Biogenesis of Mitochondria in Germinating Peanut Cotyledons[1]

R. W. Breidenbach, Paul Castelfranco, and Carol Peterson
Department of Botany, University of California, Davis, California

Received November 1, 1965.

Summary. The increase in respiratory activity in germinating peanut cotyledons has been correlated with an actual increase in mitochondria. Using sucrose gradient centrifugation, it was shown that succinoxidase activity is associated with a well-defined band of cell particulates of uniform density, size and ultrastructure. During germination the population of succinoxidase-containing particles increases, as shown by enzymatic assay, protein assay and direct particle counts with the phase contrast microscope.

It has recently been shown that the β-oxidation of higher fatty acid CoA derivatives to acetyl-CoA is catalyzed by extramitochondrial enzymes in peanut cotyledons (13, 14) and castor bean endosperm (16). The carboxyl carbon of acetyl-CoA is released as CO_2 in the presence of mitochondrial supernatant preparations obtained from either tissue; in both cases maximal rates of CO_2 production are obtained only in the presence of added glyoxylate.

When radioactive acetyl-CoA was incubated with a soluble system from castor bean endosperm (16) various organic acids became labeled. The labeling pattern obtained was consistent with that which Benedict and Beevers (2) found using buffer extracts of castor bean endosperm particulates and which they interpreted in terms of the glyoxylate bypass. The results obtained by Rebeiz et al. (13, 14) and by Yamada and Stumpf (16) with soluble systems could indicate merely that the enzymes of the β-oxidation pathway and some enzymes of the glyoxylate pathway (malate synthetase, malic dehydrogenase, and PEP carboxykinase) are easily leached from mitochondria to which they are attached in vivo. On the other hand these results could indicate that the greatest part of the enzyme activities in question are not associated with mitochondrial structures in vivo.

In an effort to elucidate the enzymatic content of the various particulate fractions obtained from fat-storing tissues of germinating seeds, we have employed the technique of centrifugation in a sucrose density gradient and have endeavored to isolate homogenous mitochondrial pellets. These isolates were assayed for succinoxidase activity and monitored by means of electron microscopy for structural homogeneity. Our results show that succinoxidase activity is associated with well-defined particles of characteristic mitochondrial structure. Succinoxidase activity is almost totally absent in the unimbibed seed and increases steadily during the first 9.5 days of germination. During this period there is also an increase in protein associated with the succinoxidase containing fraction as well as an increase in number of particles.

Materials and Methods

Materials. Reagents and materials were obtained as follows: TPP and cytochrome c from the California Corporation for Biochemical Research; ADP from Sigma Chemical Company; DPN from Boehringer and Sons. Virginia jumbo peanuts were obtained from the Suffolk Peanut Company, Suffolk, Virginia.

Preparation of the Crude Mitochondrial Pellet. Peanut cotyledons were germinated at 22 to 23° in moist perlite-vermiculite as described previously (13). Twenty-five g of cotyledon tissue were blended for 15 seconds in 100 ml of ice-cold 0.4 M sucrose, 0.2 M Tris-HCl, 0.001 M EDTA buffer at pH 7.5. To reduce damage to the isolated particles, the 110 v blender was operated at 60 v. The brei was strained through 2 layers of cheesecloth and the filtrate centrifuged at 600 × *g* for 10 minutes to sediment debris. The supernatant fraction was centrifuged at 10,000 × *g* for 30 minutes. A crude mitochondrial pellet was obtained which was either assayed directly or further purified by sucrose gradient centrifugation. No succinoxidase activity was found in the mitochondrial supernatant, which was therefore routinely discarded. Unimbibed peanut cotyledons were difficult to blend directly. Consequently, they were first shredded in a simple food mill obtained at the local hardware store, and the resulting coarse meal was then blended as described.

[1] This research was supported in part by grants GM-07532 and ES-0054-01 from the United States Public Health Service. The electron microscopy was made possible by the National Science Foundation grant G-21796 and by the personal interest of Professor T. E. Weier.

[2] We are indebted to Mr. John Lott for this electron micrograph.

