.5 and from 4.5–9.5) it becomes immediately apparent that the mitochondria at the earlier age are deficient in a type cytochromes relative to the later ages (table II).

When succinic dehydrogenase and succinoxidase activities are plotted versus age, 2 curves are obtained which are qualitatively similar except that the succinoxidase activity increases at a much

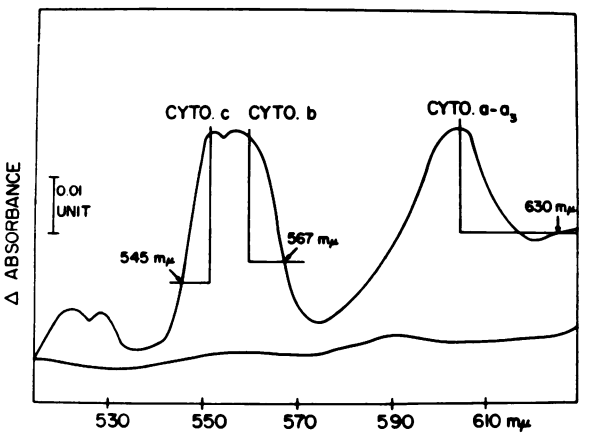

FIG. 1. Typical difference spectrum scan of cytochromes from 4.5 day old peanut cotyledon mitochondria purified by sucrose density gradient centrifugation. Each cytochrome was measured in reference to the nearest isosbestic point. To wit: cytochrome a − a_3 from 630 mμ, cytochrome b from 564 mμ and cytochrome c from 545 mμ. Lower curve shows typical background scan with contents of cuvettes identical. Upper curve is a scan of reduced vs. oxidized cytochromes.

Table I. *Increase in the Absorbancy due to Cytochromes* a–a_3, b *and* c *in Sucrose Density Gradient Preparations of Mitochondria Isolated from Peanut Cotyledons During Germination*

Differences in absorbancy at the indicated pairs of wavelengths were measured in difference spectra scans. The maximum estimated error in the individual measurements is 14 × 10^{-6} A units.

Days of germination	(A/g fr wt) × 10^6 Cyto. a–a_3 (604–630)	Cyto. b (560–567)	Cyto. c (552–545)
2.5	24	65	86
4.5	78	103	135
9.5	135	168	200

Table II. *Relative Absorbancies of* a, b *and* c *Type Cytochromes Found in Mitochondrial Pellets from 2.5 Day Old Cotyledons and Synthesized Subsequently*

Comparison of amount	Relative absorbancy of cytochromes a–a_3	b	c
Present at 2.5 days	28	76	100
Synthesized from 2.5 to 4.5 days	110	78	100
Synthesized from 4.5 to 9.5 days	88	100	100

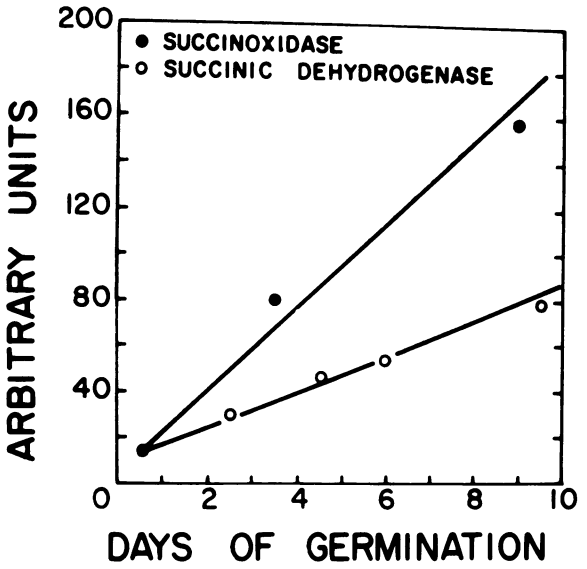

FIG. 2. Comparison of the pattern of increasing activities of succinoxidase and succinic dehydrogenase during germination of peanut cotyledons. Arbitrary units represent: Succinic dehydrogenase, $\frac{\text{m}\mu\text{moles DCPIP}}{\text{min} \times \text{g fr wt}}$; Succinoxidase, $\frac{\text{m}\mu\text{moles } O_2}{\text{min} \times \text{g fr wt}}$ $\times \frac{\text{m}\mu\text{moles DCPIP}}{\text{m}\mu\text{moles } O_2}$ at 0.5 days.

higher rate (fig 2). These findings indicate that as germination progresses the mitochondria become more efficient as judged by the succinoxidase criterion.

