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Ultrastructure of the Principal Extrafloral Nectaries of Hevea brasiliensis

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The structural changes leading to nectar secretion in the extrafloral nectaries of Hevea *were examined by light and electron microscopy. The nectary is served by vascular tissues which arise from the branch supplying to the leaves. Only the phloem traverses up to the sub-secretory cells. Ultrastructural evidence support an energy-requiring, eccrine mode of secretion, while the pathway taken by the nectar can be both apoplastic and symplastic. Release of nectar is by means of the cuticle which ruptures under pressure. The significance of nectar production to latex flow physiology is also discussed.*

The extrafloral nectaries described in this paper refer to the structures/glands, normally three in number but varying from two to four, that are borne at the point of attachment of the stalks (petiolules) of the three leaflets. Nectar is secreted from these glands exclusively during refoliation. There are also other extrafloral nectaries in *Hevea*, e.g. on the leaf axils, flowers and abaxial leaf surfaces, but their nectar secretion is minimal. A brief description of the structure of these nectaries has been given by Heusser[1] in 1920 and Frey Wyssling[2] in 1933 gave an account of their morphology, anatomy and physiology.

The recent interest in the nectaries in *Hevea* is due to the increased importance of bee keeping under rubber trees. The bees particularly depend on nectar from these extrafloral nectaries. Studies carried out in the Rubber Research Institute of Malaysia (RRIM) have shown some inter-relationship between nectar flow, development of the bee colony and honey yield[3]. In the field of ultrastructure, many workers[4-7] are also interested in establishing the various methods of nectar secretion based on cytology of the secretory cells.

This paper traces the ultrastructural changes leading to nectar secretion with special attention to the mode of secretion. The association of the latex vessel system and phloem in relation to nectar secretion is also discussed.

MATERIALS AND METHODS

Observations of nectaries were made from leaves of mature and untapped RRIM 600 trees grown in the RRIM Experiment Station at Sungei Buloh. Nectaries of various secreting stages corresponding to the different colours of the leaf related to the developmental stages, *i.e.* bronze for pre-secretory, light green for secretory and dark green for post-secretory, were sampled from three trees. These were fixed in a mixture of 3% paraformaldehyde and 2.5% glutaradehyde in 0.1 *M* phosphate buffer (pH 7.3) with 0.15 *M* sucrose added, for 2 h and then post-fixed in buffered 2% OsO_4 for another 2 h. Dehydration was in the graded ethanol series. Tissues were embedded in Epon-araldite according to Mollenhauer[8]. Ultra-thin sections were double-stained with saturated uranyl acetate and lead citrate and examined on Philips EM 300.

For light microscopic studies, semi-thin sections made from the above resin-embedded blocks were stained with 1% toluidine blue. Paraffin-embedded sections were also examined and these were stained with safranin and fast green[9].

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OBSERVATIONS

Light Microscopy

The extrafloral nectary tissue is composed of two groups of cells distinct from the neighbouring parenchyma cells *(Figure 1)*. The secretory layer, which is a modified epidermis, is made up of two to three tiers of dense columnar cells. Below them, the one to two layers of sub-secretory cells, are arranged according to the contour of the secretory layer. The other secretory cells are randomly arranged among the parenchyma cells, and are distinguished by their dense cytoplasm *(Figure 2)*.

The vascular system supplying the nectaries arises from the branch of the vascular system which supplies to the leaves *(Figure 1)*. However, only the phloem traverses as far as the sub-secretory cells, the xylem stops at the boundary between the parenchyma and the sub-secretory cells *(Figure 2)*.

The nectaries are covered with a thin cuticle which becomes more noticeable when nectar is being secreted, that is when the cuticle is detached from the surface at some points forming 'balloons'. Latex vessels have also been observed to traverse up to the sub-secretory cells and are closely associated with the phloem *(Figure 3)*.

Electron Microscopy

Pre-secretory stage. The secretory cells have very dense cytoplasm due to heavy deposits of granular material which sometimes mask the organelles (*Figures 4* and *5*). These columnar cells have thin walls, large nuclei, abundant mitochondria and dense amoeboid plastids containing very few thylakoid membranes. Endoplasmic reticulum of both the smooth and rough types are present and are mostly found close to the cell wall. Dictyosomes are relatively rare.

Few vacuoles are found in the secretory cells and they are small and often filled with stainable material of various densities and structures. Sometimes the vacuoles are completely filled with electron-dense material, possibly tannin. Plasmodesmata are common between adjacent secretory cells and between sub-secretory and secretory cells.

The sub-secretory cell is similar in appearance to the secretory cell except that the sub-secretory cell has larger and empty vacuoles and plastids containing starch grains *(Figure 6)*.

Secretory stage. The general impression given by the secretory cells during secretion is the reduction of cytoplasm, due to the presence of large vacuoles (at least one per cell). These are always filled with electron-dense material either granular in appearance or as thick deposits scattered or lining the periphery of the vacuoles *(Figure 7)* and are possibly tannins.

Besides these types of large vacuoles, smaller ones are also common and these are present in clusters or merging into bigger ones. The smaller ones are always filled with lighter stainable material. The cytoplasm remains dense as in the pre-secretory stage. Mitochondria are abundant and some plastids contain numerous plastoglobuli.

