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GLANDULAR CUTICLE FORMATION IN *CANNABIS* (CANNABACEAE)[1]

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Formation of the cuticle from components of the secretory cavity and subcuticular wall was studied by transmission electron microscopy of glandular trichomes of *Cannabis* prepared by high pressure cryofixation-cryosubstitution. Secretory vesicles in the secretory cavity resembled those localized in the subcuticular wall as well as the vesicle-related material associated with the irregular inner surface of the cuticle and appeared to provide precursors for thickening of the cuticle. Some contiguous vesicles in the secretory cavity and subcuticular wall lacked a surface feature at their point of contact, supporting an interpretation of vesicle fusion. Fibrillar matrix from the secretory cavity contributed fibrillar matrix to the subcuticular wall, and persisted as residual fibrillar matrix associated with secretory materials coalesced to the thickened inner surface of the cuticle. Elongated fibrils arranged in uniformly spaced parallel pairs contributed to the organization of fibrillar matrix in the subcuticular wall. Striae were evident in the outer portion of the cuticle, and appeared to represent sites of degraded residual fibrillar matrix associated with secretory materials coalesced to the inner cuticular surface. This study supports an interpretation that contents of secretory vesicles from the secretory cavity contribute to formation of glandular cuticle.

Glandular trichome morphogenesis in *Cannabis* includes the formation of a large secretory cavity formed by the periclinal separation of the outer cell wall of the disc cells. Separation of the wall occurs across the entire dome of disc cells composing the glandular trichome, or secretory gland. As this cavity within the wall enlarges and accumulates secretions derived from disc cells, the outer portion of the wall contributes to formation of the gland sheath, consisting of the subcuticular wall and the cuticle. Upon continued enlargement of the secretory cavity, both the cuticle and subcuticular wall components of the sheath increase greatly in surface area as well as in thickness (Mahlberg et al., 1984; Kim and Mahlberg, 1991; Mahlberg and Kim, 1991).

The mechanism by which components of the sheath increase in surface area and thickness remains to be determined. Secretions in the secretory cavity may contribute precursors for expansion and thickening of the sheath in glands of *Cannabis*. Three compartments were recognized within the secretory cavity, including the lipophilic vesicles, the hydrophilic material surrounding the vesicles, and the surface feature surrounding each vesicle (Kim and Mahlberg, 1991). Fibrillar matrix and particulate material were included among components between the vesicles. Differences in physical character and morphology of components surrounding the vesicles, as well as differences among the vesicles themselves in the cavity, have been reported (Mahlberg and Kim, 1991). The appearance of these components in the secretory cavity and their association with the cuticle and subcuticular wall appeared indicative of their involvement in sheath development (Kim and Mahlberg, 1991; Mahlberg and Kim, 1991, 1992). Numerous monoterpenes and sesquiterpenes have been identified by mass spectroscopy from these glands (Hammond, Kim, and Mahlberg, unpublished data). Essential oils and the terpenophenols represented by cannabinoids have been identified by gas chromatography and mass spectrometry following their isolation from the secretory cavity (Malingré et al., 1975; Turner, Hemphill, and Mahlberg, 1978; Lanyon, Turner, and Mahlberg, 1981).

Studies of sheath development in chemically fixed tissues reported that cuticular growth resulted from the fusion of vesicular components transported to the inner cuticular surface from the secretory cavity (Mahlberg and Kim, 1991).

In this study we used the high pressure cryofixation-cryosubstitution procedure to study the formation of the sheath and its interrelationships with components of the secretory cavity. This procedure instantaneously immobilizes and fixes structural features and physiological processes in the gland, and thereby maintains sheath and secretory cavity features in a more natural state than with chemical fixation where the fixation process extends over a period of time. Our objectives were to 1) examine the organization of the cuticle and subcuticular wall, and 2) describe the role of secretory vesicles and associated fibrillar matrix of the secretory cavity in formation of the sheath.

MATERIALS AND METHODS

Bract segments containing glandular trichomes at different stages of development were obtained from pistillate *Cannabis* plants grown under greenhouse conditions (Hammond and Mahlberg, 1973).

