DEVELOPMENTAL FLORAL AND POLLEN MORPHOLOGY

OF HIBISCUS TRIONUM L.

DISSERTATION

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By

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INTRODUCTION

The study of pollen grains, because of their size, is almost as old as microscopy itself. At first, merely as objects of beauty, they became the subject of intense study as an understanding of their essential role in the life history of the flowering plant developed.

Pollen grains possess good features for study: they are one-celled, at least at first; they are haploid; and the growth of their walls is frequently varied in structure and great in extent. Prominent among the earliest forms to be studied in detail, at first by necessity, were the large and highly elaborately walled pollen grains of the Malvaceae.

The present investigation records the origin and development of the pollen grain of *Hibiscus trionum* L. From the early growth of the floral apex it traces in particular the development of the anther and the origin of the microspore mother cells. It is offered here to provide a broad setting from which to examine the development of the pollen grains. More questions have arisen than have been answered, but perhaps this is as it should be.

*Hibiscus trionum* was chosen for several reasons: it is relatively easy to culture the year round; it flowers profusely; the flowers have many stamens; and above all, its pollen grains are large and beautifully sculptured.
MATERIALS AND METHODS

Materials for these studies were obtained from two sources: naturally occurring populations in fields and waste lots, and plants cultured from seed. Initially, the former source was used, but quite early in the study greenhouse cultured plants from seed were employed to ensure a continuous and relatively uniform source of material throughout the year. Seed obtained from the Pearce Seed Company of Moorestown, New Jersey, was found more uniform and reliable than field collected seed. Supplemental light was used to increase the photoperiod and light intensity during the fall and winter months.

Stem apices and flower buds were treated in a variety of fixatives. A Navashin fixative (1 part of a solution containing 0.5 gm. chromic acid, 5 ml. acetic acid and 95 ml. water to 1 part of a solution containing 5 ml. formalin and 95 ml. water), and F.A.A. (5 ml. formalin, 5 ml. acetic acid and 90 ml. 50% alcohol) were found the most suitable. To ensure rapid and complete fixation, material, after being placed in the fixative, was subjected to partial vacuum for from several hours to overnight. To reduce surface tension and to promote penetration of the Navashin fixative, several drops of Kodak Photo-Flo solution were added to each 50 ml. of fixative. Dehydration, clearing, and infiltration were by the usual ethyl alcohol, xylene, and paraffin series. Serial sections were cut 4-15μ in thickness, most commonly 6-8μ.
Two staining combinations and procedures were used: a quadruple stain consisting of a mixture of 2 parts Sun Yellow (C.I.No. 620), 1 part Biebrich Scarlet (C.I.No. 280), and 1 part Orange II (C.I.No. 151) counterstained in either Niagara Sky Blue (C.I.No. 520) or New Methylene Blue (C.I.No. 927) (Emig 1941) and a triple-stain procedure consisting of Safranin O (C.I.No. 841) counterstained in Fast Green FCF (C.I.No. 670) and differentiated in an Orange G (C.I.No. 27)-clove oil solution (Johnson 1959).

Whole mounts of mature pollen grains were made by the technique as described by Heilman (1949). To ascertain the extent of fixation artifacts, particularly in the cytokinesis phases, studies were made on living material mounted in a 0.05% Neutral Red (C.I.No. 825) aqueous solution.

Photomicrographs were made with the use of a Bausch and Lomb research microscope equipped with apochromatic objectives and compensating oculars. Measurements were made with a calibrated ocular micrometer.
Hibiscus trionum L., a member of the Malvaceae, commonly known as "Flower-of-an-Hour," is a native of Africa which has become naturalized in North America. It is usually considered a weed and infrequently treated as an ornamental. The numerous flowers are 5-9 cm. across. Anthesis begins in the mid-morning hours, lasting a few hours at most (Fig. 2-3). The five-cleft calyx is surrounded by 11-13 linear bracts (Fig. 1). The five light sulphur-yellow petals, blackish at the base, are exceeded by the calyx until 3-4 days before anthesis. The stamen column bears about 50 stamens on most of its outer face (Fig. 4-7). The five united styles are, for most of their length, within the hollow stamen column (Fig. 7). They are topped by capitate stigmas. The fruit is a loculicidal capsule enveloped by the inflated membranous calyx.
Figure 1. Stem with flower buds; hairy calyx and involucre bracts.

Figure 2. Flowering stem at anthesis.
Figure 3. Flower of *Hibiscus trionum* L.
Figures 4-6. Living androecia. The sepals have been removed and the petals may be seen at the base. Androecial hairs exceed anthers. Figures 5 and 6 are side and top views respectively of same androecium.
Figure 7. Longitudinal section of *Hibiscus trionum* flower, 2-3 days before anthesis.
DEVELOPMENTAL MORPHOLOGY

General Floral Development

The flowers of *Hibiscus trionum* develop from axillary buds. Both vegetative and floral apices possess a two-layered tunica. Periclinal divisions were never observed in the outermost layer of the tunica and it appears to retain its integrity throughout floral growth. Development of the floral members occurs in the following order: involucre bracts, sepals, staminal column with stamens, petals, and finally carpels. The same order has been reported for *Gossypium* (Gore 1935, Hayward 1938) and *Malva rotundifolia* (Smith 1931).

An early indication of a floral apex is the appearance of bract primordia, commonly 11-14, at the margin of the meristem (Fig. 8-9). The bract primordia have scarcely elongated or the apex broadened before the initiation of the five sepal primordia occurs (Fig. 10-11). At this time the flat remainder of the floral meristem is pentagonal in transection (Fig. 12). As both the bracts and sepals continue to elongate, the floral meristem becomes circular in transection and a central depression develops (Fig. 13). This depression is the result of more rapid cell division and enlargement at the periphery of the meristem. The depression deepens, forming a raised collar-like ring of meristematic cells which give rise to both stamen and petal primordia (Fig. 14-15).
Longitudinal sections of floral apices

Figure 8. Initiation of bract primordia (br.p.).
Figure 9. Later stage of bract primordia growth.
Figure 10. Initiation of sepal primordia (s.p.).
Figure 11. Later stage of sepal primordia growth.
At first the margins of the ring are smooth, but soon rounded groups of more deeply staining cells appear at the top and upper inside margin. These groups are the beginnings of the stamen primordia (Fig. 17). The first two or three layers of cells lining the bottom and part of the way up the inside of the depression also stain more deeply than the surrounding cells (Fig. 16-17).

