A HISTOCHEMICAL STUDY OF EMBRYO DEVELOPMENT IN *VANDA* (ORCHIDACEAE)

By

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INTRODUCTION

Most higher plants and animals originate from a single cell, the zygote. From this common source arise all of the cell types which ultimately comprise the complex, mature organism. What are the factors which govern the transformation of this general and unspecialized cell into heterogeneous, specialized groups of cells? The purpose of this investigation is to characterize the biochemical changes which occur in the major cellular constituents of the developing plant embryo with reference to the foregoing problem.

Cellular differentiation is reflected in both morphologic and physiologic characteristics and consequently the problem has been investigated from both viewpoints resulting in an impressive assemblage of facts and theories relating to the problem. In the last 15 years the mechanisms of differentiation and the acquisition of tissue specificity have been subjected to extensive investigation by animal embryologists (Brachet, 1957) and this has resulted in the basic idea that differentiation is the result of the synthesis of specific proteins. The investigations of Avery et al. (1944) and Boivin et al. (1948) led to the recognition of the genetic role of deoxyribonucleic acid (DNA) which

through ribonucleic acid (RNA) controls protein synthesis. As a result of the work of Watson and Crick, Kornberg, Nirenberg, Ochoa, and others (Taylor, 1963), the facts concerning the replication of the genetic material and the theory of the genetic code have been established. Yet, we still lack a completely coordinated explanation backed by experimental facts as to how cells of supposedly identical genetic constitution synthesize different proteins at different times thus resulting in differences between cells.

Botanical Histochemistry

The results of biochemical and physiological studies traditionally have been expressed in terms of fresh or dry weight of the tissue concerned. While such an approach has provided the basis for our understanding of fundamental biochemical reactions, it cannot yield information about the basic unit of life, the cell (Avery and Engel, 1954). As a result two other methods of approach have recently been utilized in the investigation of the problem of differentiation. The first method, quantitative histochemistry, involves the use of scaled down biochemical techniques which are applied to small samples of tissue obtained by sectioning. Quantitative histochemistry has received impetus from the efforts of Linderström-Lang *et al.* in the Carlsberg Laboratory. The second method, microscopic histochemistry, utilizes the specificity of certain dyes for specific chemical substances thus achieving intracellular

localization of substances. These latter methods have been applied extensively by zoologists in the study of differentiation of vertebrate and invertebrate embryos and have resulted in many interesting facts.

Botanists, on the other hand, have been slow to apply histochemical methods to botanical problems (Jensen, 1962). Within the last decade, however, a number of botanical investigators have applied histochemical methods to problems of cellular differentiation. Developmental studies concerning the correlation of respiration and total proteins utilizing root tip cells were conducted by Brown and Broadbent (1950). Whaley et al. (1952) investigated histochemically the cellulose patterns in root tip cells. Studies on the content and variation of nucleic acids in the nuclei of root tip cells have been numerous (Patau and Swift, 1953; Deeley et al., 1957; Holmes et al., 1955; Jensen, 1956, 1958; McLeish, 1959; Woodard et al., 1961; Das and Alfert, 1961). RNA, protein, and enzyme distribution have been investigated in the meristematic regions of both root and shoot (Jensen, 1955, 1956; Avers, 1958; Avers and King, 1960; Avers, 1961; Gifford and Tepper, 1962a, 1962b).

The Embryo as a Tissue Source

Most of the above mentioned investigations have been carried out on either shoot or root meristems which provide a convenient source of permanently embryonic tissue in plants. The chief disadvantage of meristems for the study

of cellular differentiation is that it is often difficult to trace the lineage of a given cell in a tissue section. The embryo, on the other hand, provides us with a simple, differentiating tissue in which one can, by sampling all stages of development, relate each cell to the zygote. The disadvantages of using the embryo for studies of differentiation are that it is usually quite small and is often masked by nutritive and integumentary tissue in the seed.

The Orchidaceae is particularly well suited for embryological studies since the embryo in most of the genera consists of a relatively undifferentiated group of cells contained in a thin, transparent seed coat and the endosperm is completely absent. This undifferentiated condition is also found in the parasitic and saprophytic members of the Balanophoraceae, Rafflesiaceae, Gentianaceae, Pyrolaceae, Orobanchaceae, and the Burmanniaceae (Maheshwari, 1950).

In this study the genus Vanda was chosen because the morphology of embryogenesis was well known (Swamy, 1942; Alvarez, 1962) and the embryo is relatively large. In addition each ovulary contains close to one million (Correll, 1950) developing ovules and synchronization among the ovules is high (Duncan, 1959).

Because of these advantages, the relative concentration of the insoluble polysaccharide fraction of the cell wall, total proteins, ribonucleic acid, histone, and sulfhydryl and tyrosine-containing proteins over the entire embryonic sequence of events from the Megaspore Stage

through the Protocorm Stage were investigated. It is hoped that the construction of the profile of the distribution of these major biochemical constituents during embryogeny will serve as a contribution to the ever growing body of information relating to cellular differentiation.

MATERIALS AND METHODS

The following cultivars of Vanda were used in this study: Vanda xHelen Paoa (Univ. of Fla.--UF #1109), Vanda xM. Foster X Vanda xE. Noa (UF #1115), Vanda xBurgeffii (UF #1116), and Vanda xHawaiian Blue (UF #1531). Since no differences were noted in the embryogeny of these hybrids, they will be given a common description here.

Culture Technique

Several flowers on each of the plants were pollinated with fresh pollen and the plants were kept in the greenhouse. One pod on each plant was periodically sampled to determine the degree of embryo development which had occurred. A plug of tissue containing a portion of the placental ridge was removed from the wall of the ovulary and fixed in CRAF (Johansen, 1940). The remainder of the plug was reinserted and the pod was tightly wrapped with Saran Wrap. A pod from each plant could thus be sampled repeatedly. Small fragments of the excised placental ridge were smeared in aceto-orcein and the stage of development noted.

About five days after fertilization occurred, the ovularies were excised and washed externally with a warm detergent solution. The placental ridges were aseptically

removed and each was cultured in 125 ml. Erlenmeyer flasks on the surface of a solidified culture solution (Vacin and Went, 1949) modified by the addition of 8 grams of agar and 250 ml. of coconut milk per liter of solution. The ovule cultures were kept at approximately 30°C at 120 foot candles of illumination from two cold fluorescent strip lamps.

Categorization of Developmental Stages

The developmental stages investigated were:

1. Megaspore Stage. Includes development from the completion of meiosis to the initiation of embryo sac mitosis.

2. Gametophyte Stage. Development from mitosis to fertilization.

3. Embryo Stage. Includes embryo development occurring within the ovule.

4. Protocorm Stage. Embryo development from germination up to primordial leaf and primary root formation.

Histochemical Methods and Technique

When the desired stage of development was available, fragments of the placenta with attached ovules were fixed, dehydrated in a tertiary butyl alcohol series, embedded in Parawax at 56°C for 8 to 12 hours, and sectioned to appropriate thicknesses (Johansen, 1940). The histochemical methods used for the identification and localization of particular constituents are summarized in Table 1.

Table 1--Histochemical Methods

Chemical Constituents	Method or Reagent	Fixation	Reference
Total insoluble polysaccharides	Periodic acid-Schiff's reaction (PAS)	Formalin-acetic acid-alcohol (FAA) (4 hrs.)	Hotchkiss (1948)
Starch	1% I_2KI-polarizing optics	None	Johansen (1940)
Pectins	Hot ammonium oxalate extraction	FAA (4 hrs.)	Jensen (1962)
Hemicelluloses	4% sodium hydroxide extraction preceded by pectin removal and followed by PAS reaction	FAA (4 hrs.)	Jensen (1962)
Cellulose	17% sodium hydroxide extraction preceded by pectin and hemicellulose removal followed by PAS reaction	FAA (4 hrs.)	Jensen (1962)
Total proteins	Ninhydrin-Schiff's reaction	FAA (4 hrs.)	Yasuma and Ichikawa (1953)
	Mercuric bromphenol blue		Mazia *et al.* (1953)
Ribonucleic acid (RNA)	Azure B, pH 4, following deoxyribonuclease treatment (12 hrs.)	Carnoy (3 parts alcohol: 1 part glacial acetic acid (24 hrs.)	Flax and Himes (1950)
Deoxyribonucleic acid (DNA)	Feulgen reaction	Carnoy (3:1) (24 hrs.)	Jensen (1962)
Histones	Fast green, pH 8.0	FAA (4 hrs.)	Alfert and Geschwind (1953)

Table 1, continued

Chemical Constituents	Method or Reagent	Fixation	Reference
Sulfhydryl (-SH)-containing proteins	Azo-aryl mercaptide coupling	FAA (4 hrs.)	Bennett and Watts (1958)
Tyrosine	Morel-Sisley reaction	FAA (4 hrs.)	Lillie (1957)

Method of Cell Volume Analysis

Measurements of cell size for the purpose of demonstrating the differential in cell enlargement evident between the meristematic and parenchymatous regions through all stages of development were made with a calibrated eyepiece micrometer in a Bausch and Lomb research microscope. Only the largest diameter of each cell was measured in approximately median longitudinal sections. All distinct cells in each tissue section were measured. Measurements were made in the two-cell proembryo phase of the Embryo Stage and in both meristematic and parenchymatous regions in early and late phases of the Protocorm Stage. Standard deviations for all samples were calculated and found in all cases to be small. Cell volumes were calculated using the formula $1/6\pi d^3$.

OBSERVATIONS

Background

In the Monandrae (taxa having one fertile anther), the ovulary at anthesis contains three parietal placental ridges which, upon pollination or auxin stimulus (Hubert and Maton, 1930; Magli, 1958), initiate archesporial tissue (Wirth and Withner, 1959). Further placental development has been shown in Phalaenopsis (Niimoto and Sagawa, 1962) and Dendrobium (Sagawa and Israel, in press) to consist of the formation of a system of dichotomously branched protruberences, or megasporangia, which are organized into a central column of nucellar cells surrounded by a single epidermal layer with an enlarged terminal archesporial cell which functions directly as a megaspore mother cell. Subsequent meiotic divisions of this cell give rise to four megaspores, the one nearest the chalazal region forming the eight-nucleate embryo sac surrounded by the integuments.

