

Accumulation of Cannabinoids in Glandular Trichomes of *Cannabis* (Cannabaceae)

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ABSTRACT. Sessile- and capitate-stalked secretory glands are sites of cannabinoid accumulation in *Cannabis* (Cannabaceae). Analyses show cannabinoids to be abundant in glands isolated from bracts or leaves of pistillate plants. Cannabinoids are concentrated in the secretory cavity formed as an intrawall cavity in the outer wall of the disc cells. Specialized plastids, lipoplasts, in the disc cells synthesize lipophilic substances, such as terpenes, that migrate through the plasma membrane and into the cell wall adjacent to the secretory cavity. These substances enter the cavity as secretory vesicles. An antibody probe for THC shows it to be most abundant along the surface of vesicles, associated with

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This study was supported with stipends from Konkuk University in 2001 (ESK), and Indiana University Faculty Research Fund (PGM). Drug Enforcement Administration registration number P0043113.

Journal of Industrial Hemp, Vol. 9(1) 2004
<http://www.haworthpress.com/web/JIH>

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Digital Object Identifier: 10.1300/J237v09n01_04

fibrillar material in the cavity, in the cell wall and in the cuticle; little THC was detected in the cytoplasm of disc or other cells. The phenol, phloroglucinol, is abundant in both gland types. A working hypothesis for the site of cannabinoid synthesis is proposed, and must be examined further. Knowledge of the mechanism of cannabinoid synthesis and localization can contribute to efforts to further reduce the THC content in hemp strains for potential agricultural use in the United States and elsewhere. [Article copies available for a fee from The Haworth Document Delivery Service: 1-800-HAWORTH. E-mail address: <docdelivery@haworthpress.com> Website: <<http://www.HaworthPress.com>> © 2004 by The Haworth Press, Inc. All rights reserved.]

KEYWORDS. *Cannabis*, glands, secretory cavity, cannabinoids, THC, localization

INTRODUCTION

THC (Δ^9 -tetrahydrocannabinol) is one of many cannabinoids present in *Cannabis*. However, the cellular location of THC, the major psychoactive cannabinoid, remains less generally known. Our studies have been directed to determining where cannabinoids are localized at the whole plant and the cellular levels, with a long-term objective to determine the organelle or membrane in the cell involved in cannabinoid synthesis. It also should be possible to identify the gene, or genes, responsible for synthesis of these compounds, THC in particular, and modulate them so as to develop strains of *Cannabis* with low THC levels, or no cannabinoids.

The first phase of this study was to determine localization of THC in the plant. As part of this program, we initiated an effort to accumulate a germplasm collection of various *Cannabis* strains of world-wide distribution that are utilized in hemp cultivation in the classical sense, as well as seed oil strains, and those with different levels of THC. We have utilized these strains for analyses of cannabinoids and their distribution; they also can serve as a germplasm source for future studies.

The purposes of this study are to (a) describe the development of glandular trichomes, (b) describe the distribution of cannabinoids, including THC, and related compounds in specific regions of the flowering plant, and (c) propose a possible locus for cannabinoid synthesis. This report is primarily a review of studies from the Indiana University laboratory.

MATERIALS AND METHODS

For the localization studies, typically a Mexican THC strain (~6% THC) was employed, although in some phases comparisons were made with a low THC or fiber strain (~0.4% THC), or with one possessing an intermediate level of THC.

Plants were grown from seeds in pots under greenhouse conditions. They were placed under short-day conditions (8 hr. day length) to induce and maintain flowering plants, or maintained in the vegetative condition under long-day conditions (18 or more hours of light) with supplemental lighting (Hammond and Mahlberg, 1973; Mahlberg, unpublished).

Data on morphological comparisons were obtained from populations of 10 or more plants maintained under a specific day-length condition. Since plants from the same seed sources were grown many times under these conditions we had repeated opportunities to examine their morphological features during these studies. Features examined included seedling development, leaf morphology, flowering character of both pistillate (female) and staminate (male) plants, individual flower characters, glands and hairs on leaves and floral bracts at all stages in development (Hammond and Mahlberg, 1977, 1978; Kim and Mahlberg, 1991; Mahlberg et al., 1984; Mahlberg and Kim, 1991, 1992).

Phenol contents of glands and other tissues were examined by thin layer chromatography (TLC), gas liquid chromatography-mass spectroscopy (GC-MS), and nuclear magnetic resonance spectrometry (NMR), and terpenes were analyzed by GC-MS (Hammond and Mahlberg, 1990, 1994, unpublished).

Cannabinoids were identified by gas-liquid chromatography (GC). Weighed samples of leaf material at similar stages in development from different plants, weighed samples of bract or other floral materials also at similar stages in development, isolated contents of whole glands or only the secretory contents of the non-cellular cavity of the gland were extracted with ether or chloroform and evaporated to dryness. Samples were redissolved in a given quantity of absolute alcohol containing the internal standard, androstenedione (1 mg/ml), and injected onto the GC column. Analyses were performed on a Hewlett-Packard 5710A chromatograph equipped with a hydrogen flame-ionization detector and programmed from 200-240°C (2°C/min). Nitrogen (20 ml/min) was used as the carrier gas. Injection port and detector temperatures were 250 and 300°C, respectively. Glass columns (2 mm × 2.43 m) were packed with 3% OV-1 Supelcoport (80/100 mesh). Quantitative data were deter-

mined with a Hewlett-Packard integrator (3380A), and samples were compared with cannabinoid standards obtained from the National Institute on Drug Abuse (Hemphill, Turner, and Mahlberg, 1980; Turner and Mahlberg, 1984; Turner, Hemphill, and Mahlberg, 1980).