Bracts with glands of different ages were fixed and processed by high pressure cryofixation (HPC) and cryosubstitution (CS). Small segments of bracts were placed in brass Swiss Precision planchettes and immersed in

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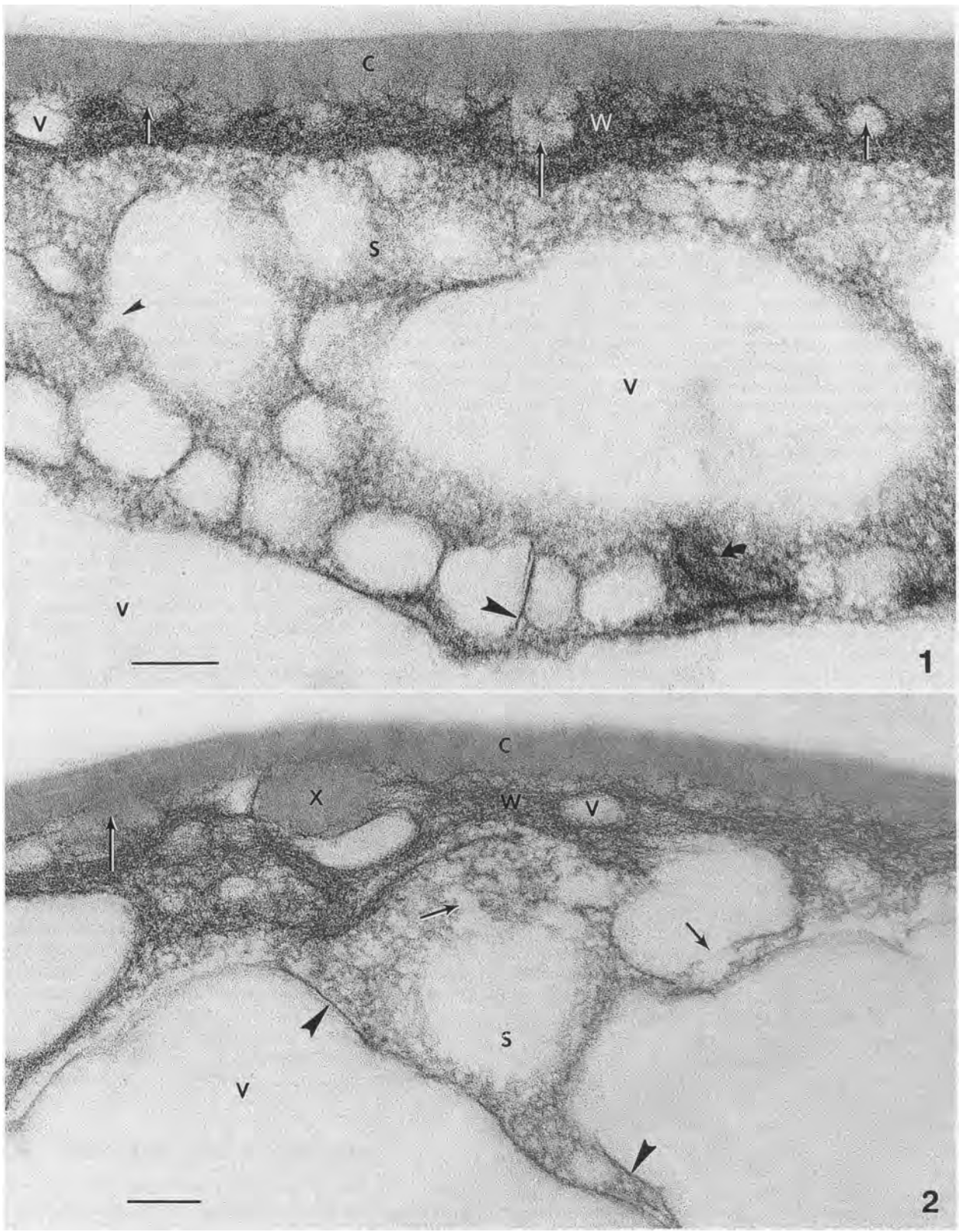

Figs. 1–2. Glandular sheath and secretory cavity in sectional view. **1.** Secretory cavity showing different-sized secretory vesicles, including very small vesicles. A distinct surface feature surrounds vesicles, and is most evident at the curvature where vesicles contact each other (large arrowhead). Some vesicles lack a surface feature at their point of contact and appear coalesced (small arrowhead). Areas of fibrillar matrix occur among vesicles in secretory cavity (curved arrow). Subcuticular wall shows dense fibrillar matrix and vesicles of different density. Some vesicles (short arrows) surrounded by fibrillar matrix appear appressed, but not fused, to lower irregular surface of cuticle. Cuticle shows vesicles, some clustered and with a central region of residual fibrillar matrix (long arrow), coalesced to cuticular surface. Bar = 100 nm. **2.** Secretory cavity shows numerous very

1-hexadecene (Sigma, St. Louis, MO) and immediately transferred to the Balzers HPM 010 instrument for cryoimmobilization under a pressure of 2100 bar. Immediately following HPC, the samples were transferred to liquid nitrogen and stored until all samples were ready for CS. The samples were quickly transferred to sieve-dried methanol containing 3% glutaraldehyde and 1% osmium tetroxide, 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 hr, and 0 C for 1 hr. Following CS the samples were transferred to fresh sieve-dried methanol over ice and subsequently brought to room temperature. Samples were immediately embedded in Spurr's low-viscosity resin (Spurr, 1969).

