Nucleic Acid, Mitochondria, & Enzyme Changes in Cotyledons of Peanut Seeds during Germination [1]

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The enzyme complement of plant cells does not remain constant; enzymic and physiological activities change with cell growth and maturation. During the germination of seeds there is a major development of mitochondria in storage and embryonic tissue (1, 2, 12, 14, 17). In the storage tissues (cotyledons or endosperm) of plants, there is about a two to threefold increase in mitochondrial nitrogen content and in respiratory activity during the early stage of germination which subsequently declines when the storage materials are depleted.

In a previous study (4) the author found that the RNA content of the peanut cotyledon doubled during the first stage of germination, followed by a sharp decline; the pattern resembled those for mitochondrial activities in storage tissues (2, 12, 14). Since the peanut cotyledon is not a growing portion of the plant and its cells do not undergo mitosis, this tissue was chosen to study the relation between the levels of nucleic acid, the activity and structure of mitochondria, and the activities of various mitochondrial and nonmitochondrial enzymes during germination. This paper is a report of our findings.

Materials & Methods

Plant Material. Virginia 56-R peanut (*Arachis hypogaea* L.) seed (thanks are due to Dr. W. K. Bailey for the seed) were lightly dusted with 2,3-dichloro-1,4-naphthoquinone, a fungicide, and germinated in a dark, humid atmosphere at 30° in vermiculite. The cotyledons of dry seed and seedlings were removed and washed prior to analysis. Dry weights were determined by drying the tissue in an oven at 110° for about six hours.

Tissue Analysis. The nucleic acids were determined by a method (5) previously found satisfactory for peanut cotyledons.

For protein determination, the tissue was homogenized in 0.5 M sucrose and the cellular debris was removed at 1500 × *g* for 10 minutes. Aliquots of the crude homogenate and debris-free homogenate were precipitated in 10 % trichloroacetic acid and the protein determined by the method of Lowry et al. (16).

Mitochondrial Respiration. Mitochondria were isolated from samples of 20 peanut cotyledons and their oxidative and phosphorylative capacities determined with the techniques and vessel additives found satisfactory for corn scutella (12).

The phosphorylation coupled to oxidation was determined by the disappearance of P_i from the vessels during the period of measured O_2 consumption (generally 30 min). One vessel of each treatment was removed when the stop cocks were closed, with respiration and final P_i being determined from two additional vessels. The method of Fiske and Subbarrow (9) was used to determine P_i.

Enzyme Assays. Ribonuclease activity was determined on homogenates of peanut cotyledons ground in 0.5 M sucrose and cleared of cellular debris. The cleared homogenates were filtered through glass wool and 1 ml aliquots were incubated in the presence of 0.25 M sucrose, 5×10^{-4} M $MgSO_4$, 5×10^{-3} M KCl, and 1 mg/ml of yeast RNA (pH 6.5) previously found satisfactory for corn scutella ribonuclease (7). Final volume was made to 2 ml, incubated for 30 minutes at 28° and the disappearance of acid insoluble RNA determined as described (7).

Succinic dehydrogenase activity was determined on acetone powder extracts of isolated mitochondria by the method of Hiatt (13). Mitochondria were isolated using the same techniques as for the mitochondrial respiration study and a 2 % suspension of 0.06 M Tris buffer (pH 7.6) of the powder was used as a source of enzyme. The rate of reduction of 2,6-dichlorophenolindophenol (13) was followed at 600 mμ during the first 2 minutes of the reaction. One unit of enzyme activity is defined as that amount of enzyme which catalyzes a decrease in optical density of 0.01 per minute under the conditions of the assay.

Cytochrome oxidase activity was also determined on the acetone power extracts by the method of Cooperstein and Lazarow (8). An aliquot of the extract was mixed in a solution containing 1.5×10^{-5} M cytochrome c (chemically reduced) and 0.03 M potassium phosphate pH 7.4; the rate of cytochrome c reduction was followed at 550 mμ. The activities given are the initial rates obtained during the interval between 15 to 45 seconds after initiating the reaction; during this interval a linear decrease in optical density with time was obtained.