Whole gland heads were removed individually from leaf or bract surfaces with a tungsten microneedle under a stereomicroscope and placed in vials containing chloroform to extract the cannabinoids. The needle was rinsed in chloroform after collecting each gland to avoid cross-contamination between collected glands. Samples of twenty to over 100 glands were collected for different sampling periods and analyzed by GC (Turner, Hemphill, and Mahlberg, 1978).

Contents of the secretory cavity were obtained by glass microcapillary micromanipulation. Bracts containing numerous glands were mounted on a special slide holder under a microscope lens. Microcapillary tubes, made with a Knopf micropipet puller and ground with diamond paste to a hypodermic tip, were mounted in a modified Leitz manipulator system under a microscope lens to probe into the secretory cavity without touching or damaging the disc cells. Microcapillary tubes with secretory cavity contents were placed in small acetone-washed test tubes containing solvent to dissolve contents, and subsequently prepared for GC analyses (Lanyon, Turner, and Mahlberg, 1981; Turner, Hemphill, and Mahlberg, 1978).

Tritium-labeled olivetol (2-pentyl 4,6-dihydroxybenzene) was fed to seedling shoot tips to examine its incorporation into cannabinoids. Its incorporation was evaluated by scintillation, GC and HPLC (Turner and Mahlberg, 1988).

For scanning electron microscopy (SEM), bracts or other tissues were fixed in a buffered glutaraldehyde solution, rinsed in buffer, dehydrated through an ethanol series to acetone and critical-point dried. Samples then were mounted on specimen holders and examined at 20 kV with an SEM (Hammond and Mahlberg, 1973). This technique was used to examine gland morphogenesis, and combined with GC to analyze gland distribution on organs and their cannabinoid composition during their morphogenesis (Hammond and Mahlberg, 1977; Turner, Hemphill, and Mahlberg, 1977).

For transmission electron microscopy (TEM), bracts with glands were prepared by high-pressure cryofixation-cryosubstitution, for the THC antibody procedure. These tissues were fixed under cryofixation-substitution conditions in glutaraldehyde alone or in glutaraldehyde and osmium tetroxide, embedded in resin, sectioned and treated with the antibody probe, stained, and examined with a TEM at 60 kV.

The monoclonal antibody for THC, obtained from Roche Diagnostic Systems, was prepared in mouse ascites; it was normally used to screen humans for drug use. We developed it as an electron microscopic probe. Antibody-treated sections were visualized for THC with protein-A bound gold particles (20-nm, Jannsen, Belgium). Controls included sections treated with antibody alone, or treated with protein-A-gold alone (Kim and Mahlberg, 1997a). For developmental studies, bracts at different stages in development were fixed in glutaraldehyde, post-fixed in osmium tetroxide and embedded in resin. Thin sections were stained with lead citrate and examined at 60 kV with a TEM (Kim and Mahlberg, 1991, 1995, 1997b, 1999; Mahlberg and Kim, 1991, 1992).

