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CHEMICAL AND MORPHOLOGICAL STUDIES ON SITES OF SESQUITERPENE ACCUMULATION IN *POGOSTEMON CABLIN* (PATCHOULI)

W. HENDERSON,* and JAMES W. HART,† P. HOW and J. JUDGE

Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford

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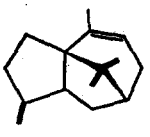
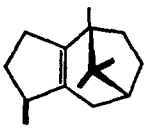
Abstract—Techniques are described for the micro-analysis of the free volatile sesquiterpenes in plant tissue by direct-loading gas liquid chromatography. Evidence is presented for the accumulation of sesquiterpenes, not only in the external glandular trichomes, but also in specialized internal accumulatory cells. The morphology and development of these external and internal structures are described as seen under light and electron microscopy. Also, while the apical dome of the stem neither shows any specialized accumulatory structures nor contains any detectable sesquiterpenes, the second pair of primordial leaves has glandular trichomes and a sesquiterpene concentration approximately twelve times higher than that in any other part of the plant.

INTRODUCTION

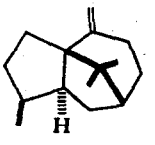
A COMMERCIALLY important essential oil is distilled from the leaves of *Pogostemon cablin* Benth. (Syn. *P. patchouli* Pellet var. *suavis* Hook).[1] The major constituents of patchouli essential oil are the sesquiterpenes α-, β-, and λ-patchoulene (I, II, III), α-guaiene (IV), α-bulnesene (V), and patchouli alcohol (VI).[2,3] In common with other members of the



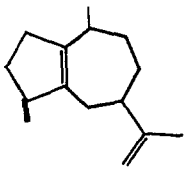
α-Patchoulene (I)



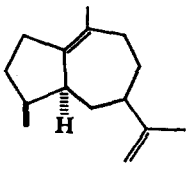
β-Patchoulene (II)



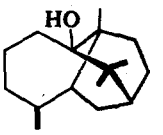
γ-Patchoulene (III)



α-Guaiene (IV)



α-Bulnesene (V)



Patchouli alcohol (VI)

* Present address: Space Sciences Lab., University of Calif., Berkeley, Calif. 94720, U.S.A.
† Present address: Dept. of Botany, University of Aberdeen, Scotland.

[1] E. GUENTHER, *The Essential Oils*, Vol. III, p. 553, Van Nostrand, New York (1949).
[2] J. A. WENNIGER, R. L. YATES and M. DOLINSKY, *Pro. Sci. Sec. Toilet Goods Assoc.* **46**, 44 (1966); N. TSUBAKI, K. NISHIMURA and Y. HIROSE, *Bull. Chem. Soc. Japan* **40** (3), 597 (1967); R. B. YATES and R. C. SLAVER, *Chem Ind.* 1715 (1962).
[3] G. BUCHI and R. E. ERICKSON, *J. Am. Chem. Soc.* **78**, 1262 (1956).

Labiatae,[4] the epidermis of *P. cablin* carries glandular hairs or trichomes. The morphology of these structures has previously been briefly described[5,6] although the development of equivalent structures in *Mentha piperita* has been more thoroughly illustrated.[7,8] However, there is little documented evidence to establish that the essential oil is, in fact, accumulated in such structures, except for a short description of their composition in *Mentha* spp.[9]

The isolation procedures of solvent extraction or steam distillation most commonly used for the collection of essential oils involve handling macro-samples of plant tissues. Analyses are therefore usually carried out on fractions which are non-specific with regard to both age and type of plant material. The technique of direct volatilization of individual pine needles to release the free terpenoid constituents for normal gas liquid chromatographic (GLC) analysis[10] seemed worthy of development for use with other types of plant tissue.

In the present report, techniques are described for the micro-analysis of plant tissue for volatile sesquiterpenoids. Evidence is presented which relates sites of sesquiterpenoid accumulation in *P. cablin* to specific external and internal structures. The morphology and development of these structures are described. The plant part with the highest concentration of sesquiterpenoids is shown to be specific primordial leaves in the stem apex.

RESULTS

Analytical Techniques

The efficiency and reproducibility of organic solvent extraction of sesquiterpenoids from plant material was examined:

(i) the reproducibility per operator was no better than ± 10 per cent;
(ii) the variation between operators could be as high as ± 30 per cent;
(iii) the recovery of methyl myristate, used as internal standard, varied between 70–90 per cent.