Thin sections of HPC-CS preparations were cut with a diamond knife on an LKB-IV Ultramicrotome and stained with uranyl acetate followed by lead citrate (Reynolds, 1963). Sections were examined and photographed with a Philips EM 300 transmission electron microscope at 60 kV. Illustrations were selected from several hundred electron micrographs derived from examination of the sheath and secretory cavity of numerous glands.

RESULTS

Secretory cavity—The secretory cavity of a gland was delimited externally by a sheath consisting of the cuticle and a subcuticular wall composed of dense fibrillar matrix (Fig. 1). Numerous vesicles of different sizes were evident in the secretory cavity under the subcuticular wall (Fig. 1). Each vesicle possessed a distinctive surface feature that delimited it from adjacent vesicles and other components in this cavity (Figs. 1–2). When sectioned in median view this surface feature appeared as a single electron-dense band with a thickness of 20 nm (Fig. 2). Its distinctness was most apparent when other components of the secretory cavity separated vesicles from each other and at junctions where neighboring vesicles contacted each other (Figs. 1–2).

Vesicles typically were round or somewhat oval in shape, and often very large in size (Figs. 1–3). Some large vesicles possessed a smooth rounded contour along their surface facing the subcuticular wall, but lobes also were present along their surface (Fig. 3).

The region between the larger vesicles was spongiose in appearance in that it consisted of numerous very small vesicles ranging in size from the visual limit of resolution to those of larger size (Figs. 1–2, 5). These very small and compactly arranged vesicles were profuse between the larger vesicles and, like larger vesicles, were delimited by a surface feature. The surface feature for many small vesicles was incomplete where they contacted each other or where a very small vesicle contacted a larger vesicle (Figs. 1–2, 5).

Fibrillar matrix often occurred in the area between vesicles in the secretory cavity (Figs. 1, 5–6). Very small vesicles also were present in this matrix. Where small masses of fibrillar matrix were evident in the secretory cavity the fibrils appeared more or less randomly arranged and individual fibrils were short in length (Fig. 6). In other regions of the secretory cavity more massive quantities of fibrillar matrix were evident between the large vesicles (Fig. 5). Fibrils in these large masses typically were oriented more or less parallel to each other and appeared longer than those in small masses of such material, although it was difficult to ascertain their length.

Fibrillar matrix in the secretory cavity also was observed to be continuous with that of the subcuticular wall and fibrils were identical in appearance with those in the wall (Figs. 5–6). At positions where fibrillar matrix of the secretory cavity contacted the subcuticular wall, immediately under the cuticle, the fibrils assumed an orientation similar to those in this wall (Fig. 5).

Subcuticular wall—The subcuticular wall consisted of a layer of compactly arranged fibrillar matrix positioned against the inner cuticular surface (Fig. 1). Fibrils related to this wall matrix were oriented more or less parallel with the general surface of the cuticle to form a distinctive wall layer (Fig. 2). However, some fibrils that surrounded vesicles, also present in this wall, were oriented in different directions, their orientation perhaps being affected by the size of the vesicle in the wall (Figs. 2–3).

Some fibrils aggregated as pairs, in sectional views, to form elongated parallel fibrillar structures measuring 160 nm or more in length; the paired fibrils measured 7 nm in width (Fig. 3). Dark dot-like areas appeared to bridge the paired fibrils along their length. These distinctive paired fibrils occurred among other fibrillar material and vesicles in this wall.

Vesicles of different sizes and densities were present in the subcuticular wall. Some vesicles lacked a surface feature at their point of contact and appeared deeply lobed (Fig. 4, at X). Vesicles in this wall typically were smaller than those in the secretory cavity, and many of them were very small (Figs. 2–3, 6). Vesicles with either light or dense contents were appressed to the cuticle but appeared separated from it by a narrow layer of fibrillar matrix (Figs. 1, 3, 5).

