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Effin T. Graham, Major Professor

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Don Williams, Gary McDaniel

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

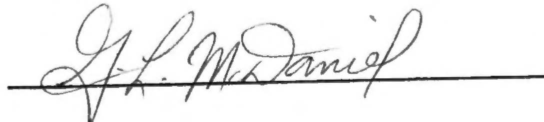
(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a thesis written by Rebecca S. Greene entitled "Histological Distribution of Orange G-Specific Cytoplasmic Globules in *Ipomoea Purpurea*." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Ornamental Horticulture and Landscape Design.

Effin T. Graham
Major Professor

We have read this thesis and recommend its acceptance:



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HISTOLOGICAL DISTRIBUTION OF ORANGE G-SPECIFIC CYTOPLASMIC GLOBULES IN *IPOMOEA PURPUREA*

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Rebecca S. Greene

December 1977

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ACKNOWLEDGMENTS

The author wishes to express her appreciation to the faculty of the Department of Ornamental Horticulture and Landscape Design and especially to each member of her committee: Dr. Effin T. Graham, my committee chairman, for all of his efforts in my behalf and especially for the support and guidance toward the completion of this study and to Dr. Don Williams and Dr. Gary McDaniel for their encouragement of my professional and educational growth.

ABSTRACT

Orange G-specific, spherical bodies, varying widely in size with some being relatively large, i.e., larger than the nuclei, and descending to the limit of resolution of the light microscope, were found in paraffin sections of shoot apices of Ipomoea purpurea Roth (Linn.) 'Heavenly Blue' when stained with safranin O followed by aniline blue-orange G. These globules only appeared when fixed in a noncoagulant, pH neutral fixative such as 5 percent glutaraldehyde in either a calcium acetate or a phosphate buffer. Visualization of the globules was improved in tissue treated with osmium tetroxide following primary fixation in gluteraldehyde and embedded in epoxy plastic. The globules appeared dense black when the osmium-treated tissue sections were stained with toluidine blue. In paraffin sections the globules were found only in dense files of cells of unknown function located in well-differentiated mesophyll and cortex tissues. In plastic they were located in the exterior layer of the pith just interior to the vascular cylinder as well as in the dense files of cells as in paraffin sections. Single giant cells throughout the mesophyll of differentiating leaves, not associated with the orange G colored globules, were also found. The cellular location of the globules was in the vacuoles. Although the globules reacted strongly with

acidic buffered orange G and with osmium tetroxide, they gave negative results to various histochemical tests for protein, lipids, nucleic acids, insoluble carbohydrates and tannin.

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CHAPTER I

INTRODUCTION

Histological investigations of plant tissues have been based largely on acidic coagulant killing-fixing fluids, such as formalin-acetic acid-alcohol (FAA) and chromium trioxide-acetic acid-formalin (CRAF), as presented in classical manuals of botanical microtechnique by Johansen (11) and Sass (16). While this type of fixation will yield a satisfactory view of the cell wall and nucleus it destroys much of the fine structure of the cytoplasm thus preventing close studies of cellular details through the light microscope, as explained by Feder and O'Brien (7) and Baker (3).

The use of the electron microscope has brought about the need for noncoagulant, pH neutral or low alkaline fixatives which retain the ultrastructure of cells, but until now these noncoagulants have been used mainly for electron microscopy. Yet, the electron microscope has some very definite drawbacks. First, the plastic method used for electron microscopy is very tedious to section, and the sample size is very restricted. Furthermore, the electron microscope does not allow for color reproduction or the differentiation which is available in light microscopy through various histological stains.

A new process for preparation of plant tissues for light microscopy has been developed recently at the University of Tennessee, Department of Ornamental Horticulture and Landscape Design (personal conversation with Dr. E. T. Graham). This procedure makes use of a noncoagulant, pH neutral killing-fixing solution which preserves the cytoplasm. It is also based on paraffin embedding which permits rapid ribbon-sectioning of large samples of tissue. In addition to these features of fixation and embedding, a new staining technique also has been added to comprise a new method for preparing histological sections.

This method has revealed numerous cytoplasmic globules showing high affinity for orange G dye, especially in the rapidly differentiating tissues of shoot apices. Several lines of investigation will be required to discover the origin, composition and function of these globules in different plants.

The objectives of this thesis were: (1) to determine the anatomical distribution of the globules in morning glory, Ipomoea purpurea Roth (Linn.) 'Heavenly Blue' (Convolvulaceae); and (2) to evaluate the adequacy of the paraffin method for preserving cytoplasmic detail in comparison with tissue processed in plastic for light microscopy.

CHAPTER II

PREVIOUS RELATED WORK

Various types of cytoplasmic and vacuolar bodies have been described. Shumway and associates (17)(18)(19) have reported vacuolar globules in apical meristem, leaves, and floral tissues of tomato, tobacco, corn and various other plants. They speculate that these cytoplasmic inclusions may play an important role in the developmental metabolism of meristematic tissue. These globules, however, have been seen mostly in electron micrographs and appear to be irregularly shaped, amorphous masses of material.

Akers, Anderson and Blum (1)(2) have reported finding spherical vacuolar bodies with clearly defined boundaries in *Spartina alterniflora*. These bodies were found in the mesophyll and outer bundle sheath cells of leaves and in parenchyma cells in roots, rhizomes, peduncles, and glumes but were not found in meristematic or very young non-differentiated tissues.