All of the preceding experimental results clearly show that there is an increase in mitochondrial constituents during germination. For this reason it seemed useful to measure the amount of mitochondrial DNA as a function of cotyledon age.

DNA purified from whole tissue homogenates formed a rather diffuse band with a shoulder on the denser side when centrifuged to equilibrium on CsCl gradients (fig 3a). The buoyant density of this band was calculated to be 1.705 g cm^{-3} using *P. aeruginosa* ^{15}N-DNA as a marker. DNA preparations from crude mitochondrial pellets exhibited the major band (ρ = 1.705 g cm^{-3}) but in addition they exhibited a satellite band with a density of 1.716 g cm^{-3}. This satellite component comprised about 10 % of the DNA present (fig 3b). Further purification of the mitochondria by sucrose density gradient centrifugation enriched the DNA with satellite so that it represented 30 % of the DNA (fig 3c). Additional evidence that the satellite is of mitochondrial origin can be drawn from the resistance to deoxyribonuclease digestion (14). When suspensions of intact mitochondria from crude mitochondrial pellets were treated with deoxyribonuclease, then extracted for DNA as described under methods and centrifuged on CsCl gradients, the major DNA component no longer formed a band, while the satellite band was unaffected (fig 3d). The resistance to deoxyribonuclease digestion exhibited by the satellite presumably results from inability of the enzyme to act on the DNA present in intact mitochondria.

Bacteria were ruled out as a source of the satellite DNA by bacterial counts made from both the original homogenate and the mitochondrial suspension from 4.5 day cotyledons. Using the value to 10^{-11} mg of DNA per bacterial cell (23),

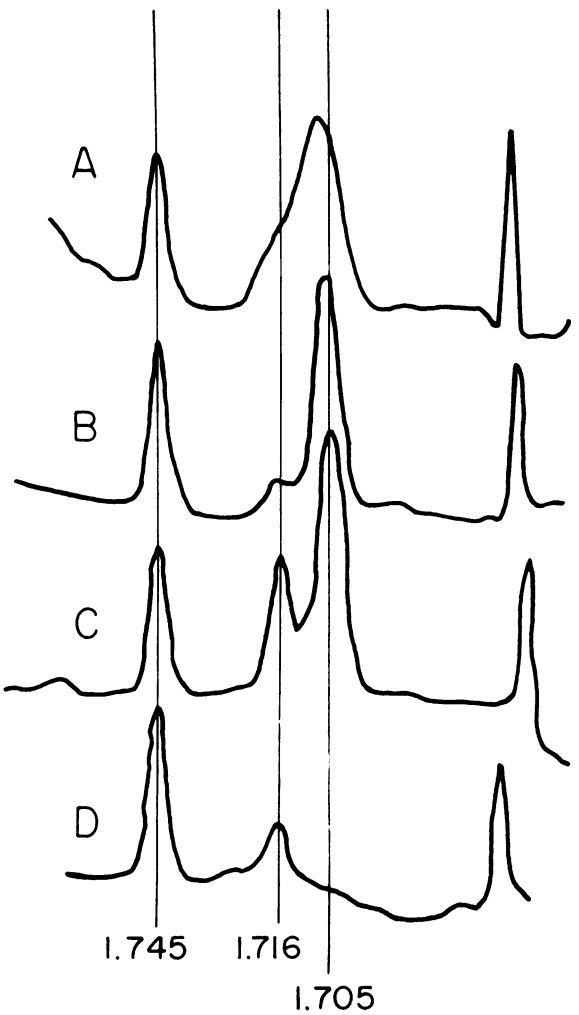

FIG. 3. Microdensitometer tracings from ultraviolet absorption photographs of DNA banded on CsCl gradients. DNA from: A) whole tissue homogenates of 4.5 day old cotyledons; B) crude mitochondrial pellet of 4.5 day old cotyledons; C) mitochondria of 4.5 day old cotyledons purified by sucrose density centrifugation; D) crude mitochondrial pellet of 4.5 day old cotyledons treated with deoxyribonuclease. The peaks represent respectively ^{15}N-DNA from *P. aeruginosa* ρ = 1.745 g cm^{-3}; mitochondrial DNA, ρ = 1.716 g cm^{-3}; cellular DNA, ρ = 1.705 g cm^{-3} and the interface at the gradient surface.

