THE MORPHOLOGY, DEVELOPMENT AND HISTOCHEMISTRY OF THE SECRETORY TRICHOMES OF *NEPETA CATARIA* L.

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CHAPTER I

INTRODUCTION

The phenomenon of separation of substances from the cell protoplast is commonly referred to as secretion and many plant types exhibit this form of cell export of materials. The secreted substances may remain within cells, internal cavities, or canals; or they may emerge from superficial secretory cells to the surface of the plant (Esau, 1960). The external secretory structures assume many forms varying from very simple to very complex. The highly differentiated secretory structures involving many cells are referred to as glands while the term glandular is applied to the less complex secretory structures such as glandular hairs or epidermis (Esau, 1960). Both the glands and glandular hairs fall into the category of plant trichomes, the term being applied to all outgrowths from the epidermis of leaves, shoots, and roots whether they be unicellular or pluricellular (Upholf, 1962).

Classification of secretory trichomes has been based on several different criteria. The three criteria most commonly used are the nature of the substance secreted, the morphology and anatomy of the secretory structure, and structure-function relationships of the secretory structure and its secretion product (Luttge, 1971). Of these three criteria, the two offering the least variability, therefore the easiest and most definite classifications, are trichome morphology and

anatomy and the nature of the secretory substance.

The secretory cell is known to have a specialized cytoplasm usually independent of the substance to be exported. The purpose of this study was to investigate the development and morphology of the secretory structures of Nepeta cataria, the histochemistry and ultrastructure of the specialized gland cytoplasm, and to observe the changes occurring in the specialized gland cytoplasm with maturation.

CHAPTER II

REVIEW OF LITERATURE

One of the earliest investigations of plant trichomes, i.e., outgrowths from the epidermis of leaves, shoots, and roots, involved the stinging hairs of the nettle plant. Hooke, in 1665, made elaborate drawings of the ventral side of nettle leaves showing the morphology of the hairs and described how their poison is injected into the skin (Upholf, 1962). In 1668, Malpighi described trichome morphology and, in relating the trichome structure to function, postulated the function as protective for emerging plant parts. Linne (1735) in writing about trichomes stated that their function was for protection of the plant from heat and cold, while Schilling (1752) regarded trichomes as "absorbing vessels," or capillary tubes (Upholf, 1962).

Classification of Trichomes and Glands

From the work done involving descriptions of trichomes and their function came attempts to classify trichomes based on their function. In 1794, Schrank made a distinction between trichome types by dividing them into hairs and glands. Glands were for the secretion of definite cell saps and hairs were thin narrow tubes of varying properties on the surface of plant parts. Some of the properties of hairs were hardness, softness, transparency, and color (Upholf, 1962). According to Upholf, today's accepted classification of trichomes involves six

categories: (1) papillae; (2) unbranched unicellular trichomes; (3) uniseriate trichomes; (4) peltate scales and stellate hairs; (5) clavate, capitate and glandular hairs; and (6) villi. The glandular hairs have been classified more on secretory activities than on structure.

Frey-Wyssling (1935) divides the phenomenon of separation of substances from the cell protoplast, commonly called secretion, into three groups: secretion, recretion and excretion. He defines secretion as the export of products of assimilative metabolism (proteolytic enzymes, polysaccharides, sucrose, hexose), recretion the export of products of dissimilative (secondary) metabolism (wax, resins, essential oils), and excretion as the export of substances in the same chemical form in which they were initially taken up by the plant, such as salts (Luttge, 1971). Esau breaks the transport process into two groups, secretion and excretion. She defines secretion as export of substances having a special physiological function after they are secreted (enzymes, hormones), and excretion as export of products of dissimilatory metabolism, substances no longer used by the plant, e.g., terpenes, resins, crystals, essential oil (Esau, 1960). For the purposes of the this work secretion will be used as a general descriptive term for the removal of substances from the cell. This is done to eliminate confusion since this type of trichome is classified generally as a "secretory trichome."

Structure and Development of Secretory Trichomes

The developmental complexity of secretory trichomes is dependent upon the mature structure. Some secretory trichomes are small, simple, unicellular structures, such as glandular epidermal cells, while a

great number are complex structures of many cells. In the Madinae (Compositae) some of the glandular trichomes are large biseriate structures having a secretory capitulum of two tiers of cells (Carlquist, 1958). The development of these structures begins with an anticlinal division of a protodermal initial yielding a biseriate condition. Divisions of these two basal cells are periclinal until the top tier of cells begins to undergo anticlinal divisions setting up the lower tier of the capitulum. The gland is then finished by one more set of periclinal divisions of the lower tier of capitulum cells, thus yielding the double-tiered capitulum.

A less complicated biseriate gland is found on the salt secreting plant Tamarix aphylla. The entire gland consists of only eight cells, two collecting cells and six secretory cells. This particular gland is not capitate but merely consists of a biseriate structure of eight cells, and then the anticlinal division of these cells setting up the eight celled biseriate structure (Campbell and Strong, 1964).

A different type of pattern is required for the glandular trichomes of Psychotria Bacteriophila which has a single stalk cell from which radiate many branch cells (Horner and Lersten, 1968). Glandular trichomes with a uniseriate stalk and a multicellular capitulum are also found on many kinds of plants. The digestive glands of the Venus flytrap consist of a single basal cell, a stalk cell, and a multicellular capitulum consisting of two tiers of cells. The first two divisions are periclinal followed by anticlinal and periclinal divisions (Scala, Schwab, and Simmons, 1968). A similar but less complicated gland is found in the Labiatae. Mentha piperita (peppermint) has two types of secretory structures, glands and glandular hairs, both of protodermal

origin, thus trichomes. The gland consists of a basal cell, a stalk cell, and eight capitulum cells. Glandular hairs in peppermint are even simpler having just a basal cell, a stalk cell and a single capitulum cell (Howe and Steward, 1962; Amelunxen, 1964, 65.)

One feature found in some trichomes but not in others is the cuticle or cuticular bladder. The presence or absence of this structure depends on the substance secreted and its possible function. Many glands entirely lack a cuticular bladder such as those found in some of the Labiatae but few entirely lack the cuticle. Some trichomes, such as the salt glands of Tamarix aphylla (Shimony and Fahn, 1968) have heavy, thick cuticles covering the entire structure and separating it from surrounding epidermal cells.

Physiological and Ultrastructural Aspects of Secretory Trichomes

Despite morphological variability in secretory trichomes, truly secretory cells appear to have several cytological features in common regardless of the nature of specific substances exported. From some of the specialized features it is possible to speculate on the physiological action of the gland. Characteristics of specialized gland cytoplasm include: small cytoplasmic vesicles or minivesicles; numerous mitochondria; dense cytoplasm; relatively large nuclei; in some cases numerous dictyosomes; and an elaborate cell wall with ingrowths into the cytoplasm (Luttge, 1971). In secretory cells the mechanism of intracellular transport depends on the presence or absence of either dictyosomes or wall ingrowths.

Small Vesicles

The salt gland of Tamarix aphylla is composed of eight cells, six of which secrete. In the secretory cells small vesicles are found to occur around the periphery of the cell in association with wall protuberances (Thompson and Liu, 1967). It is thought that the small vesicles transport materials through the cytoplasm to the wall for export from the cell. Shimony and Fahn (1969), also working with Tamarix, have also found the smaller vesicular structures in the gland cytoplasm. In Daucus carota (carrot) cell cultures, it was found that multivesicular bodies occur apparently for intracellular transport of materials to the wall during cell senescence (Halperin, 1969). Finally, the secretory trichomes of Mentha piperita (peppermint) have been found to contain many small vacuoles (Amelunxen, 1965).

Mitochondria

Mitochondria are known to be cell organelles responsible for supplying the cell with energy yielding adenosine triphosphate (ATP). An increased number of mitochondria in a cell can indicate cell functions requiring large amounts of energy, e.g., active transport. This has been found to be the case in salt glands of plants. Limonium vulgare (sea lavender) and Tamarix aphylla (salt cedar) are salt secreting plants. Ultrastructural work done on Limonium (Ziegler and Luttge, 1966) and Tamarix (Thompson and Liu, 1967) has shown that there is an increased number of mitochondria in the salt secreting cells. The reason for this increase in mitochondria is probably to provide energy to operate an ion pump for active export of salt ions (Luttge, 1971). Absorptive cells in the protoxylem of Pinus have also been

found to have increased numbers of mitochondria (Wooding, 1969). Mitochondria were found in association with the plasmalemma and the two were thought to have a functional relationship aiding in salt absorption from the xylem transpirational stream.

Dense Cytoplasm

Limonium vulgare (sea lavender) plants are reported to have glands with dense cytoplasm (Ziegler and Luttge, 1966), as do the salt glands of Tamarix aphylla (Thompson and Liu, 1967; Shimony and Fahn, 1969) and the secretory cells of lily pistils (Dashek, Rosen, and Thomas, 1971). The oil producing hairs of Mentha piperita have been found to have a dense cytoplasm which retracts irregularly from the cell wall leaving what has been termed the extraplasmatic space (Amelunxen, 1964). Amelunxen (1965) found that in secretory glands the cytoplasm and cytomembranes degenerate entirely upon ageing of the gland leaving it filled with a large vacuole. These glands have senesced and are non-living. A similar phenomenon occurs in the mucilage-producing cells of the seed coat of Plantago ovata (Hyde, 1970). These cells have dense protoplasts, but upon onset of mucilage production the protoplast separates from the cell wall, the vacuoles fuse forming an extra-plasmatic space and starch disappears.

Dictyosomes

The Golgi apparatus is known to be composed of inter-associated dictyosomes, i.e., stacks of cisternae associated with peripheral vesicles, scattered throughout the cytoplasm (Mollenhaur and Moore, 1966). In many cells having a secretory function the number of

dictyosomes has been known to be quite large. Work with rat pituitary acidophils and mouse acinar cells (Farquhar and Wellings, 1957) has shown that the number of dictyosomes has increased and it is felt that dictyosomes do indeed function in the secretory process as indicated in a quotation by the authors:

> "We believe that the Golgi apparatus could be interpreted as functioning in the building up of secretory products from smaller particles which may be manufactured and transported by other cytoplasmic constituents such as the endoplasmic reticulum together with the ribonucleoprotein particles."

Using the outer root cap cells of maize for study of the functioning Golgi apparatus, Mollenhaur and Whaley (1963) found that the individual dictyosome had a forming face and a maturing face. The maturing face was the area that produced the vesicles that became separated and moved through the cytoplasm passing their contents through the plasmalemma by fusion with that membrane. With the use of thiocarbohydrazide-osmium vapor, Hyde (1970) obtained histochemical evidence from the Golgi apparatus of Plantago ovata seed coat cells indicating the presence of polysaccharide within the vesicles. This polysaccharide (mucilage) was exported from the cell via the transporting Golgi vesicles.

Northcote (1971) described the Golgi apparatus as a part of a membrane system involved with inter- and intra-cellular transport of substances produced in cells. It should be noted, however, that this statement is generally true only with regard to carbohydrate and protein transport. Cells involved in salt export and ethereal oil secretion have not been found to have a greatly increased number of dictyosomes (Shimony and Fahn, 1969; Thompson and Liu, 1967; Amelunxen, 1965). However, glandular hairs of peppermint do have an increased

number of dictyosomes, although they are not considered to be directly involved in secretion of the essential oil (Amelunxen, 1965).