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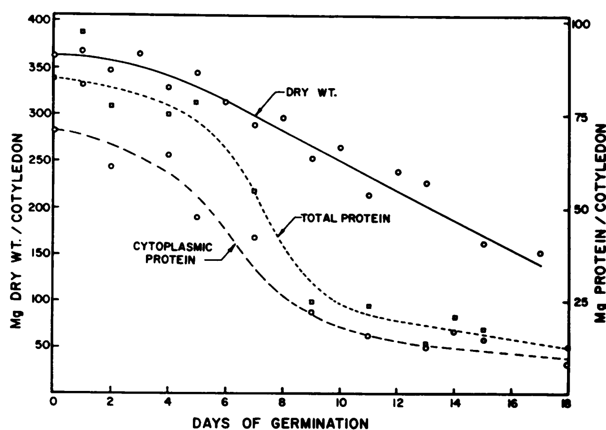
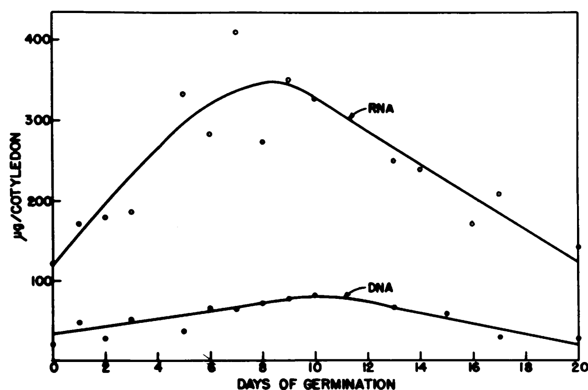
Debris-free homogenates were prepared and the following enzymes assayed by the method of Ragland and Hackett (20): DPNH oxidase, DPNH cytochrome c reductase, succinic cytochrome c reductase, and glucose-6-P dehydrogenase. The tissue was homogenized in 0.42 M mannitol, 5×10^{-3} M KCl, 5×10^{-3} M $MgSO_4$, and 2×10^{-2} M Tris buffer pH 7.5 and filtered through cheesecloth. The homogenate was cleared of cellular debris at 1500 × *g* for 10 minutes and used directly as a source of enzyme. DPNH oxidase and glucose-6-P dehydrogenase were followed by the change in absorbency at 340 mμ and the cytochrome c reductase at 550 mμ.

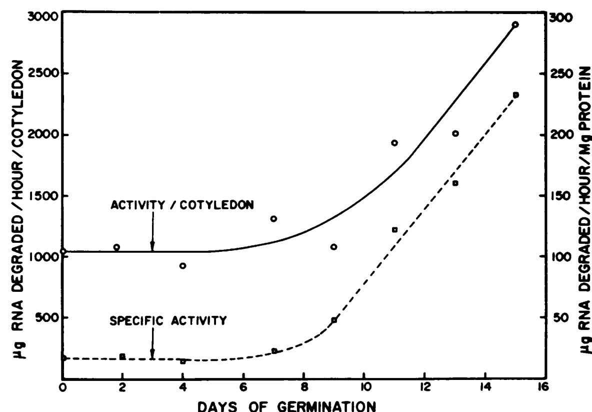
The debris-free homogenate was also assayed for cytochrome oxidase activity by the method of Cooperstein and Lazarow (8).

Isocitritase activity was determined on homogenates of peanut cotyledons prepared in 0.1 M potassium phosphate pH 7.6 (15). The homogenates were filtered through cheesecloth and the cellular debris removed at 1500 × *g* for 10 minutes. The subsequent debris-free homogenate was assayed for isocitritase activity by the method of Rao and Ramakrishnan (21). Glyoxylate was determined by the 2,4-dinitrophenylhydrazine reaction according to Smith and Gunsalus (22).

Electron Microscopy. Sedimented mitochondria from peanut cotyledons were fixed by the method of Lund et al. (17). $MgCl_2 \cdot 6H_2O$ (0.5 %) was added to the 1.5 % osmium tetroxide in veronal buffer (pH 7.6). Fixation was carried out for 2 hours at room temperature followed by dehydration in an ethanol series and two changes of absolute ethanol at room temperature. Specimens were infiltrated overnight in butyl and methyl methacrylate (4:1) containing 0.5 % dichlorobenzoyl peroxide and were subsequently polymerized at 45°. Sections (400–600 A) were counterstained with 2 % $KMnO_4$ for 15