RESULTS AND DISCUSSION

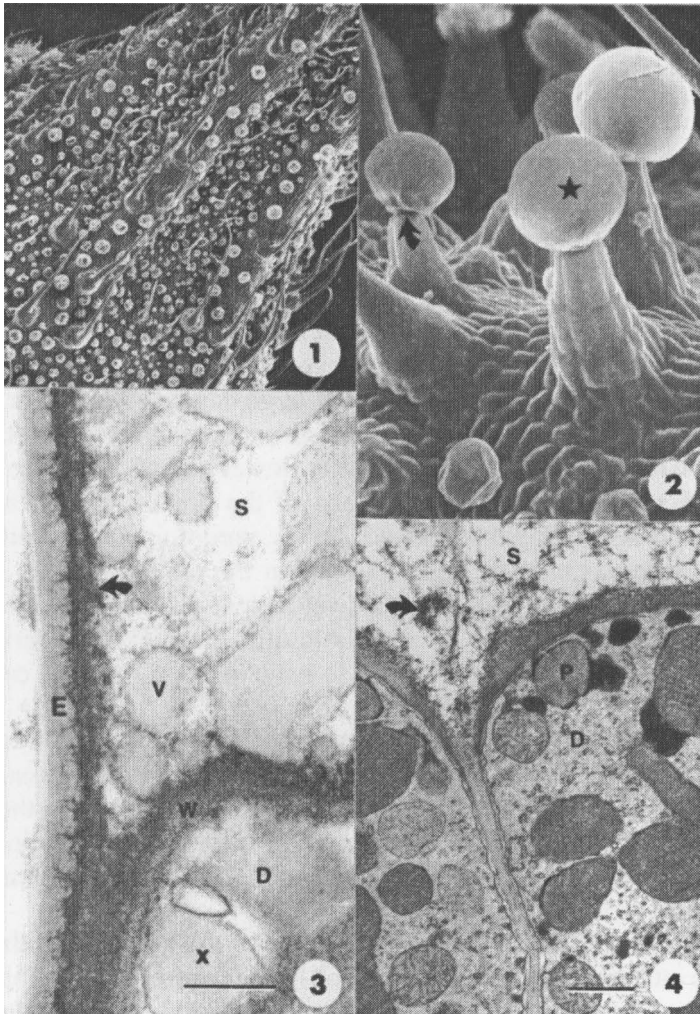
Types and Distribution of Glands

Glands cover the surface of the above ground portion of both pistillate and staminate plants, but are most abundant on bracts of pistillate plants (Figure 1). Three types are recognized: (1) the small bulbous form present on most surfaces, (2) the large capitate-sessile form also present on most surfaces, and (3) the large capitate-stalked form that develops on leaves and bracts formed after flower initiation (Figure 2). All three are present on the undersurface of the bract that subtends the flower and seed. Capitate-stalked glands predominate on veins and veinlets whereas capitate-sessile occur on the nonvein areas. Apparently some factor formed during flower formation, and most abundant along vascular tissue, provides the stimulus for stalk formation. The capitate-stalked gland is interpreted to have evolved from the capitate-sessile form (Hammond and Mahlberg, 1977).

A gland consists of a globose head attached to a leaf or bract with stipe and basal cells, the latter embedded in the leaf or bract tissue. The stalk consists of epidermal and subdermal cells, adjacent to the basal cells, that undergo multiplication and elongation to form an elongated column of cells which raises the gland head above those of capitate-sessile glands.

An abscission zone develops at the base of the head where the stipe cells are attached to the disc cells of the head (Figure 2). Disturbance of the head can result in detachment of the gland head from the plant.

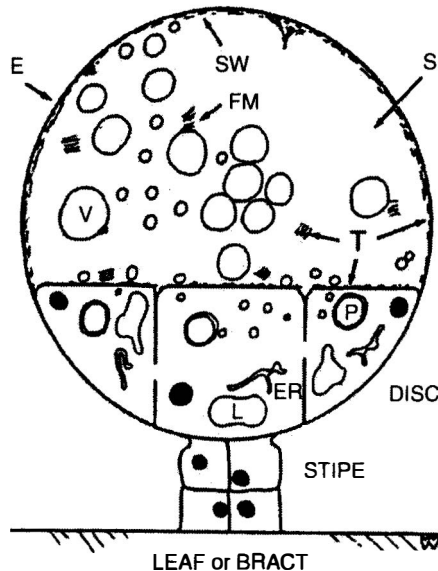
FIGURES 1-4. Secretory glands. 1. Capitately-stalked and capitately-sessile glands on underside of bract. Hairs also are present on bract $\times 35$. 2. Capitately-stalked gland showing large head (star) and abscission zone at base of gland head (curved arrow) $\times 300$. 3. Section of gland head showing relationships of secretory cavity (S), disc cells (D), cuticle (E) and subcuticular wall (arrow). Vesicles (V) occur in secretory cavity and secretions occur in disc cell just below the wall (W, and at X). Bar = 0.5 μm . 4. Portion of gland showing two disc cells, one at D, each with numerous lipoplasts (P) containing secretions (black). Portion of secretory cavity (S) is evident. Fibrillar matrix (arrow) has separated from wall. Bar = 0.5 μm .



Development of the Gland

For both capitate-sessile and capitate-stalked glands an epidermal cell enlarges and divides several times to form a tier of disc cells on a short stipe attached to basal cells in the epidermis of the leaf or bract (Diagram 1). The outer wall of the disc cells splits tangentially to initiate an intrawall cavity across the top of the entire gland surface. This cavity enlarges as secretions are accumulated in it (Figure 3). The outer portion of the wall (curved arrow) remains associated with the cuticle to form the subcuticular wall; the inner portion (W) remains associated

DIAGRAM 1. Mature secretory gland. Disc cells, attached to leaf or bract by stipe cells and basal cells (below stipe), release secretory vesicles (V) into secretory cavity (S). Lipoplasts (P) in disc cells produce secretions that accumulate outside plasma membrane, and pass through cell wall as hyaline areas (small circles) that enlarge to form secretory vesicles in secretory cavity. Fibrillar matrix (FM), derived from cell wall, contributes to surface feature of vesicles and also is dispersed throughout cavity. Vesicle content in contact with subcuticular wall (SW) contributes to thickening of this wall and cuticle (E) during enlargement of secretory cavity. THC (T) occurs in walls, fibrillar matrix, surface feature of vesicles and cuticle, but not inside vesicles; little THC is present in cytoplasm of disc cells. Nucleus, black; vacuole, L; endoplasmic reticulum, ER.

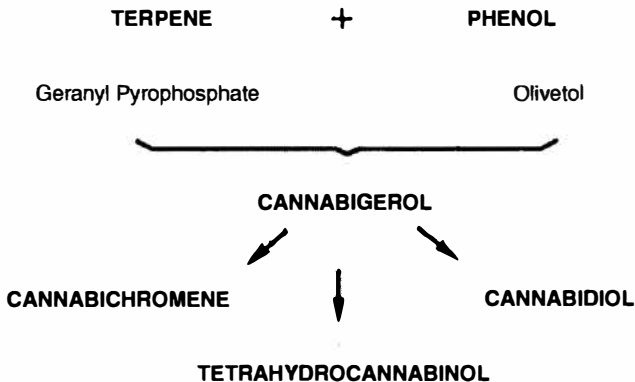


with the disc cells. Both the cuticle (E) and subcuticular wall increase in thickness as the secretory cavity (S) enlarges and, therefore, precursors for their growth must be present in the secretory cavity. Secretions as vesicles (V) are evident in the secretory cavity. Note that the secretory vesicles are similar in density (grayness) to the gray secretions (D) in the disc cell. The mechanism that controls the thickening of cuticle and subcuticular wall is as yet unknown.