Nuclei, Ribosomes and the Endoplasmic Reticulum

Nuclei in secretory cells are generally considered to be larger than nuclei of non-secretory cells based on their total volume to that of the cell (Luttge, 1971). This has been shown to be true in the salt glands of Limonium vulgare (Ziegler and Luttge, 1966) and in the seed coat of Plantago after pollination (Hyde, 1970). Associated with the nuclear membranes is the endoplasmic reticulum which is thought to originate from the nuclear membrane and which is distributed throughout the cytoplasm acting as a transport system from the nucleus through its cisternae to the cytoplasm and also functioning in synthesis of assimilates (Frey-Wyssling and Muhlethaler, 1965; Watson, 1955; Palade, 1955).

Ribosomes, i.e., submicroscopic particles consisting of protein and ribonucleic acid and aiding in protein synthesis and catalysis of synthesis of polypeptides from amino acids, are also found to increase in gland cells and are associated with the endoplasmic reticulum. The endoplasmic reticulum, when associated with ribosomes, is referred to as the rough endoplasmic reticulum. Amelunxen (1964; 1965) has found that in both the secretory gland and glandular hair of Mentha piperita (peppermint) the endoplasmic reticulum and ribosomes are of an increased density. Ribosomes and polysome numbers have also been shown to increase in the salt glands of Tamarix aphylla (Shimony and Fahn, 1969).

Cell Wall and Plasma Membrane

The cell wall of many types of secretory cells appears to be specialized because of ingrowths of the wall into the cytoplasm. This arrangement of the wall causes these cells to have protoplasts with an unusually high surface-to-volume ratio. Cells having this type of wall adaptation have been termed "transfer cells" (Gunning and Pate, 1969a). This type of wall has been found in many cells involving cell to cell short distance transport. Pericycle cells of the legume nodule have been found to have filiform branches protuberances from the wall and are thought to be involved in the symplastic lateral transfer of assimilates, from sieve elements through the pericycle, endodermis, and cortex to bacteral tissue. The apoplast within the endodermis has been found to consist largely of the pericycle labyrinthene wall and the xylem (Pate, Gunning, and Briarty, 1969).

Wooding and Northcote (1965) have found this form of secondary wall thickening in cells adjacent to the innermost xylem tracheids of the stem. They hypothesized that the "knobbly" growth would act like a sponge. They felt it possibly acted as a device to increase the surface area of the plasmalemma, thus allowing greater opportunity for selective absorption. Wooding (1969) in working with Pinus picea protoxylem found wall ingrowths with numerous mitochondria closely associated with the plasmalemma. He felt that there was a functional relationship between the association and salt absorption from the xylem transpirational stream, with the mitochondria supplying the energy required for the salt transport. The salt glands of Limonium vulgare (sea lavender) also have wall ingrowths and mitochondria associated with the plasmalemma (Ziegler and Luttge, 1966) as do the salt glands

of Tamarix (Thompson and Liu, 1967; Shimony and Fahn, 1969).

One of the most striking features of the secretory canal cells of lily pistils is the elaborate wall. Wall ingrowths are found on the side of the wall facing the canal, in the secretion zone (Rosen and Thomas, 1970; Dashek, Thomas, and Rosen, 1971). Similar elaborate wall sculpturing has been found in lower plants. In mosses these wall types have been found where the haustorial foot of the sporophyte is embedded in the gametophyte. This is an area of intensive transmembrane flux of solutes and the wall elaborations are thought to aid in the solute transport (Gunning and Pate, 1969b). These wall ingrowths also occur at sporophytegametophyte junctions in ferns, being most abundant on the donor side, i.e., the sporophyte side, in Adiantum, or on the gametophyte side in Polypodium (Gunning and Pate, 1969b). It is felt that the resulting enlargement in area of the plasma membrane of the donor cells facilitates movement of solutes into the apoplastic compartment, i.e., the non-living areas outside the living parts of the cells, between the generations (Gunning and Pate, 1969b). Receptor cells are correspondingly specialized for transport of solutes in towards the symplast, i.e., the actively metabolizing parts of plant cells. Transfer cells are common in both lower and higher plants, seemingly regardless of the reversal of relative dominance between life cycle generations. For this reason it has been proposed that the precipitating factor is a solute flux, regardless of its direction and that the wall elaboration is an adaptation for this type of transport (Gunning and Pate, 1969b). Since the protuberances are always coated with the plasmalemma, it could be concluded that the plasmalemma rather than the wall is the functional structure in respect to intensive short distance

transport (Luttge, 1971).

Plasmodesmata

Plasmodesmata are strands of cytoplasm passing through a pore in the cell wall joining the protoplasts of two adjacent cells (Esau, 1960). They have been found in many secretory-transport systems, although their ultrastructure may vary in different kinds of plants such as algae (Fraser and Gunning, 1969) or higher plants (Robards, 1968; Lopez-Saez, Gimenez-Martin, and Risueno, 1966). Plasmodesmata have been found in the secretory cells of lily pistils (Dashek, Thomas, and Rosen, 1971), the salt glands of Tamarix (Thompson and Liu, 1967; Shimony and Fahn, 1969) and in the salt glands of Limonium (Ziegler and Luttge, 1966) and many others. Plasmodesmata are generally regarded as having the function of intercellular short distance transport.

CHAPTER III

METHODS AND MATERIALS

Source of Tissue

Nepeta cataria (catnip) seeds were acquired from the Oklahoma State University Department of Biochemistry. These seeds had been harvested from field grown plants used in gas chromatograph experiments which showed cis-trans, trans-cis isomers of nepetalactone. Seeds from plants that only had the cis-trans isomer were used in a comparison of the secretory structures found on the two plant types. Seeds were germinated on damp filter paper in petri dishes and kept in a controlled environment chamber (Sherer Gillett - Model CEL 37-14). The age of the seedlings was measured from the time the emerged radicle reached a one millimeter length. Continuously propagated plants from the greenhouse were also examined.

Treatment of Tissue for Light Microscopy

Plants were harvested for study at different specific stages of development. It is known that the catnip plant begins production of the oil nepetalactone when it is 13 days old (Mitchell, 1972). Keeping this in mind, plants younger than 13 days of age and plants older than 13 days of age were used. Also used in the study were mature flowering plants. Of these plants, the stem apical region, leaves, cotyledons, and flowers were studied histologically and histochemically.

All histological work employed standard techniques. Plants were killed and fixed in Craf III (Sass, 1958), a mixture of chromic acid, acetic acid, formaldehyde and water. Then the tissues were washed in several changes of water and dehydrated with increasing concentrations of tertiary butyl alcohol (Johansen, 1940). During the last step in the dehydration series the tissue was aspirated in a vacuum oven (Precision Scientific - Thelco) for a period of not less than one hour or not more than 24 hours.

Infiltration of the tissue with Paraplast tissue embedding medium was accomplished by dissolving the Paraplast in pure tertiary butyl alcohol which is a solvent of wax and gradually increasing the concentration of the Paraplast while decreasing the concentration of the solvent by evaporation or decantation (Johansen, 1940). Infiltrated tissue was embedded by pouring melted Paraplast (55 C) into plastic boats, orienting the tissue as desired, and then letting it stand at room temperature until it was hardened. The paraffin blocks were then removed from the boats, trimmed to the desired size, mounted upon wooden blocks, and sectioned 10 micrometers thick on a rotary microtome (American Optical - Spencer 820). Sections were mounted on glass slides with Haupt's adhesive (Johansen, 1940) and allowed to set by placing the slides on a slide warmer (American Optical) set at 40 C for a period of not less than 24 hours. The slides were then stained using the quadruple stain series of Johansen (1940), mounted in Histoclad and examined with an American Optical Series 10 Microstar microscope with an ortho-illuminator.

Histochemical preparations were variously treated according to the nature of the procedure. Reagents specific for pectin required no

different procedure than the histologic preparation. The two pectin stains used were ruthenium red (Jensen, 1962) and hydroxylamine ferric chloride (Reeve, 1959). The Sudan III stain (Jensen, 1962; Horner and Arnott, 1966) specific for lipids required fresh non-fixed tissue.

Stains specific for localization of carbohydrates and protein necessitated the use of cold buffered neutral formalin (McMannis and Mowry, 1960) as a fixative. This was prepared from commercial formalin, distilled water, sodium acid phosphate monohydrate ($NaH_2PO_4.H_2O$), and disodium phosphate ($Na_2HPO_4.7H_2O$). Material was fixed and stored overnight in the formalin fixative solution. The histochemical test used with carbohydrates was the periodic acid-Schiff (PAS) reaction (Jensen, 1962). In this reaction periodic acid oxidizes 1, 2 glycol groups in carbohydrates, to produce aldehydes that are colored by the Schiff's reagent. Blockade of hydroxyl groups by acetylation with acetic anhydride in pyridine (Jensen, 1962) was used as a control. If a negative reaction was obtained from blockaded tissues and a positive reaction was given by tissues not exposed to blockade, it was assumed that the tissue contained hydroxyl and/or amino groups, since periodic acid can also oxidize amino groups in amino compounds. This procedure and all other histochemical tests were used on plants younger than 13 days of age and plants older than 13 days of age.

Protein localization procedures were those which visualize total cell proteins. The ninhydrinalloxan-Schiff's reaction was employed for this purpose (Jensen, 1962). This reaction involves the formation of an aldehyde by ninhydrin (1, 2, 3 - Triketohydrindene) or alloxan which is colored by the Schiff's reagent. Alpha-amino and alpha-carboxyl groups are necessary for a positive test. Controls in which

tissue had been treated with deamination and acetylation reagents were necessary to show that the color in test sections was the result of the staining of aldehydes produced from alpha-amino and alpha-carboxyl amino acids and to eliminate the possibility that the aldehydes were formed from saccharides by ninhydrin (Jensen, 1962).

Light microscope examination of tissue was also made of Araldite resin embedded tissue. The purpose of this was two-fold. First, light microscopic examination of tissue sectioned on the ultramicrotome allowed examination of the tissue section for the presence of the glandular hair or gland. Only sections through the gland or glandular hair could be used in electron microscopy. Second, the Araldite-embedded tissue was used for a periodic acid-Schiff stain (DiBella and Hashimoto, 1966), thus allowing for a more precise localization of carbohydrate in the "thick," i.e., about 1 micrometer, tissue sections.

The sections used for orientation of the tissue were stained with basic fuchsin (Daws, 1971) to increase visibility of the section in the resin. The procedure did not require removal of the resin embedding material. The PAS stain (DiBella and Hashimoto, 1966) incorporated the use of 0.5% periodic acid and Coleman's Feulgen reagent (Coleman, 1938) resulting in a permanent stain. The sections were mounted on glass slides with 40% acetone, stained, and covered using glycerine jelly as a mounting medium.

Electron Microprobe Examination of Tissue

Leaf material from plants younger than 13 days of age and from plants older than 13 days of age was examined with an Electron Microprobe X-Ray Analyzer-Scanning Microscope (Applied Research Laboratories)

located in the research facilities of the Continental Oil Company, Ponca City, Oklahoma. The purpose of the examination was two-fold: first, to determine the distribution of the glands and glandular hairs; and second, to allow high resolution of the secretory capitulum in order to obtain evidence of any external manifestation of pores or pore-like structures. Such structures have been reported in Mentha piperita, Labiatae, based on evidence from thin section electron microscopy (Amelunxen, 1965).