Simultaneously with or shortly after stamen initiation at the upper end of the meristematic ring, petal primordia develop at the base of the ring. The five petal primordia originate at positions alternate with the previously initiated sepals (Fig. 16). The petal primordia are more pointed than the blunt stamen primordia (Fig. 15, 22). As the upper portion of the ring, which may more properly be called a stamen column, elongates, additional stamen primordia form down the outer face of the column (Fig. 20, 22, 24). The column in transection through its middle appears circular in outline, with 10 rows of stamen primordia on its outer surface (Fig. 17, 21). A transection just below the floor of the depression reveals the five petal primordia and the central more deeply staining group of cells mentioned earlier (Fig. 16, 19).

The rows of stamen primordia are in pairs, a pair alternating with each of the five sepals (Fig. 16, 21). There are usually five or six primordia in a row. Consequently a stamen column will have from 50 to 60 stamen primordia. This agrees closely with stamen counts made of nearly mature flower buds; the number of stamens ranged from 48 to 60 per bud, the average 55. Multiplication of the number of stamens by division one or more times of already formed
Figure 12. Transection of floral apex at a stage similar to that shown in Fig. 11. Floral meristem pentagonal. Bracts (br.), sepals (s.).
Longitudinal sections of floral apex.

Figure 13. Beginning of meristematic ring (m.r.) with central depression.

Figure 14. Early stamen column (st.c.).

Figure 15. Early stamen column with stamen primordia (st.p.), and petal primordia (pp.). Figures 14-15 are of sections 72 μμ apart.
Figure 16. Transection through base of stamen column.
Petal primordia (pp.).

Figure 17. Transection 40μ above that shown in Fig. 16.
Stamen primordia in 10 rows, paired.

Figure 18. Transection 20μ above that shown in Fig. 17.

Figure 19. Transection near base of stamen column.
About same stage as that shown in Fig. 20.
primordia resulting in a large number of stamens (up to 125 per flower) has been reported in *Gossypium* spp. (Gore 1935). No indication of such multiplication was observed in the present study. As the anthers grow they become crowded; consequently their original orientation in rows is lost (Fig. 4-6).

Although bract, sepal, stamen and petal primordia appear in rather rapid succession, carpel initiation does not follow as rapidly. The relatively large size of the stamen column and the stamen primordia at the time carpel initials become evident, indicates a longer interval than that between previous floral member initiations (Fig. 20). The remainder of the floral meristem, which is at the bottom of the hollow stamen column, is flat. The first two or three cell layers have more deeply staining cytoplasm than the surrounding column cells. As the meristem enlarges and increases in diameter, the margin grows faster than the center. The result is a ridge of meristematic cells. Carpel primordia initiate at five places on the ridge (Fig. 20, 22). The outermost tunica layer undergoes only anticlinal divisions. The two layers immediately beneath divide both anticlinally and periclinaly and it is from these two layers that the body of the carpels is derived. Upward growth of the carpels at these points eventually gives rise to the stylar and stigmatic parts of the pistil which project up into the hollow stamen column (Fig. 24-25). Further carpel development was not studied.
Figure 20. Longitudinal section of flower. Initiation of carpels (c) within hollow stamen column.

Figure 21. Transection of flower midway in stamen column. The paired stamen rows alternate with the petals (p).
Figure 22. Longitudinal section of flower.
Figure 23. Transection of flower midway through stamen column. X's mark position of petals at a lower level.

Figure 24. Longitudinal section of a flower of about the same developmental stage as that shown in Fig. 23.
Figure 25. Longitudinal section of flower. Bracts, sepals, and lower portion of pistil removed.
Stamen Column and Anther Development

As mentioned earlier, stamens initiate quite soon after the meristematic ring forms. This ring eventually becomes the stamen column. The first indication of stamen initiation is the appearance of zones of more deeply staining cells along the upper margins of the ring followed by enlargement and limited cell division in these zones just below the outermost layer of cells. As a result, the meristematic ring has, in transection, an undulating margin (Fig. 17). These mounds of cells constitute the stamen primordia. With continued growth the stamen primordium changes to a more nearly bilateral shape. In transection the anther is blunt club-shaped (Fig. 23) and in longitudinal view slightly convex (Fig. 24).

The epidermal layer of an anther is a derivative of the first tunica layer of the floral meristem. During anther growth the epidermis remains distinct and undergoes only anticlinal divisions. The hypodermal layer is a derivative of the second tunica layer of the floral meristem. As an anther enlarges, hypodermal cells continue to divide anticlinally while the cells beneath divide both periclinaly and anticlinally. Soon the anther becomes broader and slightly two-lobed in transection (Fig. 26) and laterally more convex (Fig. 26).

Origin of Archesporium

A single row of hypodermal cells along the length of each anther lobe enlarges (Fig. 26). Each row constitutes an archesporium. Each archesporial cell divides periclinaly. The inner derivatives of this division—the primary sporogenous cells—function
Figure 26. Longitudinal section (somewhat oblique) of young stamen. Archesporial cell (a).

