ULTRASTRUCTURE OF EMBRYONIC PHASEOLUS VULGARIS L. AXES DURING ANHYDROBIOSIS AND THE DEVELOPMENT OF MITOCHONDRIA DURING EARLY GERMINATION

By

BETTY KAY HAMILTON

Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

1972

Submitted to the Faculty of the Graduate School
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
May, 1980

ULTRASTRUCTURE OF EMBRYONIC PHASEOLUS VULGARIS L. AXES DURING ANHYDROBIOSIS AND THE DEVELOPMENT OF MITOCHONDRIA DURING EARLY GERMINATION

Thesis Approved:

Thesis Adviser

Dean of the Graduate College

DEDICATION

To my mother, Peggy Hamilton, and to one of the best teachers I've known, Maxine Kastle. Together, they were responsible for my introduction to the microscope and the infinitely fascinating realm of science.

ACKNOWLEDGEMENTS

I feel that we live in a world full of really great people, and I know more than my share. I would like to recognize a few who have lent their support, moral and otherwise, to make the completion of this dissertation possible: Dorothy Adams, Eddie Basler, Bob Buck, Ellen Cover, Melinda Davis, Margaret Essenberg, Chuck Gardner, Jani Greene, Barbara Hamilton (my sister), Bobbye Hill, Beth Hood, Kathy Kocan, Pat Lawrence, Earl Mitchell, Maria Mottolla, Paul Richardson, Bill Sanford, Lisa Seidman, Dianna Upp, and Fred, Jack and Marge Witz (my cousins). I can never repay the kindness they have shown to me. To these special people I can only extend my heartfelt gratitude.

I must also thank my major adviser, Dr. Glenn Todd, and the advisory committee members, Dr. Eddie Basler, Dr. Paul Richardson, Dr. Earl Mitchell and Dr. Edward Grula for their help and advice in preparation of this manuscript.

It is my great pleasure to thank TOP Services Unlimited who typed both the rough and final drafts of this dissertation. Their care and patience have produced a remarkably beautiful manuscript for which I am deeply grateful.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

CW	cell wall
$\bar{D}$	mean caliper diameter
$\bar{D}_m$	mean caliper diameter of mitochondrial profiles
DMSO	dimethyl sulfoxide
DNP	2,4-dinitrophenol
HC	heterochromatin
KCN	potassium cyanide
N	numerical particle density
N_A	number of profiles observed
N_m	numerical mitochondrial density
Nu	nucleolus
P	plastid
Pb	protein body
pf	phyto ferritin
P_m	points over mitochondria
P_t	points over cytoplasm
S	starch
SHAM	salicylhydroxamic acid
V_m	volume fraction mitochondria

CHAPTER I

INTRODUCTION

Consider, if you will, the phenomenon of anhydrobiosis, the ability of some living organisms to survive loss of sufficient cellular water to cause suspension of metabolic activity. Is it not remarkable that some times in their life cycle numerous species are able to tolerate loss of all but a minimum of their cellular water? Some familiar examples include fungal spores, resurrection plants, nematodes and other soil invertebrates, brine shrimp and water flea cysts, many blue-green algae, most lichens, both the spores and vegetative thalli of many mosses and some liverworts, and the seeds of most higher plants. In some cases the potential to withstand loss of cellular water is restricted to a specific stage of the life cycle, while in other instances, organisms are able to suspend metabolic functions whenever environmental conditions demand.

The strategies adopted by diverse organisms are under study in various laboratories around the world. Current interest is apparent in recent published symposia (Grossowicz et al., 1961; Crowe and Clegg, 1973; Clegg and Crowe, 1978). While a complete understanding of this phenomenon has not yet been reached, several common trends seem to be emerging from ongoing research. In most cases, elevation of the cytoplasmic levels of polyalcohols (such as di- and trisaccharides and glycerol) seem to be required for protection from desicca-

tion damage. It is thought by some workers that the compounds may replace hydrogen-bonded water required to stabilize macromolecular and supramolecular structures. Essential components of the metabolic systems are retained during anhydrobiosis in these economical organisms although they are ametabolic due to lack of water. As water becomes available, simple enzymatic activity is initiated at about 20% relative water content, and complex processes such as protein synthesis become active at approximately 30% relative water content. Additionally, membranes seem to be conserved during desiccation and this is essential to survival of the individual.

My introduction to "anhydrobiosis" occurred when I worked on enzymes retained in desiccated Selaginella lepidophylla with Dr. Todd. We found no change in several enzyme levels using crude assays, and this discovery has left me with a permanent sense of wonder. Later, I decided to combine my love of microscopy and facination with anhydrobiotic organisms. Jim Steinle had worked on the relationship between protein synthesis and desiccation tolerance in excised green bean axes, and I decided to use this system. Initially, I decided to look at the ultrastructure of the embryo, since this tissue is able to survive extreme desiccation. This turned out to be a frustrating problem in terms of technique because conventional fixation procedures involve aqueous solutions! I tried several ideas with limited success, and then Dr. Todd relayed a procedure for "anhydrous" processing that he had heard about at the botanical congress in Moscow. The results were rather interesting although they were initially difficult to interpret. The results of this work are the subject of the second chapter of this thesis.

Interpretation of the dry bean work was still tentative when I attended a symposium entitled "Dry Biological Systems" during the AIBS meetings at Michigan State University 24-25 August 1977. It became apparent that retention of metabolic capacity and structural integrity of membranes during severe desiccation were prime characteristics of successful anhydrobiots. Discussions with other participants reinforced the feeling that my dry beans retained an impressive amount of membrane. However, this did not correspond very well to the view given in the seed literature that cells in seeds were membrane-poor following the drying stage of seed maturation. It was this conflict that lead me to undertake the study of mitochondrial development presented in the third chapter. Some concluding remarks are contained in the final chapter.

CHAPTER II

ULTRASTRUCTURE OF THE EMBRYONIC ROOT OF PHASEOLUS VULGARIS L. FOLLOWING AN ANHYDROUS FIXATION PROCEDURE

Introduction

The life cycle of higher plants is characterized by production of seeds that undergo a period of anydrobiosis prior to germination. Water content usually falls to 10-15% in mature seeds. The dry seed has little or no detectable metabolism and is able to withstand extreme environmental conditions, remaining viable for varying periods of time, depending upon the species and storage conditions. However, once environmental conditions become favorable, germination proceeds very rapidly accompanied by various metabolic activities. For instance, O_2 uptake can often be detected within minutes of the start of imbibition. This is true of the seeds of Phaseolus vulgaris (Opik, 1966; Walton, 1966), P. mungo (Morohashi and Shimokoriyama, 1972), Lactuca sativa (Eldan and Mayer, 1972) and Hordeum vulgare (Abdul-Baki, 1969). Also, protein synthesis has been reported soon after water enters the tissue in P. vulgaris (Walton et al., 1969) and Raphanus sp. (Fujisawa, 1966). In addition, other metabolic events such as ATP formation in lettuce seed (Pradet et al., 1968) and synthesis of phospholipids in pea cotyledons (Harwood and Stumpf,

1970) have been detected during the early period of imbibition. Thus, desiccation results in suspension of metabolic activity, rather than destruction of the enzymes required to resume metabolic activity. On this basis, it is reasonable to suppose that cell membranes are also preserved during seed maturation and desiccation. This expectation is not entirely supported by early ultrastructural studies of seed tissues. Reduction in the extent of membranes, especially endoplasmic reticulum, golgi bodies and mitochondrial membranes, as well as reduced membrane stainability, have been reported in the final stages of seed development (Bain and Mercer, 1966a; Klein and Pollock, 1968; Opik, 1968) and in mature, ungerminated seeds (Paulson and Srivastava, 1968; Opik, 1972). Opik (1972) suggested that populations of organelles may not decline equally in all tissues during seed maturation. Reduced contrast and difficulties in specimen preparation make comprehensive studies difficult, but the most detailed study of seed ultrastructure, that of Bechtel and Pomeranz (1978) on the ungerminated rice grain has confirmed this view.

These studies all suffer because the tissue was prepared using aqueous fixatives that allowed hydration of the tissue and changes in spatial relationships between cellular structures. The assumption made in all these studies is that these changes were, in general, not significant. However, it is difficult to imagine that imbibition, which proceeds during fixation, does not substantially alter cell structures compared to their condition during anhydrobiosis. Perner (1965) attempted to overcome this difficulty using osmium vapors. Unfortunately, because of poor penetration of the osmium, this proved satisfactory only for superficial cells of the pea radicle. Yatsu

(1965) failed in attempts to fix cotton cotyledon using osmium vapors. Freeze fracture of dry seeds (Buttrose, 1971, 1973) revealed that major alterations do occur in cell ultrastructure after even 8 s exposure to water. An alternative procedure using anhydrous organic solvents (dimethysulfoxide and chloroform) proposed by Hallam (1976) has been utilized in this study of the ultrastructure of dry P. vulgaris embryonic root.

Methods and Materials

Embryonic axes were removed from dormant seeds of Phaseolus vulgaris L. cv. Burpee Stringless Greenpod. Sections of the distal portion of the radicle, including the root tip, were dissected and processed anhydrously through a procedure modified from that of Hallam (1976). The primary fixative solution was composed of approximately 6% glutaraldehyde in dimethysulfoxide (DMSO). The glutaraldehyde was prepared by drying 2 ml 70% (EM grade, Polyscience, Inc.) over conc. H_2SO_4 in darkness for 2-5 days. It was then dissolved in the DMSO. The fixative was used immediately, for 2-3 h in darkness. Next, the tissue was washed 40 min in 2 changes of DMSO, followed by 40 min in 2 changes of chloroform. Post fixation was in 2% OsO_4 in dry chloroform for 1 h in darkness. Following post fixation, the tissue was rinsed with 2 changes of chloroform in 20 min, 2 changes of absolute ethanol in 1 h and 3 changes of propylene oxide in 1 h. Infiltration of the tissue with the low viscosity, long pot-life formulation of Spurr's resin (Spurr, 1969) was carried out over 3-5 days. Initially, the tissue was passed through 25%, 50% and 75% resin diluted with propylene oxide, 45 min per step. Final infiltration with pure

resin, 3 changes in 3-5 days, was followed by polymerization at 60 C. Throughout the procedure, care was taken to keep all solvents and reagents anhydrous. This was done by adding molecular sieves (activated, indicating, type 4A, Baker Chemical Co.) to all bottles, including epoxy resin components, and carrying out all steps in a desiccator over $CaCl_2$.

General anatomical information was obtained from 0.5-0.75 μ sections cut with glass knives and stained with azure B (bromide), pH=8 (Hoefert, 1968). For electron microscopy, thin sections were cut with a diamond knife and stained with 2% aqueous uranyl acetate and lead citrate (Venable and Coggshall, 1965). The sections were examined on a Phillips EM 200 microscope operating at 60 KeV.

In order to compare tissue processed anhydrously with tissue processed in aqueous fixative solutions, sections of root tip were also fixed by placing dry tissue into a conventional fixative composed of 2% glutaraldehyde in 0.1 M s-collidine, pH=7.4 at room temperature for 2 h. The tissue was washed 3 times, 5 min each in buffer, post fixed in 1% OsO_4 in buffer at room temperature for 1 h, and washed 3 times for 5 min each in buffer. Dehydration in a graded ethanol series and transfer to propylene oxide was followed by infiltration with Spurr's resin and polymerization at 60 C. Only superficial cells of the tissue blocks were well fixed and embedded due to poor penetration of reagents. However, the superficial layers were suitable for electron microscopy, and silver sections were cut with glass knives, stained and observed as previously described.

Results

Examination of thick, transverse sections with the light microscope disclosed three differentiating regions of the embryonic root (Figure 1). The epidermis and cortex comprise the outer layer. These cells are generally parenchymatous with large lobed nuclei, abundant protein bodies and very dense cytoplasm. The cell walls are folded, due to the effect of water loss during seed maturation, and intercellular spaces are reduced in volume. The cell walls remain in close contact with the protoplasts. Provascular tissue forms a cylinder between the cortex and the pith. These cells appear very compressed, and contain lobed nuclei with distinct nucleoli and some small protein bodies, but are characterized by their dense, darkly staining cytoplasm. The central section of the root is the pith, composed of parenchymatous cells similar to those of the cortex.

The aspect of the tissue is unusual at first glance because the cells have become contorted and folded. This occurs as a consequence of water loss and decreased volume of the tissue during seed maturation. The cell walls are still thin and will expand during seed germination as the radicle emerges from the seed coat prior to further cell divisions. Also, the tissue prepared anhydrously stains more strongly than equivalent ungerminated tissue prepared in aqueous solutions. Tissue processed anhydrously exhibits metachromatic staining with azure B. The pith and cortex stain purple-blue, while the provascular cells and a few adjacent cells stain strongly purple-red. The reason for this differential staining is not known, but variation in pH influences the stain's metachromasticity (Hoefert, 1968), and it reacts with DNA and RNA to give products with different

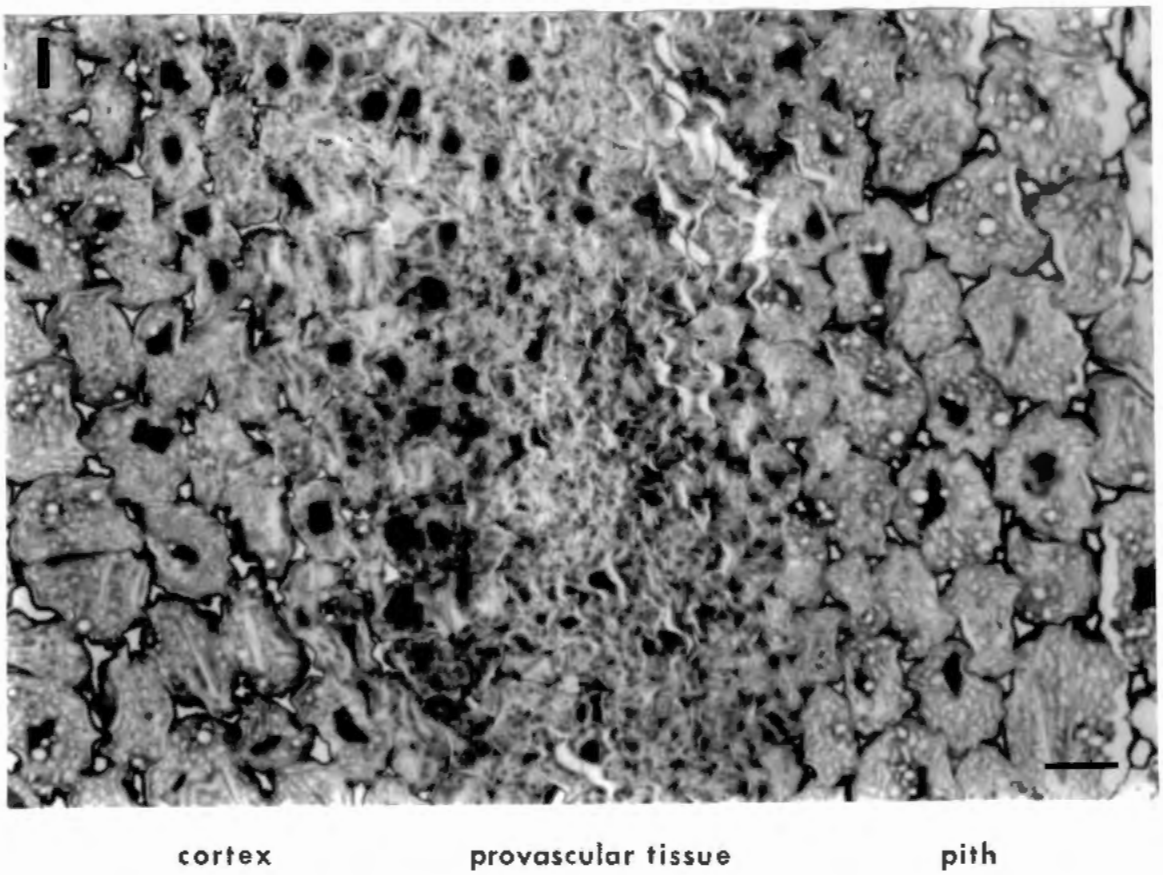


Figure 1. Light micrograph of tissue from the anhydrously prepared root of Phaseolus vulgaris. The cells are contorted due to loss of volume during seed maturation, and protein bodies are abundant within the cytoplasm. Nuclei are lobed with distinct nucleoli. Cross section stained with azure B (bromide). Magnification 100, bar represents 1000 μ.

spectral characteristics (Flax and Himes, 1952). This reaction may also reflect the inability of the anhydrous technique to extract stainable compounds when compared to conventional aqueous fixatives.