Separation of the Crude Mitochondrial Pellet on Sucrose Density Gradients. A 39 ml continuous linear gradient ranging from 0.96 to 1.9 M sucrose was prepared in 50 ml tubes. The crude mitochondrial pellet obtained from 25 g of cotyledonary tissue was resuspended in 1.0 ml of 0.96 M sucrose and layered on the surface of the gradient. The material was centrifuged for 3.5 hours in a Servall HB-4 swinging bucket rotor at 15,000 × *g*. The succinoxidase band reached the equilibrium position in about 3 hours. After centrifugation the gradient was separated into 3 ml fractions by means of a gradient sectioning device similar to the one described by Anderson (1). The whole gradient fractionation procedure was carried out at approximately 0°.

Succinoxidase Activity. Succinoxidase was assayed with an O_2 electrode (Gilson Medical Electronics, Middleton, Wisconsin, Model K Oxygraph) operated at −0.6 v. Suspensions of mitochondria or 1 ml aliquots of the gradient fractions were assayed in 2 ml of reaction mixture containing 40 μmoles of phosphate buffer pH 7.3, 5 μmoles $MgCl_2$, 1.0 μmole $MnSO_4$, 0.2 μmole TPP, 1.0 μmole ADP, 0.01 μmole cytochrome c, 0.15 μmole DPN and varying concentrations of sucrose depending on the concentration in the enzyme suspension. This cofactor mixture was used routinely for O_2 cathode assays of various primary dehydrogenases as well as for succinoxidase. The results obtained with the primary dehydrogenases are not reported in this paper. In the succinoxidase assay, only ADP and presumably P_i has any effect on the oxidation rate. Succinate-dependent O_2 uptake was stimulated about 30 % by either ADP or DNP and was proportional to enzyme concentration.

The density gradient fractions were kept at 0° and assayed as soon as possible. Storage periods were not longer than 4 hours and the preparations were stable over this period.

Protein Assay. One-tenth to two-tenths ml aliquots of enzyme suspensions 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. (8) but with the NaOH omitted from the reagents described in their procedure.

Particle Count. Particles in the fractions obtained by density gradient centrifugation were counted with a phase contrast microscope at a magnification of 1250X. An aliquot of the suspension was placed in a Howard mold counting chamber. The number of mitochondria per μl of suspension was obtained by focusing through the layer of suspension and recording all particles observed within a 79 μ^2 quadrat. The thickness of the suspension was indicated by the micrometer on the focusing knob. Thirty quadrats in 3 separate fields were counted in this manner. The particles in these suspensions appeared to be spherical and quite uniform in size.

Electron Microscopy. One ml of the gradient fraction containing the succinoxidase particles was treated with gluteraldehyde at a final concentration of 6 % for 1 hour at 0°. The sucrose concentration was then adjusted to 0.5 M by the addition of 0.1 M phosphate buffer pH 7.0 and the sample was sedimented for 30 minutes at 10,000 × *g*. The pellet was washed several times in buffer, post-fixed for 2 hours in Dalton's osmium fixative (4 % $K_2Cr_2O_7$, 3.4 % NaCl and 2 % OsO_4; 1:1:2) at 0° and washed again in buffer. The pellet was dehydrated in an acetone-propylene oxide series, infiltrated and embedded in a (6:4) Maraglas:Cardolite mixture and hardened in a vacuum oven at 70°. The embedded material was sectioned with a Servall MT-2 Porter-Blum ultramicrotome and the sections post-stained on the grids with uranyl acetate and lead citrate. The sections were viewed with a Hitachi HU-11 electron microscope.

Results

Initial studies with suspensions of unwashed mitochondrial pellets obtained from germinating peanut cotyledons showed that the level of succinoxidase activity increased progressively during germination. Figure 1 shows that as germination proceeds from

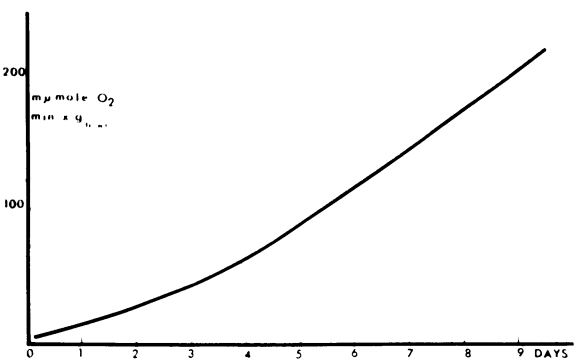

FIG. 1. Increase in succinoxidase activity in crude mitochondrial pellets (isolated by differential centrifugation between 500 × *g* and 10,000 × *g*) from germinating peanut cotyledons.