Endoplasmic reticulum is more prominent than in the pre-secretion stage but does not seem to show very much dilation. Even though sometimes located parallel to the plasmalemma, there appears to be no association with them. Dictyosomes are also rare.

An interesting feature to note at this stage is the appearance of prominent intercellular spaces especially in between the longitudinal walls of the secretory cells *(Figure 8)* and seldom between the sub-secretory cells. Some spaces would persist right up to the free surface of the secretory cells *(Figure 7)*. Stainable material is always observed in these spaces which could either be the cuticle or the nectar itself, but at a higher magnification, this material resembles the cuticle taken from the secretory cell of a bronze leaf, *i.e.* before secretion *(Figure 9)*. The cuticle is shown to be fibrillar in nature, loosely arranged unlike the cellulose walls below it. The swelling of the cuticle as observed under the light microscope

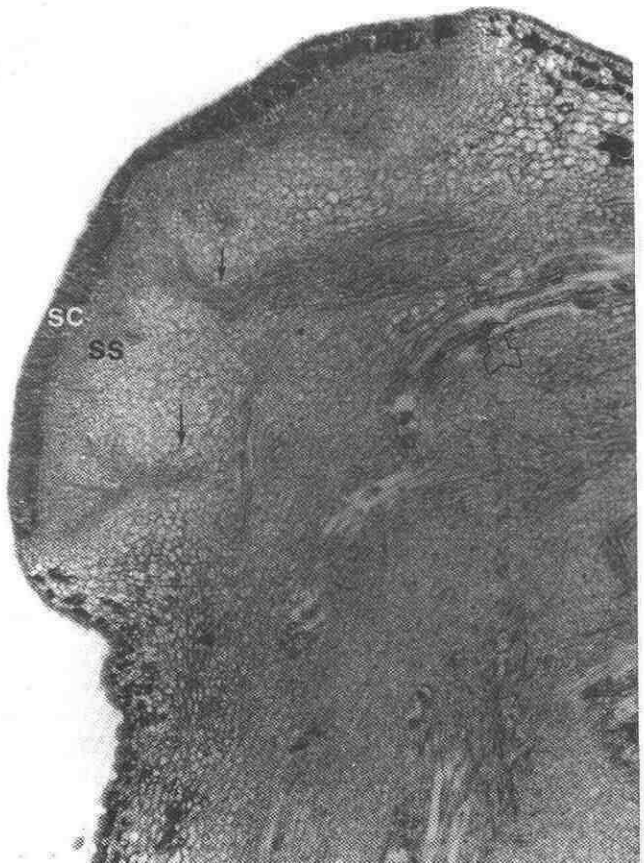

Figure 1. Nectary gland showing secretory cells (sc), sub-secretory cells (ss), vascular system supplying leaf (large arrow) and a branch of it supplying to the nectary (small arrows). Magnification 40 ×.

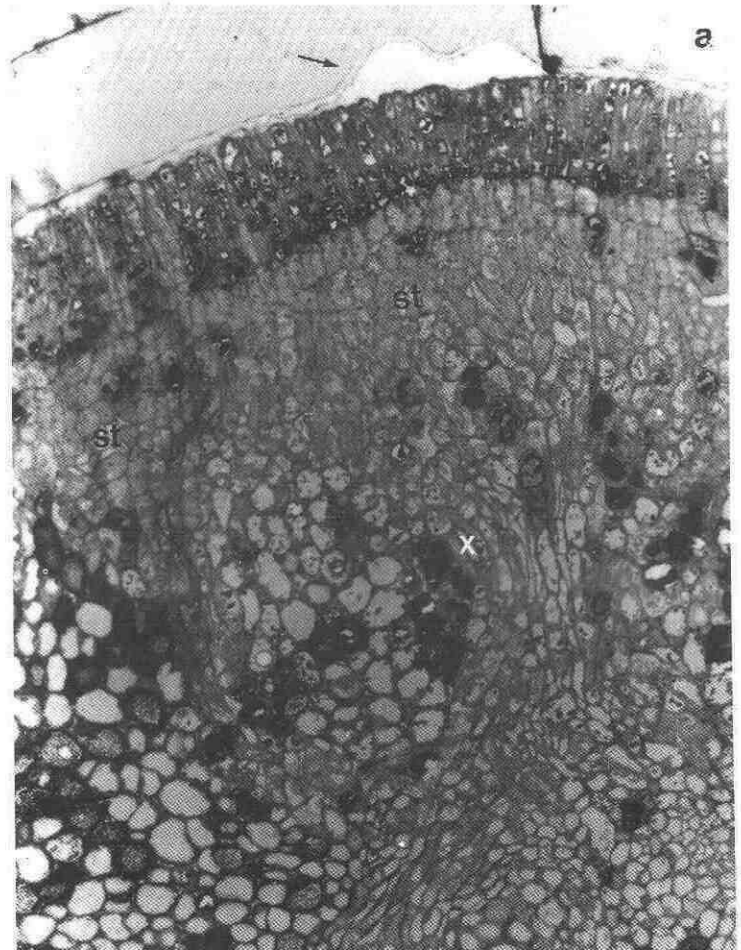

Figure 2. Nectary gland during secretion showing cuticle ballooning (arrow). Note relative position of sieve tube (st) and xylem (x). Magnification 160 ×.