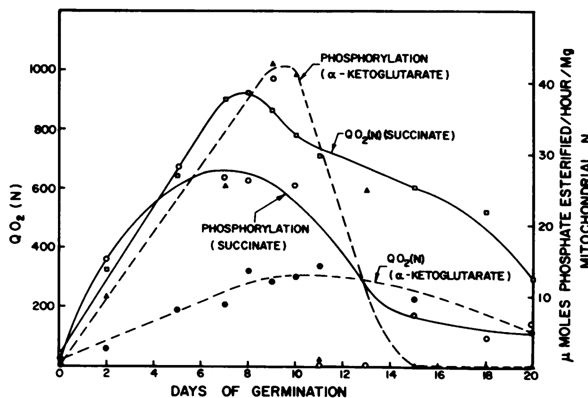


FIG. 1 (*top left*). Depletion of dry weight and storage protein in the peanut cotyledon during germination. Total and cytoplasmic protein was obtained by analysis of the total homogenate and the debris-free (nuclei-free) homogenate, respectively.

FIG. 2. (*top right*). Changes in nucleic acid (RNA & DNA) content in the peanut cotyledon during germination.

FIG. 3 (*bottom left*). Changes in ribonuclease activity in cotyledons of germinating peanuts. Activity (μg RNA hydrolyzed/hr) per cotyledon and specific activity (μg RNA hydrolyzed/hr/mg protein) are plotted.

FIG. 4 (*bottom right*). Oxygen consumption and phosphate esterification by mitochondria from cotyledons of germinating peanuts with the substrates indicated. Ten μ moles of pyruvate was added to the vessels containing 20 μ moles of succinate to spark succinate oxidation (see ref 12). No pyruvate was added to the vessels containing 40 μ moles of α-ketoglutarate. Qo_2 (N) defined as the μ liters of O_2 consumed per hour per mg mitochondrial nitrogen.

minutes and were observed with a Phillips EM-100 electron microscope.[3] All observations were made at a magnification of 18,000X.

All data were obtained from two or more experiments.

Results

Depletion of Storage Materials: During germination of peanut seed and development of the seedling plant the dry weight of the cotyledon decreases 60 % or about 220 mg per cotyledon (fig 1). This large loss of material is attributed to the disappearance of storage reserves; one of these reserves is protein. The depletion of protein from the peanut cotyledon is shown in figure 1. The protein content slightly decreased the first 5 days of germination, followed by a rapid decrease in content between 6 to 9 days. By 10 days of germination about 70 % of the protein was depleted with little loss thereafter. The depletion of total and cytoplasmic protein (debris-free) roughly paralleled each other.

Changes in Nucleic Acids. Figure 2 shows that as peanut seed germinate, the RNA content increases threefold by the eighth day followed by a rapid decline in content thereafter. The DNA content doubled by the tenth day and then declined. These data agree with previous experiments (4) with various varieties of peanut seed. However, in the previous experiments, the point of germination when the highest level of RNA was reached varied from 2 to 6 days depending on the variety and the maturity of the seed. Therefore, the changes in nucleic acids for the peanut seeds used in these experiments are presented for comparison with changes in enzymic activities.

To ascertain whether a nuclease may be involved in the pattern of RNA metabolism, the activity of ribonuclease in the peanut cotyledon was assayed. Both the specific activity (μg RNA hydrolyzed/hr/mg protein) and activity per cotyledon remained constant until about eight days of germination (fig 3). At this stage of germination ribonuclease activity increased several fold, suggesting that this enzyme is involved in the in vivo degradation of RNA after 9 days of germination.

Mitochondrial Activity. Oxygen consumption by mitochondria from cotyledons of germinating peanuts with succinate and α-ketoglutarate as substrates is shown in figure 4. Succinate was the most effective substrate; mitochondrial respiration on succinate sharply increased with germination reaching the peak of activity with a Qo_2 (N) of 900 on the eighth day and declining thereafter. Respiration on α-ketoglutarate, the less effective substrate, increased to a Qo_2 (N) of 300 on the tenth day followed by a decline activity. Phosphorylation by isolated mitochondria for both succinate and α-ketoglutarate increased from zero for mitochondria from resting seed to the highest values obtained at 5 to 10 days after planting (fig 4). After 10 days, phosphorylation for both substrates decreased to zero or nearly to zero. This pattern of phosphorylation is somewhat similar to that for respiration and is reminiscent of the pattern of RNA change (fig 2) with the peak activity or content occurring at about eight days.