THC and Other Cannabinoids

Cannabinoids are a class of specialized compounds synthesized by *Cannabis*. They are formed by condensation of terpene and phenol precursors (Diagram 2). They include these more abundant forms: Δ^9 -tetrahydrocannabinol (THC), cannabidiol (CBD), cannabichromene (CBC), and cannabigerol (CBG). Another cannabinoid, cannabinol (CBN), is formed from THC as a degradation product and can be detected in some plant strains. Typically, THC, CBD, CBC, and CBG occur together in different ratios in the various plant strains. In fiber strains, CBD/CBC are at high concentrations and THC is at a low concentration; in drug strains, THC is high and CBD/CBC may be low. An important area to be studied herein relates to the occurrence in the plant of potential precursors for cannabinoid formation and the site where synthesis occurs in the cell.

DIAGRAM 2. Cannabinoid pathway. Cannabinoids are formed from terpene and phenol precursors. Cannabigerol is interpreted to be the first cannabinoid component. It undergoes chemical change to enzymatically form cannabichromene, cannabidiol or tetrahydrocannabinol.



Cannabinoid Content of Glands

Effect of Gland Position, and of Seasonality

Both sessile and stalked glands were examined for their respective cannabinoid contents. Whole glands (20 glands) were removed from bracts and leaves of the same plant to compare their contents. They were extracted for cannabinoids and analyzed (Table 1). They also were separated as to glands over vein as contrasted to nonvein areas for the periods October and December. Results showed that stalked glands over a vein on the bract contained more THC, approximately 20 times more, than the sessile glands over a leaf vein. Similarly, stalked glands over a nonvein area contained much more THC than sessile glands over the leaf nonvein area. Surprisingly, even stalked glands on a bract varied for THC content between the vein and nonvein area. Sessile glands also were found to vary for contents between vein and nonvein areas on the leaf.

Comparison of October with December analyses showed again that stalked glands contained considerably more THC than sessile glands. For both types, however, the vein glands now showed less than the nonvein. Interestingly, the level of THC can decrease to a non-detectable level in glands on the leaf.

Sampling in this strain was repeated during the following March and, again, the stalked glands on the bract contained higher concentrations of THC than sessile glands on the leaf. Thus, a repeatable pattern appeared to occur in the plant in which stalked glands usually contained greater quantities of THC than sessile glands.

TABLE 1. THC content in capitate glands of different ages of a drug strain.

Gland type	Organ	THC/gland, ngs.	
		October	December
Capitate-stalked	Bract		
	Vein	224.00	69.30
	Nonvein	120.20	97.50
Capitate-sessile	Leaf		
	Vein	9.66	ncd
	Nonvein	6.92	6.05

ncd, no cannabinoids detected

Effect of Gland Age on Cannabinoid Content

Cannabinoid content of capitate-stalked glands was examined in glands of different ages to measure the major cannabinoid components in both a fiber and a drug strain (Table 2). Glands, viewed under a stereomicroscope, can be classified according to their secretory phases from the color of their contents. Glands most active in secretion (mature) were translucent in appearance, aged glands were yellow and senescent glands were brown. Mature glands possessed the highest content of their major cannabinoid in both the fiber and drug strains. Senescent glands possessed low levels of cannabinoids. The concentration of some components, as CBD in the drug strains, may be too low to be detectable in our analysis; similarly, for THC and CBN in the fiber strain.

It is unknown what happens to the cannabinoids during the aging process, but they may polymerize, be transported elsewhere in the plant, or volatilize from the secretory cavity into the atmosphere along with the terpenes, as noted later. Nevertheless, this phenomenon of altered cannabinoid content in glands during aging is one that should be studied to gain a more complete understanding of the secretory process in the cell and secretory cavity.

Contents of Secretory Cavity

The gland head consisted of disc cells with their cytoplasm and a non-cellular intrawall secretory cavity. The contents of the secretory

TABLE 2. Cannabinoid contents of stalked glands of different ages.

Strain	Gland age	Cannabinoids/gland, ngs.		
		CBD	THC	CBN
152 (drug)	Mature	-	57	-
	Aged	-	35	21
	Senescent	-	9	1
87 (fiber)	Mature	229	-	-
	Aged	113	-	-
	Senescent	29	-	-

cavity were examined specifically for the presence of cannabinoids. Contents of the secretory cavity, removed with micropipets, showed cannabinoids to be abundant in this cavity (Table 3). This strain was a hemp form with a predominantly CBD content. Each gland contained an average of 61.1 nanograms of cannabinoids. Each sample consisted of the contents from numerous glands, ranging in number from 80 to 350. Glands on large 9 mm bracts, samples 1 and 2, contained less total cannabinoids than did the smallest 4 mm bracts, samples 19 to 26. Most of the total cannabinoids consisted of CBD. Intermediate quantities of cannabinoids characterized the range in bract sizes from the large to small bracts except for several samples where the average content differed from the typical content of the samples. Although these differences for size classes of bracts should be expected in biological samples, they also may indicate that the cellular components that synthesize cannabinoids may not produce them at a constant rate during development of an organ.

These data show a trend that glands on younger smaller bracts contain greater quantities of cannabinoids than glands on older bracts. Mature glands appear to occur on young bracts in contrast to aged and senescent glands that occur on older bracts (Table 2).