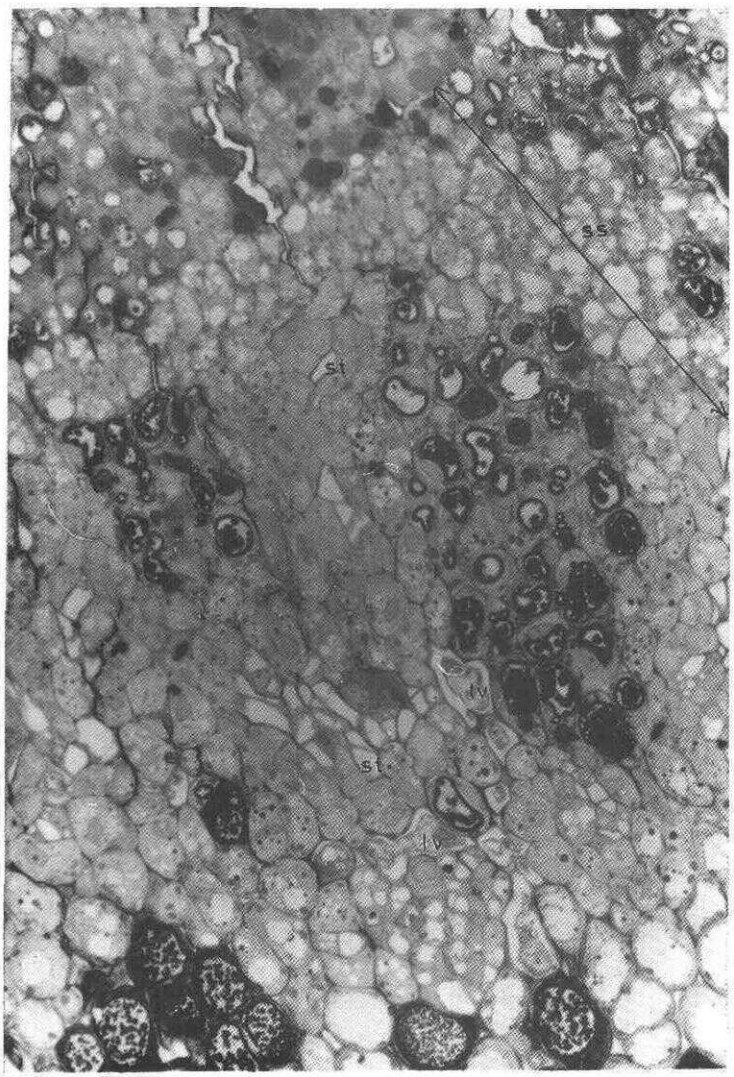

Figure 3. Sieve tube (st) and latex vessel (lv) distribution in the sub-secretory area (ss). Magnification 330 ×.

and in the scanning electron microscope *(Figure 10)* was never observed at the ultrastructural level.

Figure 11 shows a sieve tube in relation to its neighbouring cells. In most observations, the sieve tubes are about the same size or never larger than the companion cells (see *Figure 3* for overall view). Companion cells are characterised by the dense cytoplasm and smaller vacuoles compared to the parenchyma cells. Plasmodesmatal connections between sieve tubes and companion cells have also been observed *(Figure 12)*. Latex vessels are also usually located within the vicinity of the sieve tubes. In this sample preparation however, the rubber particles appear to be rather unstable.

The sub-secretory cells during secretion have smaller vacuoles and there is an increase of

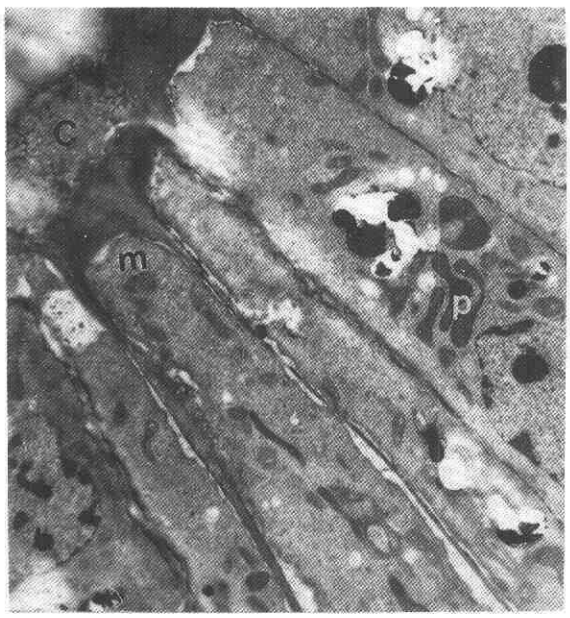

Figure 4. Secretory cells at pre-secretion showing dense cytoplasm, plastids (p), mitochondria (m) and cuticle (c). Magnification 1800 ×.