Aside from these works by Shumway et al. and Akers et al. cited above, the recent periodical research literature reveals no cellular entities comparable to the orange G-specific globules which are considered in this thesis. There are numerous cytological reports of tannin or phenolic bodies in comparable tissues of shoot apices but these would have no

special affinity for orange G according to established histochemical standards elucidated for botanical subjects by Jensen (10) or LaCour et al. (12).

The globules in question are numerous and vary widely in size with some being relatively huge, i.e., larger than the nuclei, and descending to the limit of resolution of the light microscope. Since the globules can be stained selectively and clearly with orange G dye, however, it seems unlikely that they would be confused with other known cellular components of plant tissues. No comparable cellular inclusions are discussed in recent reference treatises on plant anatomy (5)(6), plant cytology (4)(15), or general cytology (22)(23).

CHAPTER III

MATERIALS AND METHODS

Fixation and Dehydration

Tissue samples were taken from young seedlings, at various stages of development, grown under general greenhouse conditions. The tissues were immersed 12 to 24 hours in a noncoagulant killing-fixing solution made up of 5 percent glutaraldehyde in either a phosphate buffer at pH 6.8 or in 2 percent calcium acetate. The tissues were subjected to vacuum pressure early in the fixation period to eliminate trapped air bubbles and assure uniform fixation, dehydration, and infiltration of the embedding medium.

The phosphate buffer was a combination of dibasic and monobasic salts at 0.05-0.1 M concentration as commonly used for preparing tissues for electron microscopy (10). The substitution of 2 percent calcium acetate for the calcium chloride often combined with formalin solutions has been recommended for medical specimens by Lillie (14). The substitution of glutaraldehyde for formaldehyde with calcium acetate was done for the purpose of this investigation to take advantage of the cross linking property of the dialdehyde. The pH of the calcium acetate-glutaraldehyde solution was 7.0.

The fixed tissues were dehydrated in a closely graded series of ethyl and tertiary butyl alcohol mixtures, ending in 100 percent tertiary butyl alcohol which is a paraffin solvent (Table I). The tissues were left in each concentration for approximately one hour. When the sections entered 100 percent tertiary butyl alcohol they were put through three changes, apprxomately thirty minutes apart, to complete the dehydration process.

Paraffin and Wax Methods

Dehydrated tissues which were intended for paraffin processing were infiltrated with paraffin at 60°C and embedded in molds. If they were to go through a wax method they were infiltrated with diethylene glycol distearate wax, and allowed to solidify in the wax. Once solidified the tissues were trimmed of excess wax and swiftly embedded in paraffin molds, thus forming a double embedment of wax infiltrated tissue surrounded by paraffin. At this point, both wax and paraffin-embedded specimens were ready to be sectioned at 5 microns on a rotary microtome. The ribbon-sections were floated onto pools of distilled, deionized, filtered water on carefully cleaned glass slides and dried on a warming plate at least 24 hours at 50°C.

In preparation for staining, the paraffin on the slides was removed in xylene and the remaining tissue sections were rehydrated in a series of descending alcohols through

TABLE I

SERIES OF ALCOHOLS USED IN DEHYDRATION OF TISSUE SAMPLES AFTER FIXATION

% Total Alcohol	ml H_2O	ml Absolute ETOH*	ml TBA**
10	90	10	0
15	85	15	0
20	80	20	0
30	70	30	0
40	60	40	0
50	50	40	10
60	40	40	20
70	30	40	30
80	20	40	40
90	10	40	50
95	5	35	60
100	0	25	75
100	0	0	100

*ETOH — Ethyl alcohol

**TBA — Tertiary butyl alcohol

50 percent ethyl alcohol to water. The slides were then stained from one to two minutes in aqueous 0.1 percent w/v safranin O, rinsed and stained for 10 to 15 minutes in buffered acidic aniline blue-orange G at pH 3.0-3.5.

After staining, the slides were dehydrated in a mixture of 95 percent tertiary butyl alcohol and 5 percent isopropyl alcohol. Isopropyl was added to prevent the tertiary butyl alcohol from freezing. The tertiary butyl alcohol-isopropyl alcohol treatment avoids extraction of the stains, which would occur if the stained sections were dehydrated in ethyl alcohol. The slides were then placed in xylene to remove the alcohol in preparation for mounting coverglasses with a xylene-solvent resin. Once the slide was cover-glassed the result was a permanent slide for viewing under a light microscope.

A tannin test was also run by adding ferric sulphate to the glutaraldehyde and calcium acetate fixative, described above, as well as the formalin fixative suggested by Jensen (10). These tissue specimens were sectioned and mounted like other paraffin embedments, but since there was no need for additional staining, the paraffin was simply removed by xylene and the sections cover-glassed.

Plastic Method

Tissue samples to be embedded in plastic were fixed 12 to 24 hours in 5 percent glutaraldehyde in 0.067 M

phosphate buffer at pH 6.8. The tissues were rinsed in phosphate buffer, three changes during three hours, to eliminate excess aldehyde which might react with the osmium tetroxide post-fixative. The tissues were subjected to post-fixation in 2 percent osmium tetroxide for two hours followed by a water rinse for one hour and 2 percent uranyl acetate for an additional 30 minutes. The tissue samples were taken directly from the uranyl acetate and placed in the series of dehydration alcohols ending in pure tertiary butyl alcohol.

To infiltrate the specimens after dehydration, mixtures of tertiary butyl alcohol and plastic resin were used in ratios of 1:3, 1:2, 1:1 and finally pure plastic. Two low viscosity epoxy plastics were used: Spurr (21), (Polysciences Inc.) and the other according to a commercial data sheet (Ladd Research Industries). The tissue specimens were embedded in pure resin and cured in gelatin capsules at 70°C for eight hours. When the plastic had hardened, the gelatin capsules were removed by soaking in warm water, and the plastic pellet with the specimen was faced down with a razor blade ready for sectioning on the ultramicrotome.

