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Direct Demonstration of Duvatrienediol Biosynthesis in Glandular Heads of Tobacco Trichomes

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ABSTRACT

Biosynthesis of the diterpenes, α and β 4,8,13-duvatriene-1,3-diol, has been observed in detached, intact glandular heads from trichomes of *Nicotiana tabacum*, Tobacco Introduction 1068. This result shows directly that the glandular head portion of the trichome is capable of duvatrienediol biosynthesis. In additional experiments, all of the [^{14}C] duvatrienediol formed from sodium [2-^{14}C]acetate by leaf midrib sections was recovered with trichome exudate and surface washes. None was found in trichome stalk, epidermal or subepidermal tissue extracts. Also, removal of glandular heads and exudate from midrib sections reduced or eliminated duvatrienediol biosynthetic capacity. Together these results strongly suggest that glandular heads are the primary, and perhaps the only, site of duvatrienediol biosynthesis in this plant.

Incubation of detached, intact glandular heads with sodium [^{14}C]acetate in the dark or incubation in the light in the presence of DCMU reduced incorporation into duvatrienediols by 97%. These results suggest that chloroplasts which are abundant in glandular heads are involved in the biogenesis of these compounds.

Leaves, midveins, stalks, and floral parts of many *Nicotiana* species, and of certain other plants, are covered with trichomes (15), the classification of which has been simplified to include two large groups (23), (a) the glandular, exudate secreting and nonsecreting types, and (b) the nonglandular, nonsecreting types. The tendency has been to assume that glandular head cells of trichomes and not trichome stalk, epidermal, or subepidermal cells are the site of biosynthesis of terpenes accumulated in trichome exudate. However, this has not been shown directly (11). Terpenes are principal constituents of such exudate in a variety of plants. Akers *et al.* (1), in a study of the ultrastructure of exudate secreting trichomes of *N. tabacum* L. cv xanthi, noted the presence in glandular head cells of a cytoplasm containing a large nucleus, numerous mitochondria and chloroplasts and a well developed ER, suggesting intensive active secretion from these cells. These observations suggest that formation of at least some exudate components occurs in glandular head cells in this plant.

Tobacco trichome exudate, which has been suggested as a source of tobacco aroma (2), can account for 0.5 to 10% of the fresh weight of the leaf, depending on variety (9). The major diterpenes of tobacco exudate, the α and β 4,8,13 duvatriene-1,3-diols, (αDVT and βDVT, respectively) account for a large portion of surface leaf constituents (5, 17, 21). These compounds were first isolated and characterized by Roberts and Rowland (20). While their physiological functions are not established, they are shown to have plant growth inhibitor properties (13). Jackson *et al.* (16) showed convincingly that DVT[1] stimulates tobacco budworm oviposition activity. In contrast, these same compounds are shown to inhibit germination of fungal spores (12).

There is some disagreement concerning the site of formation of diterpenes in tobacco. Michie and Reid (19) using cuticle peels and trichomes shaved from *N. tabacum* cv Bergerac observed conversion of [2-^{14}C]acetate or [2-^{14}C]mevalonic acid into a terpene-containing fraction, suggesting that trichomes are the site of biosynthesis. Rates of conversion were very low, products were not well characterized, and trichome stalk cells were undoubtedly present. In contrast, Chang and Grunwald (7) using differential solvent extraction and scanning EM suggested that duvatrienediols were located on the outer surface of every epidermal cell of *N. tabacum* L. cv Kentucky 14, whether or not a trichome was present.

The presence of numerous chloroplasts in glandular head cells of tobacco suggests that this organelle may be involved in the biosynthesis of exudate constituents, either as a biosynthetic site and/or as a source of metabolic energy or carbon. There is, however, little definitive data to support this suggestion. On the basis of ultrastructural evidence Carde *et al.* (3) concluded that leucoplasts and ER are directly involved in the biosynthesis of pine monoterpenes and Vemeer and Peterson (25) came to the same conclusion in studies of terpene biosynthesis in *Chrysanthemum*. From studies involving subcellular fractionation and enzyme localization, the diterpene casbene is thought to be formed in proplastids of germinating castor bean endosperm (26).

Our work was undertaken to determine the tissue level site of, and the possible involvement of chloroplasts in, the biosynthesis of the diterpenes αDVT and βDVT in the model plant *N. tabacum*.