While much work done with the scanning type of electron microscopy involves the use of fixed, gold coated tissue, it has been reported that some types of unfixed tissue, i.e., shoot apices and developing leaves, will withstand the vacuum and electron bombardment involved in procedures used with these instruments for short periods of time, e.g., 15 to 20 minutes, without showing extensive collapse of the tissue (Falk, Gifford, and Cutter, 1971). Therefore, two types of procedures were tried and the plant material was treated in two ways. Leaves were removed from the plants and either examined after a treatment of gold evaporation over the leaf surface or the leaves were examined untreated. The material having the gold coating showed excellent detail of the epidermal surface and gland capitulum at high magnification, but caused the sterile trichomes on the leaf surface to collapse. The gold treatment did seem to greatly increase the resolution of the external structure of the glandular capitulum. The tissue left uncoated gave a better picture of the gland distribution and trichome density on the leaf surface. All material was sprayed with an antistatic compound designed to inhibit electrical charging of the tissue. Photographs were taken using a Polaroid camera fitted to the

microscopic apparatus.

Treatment of Tissue for Transmission Electron Microscopic Examination

To achieve thin sections necessary for this type of work, i.e., 40-70 nanometers (nm), material must be embedded in a hard supportive material. Araldite 502 (Ladd, Inc.) resin was used as the embedding medium. The complete resin consisted of a mixture of Araldite 502, DDSA (dodecenyl succinic anhydride), and DMP-30 (dimethyl amino methyl phenol). A stock resin mixture of Araldite and DDSA was made by using 100 grams of the Araldite resin and 78 grams of the DDSA mixed together and stored in a refrigerator. A Freon product, Blast-Off (Ladd, Inc.), was used to exclude air from the bottle in which the stock resin mixture was stored. The complete resin and 0.16 ml of DMP-30 were added with a 1 cc tuberculin syringe in preparation for embedding. The mixture was stirred for at least 5 minutes to ensure homogeneity.

Tissues analyzed were from leaves of 10 and 25 day old plants. The fixative used was 2% osmium tetroxide (OsO_4) buffered to a pH of 6.8 (Daws, 1971) with a 0.028 M sodium acetate-sodium veronal buffer (Palade, 1952). Osmium tetroxide is believed to oxidize double bonds of unsaturated fatty acids in lipid molecules resulting in the formation of a black compound of osmium showing the localized sites of lipids having double bonds; it is thus also a good lipid stain (Jensen, 1962). All material was fixed for 2-4 hours at room temperature, following a 5 minute treatment at 0 C in an ice bath.

After fixation, the material was dehydrated, infiltrated with resin and cured. Dehydration was prefaced with several changes of

buffer rinse. Following the buffer rinse dehydration of the material was achieved by passing it through successive baths of absolute alcohol beginning with 10% ethyl alcohol and progressing in concentration by 10% steps to a 100% concentration. The tissue was kept for 15 minutes in each bath, with aspiration during the 30, 40 and 50% baths. After two baths of 100% ethyl alcohol the tissue was passed through increasing concentrations of propylene oxide in absolute alcohol until a 100% concentration of propylene oxide was achieved. Five baths were used with the ratio of propylene oxide to absolute alcohol progressing from 1:3, 1:1, 3:1, and 100% (two baths) each lasting 30 minutes. The tissue was then infiltrated. The dehydration method proved unsatisfactory. It consisted of passing the tissue through two baths of absolute alcohol, 15 minutes each, and two baths of propylene oxide, each for 15 minutes.

Infiltration of the tissue began with the addition of 2 ml. of propylene oxide and 2 ml. of the complete resin. Mixing was accomplished by swirling the two components and tissue together; the material was then allowed to stand for 1 hour at room temperature. Two additional milliliters of complete resin were added, swirled, and then allowed to stand for 3-6 hours, the longer period permitting better infiltration. The tissue was then removed from the resin, drained, and placed at the top of resin-filled rubber molds.

Curing of the resin blocks was done in two ways. Some blocks were cured at 60 C overnight and others were cured by an overnight incubation at 35 C, followed the next day with incubation at 60 C. Either method is feasible. The blocks were next removed from the incubator and allowed to coll to room temperature.

Sectioning of the blocks was done using a Porter-Blum MT-2 ultramicrotome (Sorvall, Inc.). Glass knives used for sectioning were prepared by breaking glass rods ¼ of an inch in depth and 1 inch wide. An LKB 7800 Knife Breaker was used to break the glass for the knives.

After the material was sectioned, it was mounted on 400 mesh copper grids and stained using a double stain of lead citrate (Reynolds, 1963) and uranyl acetate (Daws, 1971). The grids were first stained with uranyl acetate followed by lead citrate. In the uranyl acetate staining procedure, the grids were floated section side down on the surface of the stain for 15 to 30 minutes. They were then drained on filter paper and rinsed by repeated dippings in several changes of distilled water. The lead citrate procedure also necessitated floating grids on a drop of solution and allowing them to stain for 20 minutes. Washing of grids was accomplished using sequential jets of 0.02 N sodium hydroxide alternately with jets of distilled water. The grids were allowed to dry and examined on an RCA model electron microscope.

CHAPTER IV

RESULTS AND DISCUSSION

Catnip, i.e., Nepeta cataria, plants have long been noted for their ability to produce an attractant for cats with the dominant gene for the olfactory response to the substance (Todd, 1962). It has been shown that this component of catnip is a methyl-cyclopentane monoterpenoid called nepetalactone, and that it is produced in secretory structures on the leaves, stems, and flowers of the plant (Regnier, Waller, Eisenbraun, and Auda, 1968). Early investigations indicated that the secretory structures were multicellular and of one type, a gland, but with intermediate developmental stages persistent (Regnier, et. al., 1968).

Identification of Secretory Trichomes by Light Microscopy

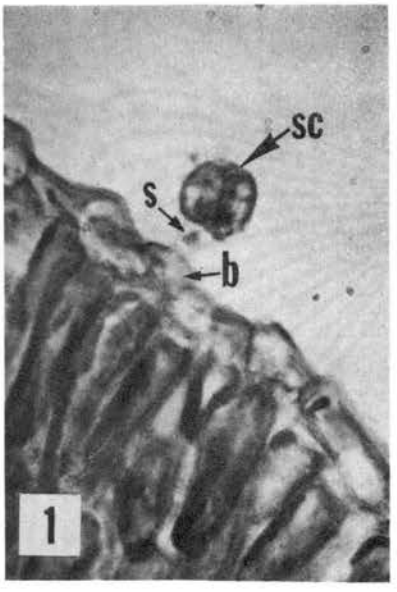
Sections taken from mature Nepeta cataria plants contained two types of secretory trichomes. Cross-sections of the leaf material revealed a gland and a glandular hair similar to those found in peppermint, i.e., Mentha piperita (Howe, 1951; Amelunxen, 1964; 1965). In cross-sections of the leaves the size and shape of the two structures could be determined. The glandular hair consisted of a basal cell, a stalk cell, and a secretory capitulum composed of two cells (Figure 1). The gland was also composed of single basal and stalk cells and a capitulum appearing to consist of two large cells (Figure 2).

Figure 1. Glandular hair in cross section showing basal cell (b), stalk cell (s), and the secretory capitulum composed of two cells (sc). Magnification: 700X.

Figure 2. Gland in cross section showing basal cell (b), stalk cell (s), and the secretory capitulum (sc). Magnification: 700X.

Figure 3. Glandular hair in paradermal section of the flower showing the two cells of the secretory capitulum (arrows). Magnification: 700X.

Figure 4. Gland in paradermal section of the flower showing the number of cells of the secretory capitulum to be four. Magnification: 700X.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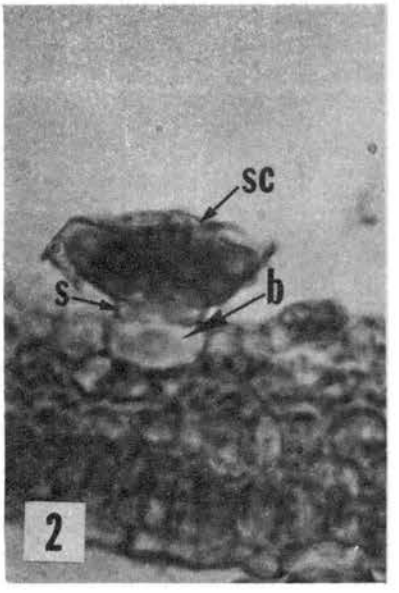
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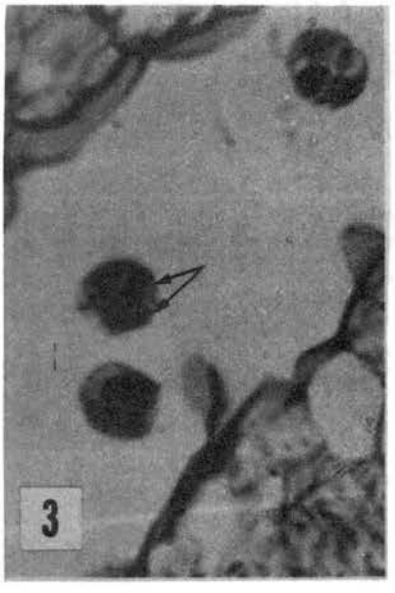
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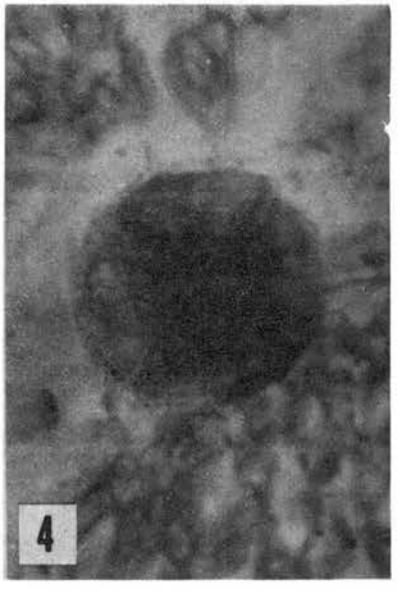
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Paradermal sections, i.e., parallel to the surface, of flower organs revealed that while the glandular hair capitulum was comprised of only two cells (Figure 3), the capitulum of the gland was comprised of four cells (Figure 4). This information concurs with that of Metcalf and Chalk (1957). It is therefore probable that the structure found and identified as an intermediate stage in gland formation by Regnier, et. al. (1968) is the stalked glandular hair and that it is a separate structure from that of the gland. Secretory structures are similar to those found on the peppermint plant, also of the Labiatae. The glandular hair of peppermint has a single-celled capitulum as compared to the two-celled capitulum found in Nepeta; the peppermint gland is comprised of eight capitulum cells, while Nepeta has only four cells in the glandular capitulum. Both types of Nepeta cataria plants, the common type secreting cis-trans and trans-cis isomers of nepetalactone and the second type secreting only the cis-trans isomer, exhibited the same types of secretory structures.

Electron Microprobe Survey of Secretory Trichomes

When mature leaves and flowers of Nepeta cataria plants were examined with an Electron Microprobe X-Ray Analyzer-Scanning Microscope, it was possible to get three-dimensional pictures of the glands, glandular hairs, and sterile hairs. A comparison was then made of leaves from plants younger than thirteen days and approximately 10 days old, and the difference in stages of development of these structures was noted. Using low magnification, i.e., 100 times, it was possible to identify glands and glandular hairs, as shown in figure 5, a view of the abaxial surface of a leaf from a mature plant. The glands are

indicated and are much larger than the indicated glandular hairs. Positive identification of the smaller structures as stalked glandular hairs is revealed by the presence of the stalk, shown in figure 6.