As would be expected from light microscopy of the thick sectioned material, the aspect of the tissue at high magnification is of cells that have contracted and become dense through loss of water (Figure 2). Some cellular organelles, such as protein bodies and nuclei are easily recognized, other organelles, such as mitochondria and plastids have become modified during seed maturation, while golgi bodies cannot be identified. All of the recognizable structures within the cell are irregular in shape due to uneven shrinkage, and any space otherwise unoccupied in the ground cytoplasm is filled with ribosomes. The cell walls are generally contiguous with the cytoplasm, however, as lipid droplets are aggregated along the cell margins, this region tends to be poorly infiltrated, often becoming stretched and distorted in the electron beam.

Following anhydrous preparation, the membranes do not appear as the classic tripartate image composed of two electron dense lines separated by a line of low electron density. Instead, they appear in negative contrast (see nuclear envelope in Figure 3), or as electron dense lines often associated with narrow electron translucent lines (Figures 4, 5, 6 and 7), or as pairs of electron translucent lines separated by an electron dense line of medium density approximately equal to the surrounding matrix (see the internal membranes of mitochondria and plastids, Figures 6, 7, and 8). Due to distortion that occurs as the tissue becomes desiccated at the end of seed formation, the organelles are literally crumpled and when sectioned their

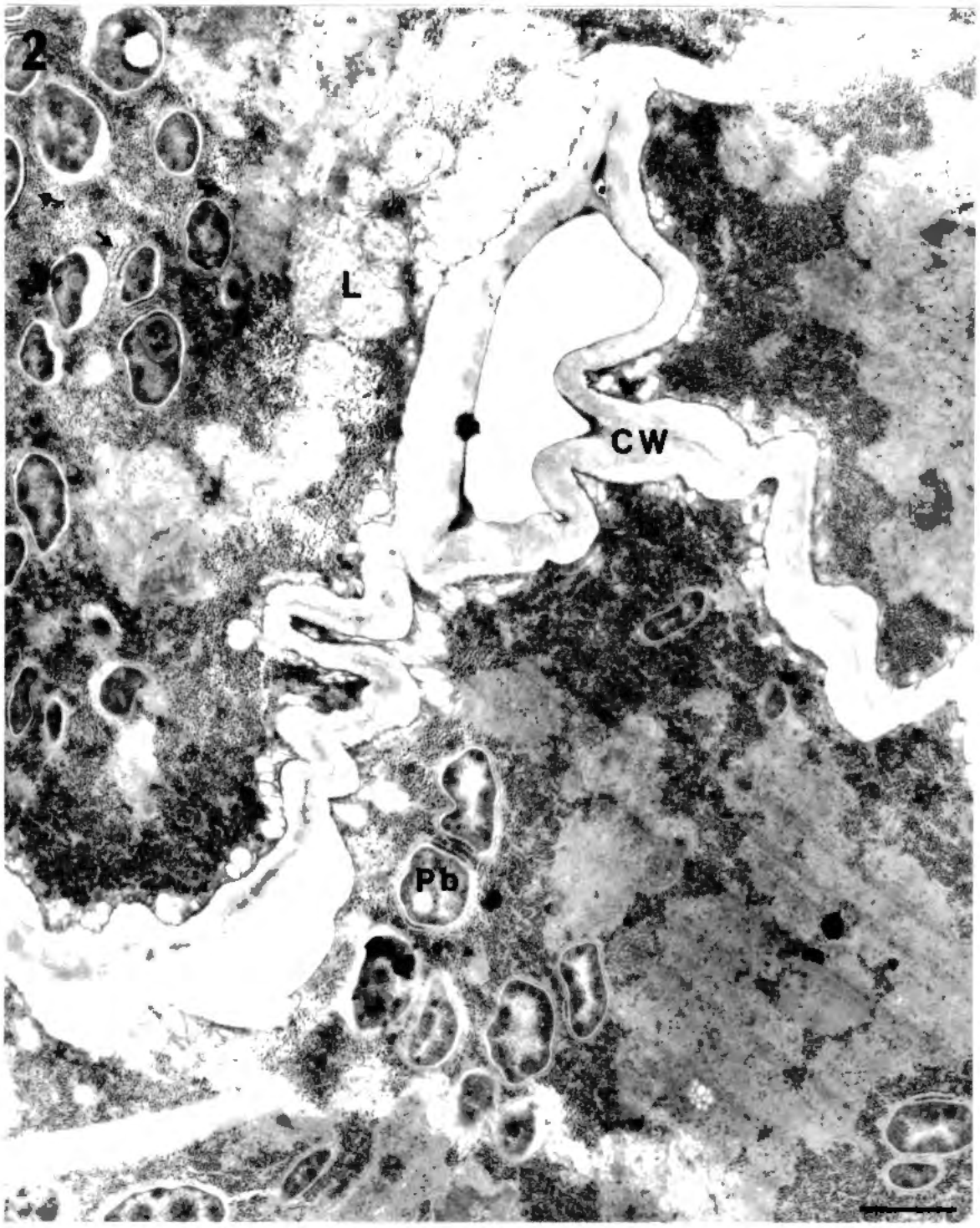


Figure 2. General aspect of anhydrously prepared tissue from the dry seed of P. vulgaris. The cell walls are folded and the cytoplasmic space is occupied by tightly packed ribosomes, protein bodies and aggregations of proplastids and mitochondria. Ribosome-membrane complexes outline some protein bodies (arrows). Magnification 16,600, bar represents 1 µm.

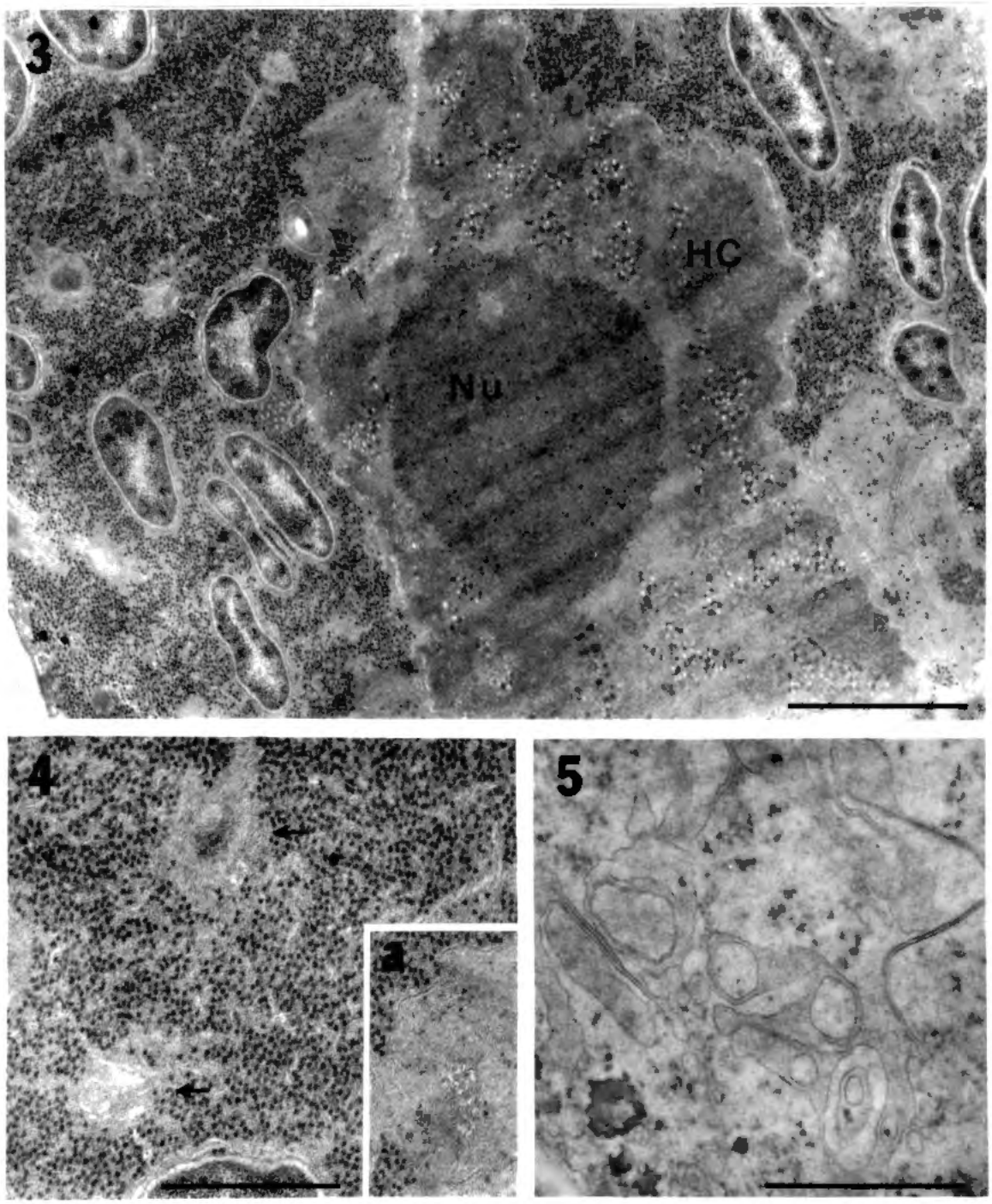


Figure 3. A nucleus with vacuolated nucleolus, condensed heterochromatin and patches of electron dense and translucent granules. Magnification 29,500, bar represents 1 µm.

Figure 4. Endoplasmic reticulum (?) in the cytoplasm (arrows), and a prolamellar body in a proplastid (inset a). Magnification 59,000, bar represents 0.5 µm.

Figure 5. Portion of a cell fixed in aqueous glutaraldehyde. Magnification 34,100, bar represents 1 µm.

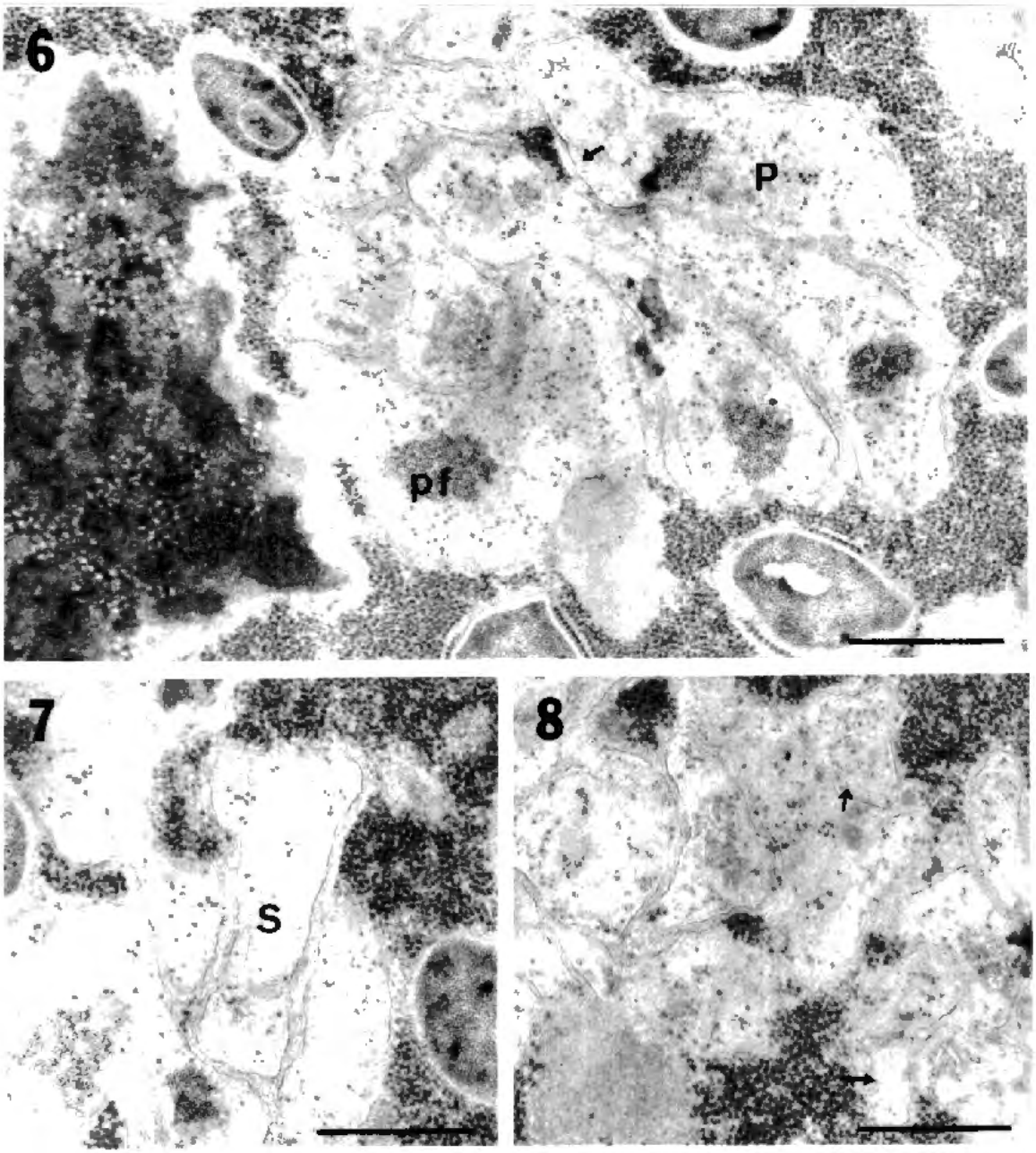


Figure 6. A cluster of proplastids with phytoferritin, faint membranes in the stroma and a few ribosomes. The outer membrane is clearly seen in negative contrast (arrows). Magnification 51,000, bar represents 0.5 µm.

Figure 7. Small clusters of starch in proplastids. Magnification 51,000, bar represents 0.5 µm.

Figure 8. Mitochondria characterized by arrays of cristae membrane in negative contrast. These are clearly seen in the lower right corner and upper center (arrows). Magnification 51,000, bar represents 0.5 µm.

membranes do not form continuous outlines. The presence of membranes in negative contrast has also been noted by Perner (1965) in pea radicle and by Morris (1968) in brine shrimp cysts following anhydrous preparation for electron microscopy. A possible explanation is reserved for the discussion.

The nucleus is the most easily recognizable cell structure. It is always irregular in outline, with arms extending into the surrounding cytoplasmic space (Figure 3). The nuclear envelope appears crinkled as if an inelastic covering has been crumpled around a shrinking core. Heterochromatic regions are prominent adjacent to the nuclear envelope. The euchromatin is differentiated into regions of medium electron density and areas containing electron dense and electron transparent granules, both larger than the ribosomes in the adjacent cytoplasm (Figures 3 and 6). The nucleolus is relatively homogeneous and is the most electron dense region of the nucleus. Roughly spherical, it appears to have nucleolar vacuoles and small electron dense grains, especially near the margins. The nucleus is very similar to that reported in the dry seed of Lactuca sativa (Paulson and Srivastava, 1968).

Protein bodies are dispersed throughout the cytoplasm. They are oval to circular, with somewhat irregular outlines (Figures 3 and 6). The contents of the protein bodies stain as electron dense granules that are most numerous at the margin, and sometimes appear to be absent at the center of large protein bodies. In unstained thick sections, observed with light microscopy, the protein bodies closest to the edge of the tissue are stained most heavily and uniformly with OsO_4 while those in cells removed from the margins of

the specimen are not stained so heavily or uniformly. Thus it appears that non-uniform staining is a result of failure on the part of the OsO_4 to penetrate evenly and does not represent substructure within the protein body. The membrane surrounding the protein bodies is indistinct and separated from the stored protein by an electron transparent corona. A similar observation was made in a freeze fracture study of wheat scutellum (Swift and Buttrose, 1972). The membrane surrounding the protein bodies was indistinct in the dry tissue, but following short inbibition became distinct. Externally, the protein bodies are often surrounded by one or more layers of double membrane (Figure 3). If several layers are present, a single layer of ribosomes will lie between each of the membrane layers forming a ribosome-membrane complex. In some sections, several layers of ribosome-membrane may lie parallel to the surface of the protein body (Figure 2), while in sections tangential to the surface, ribosomes can be seen lying along the surface of the protein body (Figure 2). Similar configurations of membranes and ribosomes have been reported in rice embryo (Opik, 1972; Bechtel and Pomeranz, 1978), rye embryo (Hallam et al., 1972) and P. vulgaris cotyledons (Opik, 1968) and it has been suggested by these authors that this may represent the arrangement in which protein for storage is synthesized prior to seed maturation. In addition to protein, cells of the embryonic axis also store lipid in droplets found at the cell periphery (Figure 2). The amount of stored lipid varies from one tissue to another. Cells of the cortex and pith regions store more lipid than provascular cells. Because DMSO is the first solvent used during fixation, most or all of the lipid is lost prior to OsO_4 fixation, and the droplets appear as

electron transparent regions of varying size. In conventionally fixed cotyledonary tissue (Mollenhauer and Totten, 1971b) the droplets are bounded by a single membrane, but such membranes cannot be discerned in the anhydrously fixed material. Areas where lipids have been extensively extracted are unstable under the beam and this accounts for the failure to observe any membrane around the lipid droplets and may also explain the failure to observe a plasma membrane in this tissue. The plasma membrane has been observed in freeze-etch preparations of dry seed tissue (Buttrose, 1973) as well as conventionally fixed tissue (Opik, 1972).