In summary: (a) capitate-stalked glands contained more THC (and total cannabinoids) than capitate-sessile glands, (b) glands at different positions on the leaf or bract can differ in cannabinoid content, (c) THC, and total cannabinoid, quantity in both gland types can differ during the year, (d) cannabinoid contents in glands decreased with aging of glands, and (e) cannabinoids occurred in the secretory cavity of the gland.

Secretory Role of Disc Cells

It is pertinent to examine the organization of the disc cells because all contents in this cavity must be derived from these cells. Cannabinoids, or their precursors, are secretions from these cells. Terpenes, both monoterpenes and sesquiterpenes, also are compounds secreted into the secretory cavity. These compounds compose the essential oils of the plant. Different combinations of terpenes in various strains contribute to odor differences among the strains. Cannabinoids are odorless to most humans.

The tier of disc cells contains a typical cell complement including the nucleus, plastids, mitochondria, endoplasmic reticulum and abundant ribosomes, as well as vacuoles (Diagram 1; Figure 4). Plastids, however, represent the unique component of these cells. They are interpreted

TABLE 3. Cannabinoid contents in the secretory cavity of capitata-stalked glands on bracts of a hemp strain.

Sample	Number of glands	Bract size, mm	Cannabinoids/gland, ngs.			
			CBD	THC	CBN	Total
1	118	9	28.1	0.6	-	28.7
2	103	9	27.9	-	-	27.9
3	200	8	37.6	-	-	37.6
4	207	8	34.5	0.3	-	34.8
5	101	8	20.9	5.8	-	26.7
6	115	8	92.2	-	-	92.2
7	350	7.8	59.5	0.9	1.8	62.2
8	100	7	57.8	4.4	-	62.2
9	175	6.8	83.2	1.2	0.1	84.5
10	198	6.5	77.8	1.0	0.2	79.0
11	109	6	70.5	2.1	-	72.6
12	198	6	48.0	0.8	0.8	49.6
13	130	5	78.2	-	-	78.2
14	103	5	85.0	-	-	85.0
15	97	5	76.4	-	-	76.4
16	160	5	40.9	-	-	40.9
17	84	5	21.7	1.1	-	22.8
18	193	5	50.2	1.4	1.8	53.4
19	103	4.5	53.8	-	-	53.8
20	80	4.5	49.7	0.8	-	50.5
21	107	4.5	77.8	1.9	-	79.7
22	100	4.5	68.4	1.3	-	69.7
23	98	4	98.9	1.3	-	100.2
24	110	4	89.3	5.6	7.9	102.8
25	113	4	76.5	1.3	-	77.8
26	200	4	38.8	-	-	38.8
Average values, \pm SD			59.4 \pm 22		61.1 \pm 23	

to be a source, perhaps the principal source, of secretions deposited in the secretory cavity. Plastids divide repeatedly to become very numerous in the disc cells. Plastids form an unusual central component, termed the reticulate body, derived from thylakoids fused into a tubular array of light and dark areas in a hexagonal arrangement (Figures 4 and 5). This large body is somewhat like a prolamellar body that forms in chloroplasts when plants are grown in the dark because it has a lattice configuration similar to a prolamellar body. But it is unlike a prolamellar body in that it persists in both the light and the dark, and it does not contribute to formation of grana membranes, as does the prolamellar body.

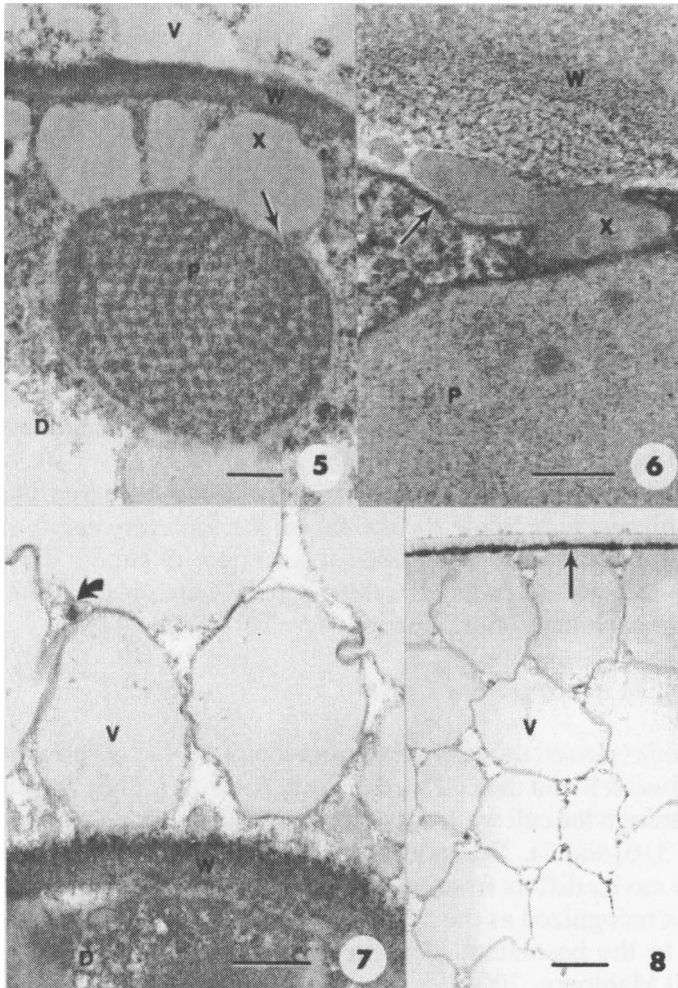
The reticulate body was associated with secretory activity. Quantities of secretions that accumulated on the surface of the plastids were continuous with the light zone in the reticulate body (Figure 5). The association of the secreted mass (X) with the light zone (arrow) on the surface of the plastid supported an interpretation that the reticulate body contributed to the synthesis of these secretions (Figure 5). Such secretions were evident on nearly all plastids in the mature gland and become so voluminous as to surround a plastid, as it appeared in an electron micrograph. These secretions were more or less round in appearance, but are undoubtedly spherical in three-dimensions. Oily in composition, they form spherical masses in the aqueous medium of the cell. These secretions may represent terpenes; plastids in other plants are reported to produce terpenes (Gleizes et al., 1983).