The shape of some vesicles positioned at irregularities along the lower cuticular surface appeared altered from a typical round form by the contour of this surface. When one or more vesicles became appressed in such an area,

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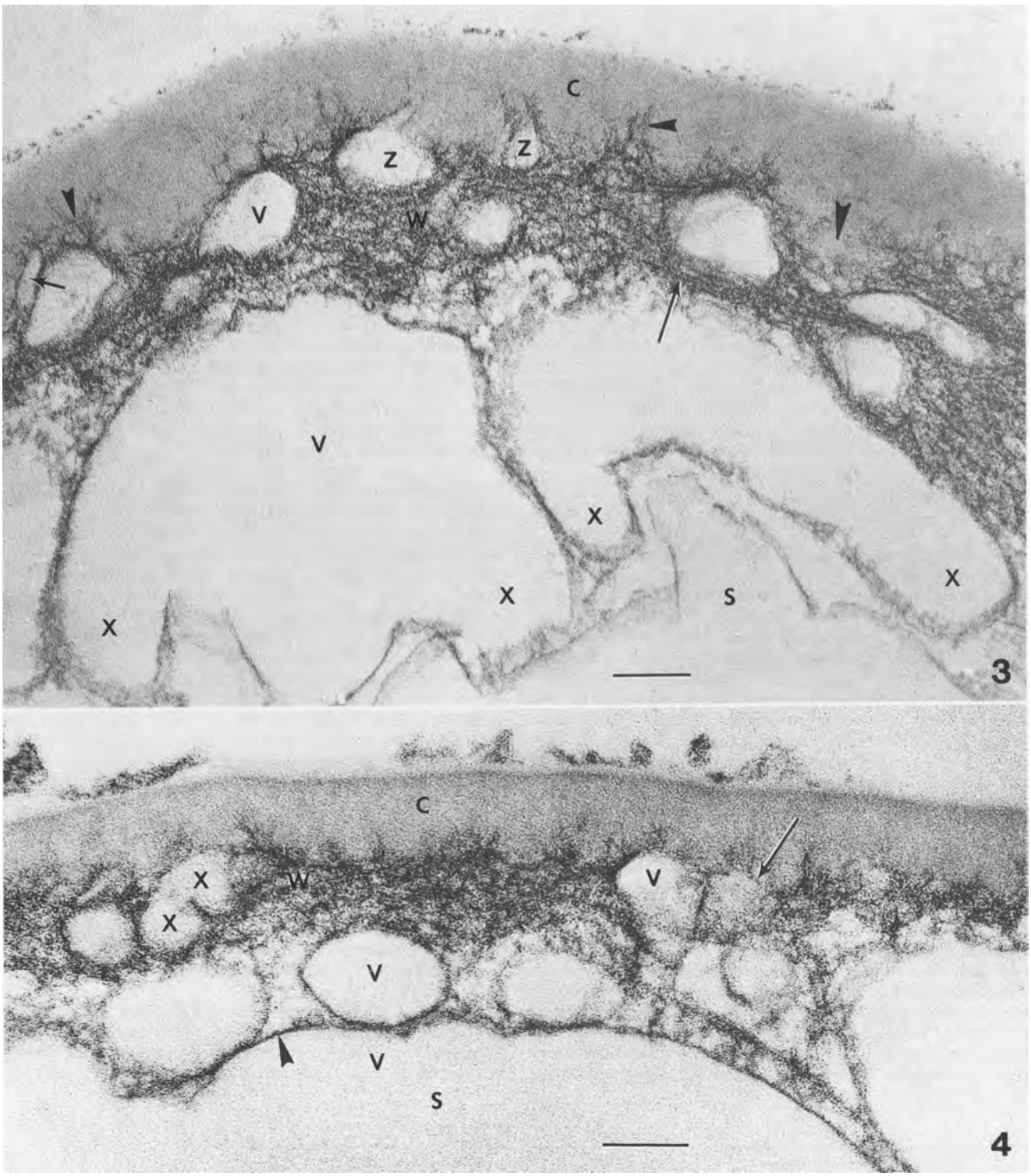
small and larger vesicles and their single surface feature (large arrowheads), as well as some vesicles lacking the surface feature at their point of contact (short arrows). Subcuticular wall shows fibrillar matrix and vesicles of different density. Cuticle shows irregular lower surface and the merger of content of a large electron-dense vesicular mass (X) with cuticular surface. Content of an electron-translucent vesicle, to left of X, appears coalesced with cuticle where it is in contact with cuticle. Other vesicular masses along irregular cuticular surface (as at long arrow) appear coalesced to cuticle. Bar = 100 nm.

Figure Abbreviations: C, cuticle; S, secretory cavity; V, secretory vesicle or vesicular content; W, subcuticular wall; bar measurements are in nanometers.