In Vanda, fertilization occurs between 60 and 70 days after pollination and shortly thereafter the outer and end walls of the outer integumentary cells become dense and the nuclei disappear. The inner integument persists until the embryo is in a multicellular stage.

The sequential steps in embryo development are illustrated in Fig. 1. The zygote divides transversely forming two cells. Subsequently, the basal (micropylar) cell divides in the same manner thus forming a two-cell proembryo and a suspensor initial. Subsequent vertical divisions of the suspensor initial result in an eight-cell suspensor apparatus. Meanwhile the two cells of the proembryo divide irregularly producing a cluster of cells. Continued cell divisions in the embryo result in the formation of an oval mass of cells surrounded by finger-like projections of the suspensor apparatus.

Growth of the embryo results in the differentiation of two intergrading regions. The cells proximal to the suspensor apparatus enlarge and become parenchymatous while those distal to the suspensor remain meristematic. Continued growth of the embryo results in the splitting of the seed coat and the protocorm either continues to adhere to the placental ridge or falls off onto the culture medium.

Soon after the embryo is free of the seed coat, rapid division of the cells of the meristematic region ensues forming an apical structure. Trichomes arise from the epidermal cells of the parenchymatous region. By this time the cells of the suspensor have become necrotic.

At this stage in the developmental sequence a primordial leaf sheath forms around the apex. The necrosis of the cells of the parenchymatous region is concomitant with the initiation of subsequent primordial leaves and is complete when the seedling is photosynthetic.

Size Relationships During Embryo Development

Differentiation of the embryo of Vanda is characterized by the formation of two regions differing primarily in cell size. Calculations of the volumes of the cells of the two regions were made in the two-cell proembryo and also in protocorms of 185 to 200 microns and 700 to 800 microns (Table 1). The cells in the two-cell proembryo are of approximately the same volume. As the embryo increases in total size, however, the difference in cell volume in the two regions increases and is maximal in the Protocorm Stage prior to the necrosis of the parenchymatous region (Fig. 2).

Measurements of cell and nuclear diameters were also made on several longitudinal sections of protocorms (Table 2). As can be seen from Fig. 3, the increase in cell and nuclear volume from the meristematic to the parenchymatous region is approximately linear.

Megaspore Stage

The megasporangium consists of an extension of the placental ridge in which the cells are organized into a nucellar row surrounded by a single epidermal layer with an enlarged terminal, tenuinucellate megaspore. Observation of this structure stained with morphological stains reveals only a difference in size and position between the megaspore and the attending tissue. Treatment of cross sections of the megasporangium with the periodic acid-Schiff's reaction reveals, however, that the inner periclinal and the

anticlinal walls of the nucellar epidermis (integumentary initials) immediately surrounding the megaspore are more highly PAS positive than the outer walls of these cells. The cell walls of the megaspore itself are also highly PAS positive indicating a high concentration of insoluble polysaccharides (Fig. 4). Efforts to determine the nature of these polysaccharides by the differential extraction of the cell wall were unsuccessful due to the extremely small size and delicate nature of the tissue.

Staining of the megasporangium with either mercuric bromphenol blue or by the ninhydrin-Schiff's reaction shows that the highest concentration of total protein in the megasporangium occurs in the megaspore and in the nucellar cells immediately surrounding the megaspore (Fig. 5). All cells of the megasporangium appear to have a higher concentration of total proteins than the cells of the placental ridge and total protein in all of the megasporangial cells is most highly concentrated in the nuclei. The nucleoli of all these cells give a very positive reaction to total protein stains and in the megaspore the chromatin is particularly evident.

Gametophyte Stage

PAS staining of the embryo sac shows a marked contrast in the concentration of insoluble polysaccharides between the components of the megagametophyte (Fig. 6). The cell walls and cytoplasm of the egg are faintly PAS

positive. Particles are often present at the base of the egg and appear to be small starch grains. These particles were never observed in the distal end of the egg which appears to be somewhat vacuolated, although the vacuole is not clearly distinct from the cytoplasm.

Markedly contrasting with the egg are the two synergids which exhibit vivid cytoplasmic staining with the PAS reaction. The stain is ubiquitously distributed in these cells and only a few PAS positive bodies are detectable in the cytoplasm. The nature of this carbohydrate is unknown and attempts at differential extraction were unsuccessful.

The cell wall at the micropylar end of the embryo sac appears greatly thickened in contrast with the rest of the wall and exhibits a high concentration of insoluble polysaccharides.

Prior to megaspore mitosis, the concentration of total protein in the cytoplasm undergoes a marked rise. The elongation and subsequent vacuolation of the megaspore prior to the first mitotic division results in the formation of a strand of cytoplasm approximately two-thirds the width of the embryo sac and extending the length of the cell (Fig. 7). The observed increase in total protein concentration in this portion of the cell probably results from a drastic decrease in total cytoplasmic volume with a concomitant uptake of water into the vacuolated portion. Nuclear total protein concentration appears to remain constant until the mitotic division ensues. During the increase in premitotic total

cytoplasmic protein concentration, the nucleolus increases to two or three times its original volume and exhibits intense staining with mercuric bromphenol blue.

At the completion of the mitotic divisions of the megaspore, the thin cytoplasmic strands which remain evident in the embryo sac show a very low concentration of total protein (Fig. 8). The polar nuclei before degeneration and the antipodal nuclei exhibit a slightly higher protein concentration than the cytoplasmic strands of the embryo sac but still appear relatively low.

The suspensor and embryo initials differ in total protein content in the two-cell phase (Fig. 11). Repeated observations indicate a somewhat higher total protein concentration in the embryo initial than in the suspensor initial. In later multicellular stages of the Embryo Stage, the difference in total protein concentration between the suspensor apparatus and the embryo proper becomes quite marked (Figs. 13, 14, 15). The cells of the suspensor appear highly vacuolated and show a very low total protein concentration. No differences with respect to this constituent, however, appear between the individual cells of the embryo proper.

An interesting phenomenon encountered in the embryo sac shortly after fertilization is the formation of an ill-defined region of very high protein concentration at the chalazal limits of the embryo sac which gradually disappears as the embryo increases in size (Fig. 16).

During the zygote and two-cell proembryo phases of the Embryo Stage, the ovule contains RNA only in the nucleoli. Repeated observations of similarly treated sections containing two-cell phases of the proembryo occasionally show the presence of a "shell" of RNA in the cytoplasm immediately surrounding the nucleus of the proembryo initial and suspensor initial. The RNA "shell" in the suspensor initial, however, was not as distinct as in the embryo initial and disappears during enlargement of the suspensors (Fig. 17).

The egg apparatus exhibits a distinct differential staining for total protein (Fig. 8) which is low in the egg cytoplasm but high in the nucleus. The egg nucleolus is also distinct, having stained darker than the nucleoplasm. In contrast, the cytoplasm of the two synergids is very high in total protein concentration and the nucleus in these cells is indistinguishable from the cytoplasm.

Treatment of longitudinal sections of the embryo sac with pH 4.0 Azure B reveals a low concentration of ribonucleic acid in the entire embryo sac. The staining was very light and limited to a small area in the cytoplasmic strands surrounding the polar nuclei (Fig. 9). Observation of early phases of the embryo sac often shows a high concentration of RNA in the polar nuclei (Fig. 9).

Embryo Stage

No difference in the amount of insoluble polysaccharides was detectable at the two and three-cell stages (Fig. 10).

When the suspensor apparatus becomes morphologically distinct, however, the cell walls of these cells become thicker and more highly PAS positive than those of the embryo proper (Fig. 11). This difference does not appear, however, until the suspensor apparatus consists of at least two cells.

In the multicellular Embryo Stage, cytoplasmic RNA concentration is low in all cells but appears to be somewhat higher in the cells of the embryo proper (Fig. 18).

Protocorm Stage

One of the characteristic features of the Protocorm Stage is the presence and distribution of starch grains throughout the tissues during the developmental sequence. These inclusions are found in large numbers in both differentiated regions but are much larger in the parenchymatous region (Fig. 19). In the later phases of the Protocorm Stage, starch grains are found only in the parenchymatous region which is becoming necrotic (Fig. 20).

These inclusions stain vividly by the PAS method, showing layering and a distinct hylum. They give a slightly positive reaction with mercuric bromphenol blue and the ninhydrin-Schiff's reaction and also with sudan III. Polarizing optics, however, show them to be birefringent.

In the early Protocorm Stage, total protein distribution is uniform in the meristematic cells and the parenchymatous cells. This result was obtained by staining with mercuric bromphenol blue and the ninhydrin-Schiff's reaction (Fig. 21).

RNA also appears to be uniformly distributed in this stage. Repeated observations, however, show what appear to be regions of high RNA concentration in the cytoplasm of the parenchymatous region. These areas are morphologically indistinguishable from the surrounding cells which exhibit lower staining intensity (Fig. 22). At this stage, two nucleoli are distinguishable in some of the nuclei of the parenchymatous cells. These appear frequently in those cells exhibiting high concentrations of cytoplasmic RNA. In the very latest phases of the Protocorm Stage, RNA concentration is distinctly higher in the meristematic apical cells, particularly in those at the base of the leaf primordium.

Hot ammonium oxalate extraction of the pectic substances from the cell walls in the Protocorm Stage with subsequent PAS staining shows that the cell walls of the meristematic cells contain greater amounts of pectic substances than those of the parenchymatous cells (Fig. 23). Further extraction of the hemicelluloses and cellulose with sodium hydroxide indicates that these constituents are in relatively higher concentrations in the cell walls of the parenchymatous cells than in the meristematic cells and that hemicellulose comprises the greatest part of the non-pectic fraction of the cell wall.