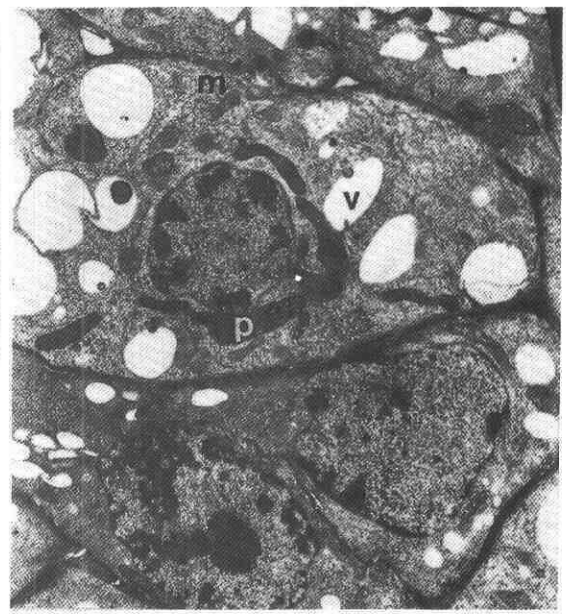

Figure 6. Sub-secretory cells at pre-secretion showing plastids (p), mitochondria (m) and vacuoles (v). Magnification 1800 ×.

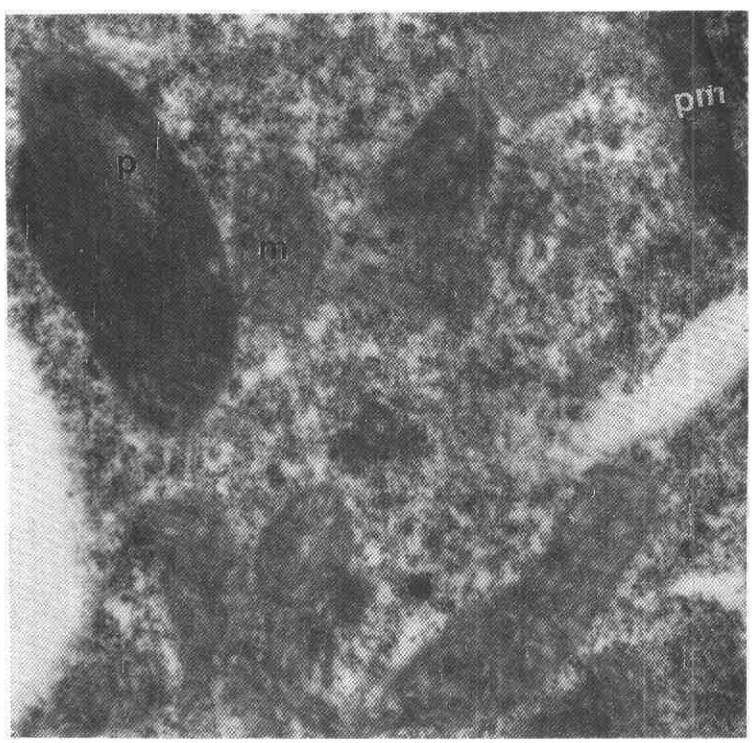

Figure 5. Cytoplasm in the secretory cell at pre-secretion masking the organelles: plastid (p), mitochondria (m). Note the plasmodesmata (pm). Magnification 27 000 ×.

Figure 7. Secretory cells during secretion showing vacuoles (v), mitochondria (m), plastid (p). Intercellular spaces (is) lined with cuticle (c) are continuous with the external surface. Magnification 4000 ×.

endoplasmic reticulum compared to the pre-secretion stage.

Post-secretion. Since samples were taken when there were no traces of nectar observed, there is no way of determining when secretion ceased. The secretory cells may therefore appear similar to those at secretion or they may have very dark cytoplasm, normally occupying a thin peripheral layer as a result of the large vacuoles present *(Figure 13)*. Mitochondria, dictyosomes, endoplasmic reticulum and small vacuoles