Figs. 3–4. Glandular sheath and secretory cavity in sectional view. **3.** Secretory cavity shows large vesicles with lobes (X) possibly representing the fusion of vesicles. Subcuticular wall shows vesicles of different density in fibrillar matrix. Some fibrils in the wall are arranged as distinctive pairs (long arrow) bridged by electron-dense particles in the core along their length. Vesicles, some appearing flattened (small arrow), are appressed to cuticle along its irregular surface, but separated from cuticle by fibrillar matrix. Other vesicles (Z) show the upper portion of their content confluent with cuticle. Cuticle possesses an irregularly thickened lower surface, the thickening representing vesicular contents derived from subcuticular wall (large arrowhead). Fibrillar matrix appears as electron-dense strands of residual matrix (small arrowheads) in cuticular layer following fusion of vesicular material with cuticle. Some electron-dense material also is evident on outer surface of cuticle. Bar = 100 nm. **4.** Secretory cavity shows

a vesicle could become partially flattened (extreme left, Fig. 3), whereas other vesicles appeared vertically ovoid and extended deeply into irregularities along the cuticular surface (as at center, Fig. 3).

Cuticle—The outer surface of the cuticle appeared as a smooth continuous layer, although in some sections small masses of dark material were evident on the surface (Figs. 3–4). The cuticular surface facing the secretory cavity, as shown in all figures, was irregular, which reflected differences in cuticle thickness that ranged from 68 nm to 143 nm or more at thickened regions.

Vesicles with contents of similar or different densities to that of the cuticle, but lacking their surface feature where they contacted the cuticle, appeared to be coalesced with the irregular cuticular surface (Fig. 2, vesicle X). The content of the light vesicle adjacent to and at the left of vesicle X also lacked its surface feature where it contacted the cuticle (Fig. 2). Similarly, the content of vesicles (Z) of light density, a portion of which projected into the cuticle, appeared confluent with the cuticle (Fig. 3). Differences in density were evident for small vesicular masses of material, and often partially outlined by fibrillar matrix, which appeared to be fused to the inner cuticular surface (Fig. 7).

Vesicles clustered together with a common border, and dark fibrillar matrix aggregated at their center, also appeared coalesced to the cuticle (Fig. 1, long arrow). Others of different sizes and densities in contact with each other appeared to lack the surface feature at their point of contact (Figs. 5–6).

Vesicular materials coalesced to the cuticle can be detected at thickened regions at various positions along the cuticular surface (Figs. 2–3, 5–6).

Dark branch-like components of fibrillar matrix, continuous with that in the subcuticular wall, extended into the cuticle and partially surrounded vesicular materials (Figs. 3, 6). These branches of fibrillar matrix in the cuticle were evident along the entire inner cuticular surface and occurred in various orientations to this surface. Similar dark strands that appeared as isolated segments in the cuticle may represent sectional views of fibrillar matrix branches (Fig. 7).

The cuticle contained numerous narrow electron-light striae that extended more or less from the center of the cuticle to its outer surface (Figs. 6–7). Striae were oriented in various directions and occurred as straight or curved features in the thick cuticle. Many striae were long, measuring up to 130 nm in these sectional views. Although they lacked a sharply defined boundary their diameter measured 6 nm. Striae occurred parallel to some dark strands of fibrillar matrix and in other views they appeared to be continuations of the matrix (Figs. 6–7).

The irregular inner surface of the cuticle consisted of distinctive masses of material, partially delimited by residual fibrillar matrix, which contributed to the increased thickness of the cuticular layer of the secretory cavity (Figs. 6–7). Such masses, made evident by their somewhat different density or by residual matrix, were detectable throughout the cuticle (Fig. 7).

DISCUSSION

The HPC-CS procedure provided a broadened insight into organization and formation of the sheath and secretory cavity, an extracellular and intrawall area, in the glandular trichome. This procedure is interpreted to retain cellular features in a more normal state of preservation with a minimum of tissue distortion than chemical fixation (CF) and to minimize artifacts attributed to CF (Hunziker, 1993).

Vesicles in the secretory cavity, examined by HPC-CS, possessed a distinctive surface feature approximately one-half the thickness of a typical bilayered membrane. In CF a bilayered membrane was detected around vesicles during the formative phase of the secretory cavity, whereas only a single-layered feature surrounded vesicles later in cavity development (Hammond and Mahlberg, 1978; Kim and Mahlberg, 1991; Mahlberg and Kim, 1991). The presence of a single-layered feature around secretory vesicles, rather than a bilayered membrane, may reflect the interaction of lipophilic contents of vesicles with other components, possibly proteins, which are reported in the cell wall (Fry, 1988). The surface feature of these vesicles may bear resemblance to that of oil bodies in plant cells or to that of very low density lipoprotein bodies in animal cells, both of which appear as a single layer and contain a proteinaceous component (Hamilton, Moorehouse, and Havel, 1991; Tzen, Lie, and Huang, 1992).