MATERIALS AND METHODS

Plant Materials and Labeling of Midvein Sections. *Nicotiana tabacum* L., Tobacco Introduction 1068 (T.I. 1068) plants were grown in the greenhouse in pots using a standard soil mixture and were fertilized (20-20-20, NPK) once per week. For all experiments, fully expanded leaves (30–40 cm length) were used from about 2-month-old plants.

In studies involving the radiolabeling of intact tissue, leaves were detached about 5 to 6 cm from the point of attachment to the stalk and discarded leaving a still attached, midvein section (having a midvein diameter of 0.5–0.7 cm) bearing a small amount of leaf material. A core (0.15 × 2 cm) was removed from the cut end of the midvein section using a glass capillary as described in detail elsewhere (G. J. Wagner *et al.*, in preparation). Liquid was removed from the tissue-vessel created by the coring and 30 μl of an aqueous solution containing about 3.5 × 10^6 dpm of either sodium [2-^{14}C]acetate (New England Nuclear, 1–

[1] Abbreviations: DVT, duvatrienediol; LC, liquid chromatography.

3 Ci/mmol) or DL[2-^{14}C]mevalonic acid lactone (Amersham, 53 mCi/mmol) was introduced. Solutions were taken up by the tissue in about 30 min and no exudation occurred from cut surfaces. Incubations were in the greenhouse unless otherwise stated.

At a prescribed time after introducing label, a wooden dowel (2 mm × 12 cm) was inserted into the cored section to facilitate handling, the section was detached from the stalk, and leaf material was trimmed and discarded. To remove exudate, the section was dipped twice (20 s each) in 6 ml of acetonitrile and the dip solution was filtered through Whatmann No. 1 paper. The extract was evaporated at 35°C and the residue dissolved in 3 ml dichloromethane and partitioned with 3 ml H_2O to remove water soluble products and unmetabolized precursor. An aliquot of the dichloromethane phase was analyzed for radioactivity and the remainder was transferred to a sealable vial, evaporated with N_2, and the vial was sealed. Samples were stored dry at −4°C until analysed. In experiments described in Table II, capillary-collected-exudate was collected prior to dipping the section in acetonitrile. This was achieved by lowering an acetonitrile-filled, 20 μl capillary pipette over individual trichome heads causing dissolution of exudate from the head without contact with the trichome stalk. Description of tissue fractionation protocols used in experiments represented by Table II and Figures 3 and 4 are described in "Results and Discussion."

Preparation and Radiolabeling of Detached Glandular Heads. Glandular heads and exudate were removed by gently touching along the midvein of undisturbed leaves with one side and one surface of 20 × 20 mm cover glasses. Generally, for one cover glass, heads were obtained from one side of the entire length of the midrib of one leaf. In inhibitor experiments, control and treated samples were obtained from opposite sides of the same midrib. Small drops of wax were applied to the corners of the cover glasses opposite from and on the same surface as that bearing the heads. Four cover glasses were inserted (head-bearing-end down) into the well (22 × 1 × 1 cm) of a plastic chamber containing 2 ml of incubation mdium. Wax drops served to keep individual cover glasses separated and allowed gas exchange and solution mixing during incubation. Except where described, a glass capillary connected to a peristaltic pump provided aeration and mixing. Light was provided by two cool-white desk lamp tubes about 10 cm above head-bearing cover glass surfaces. For dark experiments, chambers were covered to exclude light. Incubation medium consisted of 0.1 M sucrose, 20 mM Mes titrated with Tris to pH 5.0 containing about 12 × 10^6 dpm of radiolabel.

After incubation, cover glasses were rinsed in 3 separate volumes (250 ml each) of H_2O, wax drops were removed, and the cover glasses were extracted two times in 15 ml acetonitrile and the extract was filtered and prepared for HPLC as described for midvein dips.

Analysis of α and βDVT. Liquid chromatography was performed with a Varian model 2100 LC using a Micro Pak CN-10 column (30 cm × 4 mm, Varian). Solvent was prepared by mixing 3 volumes of dichloromethane-methanol (1–4, v/v) with 97 volumes of 2,2,4-trimethylpentane. Elution was isocratic at a flow of 2.5 ml/min. Detection was at 214 nm and 1.25 or 2.5 ml fractions were collected with a fraction collector. Three ml of scintillation cocktail (3a70B, Research Products International) were added to fractions and samples were counted in a quench-corrected liquid scintillation system to achieve an error level of ≤3%. Total α and βDVT were quantitated using a reporting integrator and solvents used were HPLC grade. A second HPLC system utilizing C_{18}-reverse phase gave similar separations to that of the nitrile phase system except that the order of elution of α and βDVT were reversed (data not shown).