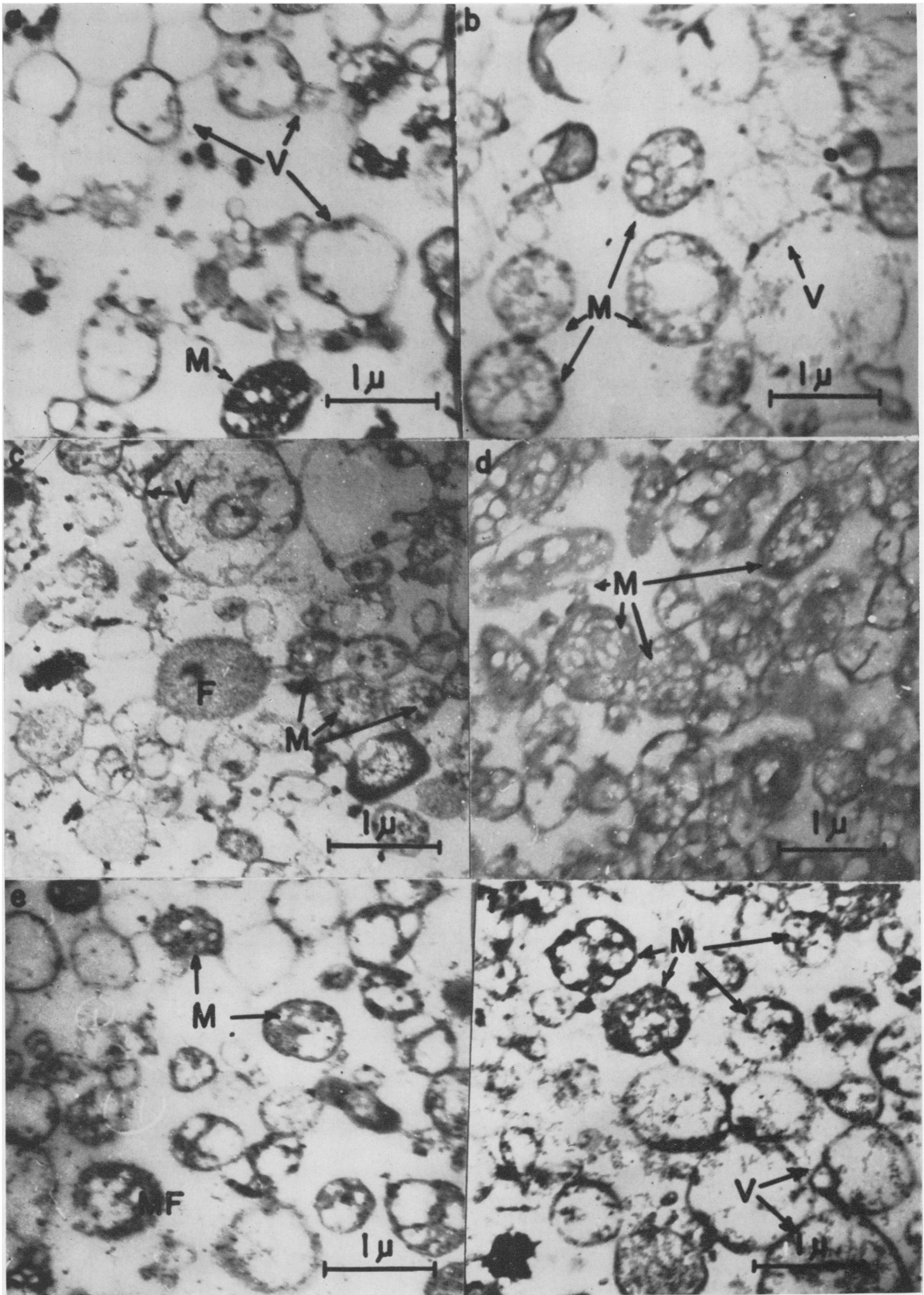
From figure 4 the P/O ratios can be calculated. Mitochondria from resting seed had a P/O ratio of zero. On the second day the P/O ratios were 0.53 with succinate and 1.85 for α-ketoglutarate which were the highest values obtained during germination. After the second day of germination the P/O ratios declined to zero or near zero by the fifteenth day.