Figure 27. Transection of young stamen in similar stage of development as that shown in Fig. 26.
directly as microspore mother cells without further division (Fig. 28, 30). The direct functioning of sporogenous cells has, according to Coulter and Chamberlain (1903), been "long known" in Malva, Datura and Chrysanthemum. Among the Malvaceae it has been reported in Gossypium hybrids (Cannon 1903), Althaea rosea (Sachs 1875), Malva sylvestris, M. neglecta (Wóycicki 1917 in Stenar 1925), Lavatera trimestris, Malva alcea, M. moschata, M. oxyloba, Sidalcea candida, Anoda cristata and Gossypium herbaceum (Stenar 1925). The present author also has found this to be the case in Althaea rosea and in Hibiscus syriacus. In contrast, Wóycicki (1917) has reported that in Althaea officinalis, there are always three to four rows of microspore mother cells and Lantis (1912) states that in Abutilon Theophrasti, each primary sporogenous cell gives rise, as a rule to four mother cells. Microspore mother cells lying in many rows have been reported in Malvastrum capense and Sida napaea (Stenar 1925).

Origin of Tapetum

The sporogenous or microspore mother cells visibly differentiate from the surrounding cells almost immediately, enlarging rapidly, and by the time the outer derivatives have divided, the sporogenous cells are several times as large as any other cell in the anther (Fig. 28-29). The outer derivatives of the archesporial division first divide either anticlinally or periclinaly (Fig. 28). The result of the first periclinal division is a primary tapetal cell to the inside and a primary parietal cell to the outside (Fig. 29). The cells immediately adjacent and to the inside of the mother cells take on the same appearance as the derivatives of the
primary tapetal cells. Whatever their origin, tapetal cells divide
only anticlinally and the tapetum remains uniseriate. A tapetal
cell early in its existence enlarges, its nucleus becomes larger
and its cytoplasm becomes more dense and deeply staining (Fig. 29).
Within the tapetal cells, shortly before or at the onset of micro-
spore mother cell meiosis, mitotic divisions begin without ac-
companying cytokinesis (Fig. 31). The tapetal cells usually remain
two-nucleate.

Multinucleate tapetal cells have been noted in other Mal-
vacean taxa: *Malva palmata*, *Malvastrum ospense*, *Sida napae*,
*Pavonia spinifex* (Stenar 1925), *Abutilon Theophrasti* (Lantis 1912),
*Althaea rosea* (Cooper 1933), *Gossypium barbadense* (Denham 1924),
*Lavateria trimestris* (Juel 1915), *Malva crispa* (Strasburger 1882).
The present investigator has observed bi-nucleate tapetal cells in
*Anoda sp.*, *Hibiscus manihot*, *H. radiatus*, and *H. syriacus*. It is
not proposed here to consider in detail number and kind of nuclear
divisions in tapetal cells. Excellent reviews of this subject have
been made by Carniel (1952) and Wunderlick (1954).
Figure 28. Transection of stamen. Early differentiation of anther tissues. Epidermis (e), outer hypodermal derivative (ohd), primary sporo-genous cell (psc).

Figure 29. Transection of stamen after division of outer hypodermal derivative into primary parietal cell (ppc) and primary tapetal cell (ptc).
Anther Wall Layers

While the tapetum is enlarging, the primary parietal cells divide both anticlinally and periclinaly. Three wall layers result: the outermost constituting the endothecium, the inner two the middle layers. The middle layers, internal to inner tapetal cells, originate from cells adjacent to the archesporium (Fig. 30-31).

Anther Dehiscence

The anthers of Hibiscus trionum are bilocular. As an anther approaches maturity, the tapetum disappears and the middle layers have become stretched and compressed. The endothecium has enlarged several-fold and U-shaped secondary wall thickenings have developed (Fig. 62). The epidermis is still intact. Prior to dehiscence the partition between the two microsporangia breaks down. Anthers dehisce by splitting along a line at the top between the two lobes, (Fig. 32). When the split is complete, the halves reflex, completely exposing the pollen grains (Fig. 33).

A mature anther contains anywhere from 100 to 150 pollen grains with an average of about 140 grains. Assuming 55 anthers in a flower, nearly 8,000 pollen grains would be produced in each flower.

Androecial Hairs

Quite early in staminal column development, hairs arise on the outer face of the column, particularly near the top. A hair originates from an individual epidermal cell (Fig. 25, 27) that enlarges considerably, becoming a long, nearly cylindrical cell
occasionally branched at the end (Fig. 4-6). The base becomes multicellular. During the first half of stamen growth, the hairs match or exceed stamen length (Fig. 4-6), later at anthesis they are shorter than the stamens, and the white hairs are found among the dark maroon filaments and stamen column (Fig. 3, 7).
Figure 30. Longitudinal section of stamen through a microsporangium showing a single row of primary sporogenous cells or microspore mother cells (mmc) surrounded by the tapetum (t).
Figure 31. Transection of anther through the two microsporangia. Some tapetal cell are already bimucleate. Division in cell marked "d." Endothecium (en), middle layers (mid).
Figure 32. Mature anthers just before dehiscence.

Figure 33. Anthers at dehiscence.
Meiosis of Microspore Mother Cells

The karyokinesis of microspore mother cells has been studied in a number of Malvacean taxa, among them *Gossypium* (Balls 1912, Beal 1928, Cannon 1903, Dehnham 1924), *Lavateria* (Byxbee 1900), *Malva* (Latter 1932) and *Thespesia* (Youngman 1927). No attempt was made in the present investigation to study karyokinesis or to make chromosome counts.

A phenomenon apparently characteristic of the Malvaceae is the appearance of so-called "perinuclear zones" during meiotic divisions. Investigators who report this, illustrate it as a densely granular zone around the spindle apparatus (Byxbee 1900 and Dehnham 1924). In *Gossypium* (Denham 1924) a zone is well developed by early prophase I. However, in *Hibiscus trionum* a perinuclear zone is not developed until late metaphase I or early anaphase I (Fig. 34). During meiosis I, a prominent spindle is evident, the ends of which terminate in this zone (Fig. 34). The area immediately around the spindle is nearly free of cytoplasm. However, by late telophase I the somewhat flattened daughter nuclei are closely surrounded by dense cytoplasm (Fig. 35). Cytoplasmic fibrils finer and more numerous than spindle fibers connect the two nuclei. Granules appear along the length of the fibrils.