The resulting sections were from 1mm square to 2 x 3mm square and 3-4 microns thick. The sections were picked up one at a time off the dry glass knife and transferred to toluidine blue O, in a citrate buffer at pH 4, stain

solution contained in 00 polyethylene embedding capsules. These capsules, each containing one section, were kept in numerical order on a rack and put in an oven at 75°C for one and one-half hours. The result being serially stained and relatively wrinkle free sections when removed from the oven.

Each section was then removed from the stain and deposited on a pool of rinse water held in microchemical spot plates. Before being transferred to a slide, the slide had to be perfectly clean, heated to approximately 100°C, and then wiped with silicone-treated lens paper. This treatment allowed water droplets to be "mounded up" and held by surface tension on a water repellent surface, so that when the rinsed tissue sections were placed on the water they would float and remain in perfect order on the slide. The slides with the tissue sections on water droplets were placed in an oven at 80°C, in order for heat to assist in flattening the sections as well as evaporate the water and allow the sections to settle onto the glass.

To cover the slides, a thin coat of immersion oil was used as the medium and the coverglass tacked with clear fingernail polish to prevent it from slipping. These slides were then maintained at room temperature, never heated. This unique method produced permanent slides with serial sections of plastic-embedded tissue specimens for viewing under a light microscope.

Other tissue samples were fixed only in glutaraldehyde with a calcium acetate buffer, and run through the dehydration series. These specimens were infiltrated and embedded in plastic in the same manner as the other plastic embedments. The specimens were also sectioned in the same way as the other embedments but they were floated on water instead of a staining solution, and heated for about two hours at 75°C to remove wrinkles.

The sections were removed from the capsules and adherred to clean slides with water. These slides were dried down and placed in a plastic solvent made up of a saturated solution of sodium hydroxide in absolute ethyl alcohol as described by Lane and Europa (13). After one hour the slides were taken from the plastic solvent, rinsed in absolute ethyl alcohol and hydrated in preparation for staining. Various stains used included: Sudan black B, periodic acid-Schiff, and aniline blue-orange G as described by Jensen (10); aniline blue black as described by Fisher (8); and toluidine blue O as suggested by Sidman et al. (20), but in this case their benzoate buffer was replaced by a citrate buffer.

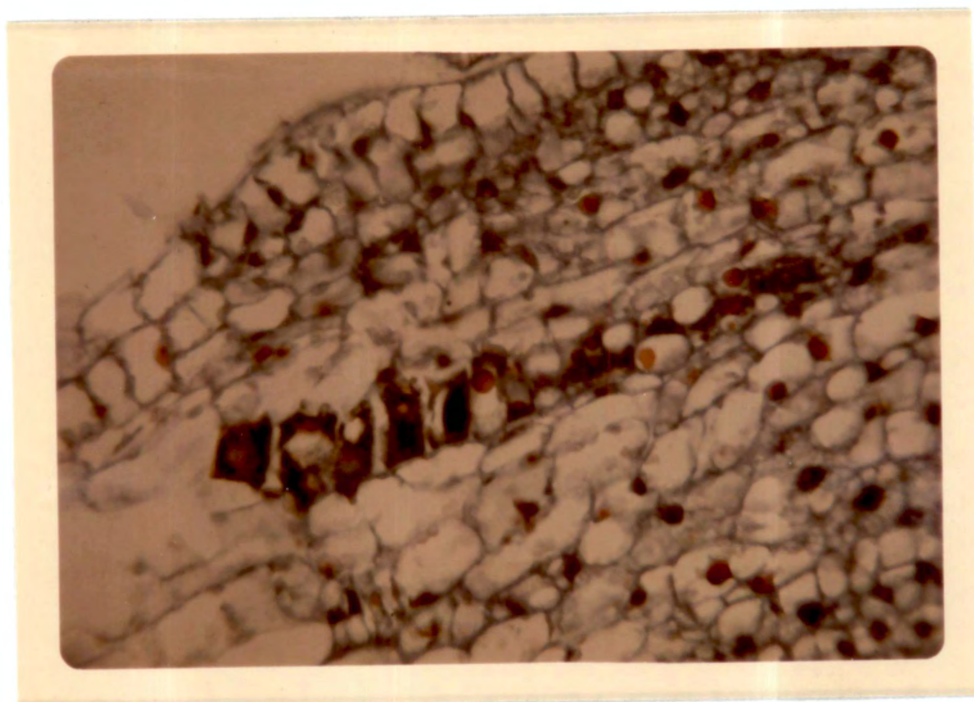
Sections stained with Sudan black B were cover-glassed with glycerin-gelatin, the others were passed through the 95 percent tertiary butyl alcohol and 5 percent isopropyl alcohol mixture followed by xylene and cover-glassed with resin.