At low magnification, the cytoplasm can be differentiated into regions with abundant, closely spaced ribosomes and regions of lower electron density containing aggregated plastids and mitochondria (Figures 2, 6, 7 and 8). The plastids are characterized by the presence of phytoferritin and occasional clusters of electron transparent starch granules. The outer membrane often appears as a dark line, and the stroma contains scattered ribosomes and a few lamellae, as well as occasional clusters of tubular membranes. The latter may be prolamellar bodies. The outlines of the plastids are relatively clear, however this is not the case with the smaller mitochondria (Figure 8). The mitochondria can be distinguished by the lack of phytoferritin and starch grains, and the presence of membranes in parallel arrays in the matrix. In plastids, membranes in the stroma always occur singly, while in the mitochondria, they most often appear as arrays of 2-3 sets of double membranes in parallel arrays, presumed to be cristae. Proplastids are frequently reported organelles in maturing, ungerminated and germinating seed tissues (Yoo, 1970; Opik,

1972; Bechtel and Pomeranz, 1978), however mitochondria are frequently reported to decline in number, size and complexity (Opik, 1974).

The remainder of the cytoplasm is filled with ribosomes. Interspersed between the ribosomes, short segments of double membrane can be seen in favorable sections (Figure 4). The membranes are distorted due to compression and shrinkage occurring during seed maturation. Although no long elements are visible in thin sections, distortion during desiccation could easily make continuous membrane segments impossible to see. While a recognizable endoplasmic reticulum is not present in the cytoplasm, these short membrane segments, together with the concentric layers of membrane associated with protein bodies may represent the endoplasmic reticulum. This interpretation is supported by the presence of abundant endoplasmic reticulum in conventionally fixed cortical cells (Figure 5). Closely packed arrays of tubular membrane are occasionally seen in cross or longitudinal section. The origin of these structures is unknown, but similar arrays have been reported in cells of ungerminated rice (Opik, 1972; Bechtel and Pomeranz, 1978) and pea root (Yoo, 1970). Golgi bodies are not recognizable in the cortex parenchyma of embryonic bean root. They are present in provascular tissue of rice (Bechtel and Pomeranz, 1978) and by the third hour of imbibition in rye (Hallam et al., 1972) and corresponding cells of P. vulgaris (Hamilton, unpublished).

Discussion

The most notable observation of this study is that membranes in this tissue do not conform to contemporary ideas about the appearance of membranes following preparation for electron microscopy.

Sharp electron dense and electron transparent lines, sometimes singly, and sometimes adjacent to one another, are seen and often the impression is of membranes in negative contrast. This phenomenon has also been observed in other anhydrobiotic tissue prepared anhydrously. In Pisum sativum cotyledons (Perner, 1965) and Artemia salina cysts (Morris, 1968) fixed with OsO_4 vapors, membranes appeared in negative contrast. In equivalent tissues, P. sativum cotyledons (Yoo, 1970) and A. salina cysts (Morris, 1968) prepared with aqueous fixatives, membranes were normal in appearance although they did not stain darkly. Reduced stainability has also been observed in other tissues with initially low water contents prepared in aqueous fixatives. This observation suggests that there is some alteration in membrane organization in anhydrobiotic tissue that is not entirely reversed upon fixation in aqueous fixatives. In the present study, the nature of the fixation procedure suggests that the electron translucent portion of the membrane image represents a region composed of lipid. The primary fixative does not stabilize lipids, while the carrier, DMSO, has a high solvent capacity for lipids. In fact, lipid in marginal lipid droplets appears to be completely extracted. If lipids are removed, proteins are the only remaining component of the membrane and would be expected to stain by H-bond formation with osmate molecules (Littman and Barnett, 1972) forming the dark lines of the membrane image. The irregular arrangement of light and dark lines, and the tendency of the dark lines to be discontinuous seems to indicate that membrane proteins, normally distributed randomly in space along the plane of the membrane have become aggregated forming patches of high protein density. The effect of such an alteration in

membrane structure upon the image seen with transmission electron microscopy is illustrated in Figure 9. Simon (1974, 1978) has recently advanced the idea that phase changes occur in membranes of organisms as they enter anhydrobiotic states. He further suggests that proteins will be displaced from their positions in the membrane. This idea can be used to explain the images of membranes seen following the anhydrous fixation used in this study.

In hydrated systems, it is now accepted that membranes are composed of a phospholipid bilayer with associated membrane proteins. Maintenance of the bilayer is primarily due to interaction of polar head groups with water (Tanford, 1973) and loss of water should alter lipid-lipid relationships. This expectation is supported by various sorts of biophysical data indicating that when water content of a membrane declines below 20%, alterations in membrane structure occur. High and low angle X-ray diffraction of isolated membranes at low water contents indicate that approximately 20% water represents essential water of hydration (Finean et al., 1966; Finean et al., 1968). At lower water contents, new reflections are seen that indicate molecular rearrangements of the lipids. Levine and Wilkins (1971) suggest that at higher water contents the alkyl chains are highly disordered and mobile within the plane of the membrane, while at low water contents mobility of the alkyl chains is greatly reduced. This transition is probably analogous to that occurring as phospholipids undergo thermal transition between the liquid crystal and crystalline gel state at the transition temperature (T_t). Differential scanning calorimetry of myelin (Ladbrooke et al., 1968) and phosphatidycholine - water lamellar systems (Chapman et al., 1977)

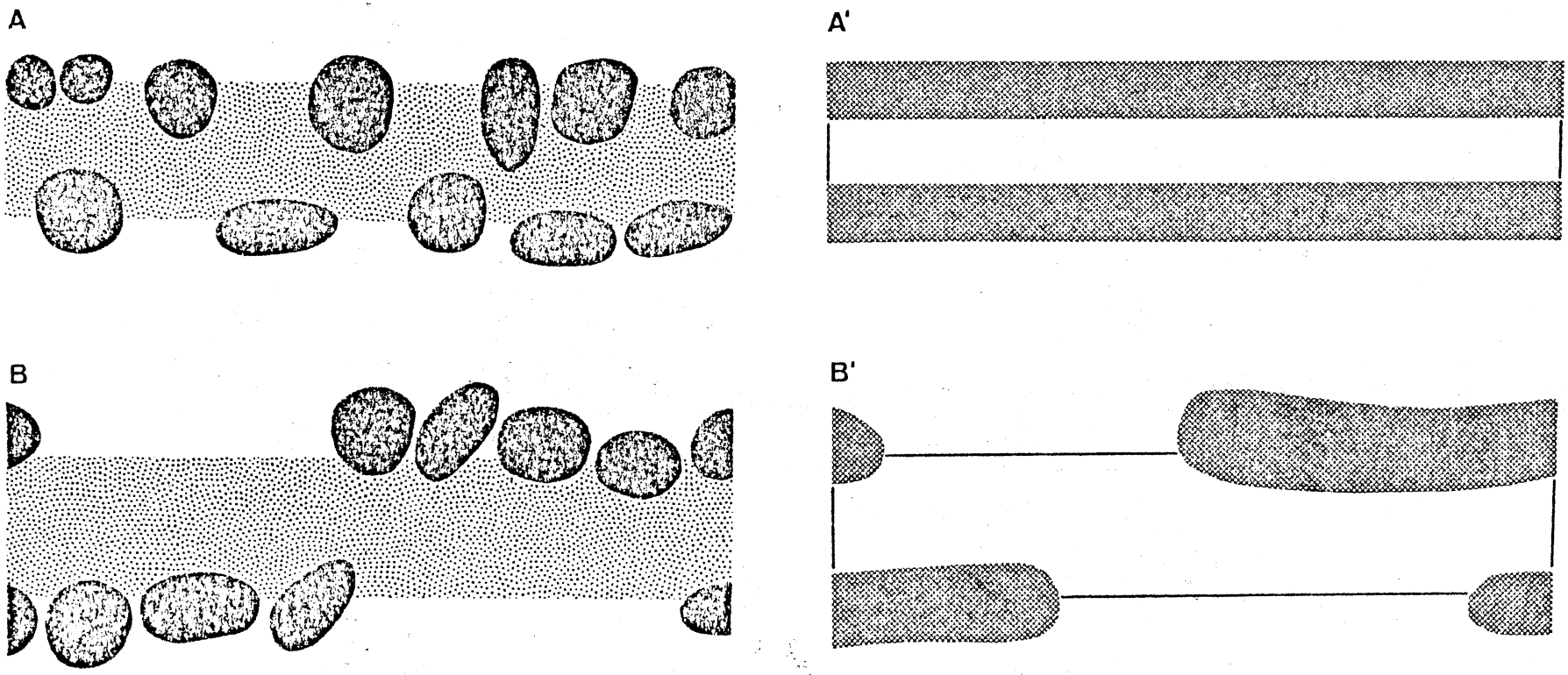


Figure 9. Diagram illustrating the relationship between the proposed alterations in membrane structure and the effect that they would have upon the image that is obtained by transmission electron microscopy. A and B represent the idealized distribution of proteins in the membrane before and after desiccation, respectively. A' and B' represent the distribution of electron density in the membrane following processing. In A and A', the proteins are distributed in a relatively even manner resulting in the classic bilayer image. In B and B', desiccation results in the disturbance of the normal protein distribution and aggregation of the proteins into patches. During the first step in the anhydrous fixation, the lipid portion of the membrane will be removed leaving only the patches of protein to stain during subsequent processing.

indicate that as water content declines, an abrupt increase in T_t occurs at 20% water associated with disappearance of the ice-melting isotherm. This is interpreted to mean that at low water contents (< 20%) only bound water remains. IR spectroscopy (Levine and Wilkins, 1971) as well as X-ray diffraction (Levine et al., 1968) indicate that all remaining water is associated with the charged polar head groups and may serve as a sort of intermolecular bridge between them.

While it is evident that decreasing water content can induce alterations in the properties of a phospholipid bilayer, the exact nature of these changes is a topic of debate. Two major proposals have been advanced. The possibility of forming hexagonal phases (specifically the hexagonal II type) was advanced by Luzzati and Husson (1962) based upon X-ray diffraction of lipid extracts from brain. Below 20% water, the original lamellar phase was replaced by a hexagonal one that probably contained water associated with reoriented polar head groups. Formation of long, tubular micells with polar groups in the center and lying in the plane of the membrane has been suggested (Finean et al., 1966). That such structures are possible is not questioned. However, the X-ray diffraction data of either synthetic or isolated natural membranes does not support this interpretation. Egg lecithin/cholesterol bilayers failed to give diffraction patterns characteristic of a hexagonal phase (Levine and Wilkins, 1971), and freeze fracture of similar preparations at low water content showed fracture faces to be of lamellar rather than hexagonal phases (Deamer et al., 1970). Alternatively, Finean (1969) proposes formation of multiple phase systems in which lamellar phases

are important. These are seen as domains of limited extent in which the conformation of the phospholipid molecules varies from those in adjacent domains. The exact nature of these domains is not specified but variation could be substantial. This proposal does not conflict with the X-ray diffraction data indicating increased order within the lipid portion of biological and synthetic membranes at low water content. It is reasonable to suggest that such phase changes also occur when a tissue, such as a seed, undergoes desiccation.

Phase transitions affect the ways that phospholipids interact with membrane associated proteins, and any factor that alters the characteristics of the lipid phase transition will also affect lipid-protein interactions (Gennis and Jonas, 1977). pH, cation composition, ionic strength, fatty acid and sterol composition, and protein content all affect lipid phase transitions. The basic effect of a phase transition upon lipid-protein associations can be visualized by freeze-etching. Thermally induced phase changes can be produced by cooling a membrane preparation to the T_t before quenching in liquid Freon 22 (-150 C). When inner mitochondrial membranes are prepared in this way prior to freeze fracture, intramembrane particles form dense aggregates within the plane of the membrane. Aggregation does not occur when similar specimens are quenched without pre-chilling (Hackenbrock et al., 1976). Aggregation is thought to result from exclusion of proteins from regions where lipids are undergoing transition from the liquid crystal to crystalline gel state. In erythrocyte ghosts, pH=5.5 results in aggregation of intramembrane particles that is reversible by either pH=7.5 or pH=9.5, and is inhibited if 0.15 M or 1.0 M NaCl is included in the buffer (Pinto da

Silva, 1972). Thus microenvironmental conditions will influence lipid-lipid and lipid-protein interactions.

In the maturing seed, during desiccation, water content declines, and the concentration of various ions increases. These factors will influence the structure of membranes, and their interactions will be complex. Regardless of the exact nature of the phase transitions that certainly occur in membranes as living tissues enter the anhydrobiotic state, proteins will undergo lateral migration in the plane of the membrane in response to altered lipid-lipid and lipid-protein interactions. The result can be visualized as proteins being frozen out of their lipid milieu to form regions of high protein density, while the phospholipids are undergoing phase transition involving increased self-association. In other words, a patchwork is formed from regions of high protein or lipid density (Figure 9). No assumption beyond the occurrence of the phase transition resulting in lateral displacement of membrane proteins is made.

Summary

The dry, embryonic root of Phaseolus vulgaris L. was prepared for electron microscopy by anhydrous techniques. The tissue appears shrunken, with folded cell walls and compactly arranged cellular components. Lipid droplets line the cell margins, plastids and mitochondria are aggregated, and irregular ovoid protein bodies are numerous and may be sheathed in layers of ribosomes and associated membranes. The nucleus contains distinct marginal heterochromatin and nucleoli. The cytoplasm is packed with ribosomes. Cellular membranes appear in negative contrast as sharp electron dense and

electron transparent lines adjacent to one another. An explanation for this appearance is advanced based upon biophysical considerations of lipid-protein interaction and the effects of drying upon membranes.

CHAPTER III

DEVELOPMENT OF MITOCHONDRIA IN THE CORTEX OF EXCISED GERMINATING *PHASEOLUS VULGARIS* L. AXES

Introduction

Mitochondria are conserved as intact entities in dry, mature seeds. Perner (1965) observed them in osmium-vapor fixed pea radicles, and Sato and Asahi (1975) isolated mitochondrial membranes from dry pea seeds. The presence of mitochondria in dry seed tissue has also been established for numerous other seeds including rice (Opik, 1972; Bechtel and Pomeranz, 1978), lettuce (Paulson and Srivastava, 1968) and rye (Hallam et al., 1972). Mitochondria are of particular interest during early germination because further development of the seed is dependent upon rapid resumption of respiratory activity during the early stages of germination. Based upon their survey of the literature Mayer and Shain (1974) suggest that mitochondria are modified in some fashion during the final stages of seed maturation. If this is so, then it is of interest to follow development of mitochondrial structure and function during germination of seed tissues.

Reduced numbers of cristae have been observed in numerous seed tissues including mature lettuce (Paulson and Srivastava, 1968) and

rice embryos (Opik, 1972), and bean cotyledons (Opik, 1968). These and similar observations have lead to the suggestion that during seed maturation mitochondrial membrane components become disorganized in some manner and must become reorganized in order to regain activity. Improved membrane definition and increased numbers of cristae are considered by most workers to be characteristic of mitochondria during germination of seed tissues. In addition, respiratory control (P/O ratio) improves during the early stages of germination, and specific activities of several respiratory enzymes increase in mitochondrial membranes (Sato and Asahi, 1975). These observations support the hypothesis that membrane reorganization occurs during early germination.