The second meiotic division is so similar in appearance to the first that the only sure way to differentiate between them is to count the number of spindles within a cell (Fig. 36). There is no trace of the cytoplasmic fibrils observed during meiosis I. The perinuclear zones are not evident after telophase I, but by
metaphase II they are well developed around each spindle (Fig. 36). The apparent interkinetic disappearance of the zones in *Malva* is attributed by Latter (1932) to light staining. She reported that perinuclear zones were clearly evident in more heavily stained preparations.

The spindles may lie at almost any angle to each other; parallel and right angle positions are not uncommon (Fig. 36). The spindles and perinuclear zones disappear soon after the four nuclei are formed. The cytoplasm becomes denser in the regions immediately around the nuclei and cytoplasmic striations appear between the nuclei (Fig. 38). If the four nuclei lie in the same plane, these striations can be seen connecting them. Within a microsporangium, division of the microspore mother cells is synchronous. Division of the microspore mother cells in the two microsporangia of an anther is synchronous or nearly so (Fig. 37).
Figure 34. Microspore mother cell in early anaphase I. Perinuclear zone well developed.

Figure 35. Microspore mother cell in late telophase I.
Figure 36. Microspore mother cell in metaphase II. Each spindle apparatus is surrounded by a perinuclear zone.
Figure 37. Transection of anther showing nearly synchronous meiotic divisions in adjacent microsporangia.

Figure 38. Microspore mother cell after meiotic divisions and before cytokinesis. Three of the four nuclei are shown.
Cytokinesis of Microspore Mother Cells

General Discussion

The cytokinesis of a microspore mother cell may be one of two types: successive or simultaneous. In successive division, a cell plate is formed immediately after meiosis I, and a cell plate in each of the daughter cells after meiosis II. Successive division is common among monocotyledons, Zea mays (Reeves, 1928) being an example. In contrast, in simultaneous division, no separation of the cytoplasm occurs until both meiotic divisions have been completed, at which time the cytoplasm is simultaneously partitioned. Cytokinesis during simultaneous division may occur in either of two general ways: by the formation of plates or by furrowing.

In 1875, Strasburger demonstrated cell plate formation as a part of cytokinesis. Since that time it has become generally accepted that this is also the manner in which all cells of higher plants undergo cytokinesis. However, Farr (1916) in his detailed study of microspore mother cells of Nicotiana, and of five other dicotyledonous genera, reported that cytokinesis was accomplished by simultaneous furrowing without the appearance of cell plates. Since 1916 other examples of furrowing have been reported. It has been noted in Magnolia (Farr 1918) that after meiosis I a furrow appears whose growth is arrested until after meiosis II is completed, when additional furrows originate and then all furrows further extend and subdivide the cytoplasm into four parts. The appearance of "transitory" cell plates after meiosis I and II, even though
actual cytokinesis is accomplished by furrowing has been reported in *Nelumbo* (Farr 1922) and *Lathraea* (Gates 1925).

Vacuoles have appeared to play a role in cytokinesis in *Melilotus alba* (Castetter 1925) and *Gentiana* (Wóycicki 1932). After meiosis II, vacuoles appear in the regions between the four nuclei. Furrows originating at the surface and extending inward soon meet the vacuoles and division of the cell is eventually accomplished when the furrows meet at the center. Mühldorf (1939, 1941) questions the existence of these vacuoles, believing they are fixation artifacts.

A relatively large number of Malvacean taxa have been studied and all have been reported to undergo simultaneous cytokinesis. However, the exact manner in which this is accomplished has been variously reported even within the same species. As early as 1850, Wimmel considered cytokinesis in *Althaea rosea* to be by some type of plate formation. Von Mohl (1853) in this same species figures constrictions both with and without plate formation. Pringsheim (1854) reported that in *A. rosea* there are formed weakly developed walls separating the four nuclei, but the walls, before they are completed, are reabsorbed and replaced by ingrowths of the mother cell, which finally divide the cell into four parts. Division by constriction beginning by the thickening of the mother cell wall and the centripetal growth of ridges which finally meet in the center was noted by Hofmeister (1867) in *A. rosea*. Sachs (1875) likewise mentions division by constriction, but in one of his figures lamellae are evident between the microspores.
Other Malvaceous plants in which simultaneous division has been indicated are *Abutilon Theophrasti* (Lantis 1912), *Lavateria* sp. (see Byxbee's (1900) figures), *Gossypium* sp. (Cannon 1903), *Malva neglecta* (Wóycicki 1917, see Stenar 1925), and *M. sylvestris* (Latter 1932). Stenar (1925) notes simultaneous division in *Malva palmata*, *Malvastrum capense*, *Pavonia spinifex*, and *Sidalcea candida* but gives no details.

The more recent studies of Mühldorf (1939, 1941) present a somewhat different view of cytokinesis in microspore mother cells. Of the utmost importance for the correct interpretation of this process is the suitable preparation of the material to be studied. With few exceptions, the observations following Farr's (1916) work, which have been interpreted as division by furrowing, have been made on fixed material. Mühldorf (1941), whose studies have encompassed nearly 300 widely distributed angiospermous species, has relied almost entirely on preparations of living material stained with Neutral Red. He distinguishes several varying types of cytokinesis. The manner in which simultaneous division occurs can be stated essentially as follows. As soon as the four nuclei take final positions within the microspore mother cell; areas of "control" around the nuclei are separated by the formation of cytoplasmic plates which arise by the intermixing of the cytoplasm at the boundary zones. The microspores are at first delimited only by these plates, then the microspore plasma forms an inner lamella around each microspore by apposition. The inner lamellae change into callose to the outside as the rounding up of the microspores is taking place.
Sometime after formation of the callose layer an inner structure is evident. The cytoplasmic plates change into middle lamellae which are particularly distinct.