CHAPTER IV

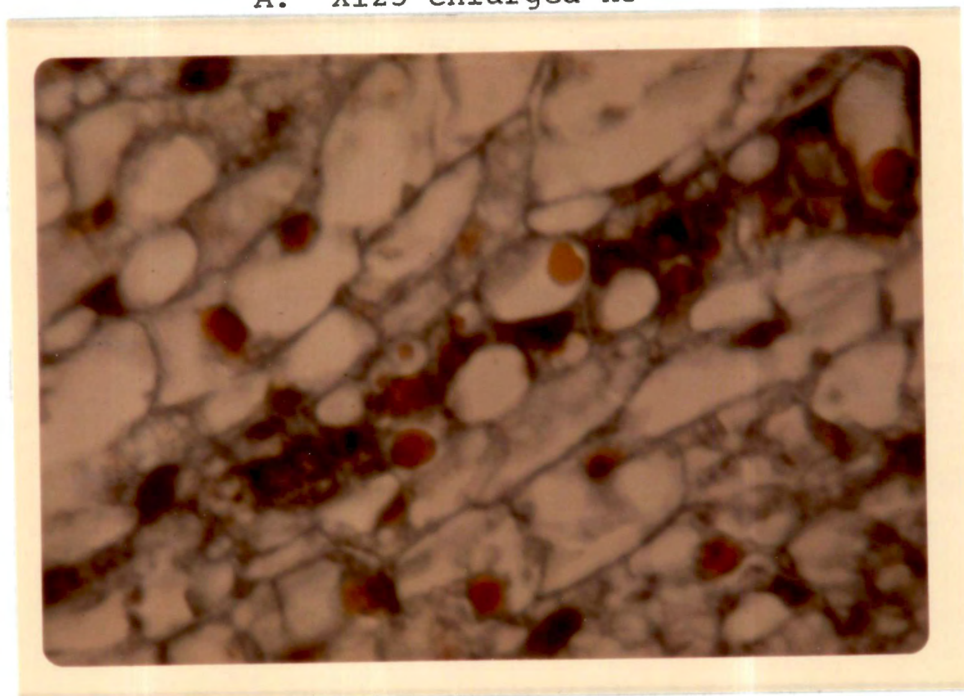
DISCUSSION OF RESULTS

Aniline blue-orange G was originated by LaCour, Chayen, and Gahan (11) and later recommended by Jensen (9) for detection of phosopholipids. However, aniline blue-orange G was applied following safranin O to yield a tri-colored general histological stain in paraffin sections. This particular combination of dyes was developed especially for this investigation. The globules exhibited a pronounced affinity for the orange G component of this combination of dyes, with other major cellular features differentiated in blue and red. Various cellular components were colored as follows: cytoplasmic globules—bright yellow to orange; interphase nucleus—blue with a red nucleolus; prophase nucleus—red; cell wall, endoplasmic reticulum, and vacuolar bodies—blue; starch—gray-blue with a bright red hilum; plastids—red to red-orange (Figures 1 and 2).

In Ipomoea purpurea 'Heavenly Blue,' globules were found only in the apex and lateral buds. In paraffin sections they occurred in files of unusually dense cells within the first or second mm from the apical meristem or leaf primordia. These files of dense cells had the singular property of occurring only in single cell widths, never as adjacent files of immediately contiguous cells (Figures 1A and 1B). These