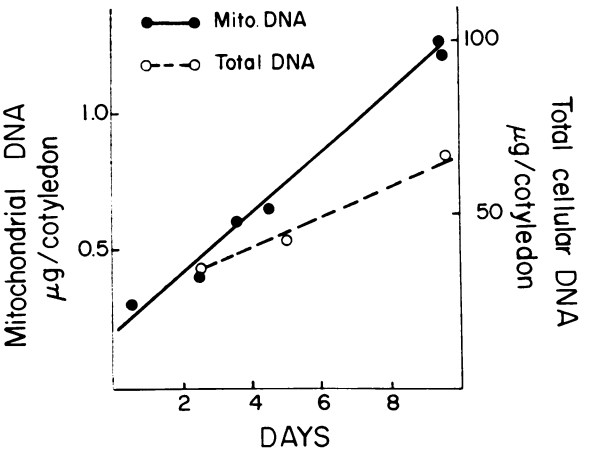

FIG. 4. Increase in the amounts of mitochondrial and total cellular DNA during germination.

the number of bacteria present (2×10^6) was 10^3 times too low to account for the amount of DNA present in the satellite band.

Having established the identity of the mitochondrial DNA band, the amount of mitochondrial DNA in preparations of crude mitochondria was measured by CsCl density gradient centrifugation at intervals during germination. The amount of mitochondrial DNA per seed increased slightly more than 4-fold during the period from 0.5 days to 9.5 days of germination (fig 4). This increase may be compared with a 13-fold increase in succinoxidase activity which was measured on the same samples. The increase in mitochondrial DNA may also be compared with the increase in total cell DNA (fig 4). The relative increase in mitochondrial DNA is nearly twice the relative increase in total DNA over a comparable germination period.

The relative increases in all of the various parameters investigated are summarized in table III. From this table it may be seen that the relative increases in the number of particles and in the amount of mitochondrial DNA are very close. The amount of cytochromes a — a_3 and the level of succinoxidase increase also, but at a rate which is much greater than the increase in the number of mitochondria and the amount of mitochondrial DNA. Succinic dehydrogenase, increases initially at an intermediate rate. All of these changes occur in tissue which is presumably nondividing (5). The total DNA, which normally bears a close correlation to the number of cells shows a 2-fold increase between 2.5 and 9.5 days.

Table III. *Comparison of Various Constitutive and Functional Changes in Peanut Cotyledon Mitochondria Occurring During Germination*

	Ratio of amount or rate at 9.5 days to that at indicated time	
	0.5 day	2.5 day
Cytochrome a–a_3	...	5.6
Succinoxidase	13	5.5
Succinic dehydrogenase	6.0	2.4
Mitochondrial DNA	4.1	3.1
No of particles	...	2.9

Discussion

The increased mitochondrial activity in germinating peanut cotyledons apparently reflects 2 superimposed phenomena; an increase in the number of mitochondria and a developmental change in the composition and respiratory capacity of the mitochondrial population. These relationships are illustrated by comparing relative increases in various mitochondrial constituents and activities.