Electron Microscopy of the Mitochondrial Pellet. The electron micrographs shown in figure 5 reveal that the material in the mitochondrial pellet is heterogeneous, containing broken fragments of protein bodies and vesicular matter some of which is probably of the endoplasmic reticulum. There are few typical mitochondria in the pellet isolated from the cotyledons of resting seed (fig 5A). The many vesicular elements contain few inclusions, are slightly larger than normal mitochondria and appear to be joined together. The mitochondrial pellet from 2- and 5-day old cotyledons (fig 5B & 5C, respectively) contain more typical mitochondria and fewer vesicular elements. A large number of typical mitochondria can be seen in the electron micrograph of the pellet from 8-day old cotyledons (fig 5D). These mitochondria are about 0.6 μ in diameter and contain a dense internal structure. It is of significance that these mitochondria also have the greatest respiratory activity (fig 4). After 8 days of germination, the mitochondria appear to swell, show a disorganization of internal structure and show a greater degree of mitochondrial disintegration (fig 5E & 5F); this increase in disorganization parallels the diminution in respiratory rate.

Enzymic Activity of Acetone Powders of Mitochondria. It was of interest to determine whether or not certain mitochondrial enzymes showed a developmental pattern of activity during germination similar to the respiratory activity of isolated mitochondria. Figure 6 shows that succinic dehydrogenase and cytochrome oxidase activities of extracts from acetone powder of isolated mitochondria do not fit the mitochondrial respiratory pattern (fig 4). Succinic dehydrogenase increased over eightfold in activity from 0 to 15 days of germination. Cytochrome oxidase also showed a major increase in activity from 0 to 13 days followed by a rapid loss in activity on the fifteenth day.

Enzymic Activity of Homogenates. Cytochrome oxidase and DPNH oxidase activity showed only slight changes with germination (fig 7) while DPNH and succinic-cytochrome reductase showed a developmental pattern of activity quite similar to that for mitochondria. In both cases the reductase activity

[3] Trade names are given as part of the exact experimental conditions and not as endorsement of the products over those of other manufacturers.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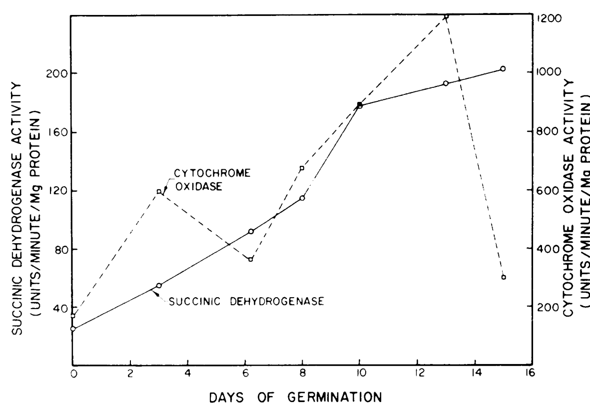

FIG. 6. Activities of two mitochondrial enzymes from the cotyledons of germinating peanuts; extracts of acetone powders of once-washed mitochondria were assayed for succinic dehydrogenase and cytochrome oxidase activity. A unit of activity is defined as the amount of enzyme which catalyzes a decrease in optical density of 0.01 per minute under the conditions of the assay.