Alternately, the number of mitochondria could decline during seed maturation, and rise during germination. Increasing numbers of mitochondria have been reported in peanut cotyledons (Breidenbach et al., 1966; Cherry, 1963) during the first days after germination, and in the early stages of germination in peanut embryos (Wilson and Bonner, 1971). Breidenbach et al. (1966) reported increased numbers of particles based on light microscopic examination of sucrose density gradient fractions, while Cherry (1963) and Wilson and Bonner (1971) base their reports on increased protein content of purified mitochondrial fractions. Increasing numbers of mitochondria have also been reported based upon ultrastructural observations of germinating rye embryos (Hallam et al., 1972). However, an attempt to detect incorporation of ^{3}H-thymidine into mitochondrial DNA has failed in pea seeds (Malhotra et al., 1973).

Respiratory activity during early germination of Phaseolus vulgaris embryos is characteristic of many legume seeds. An initial period of rapidly increasing respiration and passive water uptake is followed by a plateau period when respiratory rate and fresh weight show little increase. The plateau phase ends with a burst of respiratory oxygen consumption and water uptake (Bewley and Black, 1978). This coincides with elongation of the radicle. Increasing respiration rate in the initial phase depends primarily upon rehydration of respiratory enzymes although temperature and oxygen dependent processes also seem to be required (Morohashi and Shimokoriyama, 1977). The reason for the lag phase is unclear, but it may represent time required for synthesis of necessary enzymes or for mitochondrial repair, development or synthesis (Mayer and Shain, 1974). The present study was undertaken to gather data regarding mitochondrial development during the early stages of germination in excised embryonic axes of P. vulgaris.

Methods and Materials

Embryonic axes were removed from dry green bean seed (Phaseolus vulgaris L., cv. Burpee Stringless Greenpod). In all experiments, groups of 4 embryos were selected and the dry weight determined. Imbibition was carried out in distilled H_2O at 27 C in either small beakers (50 ml with 2 ml H_2O) or in 35 x 10 mm Falcon disposable petri plates (on filter paper with 2 ml H_2O). Care was taken that H_2O be adequate but not excessive as mild stress, either dehydration or anaerobiosis has immediate effects upon embryonic development. Fresh weights were determined after gently blotting imbibed axes with

absorbent paper. Percent weight increase was determined as (fresh weight/dry weight - 1.00) x 100.

The tissue was best preserved by a fixative composed of 3% glutaraldehyde, 1.5% para-formaldehyde and 1.5% acrolein in 0.1 M cacodylate (pH=7.2-7.4) suggested by Mollenhauer and Totten (1971a). The segment of tissue from 0.75 mm to 1.75 mm behind the root tip was removed following immersion of the intact axis in fixative, and these segments were then placed in fresh fixative for 2 h at room temperature. Because seed tissue has a high protein content, the tissue was washed in water 3 x 5 min and post fixed 20-45 min in 2% unbuffered $KMnO_4$ at 0 C. The tissue was then rinsed many times with distilled water, dehydrated in ethanol and embedded in Spurr's resin (Spurr, 1969). Thick sections (0.5-0.75μ) were cut with glass knives and stained with azure B (bromide) to select suitable areas for electron microscopy. Silver sections were cut with a diamond knife and stained with methanolic uranyl acetate and lead citrate (Venable and Coggshall, 1965). Photographs and observations were made using a Phillips 200 transmission electron microscope.

For stereological analysis, 10 sets of 4 embryos each were grown as described above for 3, 6 and 16 h. Dry weight and fresh weight were measured. From each axis, a single tissue segment was processed for microscopy and embedded individually in a BEEM capsule. No attempt was made to orient the specimens thus minimizing the possible effect of anisotropy. For each age group, one tissue segment was chosen from each of four sets. Thick sections were taken from these blocks and areas selected in the cortex of the radicle for stereological sampling. Silver sections were cut using a diamond knife. Silver

sections were collected on 200 mesh copper grids and stained with uranyl acetate and lead citrate (Venable and Coggshall, 1965). To avoid shrinkage of the sections in the electron beam, sections were lightly coated with carbon. Using a single section of good quality, a series of micrographs was recorded by a systematic sampling procedure recommended by Weibel (1973). The negative film was Kodak electron image film, and magnification of the image was 4500.

Volume fraction of mitochondria (V_m) was determined by point counting volumetry. Micrographs were printed on Kodabromide RC paper at magnification 12000. A square lattice (7 x 7 mm) of 910 points drawn on a transparent acetate sheet was laid over the micrographs, and the number of points lying over mitochondria, P_m, and cytoplasm, P_t (i.e., excluding cell wall, intercellular space, protein bodies and nucleus) was determined. For each individual measured 2500-3000 points for ground cytoplasm were accumulated. V_m was determined using $V_m = P_m/P_t$. Profile size was estimated using a series of standard circular outlines with diameters of 1, 2, ..., 12 mm drawn on a transparent acetate sheet. Estimation of mean caliper diameter of mitochondria ($\bar{D}_m$) and numerical particle density of mitochondria (N_m) are described in Results.

Measurements of oxygen uptake were made using a Clark type polarographic electrode and a YSI Model 53 oxygen monitor. Groups of four embryos were grown as described above. Following weighing, the groups of embryos were transferred to sample chambers containing 3, 4 or 5 ml distilled water at 27 C, and oxygen consumption was measured for 2 to 4 min. Potassium cyanide (KCN; 1.0 mM) and salicylhydroxamic acid (SHAM; 1.0 mM) were used to estimate relative capacities for

cyanide sensitive and insensitive respiration, respectively. 2,4-dinitrophenol (DNP; 0.375 mM), an uncoupler, was used to estimate capacity for total electron flux. Groups of embryos were treated with respiratory inhibitors at the indicated concentration for 10 min prior to measurement of oxygen consumption, and equal concentrations of inhibitors were present in the sample chambers.

Results

Respiratory uptake of oxygen by excised embryonic axes was measured using a Clark electrode. Oxygen uptake can be divided into three phases. The initial period of rapidly increasing respiratory activity parallels imbibition very closely, and may reflect, at least in part, hydration of enzymes and membranes associated with mitochondrial respiration (Figure 10). This phase lasts for approximately 1.5 h under the conditions used in these experiments. This is followed by a lag phase lasting from 1.5 to about 6 h during which little increase in respiratory activity or fresh weight occur (Figure 11). The lag phase is characteristic of many species but its biochemical basis is not well understood (Bewley and Black, 1978). The third phase is characterized by increasing oxygen consumption and fresh weight. Cell division has not begun, and no dividing cells can be seen before 24 h.

Inhibitors of respiratory activity were applied exogenously to embryos at the end of their designated imbibition periods of 1.5, 3, 6, 12, and 16 h. KCN (1.0 mM) inhibited oxygen uptake by about 60% through the first 6 h, but only 7% by 16 h (Figure 12). SHAM (1.0 mM) had little effect on oxygen uptake at any time, but the

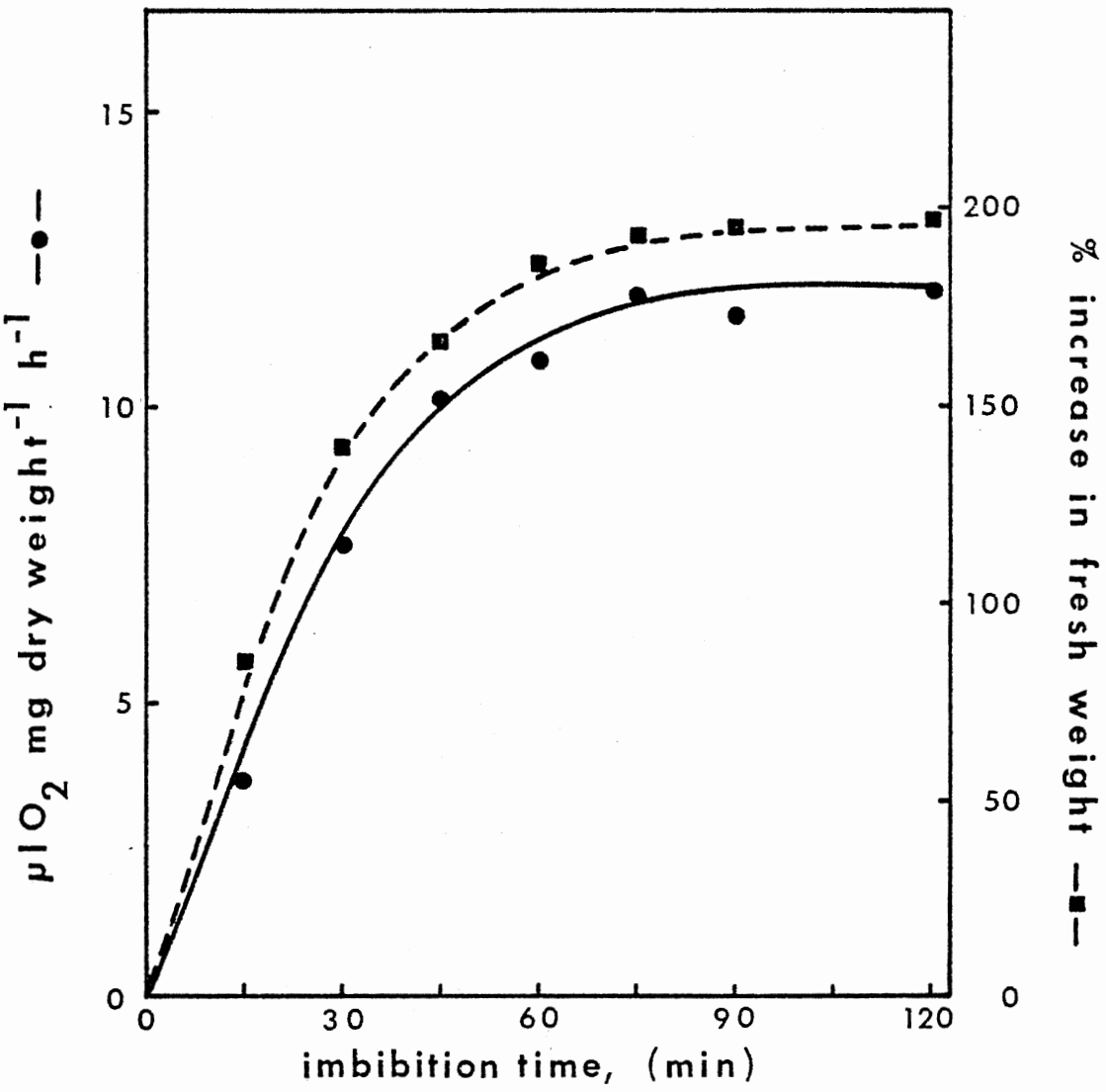


Figure 10. Respiratory activity and % increase in fresh weight during 0-120 min of imbibition of excised P. vulgaris axes. Each point represents the average from 8-11 groups of 4 embryos each.

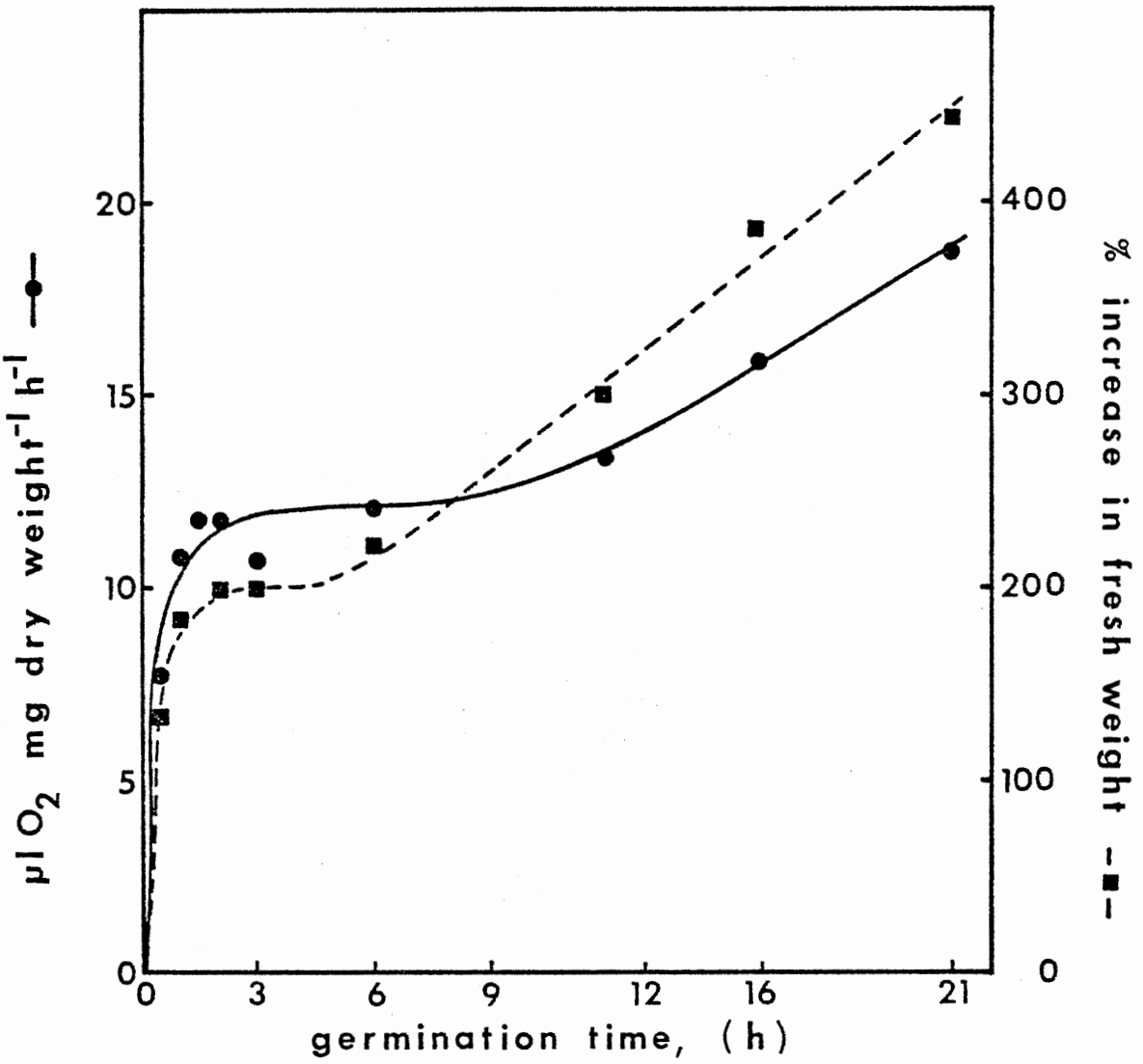


Figure 11. Respiratory activity and % increase in fresh weight during 0-21 h of germination of excised P. vulgaris axes. Each point represents the average from 8-11 groups of 4 embryos each. The values for oxygen consumption given in this graph are the control values used to calculate the effects of respiratory inhibitors presented in Figure 12.

