

IMMUNOCHEMICAL LOCALIZATION OF TETRAHYDROCANNABINOL (THC) IN CRYOFIXED GLANDULAR TRICHOMES OF *CANNABIS* (CANNABACEAE)¹

EUN-SOO KIM AND PAUL G. MAHLBERG²

Department of Biology, Konkuk University, Seoul, Korea, 133-701; and
Department of Biology, Indiana University, Bloomington, Indiana 47405

Delta 9-tetrahydrocannabinol (THC) localization in glandular trichomes and bracteal tissues of *Cannabis*, prepared by high pressure cryofixation–cryosubstitution, was examined with a monoclonal antibody–colloidal gold probe by electron microscopy (EM). The antibody detected THC in the outer wall of disc cells during the presecretory cavity phase of gland development. Upon formation of the secretory cavity, the immunolabel detected THC in the disc cell wall facing the cavity as well as the subcuticular wall and cuticle throughout development of the secretory cavity. THC was detected in the fibrillar matrix associated with the disc cell and with this matrix in the secretory cavity. The antibody identified THC on the surface of secretory vesicles, but not in the secretory vesicles. Gold label also was localized in the anticlinal walls between adjacent disc cells and in the wall of dermal and mesophyll cells of the bract. Grains were absent or detected only occasionally in the cytoplasm of disc or other cells of the bract. No THC was detected in controls. These results indicate THC to be a natural product secreted particularly from disc cells and accumulated in the cell wall, the fibrillar matrix and surface feature of vesicles in the secretory cavity, the subcuticular wall, and the cuticle of glandular trichomes. THC, among other chemicals, accumulated in the cuticle may serve as a plant recognition signal to other organisms in the environment.

Key words: Cannabaceae; *Cannabis*; cryofixation; gland; immunochemistry; tetrahydrocannabinol.

Glandular trichomes, as in *Cannabis*, produce secretions that accumulate in a specialized noncellular, intrawall secretory cavity. This cavity contains several compartments including lipophilic vesicles, the hydrophilic material in which the vesicles are suspended, and the surface feature surrounding the vesicles (Kim and Mahlberg, 1991). We are studying the development of the gland and its secretory cavity, as well as the formation and accumulation of secretory products, to further understanding of secretory processes in cells.

During gland development the progenitor cells of the secretory tier undergo anticlinal divisions to form a discoidal tier of 8–13 secretory, or disc, cells supported by a stipe attached to basal cells in the epidermis. The outer wall of the disc cells partitions transversely to initiate an intrawall secretory cavity delimited to the outside by the sheath consisting of the cuticle and the subcuticular wall. The inner portion of the wall, which contacts the disc cells, becomes thickened as the secretory cavity enlarges with its secretory contents. Secretions emitted from the disc cells appear as hyaline areas of different sizes in the thickened wall. As these secretions emerge from the wall into the cavity they become delimited by a surface feature that separates each of them from other components in the

cavity. Fibrillar matrix derived from the thickened wall also accumulates in the cavity. Both vesicles and fibrillar matrix contribute to sheath components, resulting in thickening of the cuticle and the subcuticular wall during gland development. Structural components of the subcuticular wall, when surrounded by vesicular contents, form a labyrinth of striae that extend through the thickened cuticle (Kim and Mahlberg, 1991, 1995; Mahlberg and Kim, 1991, 1992).

Gas chromatographic analyses of secretions removed from the secretory cavity by micromanipulative procedures demonstrated the presence of cannabinoids, including delta 9-tetrahydrocannabinol (THC), in this cavity (Hammond and Mahlberg, 1973; Turner, Hemphill, and Mahlberg, 1978; Lanyon, Turner, and Mahlberg, 1981). However, the organization and composition of the secretory cavity are complex, and the cytochemical distribution of a specific compound, such as THC, in the secretory cavity, as well as in disc and other cells, remains unclear.