Treatment of similar sections with hydroxylamine and ferric chloride after the method of Reeve (1959) indicates that the esterification of pectin is very low, if not indeed absent, in the cell walls of all the cells during the

Protocorm Stage. Certain sections exhibited a very weak positive reaction in the walls of some parenchymatous cells, indicating slight pectin esterification, but the results are not considered conclusive.

Staining of the early protocorm with fast green at pH 8.0 results in an equally intense stain in all nuclei indicating a proportionately higher amount of histones in the nuclei of the parenchymatous cells than in the meristematic cells (Fig. 24). In the late phases of the Protocorm Stage, however, the nuclei of the parenchymatous region stained considerably more intensely (Fig. 25) indicating a very high histone concentration. This observation is concurrent with the morphologically detectable initiation of necrosis in this region. Synchronous with this event is the appearance of slight cytoplasmic staining with fast green at pH 8.0 suggesting the presence of basic proteins in the cytoplasm.

Feulgen staining of the late protocorm shows a marked contrast between the staining intensity of the meristematic and parenchymatous nuclei, the latter being considerably darker.

Tyrosine and sulfhydryl-containing proteins are localized primarily in the nuclei of all cells and staining intensity is greatest in the nuclei of the parenchymatous cells. This relative distribution does not appear to change extensively during development (Figs. 26, 27).

Results are summarized in Tables 3 and 4.

Fig. 1--Sequence of Embryo Development

The first division of the zygote (a) is transverse producing a terminal and a basal cell (b) which also divides transversely (c). The first division of the terminal cell is vertical producing a four-cell embryo (d). A second vertical division occurs in the terminal cell at right angles to the first (e). Several successive divisions of the basal cell give rise to the globular embryo proper while continued vertical division of the terminal cells give rise to the suspensor apparatus which forms finger-like projections over the globular embryo (f).

The cells of the embryo distal to the suspensor remain meristematic while those proximal to the suspensor enlarge and become vacuolated (g). This results in the formation of intergrading meristematic and parenchymatous regions (h, i).

SEQUENCE of EMBRYO DEVELOPMENT

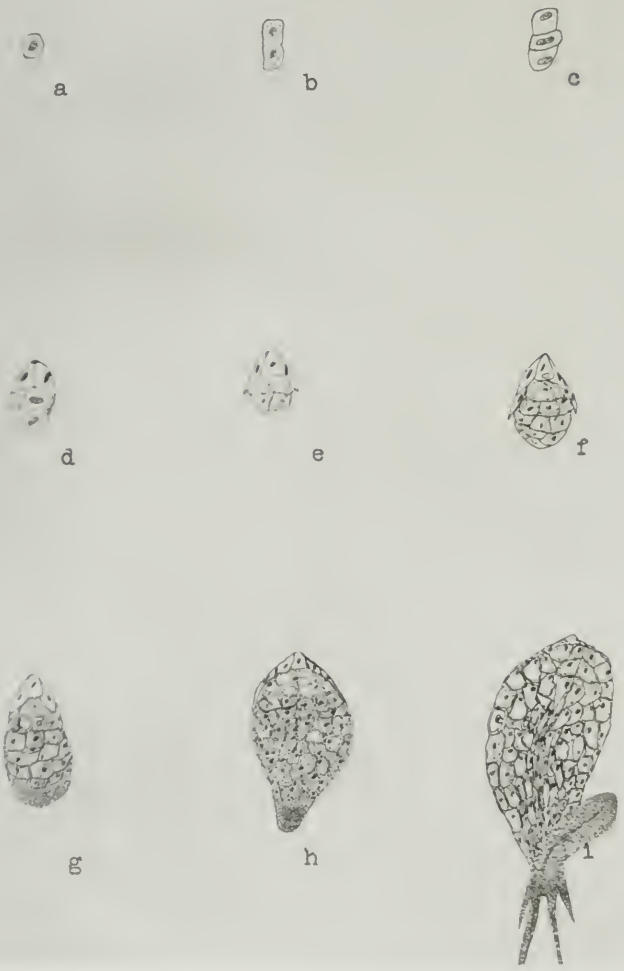

Table 2--Comparison of Mean Cell Volume ($\mu 3$) of Meristematic and Parenchymatous Cells throughout Development

Two-Cell Proembryo (Both Cells) n*=10		Early Protocorms (Total Lengths 185-200μ)		Late Protocorms (Total Lengths 700-800μ)	
		Apical Cells n=20	Parenchyma Cells n=20	Apical Cells n=20	Parenchyma Cells n=20
Mean ($\mu 3$) Volume	818	1840	15914	7800	397479
Coefficient of % Variation	25.8	23.0	28.2	22.8	14.2

*n=number of cells measured

Table 3--Changes in Mean Cell and Nuclear Volume ($\mu 3$) in 4 Quarters of Protocorms from the Apical through the Parenchymatous Regions

Segment of Protocorm (Apical to Parenchymatous)	Mean Cell Volume ($\mu 3$)	Mean Nuclear Volume ($\mu 3$)
1st Quarter	697	34
2nd Quarter	3056	112
3rd Quarter	9230	382
4th Quarter	22467	524

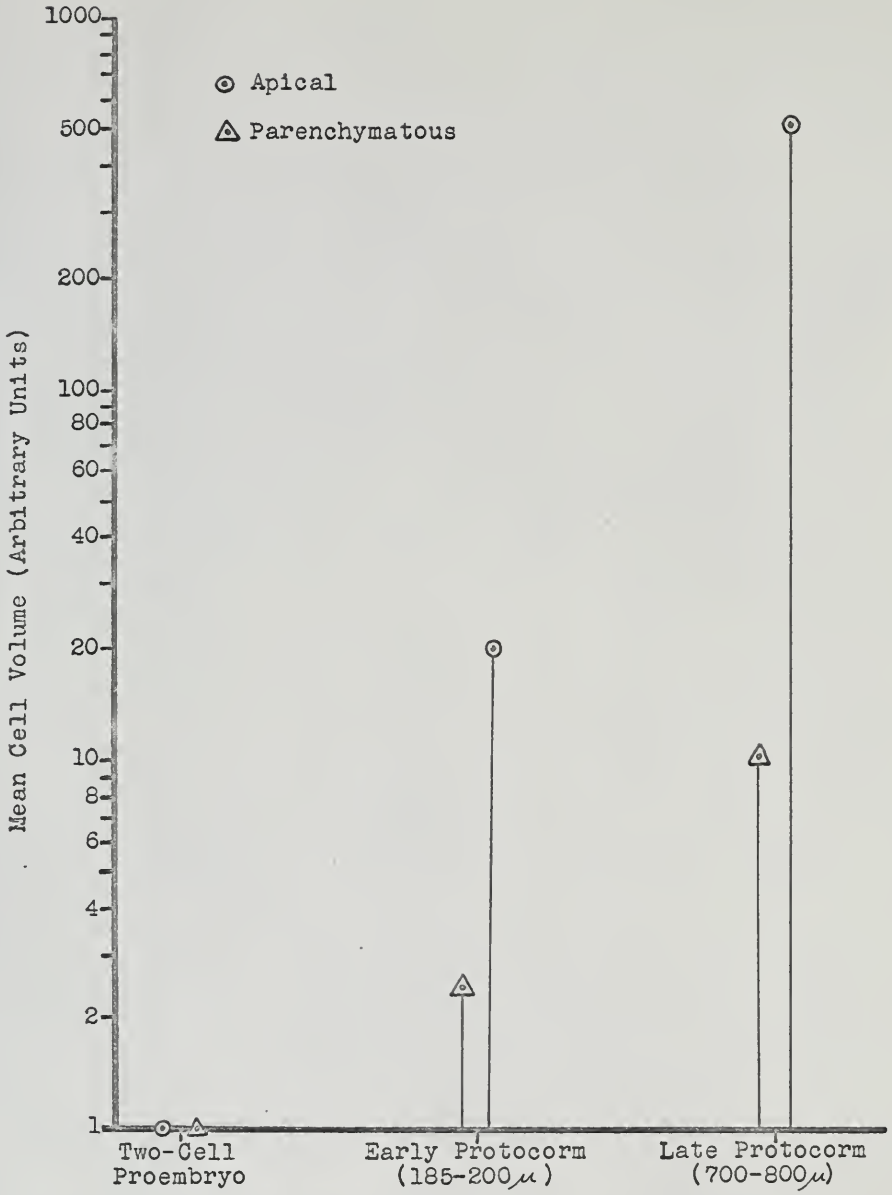

Fig. 2--Comparison of Meristematic and Parenchymatous Cell Volumes During Development