The occurance and appearance of the secretory structures in pre-secretory plants, i.e., about 10 days old, is shown in figure 7. Glands and glandular hairs have the same appearance as those from mature plants. However, the sterile hairs at 10 day are not fully elongated and are pegs sticking up above the epidermis. The young pre-secretory plants also show in the glandular hair the furrow separating the two cells of the secretory capitulum (Figure 8). The reason that this can be seen in younger plants and not in mature plants is probably due to lack of cuticular separation from the capitulum cells due to increased secretory activity in the older plants.

Examination of the capitulum of a gland from a mature plant indicated that there were bodies on the external surface of the cuticular bladder (Figure 9). When magnified 2,000 and 5,000 times, smaller bodies become apparent over the surface of the bladder, structures obviously different from the much larger bodies seen in figure 9 (Figures 10 and 11). The nature and function of these small bodies is unclear. At first it appeared they marked the locus of pores aiding in the release or evaporation of the essential oil from the bladder, as suggested in a study of the glandular structures of peppermint (Amelunxen, 1965). However, this might not be the case since studies done by the Oklahoma State University Department of Biochemistry have revealed that no nepetalactone has been found volatilized in the air surrounding the leaf, which indicates that oil loss from the bladder is by mechanical rupture of the bladder (Mitchell, 1972).

Figure 5. Electron-microprobe view of the mature Nepeta leaf surface showing sterile trichomes (t), glands (g), and glandular hairs (h). Magnification: 100X.

Figure 6. Electron-microprobe identification of the glandular hair. Note the stalk cell indicated by the arrow. Manigification: 200X.

Figure 7. Electron-microprobe survey of a leaf from a 10-day old Nepeta plant leaf. Note the appearance of the "stubby" sterile hairs which are not fully elongated (arrow). Magnification: 200X.

Figure 8. Electron-microprobe image of the 10-day old plants. Note the furrow (arrow) indicating the 2-celled nature of the secretory capitulum of the glandular hair. Magnification: 500X.


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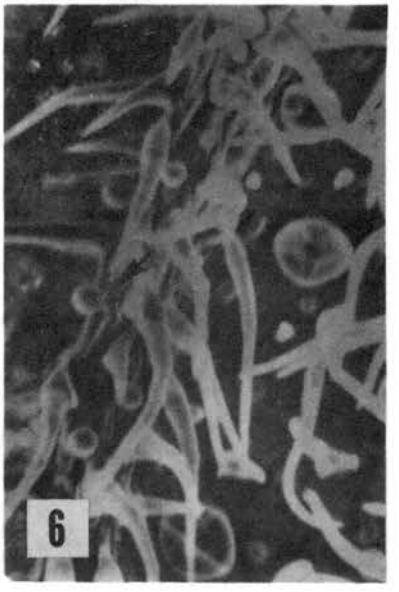
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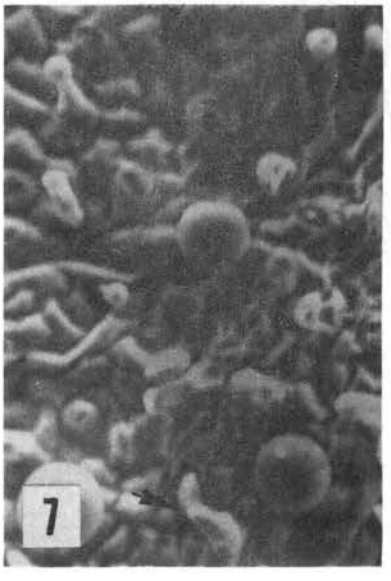
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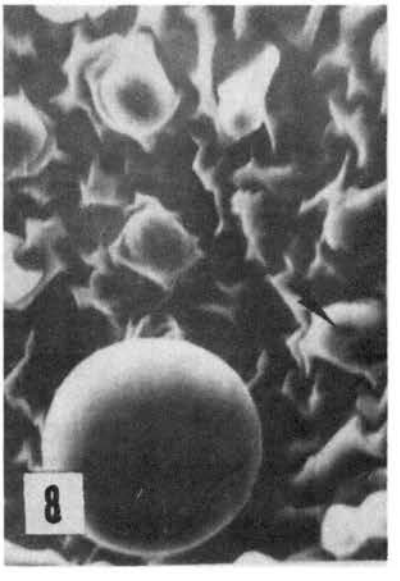
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Another piece of evidence supporting the theory that these are not pores, at least not functional pores, is the fact that in Mentha (peppermint) the glands retain full cuticular bladders even when the leaves were dry and yellowed. The bladder was not affected by dehydration of the leaf or by death of the leaf (Howe, 1951). There was no apparent loss of oil by evaporation. It is possible that if the structures are pores they may be plugged internally with pectin plugs, as was suggested by Amelunxen (1965) with regard to similar structures in peppermint. Ultrastructural examination of the secretory capitulum should reveal the presence or absence of internal pores or plugging of pores.

It is possible that the small bodies observed in figures 10 and 11 are due to uneven cuticularization or are crystals of some other substance present in the bladder that had escaped and crystalized on the gland surface. This is unlikely, however, since crystals would be larger than the bodies indicated. These structures were not found on glands of pre-secretory plants, as indicated in figure 12.

The study of the flower was done in two parts, the first part considering the sepals and the second considering the corolla tube. Gas chromatograph information indicates that the flower produces the largest quantity of oil of any of the plant parts. The microprobe examination revealed that the flower parts responsible for the major part of the oil accumulation were the sepals. The sepals had a much greater concentration of glands and glandular hairs than did the corolla tube. Figure 13 indicates the abundance of the secretory structures. The corolla tube had fewer secretory structures for two reasons. First, the abaxial surface of the corolla epidermis was

Figure 9. Electron-microprobe study of a gland. Note the scaley surface of the gland. Magnification: 500X.

Figure 10. Increased resolution of the gland capitulum; arrows indicate small bodies, possibly pores. Magnification: 2000X.

Figure 11. Greater resolution of the electron-microprobe image of the pore-like structures of the gland. Magnification: 5000X.

Figure 12. Gland from a 10-day old plant. Note the lack of surface structures. Magnification: 1000X.


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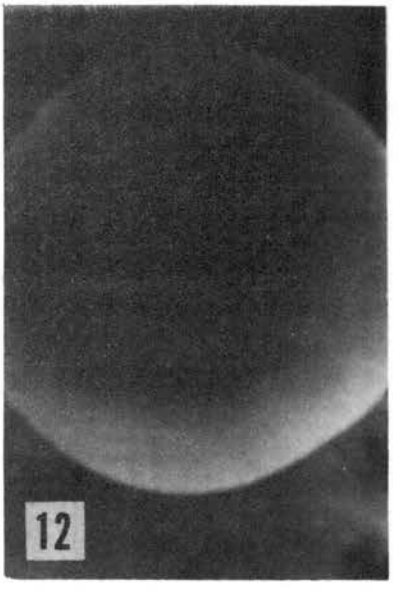
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totally devoid of trichomes of any kind, as is shown in figure 14. All of the sterile hairs and secretory structures found on the corolla were on the adaxial or inside surface of the corolla tube. Secondly, though there were secretory structures present on the adaxial surface, they seemed to be present in reduced numbers (Figure 15).

With regard to the distributional patterns of the glands and glandular hairs on the leaf surface, nothing was found to indicate that the glands or glandular hairs were concentrated in any particular area. They are found on both the leaf lamina and midrib with no apparent concentration per unit area on either midrib or lamina (Figure 5). On the other hand, the ratio of glands to glandular hairs was found to vary according to the season of the year. Greenhouse grown plants collected and studied during the late spring and early summer (May - June) have a tendency to have more glands than plants grown and studied in the winter months (December, January). However, plants grown in the winter months exhibited many more glandular hairs than glands, as seen in figure 5. The reason for this phenomenon is unknown. Since it is known that the largest quantity of oil is produced by the plant at the onset of anthesis, i.e., late spring and summer, (Regnier, et. al., 1968); and at the time of increased numbers of the larger glands, it is proposed that the morphogenic control of the plant is altered at certain times during the year and that the induction of the change toward gland and oil production and anthesis is a light or light-temperature response. The photoperiodic behavior of Nepeta has not been studied, but Allard (1941) found that many mints exhibited photoperiodic behavior with regard to flowering and long days. This being the case, it is possible that there is a correlation between flower

induction, oil production and the long day length of late spring when Nepeta is known to exhibit these phenomena.

Leaves examined from vegetative plants indicated that both glands and secretory hairs were apparently functional, which is supported by the distended cuticular bladder and densly staining cytoplasm. It can be assumed that the stalked glandular hairs, like those of figure 6, are indeed separate structures from the glands and not intermediate stages in the development of the gland as reported by Regnier, et. al. (1968).

Developmental Sequence of Trichome Development

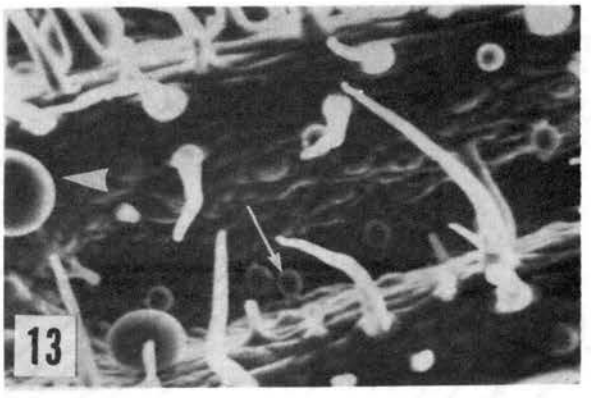
Mature trichomes are found on stems, leaves and flowers of the plant. The apical meristem was the area examined for the developmental study of the secretory trichomes. When sectioned, glands and glandular hairs were found in various stages of development on the newly forming first leaves and stem of the plant.

The development of the gland and glandular hair appears to be identical until the time that the developing gland undergoes one additional division not found in the glandular hair. This results in the four-celled secretory capitulum. Both gland and glandular hair arise from a single epidermal initial, as seen in figure 16. The epidermal cell has enlarged and protrudes above the remaining epidermal cells. The secretory structures of peppermint (Howe, 1962), Tamarix, i.e., salt cedar (Campbell and Strong, 1964), the tarweeds of the Compositae (Carlquist, 1958), venus flytrap (Scala, Schwab, and Simmons, 1968), and others (Upholf, 1962) all originate in the same manner, from a single epidermal initial in the meristematic region.

Figure 13. Microprobe image of the surface of flower sepals. Note the numerous glandular hairs and glands (arrows). Magnification: 200X.

Figure 14. Corolla tube at the apex, opening out to form the lip of the flower. Note the smooth abaxial or outer surface of the corolla (os) as opposed to the adaxial or inner surface (is) of the corolla. Magnification: 100X.

Figure 15. The inner (adaxial) surface of the corolla tube. Note the scarcity of the glands and glandular hairs. Arrows denote some of the few present. Magnification: 100X.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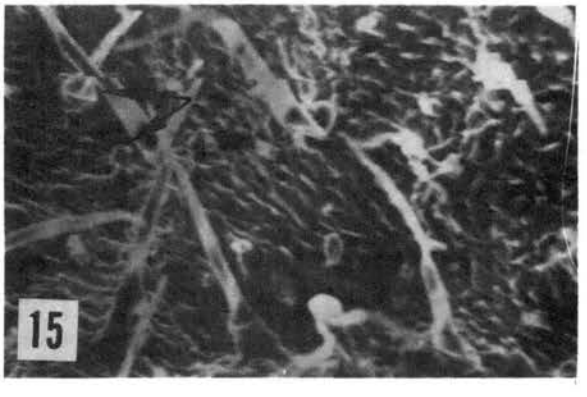
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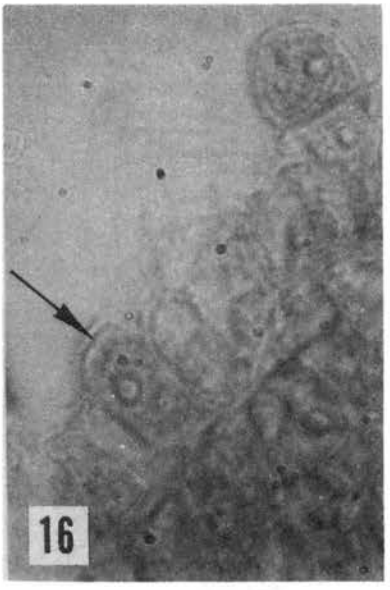
Figure 16. Epidermal initial of the secretory trichome, indicated by the arrow. Magnification: 1300X.