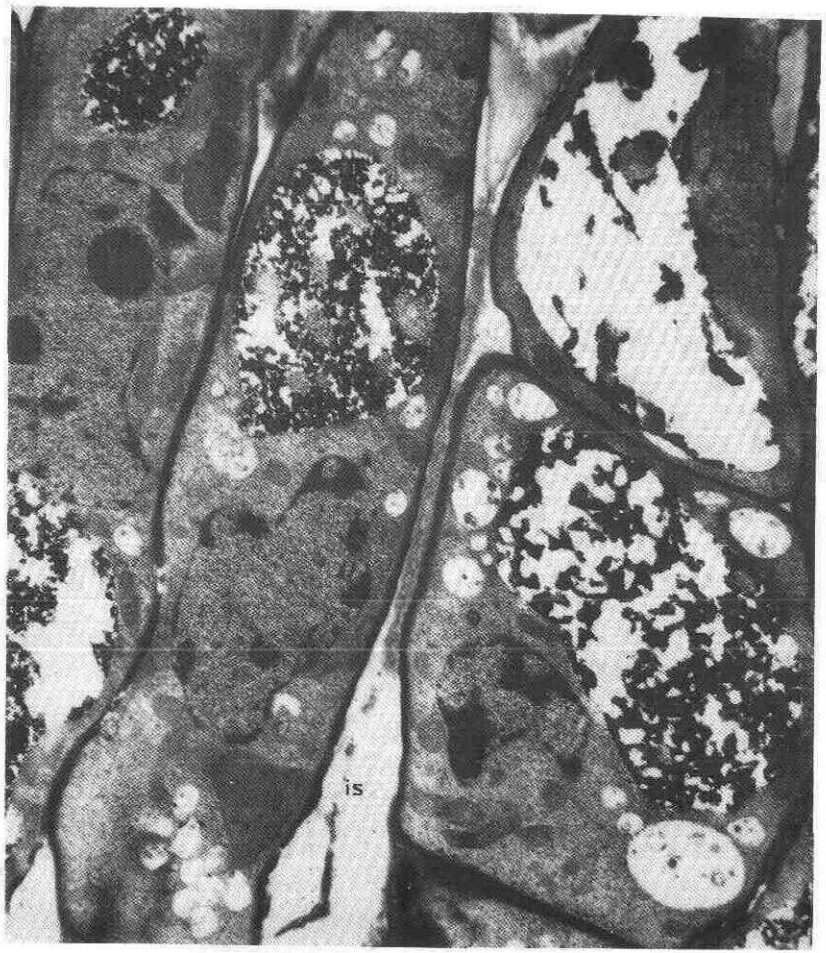

Figure 8. Secretory cells showing intercellular spaces (is) in between them. Magnification 3000 ×.

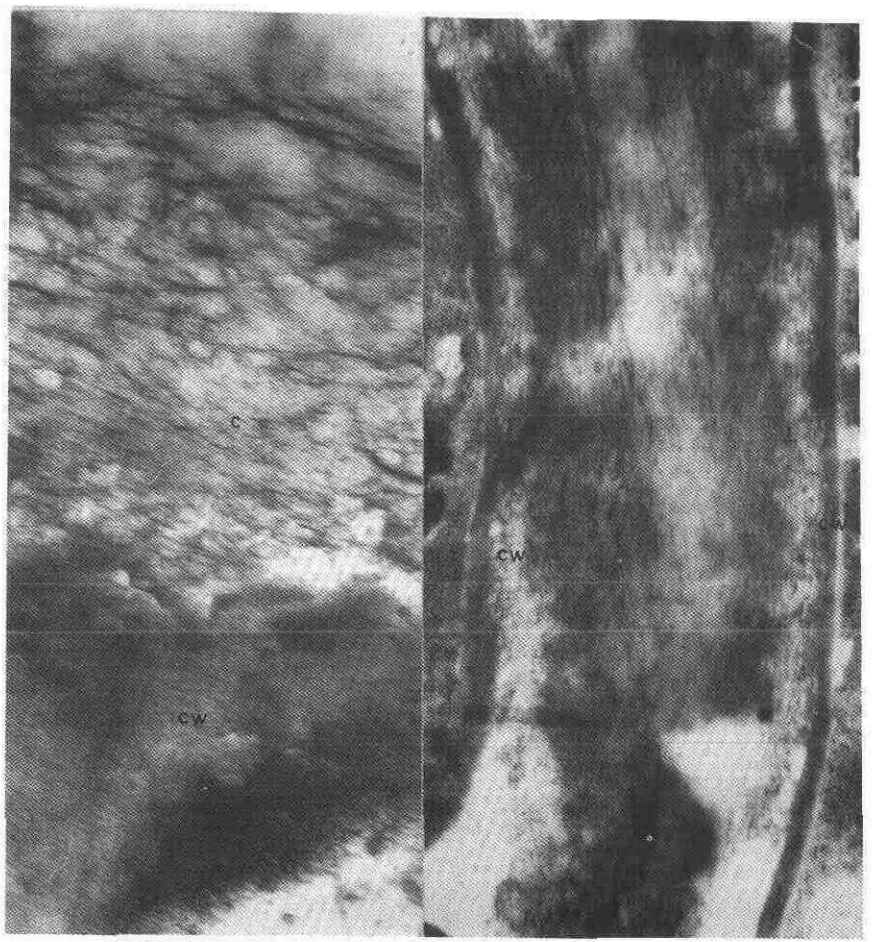

Figure 9. High magnification of cuticle (c) above the cell wall (cw) (left), compared with stainable material in the intercellular spaces (right). Magnification 27 000 ×.

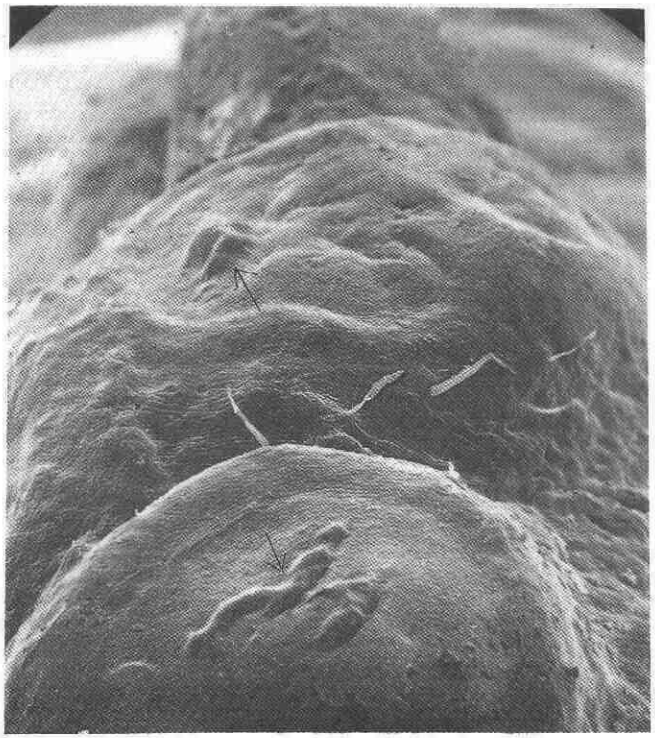

Figure 10. Scanning electron microscopy of the nectary glands showing buckling of the cuticle (arrows). Magnification 90 ×.