Vesicles in the secretory cavity could be very large, larger than those in the subcuticular wall. HPC-CS preparations provided evidence for fusion in that the absence of a surface feature between neighboring vesicles of similar or different sizes and densities in the secretory cavity and subcuticular wall was indicative of coalescence. Their large size in HPC-CS, as well as CF preparations, was indicative that their size could derive from vesicle fusion. The presence of lobes associated with large vesicles in HPC-CS preparations suggested that they may have resulted from vesicle fusion rather than represent artifactual distortion. The presence of multilobed vesicles lacking a surface feature at their points of contact also supported an interpretation of vesicle coalescence.

The thickened and irregular inner surface of cuticle was previously described to result from coalescence of vesicular contents with existing cuticle (Mahlberg and Kim, 1991). This interpretation was supported here in that vesicles of different densities were observed to lack their surface feature where they contacted the cuticle. The typically smaller size of vesicles and their more dense con-

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vesicles with single surface feature (arrowhead). Subcuticular wall shows fibrillar matrix and vesicular contents. Some vesicles (X) appear fused since they lack a surface feature at their point of contact. The irregular outline of dense vesicular material (long arrow) may represent a cluster of small vesicles, partly coalesced, appressed to cuticle. Cuticle shows irregular contour of lower surface. Some electron-dense material also is evident on outer surface of cuticle. Bar = 100 nm.