Successive and simultaneous cytokinesis, according to Mühldorf's view, are quite similar, differing primarily only in the time cell plates or cytoplasmic plates are formed. In successive division a cell plate forms in connection with spindle material after meiosis I and another one after meiosis II. In simultaneous division cytoplasmic plates arise following meiosis II, independent of karyokinesis and spindle material. A Liriodendron type in which neither type of plate is formed is the closest to furrowing that Mühldorf (1941) has found. Among Malvacean taxa, Mühldorf (1939, 1941) has studied Althaea cannabina L., A. officinalis L., A. rosea (l.) Cav., Hibiscus ternatus Cav., Kitaibelia vitifolia Wild., Lavateria thuringiaca L., and Malva sylvestris L. Cytokinesis in the above taxa is by the manner described in the preceding paragraph.

Cytokinesis in Hibiscus trionum

Cytokinesis in the microspore mother cells of Hibiscus trionum is of the simultaneous type with the formation of cytoplasmic plates. As mentioned earlier, the mother cells lie in a single row the length of the microsporangium (Fig. 30). A row of living microspore mother cells which have been squeezed out of an anther is shown in Figure 39. As the cells increase in size and approach meiosis I, their walls thicken considerably. Although no microchemical tests were made in the present study, the chemical nature of the thickened walls has long ago been identified as a
callose material by Mangin in *Althaea* (1889b) and by subsequent workers in a variety of taxa.

The origin of the callose has been much discussed. Farr (1916, 1922) and Bowers (1931) believe that it is the result of swelling of the secondary lamella of the cell wall. Beer (1906), Castetter (1925), Gates (1925), Youngman (1927), Mühldorf (1939, 1941) view the callose layer as a result of the activity of the microspore mother cell protoplast. The walls have been variously described as gelatinous, jelly-like, or firm. From observations on living cells these walls appear to be firm. Tetrads from which microspores have been expressed, except for breaks in their walls, retain their form, an indication that the wall is not fluid or gelatinous but quite firm.

The wall which surrounds a tetrad of microspores has three layers (Fig. 40). The primary layer (1M) is the chemically changed archesporial wall. Less refractive, but accounting for the thick callose deposit, is a new layer, the secondary layer (2M). To the inside of the 2M is the third layer (3M), about the same thickness as 1M. Surrounding each microspore is a relatively thick microspore chamber wall (MCW) of callose which is bounded on its inner face by an inner lamella (IL₂). Quite distinct and dividing the microspore mother cell into four parts are the middle lamellae (ML) which are derivatives of the cytoplasmic plates. The middle lamellae extend just short of the tertiary wall layer (SM). The terminology used above is that of Mühldorf (1939, 1941).
An examination of fixed and sectioned materials gives a somewhat different picture of cytokinesis, not entirely compatible with observations of living material. In such fixed and processed cells, the primary wall layer early in meiosis I frequently becomes separated from the thickening secondary wall (Fig. 35). In late tetrad stages the presence of the primary layer is doubtful. The sequence of events as represented by Figures 41 to 44, could certainly allow one to interpret cytokinesis by furrowing as described by Farr (1916). Middle lamellae were evident in only a few fixed preparations (Fig. 47). They sometimes persist even after the rest of the tetrad wall has disappeared and the microspores are free (Fig. 53). Beer (1906, 1911), Mangin (1889b) and Strasburger (1882) observed similar granular-appearing lamellae. Their origin is similar to that of the middle lamellae found in *H. triomum*. Microspore chamber walls were not visible in fixed tetrads (Fig. 45).

Tetrad Arrangement

After meiosis II, the four nuclei assume positions as far apart from each other as the confines of the microspore mother cell will permit. Two arrangements of microspores have been observed. If a microspore mother cell is not limited by adjacent cells it will assume a spherical shape. In such a case, the nuclei take positions at the four corners of a tetrahedron (Fig. 38). The eventual result is a tetrahedral tetrad of microspores (Fig. 45, 48). However, if a microspore mother cell is flattened and prevented from assuming a spherical shape (Fig. 39), the four nuclei become positioned at the four corners of a quadrilateral. The result is a quadrilateral
Figure 39. Row of living microspore mother cells expressed from anther and stained with Neutral Red.

Figure 40. Living tetrad of microspores expressed from anther and stained with Neutral Red. See text for details.
Figures 41-44. Sequence of stages of cytokinesis as they appear in fixed and sectioned material.
tetrad of microspores all in the same plane (Fig. 40, 44). What arrangement is attained depends on how closely and for how long the microspore mother cells are packed in the row during nuclear positioning and cytokinesis. In some microsporangia only a few tetrahedral tetrads are found, in others, nearly half of the tetrads may be tetrahedral.

For critical reviews of cytokinesis literature see Farr (1916), Yamaha (1926), Schnarf (1929, 1931), and Mihldorf (1941).
Figure 45. Tetrahedral tetrad of microspores. Three of the four microspores shown. Absence of tetrad wall layers in fixed material. Tapetal cells bimucleate.
Microspore and Pollen Grain Development

Some pollen grains possess walls of unique structure. Therefore, it is not surprising that a number of especially large and elaborately sculptured forms have long been objects of study. The historical review by Wodehouse (1935) is an almost definitive survey of the work on pollen grains up to that time. Recent studies of pollen wall ultrastructure have been reviewed by Steffen (1955), Afzelius (1956) and Faegri (1956). Within the Malvaceae, extensive wall studies have been made by Strasburger (1882, 1889, 1898) and Wóycicki (1911).

Terminology of Wall Layers

The covering or sporoderm of the mature pollen grain of Hibiscus trionum is composed of a number of layers (Fig. 46). In Erdtman's terminology (Erdtman 1952, Erdtman and Vishnu-Mittre 1958) the sporoderm consists of exine and intine. The exine is further subdivided into a thick inner layer, apparently homogeneous, the nexine, and an outer sculptured layer, the sexine. The sexine consists of a layer of radial rods or bacula (endosexine) overlain by a thin tegillum (ectexine) more or less parallel to the nexine. Large spines appear to have their bases on the nexine. The exine is interrupted at places by germ pores.