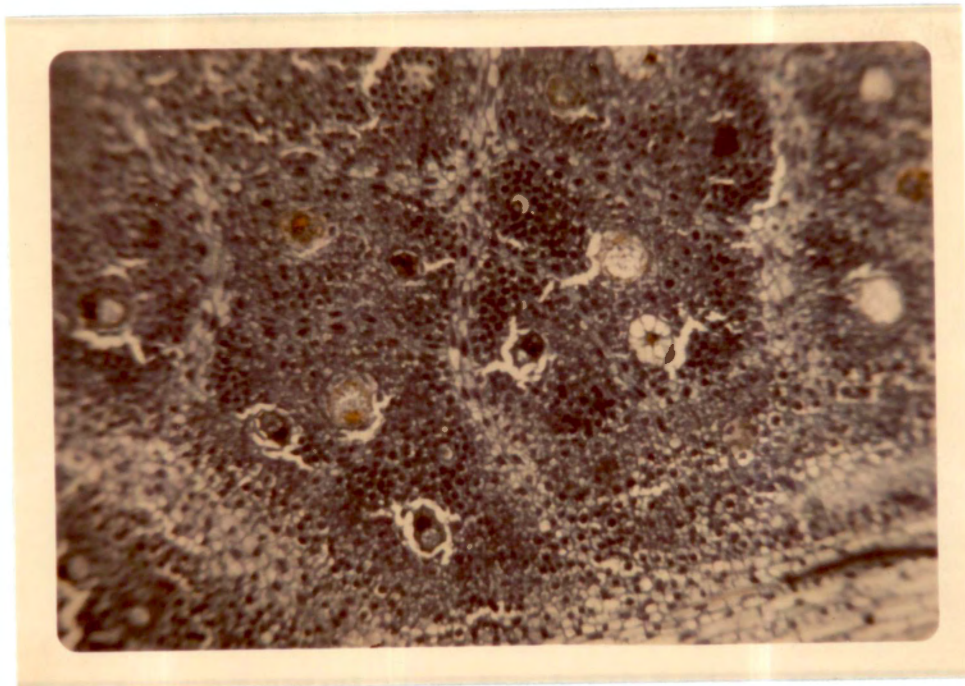


A. X125 enlarged X3

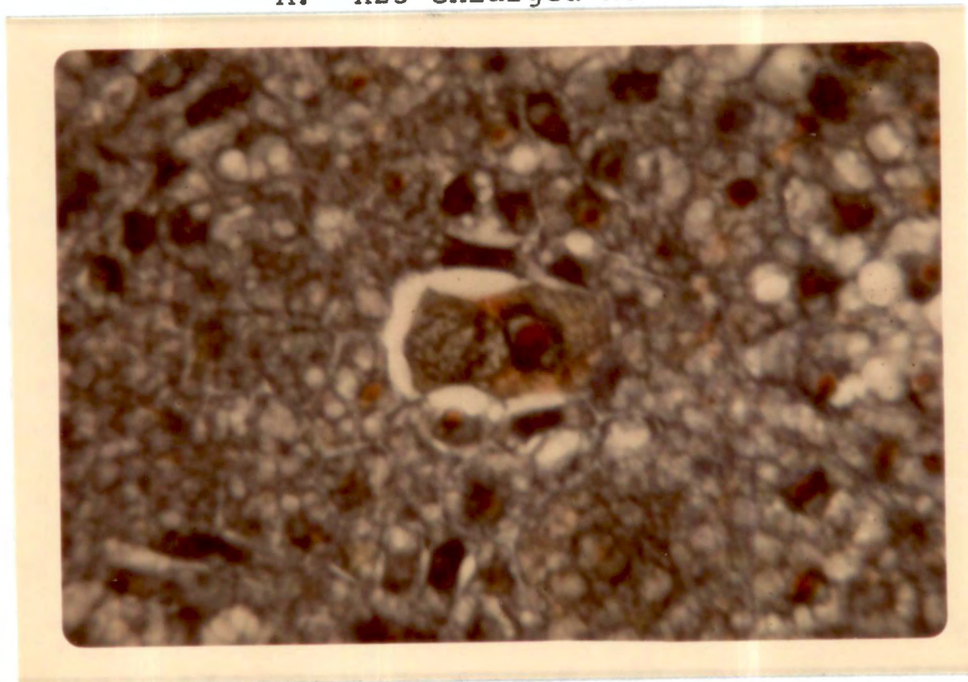


B. X250 enlarged X3

Figure 1. Longitudial apex section of _Ipomoea purpurea_ showing long dense cells in paraffin, stained with safranin O and aniline blue-orange G.



A. X25 enlarged X3



B. X125 enlarged X3

Figure 2. Leaf section of *Ipomoea purpurea* in paraffin showing single large dense cells stained in safranin O and aniline blue-orange G.

files of dense cells had an appearance of being glandular or laticifer structures, and they appeared in the mesophyll of differentiated leaves and the cortex of the stem area. Cells comprising the files contained other unidentified inclusions aside from the globules. No similar structures have been indicated in recent reference manuals of plant anatomy (5)(6) and cytology (4)(15)(22)(23), and this is apparently the original report of the description of the structure in Ipomoea.

In addition to these unusual files of dense cells, other large single dense cells were present in the leaves of I. purpurea (Figures 2A and 2B, page 14). These previously unreported cells were gigantic with nucleoli larger than whole nuclei in adjacent cells. They had a most unusual structure and staining affinity which varied from cell to cell, no two being identical. Each cell appeared to have a scollop-shaped heavy cell wall giving the appearance of a spider's web. They usually contained a dense staining material with the colors varying from bright yellow and red to gray-blue and black, when stained in safranin O and aniline blue-orange G. The nucleus and nucleolus were always very prominent in each cell and tended to stand out vividly. Globules with high affinity for orange G were not associated with the giant cells in paraffin sections.

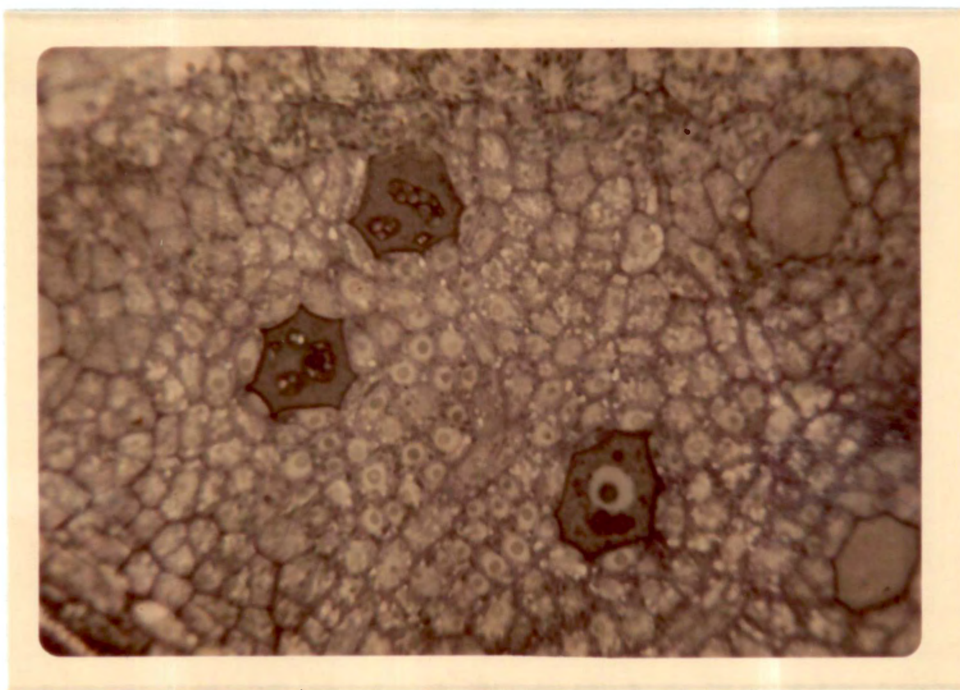
In plastic, the giant cells, when stained with osmium tetroxide and toluidine blue, had a more uniform appearance

than in paraffin sections. The cytoplasmic material stained a medium blue with deep purple bodies, which varied from tiny grains to irregular masses. The nucleus stained a pale blue-green with a deep blue-green nucleolus (Figures 3A and 3B). These giant cells, numerous in count, might be a type of glandular body; but their composition and function is not yet known.