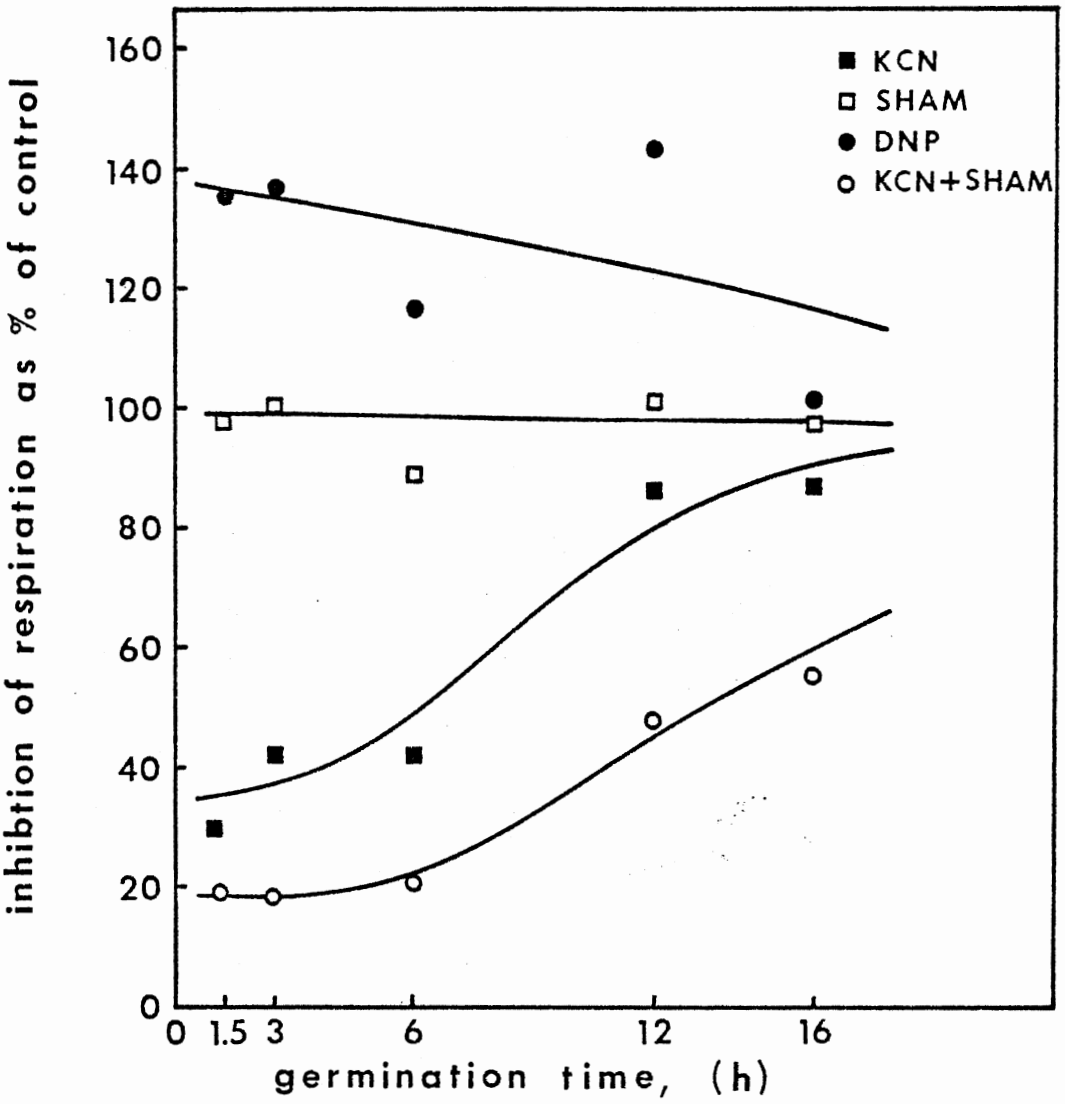


Figure 12. The effects of KCN, SHAM, DNP and KCN+SHAM upon respiration of excised embryonic axes of P. vulgaris. Each inhibitor treatment was used for 8-11 groups of embryos consisting of 4 embryos each. The average for each treatment was determined, and this value compared to the control to give % of control.

combination of KCN and SHAM depressed uptake by approximately 80% during the first 6 h. At 12 h the effectiveness of the combination of the two inhibitors was declining, and at 16 h the rate of oxygen consumption was 50% of the untreated axes. The effect of DNP (0.375 mM) is to increase the rate of oxygen consumption during the first 6 h 20-30% above the controls, and the rate of DNP-stimulated uptake remained higher than the controls at 12 and 16 h.

Respiration during the early germination of P. vulgaris appears to be primarily CN^--sensitive. Lack of significant CN^--insensitive respiration during the early phases of germination has also been reported in pea cotyledons (James and Spencer, 1979) and Cicer arietinum (Burguillo and Nicolas, 1977). This implies that a functional electron transport system is present in dry seeds although early deficiencies in cytochrome c (Wilson and Bonner, 1971) and cytochrome $a-a_3$ (Breidenbach et al., 1967) have been reported in peanut embryos, and pea cotyledons appear deficient in cytochrome $a-a_3$ (Kolloffel and Sluys, 1970; Solomos et al., 1972). The increase in oxygen uptake stimulated by the uncoupler DNP indicates that the capacity to transport electrons to oxygen is not the rate limiting step of respiration at this time. After 6 h imbibition, there is a pronounced increase in the ability of oxidases other than cytochrome $a-a_3$ to transfer electrons to oxygen (Figure 12). Although CN^--insensitive respiration increases, it is difficult to estimate the true partitioning of electron flow. The data simply indicate that the potential activity of the alternate oxidase(s) has increased sufficiently to accept a large proportion of the total electron flow through the system. The increase in oxygen consumption that is not

inhibited by KCN+SHAM could be due to a failure of the inhibitors to penetrate the tissue, but is more likely due to the increasing levels of other oxidases such as lipoxygenase (Parrish and Leopold, 1978). It is doubtful that these nonrespiratory oxidases would account for a large proportion of the oxygen consumed in the control axes because the affinities of these enzymes for oxygen are much lower than that of cytochrome a-a_3.

The primary purpose of the respiration experiments was to determine appropriate times for the stereological analysis. We hoped to choose times that would represent undeveloped, developing and mature mitochondrial stages. Because it is near the end of the lag phase, 6 h was chosen. If reorganization of membrane components and increased amounts of inner membrane are required before respiratory activity can increase, it is logical to expect changes to begin before the end of the lag phase. Since we wished to avoid possible effects on mitochondrial structure as a result of cell division, and at the same time use the oldest tissue possible, 16 h was chosen as the last point. If either mitochondiral numbers or inner membrane area is increasing this change should be evident by this time. The early point was more difficult to chose. For the earliest point in the analysis, we wanted to see mitochondria before extensive changes could occur, and 2 h was chosen to avoid any initial difficulties associated with incomplete hydration of the tissue. However, preliminary attempts of fix root tissue following 2 h imbibition were very unsatisfactory, and we subsequently succeeded in fixing 3 h tissue, so this became the initial point in the stereological analysis.

To evaluate the possibility that morphological alterations of mitochondrial structure may occur during early germination, a simple stereological analysis was performed. In order to obtain a random sample, ten groups consisting of four embryonic axes each were grown for 3, 6 and 16 h. A single segment of root was removed from each axis and processed for electron microscopy. One segment from each of four groups in each age class was selected for stereological analysis so that n = 4 for 3, 6, and 16 h. Volume fraction of mitochondria (V_m) in the cytoplasm was determined for each individual. Profile size distributions, mean caliper diameter of mitochondrial profiles (D_m) and numerical density of mitochondria (N_m) were estimated by combining data from the four individuals that constituted the samples at 3, 6 and 16 h. This combination of parameters yields general information about the mitochondrial populations present in parenchyma cells of the root cortex during early germination. V_m, D_m and N_m are summarized in Table I. Profile size distributions are given in Figure 13.

Mitochondria occupy a small proportion (about 6-7%) of the cytoplasmic volume of the root cortex cells. V_m does not appear to change during the first 16 h of germination. The value of V_m at 6 h is slightly higher than at 3 or 16 h, and this agrees with subjective observation. Alternatively, the increased V_m may be associated with random error associated with the small sample size (n = 4). However, based on calculations by Weibel (1969) it is estimated that the expected relative error in determination of the V_m for each individual axis is 5-7%. Thus the data should be accurate enough to reveal changes in V_m.

TABLE I

RESULTS OF STEREOLOGICAL ANALYSIS OF MITOCHONDRIAL DEVELOPMENT DURING EARLY GERMINATION OF EXCISED PHASIOLUS VULGARIS EMBRYONIC AXES

Morphometric Parameter	Inhibition Time		
	3 h	6 h	16 h
Volume Fraction			
V_m, $\bar{X}$ (n = 4)	0.060(±0.010)*	0.068(±0.026)	0.062(±0.010)
Individual Values	0.0575	0.0714	0.0655
	0.0628	0.0761	0.0570
	0.0552	0.0695	0.0633
	0.0648	0.0569	0.0611
Mean Caliper Diameter **			
$\bar{D}_m$ (Weible and Gomez, 1970)	7.29	7.39	6.81
(Henning and Elias, 1971)		6.00±0.25 in all cases	
Numerical Density***			
N_m (particles/cm^3)	6934	7460	8758

*Confidence intervals calculated at P = 0.10

**Numerical values based upon the standard diameters in mm used to determine profile size distribution.

***Calculated using $\bar{D}$ calculated by method of Weibel and Gomez (1970). These values estimate particle number based on data from $\sim 3.5 \times 10^5$ μ^2 cytopasmic area per germination time.

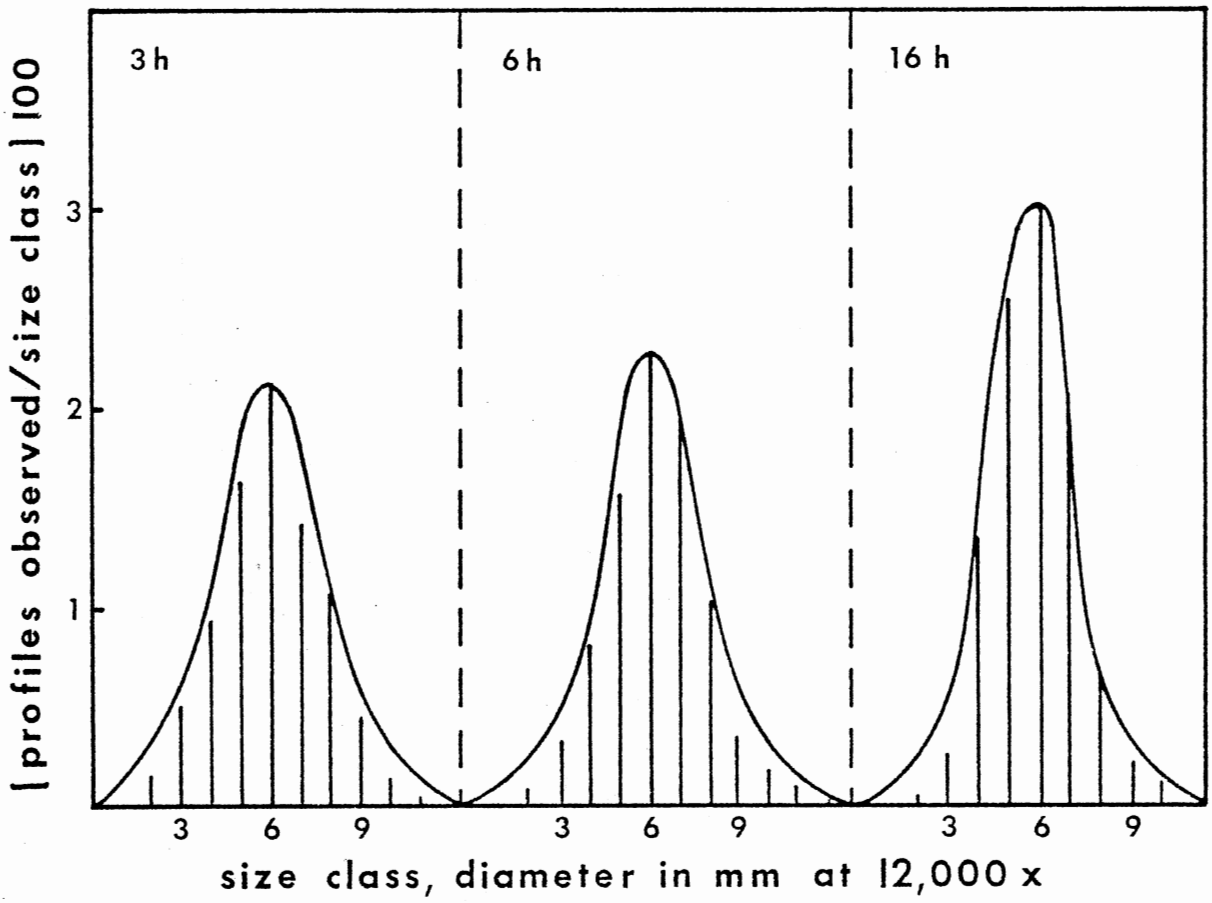


Figure 13. Profile size distributions for 3, 6 and 16 h. The number of mitochondrial profiles represented is 906, 954 and 1205, respectively. The smooth curves were drawn for determination of $\underline{D}_m$ by the method of Henning and Elias (1971).

Size class distributions were determined using a standard set of circles to estimate each mitochondrial profile area. Thus, profiles of similar area are recorded in a single size class. Data from all profiles appearing in the micrographs used to determine V_m were pooled resulting for a single size class distribution for each age group. Size class distributions are useful in approximating D_m and N_m. In addition, the shape of the distribution provides information about the population of particles being sampled. In this case a particle is synonymous with a mitochondrion, and a profile is a two dimensional segment of a mitochondrion. In this case, the distributions resemble gausian curves (Figure 13). This is strong evidence that the observed profiles do not represent a population of mitochondria of uniform size. A uniform population of mitochondria would have generated a distribution strongly offset toward the largest size classes. Because of the ambiguities involved in determining D_m from a non-uniform population of mitochondria, the method of Henning and Elias (1971) was applied. These authors have constructed several theoretical populations of spherical particles having different distributions of particle diameter and calculated the theoretical size distribution of the profiles that would be generated from each population. To use this method, smooth curves are constructed to fit observed profile size distributions (Figure 13). The constructed curves are then compared with the theoretical curves. The shapes of the theoretical curves are characteristic of the particle diameter distributions, and the position of the peak in the distribution of profile sizes in relation to the center of the size class range is indicative of mean caliper diameter ($\bar{D}$) for a given population of

particles. The observed mitochondrial profile distributions are characteristic of non-uniform populations whose $\bar{D}$ is near the center of the size class range. In the case of the distributions for 3, 6 and 16 h, $\bar{D}_m$ appears to be very close to 6. Secondly, the steepness of the slopes of the distributions indicates that the variance in particle diameter is rather small. $\bar{D}$ can also be estimated by the method of Giger and Riedwyl (1970, see Weibel, 1973). In this procedure, the profile size distribution is corrected for loss of small profiles that are hard to identify, and a mean value ($\bar{d}$) calculated. The equation $\bar{D} = \frac{4}{\pi} d$ then gives an estimate of $\bar{D}$. Using this method, $\bar{D}_m$ values at 3, 6 and 16 h are somewhat higher (Table I).

Numerical density of particles is a difficult value to determine with any degree of accuracy. Because it was impractical to determine $\bar{D}$ directly, it was decided that the method of Weibel and Gomez (1962) would be used to estimate N_m. This involves two constant factors, K (dependent upon distribution of particle size, K = 1.07 where the standard deviation of particle diameters is $\leq$ 25% of the mean) and β (a shape factor, 1.5 where length/diameter ~ 1.7). N_A (number of profiles observed) and V_m were directly determined. The formal relationship is $N_m = (\frac{K}{\beta}) \cdot \frac{N_A}{V_m^{1/2}}$. Calculated values of N_m are given in Table I. These indicate that mitochondrial numbers are increasing However, several workers have reported that no replication of mitochondrial DNA occurs during this period of germination in pea cotyledons (Malhotra et al., 1973) based on ^{3}H-thymidine incorporation. This indicates that the increase in N_m may not represent mitochondrial biogenesis by mitochondrial division. Further the increase in N_m may be artifactual because of inadequate understanding of mitochon-

drial shape. The accuracy of estimating both $\bar{D}_m$ and N_m is dependent upon the assumption that the shape of individual mitochondria in the population sampled is relatively uniform and can be approximated in geometric terms, and that each profile observed represents a single particle. This may or may not be the case in the present study.

The morphometric data seem to indicate that the mitochondrial population remains stable during the first 16 h of germination. V_m does not increase, $\bar{D}_m$ appears stable, and the size distribution of profiles is indicative of very similar distributions of diameter throughout the period of the experiment. The increase in N_m at a time when mitochondrial division is not occurring indicates that some change in mitochondrial shape is occurring.

During the process of determining V_m, it became apparent that the spatial distribution of mitochondrial profiles does not fit expectations for a randomly dispersed population of particles. Relatively few mitochondria appeared to be separate from neighboring mitochondria, and most appeared in groups ranging from 2 - 6. Occasional groups of 7, 8 and 9 mitochondria were observed, and in two exceptional cases, 11 and 14 mitochondrial profiles were observed in close proximity to one another. We attempted to estimate the extent of spatial association in the following manner. The arbitrary decision was made that if the boundaries of two mitochondrial profiles approached one another at distances of $< \bar{D}_m = 6.5$ they would be considered to be "associated." Using this criterion, the number of individual profiles and profile associations was determined for the same set of micrographs used to determine V_m. Isolated profiles were assigned an association number of 0, groups of two mitochondrial

profiles had an association number of 1, etc. The data from 3, 6 and 16 h are very similar and so have been added together. For each association number, the total number of groups of that size was determined, and the number of mitochondrial profiles thus accounted for was calculated by multiplying (number of groups per association number) x (association number + 1). Then the proportion of the total population of profiles was calculated and plotted in Figure 14. The most striking aspect of these data is that approximately 0.4 of the profiles appear individually, but approximately 0.5 of the total appear in groups with association numbers of 1-5. For small particles occupying only a small portion of the cytoplasmic volume, this certainly reflects non-random distribution in space.