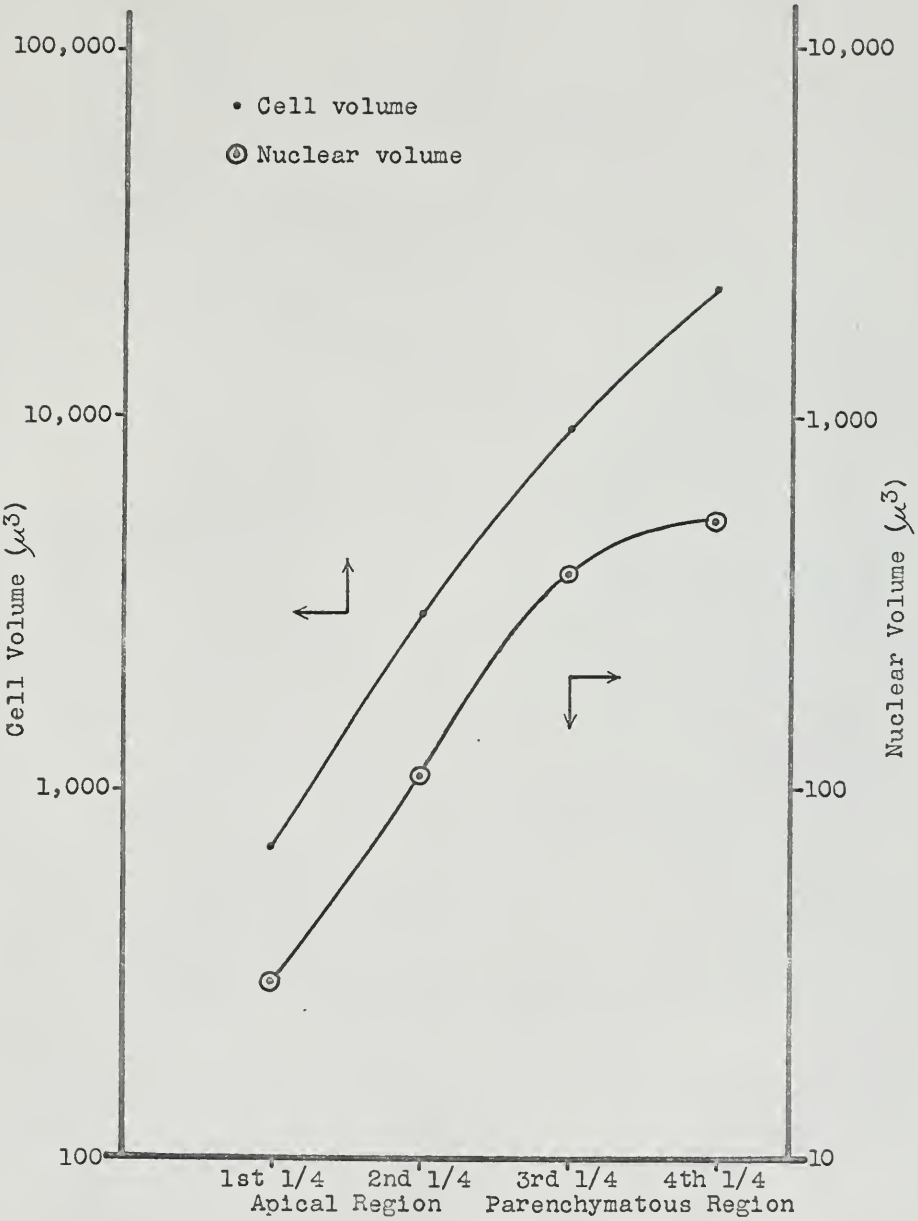

Fig. 3--Comparison of Cell
and Nuclear Volumes in the Protocorm

Table 4--Summary of Results in Megaspore and Gametophyte Stages

Stage	Concentration or Presence of Compounds		
	Insoluble Polysaccharides	Total Proteins	Ribonucleic Acid
Megaspore	Megaspore wall: anticlinal nucellar epidermal walls.....HIGH Megaspore and nucellar epidermis cytoplasm.....LOW	Megasporangium distal end.....HIGH Proximal end.....LOW	(NOT TESTED FOR)
Gametophyte	Synergid cells..HIGH (uniform distribution) Egg cell.....LOW (slightly higher in nucleus) Embryo sac cytoplasm.....LOW Polar nuclei.....LOW Antipodal cells.....LOW	Embryo sac cytoplasm prior to mitoses.....HIGH (concentrated in thin central strip) After mitoses.....LOW Egg cell.....LOW Polar nuclei.....LOW Antipodal cells.....MEDIUM Synergid cells.....HIGH (uniform distribution)	Embryo sac cytoplasm.....LOW Egg.....LOW Polar nuclei..MEDIUM (in early phases--gradually diminishes) Antipodal cells.....LOW Synergid cells.....LOW

Table 5--Summary of Results in Embryo and Protocorm Stages

Stage	Concentration or Presence of Compounds					
	Insoluble Polysaccharides	Total Proteins	Ribonucleic Acid	Histones	Sulfhydryl-Containing Proteins	Tyrosine-Containing Proteins
Embryo	Suspensor initial...LOW Embryo initial...LOW Suspensor cell walls (multicellular)...HIGH	Suspensor initial..LOW Embryo initial.HIGH Mature suspensor apparatus..LOW Multicellular embryo..HIGH	Suspensor initial..LOW Embryo initial..MED. Mature suspensor apparatus..LOW Multicellular embryo..HIGH	(Not tested for)	(Not tested for)	(Not tested for)
Protocorm	Enlarging cells pectins.HIGH hemicelluloses..LOW Enlarged cells pectins..LOW hemicelluloses.HIGH All cell walls methyl esterification...LOW	Meristematic region..HIGH Parenchymatous region..HIGH	Meristematic region...MED. Parenchymatous region...MED. Regions of vascularization..HIGH	Early protocorm meristematic.....LOW parenchymatous...LOW Late protocorm meristematic.....LOW parenchymatous..HIGH	Meristematic region...MED. Parenchymatous region...MED.	Meristematic region..MED. Parenchymatous region..MED.

Fig. 4--PAS Stained Cross Section
through the Megasporangium

The anticlinal and inner cell walls of the nucellar epidermis surrounding the megaspore as well as the walls of the megaspore are thicker and more highly PAS positive than the other cell walls of the megasporangium.

Fig. 5--Protein Concentration Gradient
in the Megasporangium

The megaspore and surrounding nucellar cells exhibit a higher total protein concentration than the basal nucellar epidermis and axial row.

Fig. 6--PAS Stained Longitudinal
Section of the Embryo Sac

The synergid cells show a high, uniform concentration of insoluble polysaccharides. All other embryo sac components are low in insoluble carbohydrates.

Fig. 7--Protein Localization in a Megasporangium
with a Pre-mitotic Megaspore

Immediately prior to mitosis the megaspore elongates and becomes laterally vacuolated while protein concentration in the cytoplasm increases.

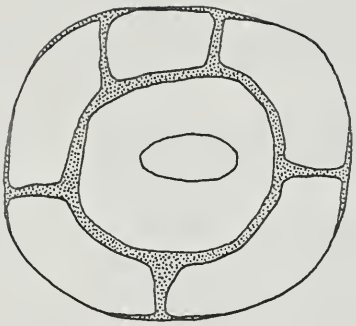

FIG. 4

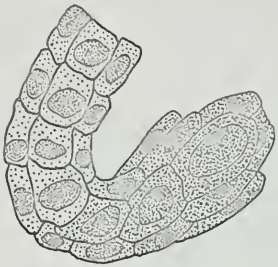

FIG. 5

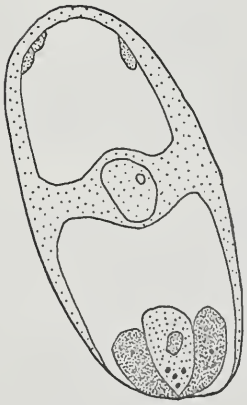

FIG. 6

FIG. 7

Fig. 8--A Longitudinal Section of
an Embryo Sac Stained for Protein

Total protein concentration is high and uniform in the synergid cells. Total protein concentration in the polar nuclei diminishes as the embryo sac matures.

Fig. 9--A Longitudinal Section of
an Embryo Sac Stained for Ribonucleic Acid

RNA is low in all embryo sac components except the polar nuclei.

Fig. 10--A Longitudinal Section of
a Three-cell Embryo Stained with PAS

No differences in insoluble polysaccharides are evident between the cells at this stage. Note the thick, highly PAS positive cell wall at the micropylar end of the embryo sac.

Fig. 11--A Longitudinal Section of
a Multicellular Embryo Stained with PAS

At this stage the cell walls of the suspensor apparatus are thicker and more highly PAS positive than the cell walls of the embryo proper.

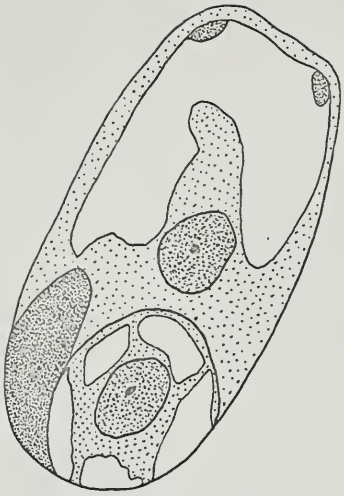

FIG. 8

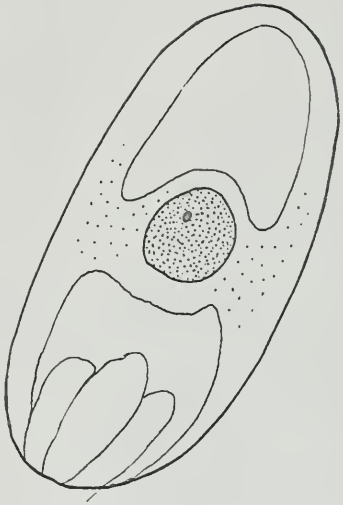

FIG. 9

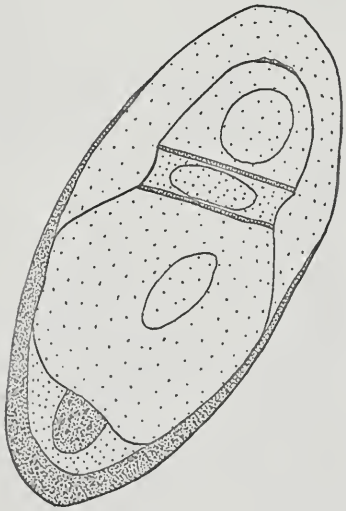

FIG. 10

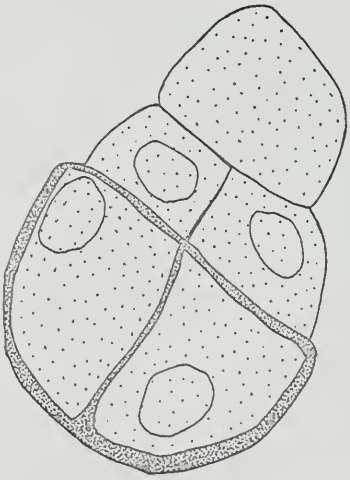

FIG. 11

Fig. 12--A Longitudinal Section of
a Three-cell Embryo Stained for Total Protein

As early as the two-cell stage, the embryo initial exhibits a higher total protein concentration than the suspensor initial.