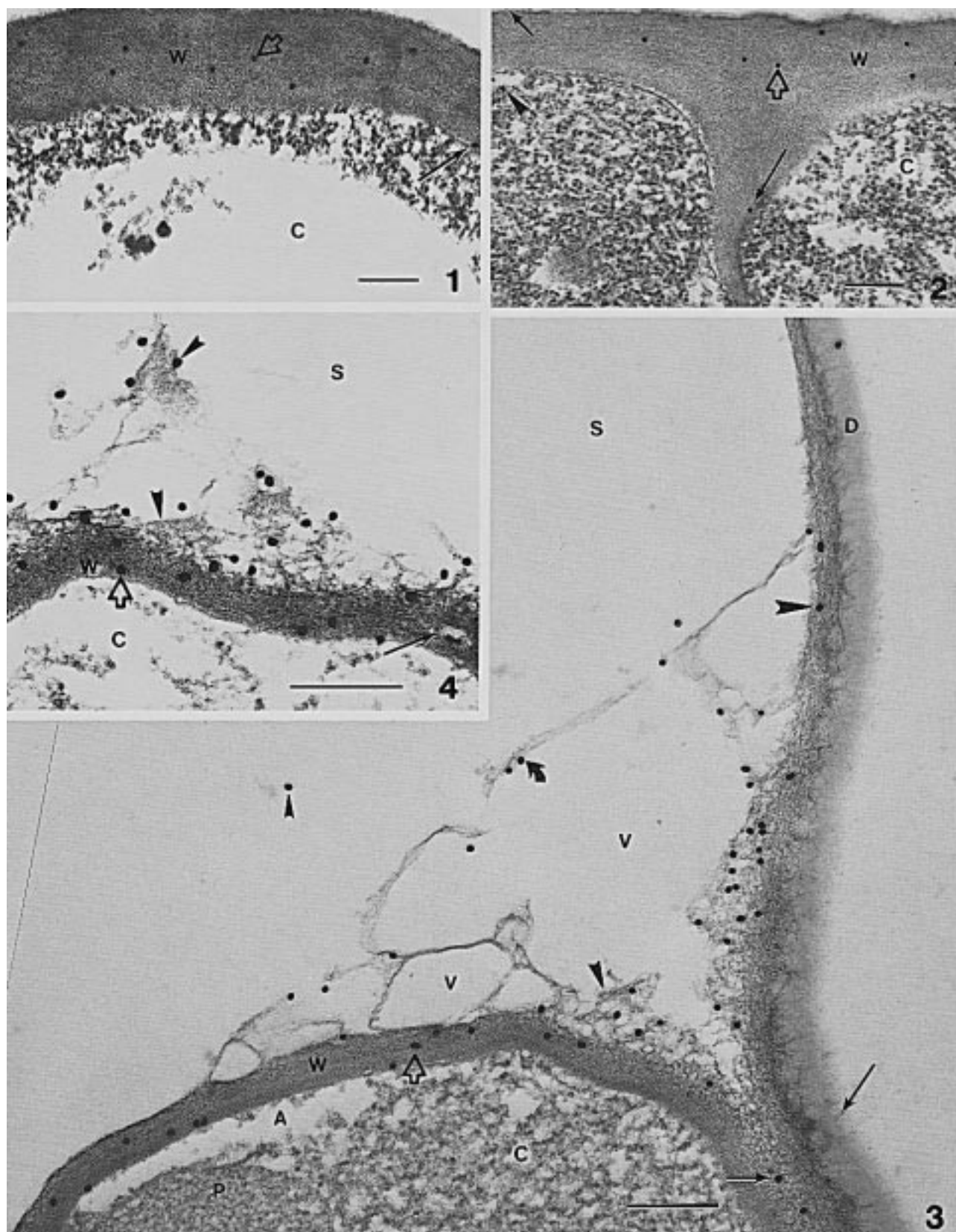
Our observations on the secretory process and fate of substances in the secretory cavity support a thesis that the disc cells contribute to the continuous production of secretions that flow or are transported throughout the secretory cavity for incorporation into the cuticle. We have hypothesized that the striae in the cuticle may serve to facilitate passage of compounds from the secretory cavity through the sheath to the gland surface from which they volatilize into the atmosphere (Mahlberg and Kim, 1992; Kim and Mahlberg, 1995).

In this study we use a monoclonal antibody for THC to examine the distribution and interpret the possible transport of this cannabinoid in glandular tissues prepared by high pressure cryofixation–cryosubstitution. Our ob-

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² Author for correspondence.