These results suggest that solvent extraction is not a satisfactory method for establishing a correlation between sesquiterpenoid accumulation and specific plant parts on a microgram scale. Therefore, a more reproducible analytical procedure was developed which minimized the variations introduced by operator handling and eliminated solvent extraction and evaporation. Fresh plant tissue was volatilized in a heated unit and the effluent from this unit was swept directly, by carrier gas, into a gas chromatograph. Figure 1 shows the volatilization unit developed for use with a Pye 104 GLC and 4 mm i.d. packed glass columns for direct GLC analysis of fresh plant tissue.

The optimal conditions for reproducible release of the volatile constituents were determined: a volatilization temperature of 160° gave good release, with minimal charring and pyrolysis typical of higher temperatures, yet without the deterioration of GLC peak resolution obtained at lower temperatures. The volatilized mixture was swept on to the top of the GLC column, maintained at room temperature, by purging the unit with nitrogen at a flow rate of 13 ml/min throughout the duration of the volatilization.

[4] K. ESAU, *Plant Anatomy*, John Wiley, New York (1953).
[5] W. BRANDT, *Proc. Intern. Cong. Plant Sci.* p. 1362, Ithaca, New York (1929).
[6] G. GOGROF, *Pharmazie* **12**, 38 (1957).
[7] K. J. HOWE and F. C. STEWARD, *Cornell Univ. Agric. Exp. St. Memoir* 379, Part II, p. 11, New York State Coll. of Agric., Ithaca, New York, (1962).
[8] F. AMELUNXEN, *Planta Med.* **13**, 457 (1965).
[9] F. W. HEFENDEHL, *Naturwiss* **54**, 142 (1967).
[10] E. VON RUDLOFF, *J. Gas Chromatog.* **3**, 390 (1965).

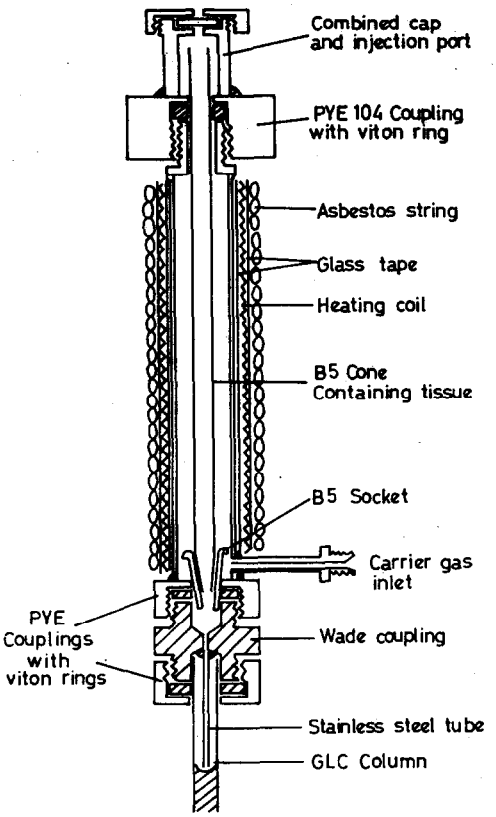


FIG. 1. VOLATILIZATION UNIT USED FOR DIRECT ANALYSIS OF THE VOLATILE SESQUITERPENOID CONSTITUENTS OF FRESH PLANT TISSUE, AS DESIGNED FOR USE WITH A PYE 104 GAS CHROMATOGRAPH EQUIPPED WITH DUAL PACKED COLUMNS.

No significant deterioration of GLC resolution, compared with on-column injection of a sample of commercial Patchouli oil was observed. The high percentage of water in fresh plant material did not appear to affect GLC resolution, nor did it cause deterioration of column performance over a period of months. The technique is reproducible and variations in the pattern of peaks or in the relative abundances could be attributed to the particular biological system. Specimens of plant tissue of 10–100 mg fr. wt. of Patchouli plant produced significant GLC traces: $2{\cdot}5 \times 10^{-10}$ g per component could be detected.