Figure 17. 2-cell stage of the developing secretory trichome. Note the paraclinal wall (arrow) cutting off the outer stalk cell initial (i). Magnification: 1300X.

Figure 18. Second paraclinal division (arrow) of the developing trichome resulting in the 3-cell stage of the glandular hair; indicated are the basal cell (b), stalk cell (s), and capitulum initial (ci). Magnification: 700X.

Figure 19. Second paraclinal division (arrow) of the developing trichome resulting in the 3-cell stage of the gland; indicated are the basal cell (b), stalk cell (s), and capitulum initial (ci). Magnification: 700X.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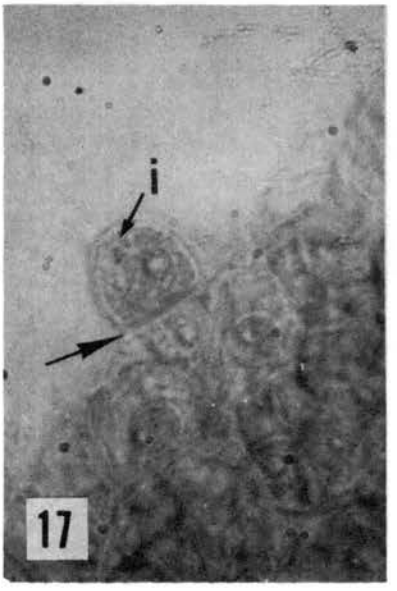
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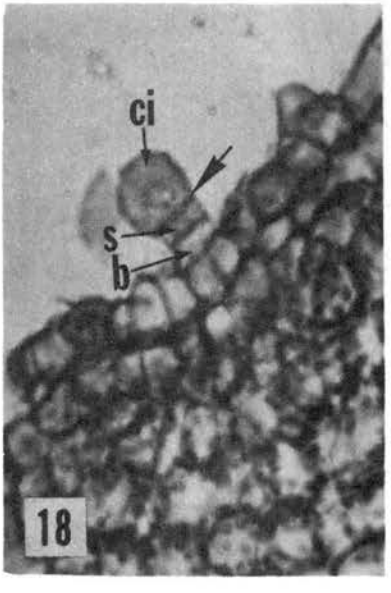
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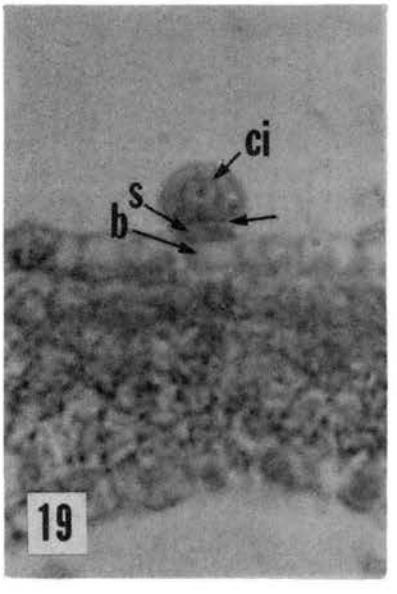
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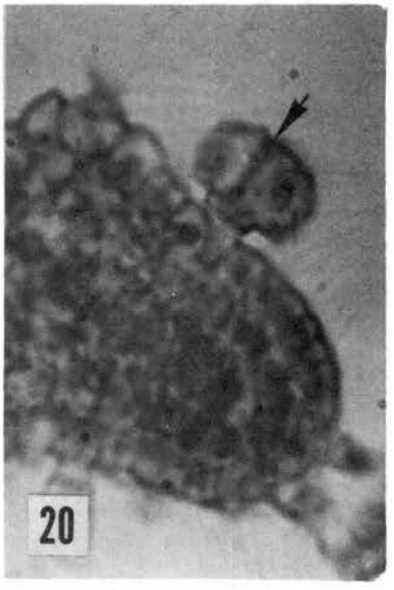
The first division of the epidermal initial is paraclinal, i.e., parallel to the surface of the stem or leaf, setting up a two-celled stage consisting of a basal cell and a stalk cell initial (Figure 17). The second division, a division of the stalk cell initial, involves the formation of the capitulum initial and is again paraclinal in nature. The uniseriate, i.e., consisting of a single layer of cells, tier of cells is composed of the basal cell, the stalk cell, and the capitulum initial (Figures 18 and 19). The third division to occur is anticlinal in nature, i.e., perpendicular to the plane of the leaf or stem axis, setting up the two-celled capitulum (Figures 20 and 21). It is at this point that development of the gland and glandular hair differs. For the glandular hair this is the final division and the structure will begin its secretory activity in this form and remain in this form. The gland, on the other hand, will undergo one additional anticlinal division resulting in a four-celled capitulum (Figure 22), and it is in this state that the structure begins its secretory activity. Previous to capitulum initial formation, it is impossible to distinguish between gland and glandular hairs. The stalk cell of the mature gland is always very compressed, broad, and densly cytoplasmic (Figure 2), while the stalk cell of the glandular hair is usually more vertically elongate and does not exhibit a densly staining cytoplasm (Figure 1). Also, the basal cell of the gland is more embedded in the epidermal layer, while the basal cell of the glandular hair protrudes above the epidermis. Based on these observations, it is possible, within limits, to distinguish between the developing glandular hair and gland once development reaches the capitulum initial formation stage. Examples of these characters displayed in the three-celled

Figure 20. Gland with capitulum composed of two cells following the first anticlinal division (arrow). Magnification: 700X.

Figure 21. Glandular hair following the anticlinal division (arrow) that set up the 2-cell secretory capitulum. Magnification: 700X.

Figure 22. Second, and last, anticlinal division of the gland resulting in the 4-cell secretory capitulum. Magnification: 700X.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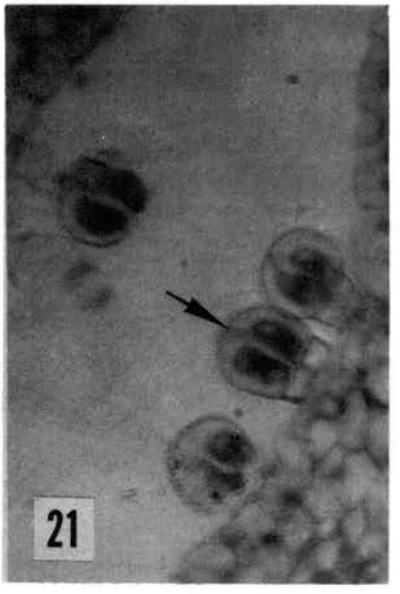
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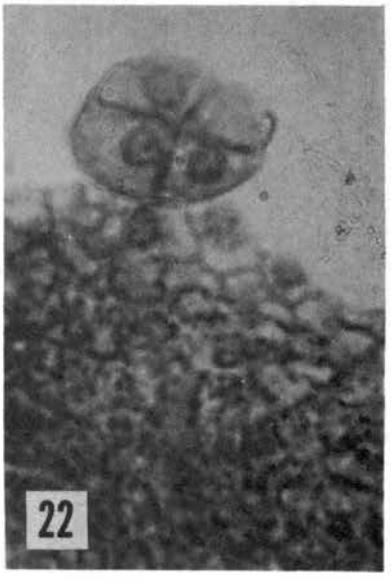
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stage are shown in figures 18 and 19.

One additional thing should be mentioned, and that is the general tendency for the gland to mature before the glandular hair. On developing stems and leaves maturing glands always appear earlier than the glandular hairs. This phenomenon has also been shown to occur in the tarweeds of the Compositae (Carlquist, 1958) and in peppermint (Howe, 1962). The more complex gland apparently starts developing earlier than the less complex secretory hair resulting in the maturation of both structures at approximately the same time. This might also be used in distinguishing between developing secretory hairs and glands. Developing structures found in the meristematic region, with no indication of maturing glands present, should be developing glands. When developmental stages are found in the presence of already developing glands, these then might be stages in the development of the secretory hair. This method of identifying developmental stages in unacceptable because by itself offers no tangible evidence for separating the developmental stages of the two structures.

The onset of secretory activity is indicated by a distention and separation of the cuticle from the cell wall forming the thin cuticular bladder into which the secretory products, including nepetalactone, are passed. It has been reported in Mentha (Howe, 1951) that, at least during certain phases of the life cycle, substances other than the essential oil are also secreted. This phenomenon of the secretion of substances varying in oil concentration from 100 per cent to something considerably less than 100 per cent may occur in Nepeta as it does in Mentha. This has been indicated by nepetalactone biosynthesis studies (Regnier, et.al., 1968) and will be mentioned in the discussion

on the histochemistry of the secretory structures. The bladder distention found in the gland is very pronounced while in the glandular hair it is very slight. Some additional indication of the differences between the two secretory structures was sought using electron microscopy and the results are discussed in the section on electron microscopy.

There is some disagreement concerning the nature of glandular capitulum cells after the onset of secretion and the filling of the cuticular bladder. Howe and Steward (1962) reported that while the cells in the capitulum of Mentha appeared to become disorganized and degenerate in light microscope preparations study of live material indicated that the cells of the capitulum may remain alive and intact as long as the cuticular bladder remained intact. On the other hand, Amelunxen (1965) reported that when studied with the electron microscope the contents of the gland capitulum cells of Mentha became disorganized and eventually became filled with a homogenous substance. The ultrastructure of the mature gland cell of Nepeta will be discussed as part of the ultrastructural analysis of the glandular capitulum. Figure 23 shows, diagrammatically, the developmental process of the glandular hair and gland.

Histochemical Study of Secretory Trichome Composition

After observing the structural changes occurring in the gland and glandular hair as maturation and the onset of secretion approached, an attempt was made to identify changes in the classes of macromolecular compounds. Microscopic histochemistry was employed in this study for the localization and identification of protein, carbohydrate, lipid,

Figure 23. Diagramatic Representation of the Developmental Sequence of the Gland and Glandular Hair.