zero to 9.5 days, the enzymatic activity recovered in the pellet from 1 g fresh weight of cotyledon tissue increased approximately 20-fold. Imbibition is complete within 1 day when the cotyledons have taken up an amount of water roughly equivalent to the weight of the unimbibed seed. When crude mitochondrial pellets were subjected to density gradient purification the succinoxidase activity was confined to a single band (fig 2). In agreement with the observations on crude mitochondrial suspensions (fig 1), the activity of succinoxidase in this band was found to increase with age (fig 2). A major protein band was shown to correspond with the succin-

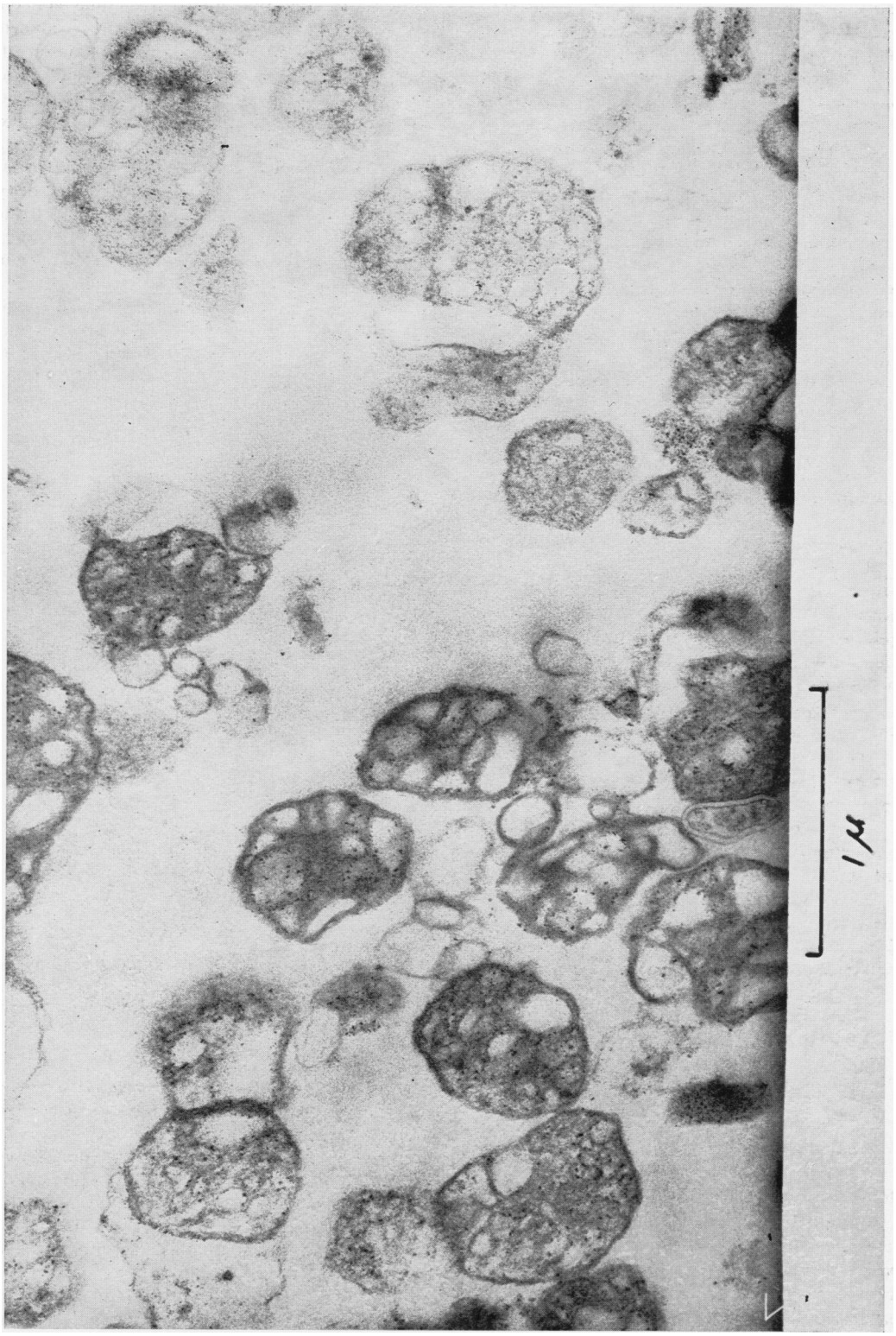

FIG. 4. Electron micrograph of the mitochondrial band obtained by sucrose density gradient centrifugation of mitochondria from 4.5-day-old cotyledons. Magnification, 32850×.