However, for the studies reported here on analyses of glandular trichomes and internal accumulatory sites, even greater sensitivity was required. Support coated open tubular (SCOT) capillary columns (Perkin–Elmer Ltd.), in which an inert solid support is fixed to the walls of capillary tubing and the liquid phase coated onto this layer, represent an attempted compromise between packed and capillary columns. The high plateage characteristics of capillary columns are retained (17,500–20,000) and the greater surface areas allow larger concentrations of mixtures to be analysed at high efficiencies with relatively short retention times, due to the high linear gas velocity through the column.

It was found possible to "direct-load" fresh Patchouli tissue into a heated inlet and volatilize the low-boiling point constituents on to a SCOT capillary column. The inlet arrangement is shown in Fig. 2, which is a standard part of the Perkin–Elmer capillary

column F-11 gas chromatograph. In our studies, less than 1·0 mg fr. wt. of Patchouli tissue could be successfully analysed. Since the support coated capillary columns have a much higher surface area, it should be possible to dispense with the pre-column splitting assembly and thus increase the sensitivity of the method × 15, making possible analyses of specimens of 10–100 μg fr. wt. The details of these techniques are given in the Experimental section.

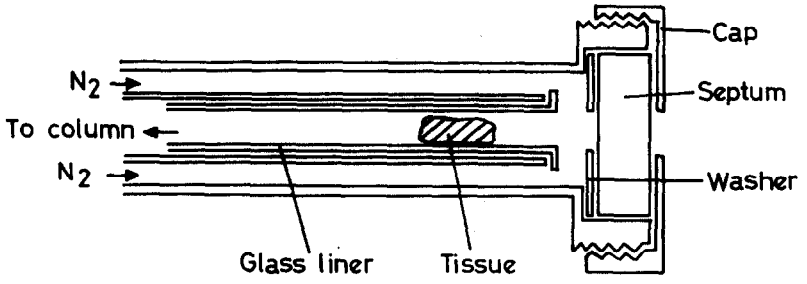


FIG. 2. HEATED INJECTION INLET OF A PERKIN–ELMER CAPILLARY F-11 GAS CHROMATOGRAPH AS USED FOR DIRECT ANALYSIS OF THE VOLATILE CONSTITUENTS OF A FRESH PLANT TISSUE WITH SUPPORT COATED CAPILLARY COLUMNS.

Whole Plant Analysis

The chemical composition of the commercial Patchouli essential oil was investigated by gas chromatographic and mass spectrometric techniques. Similarly, an organic solvent extract of *Pogostemon cablin*, grown under our conditions, was subjected to the same examination. Figures 3(a) and (b) show the capillary GLC records obtained for the two samples. The composition of the separated mixture in each case is virtually identical. Characterization of the individual peaks by combined gas liquid chromatography–mass spectrometry and co-injection of mixed samples showed the major constituents to be α-, β-, and λ-patchoulene, α-guaiene, α-bulnesene and Patchouli alcohol. These results are in agreement with previous workers.

TABLE 1. TOTAL SESQUITERPENE CONTENT AND PERCENTAGE ABUNDANCE OF INDIVIDUAL SESQUITERPENE CONSTITUENTS DERIVED FROM THE DIFFERENT PLANT PARTS OF PATCHOULI BY DIRECT LOADING GLC AND SUBSEQUENT PEAK AREA MEASUREMENT

Patchouli plant parts	Total sesquiterpene content per mg fr. wt. tissue	% Abundance of the constituent sesquiterpenes						
		Peak 1†	Peak 2 (II)*	Peak 3 (IV)*	Peak 4 (I, III)*	Peak 5 (V)*	Peak 6 (VI)*	Peak 6'†
1-cm leaf	7·0 μg	0·7	1·6	19·5	13·4	27·9	37·0	0
7-cm leaf	11·4 μg	0	1·9	20·5	12·0	29·0	36·7	0
11-cm leaf	4·2 μg	0·6	1·8	21·0	12·6	26·5	37·4	0
Outer cortex	1·9 μg	2·75	2·3	20·1	13·2	28·4	33·4	0
Inner stele	0	0	0	0	0	0	0	0
(Lateral) root	0·1 μg	0	9·3	22·0	14·8	22·3	22·8	8·5
(Adventitious) root	1·2 μg	0	12·9	20·6	14·8	18·7	23·6	9·9

* These peaks correspond to the structures shown in the Introduction, as identified by GLC and mass spectrometry.

† Peaks 1 and 6' are unidentified sesquiterpenoids of *P. cablin*.