For HPLC analysis, samples were dissolved with dichloromethane, an aliquot was assayed for radioactivity, and the remainder was introduced into the injector sample loop (0.5 ml) followed by a 50-μl wash of the sample vial. At the end of the run, injection syringe, sample loop, and column were rinsed with 95% 2,2,4-trimethylpentane, 5% dichloro-methane-methanol (1–4, v/v) and the rinses analyzed for radioactivity. Recovery of activity in LC profiles was approximately 80%.

To directly compare the retention times of radiolabeled and unlabeled α and βDVT in an LC separation, 1.25 ml fractions were collected and 100 μl aliquots of each fraction was removed and the remainder was analyzed for radioactivity. One hundred μl of 0.05% butylboronic acid in pyridine was added to each separated aliquot to form the butyl boronate derivatives of DVT, essentially as described by Chang and Grunwald (6). Analysis of these was made by GC, essentially as described by Gamou and Kawashima (14).

For crystallization experiments (Table I), four to five preparations from labeled midvein sections were used. Fractions from LC runs corresponding to βDVT were pooled, evaporated, resuspended in 5 ml of heptane, and 8.9 mg of authentic unlabeled βDVT was added and dissolved prior to filtration of the solution. Crystals were allowed to grow at 10°C, were recovered, and were washed with cold heptane prior to sampling for label and melting point (Fisher-Johns apparatus). Subsequently they were solubilized in heptane, recrystallized, and reexamined for specific radioactivity and melting point. Samples for radioactivity determination (1–1.2 mg) were counted to achieve ≤3% error. The high purity of βDVT recovered from HPLC greatly facilitated crystallization experiments.

RESULTS AND DISCUSSION

While the logical and often assumed site of trichome exudate diterpene biosynthesis is the glandular head of exudate secreting trichomes, this is not established. Croteau and Johnson (11) discussed the sites of terpene biosynthesis in various tissues and summarized the indirect evidence which implicates the glandular heads in this process in glandular trichome-bearing systems. Here we used tobacco, T.I. 1068, which bears highly exudated glandular trichomes to determine if glandular head cells and/or other surface structures are involved in the biosynthesis of secreted diterpenes, specifically the α and β isomers of 4,8,13-duvatriene-1,3-diol.

In all experiments described here, acetonitrile was used to remove trichome exudate (Fig. 1). In contrast to the damaging effects of dichloromethane and acetone (not shown), acetonitrile treatment did not alter apparent surface morphology. Acetonitrile solubilized components were fractionated and analyzed by liquid chromatography on a nitrile phase column which separates compounds of medium polarity (Fig. 2). In a typical experiment, 72 h after labeling, radiolabel recovered in α and βDVT accounted for 0.025 and 0.01%, respectively, of the label introduced into midvein sections. Specific radioactivity of α and βDVT, assessed from radioactivity and UV measurements in LC profiles—after calibration of UV response *versus* DVT concentration—was about 1.5 to 3 × 10^5 dpm/mmol α or βDVT. Low specific radioactivity was due to the presence of large amounts of DVT accumulated in exudate prior to labeling. Attempts to remove exudate prior to labeling prevented formation of labeled DVT (G. J. Wagner, unpublished data). A ratio of α to βDVT of about 2.5 to 1 was observed for total α and βDVT—principally accumulated prior to labeling—and for radiolabel in α and βDVT (Figs. 2 and 6).

The bulk of the label introduced into midvein sections remained in acetonitrile dipped sections in the form of $CHCl_3$:methanol soluble and insoluble components (G. J. Wagner *et al.*, in preparation). Thus, [^{14}C]acetate was incorporated into α and βDVT but only at low levels, levels similar to those observed by others in attempts demonstrate conversion of ter-