Moore (1976) has discussed a similar phenomenon, "connectivity," in statistical terms based on mathematical models. Connectivity is defined as the approach of two particles within a containing volume, so that their center to center distance is 4 - 2r, and values are calculated in a fashion similar to that described previously for mitochondrial profile association. In the case of mitochondrial profiles, a greater relative approach distance was used, but since we are dealing with two-dimensional space rather than three-dimensional space, this is probably not a serious error. In order to compare expected and observed levels of connectivity and association, respectively, the expected distribution of groups in three-dimensional space has been plotted with the observed data from two-dimensional space in Figure 14. This reveals that there are more profiles associated in relatively large groups than would be expected, even in three-dimensional space and fewer unassociated particles. Thus it appears

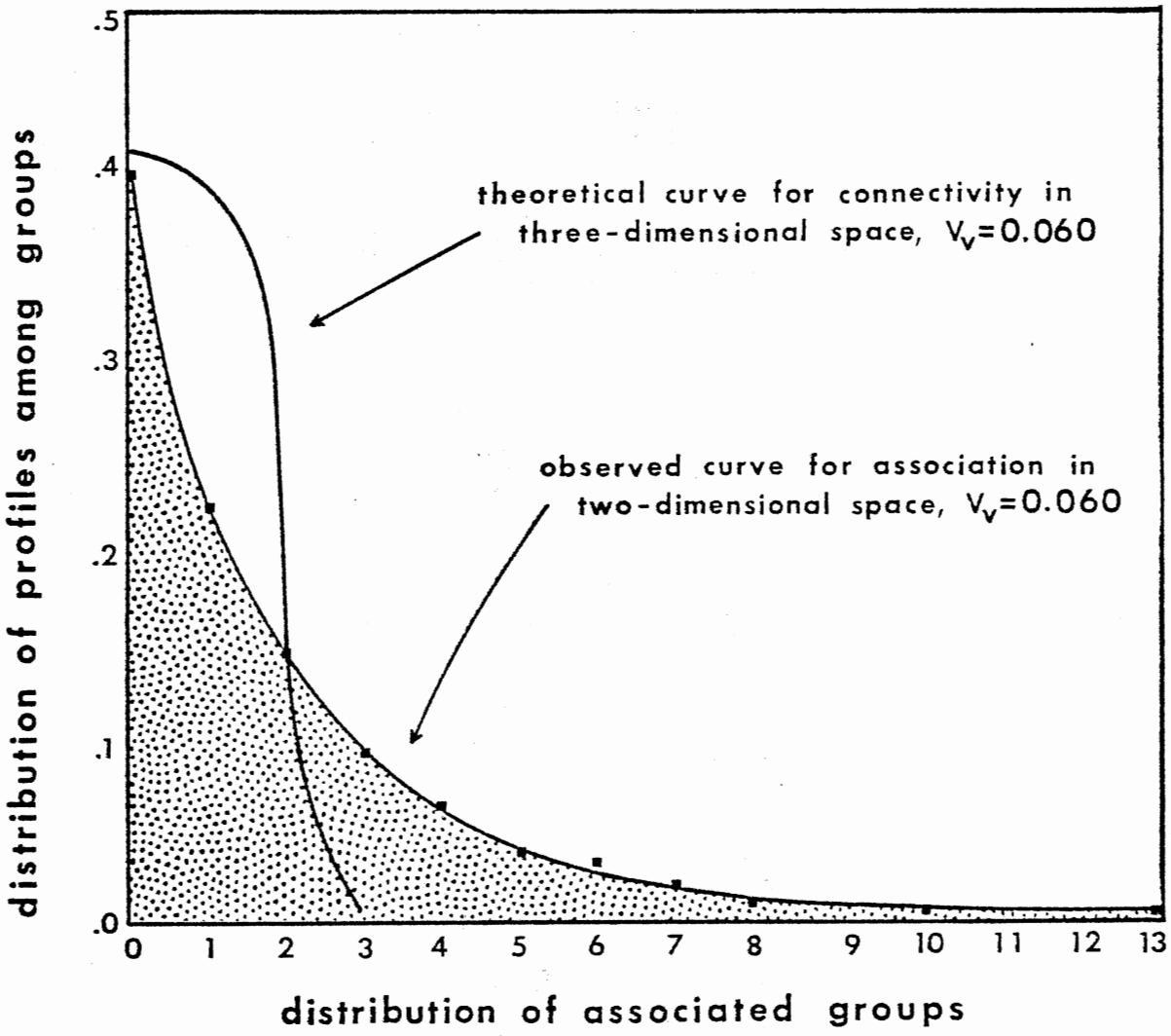
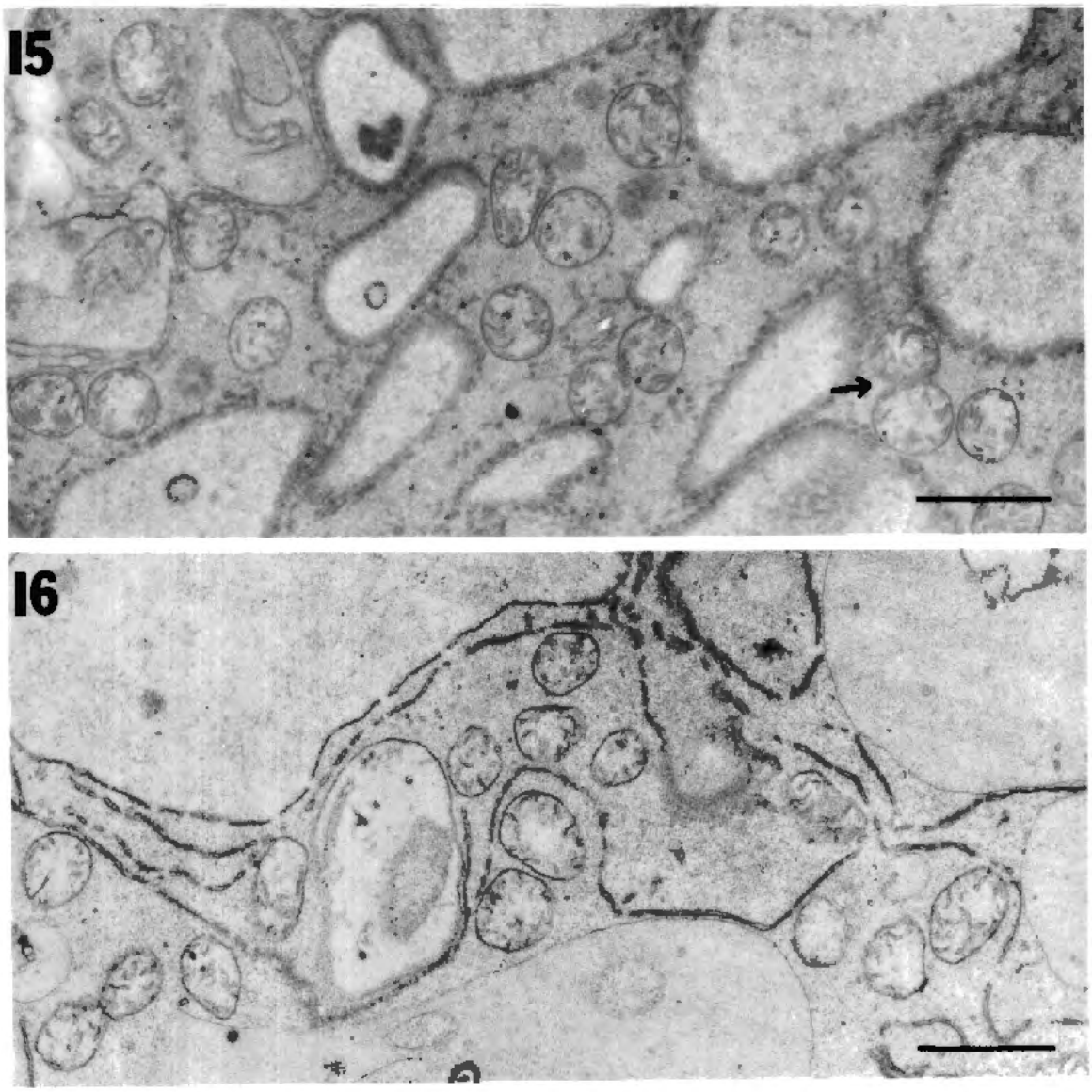


Figure 14. Distribution of mitochondrial profiles within the different association groups. The theoretical curve was derived from the expected values computed by Moore (1976) in his study of connectivity of discrete particles in three-dimensional space.

that at least a portion of the profiles observed represent mitochondria that are structurally related to one another in some way. It seems very likely that the simple condition of individual, discrete mitochondria whose shape is spheric to rod-like may not represent the shape of many of the mitochondria present in these cells. Rather, mitochondria may have a rather complex morphology at the beginning of germination perhaps forming branched, ameboid structures (Figures 15 through 18). In the mitochondrial cluster seen in Figure 17 there are several "individual" profiles that appear to be connected. The entire cluster of profiles could easily represent a single plane passing through a large structure composed of elongated branches. Although no other large groups are seen in the other micrographs pairs of connected profiles can also be seen in Figures 15 and 16. In Vicia faba, Harris (1979) has reported dumbell shaped mitochondria during the late stages of protein deposition in maturing cotyledons. More complex mitochondria have been reported in yeast (Hoffman and Avers, 1973) and in various green algal species (Gaffal and Kruetzer, 1977) during specific periods of the cell cycle. Although we have not attempted more complex analyses of mitochondrial structure in this system, it appears likely that the structural complexity observed in some single celled eukaryotes may also occur in some more highly differentiated tissues such as the root cortex cells of P. vulgaris embryos.

The area of the inner mitochondrial membrane and especially the cristae membrane is a very important parameter in evaluation of mitochondrial development. This parameter can be evaluated by calculating the membrane area as a function of the volume. However, it was felt



Figures 15-18. Illustrations of mitochondrial ultrastructure in root cortex cells of embryonic P. vulgaris. Tissue was post fixed for 40-45 min with permanganate. Staining and section thickness are equivalent for each specimen, and all negatives were printed on grade 3 paper. Magnification 21,000, bar represents 1 µm.

Figure 15. Root cortex cell imbibed 30 min at 4 C. Mitochondria are clustered, and at least one pair of profiles are connected (arrow). The tubular cristae are tightly appressed and all membranes are indistinct.

Figure 16. Root cortex cell after 3 h germination. Cristae membranes are slightly dilated and more distinct than in Figure 15. Overall the contrast of other membranes is also higher.

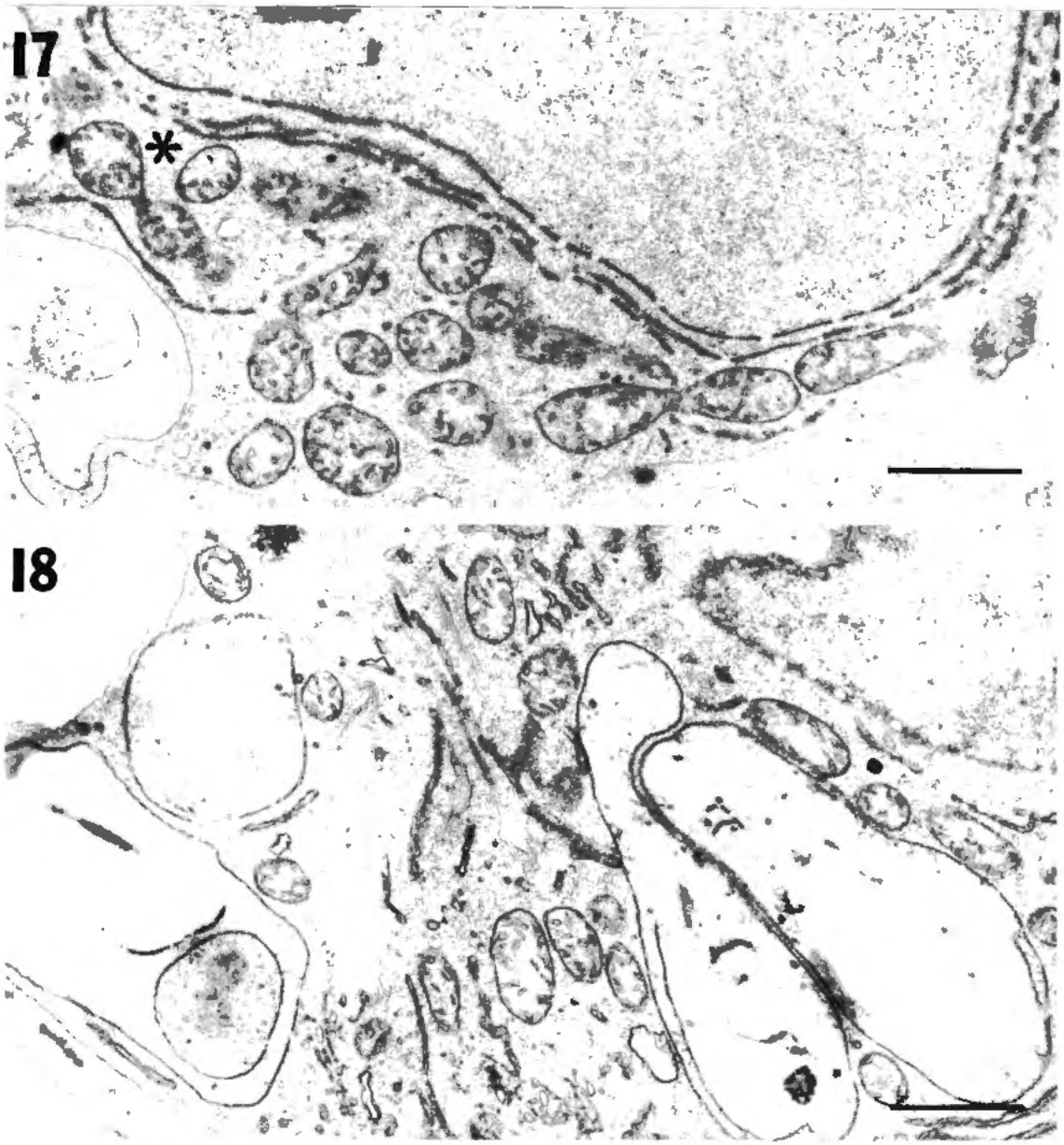


Figure 17. Root cortex cell after 6 h germination. Complex mitochondrial morphology is suggested by clusters of mitochondrial profiles such as this one. At the upper left (*) four profiles form a line. The central pair are clearly connected, while at the ends are two cap sections. Other interconnections are visible. At this time the mitochondrial matrix appears to be more strongly stained than the cytoplasm.

Figure 18. Root cortex cell after 16 h germination. Mitochondria have slightly dilated, distinct cristae and mitochondrial matrix that is no more densely staining than the ground cytoplasm. These mitochondria appear to be mature ultrastructurally.

that because of the tubular nature of the cristae and their small size in relation to section thickness, stereological estimation of membrane surface would be ambiguous. However, based upon the appearance of the mitochondria observed in the stereological analysis of V_m, it is apparent that there is no dramatic increase in the number or length of the cristae (Figures 16, 17 and 18) between 3 and 16 h. Since membrane formation could occur before 3 h, excised axes were imbibed for 30 min in water at 4 C prior to fixation (Figure 15). Morohashi and Shimokoriyama (1977) report that this treatment prevents normal initiation of respiratory development. Further, it is unlikely that membranes could be reformed during such treatment. The mitochondria in these embryos are almost indistinguishable from those of 16 h embryos. The mitochondrial profiles seen at 16 h are generally circular to ovoid, and the outer membrane is closely appressed to the marginal portion of the inner membrane. The cristae are slightly dilated and appear to be randomly distributed within the mitochondrial matrix. When embryos are imbibed for 30 min in cold water, the mitochondria tend to be circular in shape with closely appressed marginal membranes, the cristae are numerous and do not appear to be dilated. At 30 min the membranes are very fuzzy, and lack the contrast seen in the mitochondria after 16 h. The mitochondria at 3 and 6 h have membranes that show increasing definition and contrast.

Discussion

That mitochondria are retained in dry, mature seed tissues is now accepted by most workers. However, their condition in dry seeds

and their development during early germination is still a matter of some controversy. For instance Opik (1974) feels that cristae are reduced in number but that all basic structural elements are retained. Mayer and Shain (1974) suggest that mitochondria in dry seeds are not normal, and that rapid changes occur during germination, perhaps including structural changes in mitochondrial membranes. Although various workers feel that either mitochondrial biogenesis or mitochondrial repair occur during early germination, Bewley and Black (1978) suggest that no substantial evidence has been produced either way. Certainly the literature is voluminous and contradictory.