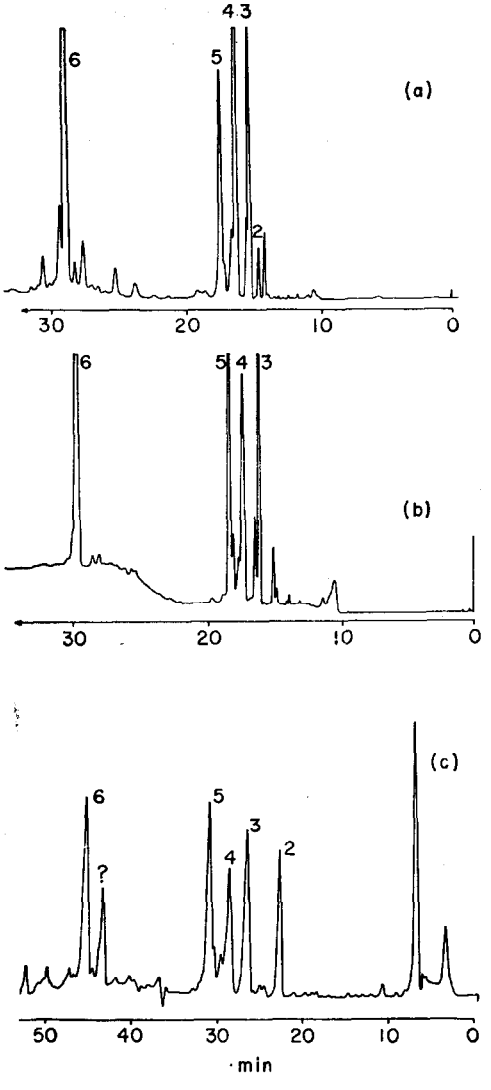


FIG. 3. GAS CHROMATOGRAPHIC RECORDS.

Obtained from: (a) 0·1 μl commercial Patchouli oil on Perkin–Elmer capillary F.11 on a support coated FFAP capillary column, attenuation × 200, carrier gas flow rate 2 ml/min, treated as per the plant tissue, i.e. sample volatilized at 160° for 10 min in the injection unit with the GLC column at room temperature, then rapidly heated to 160° and maintained isothermally for 12 min followed by linear temperature programming from 160° to 200° at 10°/min. (b) 0·1 mg fr. wt. *P. cablin* leaf on F.11 support coated FFAP capillary column, as for (a) above. (c) 3·5 mg fr. wt. *P. cablin* root tissue on Pye 104 GLC with dual packed columns, 4 mm × 9 ft (10% FFAP coated on celite, 100 mesh), direct loaded as for (a) and (b) above, temperature programmed from 50° to 250° at 3°/min, carrier gas flow rate, 60 ml/min, attenuation 1×10^3. The peak numbers shown correspond to those of Table 1.

Systematic analyses of various plant parts, described in Table 1, were carried out by packed column GLC. The results show that the sesquiterpenoid composition is uniform throughout the whole plant, with the exception of the roots which have an unidentified ses-

quiterpene in addition to the characteristic sesquiterpenoid pattern; the GLC record obtained after packed column GLC of the roots is shown in Fig. 3(c). Table 1 shows the levels of the total and the individual sesquiterpenes in the various plant parts. No sesquiterpenes could be detected in the woody xylem. The roots contained significant amounts and the outer stem tissue (bark and cortex) even higher levels. It was impossible to obtain accurate peak area measurements for peak 2 in the root samples examined due to elution of a non-sesquiterpenoid peak with almost the same GC retention time as the sesquiterpenoid peak 2 (see Fig. 3). Therefore the peak area data for peak 2 (roots) in Table 1 are false and probably are in fact similar to the results obtained for the other samples.

Glandular Trichomes

The aerial epidermis of *P. cablin*, with the exception of the stem apices which will be discussed separately, bears various specialized structures in addition to stomata. These trichomes[4] are of two types: profuse multicellular hairs and less numerous glandular structures consisting of stalk cells surmounted by enlarged head cells (Plate 1). Low-power microscopic examination showed that there are approximately ten times more glandular trichomes on the leaf lamina than on the petiole or stem; the abaxial epidermis of the leaf has twice as many as the adaxial epidermis. Although most densely distributed along the veins, the glandular trichomes are present over the whole leaf surface with decreasing frequency towards the leaf edges. An interesting point to emerge from these observations is the fact that the numbers of glandular trichomes for different leaf sizes remain remarkably constant; for example, an abaxial epidermis of a 2 sq. cm leaf has 22,000/sq. cm compared with 95/sq. cm on a 350 sq. cm leaf. There are no glandular trichomes on the root epidermis.