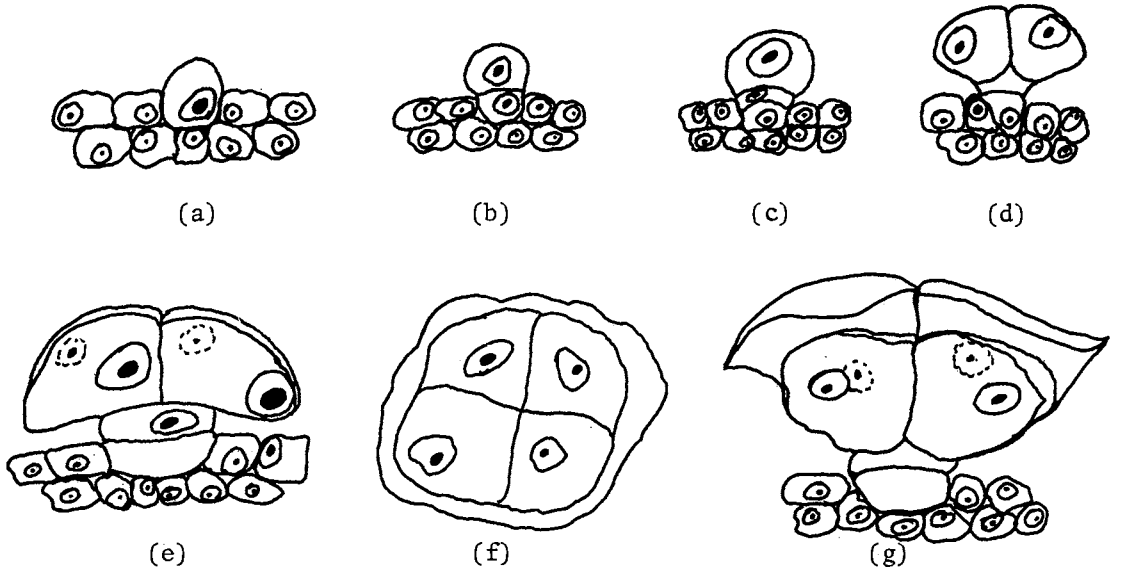
Gland Developmental Sequence:

(a) enlarged epidermal initial protruding above the epidermis.
(b) first paraclinal division of the initial creating the two-celled stage of the gland.
(c) second paraclinal division creating the three-celled stage composed of basal cell, stalk cell, and capitulum initial.
(d) first anticlinal division creating a two-celled capitulum.
(e,f) second anticlinal division setting up the four-celled secretory capitulum (l.s., c.s.).
(g) separation and distention of the cuticule to form the oil containing bladder.

Glandular Hair Developmental Sequence:

(a) protrusion of epidermal initial.
(b) first paraclinal division creating 2-celled stage.
(c) second paraclinal division creating 3-celled stage.
(d,e) first, and only, anticlinal division creating the glandular hair with a 2-celled capitulum (l.s., c.s.).

Gland Developmental Sequence



Glandular Hair Developmental Sequence

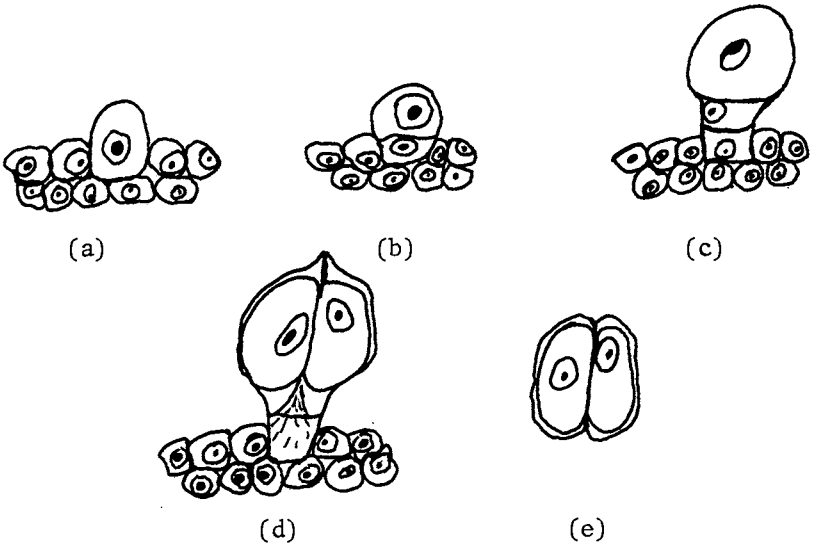


Figure 23

and pectin. Pre-secretory plants 10 days old were examined and compared with secretory plants 13 or more days of age. The presence or absence of the macromolecular components was noted using histochemical tests based on color identification of the compounds.

Carbohydrates

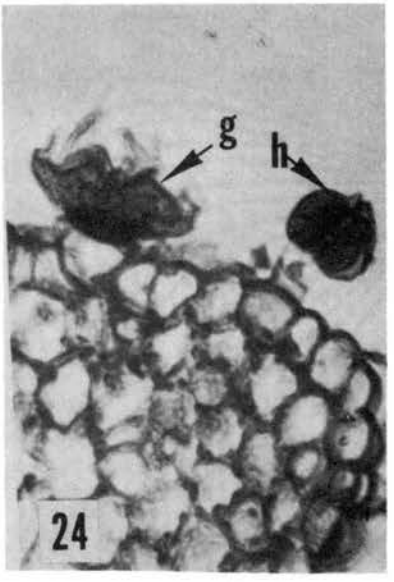
Localization of carbohydrates in microscopic histochemistry involves staining of insoluble polysaccharides since there is no procedure for the localization of soluble sugars in fixed tissue. The method used was the periodic acid-Schiff's reaction. In plant tissue 10 days of age the test for carbohydrates was positive, indicating the presence of polysaccharide (Figure 24). The corresponding acetylation blockade control gave a negative reaction, or rather, the tissue remained unstained (Figure 25). In this procedure the cytoplasm is supposed to remain colorless, the nuclei staining only lightly, the walls staining, and starch reacting very strongly. As can be seen in figure 24, the cell walls do stain strongly and the cytoplasm of cells around the glandular structures is clear. However, the secretory capitula of both gland and glandular hair are very densly staining structures. The gland shown in figure 24 has what appears to be an already functioning secretory capitulum, as noted by the distended cuticular bladder. The same observation can be made concerning the glandular hair. Two questions now arise concerning these observations. First, what is the composition of the densly staining material in the secretory capitulum; and second, are these structures functioning at this age? The densly staining material may be starch. It presumably cannot be composed of soluble carbohydrates since they are lost during tissue preparation.

Figure 24. Leaf tissue, in cross section, of a 10-day old plant stained for carbohydrate with the periodic acid-Schiff's test. Glandular hair (h) and gland (g) indicated show a positive reaction by staining intensely red. Note that the stalk cell of the gland stains while the stalk cell of the hair stains only slightly. Magnification: 700X.

Figure 25. 10-day old leaf tissue is cross section stained for carbohydrate after first being subjected to an acetylation blockade. This was used as a control. Note that the gland and glandular hair remain unstained. Magnification: 700X.

Figure 26. Carbohydrate test run on 20-day old plant tissue. Note the staining of the glandular hair cell walls and capitulum, and the staining of the stalk cell of the gland as well as the secretory capitulum (arrows). Magnification: 300X.

Figure 27. Acetylation blockade for the carbohydrate stain on plants 20-days old showing positive blocking of the carbohydrate aldehyde groups. Note that the glandular hair and leaf tissue remains unstained. Magnification: 700X.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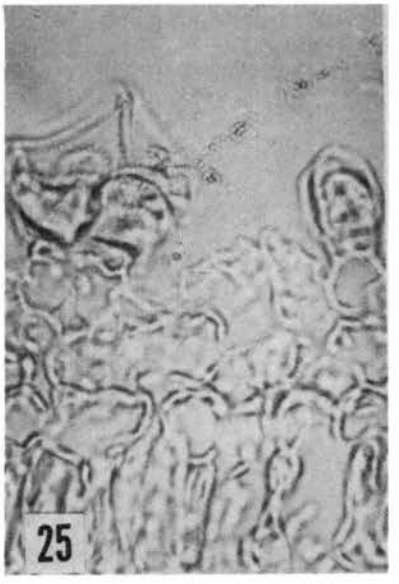
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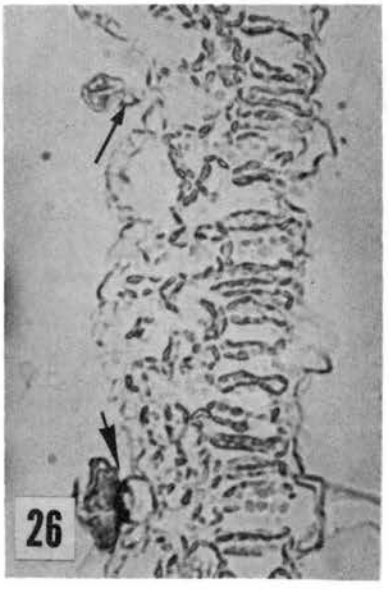
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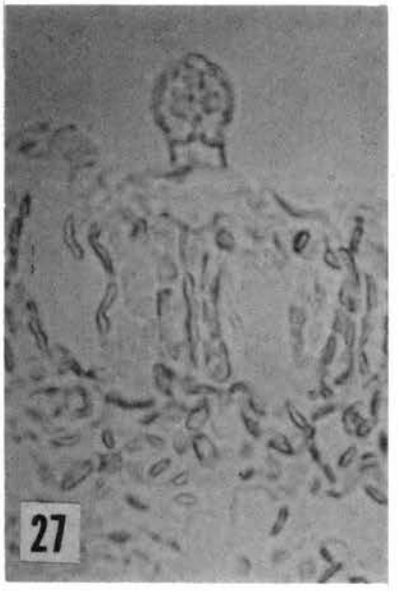
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The substance is a carbohydrate because the tissue, when first subjected to a acetylation blockade (Figure 25) before staining, gave a negative response. Since there are no additional histological tests for carbohydrate, the densly staining material is as yet unidentified. If the material is starch, a possible function would be to serve as an energy source for the secretion process.

In regard to the second question, it is known that the catnip plant begins the secretion of the oil, nepetalactone, at the thirteenth day after germination. However, this does not exclude the possibility of secretion of some other substance, possibly nepetalactone precursors, or the distended bladder might be an artifact of fixation, although this is unlikely based on the ultrastructural evidence obtained in a study on Mentha (Amelunxen, 1964). Once it is known where the final conversion of nepetalactone precursors into nepetalactone occurs, either in the surrounding plant tissue, in the glandular capitulum, or subcuticular space of the bladder, it might then be possible to identify this substance that is secreted prior to the appearance of nepetalactone at day 13.

Plants older than 13 days gave the same staining pattern as did the younger plants (Figure 26). The cell walls stained as expected, and the cells of the secretory capitulum stained intensely for carbohydrate. This effect was again negated when previous treatment with an acetylation blockade was administered (Figure 27, 28).

The periodic acid-Schiff's test run on Araldite embedded tissue did not give results different from those given from paraffin embedded tissue.

Protein

Protein localization was accomplished with the use of a procedure which visualized all proteins of the cell. Since the procedure was done on fixed and embedded tissue, all free amino acids must be assumed to have been lost. The results of the test, the ninhydrin-alloxan-Schiff's reaction, for the 10-day old plants were positive for protein, indicated by a bright red stain (Figure 29 and 30). The reaction wasn't as intense as the carbohydrate stain was, but the tissue did show a positive reaction. When acetylation (Figure 31) and deamination (Figure 32) blockades were first run before treating with the Schiff's reagent, a negative result occurred. Since these blockades are specific for the ninhydrin produced aldehydes, it is apparent that protein is present. The protein is localized in the secretory capitulum and stalk cell of the gland and in the capitulum of the glandular hair. Since nothing present in the cuticular bladder stained, it must be concluded that no proteins are present in the bladder under these conditions. Again, as found in carbohydrates, the soluble component, amino acids are lost during fixation and embedding. Since there appears to be no protein present in the cuticular bladder, it is probable that the enzymes responsible for the conversion of precursors into nepetalactone does not occur in the cuticular bladder.