Figs. 13, 14, 15--Total Protein Distribution
in Early Multicellular Embryos

As development proceeds, the higher total protein concentration in the embryo proper versus the suspensor apparatus becomes more marked.

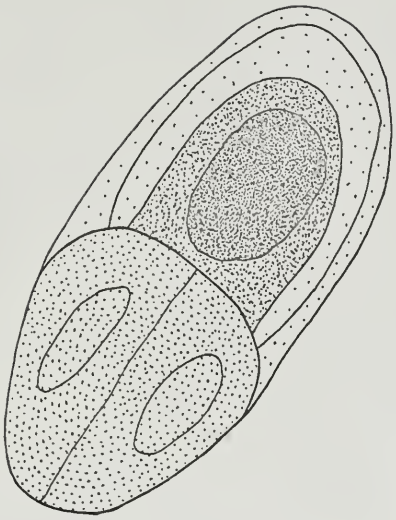

FIG. 12

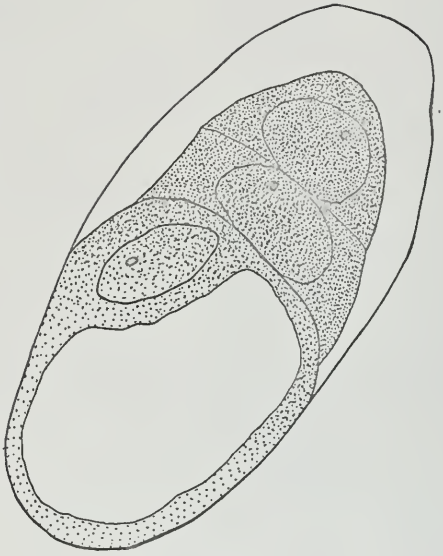

FIG. 13

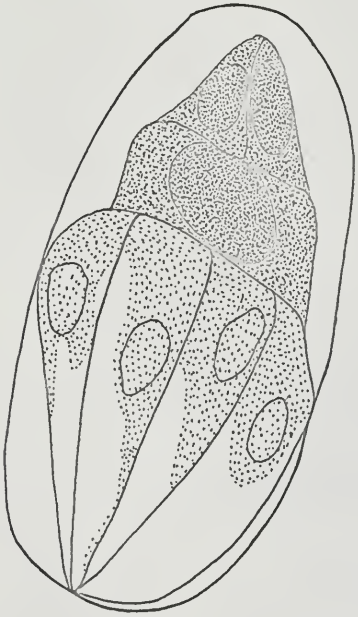

FIG. 14

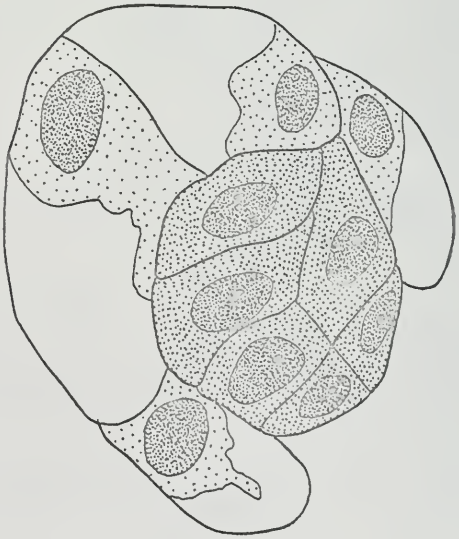

FIG. 15

Fig. 16--A Longitudinal Section of an Embryo Sac Showing a Post Fertilization Total Protein Increase at the Chalazal End

Shortly after fertilization the concentration of total protein at the chalazal end of the embryo sac increases markedly.

Fig. 17--A Longitudinal Section of a Two-cell Embryo Stained for Ribonucleic Acid

A marked basophilia is exhibited at the periphery of the nuclei at the two-cell stage. This reaction disappears from the cells of the suspensor apparatus during subsequent development.

Fig. 18--A Longitudinal Section of an Early Multicellular Embryo Stained for Ribonucleic Acid

RNA distribution in the multicellular embryo parallels closely that of protein.

Fig. 19--A Longitudinal Section of an Early Protocorm Stained with PAS

Note that starch grains are present in both meristematic and parenchymatous cells.

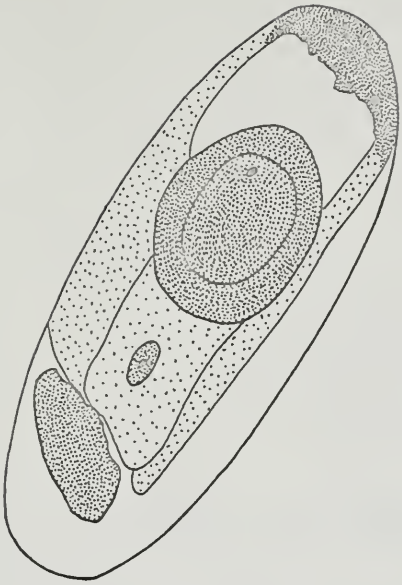

FIG. 16

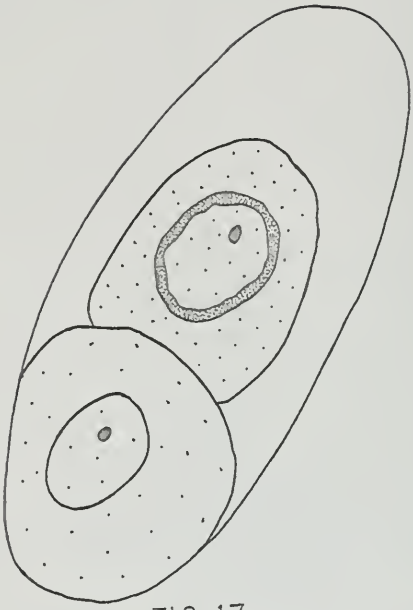

FIG. 17

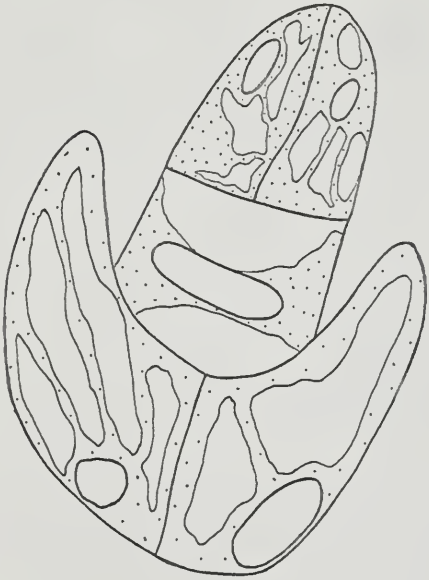

FIG. 18

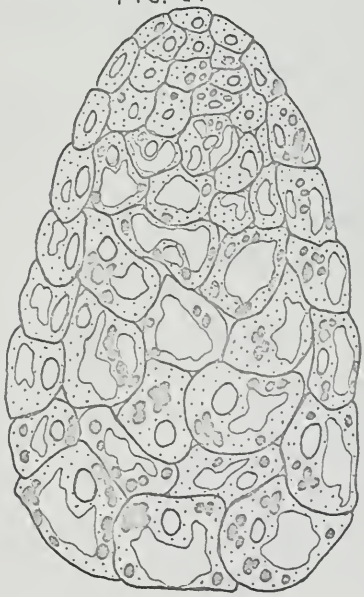

FIG. 19

Fig. 20--A Longitudinal Section of
a Late Protocorm Stained with PAS

During late phases of the Protocorm Stage, starch grains become limited to the parenchymatous region.

Fig. 21--A Longitudinal Section of
an Early Protocorm Stained for Total Protein

Note that the total protein concentration is the same in the meristematic and parenchymatous cells at stage of development.

Fig. 22--A Longitudinal Section of
an Early Protocorm Stained for Ribonucleic Acid

Certain cells in the inner parenchymatous region often exhibit high basophilia. These cells appear to be involved in the formation of vascular tissue.

Fig. 23--A Longitudinal Section of
an Ammonium Oxalate Extracted Early Protocorm
Showing Pectin Distribution

The cell walls of the meristematic cells contain proportionately greater amounts of pectic substances than the parenchymatous cells.

FIG. 20

FIG. 21

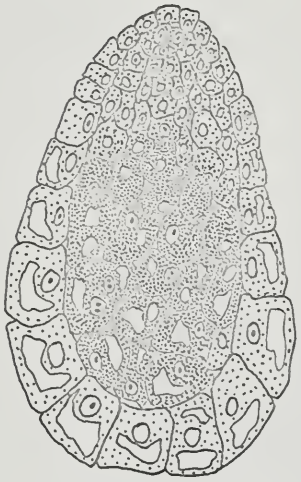

FIG. 22

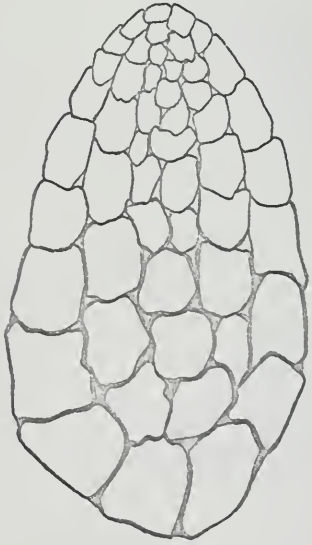

FIG. 23

Figs. 24, 25--Longitudinal Sections of Early and Late Protocorms Showing Changes in Histone Concentration

In the early protocorm stage (Fig. 24), histone concentration in the nuclei of the meristematic and parenchymatous cells is the same. In the late protocorm (Fig. 25), however, histone concentration rises markedly in the parenchymatous nuclei.