In the Labiatae, the morphology and development of the glandular trichomes of *Mentha piperita* have been most extensively studied.[7,8] The ontogeny of equivalent epidermal structures in *P. cablin* is basically similar. Plate 1 shows the range of types of glandular trichomes found on *P. cablin* and these probably correspond to a developmental sequence:

(i) division of an initiating epidermal cell in a plane parallel to the surface;
(ii) a second division of the distal cell in a similar plane;
(iii) enlargement of the distal cell to form a head cell on a two-celled stalk (Plate 1a);
(iv) division of the head cell to give four cells, with concomitant distortion of the stalk cells (Plate 1b, c, d and g);
(v) at any of these stages the internal organization of the cells can become indistinct and the whole structure appear saucer-shaped (Plate 1e);
(vi) finally, the "cuticle" separates from the head cells, the space becoming filled with fluid (Plate 1f).

The final stages in the development have been previously described.[5,6] All stages of the development are present on every type of leaf.

Capillary GLC analyses of epidermal tissue showed a sesquiterpenoid composition identical to that of the whole plant. The variation in sesquiterpenoid concentration over the whole leaf surface corresponded very well with the variation in glandular trichome distribution described above. Capillary GLC analyses of sections of the abaxial epidermis with microscopically determined numbers of glandular trichomes showed a good positive correlation between total sesquiterpenoid concentration and number of glandular trichomes (Fig. 4). In these analyses it was ensured that the correlation was truly with glandular trichome number rather than with tissue size. Also, using the technique of polyvinyl-alcohol

film stripping of leaf surfaces,[9] the presence of sesquiterpenoids in glandular trichomes was confirmed. (This technique, because of the profuse covering of hairs on *P. cablin* epidermis, was not completely satisfactory in yielding quantitative data.) From Fig. 4 it can be calculated that, under our growing conditions, the sesquiterpenoid concentration is 2×10^{-9} g per glandular trichome in *P. cablin* abaxial epidermis.

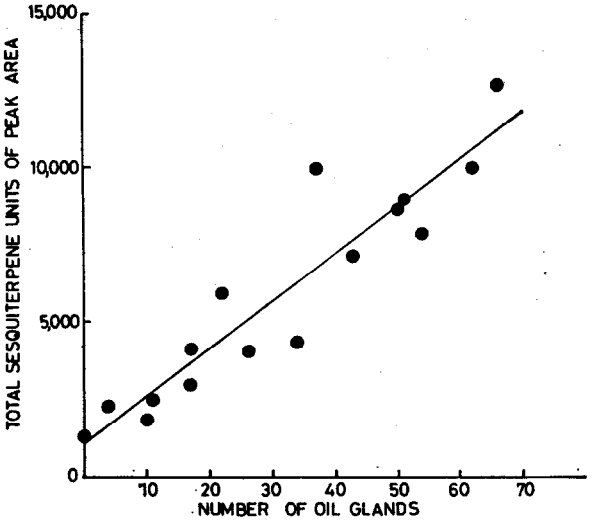


FIG. 4. CORRELATION OF NUMBERS OF GLANDULAR TRICHOMES (EXTERNAL OIL GLANDS) WITH TOTAL SESQUITERPENOID CONTENT (UNITS OF PEAK AREA), AS OBTAINED BY CAPILLARY GLC OF VIBROTOME SECTIONS OF *P. cablin* EPIDERMAL TISSUE. GLC CONDITIONS AS DESCRIBED IN FIG. 3(b).