Figs. 1–4. Monoclonal immunolabel for THC in glands of *Cannabis*. No label occurred outside of gland tissue. **1.** Immunolabel (open arrow) in disc cell prior to secretory cavity formation showing gold grains in outer cell wall and between the plasma membrane (arrow) and cell wall. Bar = 0.2 μ m. **2.** Junction region of two disc cells before cavity formation showing label in outer wall (open arrow), on surface of cuticle (short arrow), and along anticlinal walls between adjacent disc cells (long arrow). A gold grain was evident among ribosomes (arrowhead). Bar = 0.2 μ m.

jectives include determination of THC distribution (1) in the different compartments of the secretory cavity, (2) among cytoplasmic components in disc cells, and (3) in other cells adjacent to the gland.

MATERIALS AND METHODS

Cryofixation–cryosubstitution—Bract segments containing glands of different developmental ages, obtained from pistillate *Cannabis* plants grown under greenhouse conditions (Hammond and Mahlberg, 1973), were fixed and processed by the Balzers high pressure cryofixation (HPC) and cryosubstitution (CS) procedure. Small segments of fresh bracts were placed in brass Swiss Precision planchettes, immersed in 1-hexadecene (Sigma, St. Louis, MO), and immediately transferred to a Balzers HPM 010 for cryoimmobilization under a pressure of 2100 bar (Hunziker and Schenk, 1984). Immediately following HPC, the samples were transferred to liquid nitrogen and stored briefly until all samples were ready for CS. The planchettes were quickly transferred to sieve-dried methanol containing 3% glutaraldehyde and immediately placed in the Balzers FSU automated CS instrument. CS proceeded uninterrupted from -90°C to -60°C to -30°C , each for 8 h, and at 0°C for 1 h. Following CS the samples were transferred to fresh sieve-dried methanol over ice, subsequently brought to room temperature, and immediately embedded in Spurr's low-viscosity resin (Spurr, 1969).

Preparation of antibody—The monoclonal antibody for THC was prepared in mouse ascites (11.1H2) by Roche Diagnostics Systems, Branchburg, NJ.

Preparation of protein A-gold complex—Binding of protein A to gold particles (20-nm gold particles, Jannsen, Belgium) was carried out using the protocol of Roth (1982).

Immunogold labelling—Thin sections of HPC-CS preparations were cut with diamond or glass knives on an LKB-IV Ultramicrotome and mounted on 200-mesh nickel grids. Immunochemical labelling was performed by floating grids serially, section surface down, on 20- μL drops of solutions placed on parafilm in plastic petri dishes. Sections were subjected to a pretreatment to block nonspecific binding sites and quench aldehydes on the section surface by placing a grid for 15 min on a drop of blocking buffer [1% bovine serum albumin, 0.02% sodium azide, and 1% Tween 20 in Tris-buffered saline solution (TBS:10 mmol/L Tris-HCl, pH 7.4, plus 150 mmol/L NaCl)] followed by two rinses in the buffer. Sections were incubated overnight at 4°C on drops of 1:20 monoclonal antibody (TBS/BSA) followed by six 1-min rinses with TBS to remove unbound antibody. The grids then were floated on drops of diluted (1:10) protein A-gold, and rinsed with six 1-min changes with TBS in small dishes, while slowly stirred with mini-stirring bars, followed by a rinse in deionized water. Excess water was removed from the grids with filter paper. Controls included sections treated with antibody alone, or treated with protein A-gold alone.

Staining—Grids were stained in 1% aqueous uranyl acetate for 20 min followed by Reynold's (1963) lead citrate for 10 min. Sections were examined and photographed with a Philips EM 300 transmission elec-

tron microscope at 60 kV. Illustrations were selected from several hundred electron micrographs derived from examination of numerous glands and adjacent tissues.

RESULTS

Presecretory cavity stage—Monoclonal antibody specific for THC labeled the outer wall of the disc cells prior to formation of the secretory cavity (Fig. 1). Immunolabel was distributed throughout the thickened outer wall of the disc cells. Occasionally a gold grain was found between the plasma membrane and cell wall. Gold grains were consistently present throughout the outer wall (Fig. 2). Some grains also appeared on the cuticle. Label also was present along the thinner anticlinal walls and middle lamella of adjacent disc cells, although fewer grains were evident over these walls than the thickened outer wall of the gland. The incipient secretory cavity, formed by tangential separation of the outer wall giving rise to the subcuticular wall, contained no gold grains (not shown).