Fig. 26--A Longitudinal Section of a Protocorm Stained for Tyrosine-containing Proteins

Tyrosine-containing protein concentration is higher in the nuclei of the parenchymatous cells.

Fig. 27--A Longitudinal Section of a Protocorm Stained for Sulfhydryl-containing Proteins

Sulfhydryl-containing protein concentration is higher in the nuclei of the parenchymatous cells.

FIG. 24

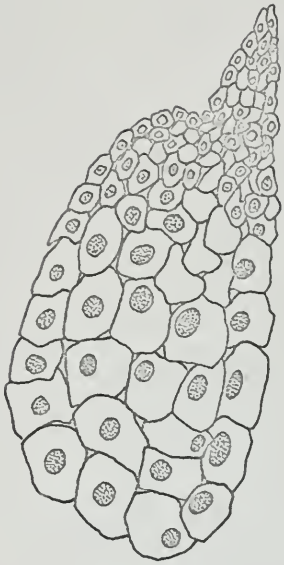

FIG. 25

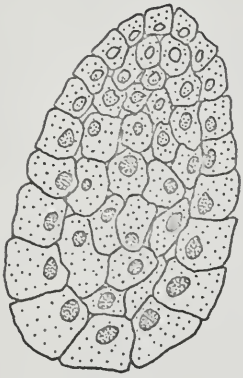

FIG. 26

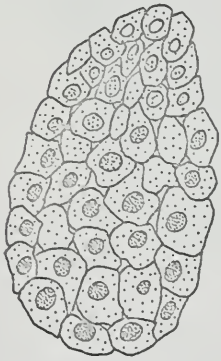

FIG. 27

DISCUSSION OF OBSERVATIONS

The Cell Wall

Early studies of the cell wall were limited primarily to the definition of certain gross properties such as swelling (Naegli, 1864), birefringence (von Mohl, 1859), and cytoplasmic proximity (Fitting, 1900). From these studies evolved the inquiries resulting in the extensive assays of the chemical nature of the cell wall during the early to mid nineteen hundreds (Katz, 1924; Ambronn and Frey, 1926; Frey-Wyssling, 1935, 1936). Following the advent of the electron microscope, emphasis was placed upon the correlation of chemical and physical characteristics, obtained with polarizing optics and x-ray defraction techniques, with the observable ultrastructure as revealed by ultrathin sectioning (Whaley *et al.*, 1952; Scott *et al.*, 1956). In recent years, emphasis in cell wall studies has centered around the elucidation of the mechanisms involved in cell expansion (Green, 1958) and on the distribution and biosynthesis of cell wall constituents during cellular differentiation (Bishop *et al.*, 1958; Jensen, 1960; Flemion, 1961). As a result of the dominant role played by the cell wall in hormonal regulation of plant cell growth, the effect of auxin treatment on wall biosynthesis has also received extensive

investigation (Ordin et al., 1955; Bonner et al., 1955; Lamport, 1963). For recent reviews on this aspect see Audus (1959), Thimann (1960), Galston and Purves (1960), and Ray (1961). For general reviews on the cell wall, Preston (1959), Mühlethaler (1961), and Northcote (1963) may be consulted.

The investigation described herein reveals that the cell wall and other insoluble polysaccharide structures undergo ordered changes in distribution and concentration during embryogenesis. These shifts in distribution appear to be closely associated with cellular growth and differentiation.

The Cell Wall in the Megaspore and Embryo Stages

During the Megaspore Stage the cell walls of the megaspore and the anticlinal walls of the surrounding nucellar epidermis appear considerably thicker than those of the other megasporangial cells. Similar changes also occur in the cell walls of the suspensor apparatus during the marked enlargement of these cells in the Embryo Stage. Evidence of the loss of plasmadesmatic connections between the megaspore mother cell and the surrounding nucellar cells has been obtained with the electron microscope in Dendrobium (Israel, 1962). Thus, while thickened cell walls do not necessarily indicate few plasmadesmata, they do serve to emphasize the fact that the megaspore is embarked on a course of development different from the cells around it and that the cell wall is at least involved in such development.

The Cell Wall in the Protocorm Stage

An interesting correlation exists between the observed cell wall thickening of the megaspore and the suspensor apparatus and the cell walls of the meristematic cells of the protocorm. Differential extraction of the cell walls of the protocorm reveal that the pectic substances are contained in higher amounts in the growing cells of the meristematic region than in the cells of the parenchymatous region. Jensen (1960) reports similar results in the differentiating protodermal cells of the onion root tip. Methyl esterification of the pectins, however, is practically absent in all of the cell walls of the protocorm. This finding conflicts with the proposition of Ordin *et al.* (1955) that cell wall plasticity is the result of the formation of methyl bridges between carboxyl groups which prevent or reduce cross linkage of the molecules of galacturonic acid by ionic binding of calcium ions and that this biosynthesis is increased by indole-3-acetic acid. Jansen *et al.* (1960), however, report that while the hot-water soluble pectin is almost fully esterified, the residual insoluble "protopectin," which comprises 80 percent of the pectic substance, is only approximately 30 percent esterified. Since little reaction for methyl esterification was obtained in the protocorm, it is possible that the esterified hot-water soluble fraction was not present since the tissue was not in contact with hot water at any time during the localization procedure. It is considered possible, however, that hot paraffin immersion

may have caused the removal of the hot-water soluble fraction. But regardless of this, the pectin fraction detected after ammonium oxalate extraction--probably protopectin--is proportionately higher in the expanding cells which concurs with the idea proposed by Albersheim and Bonner (1959) that the increased wall plasticity resulting from auxin stimulus is due to an over-all softening of the cell wall by the addition of pectic material.

Further extraction of the cell walls of the protocorm indicates that the major non-cellulosic component of the walls of all the cells is hemicellulose. This finding is in agreement with the suggestion of Jensen (1960) that the non-cellulosic polysaccharides may play a major role in determining the characteristics of the cell wall. It appears that cell wall thickening in the megaspore and suspensor apparatus may also be due to the same phenomenon observed in the expanding cells of the protocorm. This increase in cell wall polysaccharide content without secondary wall formation seems to be characteristic of expanding cells.

Another hypothesis regarding cell wall extensibility that merits discussion is that proposed by Lamport (1963). Until recently there was little support for the view that protein might be the wall component which controls its plasticity since the amount of protein in the cell wall had been shown to be quite small; 4 to 12 percent by weight. Lamport, however, has implicated the amino acid hydroxyproline in the structure of a cell wall protein which he refers to as

"extensin." He speculates that this protein is directly involved in controlling cell wall plasticity by providing a network of labile cross-linkages between the cellulose microfibrils. The existence of hydroxyproline in peptide linkage in the cell wall has been demonstrated by Lamport. This protein is soluble when first synthesized but later becomes firmly attached to the cellulose, perhaps through a covalent protein-cellulose link. Lamport has further shown that the oxygen of the hydroxyproline is derived from atmospheric oxygen and he proposes experiments in which the oxygen content of a tissue is varied--but never allowed to become limiting to the function of the cytochrome system--to determine what, if any, effect the presence of oxygen has on cell expansion. Such an experiment might be conducted utilizing the protocorm of Vanda, since in this tissue cell expansion is a normal function of embryo growth and no other complicating paths of differentiation are present nor is auxin stimulus necessary.

The Synergids

The synergid cells are usually ephemeral structures in the angiosperm gametophyte which disappear soon after or even before fertilization (Maheshwari, 1950). In some cases, however, as in Vanda, one or both of these cells may persist and show considerable activity. A similar condition has been observed in the cases of Allium unifolium and A. rotundum (Weber, 1929). While the synergids are usually considered to be morphological remnants of the archegonium

(Fuller and Tippo, 1954), the unusually high concentration of insoluble carbohydrates and total protein, as well as the ubiquitous distribution of these classes of compounds in the cytoplasm, suggests the possibility of a nutritional role for these cells in support of the egg and early embryo. Similar results and conclusions were obtained by Pritchard (1962) in his histochemical study of the embryogeny of Stellaria media.

Unlike Stellaria, the synergids in Vanda also exhibit a high concentration of total protein, the distribution of which is similar to the polysaccharide distribution observed in these cells. Since the embryo sac and, indeed, the ovule at this stage, is very small, differential extraction of tissue sections was not successful. While discrete PAS positive particles are also found in these cells as well as in the basal portion of the egg, and while the presence of starch has also been reported in other angiosperm embryo sacs (Maheshwari, 1950), and because of its uniform distribution, it seems likely that the PAS positive material in the case of Vanda is more in the nature of a mixture of hemicelluloses such as the mannans found in the seed of the date palm or the hexosans in leguminous seeds which have been shown to function as reserve foodstuffs (Mühlethaler, 1961). In the above cited cases, however, the hemicellulosic compounds are contained as a part of the cell wall. The possibility of a mucopolysaccharide nature of this substance is alluded to by the similarity of the distribution of the

protein and the PAS positive material although no analagous cases can be found in the literature.

Starch Distribution

The early Protocorm Stage is characterized by the presence of starch grains in the cells of both the meristematic and parenchymatous cells. In the later phases of protocorm development, however, starch grains become confined to the parenchymatous portion where they become progressively smaller and ultimately disappear subsequent to the initiation of necrosis of the parenchymatous region. In this phase of development, the remaining starch grains appear in greater number in the cells in the central portion of the protocorm which appear as vascular initials. Since this occurs prior to the establishment of full photosynthetic activity, as evidenced by the external greenness of the plant, it appears that the entire parenchymatous region serves as a store of nutrients for the actively dividing cells of the meristem.

Proteins and RNA

The correlation of the activity of RNA and protein metabolism has been the subject of intensive investigation in recent years and the literature is far too extensive to review here. For general reviews consult Brachet (1957) and Zalokar (1961).