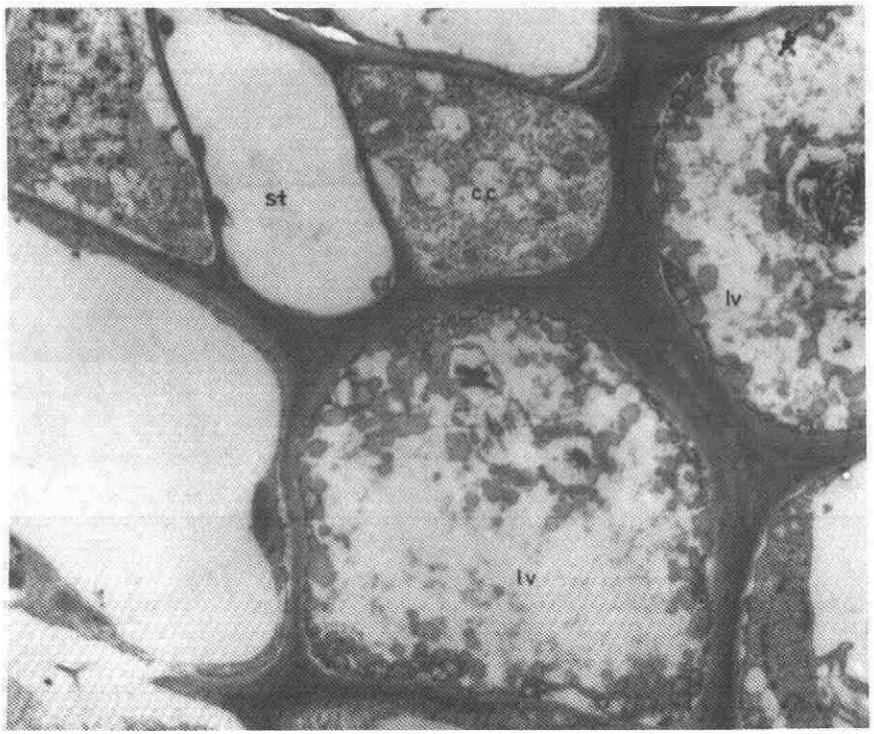

Figure 11. Sieve tube (st) in relation to companion cell (cc) and latex vessels (lv). Magnification 4000 ×.