Internal Sites of Accumulation

The presence of sesquiterpenoids in the roots, together with the absence of epidermal appendages, stimulated a search for possible internal sites of essential oil accumulation. These were found not only in the roots but also in all other parts of the plant except the woody xylem and the stem apex. In the stem and roots they are present in the cortex parenchyma; in the leaves, in the spongy mesophyll. Previous workers[5,6] have noted these structures only in leaves of *P. cablin*. These internal structures occur with approximately three times greater frequency than the epidermal structures. They are similar in size but not shape; $60 \times 40\,\mu$ for the internal structures as opposed to $60 \times 60\,\mu$ for the glandular trichomes. All the internal structures were composed of a single head cell located in the mesophyll with a two- or three-celled stalk, the foot-cell adjoining a palisade cell (Plate 2). No developmental sequence was observed in the arrangement of the cells, although the subcellular structure did show changes (Plate 3): "young" head cells have dense cytoplasm containing golgi bodies, many mitochondria, many whorled membranous structures, degenerating chloroplasts with starch grains and many small vacuoles; as the head cell "ages" the vacuoles seem to coalesce with subsequent disruption of the cytoplasm and its organelles to result in a large sac-like cell filled with many small vesicles, the cytoplasm being confined to a region adjacent to the stalk cells (Plate 3b).

GLC analyses were carried out by capillary GLC on specimens of internal tissue prepared by vibrotome sectioning. The sesquiterpenoid composition was identical to that of the whole plant. Capillary GLC analyses of mesophyll containing microscopically determined numbers

of internal glands again showed a positive correlation between sesquiterpenoid concentration and numbers of internal accumulatory structures. Under our growing conditions, the sesquiterpenoid concentration is 6×10^{-10} g per internal structure, that is, approximately three times less than in the glandular trichomes.

Stem Apex

The stem apex of *P. cablin* is typical of dicotyledons[4] with an apical dome. The dome is protected by two to three pairs of primordial leaves arranged in four orthostichies corresponding to the strongly four-angled stem of labiates. This arrangement can be seen in the longitudinal and transverse sections shown in Plate 4. There are no glandular trichomes or internal glands present on or in the apical dome; capillary GLC analysis did not detect any sesquiterpenes in this region. The first pair of primordial leaves have glandular trichomes of all types (except stage f, Plate 1) but no internal glands; capillary GLC analysis did not detect any sesquiterpenes in these leaves. The second pair of primordial leaves have glandular trichomes of all types but still no internal glands although they show typical leaf vascularization; capillary GLC analysis showed the characteristic pattern of sesquiterpenes found in the whole plant. The total concentration of sesquiterpenoids in this second pair of primordial leaves is twelve times greater than in any other part of the plant; for example, a GLC integrated output equivalent to 1·2 μg sesquiterpenes was obtained from 10 μg "dry wt." of primordial leaves compared to 1·5 μg from 150 μg "dry wt." of fully developed leaf (the "dried" tissue from the capillary GLC analyses of 30 min × 160° being weighed on an ultrabalance). Although this high concentration may be due to the high population of glandular trichomes, this does not detract from the fact that there is a high concentration of sesquiterpenes per unit weight of this tissue.

DISCUSSION

It would therefore appear that in *Pogostemon cablin* the metabolites of a specialized aspect of metabolism accumulate in specialized regions of the plant. This is supported by the numerous correlations of sesquiterpene content with visible structures throughout the whole plant and within specific organs. The presence of internal sites of lower terpenoid accumulation does not seem to be common to other intensively studied essential oil-producing species, for example *Mentha piperita*,[7,8] but terpenoids have been found in the roots of other species[11] without mention being made of specific sites of accumulation.

Although regions of accumulation in *P. cablin* have been described, it is not yet possible to distinguish between sites of biosynthesis, secretion and storage. The dense cytoplasmic contents and clearly defined organelles of the accumulatory structures suggest, at least in their early stages of development, a high capacity for metabolic activity. Aberrant chloroplasts containing starch grains are an obvious feature of the stalk cells of the internal glands of *P. cablin*. If such chloroplasts are found similarly associated with the glandular trichomes of *M. piperita* then the high incorporation of ^{14}C from $^{14}CO_2$ into the monoterpenes of peppermint[12] could be readily explained. However, the high concentration of sesquiterpenes in the apical organs of *P. cablin*, a metabolic sink of high capacity, might suggest that these compounds accumulate as waste or storage products. It would seem reasonable to carry out studies aimed at demonstrating biosynthesis or degradation of sesquiterpenes in *P. cablin*

[11] E. GUENTHER, G. GILBERTSON, R. T. KOENIG, *Anal. Chem.* **41**, 41R-58R (1969), and references therein.
[12] W. D. LOOMIS, *Terpenoids in Plants* (edited by J. B. PRIDHAM), p. 59, Academic Press, New York (1967).

using tissue from the apical region, rather than on fully expanded leaves, since glandular trichomes seem to be present prior to sesquiterpene accumulation.