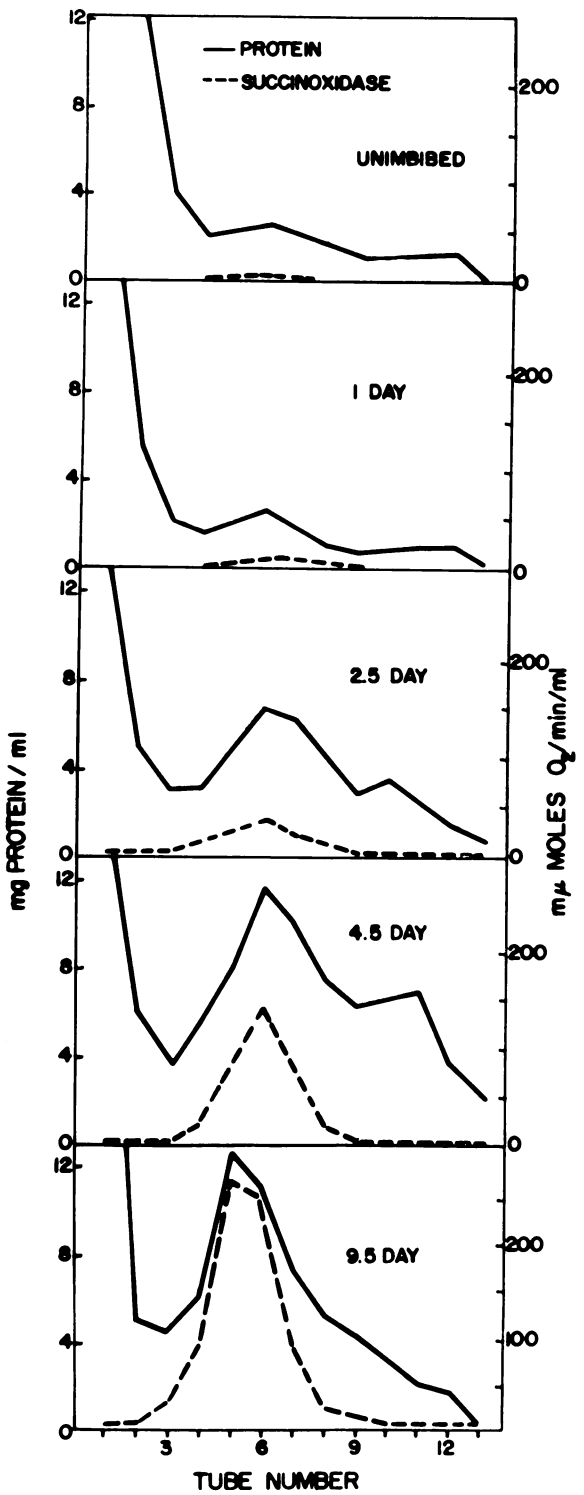

FIG. 2. Distribution of protein and succinoxidase activity after sucrose density gradient centrifugation of mitochondria from germinating peanut cotyledons. From top to bottom; unimbibed seed, 1 day, 2.5 days, 4.5 days, and 9.5 days of germination.

oxidase-containing particles, and this band also increased with germination time.

The crude mitochondrial pellet contains other proteinacious particles both lighter and heavier than the succinoxidase-containing band (fig 2). The lighter particulates account for most of the protein in the unimbibed cotyledons, but during imbibition the protein in this fraction falls to a relatively constant value. A particulate fraction which is more dense than the succinoxidase-containing particles increases rapidly from zero to 4.5 days but decreases slightly thereafter.