Early Exine

Microspores may be said to come into existence as soon as the protoplast of the microspore mother cell has become partitioned by the formation of cytoplasmic plates. Quite early, each microspore
Figure 46. Transection of sporoderm of nearly mature pollen grain. Bacula (bac), cytoplasm (cyt), germ pore (gp), germ pore plug (gpp), intine (i), nexine (n), tapetal periplasmodium (per), sexine (sex), spine (sp), and tegillum (teg).
becomes surrounded by an inner lamella which in turn is surrounded by a relatively thick callose layer (Fig. 40). By this time a limiting microspore plasma membrane, independent of the lamella, has developed. Soon, and while still within the tetrad wall layers, the membrane becomes thicker (about 0.5μ), appearing as a double line and readily seen when the protoplast has plasmolyzed (Fig. 40). This membrane, the exine, is not visibly stratified. It is apparently of some rigidity, for microspores expressed from within tetrads, maintain their original shape (Fig. 49). Microspores up to 30μ in diameter may be found still within the tetrad walls.

Spine Initiation

While no direct evidence of spines was seen in microspores still within the tetrad walls, their initiation occurs very early in exine growth. Living microspores, 33μ in diameter and free of the tetrad, possess spines of the order of 0.5μ diameter at the base (Fig. 51). Free, fixed microspores of only 25-27μ diameter have an exine thickness of 0.5-0.7μ and spines of at least this length (Fig. 52). Germ pores and plugs are also present. It is apparent that exine, spines, germ pores and plugs initiate early while the microspores are still within the tetrad walls.

Breakdown of Tetrad Wall.

When the tetrad wall begins to disorganize, the microspore exine is still not visibly stratified into sexine or nexine. Tetrad wall breakdown is rapid, for microspores of equal size may or may not be enclosed in the callose layer. Dissolution of the
Figure 47. Tetrad of fixed microspores showing middle lamella (ml).

Figure 48. Living tetrahedral tetrad of microspores stained with Neutral Red. Middle lamella (ml), exine (ex).
tetrad wall progresses from the outer layers inward. The only portion which persists for any length of time is the middle lamella (Fig. 53). It is not possible to say with any assurance whether any of the tetrad wall material is utilized in the rapid increase in exine growth which follows. Beer (1911) from his study of Ipomaea believes that this material is not used in exine growth while Mühldorf (1941) states that the callose substance may be utilized as a "reserve" material in further exine development.

Exine Differentiation

Differentiation of the exine into sexine and nexine is rapid. This is apparent from a comparison of microspores of about equal size. Within the size range of 30-35μ diameter one can find a series ranging from microspores with just the beginning of spines and pores and no exine stratification (Fig. 51) to microspores with relatively large spines, well developed pores and plugs, sexine and nexine (Fig. 54). Microspores of a slightly larger size (35-40μ) possess all the wall layers of a mature pollen grain wall except the intine (Fig. 56), spines about a third of their final length, a tegillum underlain by bacula, and a relatively massive nexine, are clearly evident.

Origin of Wall Material

The increase of nearly five-fold in diameter and over twenty-fold in wall thickness, from microspore to pollen grain is evidence that considerable growth has taken place. The problem as to the origin of the material which accounts for this growth is one
Figure 49. Living microspore expressed from tetrad and stained with Neutral Red.

Figure 50. Living tetrad of microspores still within tetrad wall. Initiation of germ pores and plugs (gp). Stained with Neutral Red.
Figure 51. Living microspore free of tetrad. Left: optical section. Right: surface view. Stained with Neutral Red. Exine (ex), germ pore (gp), and spine (sp).

Figure 52. Fixed microspore free of tetrad. Exine (ex), germ pore (gp), and spine (sp).
Figure 53. Transection of anther. Remnant of middle lamella between microspores. Tapetal breakdown has begun.
Figure 54. Microspore with well-developed germ pore plugs. Exine clearly differentiated into nexine and sexine. Epidermis (e), endothecium (en), middle layers (mid), and tapetal cell (t).
Figure 55. Longitudinal section of anther showing tapetal breakdown.
Figure 56. Microspore from anther section shown in Fig. 55. All sporoderm layers except intine have differentiated. Tapetal material on surface of sexine.
which as yet is not completely solved. Two sources which have been suggested are the microspore protoplast and the tapetum.

There seems little doubt that at least the original thin membrane surrounding the microspore is a product of its protoplast. The protoplast during the early stages is in close contact with the developing wall (Fig. 51). The microspore, up to the time when all wall layers except the intine have differentiated, is filled with moderately densely-staining cytoplasm (Fig. 53). However, midway in microspore development, the cytoplasm becomes highly vacuolated and ceases to fill the cell (Fig. 57). It is difficult to say whether there is an actual loss of cytoplasm or merely a decrease in cytoplasmic synthesis relative to increase in microspore size. In any case, the relative amount of cytoplasm continues to decrease as the wall thickens and the microspore enlarges. Microspores in the late stages of development are almost devoid of cytoplasm, which consists of little more than a shell concentrated mostly near the nucleus. Shortly before or after mitosis of the microspore nucleus occurs; there is a rapid increase in the amount of cytoplasm. Most two-nucleate pollen grains are filled with cytoplasm, some completely packed with dense granular material (Fig. 58), others vacuolated (Fig. 62).

Reports of similar cytoplasmic behavior during microspore growth have been noted for Malva silvestris (Wóycicki, 1911), Malope trifida (Luxenburg 1927), Malva crispa, Althaea rosea (Strasburger 1882, 1898), Gossypium barbadense var. maritima, Watt (Denham 1924), and Ipomoea purpurea Roth. (Beer 1911). In these
cases, the investigators believe that at least in the early stages of wall development, the protoplast is a source of wall material.

Investigations of abortive grains are the best evidence to date that the exine develops independently of the protoplast. Tischler's (1908) work on sterile Mirabilis hybrids indicates that if the microspore cell dies after only the first rudiments of the sculpturing are laid down, the exine continues to develop a form similar to that of normal functioning pollen grains. Wodehouse (1929) has reported a similar case in a Cichorium intybus strain.