The secretions on the surface of plastids were observed in contact with the plasma membrane surface facing the secretory cavity (Figures 5 and 6). Similarly, other quantities of secretions of similar density, but not in contact with a plastid in a thin section, were observed in contact with the plasma membrane (Figure 3).

Secretions in the Secretory Cavity

Secretions passed through the plasma membrane to accumulate in the space between it and the cell wall (Figure 6, arrow). They subsequently passed through the cell wall, appearing as small hyaline areas in the wall (Figures 3, 6, and 7). That portion of the plasma membrane facing the secretory cavity differs from other regions in its greater secretory activity, and is recognized as the apical domain region of this membrane, in contrast to the basolateral domain region elsewhere around the cell (Kim and Mahlberg, 2000).

FIGURES 5-8. Secretory glands. **5.** Lipoplast (P) showing secretion (X) on its surface and region where secretion joins reticulate body (arrow). D, disc cell; V, secretory vesicle. Bar = 0.2 μm . **6.** Lipoplast (P) near plasma membrane (arrow). Portion of secretion (X) in contact with and passing through plasma membrane. It will accumulate between the membrane and cell wall (W). Bar = 0.1 μm . **7.** Secretions, small light areas in wall (W), emerge as vesicles (V) in secretory cavity. Fibrillar matrix (arrow) in intervesicular zone was released into secretory cavity from wall. Secretions, light areas, are evident in cytoplasm adjacent to the plasma membrane in disc cell. Bar = 0.2 μm . **8.** Outer sheath of secretory cavity (arrow) consists of cuticle and subcuticular wall. Secretory vesicles (V), each surrounded with surface feature, fill secretory cavity. Bar = 0.1 μm .



Secretions emerged into the secretory cavity as small accumulations on the wall surface facing the cavity (Figure 3). Some small gray vesicles were partly embedded in the wall (W) near the “corner” of the secretory cavity. They have the same gray density as secretions in the disc cell (Figure 7). Upon passing through the cell wall, the secretions accumulated as secretory vesicles on the wall surface facing into the secretory cavity. As these new vesicles emerged into the secretory cavity they became surrounded with a surface feature about one-half the thickness of a typical membrane (Figures 3 and 7).

Secretory vesicles, upon being released from the wall surface, aggregated in the secretory cavity (Figures 3, 7, and 8). As new secretory vesicles were deposited in the secretory cavity, existing vesicles were redistributed throughout the secretory cavity. Some of these vesicles were transported to the subcuticular wall surface, and released their contents into this wall (Figure 8, arrow). These contents moved through the subcuticular wall to the cuticle where they contributed to thickening of the cuticle (Figures 3 and 8). The irregular contour of the inner surface of the cuticle, with dark fiber-like extensions called striae, was derived from the fusion of quantities of vesicular material with existing cuticle. The surface feature of these vesicles contributed to the formation of a strial network that permeates the cuticle. Striae appeared to form microchannels in the cuticle that may facilitate passage of secretions out of the secretory cavity.

Fibrillar matrix was released from the surface of the disc cell wall into the secretory cavity. It was evident throughout the wall, as well as in the secretory cavity (Figures 4 and 7, curved arrow). This matrix was transported to and became incorporated into the subcuticular wall thereby contributing to the thickening of this wall. The mechanisms controlling deposition of fibrillar matrix in the subcuticular wall, as well as the deposition of vesicular materials into the subcuticular wall and cuticle, remain to be studied. Since the sites of fibrillar matrix deposition were very distant from the origin of the material in the disc cells, we speculate that the control mechanism for its transport somehow resided in the non-cellular secretory cavity.

In summary: (a) plastids produce a major quantity of secretions that are released from their surface to pass through the plasma membrane and outer wall into the secretory cavity, (b) secretions accumulate in the secretory cavity as secretory vesicles, whose contents contribute to thickening of the cuticle, (c) volatile components of secretory vesicles may volatilize into atmosphere and contribute to plant odor, (d) fibrillar matrix and other material released from the wall surface surround the

secretory vesicles with a surface feature of yet unknown composition, and (e) fibrillar matrix contributes to thickening of the subcuticular wall and to the formation of the strial network in the cuticle.

Localization of THC Within the Gland

The purpose of this phase of study was to determine the site of THC localization in the gland using a monoclonal antibody to detect this cannabinoid. Fresh glands were instantaneously frozen with high-pressure cryofixation and then processed by cryosubstitution to prevent movement of THC in the gland during the fixation process. A monoclonal antibody for THC was prepared as a probe by attaching small gold particles to it so that it would be visible under the TEM. Thin sections of glands were treated with the antibody probe; this antibody would attach to any THC present in the tissue.