One major point of discussion is whether the cristae membranes are retained or diminish in area during seed maturation. Ultrastructural observations are numerous and for convenience, we have arranged the reports of many workers into Table II according to species and tissue. This list is not complete, but is intended to serve as an overview of the literature. It is clear that broad generalizations are difficult on the basis of these observations. For instance, in A. hypogaea cotyledons, Bagley et al. (1963) could not identify mitochondria in intact tissue although mitochondria could be isolated from the cotyledons (Cherry, 1963). Bain and Mercer (1966a) reported that mitochondria were lost in mature P. sativum cotyledons, while Solomos et al. (1972) observed mitochondria with a few cristae in dormant seeds. Two studies (Perner, 1965; Yoo, 1970) report that mitochondria in P. sativum embryos from dry seeds have nearly normal numbers of cristae. The present study establishes that abundant cristae are present in 30 min cold imbibed radicles of P. vulgaris, and Klein et al. (1971) report similar observations in the

TABLE II

SOME ULTRASTRUCTURAL AND ASSOCIATED PHYSIOLOGICAL OBSERVATIONS OF MITOCHONDRIAL STRUCTURE IN VARIOUS SEED TISSUES

Species Tissue	Respiratory Lag Phase?*	Observation of Cristae or Mictochondria	Citation
Archis hypogaea			
mature cotyledon		mitochondria not seen	Bagley, et al., 1963
isolated mitochondria		cristae scarce	Cherry, 1963
Cucurbita maxima			
germinating cotyledon		more cristae in veins than midveins, 3 days	Lott and Castel-franco, 1970
Glycine max	Yes		
mature cotyledon		mitochondria not seen	Treffery et al., 1967
Gossypium hirstum			
mature cotyledon		cristae appear normal	Yatsu, 1965
Hordeum vulgare	No		
scutellum		cristae scarce	Nieuwdorp, 1963
aleurone cells		initially few cristae increasing with germination	Eb and Nieuwdorp, 1967
aleurone cells		mitochondria indistinct	Paleg and Hyde, 1964
aleurone cells		cristae present, increasing with germination	Jones, 1969
Lactuca sativa	Yes		
embryonic axis		cristae scarce, increasing with germination	Paulson and Srivastava, 1968

TABLE II (Continued)

Species Tissues	Respiratory Lag Phase?*	Observation of Cristae or Mitochondria	Citation
Medicago sativa			
radicle		few internal membranes	Singh, 1977
hypocotyl		mitochondria indistinct	Singh, 1977
cotyledon		cristae scarce	Singh, 1977
Oriza sativa	No		
mature coleoptile		cristae scarce, indistinct	Opik, 1972, 1973
mature coleoptile		cristae scarce, indistinct	Ueda and Tsuji, 1971
mature embryo		mitochondria indistinct	Bechtel and Pomeranz, 1978
Pisum sativum	Yes		
mature radicle		cristae normal	Perner, 1965
mature radicle		cristae normal, becoming more distinct during germination	Yoo, 1970
mature cotyledon		mitochondria not seen	Bain and Mercer, 1966a
mature cotyledon		cristae scarce	Solomos et al., 1972
Phaseolus lunatus	Yes		
developing embryo		cristae not well resolved	Klein and Pollock, 1968
mature and germinating embryo		cristae reduced, increasing during germination	Klein and Ben-Shaul, 1966
mature and germinating embryo		cristae abundant, not increasing at 16 h of germination	Klein et al., 1971

TABLE II (Continued)

Species Tissues	Respiratory Lag Phase?*	Observation of Cristae or Mitochondria	Citation
Phaseolus vulgaris	Yes		
mature radicle		abundant cristae, not increasing at 16 h germination	(Present Study)
mature cotyledon		cristae scarce	Opik, 1968
germinating cotyledon		cristae increasing during germination	Opik, 1965, 1966
Secale cereale	No		
mature embryo		cristae scarce, increasing during germination	Hallam, 1972; Hallam et al., 1972
Sinapsis alba	No		
mature cotyledon		mitochondria indistinct	Rest and Vaughan, 1972
Triticum vulgare			
mature shoot apex		cristae scarce, increasing during germination	Marianos and Fife 1972
scutellum		cristae increase from 6 h to 24 h	Swift and O'Brien, 1972
Zea mays	No		
mature radicle		cristae scarce	Crevecoeur et al., 1976

*According to Bewley and Black (1978).

embryonic axes of P. lunatus. However, Opik (1968) reports that cristae are scarce in mature cotyledons of P. vulgaris (Opik, 1966). However, mitochondria in all tissues of another legume seed (Medicago sativa; Singh, 1977) appear to have little internal membrane. Thus, although embryonic axes of legumes seem to retain cristae membranes, this is not always observed. Further, although cristae seem to be reduced in cotyledons, this is not observed in all species (i.e., Gossypuim hirsutum; Yatsu, 1965). Thus, it appears that the inner mitochondrial membrane is retained in dry seeds, but that the amount of membrane retained may vary.

The possibility does exist that a general characteristic of respiratory development, the presence or absence of a lag phase, may be related to observed changes in mitochondrial structure. Bewley and Black (1978) list a number of species as to the presence or absence of a lag phase, and where it is applicable these data are included in Table II. In all cases, species reported to lack a lag phase also are reported to have poorly developed mitochondrial structure at the beginning of germination. Interestingly, this group of species is composed of grasses. In Hordeum vulgare aleurone (Jones, 1969; Eb and Nieuwdorp, 1967) and Triticum vulgare scutellum (Swift and O'Brien, 1972) cristae seem to increase as germination proceeds. Cristae also increase in the embryonic shoot apex of T. vulgare (Marianos and Fife, 1972) the embryonic radicle of Secale cereale (Hallam et al., 1972) and the coleoptile of Oriza sativa (Ueda and Tsuji, 1971; Opik, 1973) during germination. Ueda and Tsuji (1971) have proposed that structural changes observed in anaerobically germinating rice coleoptiles may be the result of reorganization of

membrane components retained during seed maturation. Initially, cristae membranes are very indistinct and few can be observed. However, flocculent patches of electron dense material can be seen in the matrix. At 12 h germination, cristae with fuzzy membranes can be seen associated with the flocculent material, and by 24 h the flocculent material has disappeared from the mitochondrial matrix. Taken literally, this proposal indicates that structural reorganization of the inner mitochondrial membrane is associated with increasing respiratory activity.

In seeds with a respiratory lag phase in early germination several workers report increasing mitochondrial structure during germination of P. sativum cotyledons (Solomos et al., 1972) and P. vulgaris (Opik, 1966) cotyledons and Lactuca sativa embryo (Paulson and Srivstava, 1968). In fact comparison of the micrographs in these studies with the micrographs published in Ueda and Tsuji (1971), Marianos and Fife (1972) and Hallam et al. (1972) as well as other workers reveals a remarkable similarity between the results of many of these studies. There is a characteristic increase in contrast and definition of the inner membrane during germination. In many cases, the mitochondrial matrix is dense or contains irregular areas of electron dense material. In some cases, this electron dense material could easily obscure the small, tubular membranes of the cristae. As germination proceeds, the irregularly dense material seen in the matrix declines, the cristae become slightly dilated and their membranes are more distinct. Careful observation by these authors often fails to reveal major increases in cristae membranes in the published micrographs if the dark flocculent patches are taken

into account. This is, of course, a subjective observation. However, our experience with the embryonic axis of P. vulgaris indicates that the procedure used to process such tissue can dramatically influence the amount of detail that is visible using transmission electron microscopy.

Initially we fixed 2 h germinating axes using a conventional procedure similar to that used by Opik (1966, 1968). The results were very similar to hers. However, as mentioned previously, this was not considered adequate for the purpose of this study. Next we tried a mixed glutaraldehyde and osmium fixation, with less success. In this case, using 2 h germinated axes, it was difficult to see any cytoplasmic membranes because the cytoplasm and its abundant ribosomes were very densely stained, and the mitochondrial matrix was virtually without detail because it too was densely stained. At this point it appeared that few membranes were retained in the embryonic root. The nuclear membrane and plasma membrane were difficult to distinguish, and very little endoplasmic reticulum could be recognized. Use of permanganate for post fixation (Mollenhauer and Totten, 1971a) was the only type of treatment that would reduce the background staining enough to allow visualization of detail within the cytoplasm. The time allowed for the post fixation was also found to be critical. A time of 20-30 min was insufficient and little detail was visible in the sections, while 45 min of post fixation produced distinct membranes (Figures 15-18). It is interesting to note that when other workers used permanganate as a primary or post fixative, the clarity and amount of inner membrane that they observed tended to increase regardless of the tissue (Yatsu, 1965; Jones, 1969;

Klein et al., 1971). It is interesting to speculate about the possible appearance of other seed tissues if fixed using similar procedures.

Not only does the contrast (signal/noise ratio) of mitochondrial membranes improve, their stainability also increases during germination. In other words, they stain more readily with heavy metal ions implying that an increased number of ionic sites are available on the membrane. The most direct way to increase stainability of a membrane is to increase the protein content.

Increased protein content of mitochondrial membranes has been reported by Sato and Asahi (1975) in P. sativum cotyledons during the early hours of germination. Initially they observed a heterogenous population of mitochondria using sucrose density gradients, but within 6 h of imbibition denser fractions having high protein/lipid ratios were converted into a lighter population of mitochondria. These mitochondria were enriched in higher molecular weight peptides and had somewhat improved respiratory activity. This initial development of mitochondrial membranes does not depend upon de novo protein synthesis (Nawa and Asahi, 1973). Incorporation of ^{14}C-leucine into mitochondrial proteins of germinating P. sativum cotyledons (Malhotra et al., 1973; Malhotra and Spencer, 1973) and ^{3}H-leucine into germinating P. lunatus axes (Klein et al., 1971) begins near the end of the lag phase. These proteins are primarily synthesized on cytoplasmic ribosomes, and treatment of the tissue with cyclohexamide prevents incorporation of labeled proteins and any increase in respiratory rate. This has been interpreted to mean that the end of the lag phase is dependent upon protein synthesis.

However, it is not clear whether respiratory activity could increase when protein synthesis, a primary sink for ATP and source of ADP, does not function. Regardless of the functional implications of the incorporation of protein into mitochondrial membrane, it is clear that stainability of these membranes should increase as germination progresses. This occurred in this study, and has been commonly reported by other workers.

It is sometimes assumed, particularly on the basis of ultrastructural observations, that development of mitochondrial structure is synonymous with increased inner mitochondrial membrane area. Studies reporting incorporation of proteins into these membranes tend to reinforce this idea. This is not always the case. In Chlorella there is temporal separation of inner mitochondrial membrane biosynthesis (increasing surface area), synthesis of mitochondrial membrane proteins and intercalation of these proteins into the membrane (Forde et al., 1976). In synchronously dividing culture, the area of inner mitochondrial membrane increased steadily through the first half of the cell cycle. During this time, the ratio of enzyme activity of both succinate dehydrogenase and cytochrome oxidase to membrane area declined. In the second half of the cell cycle, the area of the inner membrane remained fairly stable while the activity of succinate dehydrogenase and cytochrome oxidase increased. Although they did not look at the problem in this way, Klein et al. (1971) observed that incorporation of protein into the mitochondrial membranes was not accompanied by an increase in membrane area. Although we have not measured protein content of mitochondrial membranes in P. vulgaris,

a similar course of events probably takes place in this system as well.

Several authors have observed changes in the buoyant density of mitochondrial populations during early germination (Nawa and Asahi, 1971; Sato and Asahi, 1975; Solomos et al., 1972; Solomos and Spencer, 1973; Wilson and Bonner, 1971). Immature mitochondria are slightly denser than more mature mitochondria. This would be expected if the relative protein content of the membrane is increasing and suggests that proteins are being intercalated into pre-existing membrane area. This was predicted by Forde et al. (1976) on the basis of their studies.

Thus, it appears that mitochondrial development proceeds by incorporation of proteins into probably pre-existing membranes. At least early in germination these proteins are present in the cell (and perhaps in the mitochondria) at the start of germination. Later, newly synthesized proteins are also incorporated into these membranes. In the present study and the study of Klein et al. (1971) neither the inner mitochondrial membrane nor mitochondrial number appeared to increase. This agrees well with the report that mitochondrial DNA is not replicated at this time in germination of P. sativum cotyledons (Malhotra et al., 1973). This further substantiates the general view that no net increase in mitochondria occurs at the beginning of germination.

Summary

The development of mitochondria in excised germinating embryos of Phaseolus vulgaris has been followed during early germination. Three

phases of oxygen consumption characterize early respiratory development. These are an initial phase of rapid uptake of water and increasing oxygen consumption followed by a lag phase in respiration and increasing fresh weight. The lag phase terminates when elongation of the radicle begins and both respiration and fresh weight increase. Stereological analysis was performed at 3, 6 and 16 h to determine mitochondrial volume (V_m), diameter ($\bar{D}_m$) and numerical density (N_m). These parameters remain stable from 3 to 16 h of germination indicating that mitochondrial division is not occurring. There is evidence that mitochondrial morphology is complex at this time, and clusters of mitochondrial profiles are often observed. The area of the inner mitochondrial membrane does not appear to increase during the period of germination studied, and cristae are numerous even after 30 min imbibition in cold water. The mechanism(s) by which mitochondrial development may occur are discussed.

CHAPTER IV

CONCLUSIONS

Ching (1972, p. 121) has noted that ". . . preservation of mitochondria in seeds through dehydration and extended periods of dry storage appears to be a sheer wonder of nature." It is certainly an important observation regarding the potential stability of biological membranes and the living systems of which they are a part. There is evidence that the composition (protein/lipid) of membranes may change. Whether this is a response to reduced cellular water or a protective mechanism useful in preserving basic bilayer integrity during stress is not certain. The retention of membranes in various anhydrobiotic systems is well documented (see Clegg and Crowe, 1978) and reflects the basic economics of life in an unstable and inhospitable environment. It is rather awesome to realize that our existence as human beings is utterly dependent upon the ability of seeds and other life forms to endure loss of substantial amounts of cellular water and suspension of metabolic activity. To achieve a more complete understanding of this characteristic of seeds, such as Phaseolus vulgaris, and other anhydrobiots, is to move closer to a mystery as complex and important as the genetic code and regulation of cellular activity.