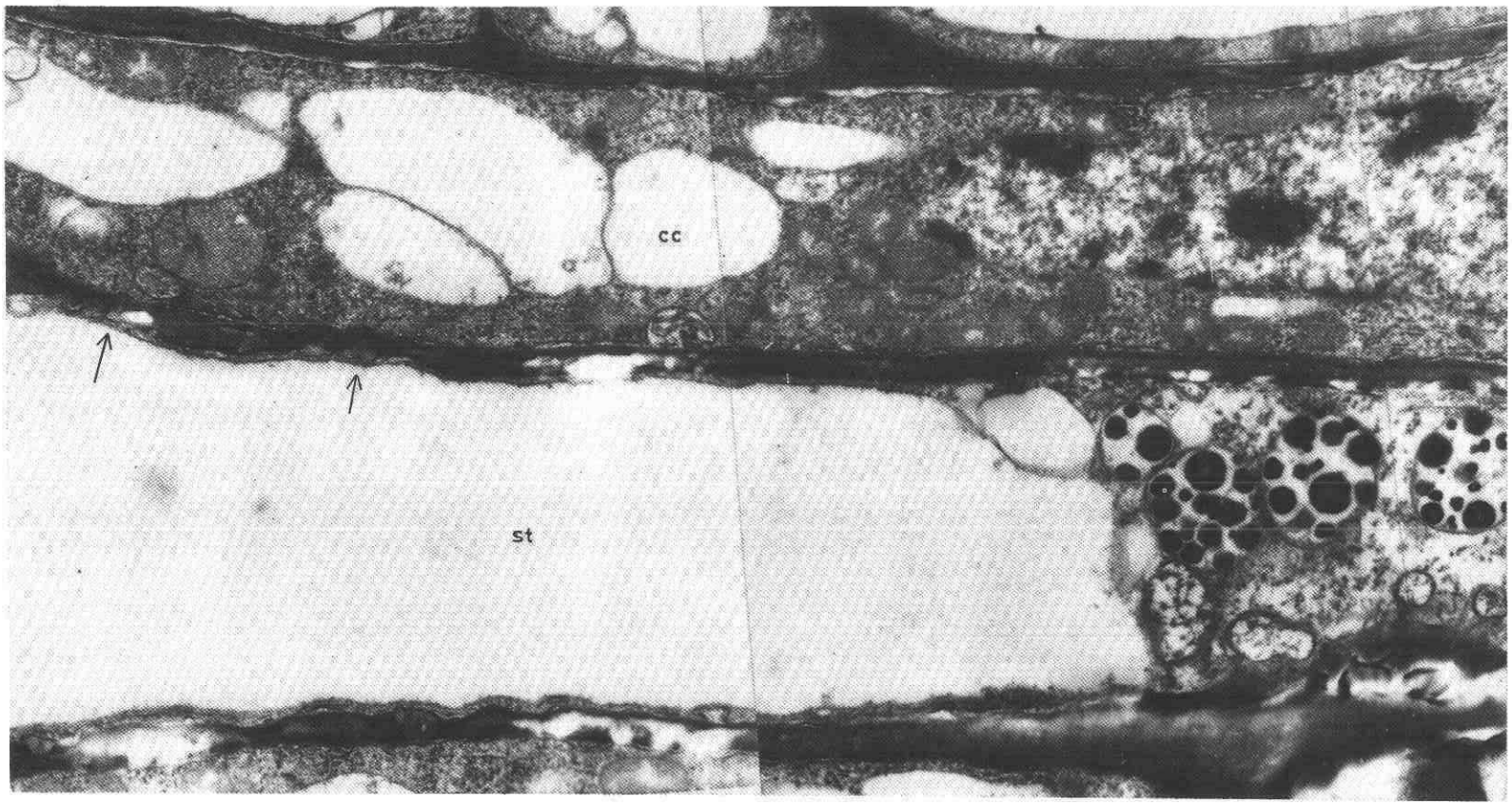

Figure 12. Longitudinal section of sieve tube (st) and companion cell (cc) showing the plasmodesmatal connection (arrow) between them. Magnification 4000 ×.

containing vesicles are also present *(Figure 14)*. Plastids have very well-developed thylakoid membranes.

The cuticle ultrastructure is no longer fibrillar but has become rather thick and dense. It is possible that the microstructure has been altered. Continuity of the cuticle is also broken in some parts of the nectary surfaces.

DISCUSSION

The ultrastructure of the nectary tissue of *Hevea* observed during secretion is typical of most nectary cells[4-7], *i.e.* dense cytoplasm, abundant endoplasmic reticulum, mitochondria, small vacuoles and plasmodesmata. Dictyosomes are also present but relatively rare. An exception perhaps is the presence of large vacuoles containing dark-staining material, possibly tannin, which reduces the cytoplasm. The definite role of these large vacuoles at this stage is unknown but these are perhaps a result of normal cell maturity.

The presence of intercellular spaces and plasmodesmata could indicate an apoplastic and symplastic route respectively for the nectar secretion. Nectar which has been shown to be secreted phloem sap[10] is transported through the symplasm *via* the plasmodesmatal connections that exist right through to the secretory cells. The enlarged companion cells could also play a role in the short-distance transport from the sieve tubes to the sub-secretory cells as was suggested for the extrafloral nectaries of *Passiflora*[5]. On the other hand, an apoplastic route would also be possible especially at the secretory cells where large spaces were observed during secretion. The lining of the spaces with cuticle and its continuity with the external surface have also been shown in *Brassica*[6], *Vinca* and *Citrus*[7]. In these plants however, the spaces are in continuity with the stomatal spaces, the stomata being the exit zone for the nectar. In *Hevea*, nectar is probably released through the cuticle which breaks up upon pressure building up, resulting from nectar accumulation beneath the cuticle. This is seen in the swelling of the cuticle and its discontinuity under the electron microscope.

As for the mode of secretion, the ultrastructural evidence such as abundance of mitochondria and lack of dictyosomes and dilated endoplasmic reticulum seem to indicate an active eccrine machanism rather than the granulocrine. A similar conclusion based on the above observations was also made for *Brassica napus*[6] and *Passiflora*[5]. The abundance of endoplasmic reticulum and mitochondria after the cessation of secretion however may indicate a different role for them. These may be involved in the lysis of cytoplasm as demonstrated in *Sambucus*[4], which was inferred to result in the darkening of the cytoplasm.

The occurrence of phloem and the absence of xylem in the sub-secretory cells are significant to the sugar content of the nectar. The mean of 13% sugar content obtained in an untapped ten-year-old tree[11] is quite low as compared to the values (up to 50% sugar) given by Frey-Wyssling[10] in nectaries solely supplied by the phloem. Our values obtained were measured quite early in the morning and it is possible that evaporation had not taken place yet.

The presence of nectar is significant in *Hevea*. It not only provides nectar for the bees and other nectarphillic insects, but is also related to other physiological functions. Available data has shown that the volume of nectar and its sugar content were reduced when the trees are tapped[11]. It is possible that nectar production and latex flow are inter-related. Studies by Low and Gomez[12] concluded that the increase of sucrose in the second tapping of trees tapped twice daily was due to the import of sugars from remote regions of the panel. Hence, if trees are tapped, there is a possibility that sugar meant for the nectar is mobilised to the tapping panel. The reduction in volume of sugar in the nectar can also be explained by the effect caused on the phloem, as a result of the suction pressure created when turgor is lost during tapping[13].