The succinoxidase particles appear to become slightly less dense as germination proceeds, since the peak of enzyme activity shifts from fraction 6 (average density, 1.21) at 4.5 days to fraction 5 (average density, 1.20) at 9.5 days.

The relationship between the changes in protein and succinoxidase are shown in figure 3. Not only does the total activity of succinoxidase increase with germination time, but the specific activity expressed in units of succinoxidase activity per mg protein, also increases.

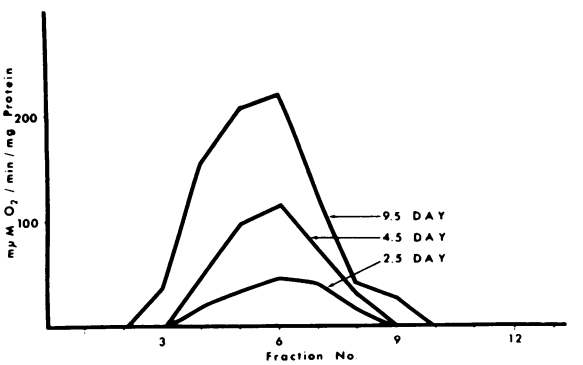

FIG. 3. Increase in the specific activity of succinoxidase during germination (in the mitochondrial band obtained by sucrose density gradient centrifugation).

The general appearance of the particles in the succinoxidase band has been monitored by electron microscopy. Figure 4 shows a typical electron microscope field of a succinoxidase band from 4.5-day-old germinating peanut cotyledons.[2] Except for some small vesicles which are probably mitochondrial fragments or tangential sections of whole mitochondria, the particles are uniform in size (ca. 0.7 μ) and of characteristic mitochondrial appearance. The mitochondrial band obtained from unimbibed cotyledon tissue showed a considerably greater proportion of recognizable contaminants. However monitoring revealed approximately uniform purity of the mitochondrial band at all stages after imbibition. This being the case, the number of particles observed with the phase contrast microscope in each of the density gradient fractions should reflect the number of mitochondria present. Actual counts show that the distribution of particles in fractions 4 to 7 closely

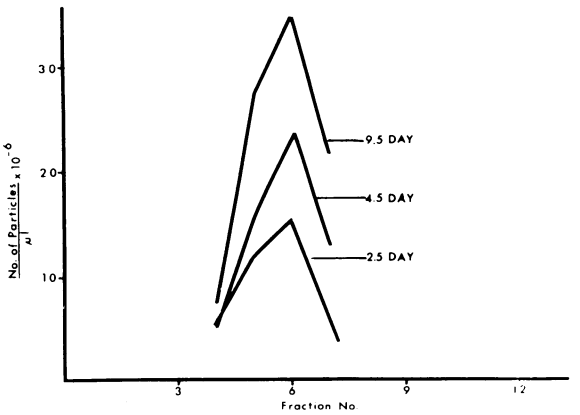

FIG. 5. Particle distribution in the sucrose density gradient. Particles in fractions 4, 5, 6 and 7 were counted with a phase contrast microscope as described under Materials and Methods.

follows the distribution of succinoxidase (fig 5). Furthermore, the number of particles in the band increases markedly with the age of the tissue just as does the total succinoxidase activity.

Discussion

The experimental results clearly indicate that a synthesis of succinoxidase-containing particles occurs in germinating peanut cotyledons. The particles are presumed to be mitochondria, since the succinoxidase system is traditionally associated with mitochondria. This activity was not detected in any other cell fraction. Preparations purified by sucrose density gradient contain increasing numbers of particles, increasing protein and increasing succinoxidase activity as germination proceeds.

It is not known whether the synthesis of mitochondria which we have measured occurs uniformly throughout the cotyledon. However, preliminary histochemical observations with vital redox indicators have shown that succinic dehydrogenase activity is present in both the vascular bundles and the storage parenchyma.

Initially the succinoxidase-containing band represents less than 10 % of the total protein associated with the crude mitochondrial pellet, but after 9.5 days of germination the succinoxidase band contains approximately 42 % of the total protein in the crude pellet. We feel that the particulates which lack succinoxidase activity cannot be regarded as mitochondria in a proper biochemical sense. Eventually we plan to undertake a detailed study of the nonmitochondrial particulates and of the enzymatic activities associated with them.