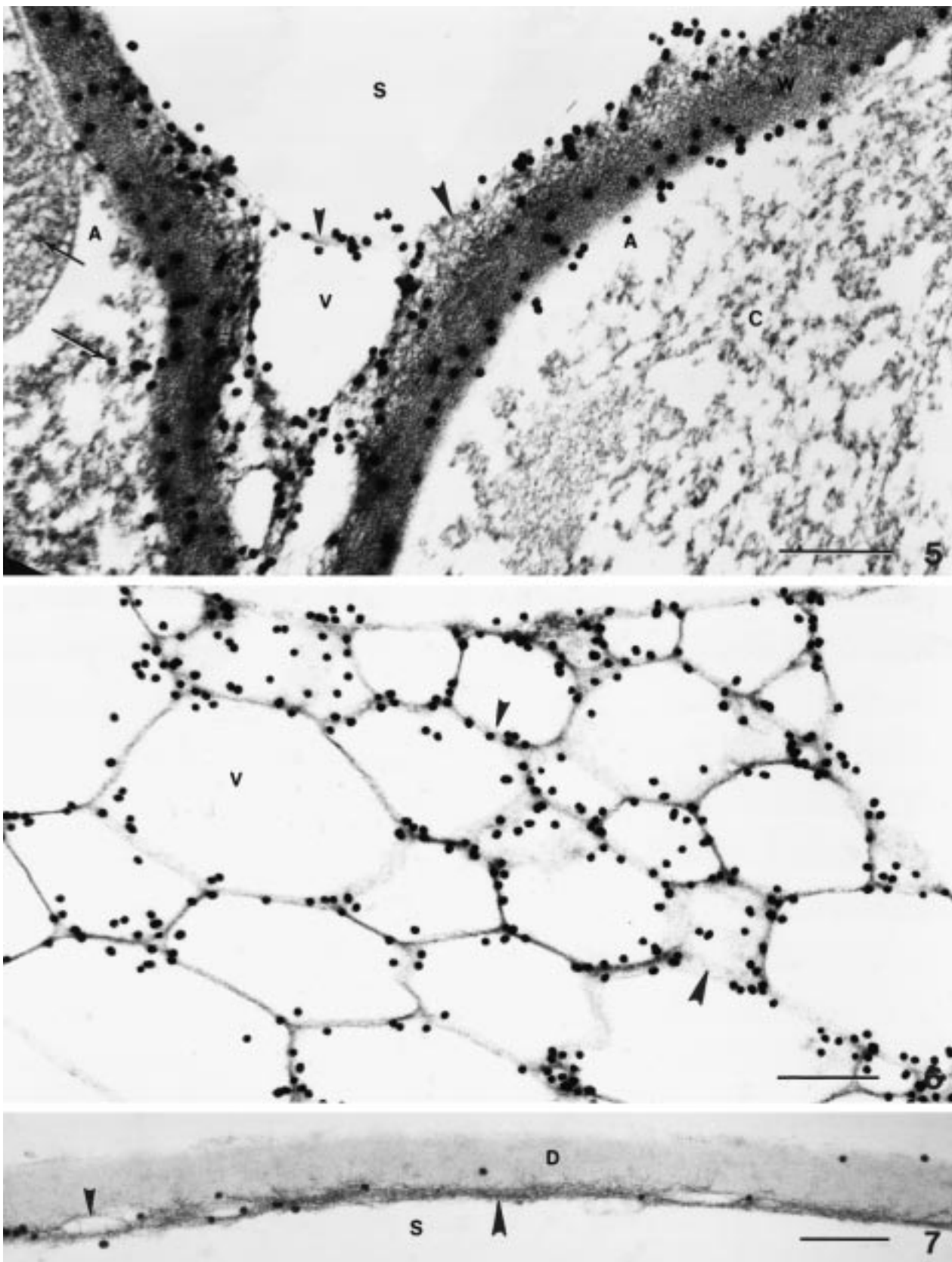
Immunolabel was only occasionally detected in the cytoplasm of the disc cells appearing among ribosomes, or over an organelle or cytomembrane (Fig. 2).