LITERATURE CITED

Abdul-Baki, A.A.: Metabolism of barley seed during early hours of germination. Plant Physiol. 44, 733-738 (1969)

Atkinson, A.W., John, P.C.L., Gunning, B.E.S.: The growth and division of the single mitochondrion and other organelles during the cell cycle of Chlorella, studied by quantitative stereology and three-dimensional reconstruction. Protoplasma 81, 77-109 (1974)

Bagley, B.W., Cherry, J.H., Rollins, M.L., Altschul, A.M.: A study of protein bodies during germination of peanut (Arachis hypogaea) seed. Am. J. Bot. 50, 523-532 (1963)

Bain, J.M., Mercer, F.V.: Subcellular organization of the developing cotyledons of Pisum sativum L. Aust. J. Biol. Sci. 19, 49-67 (1966a)

Bain, J.M., Mercer, F.V.: Subcellular organization of the cotyledons in germinating seeds and seedlings of Pisum sativum L. Aust. J. Biol. Sci. 19, 69-84 (1966b)

Bechtel, D.B., Pomeranz, Y.: Ultrastucture of the mature ungerminated rice (Oriza sativa) caryopsis. The germ. Am. J. Bot. 65, 75-85 (1978)

Bewley, J.D., Black, M.: Physiology and biochemistry of seeds in relation to germination, vol. 1, development, germination and growth. Berlin, Heidelberg, New York: Springer 1978

Breidenbach, R.W., Castelfranco, P., Peterson, C.: Biogenesis of mitochondria in germinating peanut cotyledons. Plant Physiol. 41, 803-809 (1966)

Breidenbach, R.W., Castelfranco, P., Criddle, R.S.: Biogenesis of mitochondria in germinating peanut cotyledons II. Changes in cytochromes and mitochondrial DNA. Plant Physiol. 42, 1035-1041 (1967)

Briarty, L.G., Coult, D.A., Boulter, D.: Protein bodies of developing seeds of Vicia faba. J. Exp. Bot. 20, 358-372 (1969)

Briarty, L.G., Coult, D.A., Boulter, D.: Protein bodies of germinating seeds of Vicia faba. Changes in fine structure and biochemistry. J. Exp. Bot. 21, 513-524 (1970)

Burguillo, Placido de la Fuente, Nicolas, G.: Appearance of an alternate pathway cyanide-resistant during germination of seeds of Cicer arietinum. Plant Physiol. 60, 524-527 (1977)

Buttrose, M.S.: Ultrastructure of barley aleurone cells as shown by freeze-etching. Planta 96, 13-26 (1971)

Buttrose, M.S.: Rapid water uptake and structural changes in imbibing seed tissues. Protoplasma 77, 111-112 (1973)

Chapman, D., Peel, W.E., Kingston, B., Lilly, T.H.: Lipid phase transitions in model biomembranes. The effect of ions on phosphatidylcholine bilayers. Biochim. Biophys. Acta 464, 260-275 (1977)

Cherry, J.: Nucleic acid, mitochondria, and enzyme changes in cotyledons of peanut seeds during germination. Plant Physiol. 38, 440-446 (1963)

Ching, T.M.: Metabolism of germinating seeds. In: Seed biology, vol II, pp. 102-218, Kozlowski, T.T., ed. New York, London: Academic Press 1972

Clegg, J.S., Crowe, J.H., eds.: Dry biological systems. New York, San Francisco, London: Academic Press 1978

Crevecoeur, M., Deltour, R., Bronchart, R.: Cytological study on water stress during germination of Zea mays. Planta 132, 31-41 (1976)

Crowe, J.H., Clegg, J.S., eds.: Anhydrobiosis. Stroudsburg, Pennsylvania: Dowden, Hutchinson and Ross, Inc. 1973

Deamer, D.W., Leonard, R., Tradieu, A., Branton, D.: Lamellar and hexagonal lipid phases visualized by freeze etching. Biochim. Biophys. Acta 219, 47-60 (1970)

Eb, A.A. Van Der, Nieuwdorp, P.J.: Electron microscopic structure of the barley aleurone cells of barley during germination. Acta Bot. Neerl. 15, 690-699 (1967)

Eldan, M., Mayer, A.M.: Evidence for the activation of NADH-cytochrome c reductase during germination of lettuce. Physiol. Plant. 26, 67-72 (1972)

Finean, J.B.: Biophysical contributions to membrane structure. Quart. Rev. Biophys. 2, 1-23 (1969)

Finean, J.B., Coleman, R., Green, W. G., Limbrick, A.R.: Low-angle X-ray diffraction and electron microscope studies of isolated cell membranes. J. Cell Sci. 1, 287-296 (1966)

Finean, J.B., Coleman, R., Knutton, S., Limbrick, A.R., Thompson, J.E.: Structural studies of cell membrane preparations. J. Gen. Physiol. 51, 19s (1968)

Flax, M.H., Himes, M.H.: Microspectrophotometric analysis of metachromatic staining of nucleic acids. Physiol. Zool. 25, 297-311 (1952)

Forde, B.G., Gunning, B.E.S., John, P.C.L.: Synthesis of the inner mitochondrial membrane and the intercalation of respiratory enzymes during the cell cycle of Chlorella. J. Cell Sci. 21, 321-340 (1976)

Fujisawa, H.: Role of nucleic acid and protein metabolism in the initiation of growth at germination. Plant Cell Physiol. 7, 185-197 (1966)

Gaffal, K.P., Kreutzer, D.: The mitochondria of Polytoma papillatum at two different stages of the vegetative cell cycle. Protoplasma 91, 167-177 (1977)

Gennis, R.B., Jonas, A.: Protein-lipid interactions. Ann. Rev. Biophys. Bioenerg. 6, 195-238 (1977)

Grossowicz, N.S., Hestrin, N.S., Keynan, A., eds.: Cryptobiotic stages in biological systems. Amsterdam, New York: Elsevier 1961

Hackenbrock, C.R., Holchli, M., Chau, R.M.: Calorimetric and freeze fracture analysis of lipid phase transitions and lateral translational motion of intramembrane particles in mitochondrial membranes. Biochim. Biophys. Acta 455, 466-484 (1976)

Hallam, N.D.: Embryogenesis and germination in rye (Secale cereale L.) I. Fine structure of the developing embryo. Planta 104, 157-166 (1972)

Hallam, N.D.: Anhydrous fixation of dry tissue using non-aqueous fixatives. J. Microscopy 106, 337-342 (1976)

Hallam, N.D., Roberts, B.E., Osborne, D. J.: Embryogenesis and germination in rye (Secale cereale L.) II. Biochemical and fine structural changes during germination. Planta 105, 293-309 (1972)

Harris, N.: Endoplasmic reticulum in developing seeds of Vicia faba. A high voltage electron microscopic study. Planta 146, 63-69 (1979)

Harwood, J.L., Stumpf, P.K.: Fat metabolism in higher plants XL. Synthesis of fatty acids in the initial stage of germination. Plant Physiol. 46, 500-508 (1970)

Henning, A., Elias, S.: A rapid method for visual determination of size distribution of spheres from the size distribution of their sections. J. Microscopy 91, 101-107 (1971)

Hoefert, L.L.: Polychromatic stains for thin sections of Beta embedded in epoxy resin. Stain Technol. 43, 145-151 (1968)

Hoffman, H.P., Avers, C.J.: Mitochondrion of yeast: ultrastructural evidence for one giant, branched organelle per cell. Science 181, 749-751 (1973)

James, T.W., Spencer, M.S.: Cyanide-insensitive respiration in pea cotyledons. Plant Physiol. 64, 431-434 (1979)

Jones, R.L.: The fine structure of barley aleurone cells. Planta 85, 359-375 (1969)

Klein, S., Barenholz, H., Budnik, A.: The initiation of growth in isolated lima bean axes. Physiological and fine structural effects of actinomycin D, cyclohexamide, and chloramphenicol. Plant Cell Physiol. 12, 41-60 (1971)

Klein, S., Ben-Shaul, Y.: Changes in cell fine structure of lima bean axes during early germination. Can. J. Bot. 44, 331-339 (1966)

Klein, S., Pollock, B.M.: Cell fine structure of developing lima bean seeds related to seed desiccation. Am. J. Bot. 55, 658-672 (1968)

Kolloffel, C., Sluys, J.V.: Mitochondrial activity in pea cotyledons during germination. Acta Bot. Neerl. 19, 503-508 (1970)

Ladbrook, D.B., Jenkinson, T.K., Kamat, J.B., Chapman, D.: Physical studies on myelin I. Thermal analysis. Biochim. Biophys. Acta 164, 101-109 (1968)

Levine, Y.K., Bailey, A.J., Wilkins, M.H.F.: Multilayers of phospholipid bimolecular leaflets. Nature, 222, 577-578 (1968)

Levine, Y.K., Wilkins, M.H.F.: Structure of oriented lipid layers. Nature New Biol. 230, 69-72 (1971)

Littman, R.B., Barnett, R. L.: The mechanism of the fixation of tissue components by osmium tetroxide via hydrogen bonding. J. Ultrastruct. Res. 38, 63-85 (1972)

Lott, J.N.A., Castelfranco, P.: Changes in cotyledons of Cucurbita maxima during germination II. Development of mitochondrial function. Can. J. Bot. 48, 2233-2240 (1970)

Luzzati, V., Husson, F.: The structure of the liquid-crystalline phases of the lipid-water systems. J. Cell Biol. 12, 207-219 (1972)

Malhotra, S.S., Solomos, T., Spencer, M.: Effects of cyclohexamide, D-threo-chloramphenicol, erythromycin and actinomycin D on de novo synthesis of cytoplasmic and mitochondrial proteins in the cotyledons of germination pea seeds. Planta 114, 169-184 (1973)

Malhotra, S.S., Spencer, M.: Structural development during germination of different populations of mitochondria from pea cotyledons. Plant Physiol. 52, 575-579 (1973)

Marianos, N.G., Fife, D.N.: Ultrastructural changes in wheat embryos during a "presowing drought hardening" treatment. Protoplasma 74, 381-396 (1972)

Mayer, A.M., Shain, Y.: Control of seed germination. Ann. Rev. Plant Physiol. 25, 167-194 (1974)

Mollenhauer, H.H., Totten, C.: Studies on seeds I. Fixation of seeds. J. Cell Biol. 48, 378-394 (1971a)

Mollenhauer, H.H., Totten, C.: Studies on seeds II. Origin and degradation of lipid vesicles in pea and bean cotyledons. J. Cell Biol. 48, 395-405 (1971b)

Moore, G.A.: Connectivity of "dispersed" particles: a probabilistic computation. In: Fourth international congress of stereology, National Bureau of Standards Special Publication 431, pp. 41-44, Underwood, E.E., deWitt, R., Moore, G.A., eds. Washington, D.C.: U.S. Government Printing Office 1976

Morohashi, Y., Shimokoriyama, Y.: Physiological studies on germination of Phaseolus mungo seeds I. Development of respiration and changes in the constituents in the early stages of germination. J. Exp. Bot. 23, 45-53 (1972)

Morohashi, Y., Shimokoriyama, Y.: Water content and mitochondrial activities in the imbibitional phase of germination of Phaseolus mungo seeds. Z. Pflanzenphysiol. 82, 173-178 (1977)

Morris, J.E.: Dehydrated cysts of Artemia salina prepared for electron microscopy by totally anhydrous techniques. J. Ultrastruct. Res. 25, 64-72 (1968)

Nawa, Y., Asahi, T.: Rapid development of mitochondria in pea cotyledons during the early stage of germination. Plant Physiol. 48, 671-674 (1971)

Nawa, Y., Asahi, T.: Biochemical studies on development of mitochondria in pea cotyledons during the early stages of germination. Effects of antibiotics on the development. Plant Physiol. 51, 833-838 (1973)

Nieuwdorp, P.J.: Electron microscopic structure of the epithelial cells of the scutellum of barley. Acta Bot. Neerl. 12, 295-301 (1963)

Opik, H.: Respiration rate, mitochondrial activity and mitochondrial structure in the cotyledons of Phaseolus vulgaris L. during germination. J. Exp. Bot. 16, 667-682 (1965)

Opik, H.: Changes in the cell fine structure in the cotyledons of Phaseolus vulgaris L. during germination. J. Exp. Bot. 17, 427-439 (1966)

Opik, H.: Development of cotyledon cell structure in ripening Phaseolus vulgaris seeds. J. Exp. Bot. 19, 64-76 (1968)

Opik, H.: Some observations on coleoptile cell ultrastructure in ungerminated grains of rice (Oriza sativa L.). Planta 102, 61-71 (1972)

Opik, H.: Effect of anaerobiosis on respiratory rate, cytochrome oxidase activity, and mitochondrial structure in coleoptiles of rice (Oriza sativa L.). J. Cell Sci. 12, 725-739 (1973)

Opik, H.: Mitochondria. In: Dynamics of plant ultrastructure, pp. 52-83, Robards, A.W., ed. San Francisco, New York: McGraw Hill 1974

Paleg, L., Hyde, B.: Physiological effects of gibberellic acid VII. Electronc microscopy of barley aleurone cells. Plant Physiol. 39, 673-680 (1964)

Parrish, D.J., Leopold, A.C.: Confounding of alternate respiration by lipoxygenase activity. Plant Physiol. 62, 470-472 (1978)

Paulson, R.E., Srivastava L.M.: The fine structure of the embryo of Lactuca sativa I. Dry embryo. Can. J. Bot. 46, 1437-1446 (1968)

Perner, E.: Electron-microscopic investigations of cells of embryos in the state of the complete rest period of the seed. I. Communication: The order of cellular structure in the radicula of air-dried seeds of Pisum sativum. Planta 65, 334-357 (1965)

Pinto da Silva, P.: Translational mobility of the membrane intercalated particles of human erythrocyte ghosts. pH dependent, reversible aggregation. J. Cell Biol. 53, 777-783 (1972)

Pradet, A., Armugkannud, A., Vermeersch, J.: Plant tissue AMP, ADP, and ATP III. Energy metabolism during the first stages of germination of lettuce seeds. Bull. Soc. Fr. Physiol. Veg. 14, 107-114 (1968)

Rest, J.A., Vaughan, J.G.: The development of oil bodies in the seed of Sinapsis alba L. Planta 65, 245-262 (1972)

Sato, S., Asahi, T.: Biochemical properties of mitochondrial membranes from dry pea seeds and changes in their properties during imbibition. Plant Physiol. 56, 816-820 (1975)

Simon, E. W.: Phospholipids and plant membrane permeability. New Phytol. 73, 377-420 (1974)

Simon, E. W.: Membrane structure and function in dried seeds. In: Dry biological systems, pp. 204-225, Clegg, J.S., Crowe, J.H., eds. New York, San Francisco, London: Academic Press 1978

Singh, A.P.: Fine structure of the dormant embryo of Medicago sativa. Am. J. Bot. 64, 1008-1022 (1977)

Solomos, T., Malhotra, S.S., Prasad, S., Malhotra, S.K., Spencer, M.: Biochemical and structural changes in mitochondrial and other cellular components of pea cotyledons during germination. Can. J. Biochem. 50, 725-737 (1972)

Spurr, A.R.: A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26, 31-43 (1969)

Swift, J.G., Buttrose, M.S.: Freeze etch studies of protein bodies in wheat scutellum. J. Ultrastruct. Res. 40, 378-390 (1972)

Swift, J.G., O'Brien, T.P.: The fine structure of wheat scutellum during germination. Aust. J. Biol. Sci. 25, 469-486 (1972)

Tanford, C.: The hydrophobic effect. New York: Wiley and Sons, Inc. 1973

Treffery, T., Klein, S., Abrahamsen, M.: Studies of fine structure and biochemical changes of germinating soybeans. Aust. J. Biol. Sci. 20, 859-868 (1967)

Ueda, K., Tsuji, H.: Ultrastructural changes in coleoptile cells during anaerobic germination of rice seeds. Protoplasma 73, 203-215 (1971)

Venable, J.H., Coggshall, R.: A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. 25, 407-408 (1965)

Walton, D.C.: Germination of Phaseolus vulgaris I. Resumption of axis growth. Plant Physiol. 41, 298-302 (1966)

Walton, D.C., Soofi, G.S.: Germination of Phaseolus vulgaris III. The role of nucleic acid and protein synthesis in initiation of axis elongation. Plant Cell Physiol. 10, 307-315 (1969)

Weibel, E.R.: Stereological principles for morphometry in electron microscopic morphometry. Int. Rev. Cytol. 26, 235-302 (1969)

Weibel, E.R.: Stereological techniques for electron microscopic morphometry. In: Principles and techniques of electron microscopy. Biological applications, vol. 3, pp. 237-269, Hyatt, M.A., ed. New York, Cincinnati, Toronto, London, Melbourne: Van Nostrand Reinhold 1973

Weibel, E.R., Gomez, D.M.: A principle for counting tissue structures on random sections. J. Applied Physiol. 17, 343-348 (1962)

Wilson, S.B., Bonner, W. D., Jr.: Studies of electron transport in dry and imbibed peanut embryos. Plant Physiol. 48, 340-344 (1971)

Yatsu, Y.L.: The ultrastructure of cotyledonary tissue from Gossypium hirsutum L. seeds. J. Cell Biol. 25, 193-199 (1965)

Yoo, B.Y.: Ultrastructural changes in cells of pea radicles during germination. J. Cell Biol. 45, 158, 171 (1970)

VITA 2

Betty Kay Hamilton

Candidate for the Degree of

Doctor of Philosophy

Thesis: ULTRASTRUCTURE OF EMBRYONIC PHASEOLUS VULGARIS L. AXES DURING ANHYDROBIOSIS AND THE DEVELOPMENT OF MITOCHONDRIA DURING EARLY GERMINATION

Major Field: Botany

Biographical:

Personal Data: Born in Stillwater, Oklahoma, March 14, 1950, the daughter of Mr. and Mrs. Thomas Hamilton.

Education: Graduated from Perkins High School, Perkins, Oklahoma, in May, 1968; received a Bachelor of Science degree in Botany and Plant Pathology, with University Honors from Oklahoma State University, Stillwater, Oklahoma, in 1972; attended Southern Illinois University at Carbondale, Carbondale, Illinois, from 1972 to 1974; fulfilled the requirements for Doctor of Philosophy in Botany at Oklahoma State University, Stillwater, Oklahoma, in May, 1980.

Professional Experience: Teaching Assistant at Southern Illinois University, General Biology, 1973-1974; Teaching Assistant at Oklahoma State University in General Botany, Fall 1974 and in Cellular and Molecular Biology Laboratory, Spring 1975 through Spring 1976; Technician II in Dr. Margaret Essenberg's laboratory, Department of Biochemistry, Oklahoma State University, January 1977 through March 1980.

Professional Organizations: Oklahoma Society for Electron Microscopy, Oklahoma Academy of Science, Sigma Xi, Phi Kappa Phi.