Gold particles identified THC in the cell wall facing the secretory cavity (W), and in the subcuticular wall (arrowhead) under the cuticle (Figure 9). It was also present in the fibrillar matrix (open arrow) being released into the cavity from the disc cell wall. It was along the surface feature (short arrow) surrounding the large secretory vesicles in the cavity. It was also in the cuticle (long arrow).

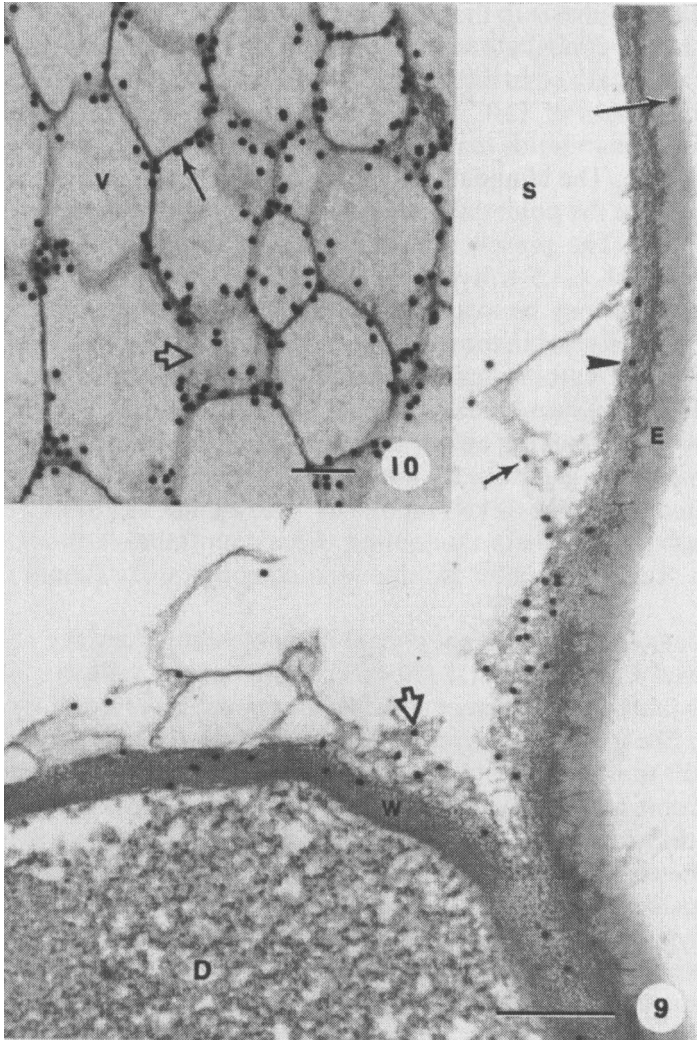
Deep within the cavity, THC was associated with the surface feature around the numerous secretory vesicles, but it was not present inside the vesicles (Figure 10, arrow). Some secretory vesicles were cut with their surface feature in planar view (slightly gray appearance at open arrow), and gold particles also were evident on this area of the surface feature. The contents of these vesicles (clear area) were interpreted to be the site of lipophilic terpene accumulation in the secretory cavity.

Surprisingly, THC was not in the cytoplasm of the disc cells (Figure 9, D). It was detected only along the plasma membrane and in the wall proper of the disc cell. Only an occasional gold particle was detected in the cytoplasm, either over a plastid, or mitochondrion or among ribosomes. If cannabinoids were synthesized in the cytoplasm of disc cells, there should be an abundance of gold particles at sites of THC synthesis and accumulation.

In other cells, as epidermal cells, some THC was present in the wall, but in smaller quantities than in walls of the disc cell. Few or no gold particles were present in the cytoplasm or vacuoles of other cells.

Controls included sections treated with antibody alone, or treated with the protein A-gold alone. Controls showed no antibody in the disc cells, or other cells. No antibody was detected outside of the cells.

FIGURES 9-10. Secretory glands. **9.** Sites of THC accumulation are evident as black dots representing gold attached to THC antibody. THC is present in the disc cell wall (W), subcuticular wall (arrowhead), along surface features around secretory vesicles in cavity (short arrow), in fibrillar matrix being released from disc cell wall (open arrow) and in cuticle (long arrow). No gold particles were evident outside gland. Bar = 0.2 μm . **10.** THC is abundant along surface features (arrow) of numerous secretory vesicles (V in secretory cavity, but absent from center of vesicles). Where the surface feature is sectioned in surface view, THC appears over entire surface (open arrow). Bar = 0.1 μm .