Within recent years, the role of RNA and protein in plant morphogenesis has emerged as a leading factor and numerous studies concerning the synthesis, activity, and

distribution of these compounds have been forthcoming. Outstanding among these studies is the work of Sunderland et al. (1957) in which they correlated protein concentration and respiration in the various tissues of the apical meristem of Lupinus alba. Also noteworthy are the studies of Taylor (1958, 1959) on the synthesis and distribution of nucleic acids during microsporogenesis in Lilium and Tulbaghia.

The metabolism of the nucleic acids and proteins was recently investigated in seedlings of barley by Ledoux et al. (1962) in which they followed changes in the content of these substances between the various organs of the seedling and the endosperm during early development. The distribution of RNA and proteins in the apical meristem has also been studied by Gifford and Tepper (1962a, 1962b) and these authors were able to correlate fluctuations in these classes of compounds in the various histologically differentiated regions of the shoot apex during floral induction.

All of the above cited investigations, together with the impressive number of studies of the interrelation of the metabolism of these compounds in animal tissue, point to the conclusion that the control of protein synthesis resides in the nucleus and that this control is mediated to the cytoplasm via RNA. The strongest support for this view is summarized in the following facts: 1. RNA is required for protein synthesis to occur in the cytoplasm. 2. The RNA fraction of the Tobacco Mosaic Virus determines what type of protein is made in cases of virus infection. 3. The rate of

incorporation of RNA precursors is much higher in the nucleus than in the cytoplasm. 4. Transplantation experiments have shown that labelled RNA can pass from the nucleus to the cytoplasm (Mirsky and Allfrey, 1958).

In the investigation described herein, all of the findings relating to RNA and protein distribution are in agreement with this fundamental idea.

The Megasporangium

Observation of the megasporangium stained for total protein shows that the concentration is considerably higher in the cells of the distal end, particularly in the megaspore and in the nucellar cells immediately surrounding it. Observation of the megaspore itself between the time of its formation until the beginning of the mitotic divisions reveals no striking increases in the protein concentration of the cell. In fact, after the enlargement of the embryo sac following the mitotic divisions of the nucleus, protein concentration in this structure appears to be considerably reduced.

Ultrastructure studies of megasporogenesis in Dendrobium (Israel, 1962) have shown that the megaspore mother cell actively absorbs the surrounding nucellus and this may be a mechanism for the rapid increase of protein in a rapidly enlarging cell. The occurrence of this phenomenon was alluded to by Esau (1953) and the active uptake of intact protein has been demonstrated in vertebrates (Ebert, 1954), in tissue culture (Francis and Winnick, 1953), and in plant cells (Jensen and McLaren, 1960). In view of the above, it appears

that the elevated protein concentration observed in the nucellus of the distal tip is a remnant of the condition of active intact protein absorption, which existed during megasporogenesis, but which is not functioning in the Megaspore Stage.

The Gametophyte

Observation of the embryo sac before fertilization indicates that the concentration of total proteins and RNA is low in all components except the synergids which have already been discussed. The polar nuclei and the antipodal cells appear to have a higher concentration of these classes of compounds during the early phases of the Gametophyte Stage. The endosperm nucleus disappears, however, during later embryo sac development.

In the case of the polar nuclei, RNA appears to be confined entirely to the nucleus and its distribution therein is uniform except for a deeper stain in the nucleolus. RNA was never observed in the thin cytoplasmic strand surrounding the secondary nucleus. Any explanations given for the observed decrease in the protein and RNA content of the polar nuclei would be purely speculative since comparable studies are nonexistent. However, Ledoux *et al.* (1962) report that in the endosperm of barley, RNA'ase activity increases as RNA in this tissue decreases. Thus one might speculate a similar occurrence in *Vanda* but at an earlier phase. This condition might be related to the increase in ploidy in the

secondary nucleus resulting from the fusion of the polar nuclei in which a factor, or factors, for RNA'ase production may be contained in double dose. In most angiosperms, the polar nuclei lie just above the egg and are connected to it by a conspicuous cytoplasmic strand (Maheshwari, 1950). In the embryo sac of Vanda, however, the polar nuclei are located in the center of the embryo sac and appear to be suspended between two large vacuoles by thin cytoplasmic strands emanating from the long sides of the embryo sac. Possibly this position makes access to the precursor ribose nucleotides difficult (Ambellan, 1955).

Certainly a cytochemical comparison of the embryo sac of Vanda with a species in which the endosperm is well developed would be enlightening.

Shortly after fertilization, the chalazal end of the embryo sac shows a small region of intense protein staining. As the embryo enlarges, this region increases in area and in staining intensity. When the embryo nearly fills the embryo sac, the region begins to disappear. This activity appears to be associated with the antipodal cells.

The role of the antipodals in angiosperm embryology is not clear. Phylogenetically, they have been regarded as vestiges of the prothalial tissue (Fuller and Tippo, 1954).

In the Gramineae, Gentianaceae, and Compositae, the antipodals frequently show a considerable increase in size or number (Maheshwari, 1950). This event is sometimes accompanied by an increase in ploidy in these cells and is

indicative of their high metabolic activity. The increase in protein staining observed in Vanda suggests the possibility of an increase in the number of these cells. Feulgen staining of the embryo sac does not indicate, however, any increase in DNA in this region. In cases of an increase in cell number, the cells maintain their individuality. In Vanda, on the other hand, the region giving the intense protein reaction does not appear to be composed of individual cells. One may speculate that the antipodal cells in Vanda do increase in number, but that the cellular membranes break down liberating their cellular content into the cytoplasm of the embryo sac, thus making added protein available to the growing embryo. The possibility also exists that this region is nothing more than the cytoplasm of the embryo sac which has been pushed up against the chalazal limits by the growing embryo resulting in an increase in total protein concentration due to a reduction in volume.

The Embryo

The first division of the zygote results in a two-cell proembryo. At this early stage the morphological difference between the two cells is slight. As development progresses, however, the micropylar cell gives rise to the suspensor apparatus and the chalazal cell to the embryo. These two portions of the embryo undergo very different courses of cytodifferentiation. The cells of the suspensor apparatus enlarge considerably and become highly vacuolated while those

of the embryo proper remain small, non-vacuolated, and typically meristematic. The cells of the suspensor begin to disappear soon after germination of the embryo.

If one examines the two-cell proembryo for its distribution of total protein and RNA, it becomes evident that the two cells differ chemically from each other, if not structurally, even at this early stage. The embryo initial shows a markedly higher concentration of total protein in its nucleus and cytoplasm and a "shell" of RNA appears around the nuclear membrane. While this accumulation of basophilic material is also present in the suspensor initial, it soon disappears. Thus, in the cell forming the dividing tissue, RNA and total protein is high in both nucleus and cytoplasm, while in the cell destined to form cells enlarging primarily as a result of water uptake, these constituents are low.

Such a condition is what one would expect to find if, indeed, protein synthesis is under nuclear control via RNA, and correlates well with the findings of Jensen (1958) in the root tip. Differentials in RNA and protein activity in early embryo development are well known in amphibian embryos where it has been shown that the incorporation of RNA and protein precursors occurs primarily in the nucleus during segmentation but increases in the cytoplasm following gastrulation resulting in the formation of dorso-ventral gradients of activity (Fico, 1954; Sirlin, 1955; Brachet and Ledoux, 1955). In the sea urchin egg, RNA synthesis is detectable in the blastula stage (Hultin, 1950) but increases markedly during gastrulation (Elson, Gustafson, and Chargaff, 1954).

The fact that the content of RNA in an initial determines the subsequent growth of a cell was illustrated recently by Woodstock and Skoog (1962) in Zea. Nuclear modification during early differentiation in specialized cells, such as in secretory cells of the pancreas, has been amply demonstrated in animals (Zalokar, 1961). All of the above provide further evidence for the direct relationship existing between RNA, protein synthesis, and morphogenesis.

What, then, is the controlling mechanism which enables one cell in a two-cell system to synthesize and elaborate RNA into the cytoplasm and prevents a morphologically and, indeed, genetically identical cell from doing the same? Does this control reside in the genetic material of these cells, or is it mediated by virtue of the environment of the cell?

The only readily detectable differences between the suspensor initial and embryo, aside from their protein and RNA content, is the position that they occupy in the embryo sac. The suspensor initial lies in intimate contact with the synergids while the embryo initial protrudes into the cytoplasm or possibly into the vacuole of the embryo sac. If one considers the hypothesis proposed by Markert (1958) that the activation of a gene or gene complex is dependent on the condition of the cytoplasm, it follows that possibly RNA synthesis in the cells of the embryo occurs only in the absence of certain substrates in the cytoplasm. In the suspensor initial, such substrates might be furnished by the

synergids, whereas in the case of the embryo initial, these substrates may be lacking due to the slow rate of movements of macromolecules through the cytoplasm of the suspensor initial. Thus certain gene groups may be activated in this cell resulting in the synthesis of specific proteins. In view of the increased thickness of the cell wall of the suspensor apparatus, which is apparent when it consists of at least two cells, one might further speculate that transfer of macromolecules between the embryo and suspensor becomes increasingly difficult, ultimately resulting in the necrosis of the suspensor following the demise of the synergids. Such a proposal is, of course, purely speculative but is in agreement with the observations and with current hypotheses of the mechanisms involved in cellular differentiation.

The Protocorm

The Protocorm Stage is characterized morphologically by the establishment of two intergrading regions of cell types; the meristematic and parenchymatous regions. During the Embryo Stage, all of the cells of the embryo proper are similar morphologically and in protein, RNA, and insoluble polysaccharide distribution. Shortly after emergence of the embryo from the seed, however, the cells proximal to the suspensor apparatus begin to enlarge and become distinctly vacuolated, while those distal to the suspensor remain meristematic. Growth of the protocorm results from the continued enlargement of the subapical meristematic cells.