**Tapetal Periplasmodium**

The tapetum is of considerable physiological importance since all the material entering the sporogenous cell and later the pollen grains must pass through it. Apparently characteristic of the Malvaceae is a tapetum in which the cell walls break down, but the protoplasts remain intact, protrude and "wander" within the loculus. Frequently the protoplasts may coalesce and form a periplasmodium. Periplasmoidal formation has been reported for the following Malvacean taxa: *Althaea rosea*, *Malva rotundifolia*, *M. crispa* (Strasburger 1882, 1889), *M. sylvestris* (Wójcicki 1911), *Lavateria trisemtris*, *Hibiscus rosa-sinensis* (Juel 1915), *Kitaibelia vitifolia*, *Malva moschatê*, *M. crispa*, *M. neglecta*, *M. oxyloba*, *M. parvifolia*, *M. palmata*, *M. heterophylla*, *M. limensis*, *M. aloea*, *M. waltherifolia*, *Malvastrum capense*, *M. peruvianum*, *Lavateria arborea*, *Pavonia spinifex*, *Sidalcea candida*, *Sida napaea*, and *Hibiscus trinomum* (Stenar 1925).
As early as the meiotic divisions of the microspore mother cells, considerable change within the tapetal cells becomes evident. In fixed tapetal cells the protoplasts have shrunk away from the cell walls (Fig. 37-38), the cytoplasm in some cells is particularly dense and deeply staining (Fig. 37) and in others it is vacuolated (Fig. 45). When the microspore walls begin their rapid thickening, the tapetal walls disappear, although the protoplasts retain their individuality. A typical fixation image is a ring of tapetal protoplasts separated from the sporangium wall and surrounding, but not touching, the microspores (Fig. 53, 55). The cytoplasm to the inside, facing the microspores, appears to break down into fine granules. In some microsporangia this granular material accumulates on the surface of the microspores while (Fig. 56) in others little or none is evident (Fig. 54).

About the time the microspore nucleus divides the tapetal protoplasts lose the last vestiges of their original form and become ameoboid. The tapetal nuclei are still distinct and there is no general coalescence of the protoplasts (Fig. 58). The protoplasts are found throughout the loculus, among the pollen grains. Frequently tapetal material is found over the surface of the grains (Fig. 46, 58). Little if any trace of tapetal material remains by the time the anther dehisces.

While there is no doubt that the tapetum plays an important role in the development of the pollen grains, specifically what this may be is not clear. Rowley (1959) suggests the development of the pollen grain wall might be compared to that of the cutinized
Figure 57. Microspores with cytoplasm becoming depleted. Endothecium (en) enlarging.
Figure 58. Pollen grains with all sporoderm layers, generative nucleus (g.n.) and vegetative nucleus (v.n.) present. Tapetal periplasmodium (per) has formed.
epidermal cell. Taylor (1959) in his autoradiographic studies in Lilium found that synthesis of labeled proteins occurs in tapetal cells prior to each mitosis and suggests it may be necessary for the secretory activity of the tapetum which occurs following meiosis when the thick microspore walls form. Large quantities of labeled material accumulate in microspore walls but not in their protoplasts, along the inner walls of the tapetum, and within the anther loculus. Taylor concludes that the primary function of the tapetum appears to be secretion of microspore wall material. Further isotopic and culture studies will be necessary before an answer will be found.

Germ Pores and Plugs

Germ pore initiation occurs in microspores still within the tetrad wall. This was observed in microspores of about 30μ in diameter in which the exine was little more than a thin membrane (Fig. 50). The material which accumulates at pore sites stains with Neutral Red in living cells. Cannon (1903) describes in Gossypium "colorless accretions" which respond to the test for cellulose on the periphery of very young microspores. The places where the accretions first appear mark the location of the germ pores. In Hibiscus trionum these sites are distributed in a spiral pattern over the surface of the microspore. A portion of the spiral is evident in Figure 50.

In fixed and sectioned microspores of about 25-30μ in diameter, projections of deeply staining homogeneous material occur, extending inward from the pore positions, while the exine is still
relatively thin (Fig. 52). As the exine differentiates and thickens, these projections or germ pore plugs continue to enlarge and project inward beyond the nexine (Fig. 54). As the microspore enlarges, accumulation of plug material continues for a time to match the progress of exine growth in all directions. In time, however, the plugs become embedded within the thickening exine and no longer project inward beyond the nexine (Fig. 56-57). In nearly mature pollen grains the plug material is pushed to the outside end of the pore by the intine (Fig. 46).

Wóycicki (1911) in his study of *Malva sylvestris* illustrates, though poorly, structures similar to the present pore plugs, developing early in microspore growth and prior to spine formation. In *Gossypium*, Denham (1924) describes "extra-nuclear bodies" which he believes are concerned with the formation of germ pores "since they are always found opposite these pores at a later stage." Lens-shaped thickenings beneath the germ pores, common in the Malvaceae, were called "Zwischenkörper" by Fritzsche in 1837 (Wodehouse 1935). Mangin (1889a) concluded that under the pores where the intine is usually greatly thickened, the thickenings consist principally of pectose. Strasburger (1898), from his studies of *Althaea rosea* believed that an intine plug, with its outer part a cutinized cap projects up into the pore.

In *Hibiscus trionum*, *H.* manihot, *H.* radiatus, and *H.* syriacus, germ pore plugs are evident prior to the appearance of the intine. There is no indication that plug material disappears and is replaced entirely by an intine plug. There appears to be no
decrease in the amount of plug material during later stages of microspore development although further accumulations may cease. Instead the original plug material is forced by the developing intine to the outside end of the enlarged pore. It is not unreasonable to assume that the very early accumulation of plug materials, before the exine becomes more than a thin membrane, prevents further exine development at those places. Thus exine and germ pores would develop simultaneously. In mature grains a thin membrane flecked with granules and staining the same as the exine can be seen covering the germ pores, which are 5-6 μ in diameter.