Possible Site of Cannabinoid Synthesis

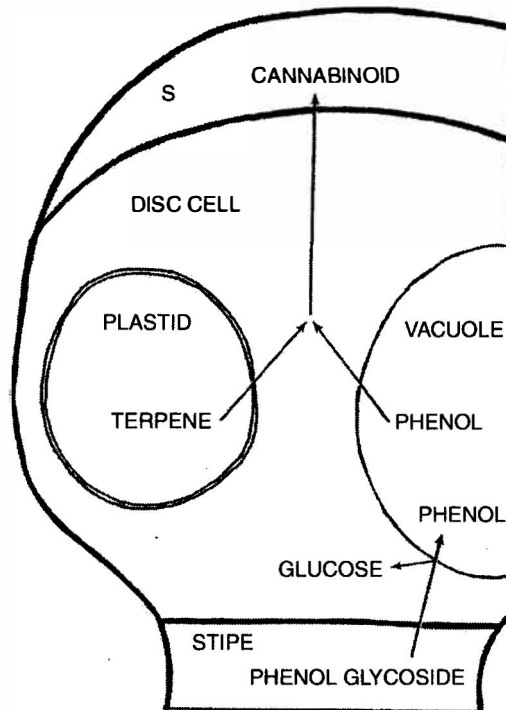
The chemistry of cannabinoids has been described, and a possible pathway for cannabinoid biosynthesis has been proposed (Diagram 2). Precursors contribute to the formation of cannabigerol (Mechoulam, 1970; Shoyama et al., 1975). Enzymes specific for CBC, CBD, and THC products have been described (Morimoto et al., 1998; Taura et al., 1995, 1996). However, definitive information is lacking on the precise site of their synthesis in the cell or in the gland.

Our studies contribute several pieces to the puzzle on where cannabinoids are localized in the plant. Data from the antibody probe studies show only traces of THC to be present in the cytoplasm, which suggests that cannabinoids may be formed at or outside the plasma membrane surface. The abundance of secreted terpenes in the disc cells is indicative that the potential source for a terpene precursor is available in the gland. The presence in the gland of simple phenols such as phloroglucinol, 1,3,5-trihydroxybenzene, is indicative that a phenolic precursor also may be available for cannabinoid synthesis. We have not detected olivetol in the gland; it differs from phloroglucinol by the presence of a 5-carbon chain at the 2 position. However, we have demonstrated that tritium-labeled olivetol, 2-pentyl 4,6-dihydroxybenzene, is absorbed by seedling apices and incorporated into all cannabinoids of this pathway (Diagram 2). It is presently assumed that the plant incorporated the entire olivetol molecule into cannabinoids and not just the 5-carbon side chain containing the tritium label, although additional studies using other isotope-labeled compounds should be pursued.

Our detection of abundant phenol in whole glands and the secretory cavity, and knowledge that phenols accumulate in vacuoles of cells, suggests that this component also may be available for the biosynthetic process. Phenols are transported in the plant as glycosides, which enable them to be soluble in the aqueous system. Upon entering a cell, they become localized in the vacuole. As they enter a vacuole the sugar moiety dissociates from the phenol making the phenol less soluble in the aqueous system, and the sugar is returned to the cell cytoplasm where it can serve as an energy source.

We hypothesize that terpenes and phenols, when released from their respective lipoplast and vacuole compartments, accumulate at the plasma membrane and cell wall interface, where enzymes combine these precursors into cannabinoids (Diagram 3). Enzymes specific for CBC,

DIAGRAM 3. Gland illustrating possible process of cannabinoid localization in secretory cavity. Phenol glycosides are transported into vacuole of disc cell where phenol components are stored and glycoside moiety returned to cytoplasm. Terpenes are synthesized by lipoplasts. Terpene and phenolic precursors react to form cannabinoids at the plasma membrane surface or in the wall whereupon they appear in the secretory cavity (S).



CBD, and THC products have been described (Morimoto et al., 1998; Taura et al., 1995, 1996). These enzymes, possibly, could be prepared as antibody probes and used to identify more precisely the locus of cannabinoid synthesis. The involvement of a simple phenol or related precursor in this pathway makes it unusual in plants and may be the basis for this pathway being unique to *Cannabis*.

Glands represent unique structures that can be utilized to broaden our understanding of cannabinoid synthesis and aid in our effort to reduce the cannabinoid content of *Cannabis* strains for application to the hemp industry.

CONCLUSIONS

THC, and other cannabinoids, accumulated in abundance in the secretory cavity where it was associated with the cell walls, the surface features of secretory vesicles, fibrillar material released from disc cell wall, and the cuticle. It was not associated with the contents of the secretory vesicles. The association of THC with structural components, particularly the wall, fibrillar matrix and surface feature of vesicles, suggests that it may be chemically bound to them rather than being free in the cavity. If THC and other cannabinoids are bound to components in the cavity, their presence and movement may require a source of energy in the cavity. Additional studies are necessary to determine their bound or free status.

Little or no THC was detected in the cytoplasm of the disc cells. However, the presence of both terpenes and phenols in the disc cells suggest that precursors for cannabinoid synthesis are present in the gland. We propose, as a working model, that the biosynthesis of cannabinoids occurs at the surface of the plasma membrane or in the cell wall facing the secretory cavity.

Some THC was present in the cell walls of other cells indicating that genes for cannabinoid synthesis are present in all cells of the plant, but tissues other than glands produce low levels of these compounds. The pathway for cannabinoid synthesis is controlled genetically; glands are specialized to synthesize high levels of cannabinoids. Thus, a glandless plant can be expected to synthesize very low levels of cannabinoids.

The roles of one or all cannabinoids in the glands or plant are unknown. They may be involved in some way with "protection" (Pate, 1994), signal chemicals (semiochemicals) in the environment, or other role. The absence of glands may not alter these possible functions, as other cells also synthesize these compounds, at very low levels, and this quantity may be sufficient to perform those roles. Therefore, a glandless mutant(s) could serve to reduce the THC content of *Cannabis* for utilization of such strains in the hemp industry.

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RECEIVED: 25 April 2003

ACCEPTED IN REVISED FORM: 14 November 2003