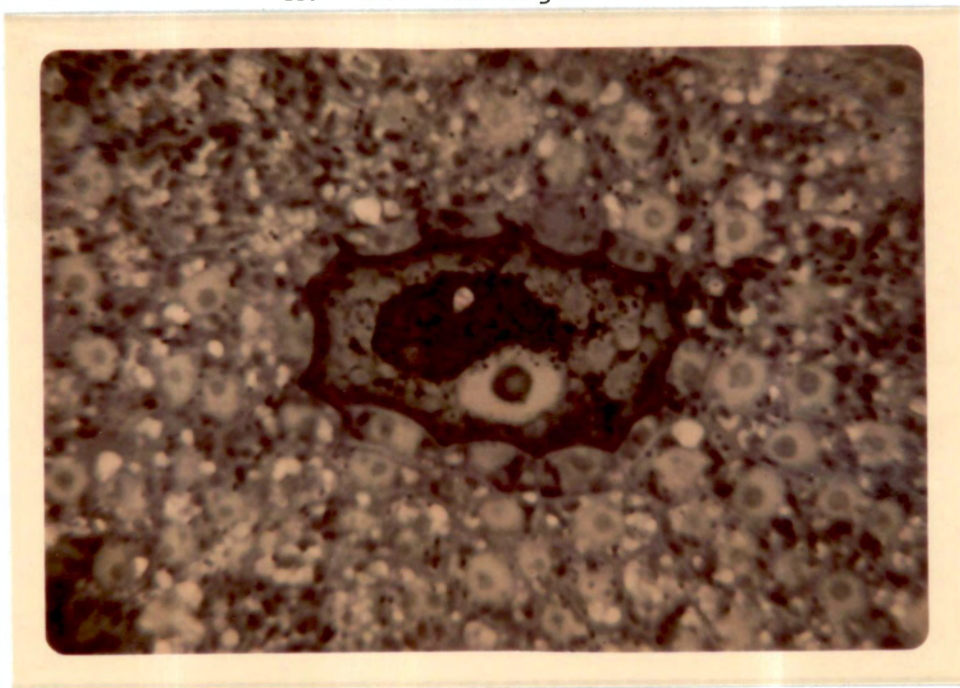
The globules appeared jet black in plastic sections fixed in osmium tetroxide and stained with toluidine blue. They occurred in the exterior layer of the pith just interior to the vascular cylinder (Figures 4A and 4B). Globules also were observed in the same files of dense cells as seen in paraffin sections (Figures 5 and 6). In both cases the cellular sites of the globules were in vacuoles, but this was much more clearly visible in plastic than in paraffin sections.

In the slides in which the plastic was removed, the sections were subjected to various stains to test for their composition. When subjected to the safranin O and aniline blue-orange G stain, some of the globules, but not all, took up the orange stain. On the other hand, when subjected to Sudan black B, which stains general lipids, there was no reaction. This result eliminated phospholipids as a major component of the globules, which had been indicated as a possibility by their uptake of orange G.

Other negative histochemical results were also obtained as follows: aniline blue black for protein; acidic buffered