Our observations made with purified mitochondrial preparations agree with most of the results reported by Cherry (3). Nearly all of the electron transport and citric acid enzymes which he measured increased in activity during the portion of the germination period comparable to ours. In fact, Cherry suggested that there is an increase in mitochondria during germination.

Other enzymes usually considered to be mitochondrial have been measured in peanut preparations. Results indicate that they too increase during germination (3, 4, 12). However, as was pointed out for the β-oxidation pathway, the presumption of a given enzyme's localization based on experimental findings with different biological materials is not necessarily valid.

Biogenesis of mitochondria has been studied in several biological systems (5), particularly rapidly-growing cultures of yeast (6, 7, 15) and *Neurospora* (9, 10, 11). One difference between cotyledon tissue and these materials is noteworthy: germinating peanut cotyledons have a very low rate of cell division: Cherry (4) failed to discover any mitotic figures in this tissue upon anatomical examination. Therefore, we hope that a study of mitochondrial biogenesis in a tissue in which nuclear events are probably minimal may yield information concerning nucleus-organelle interactions that could not be obtained in rapidly dividing cell populations.

Literature Cited

1. ANDERSON, N. G. 1956. Techniques for the mass isolation of cellular components. In: Physical Techniques in Biological Research. Vol. III. G. Oster and A. W. Pollister, eds. Academic Press, New York. p 337.
2. BENEDICT, C. R. AND H. BEEVERS. 1962. Formation of sucrose from malate in germinating castor beans. II. Reaction sequence from phosphenolpyruvate to sucrose. Plant Physiol. 37: 176–78.
3. CHERRY, J. H.. 1963. Nucleic acid, mitochondria and enzyme changes in cotyledons of peanut seeds during germination. Plant Physiol. 38: 440–46.
4. CHERRY, J. H. 1963. Protein bodies, nucleic acids, and enzymes of peanut cotyledons: changes on germination. In: Proceedings, Seed Protein Conference; January 21-23, 1963. Southern Utilization Research and Development Division, New Orleans, Louisiana. p 115–44.
5. HANSON, J. B., A. E. VATTER, M. E. FISHER, AND R. F. BILS. 1959. The development of mitochondria in the scutellum of germinating corn. Agron. J. 51: 295–301.
6. EPHRUSSI, B. 1950. The interplay of heredity and environment in the synthesis of respiratory enzymes in yeast. The Harvey Lectures, 1950-1951. Charles C. Thomas, Publisher. Springfield, Illinois.
7. LINNANE, A. W., E. VITOLS, AND P. G. NOWLAND. 1963. Studies on the origin of yeast mitochondria. J. Cell Biol. 13: 345–50.
8. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 193: 265–75.
9. LUCK, D. J. L. 1963. Genesis of mitochondria in *Neurospora crassa*. Proc. Natl. Acad. Sci. 49: 233–40.

10. LUCK, D. J. L. 1965. The influence of precursor pool size on mitochondrial composition in *Neurospora crassa*. J. Cell Biol. 24: 445–60.
11. LUCK, D. J. L. 1965. Formation of mitochondria in *Neurospora crassa*: A study based on mitochondrial density changes. J. Cell Biol. 24: 461–70.
12. MARCUS, A. AND J. VELASCO. 1960. Enzymes of the glyoxylate cycle in germinating peanuts and castor beans. J. Biol. Chem. 235: 563–67.
13. REBEIZ, C. AND P. CASTELFRANCO. 1964. An extra-mitochondrial enzyme system from peanuts catalyzing the β-oxidation of fatty acids. Plant Physiol. 39: 932–38.
14. REBEIZ, C., P. CASTELFRANCO, AND A. H. ENGELBRECHT. 1965. Fractionation and properties of an extra-mitochondrial enzyme system from peanuts catalyzing the β-oxidation of palmitic acid. Plant Physiol. 40: 281–86.
15. SCHATZ, G. 1963. The isolation of possible mitochondrial precursor structures from aerobically grown (baker's) yeast. Biochem. Biophys. Res. Commun. 12: 448–51.
16. YAMADA, M. AND P. K. STUMPF. 1965. Fat metabolism in higher plants. XXIV. A soluble β-oxidation system from germinating seeds of *Ricinus communis*. Plant Physiol. 40: 653–58.