Secretory cavity stage—Upon development of the secretory cavity and at the juncture of wall separation at the base of the cavity, the THC label was present in the disc cell wall facing this cavity (Fig. 3). It also was present in the wall along the periphery of the gland where the middle portion of the wall appeared somewhat electron-transparent and not yet cavitated. Gold grains were evident throughout the subcuticular wall, which was continuous with the cell wall along the periphery of the cavity, as well as over fibrillar matrix extending into the cavity from the thickened wall of the disc cell. Cuticle also contained gold deposits, but they were fewer in number than in the subcuticular wall at this position along the gland surface. In the secretory cavity gold grains were associated with fibrillar matrix that extended between the disc cell wall and the subcuticular wall. Even small fragments of this matrix in the cavity were labeled with the gold probe.

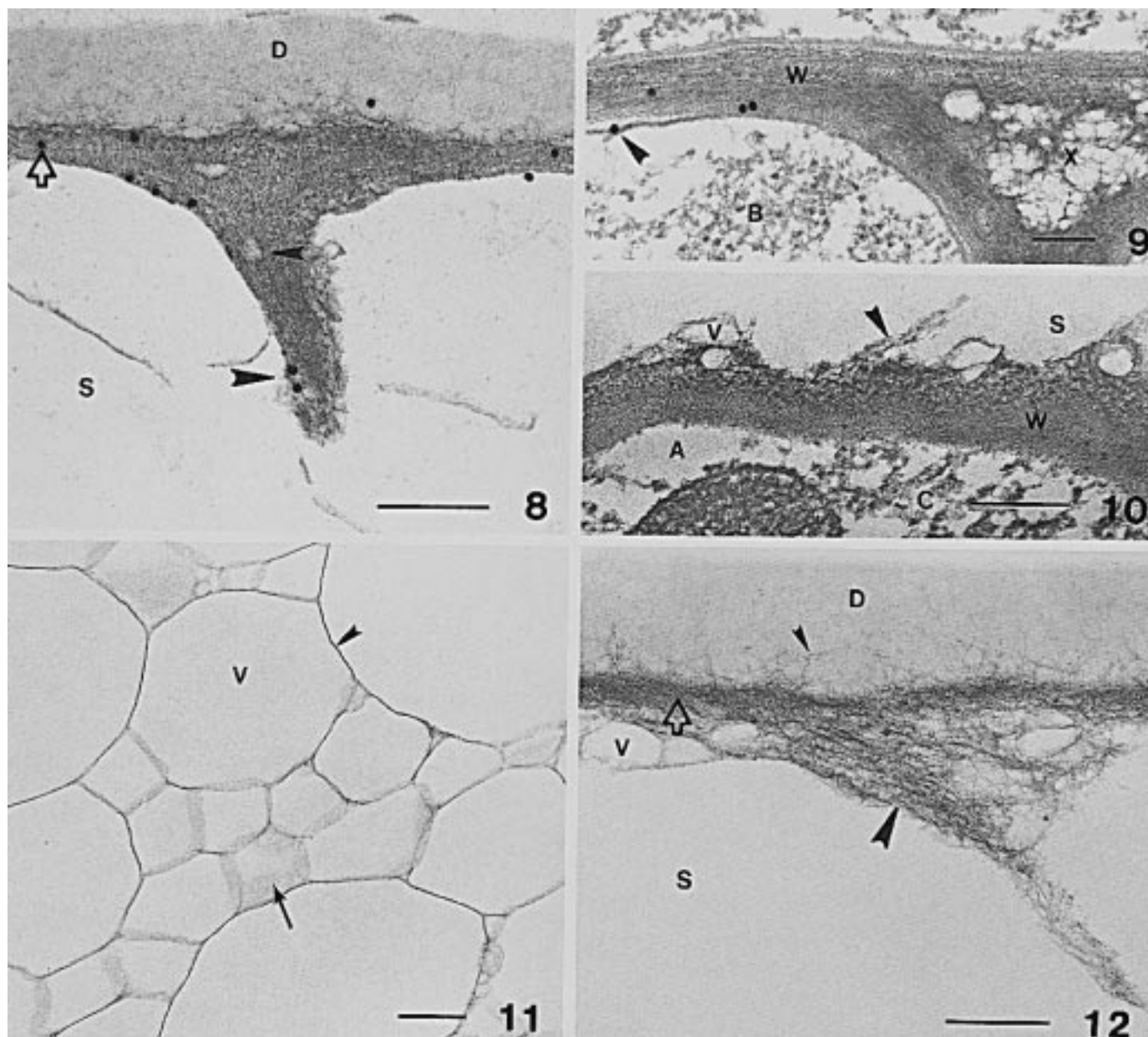
In glands with an enlarged secretory cavity the THC label was abundant throughout the disc cell wall (Fig. 4). The wall differed somewhat in electron density from the plasma membrane side to the surface facing the secretory cavity. Gold grains were distributed throughout the wall, including some in the proximity of the plasma membrane. No grains were present over hyaline, or light, areas in the wall. The THC label was positioned directly over or in immediate association with fibrillar matrix extending from the wall into the secretory cavity. Gold grains also were present over fragments of fibrillar matrix in the se-