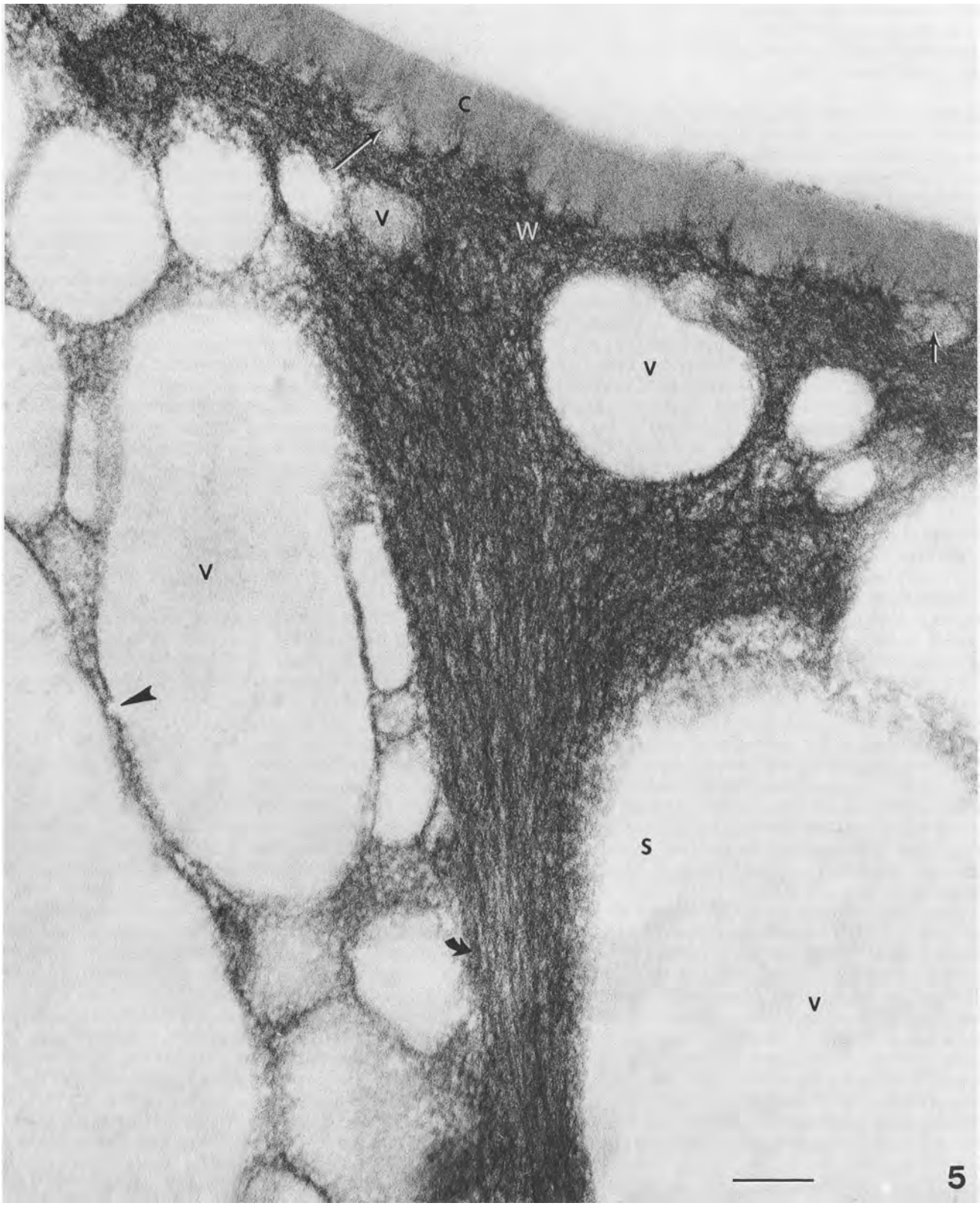
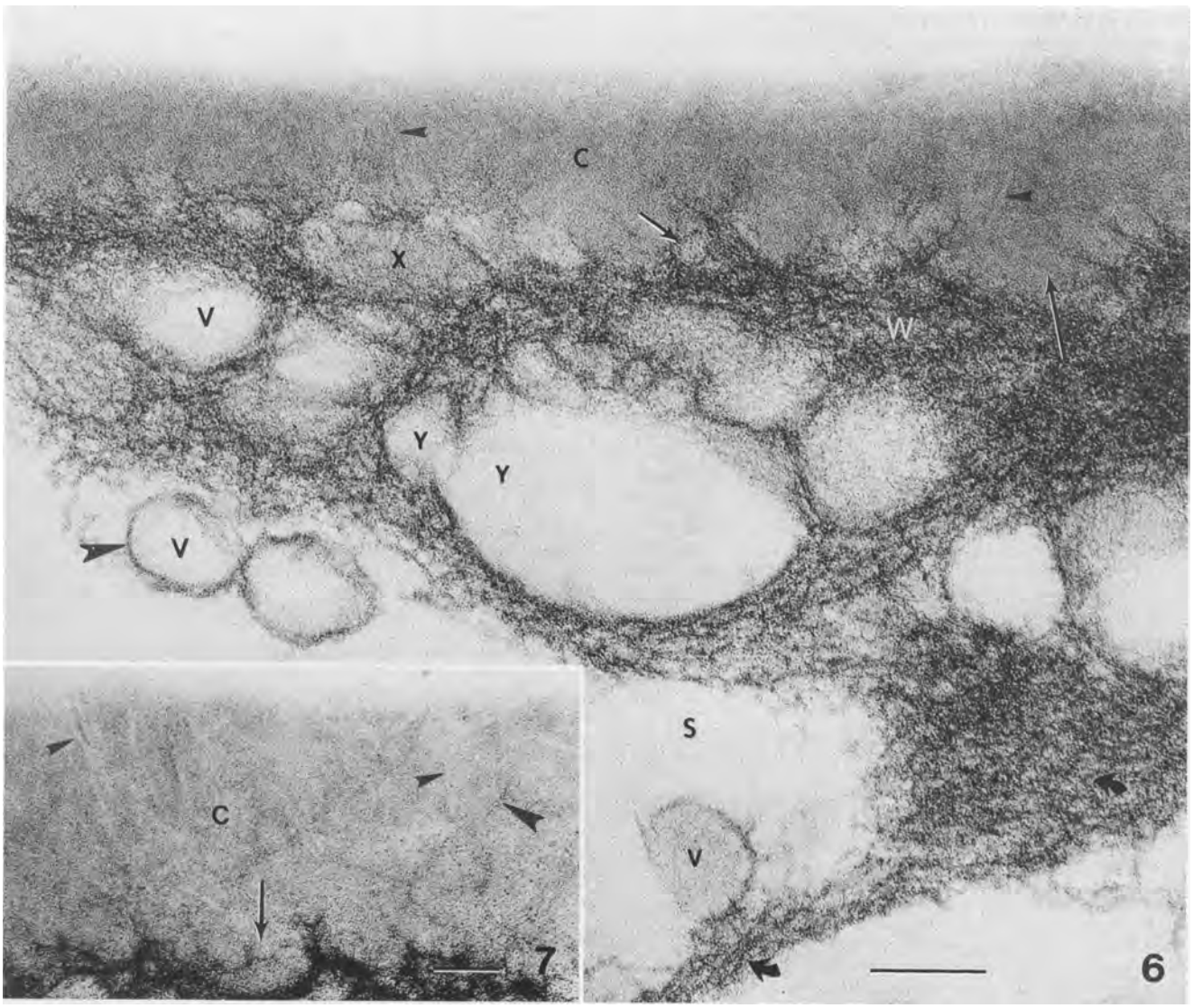