The increase in mitochondrial DNA parallels in a striking manner the increase in numbers of particles obtained by direct count. This relationship strongly suggests that the new particles are mitochondria containing a new complement of DNA. It can be calculated from our data that each mitochondrion contains 4×10^{-11} μg of DNA. In comparison, Suyama and Preer (22) made an estimate of 3.7×10^{-10} μg of DNA per *Tetrahymena* mitochondrion and Suyama and Bonner (21) estimated 5.0×10^{-10} μg per mung bean mitochondrion. In these papers it is not clear what method was used to assay the amount of mitochondrial DNA. If this value was obtained by measuring the 260 mμ absorbancy of DNA extracts from deoxyribonuclease treated mitochondrial preparations, it is possible that the DNA was overestimated. During the course of our investigations it was found that, although the nuclear DNA band on CsCl gradients disappeared after deoxyribonuclease digestion, 50 % of the amount of DNA present in undigested mitochondrial suspensions is still present as diphenylamine-reactive non-dialyzable polydeoxynucleotides. Since 90 % of the amount of DNA present in crude mitochondrial preparations is probably of nuclear origin, measurements of ultraviolet absorption would be 4 to 5 times too high. Even after sucrose density gradient purification of the mitochondria, only 30 % of the DNA is of mitochondrial origin (fig 3c). These observations suggest that estimates of mitochondrial DNA by chemical methods or ultra-violet absorbancy may be too high unless precautions are taken to insure that all contaminating DNA and oligonucleotides are removed.

Our estimate of the amount of DNA present in peanut cotyledon mitochondria agrees with the findings of Corneo, et al. (6). These workers estimated that each beef heart mitochondrion contains 5×10^{-11} μg of DNA. If the amount of DNA present in a peanut mitochondrion existed as 1 double-stranded molecule it would have a molecular weight of 24 million. This value is comparable to the value of 16 million obtained by Luck and

Reich (14) from measurements of the length of *Neurospora* mitochondrial DNA strands by electron microscopy.

The DNA content of peanut mitochondria is similar to that of bacteriophages. If it possessed genetic properties similar to the double-stranded DNA in other systems, it could code for nearly 200 proteins with an average molecular weight of 10^5. Therefore it is at least possible that mitochondrial DNA could direct to a large extent the synthesis and organization of mitochondrial proteins.

The results obtained in this study by measuring mitochondrial and total DNA in germinating peanut cotyledons show that the relative increase in mitochondrial DNA is approximately 50 % greater than the relative increase in total DNA. This observation is certainly not conclusive but does suggest that synthesis of mitochondrial DNA is in some way distinct from the general synthesis of DNA. This conclusion is also in agreement with the difference in turnover rates of ^{15}N during the semiconservative replication of nuclear DNA and mitochondrial DNA reported by Corneo, et al. (6).

During the early stages of germination the particles isolated from cotyledon tissue have a low oxidative capacity. This low oxidative capacity seems to be related to a low cytochrome content and perhaps in particular to a deficiency in cytochromes a − a_3. As germination proceeds the relative increase in succinoxidase activity and cytochromes exceeds the increase in succinic dehydrogenase. These findings bear a striking resemblance to results that have been obtained with yeast grown under aerobic and anaerobic conditions. When yeast cells are grown anaerobically on glucose they have no oxidative capacity and lack cytochromes b and a − a_3 (7). Particles can be isolated from anaerobic yeast cells and, although they contain no cytochrome b or a − a_3 and possess no oxidative activity, they do have relatively high succinic dehydrogenase activity (12, 17, 18, 24). After the yeast cells have been adapted to aerobic conditions, mitochondria with a full complement of cytochromes and enzymatic activities can be isolated. It is presumed that these succinic dehydrogenase containing precursors develop into typical mitochondria (17, 18).

The relationships found in this study may reflect either a uniform change in composition and functional capacity throughout the mitochondrial population or the contributions of a new subpopulation of more active mitochondria. It is not known whether the increase in mitochondrial numbers and the developmental changes in composition and functional capacity of mitochondria occur throughout the cotyledon tissue. Preliminary histochemical determinations of succinic dehydrogenase by Lott (personal communication) indicate that through the first 5 or 6 days of germination the enzyme is present throughout the tissue. The vascular tissue is always more active than the storage tissue but for the first 5 to 6 days the relative increase in both tissues appears to be the same. Later on, the vascular tissue has most of the staining. These observations emphasize the uncertainty of the mechanism by which the composition and functional capacity of the mitochondrial population is altered. Obviously further critical study of the histological distribution of mitochondrial enzymes during germination is needed.

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