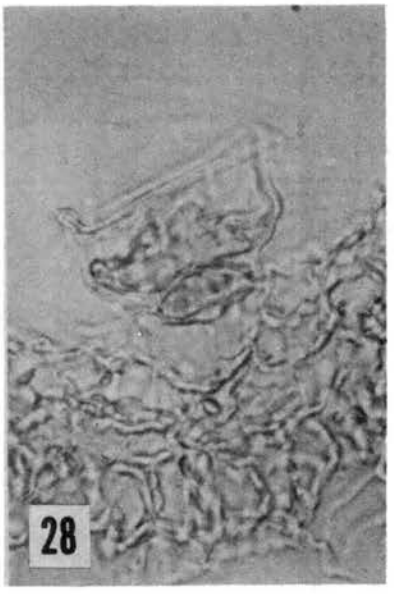
Plants that were older than 13 days exhibited the same pattern of staining as did the 10 day, pre-secretory plants. The glandular hairs reacted more strongly (Figure 33) than did the hairs of the younger plants, but this could be due to procedural variables rather than increases of protein present in the cell. The gland (Figure 34) stained heavily in the stalk cell and capitulum cells, as did the

Figure 28. Acetylation blockade for carbohydrate stain on plants 20-days old showing positive blocking of the carbohydrate aldehyde groups. Note that the gland remains unstained. Magnification: 700X.

Figure 29. 10-day old tissue stained for protein with the ninhydrin-Schiff's reaction; note the moderate staining of the secretory capitulum and stalk cell of the gland (arrow). Magnification: 700X.

Figure 30. Glandular hair of 10-day old leaf tissue stained for protein; the stain indicates the presence of protein in the secretory capitulum cells. Magnification: 700X.

Figure 31. Acetylation blockade run as a control on 10-day old tissue for the protein test. Note that the glandular hair (arrow) did not stain, as expected, but that the gland appears to have stained. The gland did not, however, stain the purplish-pink color indicative of the Schiff's stain but appears dark due to dense cytoplasmic composition. Magnification: 300X.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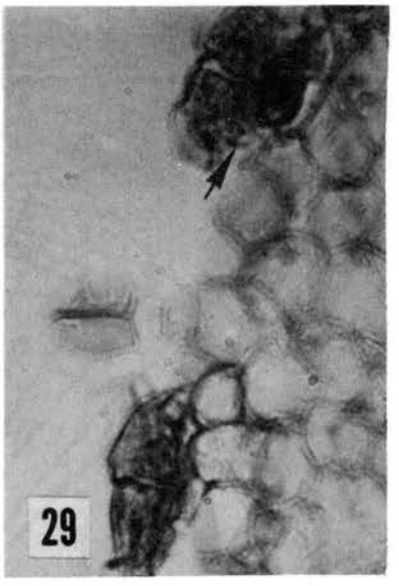
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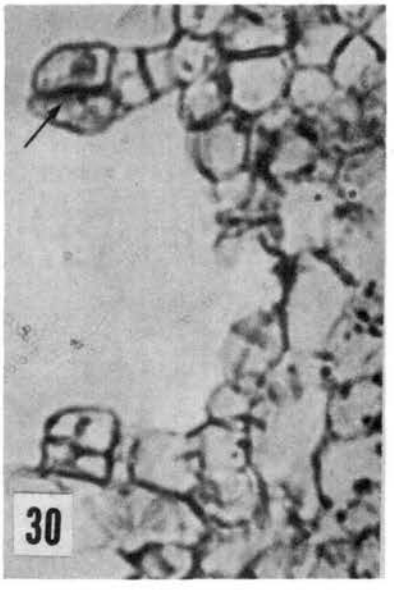
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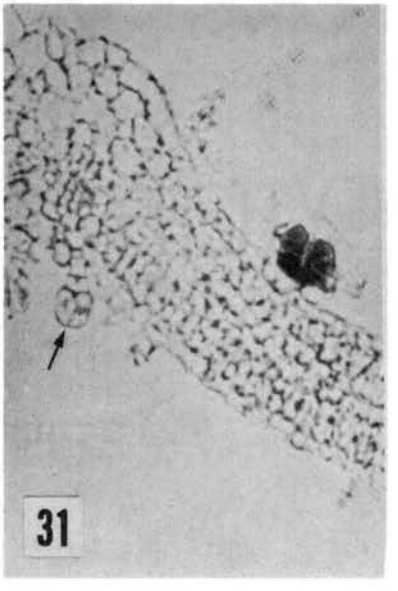
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younger plant glands. This may be indicative of a more active role of the gland in secretion as compared to the glandular hair. Deamination and acetylation blockade control tests were run and resulted in complete blocking of the staining action (Figures 35, 36, 37). Nothing in the cuticular bladder stained for protein.

It must be stressed at this point that the procedures used for protein localization are not fully understood in their action on plant tissues (Jensen, 1962). It is for this reason that results of these procedures should be used with caution and not relied upon too strongly. The intent of this work was to identify relative changes in amounts or presence of these compounds.

Pectin

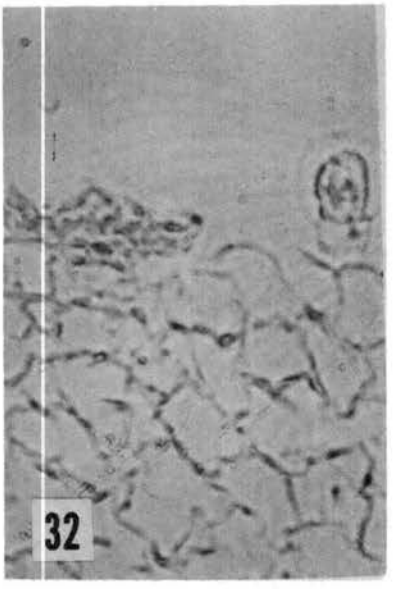
Pectin substances are some of the more important chemical components of the cell wall. Localization techniques tried were the standard ruthenium red stain and the newer method employing hydroxylamine-ferric chloride as the stain. In the latter staining procedure the amount of color in the tissue depends on the amount of esterified pectins and on the degree of esterification. The results obtained with the ruthenium red stain were positive. Figure 38 shows a glandular hair with the walls stained heavily. The stain gives a rather grainy appearance to the material. It does not stain smoothly as does the PAS stain. A gland is shown in figure 39 with cell wall reacting and a more heavily stained region in the secretory capitulum. Since the basis of this reaction is only partly understood, it can be considered only partially specific (Jensen, 1962).

Figure 32. Deamination blockade used as a control in the ninhydrin-Schiff's test for protein in 10-day old tissue. Note that the stain was effectively blocked by the control procedure, thus indicating that the positive reaction in figures 29 and 30 was due to the staining of protein. Magnification: 700X.

Figure 33. Protein-stained tissue, 20-days old. Note the staining of the glandular hair capitulum (arrow). Magnification: 300X.

Figure 34. Cross section through 20-day old leaf tissue stained for protein localization. Note the staining of the gland capitulum and stalk cell (arrow). Magnification: 700X.

Figure 35. Deamination blockade used as a control for the protein test in 20-day old plant tissue. Note that the gland stalk cell has apparently stained (arrow). This is not the case, however, since the structure did not stain the redish-purple of the ninhydrin-Schiff's stain, but appears dark due to the natural density of the cell cytoplasm. The glandular hair remained unstained also. Magnification: 300X.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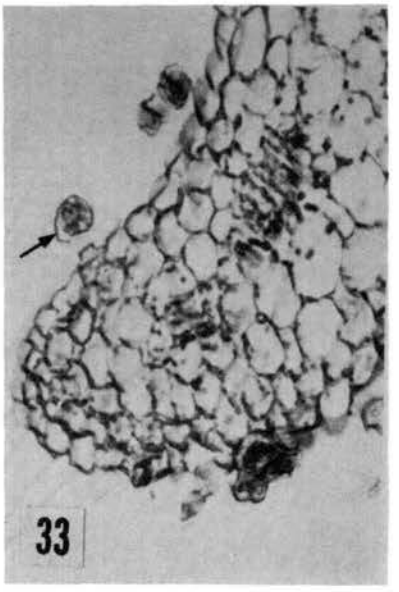
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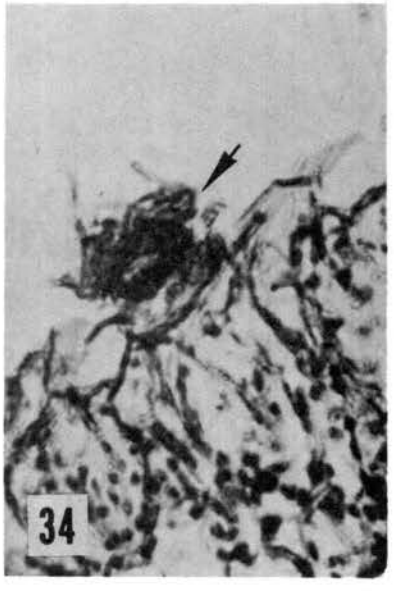
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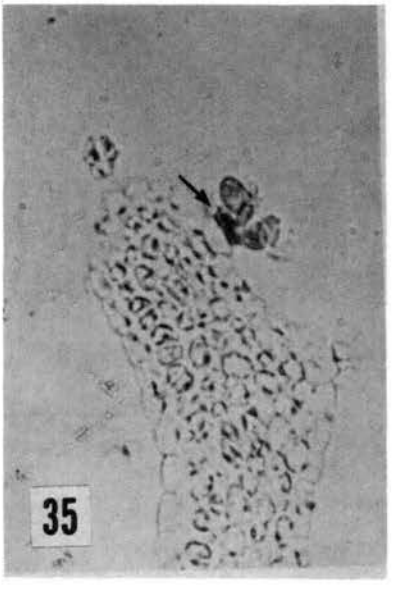
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Results from the more pectin-specific reaction employing hydroxylamine-ferric chloride were disappointing. The material used was fixed in cold neutral buffered formalin and embedded in paraffin; and due to this pre-fixation, the stain is an unstable one as far as giving positive or negative results. The presence or absence of a pectin layer in the walls of the glandular cells should be verified by electron microscopy.

Lipid

The oil soluble colorants provide the most widely used general test for lipids even though not all free lipids can be demonstrated (Casselman, 1959). The coloration of the lipids by the colorant depends upon the reagent dissolving in the lipids, thus the lipids having lower melting points color more readily than do lipids having higher melting points. The Sudan dyes are among the most frequently used oil-soluble colorants. Sudan III was used in this study.

In plants 10 days of age the test for lipid was negative for glandular hair and gland bladder contents. The cuticle of these plants, however, did react positively. Figures 40 and 41 show the presence of gland and glandular hair with definition of the structures due to the Sudan staining of the cuticle. In figure 40 the gland shows a distended cuticular bladder devoid of any globlets inside the bladder. The grainy appearance of the cytoplasm of the gland and glandular hair in figures 40 and 41 is due to the normal cytoplasmic density since it occurs even in unstained tissue.

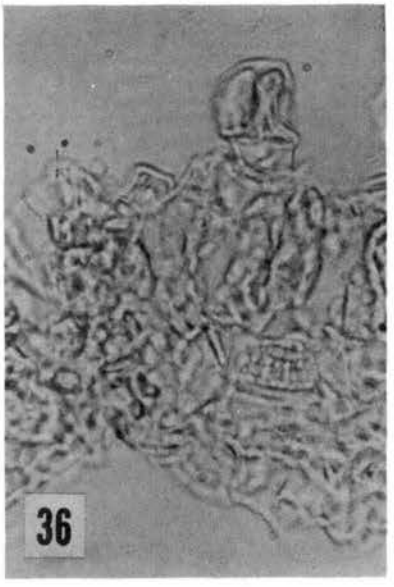
In plant tissue taken from mature flowering plants glandular hairs again give a negative reaction, as seen in figure 42. The cuticle

Figure 36. Acetylation control for the protein test in 20-day old tissue. Note that the control was effective in blocking the staining of the glandular hair. Magnification: 700X.