The spiral pattern of the pores which is evident from the very beginning of wall development is maintained in the mature pollen grains (Fig. 59). Similar pore patterns are found in H. moscheutos, H. radiatus, H. rosa-sinensis and H. syriacus. Such a pattern cannot be explained on the bases which Wodehouse (1929, 1935) has so successfully used in accounting for regular polygonal pore and furrow arrangements.

Intine

In H. triornum the intine is the last layer of the sporoderm to develop. It is not clearly evident until late microspore or early pollen grain stages of development. In older pollen grains the intine is thick under and adjacent to pores, but between pores it is a thin layer (Fig. 46, 58). Sitte (1953) in his ultrastructure studies confirms Strasburger's (1898) conclusion that the intine is made up of cellulose, finding that it is frequently combined with small amounts of pectin.
Figure 59. Mature pollen grains showing spiral pattern of germ pores.
Mature Exine Structure

Mature pollen grains at anthesis, not including the spines, measure from 100-120µ in diameter, averaging about 110µ. The spines from the surface of the tegillum to their rounded ends measure 13-15µ. A spine at its widest point may be 5-7µ (Fig. 61). Its base is made up of 5-7 rod-like structures which are about twice the size of the surrounding bacula (Fig. 60). The upper part of a spine appears homogeneous. The base is embedded in the endosexine and may be considered a part of it, for when one is removed (Fig. 60) a cavity is made in the endosexine, but not in the nexine. The bacula are approximately 1.5µ long and 0.3-0.7µ in diameter and are spaced from 1-2µ apart (Fig. 60-61). The nexine, the thickest wall layer, is 4-7µ thick. The pollen grains at anther dehiscence are covered with a golden-yellow oil-like substance which is soluble in xylene but not in 95% alcohol. The chemistry of pollen exines is little known. The great durability of exine, which makes pollen analysis possible, is attributed to a unique substance sporopollenine. Speropollenine has been likened to cutin and to complex high polymer terpenes.

Generative and Vegetative Nuclei

About the time the periplasmodium has formed, the microspore nucleus divides resulting in a generative nucleus and a vegetative nucleus (Fig. 62). Actual division figures of the microspore nucleus were never observed. According to Guignard (1904) the larger of the two is the vegetative nucleus and division of the
Figure 60. Section of nearly mature sporoderm parallel to and at level of bacula layer. Bacula (bac), germ pore (gp), base of spine (b. sp), place where spine has been removed (x).

Figure 61. Transection of sporoderm of nearly mature pollen grain. Bacula (bac), intine (i), nexine (n), spine (sp), tapetal periplasmium (per), tegillum (teg), and cytoplasm (cyt).
generative nucleus does not occur until after pollination. The vegetative nucleus is much the larger, frequently irregular in outline and lighter staining. The generative nucleus is commonly flattened, sometimes lens-shaped.

Figure 62. Transection of pollen grain. Generative nucleus (g.n.), vegetative nucleus (v.n.), tapetal periplasmodium (per), and endothecium (en).
SUMMARY

Flowers of *Hibiscus trionum* L. originate from axillary buds. Development of the floral members occurs in the following order: bracts, sepals, stamen column with stamens, petals, and carpels. A meristematic collar gives rise to the staminal column upon which the stamens form. The carpels initiate at five places from within the meristematic collar. The outermost tunica layer undergoes only anticlinal divisions. The two layers immediately beneath divide both anticlinally and periclinaly and it is from these layers that the body of the carpels is derived.

Anthers are bilocular. The archesporium is a single row of hypodermal cells extending the length of the microsporangium. Each cell divides periclinally and the inner derivative—the primary sporogenous cell—functions directly as the microspore mother cell without further division. The outer derivative divides periclinally resulting in a primary tapetal cell to the inside and a primary parietal cell to the outside. The primary tapetal cells divide only anticlinally; the tapetum remains uniseriate. Tapetal cells become binucleate. The primary parietal cells divide both anticlinally and periclinally. Three wall layers result: the outermost, the endothecium, and two inner middle layers.

As an anther approaches maturity, the endothecium enlarges and U-shaped thickenings develop. The anthers dehisce by splitting.
along the top between the lobes after which the walls reflex completely, exposing the pollen grains.

The two meiotic divisions of the mother cells are similar; perinuclear zones form at each division. Cytokinesis of the mother cells is the simultaneous type; partition of the proplasts is by the formation of cytoplasmic plates. The early microspores within the tetrad are surrounded by thick callose layers. Microspore tetrads are either tetrahedral or tetragonal in arrangement.

The mature pollen sporoderm consists of intine, nexine, bacula, and tegillum. Large spines appear to be based on the nexine. The exine is interrupted at places by germ pores. The exine, spines, germ pores and plugs initiate simultaneously while the microspore is still within the tetrad wall. The early exine is a product of the microspore protoplast. Germ pore plug material prevents exine development at pore sites. At pollen maturity, original plug material occupies the upper end of the enlarged pore, the late-developing intine fills the remainder of the pore. The pores are in a spiral pattern. Differentiation of the exine into sexine and nexine is rapid. Sporoderm development is probably the result of both microspore and tapetal cell activity. The tapetum, midway in spore development, forms a periplasmodium which has disappeared at anthesis. The increase of nearly five fold in diameter and over twenty fold in wall thickness, from microspore to pollen grain, is evidence of considerable growth.
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AUTobiography

I, Alan Smith Heilman, was born in Pittsburgh, Pennsylvania, December 23, 1927. I received my primary and secondary education in the public schools of Dormont, Pennsylvania, and my undergraduate training at the University of Pittsburgh, which granted me the Bachelor of Science degree in 1949. From the University of Pittsburgh, I received the Master of Science degree in 1951. While there, I was in turn, graduate teaching assistant, research assistant, and lecturer in the Department of Biological Sciences. In September, 1952, I entered The Ohio State University and was appointed a research assistant in the Department of Botany and Plant Pathology, a position I held for four years. In September, 1956, I was appointed an assistant instructor for one year. This was followed by three years as technical assistant in the department.