During this enlargement, the protein concentration in the parenchymatous cells appears to remain nearly constant. Thus while these cells are enlarging ultimately to roughly four times their original diameter, the relative concentration of total proteins remains very nearly the same as in the initials from which they derived. This observation indicates that while the major cause of enlargement in these cells, as well as in the premitotic megaspore, is due primarily to the vacuolation of the cytoplasm resulting from increased water uptake, protein synthesis is occurring at a rate sufficient to maintain the concentration of total protein roughly equal to that of the actively dividing meristematic cells.

These findings are partially in conflict with the generalization pertaining to plant cell growth voiced recently by Ray (1961) in which he states: "Unlike typical growth in animals or microorganisms, plant cell growth does not consist primarily of protein synthesis. While cell enlargement in the intact plant is normally accompanied by vigorous synthesis of proteins, this synthesis commonly is far from sufficient to keep pace with the volume increase of the cell." Jensen (1955), in his morphological and biochemical analysis of root tip growth in Vicia faba, also has shown that while during the stage of radial enlargement protein synthesis does occur, elongation of these cells is marked by a cessation of protein synthesis accompanied by an increase in water uptake. Avery and Engel (1954), on the

other hand, found that in the Avena coleoptile total nitrogen continues to increase when section growth is dependent solely upon enlargement of the existing cells. These authors found that this increase is roughly proportional to the increase in cell volume. They conclude that: "Cell enlargement thus is not solely a matter of water uptake, but is accompanied by the synthesis of additional protoplasm." This condition certainly appears to be the case in the protocorm of Vanda.

Further evidence for the occurrence of protein synthesis in the enlarging parenchymatous cells of the protocorm is provided by the fact that cytoplasmic RNA is evident and the relative concentration of this compound, like protein, is roughly equal in the two regions of the protocorm. The cytoplasmic RNA disappears from the parenchymatous region after the completion of cytodifferentiation of the component cells. This latter observation is in accord with the findings of Brachet (1950) on early developing amphibian embryos in which he reports that RNA increases in every organ just before differentiation but decreases after differentiation is complete unless the organ in question is active in protein synthesis.

Observation of longitudinal protocorm sections reveals the presence of groups of cells in the parenchymatous region in which the concentration of cytoplasmic RNA is considerably higher than in the surrounding cells. These groups of cells appear to occur at random in the parenchymatous region but are usually located toward the center of the parenchymatous

mass and usually encompass those cells which form the vascular strands. Similar results have been obtained in embryos of Capsella bursa-pastoris by Pritchard (personal communication). The formation of vascular tissue from parenchyma involves cell elongation. Thus it appears that elongation of these cells requires protein synthesis at least during the initial stages of differentiation. The possibility exists, however, that the observed increase in concentration of cytoplasmic RNA results from a reduction in cytoplasmic volume due to plasmolysis induced by fixation. However, in view of the fact that cells surrounding the region of high cytoplasmic RNA concentration exhibit similar degrees of vacuolation without noticeable change in RNA distribution, the likelihood of fixation artifact seems remote.

The Histones

Longitudinal sections of protocorms show that during early phases of protocorm development the relative concentration of histones in the nuclei of the two regions is approximately equal. In later phases, however, the relative concentration of histones in the large nuclei of the parenchymatous region is seen to increase. This increase in histone staining occurs immediately prior to the initiation of visible signs of the necrosis of this region. After the increase in histone concentration, cytoplasmic RNA and total protein decrease until the withering of the entire region is complete.

Stedman and Stedman (1950) first proposed that histones might act as regulators of genetic activity. Huang and Bonner (1962) presented evidence that DNA bound to histones is inactive in the support of DNA-dependent RNA synthesis and these authors speculated that histones might act as gene regulators. Allfrey et al. (1963) have further shown that histones, protamines, and polylysine inhibit the rate of incorporation of C^{14}-adenosine into RNA and of C^{14}-labelled amino acids into protein in isolated thymus nuclei. These authors conclude that histones inhibit a large number of nuclear biosynthetic activities, probably by interference with nuclear ATP synthesis. Thus the mechanism of repression by histones, if it indeed exists, is probably quite complex. As pointed out by Barr and Butler (1963), any model for the mode of action of gene repression by histones would have to include a mechanism which would insure the "presence and removal of particular histone molecules at appropriate times."

The possibility exists that the increased histone staining observed in the nuclei of the protocorm is due to the depolymerization of the nucleoproteins during pycnotic nuclear degeneration as reported by Leuchtenberger (1950). Three basic changes in nuclear morphology may be observed during the death of a cell: 1. pycnosis, which involves a decrease in the size of the nucleus and a concentration of the chromatin; 2. karyorrhexis, in which fragmentation of the chromatin occurs; and 3. karyolysis, in which chromatin

basophilia decreases markedly. Examination of the cells of the parenchymatous region during the developmental phase of the protocorm in which an increase in histones is apparent does not reveal any of the above listed symptoms of a dying cell; in fact, the nuclei are large and exhibit a high degree of basophilia with the Feulgen stain. Thus it appears that the increase in nuclear histones observed in this region accompanies an increase in the amount of DNA in these nuclei, probably as a result of polyploidy.

SUMMARY

The purpose of this investigation was to examine and correlate the changes in relative concentration and distribution of total proteins, ribonucleic acid, insoluble polysaccharides, histones, and sulfhydryl and tyrosine-containing proteins throughout the development of the embryo sac and embryo of Vanda. In this investigation, emphasis was placed on the elucidation of the relationship between the biochemical composition of a structure and its form. Below are listed the major results and interpretations derived from this study.

Megaspore Stage

1. The inner and anticlinal primary walls of the integumentary cells surrounding the megaspore and the walls of the megaspore proper are thicker and richer in insoluble polysaccharides than those of the other megasporangial cells. The loss of plasmadesmatic connection between the megaspore mother cell and the surrounding nucellar epidermis in Dendrobium, and the unusually thick primary wall observed, suggest that this condition might be peculiar to cells embarked on a course of development quite unlike those of its immediate neighbors.

2. There is a concentration gradient of total proteins in the megasporangium, higher at the distal than proximal end. It appears that this is a remnant of the condition of active protein absorption through rhopheocytosis previously described in Dendrobium.

Gametophyte Stage

1. Only the synergid cells have a high concentration of insoluble polysaccharides. They also exhibit a high concentration of total protein. Both of these classes of compounds are ubiquitously distributed in the cells. It was proposed that the synergids serve a nutritional role in support of the egg, since no endosperm is present and the egg is low in these compounds.

2. RNA concentration is low in all parts of the embryo sac. The highest concentration appears in the polar nuclei. After fertilization, the concentration of RNA in the endosperm nucleus becomes progressively lower until it is no longer detectable.

3. During the initiation of megaspore mitosis, the cytoplasm becomes confined to a central strip bordered by vacuoles, the concentration of total protein in the cytoplasm rising markedly to a peak and then decreasing again during cell enlargement. The process of embryo sac enlargement appears to consist primarily of an increased water uptake by the megaspore during mitotic division, protein synthesis being minimal.

4. Around the time of fertilization, the chalazal region of the embryo sac shows a high concentration of total protein, which diminishes after the enlargement of the embryo. This localized increase in total protein is postulated to be due to an increase in the number of antipodal cells followed by their breakdown. This process liberates protein into the embryo sac which may then be taken up by the embryo.

Embryo Stage

1. There is no difference in insoluble polysaccharides between the suspensor initial and embryo initial in the two-cell stage. When the suspensor apparatus consists of two or more cells, however, the cell walls of the suspensor become thicker than those of the embryo.

2. There is a difference in total protein and RNA concentration between the suspensor initial and embryo initial at the two-cell stage. The embryo proper exhibits a higher concentration of both these classes of compounds and this difference persists until the demise of the suspensor apparatus.

Protocorm Stage

1. The Protocorm Stage is characterized by the presence of starch grains in the parenchymatous region, which diminish in size as development progresses. It was proposed that the parenchymatous region serves as a storage tissue for the active meristematic portion in lieu of an endosperm tissue.

2. The differentiation of a meristematic cell into a parenchymatous cell involves an increase in over-all size. This increase is due partially to vacuolation. Thus this growth involves water uptake accompanied by protein synthesis at a rate sufficient to maintain a constant protein concentration.

3. RNA distribution in the protocorm parallels that of protein. However, in the parenchymatous region, groups of cells associated with the formation of vascular tissue exhibit inordinately high concentrations of cytoplasmic RNA.

4. Differential extraction of longitudinal sections of the protocorm for cell wall constituents reveal that: A. Methyl esterification of the pectins is low in all cells. B. The walls of the meristematic cells contain proportionately higher amounts of pectic material than the parenchymatous cells. C. The major non-pectic components of the cell wall of all cells in the protocorm are the hemicelluloses.

5. Concentration of histones in the nuclei of the meristematic and parenchymatous cells appears approximately equal in the early phases of the Protocorm Stage. Immediately prior to the morphologically detectable initiation of necrosis of the parenchymatous region, the concentration of histones in the nuclei of these cells increases markedly. The question of the role of histones as gene repressors in connection with this phenomenon was discussed.

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BIOGRAPHICAL SKETCH

Marvin Ray Alvarez was born April 3, 1936, at Tampa, Florida. In June, 1954, he was graduated with honors from Thomas Jefferson High School. He received the degree of Bachelor of Science with a major in biology and a minor in chemistry from the University of Tampa in June, 1958. From 1958 to 1961, Mr. Alvarez taught biology and chemistry at Hillsborough High School in Tampa. In 1960, he was awarded a National Science Foundation summer institute fellowship to pursue graduate study at the University of Florida.

In September, 1961, he enrolled at the University of Florida as a graduate student. He received the degree of Master of Science and has since been pursuing his work toward the degree of Doctor of Philosophy. During this time, Mr. Alvarez has held a Graduate Teaching Assistantship, a Research Assistantship, and was awarded a Graduate School Fellowship.

Marvin Ray Alvarez is married to the former Delma Barbara Flores and is the father of two children. He is a member of the Phi Sigma Biological Society and the Botanical Society of America.

This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

June
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