3. Disc cell at periphery of enlarged secretory cavity showing gold grains (open arrow) in thickened cell wall (W), and in unseparated region of wall (short arrow) at periphery of cavity (long arrow). Label is evident in subcuticular wall (large arrowhead) and in cuticle (above D). Immunolabel appears in fibrillar matrix (medium arrowhead) adjacent to disc cell wall and in surface feature (curved arrow) surrounding vesicles in secretory cavity. Label also is associated with the portion of fibrillar matrix in sectional view deep in the cavity (small arrowhead). No label is found in plastids or other cytoplasmic components in disc cell. Bar = 0.2 μm . 4. Disc cell showing immunolabel (open arrow) and its distribution in wall facing secretory cavity and in fibrillar matrix (arrowheads) extending into this cavity. A hyaline area is evident in wall (arrow). No label is present elsewhere in cavity or in disc cell. Bar = 0.2 μm .

Figure Abbreviations: A, secretory product; C, disc cell; D, cuticle; P, plastid; S, secretory cavity; V, secretory vesicle; W, cell wall.



Figs. 5-7. Monoclonal immunolabel for THC in glands of *Cannabis*. No label occurred outside of gland tissue. 5. Junction region between two adjacent disc cells showing label distribution in cell wall, in fibrillar matrix (large arrowhead) extending into the secretory cavity and in surface feature of vesicle (small arrowhead) positioned between the walls of the two cells. Some gold grains are positioned to inside of cell wall (disc cell



Figs. 8–12. Monoclonal immunolabel for THC in glands of *Cannabis*. No label occurred outside of the gland tissue. **8.** Sheath showing grains in cuticle and subcuticular wall (open arrow), and in fibrillar matrix (arrowhead), which is continuous with subcuticular wall. Several small vesicles (at and opposite small arrowhead) are evident in this matrix, which is in contact with the sheath. Bar = 0.2 μm . **9.** Portions of three parenchyma cells (one represented by B) showing immunolabel over wall but not intercellular space (X) between cells. A gold grain is positioned between plasma membrane and cell wall (arrowhead). Bar = 0.2 μm . **10.** Control. Disc cell wall showing no immunolabel over wall, fibrillar matrix (arrowhead), surface feature of vesicles, in secretory cavity, or in cytoplasm of disc cell. Bar = 0.1 μm . **11.** Control. Vesicles in secretory cavity showing no immunolabel on surface feature (arrowhead) or in their contents. Surface feature of some vesicles appears in face view as gray area (arrow). Bar = 0.3 μm . **12.** Control. Sheath showing no immunolabel in fibrillar matrix (arrowhead), secretory vesicles, subcuticular wall (open arrow), or cuticle. Fibrillar matrix residues that contribute to striae are evident in cuticle (small arrowhead). Bar = 0.2 μm .

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at right); electron-transparent area (A) represents secretory product in cell. In disc cell at left, a gold grain is located in an organelle (short arrow) and several grains occur in cytoplasm adjacent to cell wall (long arrow) and between plasma membrane and cell wall (above A). Bar = 0.2 μm . **6.** Secretory vesicles in cavity showing immunolabel associated with the surface feature (small arrowhead and elsewhere), but not with contents, of vesicles. Gold grains are evident over the face view of several vesicles (electron-gray appearance; large arrowhead). Bar = 0.2 μm . **7.** Sheath showing gold label in the cuticle, including outermost edge (extreme right). Immunolabel also is present over subcuticular wall (large arrowhead). Small secretory vesicles occur in subcuticular wall (small arrowhead). Bar = 0.2 μm .

cretory cavity. No gold grains were present in the clear areas, or in secretory vesicles, in the cavity.