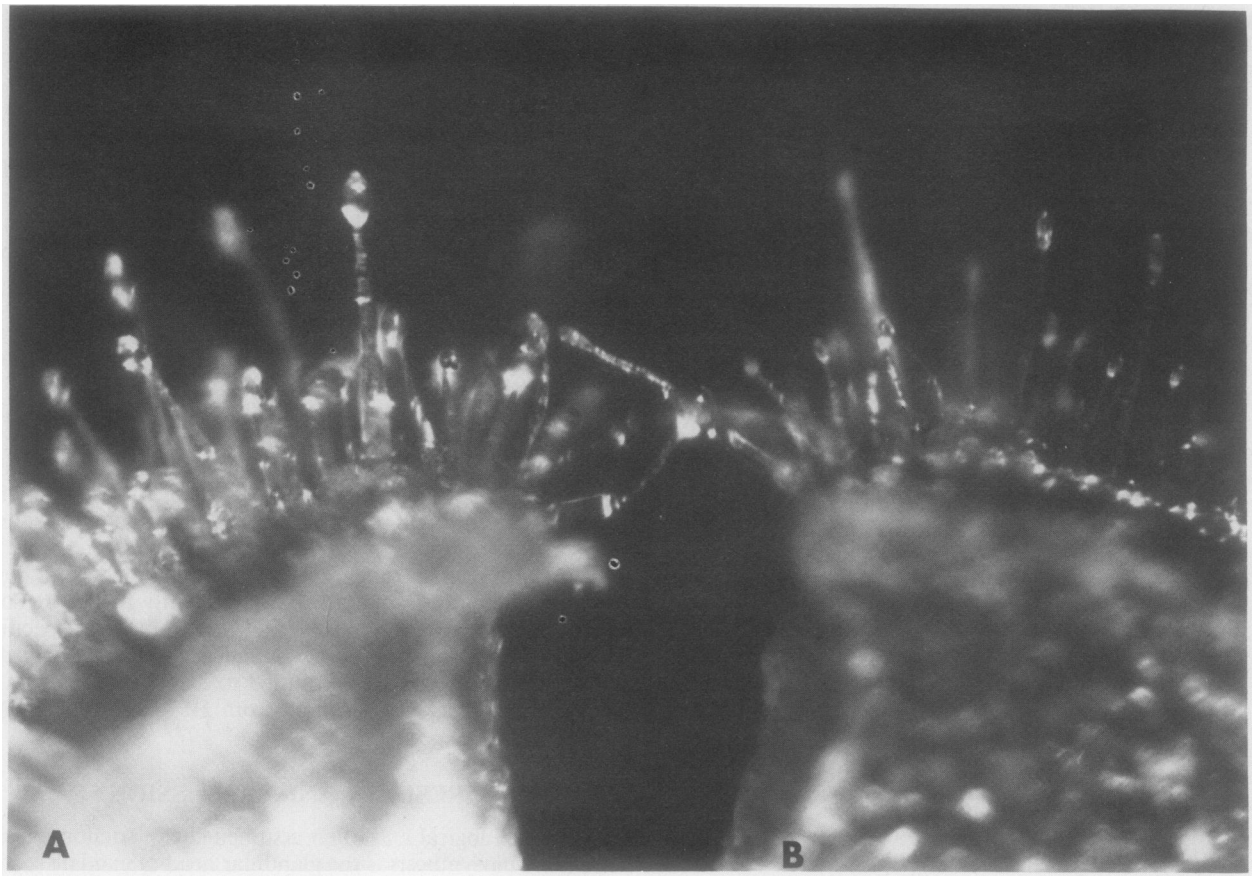

FIG. 1. Midvein sections of T.I. 1068 showing trichome structures: A, with exudate intact and B, with exudate removed by dipping section for 20 s in acetonitrile.

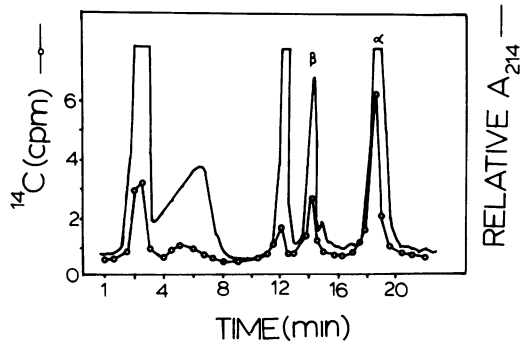

FIG. 2. Liquid chromatogram of acetonitrile extract of sodium [2-^{14}C]acetate-labeled T.I. 1068, midvein section. Absorption at 214 nm is primarily due to exudate present before radiolabeling.

pene precursors in higher plants (10, 11, 18). Low efficiency of conversion of labeled precursors into terpenes of higher plants has been attributed to permeability barriers, competing pathways, distance of the biosynthetic site from the site of label, and inhibition of participating enzymes by the high concentrations of precursurs administered (11, 18).