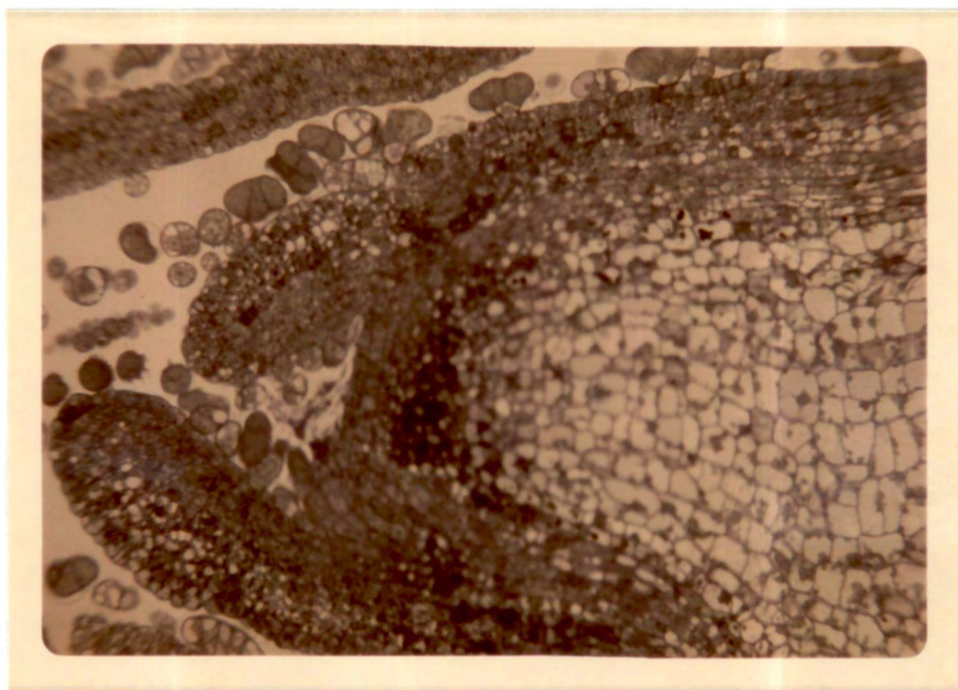


A. X50 enlarged X3

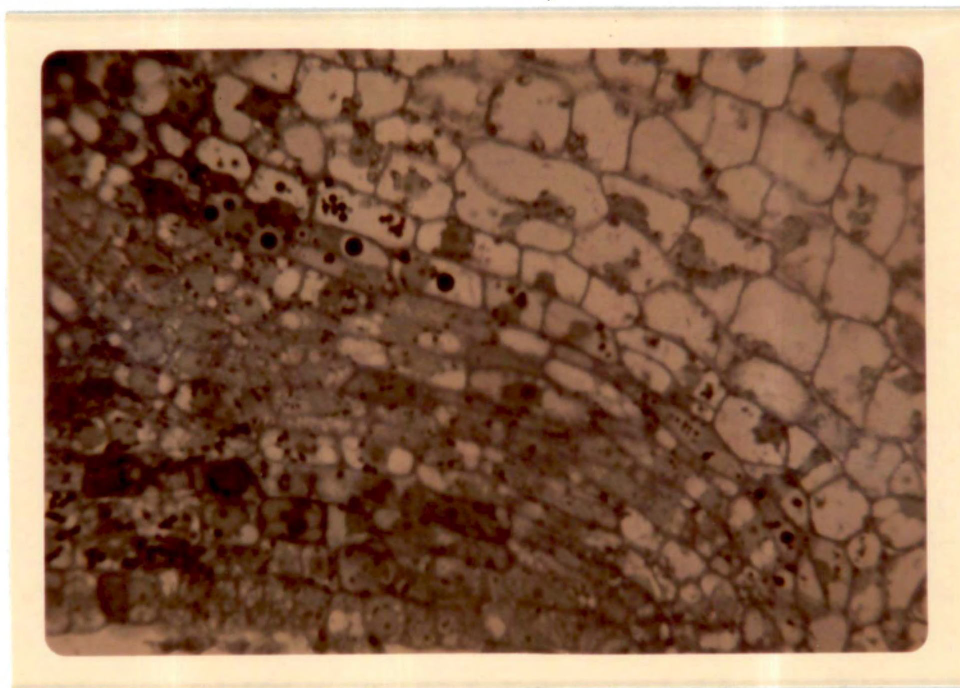


B. X125 enlarged X3

Figure 3. Leaf sections of Ipomoea purpurea in plastic showing single large dense cells stained in toluidine blue and osmium tetroxide.



A. X50 enlarged X3



B. X125 enlarged X3

Figure 4. Plastic embedded longitudial apex section of Ipomoea purpurea showing globules in cells at edge of pith cylinder stained with osmium tetroxide and toluidine blue.

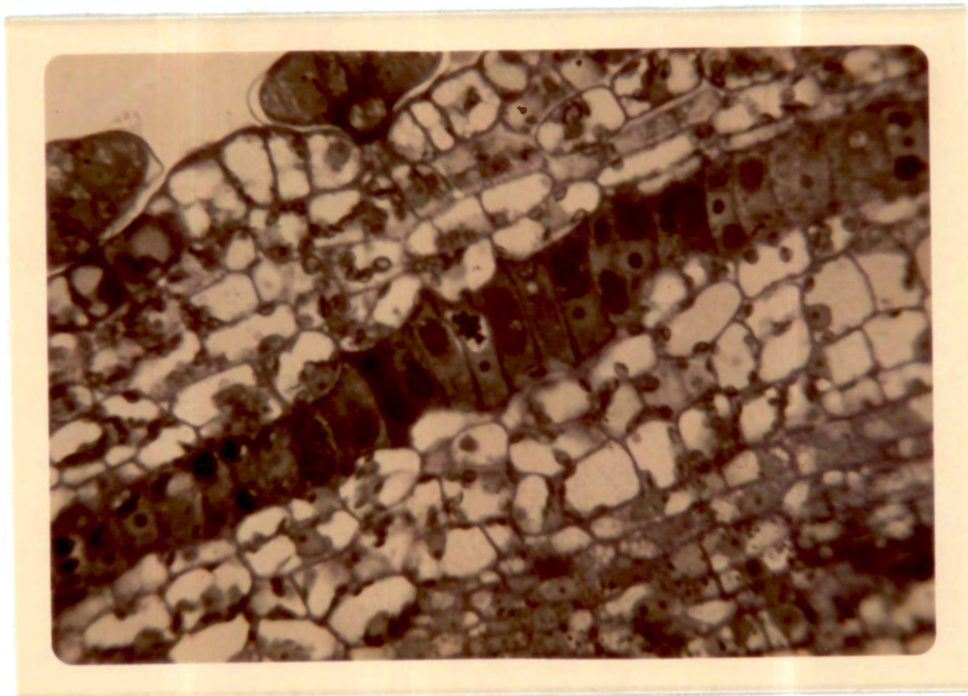


Figure 5. Long file of dense cells in leaf of Ipomoea purpurea embedded in plastic and stained with osmium tetroxide and toluidine blue.