Fig. 5. Glandular sheath and secretory cavity in sectional view. Secretory cavity shows numerous vesicles of different sizes and density, and a large mass of fibrillar matrix (curved arrow) that is continuous with matrix in subcuticular wall. Vesicles lacking a surface feature at their point of contact (arrowhead) appear to have coalesced. Subcuticular wall shows fibrillar matrix continuous with matrix in secretory cavity, and vesicles interspersed in the matrix. Cuticle inner surface appears irregularly thickened. Vesicular material, some of which appears to consist of several fused vesicles (short arrow), is partially coalesced to inner cuticular surface. Elsewhere along cuticular surface vesicular material (long arrow) appears fused to cuticle. Bar = 100 nm.



Figs. 6–7. Glandular sheath and secretory cavity in sectional view. **6.** Secretory cavity shows fibrillar matrix (curved arrows) and vesicles of different sizes and density, which possess a single surface feature (large arrowhead). Absence of surface feature between neighboring vesicles (Y) suggests their coalescence. Subcuticular wall shows vesicles in fibrillar matrix. Some vesicles are appressed to cuticle (short arrow). Cuticle shows vesicular material (X) appressed to its inner surface. Thickening along inner irregular cuticular surface derives from vesicular materials coalesced to this inner surface (long arrow). Numerous striae (small arrowheads) are evident throughout cuticle. Bar = 100 nm. **7.** Cuticle showing coalesed vesicular materials (arrow and surrounding area) to form irregular inner surface, and residual fibrillar matrix (electron-dense strands), partially surrounds vesicular materials fused to the cuticle. Some residual fibrillar matrix (large arrowhead) may persist deep in cuticle. Striae (small arrowheads), evident throughout cuticle, extend toward outer surface of cuticle and appear related to sites occupied by residual strands of fibrillar matrix prior to its disappearance from the cuticle. Bar = 100 nm.

tents, resembling that of the cuticle, suggested that these contents may in some way become concentrated prior to coalescence with the cuticle. Decrease in vesicle size and concentration of their contents, a phenomenon that must be studied further, may be related to the release of volatile monoterpenes from the cuticular surface of a gland as plant fragrances (Tyson, Dement, and Mooney, 1974; Malingré et al., 1975; Hammond, Kim, and Mahlberg, unpublished data).

Traces of fibrillar matrix associated with vesicles in the subcuticular wall appeared as residual fibrillar matrix around vesicular material coalesced with the cuticle. Residual matrix in the cuticle also was evident in CF preparations (Mahlberg and Kim, 1991). During continued thickening of cuticle, as evident in HPC-CS preparations, these fibrillar strands became localized more deeply in the cuticle by deposition of additional vesicular material to the inner cuticular surface. Although the fate of these strands of residual matrix in the cuticle is uncertain, the sites of the strands lacking matrix closely resembled striae in diameter, length, and orientation. Thus, residual fibrillar matrix, most evident in the inner portion of the

cuticle, is interpreted to be degraded somehow to reveal in outline the striae that are most evident in the outer portion of the cuticle.

The orientation of wall fibrils into paired arrays represents a level of wall organization not previously detected. The factors controlling the organization of these arrays as well as their function are yet to be determined. The presence of these arrays in the wall suggests that they may perform a role in controlling the distribution of secretory vesicles along the irregular cuticular surface to maintain a more or less uniform thickness of the cuticle during its development.

Although there is a general interest in cuticle organization on epidermal cells (Uphof, 1962; Martin and Juniper, 1970; Lyshede, 1978; Baker, 1982; Cutler, Alvin, and Price, 1982; Hallam, 1982; Holloway, 1982; Rodriguez, Healey, and Mehta, 1984; Miller, 1985), few studies have been directed to the cuticle of the gland. However, the gland can serve as a model for studying the cuticle formation because of the visualized clarity of the enlarged secretory cavity, the progressive enlargement of the subcuticular wall, and the progressive thickening of the cuticle proper with secretions from the secretory cavity compared to less evident observable events associated with cuticle formation on epidermal cells. Thus, studies of glands can provide a more complete understanding of cuticle formation not only on the gland but on plant cells in general.

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