At junctures of adjacent disc cells facing the secretory cavity the probe identified THC along the entire wall surface facing the cavity, including the juncture region between cells where the walls now were somewhat separated from each other (Fig. 5). Fibrillar matrix bridging the walls of the adjacent cells, in the region of the former middle lamella, also possessed gold grains. Similarly, grains of the probe were present elsewhere throughout the fibrillar matrix extending into the cavity along the entire cell wall. The probe was present in the area of the plasma membrane and, where the membrane is evident, most of the grains appeared to be outside of this membrane. Secretory vesicles, which appear as clear circular areas between the adjacent walls and in the secretory cavity, contained no gold grains. The delimiting surface feature on these vesicles contained gold grains.

Immunolabel was distributed along the surface feature of all of the numerous secretory vesicles in the secretory cavity (Fig. 6). In contrast, no gold grains were found in the clear more or less round areas representing vesicular contents. The surface features of most vesicles were sectioned so that their margins appeared in cross-sectional view. However, for some vesicles the surface feature also appeared partially in face view as a gray area with gold grains present on it.

Immunolabel was present over the fibrillar matrix contiguous with the subcuticular wall as well as in the cuticle, including the outermost edge of the cuticle (Fig. 7). No gold grains were evident in the small secretory vesicles in the fibrillar matrix adjacent to or in this wall. Subcuticular wall components, both the fibrillar matrix and small secretory vesicles, appear to be derived from these components in the secretory cavity (Fig. 8). Gold grains also were present throughout the fibrillar matrix in the secretory cavity in continuity with this matrix component in the subcuticular wall.

Little or no immunolabel for THC was found in the cytoplasm of the disc cells. Some gold grains were present over the plasma membrane (Fig. 4) or in close proximity to it in the subjacent cytoplasm (Fig. 5). A gold grain occasionally was detected over a plastid or other cytoplasmic component in the disc cells (Fig. 5).

Immunolabel in other cells—Immunolabel was consistently found in the walls of epidermal and parenchymal cells of the bract (Fig. 10). Grains were only occasionally evident over cytoplasmic components in these cells.

Controls—No label was detected in the cytoplasm of the disc cells or wall facing the secretory cavity (Fig. 10), on the surface feature or in secretory vesicles in the secretory cavity (Fig. 11), or in the subcuticular wall and cuticle (Fig. 12), or in parenchymal cells (not shown) in control treated separately with antibody or with protein A-gold alone.

DISCUSSION

The HPC-CS procedure instantaneously immobilizes activities and substances in tissues (Hunziker, 1993) and, therefore, is interpreted to represent the preferred proce-

cedure to retain secondary compounds, such as THC, in situ with minimal redistribution during tissue processing. The monoclonal antibody probe for THC demonstrated the presence of this cannabinoid in the gland. Its presence in the cell wall and secretory cavity of the gland and cell wall of bracteal mesophyll indicated that this cannabinoid is secreted from the cell. The greater number of gold grains in the secretory cavity and in the walls of disc cells than mesophyll cells is indicative of the greater involvement of disc cells in THC secretion. The reported abundance of cannabinoids in glands supports this interpretation (Turner, Hemphill, and Mahlberg, 1978; Lanyon, Turner, and Mahlberg, 1981).

THC is selectively distributed in the secretory cavity occurring in association with the fibrillar matrix and the surface feature of vesicles. It was not detectable in vesicles or in the space around the fibrillar matrix or vesicles. The surface feature of vesicles has been shown to be derived from components in the wall during vesicle emergence into the cavity (Mahlberg and Kim, 1992). The consistent association of THC with wall and matrix materials suggests that THC may be bound in some way to the fibrillar matrix and surface feature following its secretion into the wall. Since both the fibrillar matrix and surface feature of vesicles become incorporated into the sheath, it is possible for the THC to be transported throughout the secretory cavity.

Detection of a relatively high concentration of labeled THC in the loosened wall-derived fibrillar matrix in the secretory cavity at different phases in gland enlargement indicates that THC formation and deposition in the wall is a continuing process during development of the cavity. Its association with the fibrillar matrix throughout the cavity and with the subcuticular wall indicates that it is transported into the cuticle where it can occur on the outer surface of the sheath. It is not yet clear whether THC, like volatile monoterpenes, can volatilize from the surface of the cuticle. However, the accumulated constellation of chemicals, including THC, in the cuticle may serve as a recognition signal to other organisms in the environment.