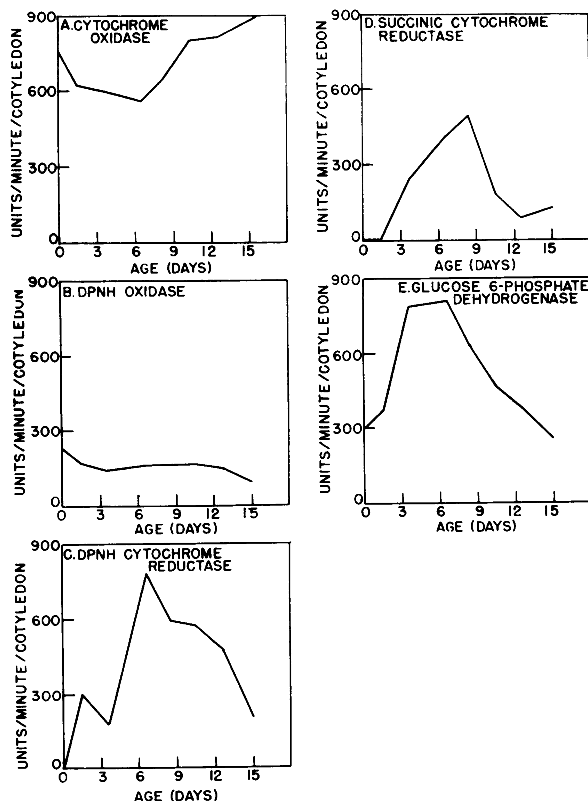

FIG. 7. Changes in activities per cotyledon of various enzymes of germinating peanuts. Homogenates were assayed for the enzymes indicated and a unit of activity expressed as a change in optical density of 0.01 per minute.

was zero in resting seed and rapidly increased in activity with germination to its peak at 7 to 8 days of germination. Subsequent germination resulted in a decline in reductase activity. Seed possess a fairly active glucose-6-P dehydrogenase. During germination the enzyme activity rapidly increases to a maximum at 4 to 7 days and subsequently followed by a sharp drop with age.

The specific activities (activity/mg protein) of the five above-mentioned enzymes during germination of peanut seed show the same pattern of changes as when the activity per cotyledon was plotted. One exception is that the oxidase appear to increase in activity similar to the pattern for succinic dehydrogenase and cytochrome oxidase of acetone powders of mitochondria (fig 6).

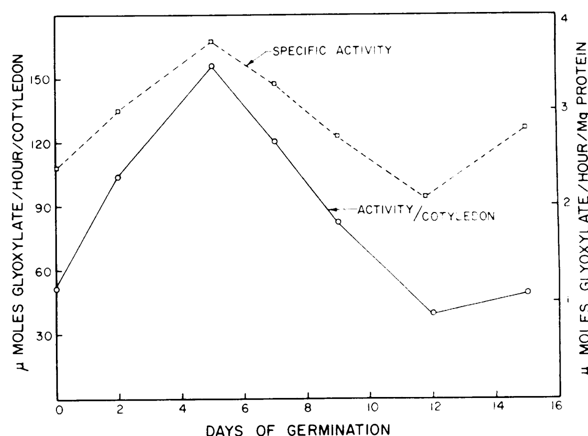

FIG. 8. Changes in isocitritase activity from cotyledons of germinating peanuts. Activity per cotyledon and specific activity are plotted.

The germination of fat-containing seed, such as those of peanut, is accompanied by a decrease in the content of fat and an increase in carbohydrate (19). Kornberg and Beevers (15) have shown that the conversion of fat to carbohydrate may occur by the glyoxylate cycle. One of the enzymes of this cycle is isocitritase; and Beevers (3), and Marcus and Veslasco (18) have shown that this enzyme has a developmental pattern of activity in germinating pumpkin seed and peanut seed, similar to other enzymes reported in this paper. Therefore, it was desirable to assay the activity of isocitritase in the cotyledons of germinating peanuts. Figure 8 shows the change in activity of this enzyme during germination. Both the activity per cotyledon and specific activity show an increase from 0 to 5 days followed by a decline with subsequent germination. As with

FIG. 5. Electron micrographs of once-washed mitochondria isolated from peanut cotyledons. Micrographs of the mitochondrial pellet from cotyledons after 0, 2, 5, 8, 12, and 16 days of germination are shown in A, B, C, D, E, and F, respectively. The magnification in all micrographs is 18,000 ×. M-mitochondria, V-vesicular elements, F-fragment of protein body, MF-mitochondrial fragment.

glucose-6-P dehydrogenase (fig 7), the resting seed contained a fairly active isocitritase and the peak activity was reached earlier than with the respiratory activity of isolated mitochondria (fig 4). However, Beevers (3), and Marcus and Veslasco (18) have previously shown that resting pumpkin and peanut seeds contain little or no isocitritase activity.