Sections of nectary glands have also been seen to harbour fungal colonies which may cause leaf diseases[14]. It is possible that the fungi use the nectar as their substrate and hence the glands act as the point of entry of pathogenic organisms. Based on the above

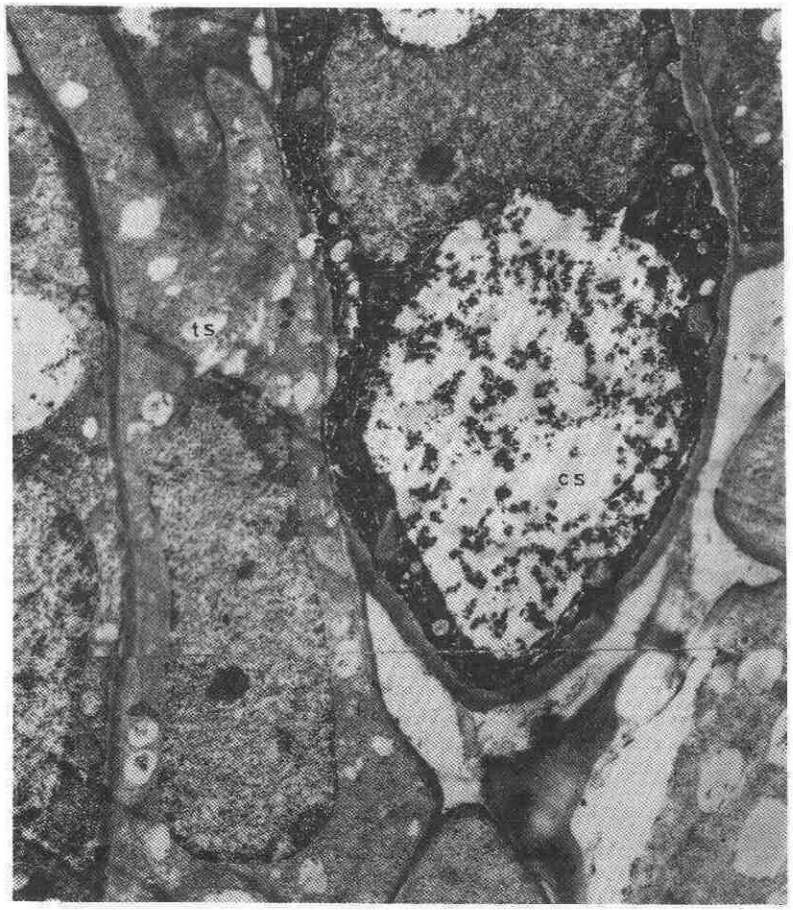

Figure 13. Secretory cells at post-secretion showing both typical secretory cell (ts) and cell undergoing cytolysis (cs). Magnification 4000 ×.

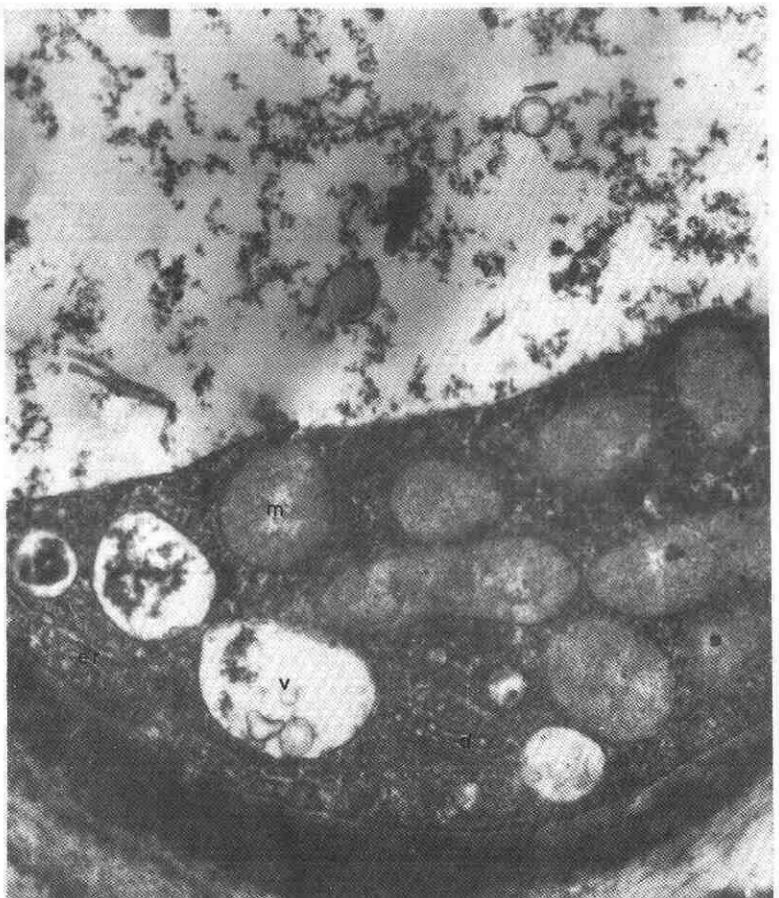

Figure 14. Secretory cell with darkened cytoplasm showing mitochondria (m), dictyosome (d), vacuoles (v) and endoplasmic reticulum (er). Magnification 27 000 ×.

observation it would be interesting to speculate whether the removal of nectar by nectarphillic insects would reduce the incidence of leaf diseases.

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