Absence or only occasional detection of the probe in the cytoplasm of disc and parenchymal cells supports an interpretation that THC is secreted from the cell to accumulate outside the plasma membrane in the wall or in the enlarged intrawall cavity of the secretory gland.

Our successful application of an antibody probe for a presumptively soluble molecule, such as THC, provides a basis for developing probes for other cannabinoids and their precursors to study the biosynthesis of these compounds in the plant. In a similar way, it may be possible to develop probes to examine the localization of other secondary compounds in biological tissues.

Although it has been shown that THC and other cannabinoids do accumulate in the secretory cavity, the mechanism of their transport from the disc cell and through the plasma membrane and cell wall are unknown (Malingré et al., 1975; Turner, Hemphill, and Mahlberg, 1980; Lanyon, Turner, and Mahlberg, 1981). The areas of different density in the cytoplasm and cell wall may be secretions associated with the secretory process (Kim and Mahlberg, 1991; Mahlberg and Kim, 1992). Further study of secretions in disc cells and the transport of these

substances into the secretory cavity will expand our understanding of the secretory process in glands as well as in other plant cells (Mahlberg and Kim, 1992; E. S. Kim and P. G. Mahlberg, unpublished data).

LITERATURE CITED

- HAMMOND, C. T., AND P. G. MAHLBERG. 1973. Morphology of glandular hairs of *Cannabis sativa* L. from scanning electron microscopy. *American Journal of Botany* 60: 524–528.
- HUNZIKER, E. B. 1993. Application of cryotechniques in cartilage tissue preservation and immunoelectron microscopy: potentials and problems. *Microscopy Research and Techniques* 24: 457–464.
- , AND R. K. SCHENK. 1984. Cartilage ultrastructure after high pressure freezing, freeze substitution and low temperature embedding. 2. Intercellular matrix ultrastructure: preservation of proteoglycans in their native state. *Journal of Cell Biology* 98: 277–282.
- KIM, E. S., AND P. G. MAHLBERG. 1991. Secretory cavity development in glandular trichomes of *Cannabis sativa* L. (Cannabaceae). *American Journal of Botany* 78: 142–151.
- , AND ———. 1995. Glandular cuticle formation in *Cannabis* (Cannabaceae). *American Journal of Botany* 82: 1207–1214.
- LANYON, V. S., J. C. TURNER, AND P. G. MAHLBERG. 1981. Quantitative analysis of cannabinoids in the secretory product from capitate-stalked glands of *Cannabis sativa* L. (Cannabaceae). *Botanical Gazette* 142: 316–319.
- MAHLBERG, P. G., AND E. S. KIM. 1991. Cuticle development on glandular trichomes of *Cannabis sativa* (Cannabaceae). *American Journal of Botany* 78: 1113–1122.
- , AND ———. 1992. Secretory vesicle formation in glandular trichomes of *Cannabis sativa* (Cannabaceae). *American Journal of Botany* 79: 166–173.
- MALINGRÉ, T., H. HENDRIKS, S. BATTERMAN, R. BOS, AND J. VISSER. 1975. The essential oil of *Cannabis sativa*. *Planta Medica* 28: 56–61.
- REYNOLDS, E. 1963. The use of lead citrate at high pH as an electron opaque stain for electron microscopy. *Journal of Cell Biology* 17: 208–212.
- ROTH, J. 1982. The protein A-gold (pAg) technique—a qualitative and quantitative approach for antigen localization on thin sections. In G. R. Bullock and P. Petrusz [eds.], *Techniques in immunocytochemistry*, vol. 1, 107–133. Academic Press, London.
- SPURR, A. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructural Research* 26: 31–43.
- TURNER, J. C., J. K. HEMPHILL, AND P. G. MAHLBERG. 1978. Quantitative determination of cannabinoids in individual glandular trichomes of *Cannabis sativa* L. (Cannabaceae). *American Journal of Botany* 65: 1103–1106.
- , ———, AND ———. 1980. Trichomes and cannabinoid content in developing leaves and bracts of *Cannabis sativa* L. (Cannabaceae). *American Journal of Botany* 65: 140–151.