Discussion

The data presented here unequivocally shows that as peanut seed germinate and deplete their storage materials, there is an increase in enzyme and mitochondrial activity to about eight days followed by a reduction in activity thereafter. This pattern of enzymic change closely resembles the levels of RNA during germination. From these results one cannot directly relate contents of RNA in the cotyledon to enzyme formation or activation. But from present knowledge of the role of RNA in protein synthesis, it seems likely that RNA could be involved in the observed pattern of enzyme formation or activation. These studies do not show if a specific type of RNA (template) is formed, nor whether it is localized in a particular subcellular organelle.

Akazawa and Beevers (2) and Cherry and Hageman (6) showed that there is an increase in mitochondrial RNA in the castor bean endosperm and corn scutellum, respectively, during germination. This increase in RNA is not only a result of a larger mitochondrial pellet but is also due to an increase in RNA content. Mitochondria with a high RNA content may be related to an increased respiratory activity. The participation of RNA in oxidative phosphorylation is not clear; however, Hanson (10, 11) showed that treatment of mitochondria with ribonuclease destroys activity and causes the formation of holes in the mitochondrial membrane. The electron micrographs (fig 5) show an increase in mitochondrial structure and organization during the first 8 days of germination; subsequent germination results in mitochondria disintegration and loss of activity. The mitochondria from the last stage of germination (fig 5E & 5F) appear similar to those of Hanson (11) which were treated with ribonuclease. This agrees with the observation that after 8 days of germination the ribonuclease activity increases severalfold (fig 3) and thus the in vivo action of ribonuclease may result in mitochondrial disintegration and a loss of some activity.

The in vivo degradation of RNA (fig 2) closely agrees with the enhanced activity of ribonuclease (fig 3). If ribonuclease is actually involved in the disappearance of RNA from the cotyledon, then one of the physiological roles of this enzyme may be to degrade functional RNA after the necessary enzyme complement is obtained. One question that is not answered here is what triggers the formation of ribonuclease which apparently degrades RNA and thus stops other enzyme formation.

Most of the enzymes of the homogenate, and the respiratory activity of mitochondria assayed in this study follow a general pattern of an increase followed by a decrease in activity during germination. This is true for all enzymes or enzyme systems studied except for DPNH oxidase and cytochrome oxidase of the homogenate, and succinic dehydrogenase and cytochrome oxidase of the mitochondrial pellet. This fact points out that certain enzymes, even though involved in a metabolic pathway, may not be affected the same way as other enzymes of the same pathway. Therefore, it is not surprising that changes in the activity of the complex oxidase systems do not exactly parallel the changes in certain individual enzymes. The respiratory and phosphorylative activities of mitochondria not only depend on the presence of essential enzymes but also on the integrated systems and structure of the mitochondria. It appears that during the senescence period of peanut seedlings the loss of mitochondrial activity can be attributed to the loss of some enzyme activities and structural properties.

Summary

A study of the contents of nucleic acid and activities of several enzymes and mitochondria of the peanut cotyledon was made during germination. The following observations were noted:

I. During the germination of peanut seed over 60 % of the dry weight of the cotyledon and 70 % of the protein is depleted.

II. RNA content of the cotyledon triples from 0 to 8 days of germination; subsequent germination results in a rapid loss of RNA. Concomitant with the in vivo degradation of RNA the ribonuclease activity increases several-fold.

III. DNA content of the cotyledon doubles by the tenth day followed by a reduction in content thereafter.

IV. Oxidative and phosphorylative activities of isolated mitochondria showed an increase during germination with their peak in activity occurring at about 8 days; subsequent germination resulted in a decline in activity. The P/O ratios with succinate and α-ketoglutarate as substrates declined with seedling age.

V. Electron micrographs showed that the cotyledon of resting seed contain few typical mitochondria, but many vesicular membranes. During the first 8 days of germination the mitochondria appear to increase in structure and internal organization. As the germination process proceeds the mitochondria swell and there is a large degree of disintegration.

VI. DPNH cytochrome c reductase, succinic cytochrome c reductase, glucose-6-P dehydrogenase, and isocitritase of homogenates of cotyledonary tissue increased in activity to about the fifth to eighth day of germination followed by a rapid reduction in activity thereafter.

VII. DPNH oxidase and cytochrome oxidase of

homogenates showed slight changes with germination, while succinic dehydrogenase and cytochrome oxidase of acetone powders of mitochondria increased in activity with seedling age.

Acknowledgments

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