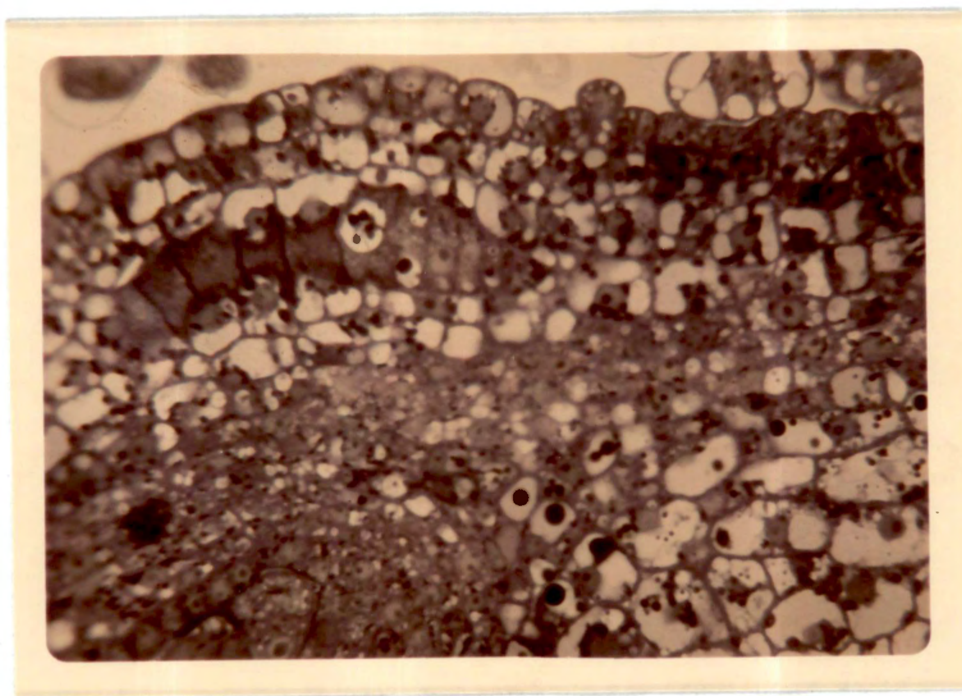


Figure 6. Plastic embedded section of leaf primordia of Ipomoea purpurea showing file of dense cells stained in osmium tetroxide and toluidine blue.

toluidine blue for nucleic acids; the periodic acid-Schiff reaction for insoluble carbohydrates or polysaccarides; and the ferric sulphate test for tannins.

Ipomoea noctiflora was also sectioned in the same way as I. purpurea and similar results were found. The globules were in the same general locations but they were fewer and smaller. Files of dense cells and individual dense cells, like those found in I. purpurea, were also present and numerous.

It must be emphasized that the globules could not be found in tissue which had been fixed in formalin-acetic acid-alcohol (FAA). While their image sites were apparent in tissue fixed in chromium trioxide-acetic acid-formalin (CRAF), the presence of the chromium prevented specific staining with orange G and generally made for poor color discrimination among the various cellular components. Since FAA and CRAF are the standard fixatives which have been used in the past for light microscopy of plant tissues (11)(16), the nature of fixation is most probably the major reason these globules have not been reported previously.

Primary fixation in glutaraldehyde preserved at least some of the globules, such as those in the files of dense cells, so that they survived the process of paraffin preparation. Other globules, however, appeared to be more fragile and were eliminated as discrete bodies. This

apparently was the case with those globules lying at the exterior of the pith cylinder subjacent to the apical meristem. Glutaraldehyde also gave more satisfactory fixation of other structures such as the dense files of cells and individual giant cells than was possible with the FAA and CRAF. Thus, the glutaraldehyde-calcium acetate fixation offered considerable improvement in the paraffin method when compared with the traditional procedures of botanical histological technique as presented by Johansen (11) and Sass (16).

Although the results obtained in glutaraldehyde-fixed paraffin sections were clearly more satisfactory than would have been possible with standard botanical fixation, they were still not good enough to permit visualization of much fine detail. A complete picture of the location of the globules can be seen only in tissues fixed first in buffered glutaraldehyde, post-fixed in osmium tetroxide and embedded in epoxy plastic. Acidic toluidine enhances the differentiation of the osmium stained globules in plastic sections. This also applies to other cellular details such as plastids and mitochondria as well as the globules.

A very favorable reason for using toluidine blue and other thiazine-derivative dyes, such as azure A and azure B, was that they stained the tissue through the plastic without staining the plastic. Most common histological dyes would

not have this important property, and would tend to stain the plastic, thus preventing clear optical differentiation of cellular details. Unfortunately, either safranin or orange G when applied to the plastic stained both the plastic and the tissue. Aniline blue penetrated the plastic so slowly as to preclude its practical use. Thus, the combination safranin-aniline blue-orange G, which was excellent for paraffin sections, was useless for plastic sections. Removal of the plastic with the strongly alkaline sodium ethoxide altered the dye affinity of the tissue so that the absence of the plastic offered no improvement over the presence of plastic as far as use of safranin-aniline blue-orange G was concerned.

The paraffin method of embedding is relatively fast and useful for work not needing much detail. The expansion and contraction which occurs when the paraffin melts and solidifies has a tendency to pull the cells apart and destroy much of the cell structure. The wax embedment reduces some of the shrinkage which occurs in the paraffin, but the quality is not so much improved that the trouble taken to use it would be justified. The wax is very difficult to get to ribbon on the microtome.

The plastic embedded specimens were superior for the purpose of this investigation. The cells appeared to be perfectly intact and much of the material was preserved in

plastic which was lost in the paraffin method. The plastic had one serious disadvantage, however, since it took much time and practice to get specimens prepared, sectioned, and attached to slides.

It is my feeling that paraffin would be perfect for any preliminary work where detail is not important and great amounts of time can be saved. It also would be of value in staining where dye colors are essential. The plastic embedments, on the other hand, are much more efficient and effective for work in which details may be crucial.

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