Figure 37. Acetylation control for the protein test in 20-day old tissue. Note that there is no staining of the gland. Magnification: 700X.

Figure 38. 10-day old tissue stained for pectin. Note the staining of the cell walls of the glandular hair (arrow). Magnification: 300X.

Figure 39. 10-day old tissue stained for pectin. Note the staining of the cell walls of the gland (arrow). Magnification: 300X.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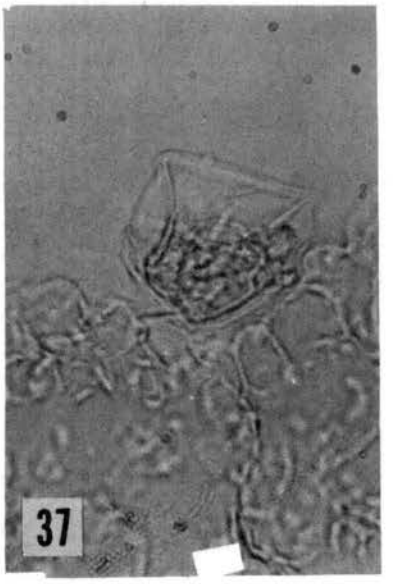
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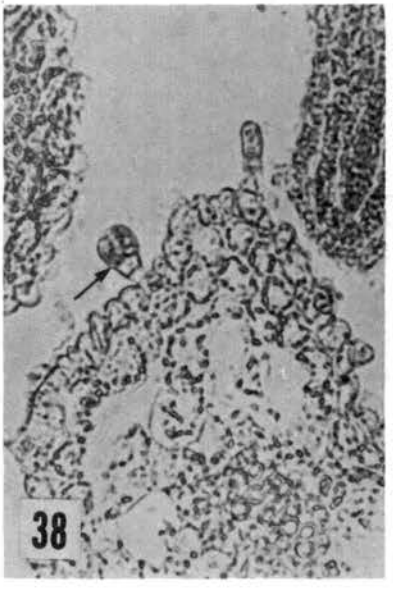
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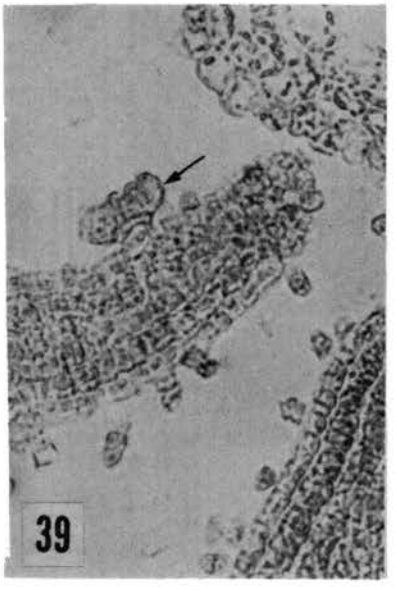
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reacts slightly but cuticular bladder contents indicate that no lipid droplets are present. The cytoplasm of the glandular hair is noticeably contracted into the middle of the capitulum leaving the space suspected of containing nepetalactone to the periphery of the capitulum cells. The reaction of the Sudan stain in the gland, however, is positive, as seen in figures 43 and 44.

Figure 43 indicates a gland with obviously staining lipid as shown by the droplets present in the cuticular bladder. Figure 44 shows the gland in the paradermal plane and indicates the presence of lipid in the bladder.

Nepetalactone, a terpenoid, is not stained by the lipid stains, although it is called an "essential oil." There appears to be no correlation between nepetalactone occurrence and the presence of lipid, since the glandular hairs of both pre- and post-secretory tissue gave negative staining results. However, since there is apparently no lipid present in the pre-secretory tissue, it is most unlikely that the positive reaction of the periodic acid Schiff's test for carbohydrates was caused by the staining of aldehydes produced from the air oxidation of unsaturated fatty acids.

Ultrastructural Aspects of the Secretory Trichomes

It was the purpose of this part of the study to observe the presence of cellular organelles at the ultrastructural level and to note the presence or absence of certain organelles and their distribution within the secretory gland cytoplasm. Several different methods were employed in the preparation of the tissue for ultrastructural examination. However, none of the methods proved sufficient for solving

Figure 40. Sudan III stain for lipid in 10-day old plant tissue. The cuticle covering the epidermis and comprising the cuticular bladder of the gland stain positively for the presence of lipid. Note, however, that contents of the bladder do not stain (arrow). Magnification: 700X.

Figure 41. Sudan III stain for lipid in 10-day old plant tissue. Again note the staining of the cuticle. The glandular hair at this stage does not have a distended cuticle surrounding the capitulum and there is only staining of the cuticle around the glandular capitulum. Magnification: 700X.

Figure 42. Sudan III stain for lipid on tissue from a mature flowering plant. Note the glandular hair with the degenerating cytoplasm; there does not appear to be any lipid present taking up the Sudan stain. Magnification: 700X.

Figure 43. Sudan III stain on tissue from a flowering plant. Shown are the lipid globules present in the gland bladder (arrows). Magnification: 700X.


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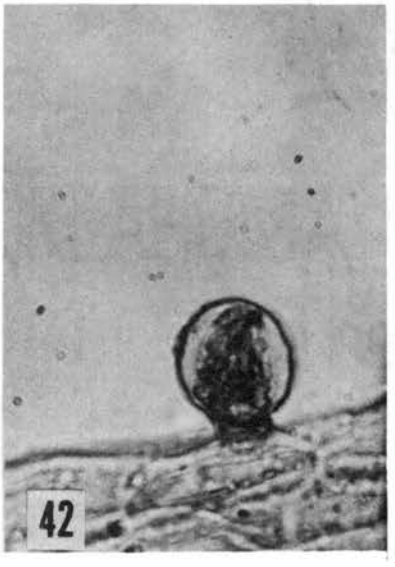
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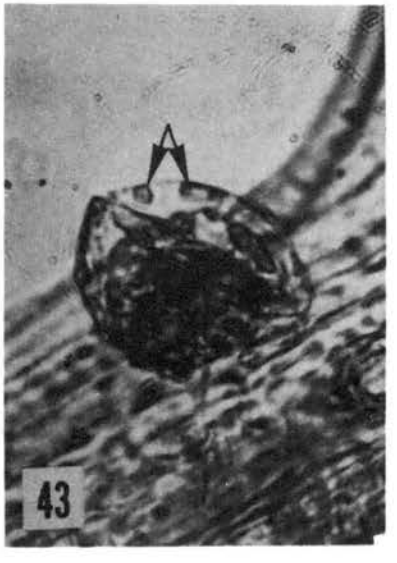
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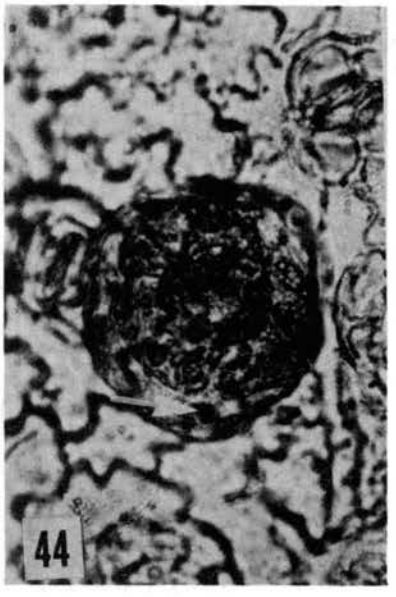
Figure 44. This is a paradermal view again showing the presence of the lipid in the gland bladder (arrow). Magnification: 700X.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problems incurred. The major problem incurred was in dealing with the infiltration and embedment of the tissue. Araldite 502 resin was used as the embedding medium, and due to its viscosity infiltration of the resin into the tissue was not achieved. The consequence of only partial embedment of the tissue was that during thin sectioning the tissue tore out of the resin due to its uneven hardness in the resin. To correct the situation, low heat was applied during infiltration under a vacuum. The result was a partial polymerization of the resin due to both the temperature and the evaporation under the vacuum of the solvent for the resin. When infiltration was attempted using no heat and a vacuum or done at room temperature, penetration was not uniform throughout the tissue.

Possible reasons for the embedding difficulties could stem from the fact that the relatively young 10 and 20 day old tissue had cuticle present in amounts that would disallow infiltration of the tissue with a medium of this viscosity. Another possibility, although unlikely is that complete fixation of the tissue was never achieved. This was probably not the case, however, since the tissue did become blackened due to the disposition of osmium. This is usually an indication of proper fixation. It is recommended that for the continuation of this study of the ultrastructural aspects of the catnip gland cytoplasm an embedding medium of a lower viscosity be employed and thus avoid embedding difficulties.

CHAPTER V

SUMMARY AND CONCLUSIONS

To study the development and histochemistry of the secretory trichomes of Nepeta cataria, the use of paraffin as an embedding medium proved reliable in all cases except for lipid histochemistry which necessitated the use of fresh tissue. All of the histological work was done using Johansen's quadruple stain and the histochemical work was done using standard reagents specific for the desired compounds to be stained.

The study showed that there are two separate secretory structures present on the stems, leaves, and flowers of the catnip plant. The larger of the two structures is a gland composed of a basal cell, a stalk cell, and a secretory capitulum composed of four cells surrounded by a raised cuticle forming a cuticular bladder. The second structure is the glandular hair also having basal and stalk cells but having a capitulum composed of only two cells. The development from a single protodermal cell to the two-celled capitulum stage proceeds identically in both structures, the gland then proceeding one division more forming the four-celled capitulum. The sequency of development was found to consist of a primary periclinal division of a protodermal cell yielding the two cell stage. The second periclinal division occurred in the most distil of the two cells forming a three-cell stage consisting of the basal cell, stalk cell and capitulum initial cell. The glandular

hair was found to undergo one anticlinal division to form the two-celled capitulum. The gland was found to undergo two anticlinal divisions to form the four-celled capitulum.

Histochemical studies were done for the presence of carbohydrates, proteins, pectin, and lipids. Changes in their presence as plants matured from the pre-secretory to the secretory stage were noted. Carbohydrate protein and pectins were all found present in both pre-secretory and secretory structures with no noticeable changes in amounts with maturity. The presence of lipids was only noted in the cuticle in both age-groups and in the cuticular bladder of the secretory plants. No lipids were found in the bladder of pre-secretory plants.

With the aid of an electron microprobe X-ray analyzer scanning microscope a study was made of the secretory structures in regard to distributional patterns and the cuticular bladder morphology. It was found that both glands and glandular hairs occur on the leaves and stems with no apparent concentration in any particular area, e.g., along veins. While both structures were found to be present on the flower sepals and petals, there were many more of both structures on the sepals. They were densely covered with glandular hairs and glands. The corolla tube was found to be totally devoid of any trichomes on the abaxial surface while there are many sterile hairs and a few glands and glandular hairs scattered over the adaxial surface and down into the tube. The gland capitulum of secretory plants was found to have, at high magnification, small bodies that could be pores. These structures were not found on pre-secretory gland capitula.

Attempts were made to study the ultrastructure of the secretory

structures and observe changes in the cytoplasm as maturity was achieved. However, due to problems involved with embedding the material in resin, no good results were achieved.

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