The lower terpenoids appear to be stored extra-cellularly in glandular trichomes in both *P. cablin* (Plate 1) and *M. piperita*,[7,8] that is between the cell membrane and the cuticle. For transport through the membrane the forms of terpenoids bound to sugar moieties, which have been reported in other species[13] and detected in *P. cablin* (W. Henderson, unpublished data), may be necessary. If, in fact, these are the transportable forms, the golgi bodies may be the site of the sugar–terpene complex formation in a system analogous to glycoprotein formation and secretion.[14] Also, if the sugar–terpene complex is the transportable form, a high glycosidase activity should be characteristic of the storage areas.

It can thus be seen that systematic study of isolated accumulatory structures, at all stages of development, would provide information, not only concerned with sesquiterpene biosynthesis, but also about wider aspects of transport and permeability.

EXPERIMENTAL

Plant material. Plants of *Pogostemon cablin* were obtained as cuttings from a commercial plantation in Malaya and grown clonally in greenhouse conditions under natural day length supplemented to 12 hr in winter and a temperature of 16° min. Cuttings were propagated vegetatively at regular intervals of 4 months such that the plants used experimentally were uniform. The choice of plant parts for examination was as described in the Results.

Microscopy. Fresh material for light microscopy was vacuum infiltrated with water before being sectioned on an Oxford Vibrotome. They were then mounted in water or methylene blue for examination and photography on a Vickers 55 microscope.

For more detailed microscopy, specimens were fixed in glutaraldehyde/osmium, embedded in Epon 812 (Taab Laboratories) and sectioned on an ultramicrotome (LKB Mk. III). Light examinations were carried out on a Cooke, Troughton & Sims microscope, modified for photomicrography. Specimens for demonstrating internal accumulatory structures, after lead citrate staining,[15] were examined on a JEM IV electron microscope.

Analytical methods. The analyses were carried out solely on the free volatile sesquiterpenes. The GLC loading modifications are shown in Figs. 1 and 2.

1. Packed column GLC analysis of tissue specimens 10–100 mg fr. wt.:

A Pye 104 gas chromatograph with dual flame ionization detectors was used with dual packed columns (9 ft × 4 mm coated with 10% FFAP). Specimens were loaded into a pre-column heating unit at 160° for 10 min under a nitrogen flow of 13 ml/min which swept the volatilized constituents on to the column which was maintained at room temperature during this collection period. The columns were then rapidly heated to 50° and normal GLC analysis followed, i.e. linear temperature programmed from 50–250° at 3°/min with a carrier gas flow of 60 ml/min.

Tissue which had been subjected to this treatment was shown upon subsequent solvent extraction to have retained negligible quantities of free sesquiterpenes.

2. Capillary GLC of vibrotome and microtome sections:

A Perkin–Elmer F.11 Mk. II gas chromatograph with a flame ionization detector was used with a support coated open tubular capillary column[16] (50 ft × 0·02 in. coated with FFAP). Specimens were loaded into glass liner tubes (Perkin–Elmer Ltd.) and placed inside the assembly of pre-column injection splitter and heated injection block at 160° for 10 min under a nitrogen flow of 2 ml/min which swept the volatilized constituents on to the column, maintained at room temperature. The column was then rapidly heated to 160° and an initial isothermal period of 11 min at 160° was followed by linear temperature programming from 160–200° at 10°/min. The carrier gas flow was maintained at 2 ml/min.

[13] M. J. O. Francis and C. Allcock, *Phytochem.* **8**, 1339 (1969) and references therein.
[14] E. Schnepf, *Planta* **79**, 22 (1968).
[15] E. S. Reynolds, *J. Cell. Biol.* **7**, 208 (1963).
[16] L. S. Ettre, J. E. Purcell and K. Billeb, *J. Chromatog.* **24**, 335 (1966).

For packed column analyses, peak areas were measured manually. For capillary GLC analyses, a Kent Chromalog Mk. II digital integrator established the peak areas. From the peak area data, the total sesquiterpenoid content per fr. wt. of tissue and the percentage abundances of the individual sesquiterpenes were calculated.

Acknowledgement—We thank J. Ward for help with the observations on the internal glands.