Unequivocal evidence that radiolabel identified as α and βDVT occurs in these molecules is shown in Table I where radiolabel ascribed to βDVT was shown to co-crystallize with authentic βDVT. The melting point obtained for βDVT was in agreement with that reported by Cutler *et al.* (13). The α isomer, while more prominent than βDVT, is more difficult to crystallize (20) and was not examined in this aspect of the study. The retention times of both α and βDVT and [^{14}C]acetate eluting in the region of these compounds were found to be exactly coincident on analysis by GC of butyl boronate derivatives prepared as described in "Materials and Methods." Cis-abienol, another major constituent of T.I. 1068 exudate (17) eluted earlier than α or βDVT in the LC system used. A radioactive peak was consistently observed in the region of cis-abienol (4–5 min; Figs. 2 and 6). Reduction of activity in this peak due to dark treatment or to the presence of DCMU or $HgCl_2$ (detached head experiments) was comparable to the reduction in DVT observed (not shown).

Table I. *Crystallization of Tobacco-Trichome derived βDVT to Constant Specific Radioactivity*

Crystallization and recrystallization from N-heptane except for 3rd recrystallization which was from hexane.

	Specific Radioactivity	Melting Point
	dpm/mg	*°C*
1st Crop	260	122–123
1st Recrystallization	300	123
2nd Recrystallization	260	123
3rd Recrystallization	260	123

Having achieved reproducible and adequate levels of DVT labeling, we determined which portions of midvein sections were capable of DVT biosynthesis. Six d after labeling with sodium [2-^{14}C]acetate, midvein sections were separated into the fractions listed in Table II. Only capillary-collected-exudate and that subsequently removed by dipping in acetonitrile contained labeled (or unlabeled) α and βDVT, indicating that accumulation of synthesized α and βDVT was principally associated with exudate. Qualitatively similar results were obtained in a tissue distribution

Table II. *Distribution of Sodium [2-^{14}C]Acetate-derived DVT in Components of Labeled Midvein Section*

Fractions consisted of: 1) exudate collected directly from about 200 individual trichrome/heads using a 20 μl micropipette filled with acetonitrile; collections were made under a dissecting microscope; 2) subsequent dip of section for 20 s in 15 ml acetonitrile to remove residual exudate; 3) trichomes—about 200—mechanically removed with tweezers; 4) subsequent dip in acetonitrile; 5) about 30% of epidermis; 6) subepidermal tissue. Fractions 3, 5, and 6 were homogenized with acetonitrile.

Fractions	αDVT	βDVT
	dpm	
1) Capillary-collected-exudate	260	114
2) Subsequent acetonitrile wash	4495	1740
3) Trichomes	N.D.[a]	N.D.
4) Subsequent acetonitrile wash	N.D.	N.D.
5) Epidermal peels	N.D.	N.D.
6) Subepidermal tissue	N.D.	N.D.

[a] N.D., not detected.

study using [2-^{14}C]mevalonic acid lactone (data not shown). These results, obtained 6 d after introducing label, show that accumulated [^{14}C]DVT is primarily if not exclusively associated with exudate that surrounds glandular trichome heads.

To determine if biosynthesis could proceed in the absence of glandular heads, we removed, by brushing gently with a camel's hair brush, the majority of heads and exudate from intact midvein sections, and epidermal strips of sections, prior to labeling. Similar methods have been used previously to remove trichomes from frozen leaves (8) and on a large scale Chakraborty and Weybrew (4) used a machine with brushes to remove trichomes and exudate for chemical analysis. As shown in Figure 3, brushing removed the vast majority of heads from trichomes but apparently left most stalk cells intact (verified by rejection of Evan's blue dye (not shown). Twenty-four h after introducing [^{14}C]acetate, comparison of [^{14}C]DVT labeling in brushed *versus* nonbrushed midvein sections and epidermal strips from sections (the latter floated on buffered 0.1 M sucrose containing label) showed that brushing reduced incorporation 85 to 95% (Fig. 4). Since brushing quantitatively lowered total α and βDVT and [^{14}C] α and βDVT by the same amount it was concluded that complete removal of heads by more vigorous brushing would eliminate conversion. DVT biosynthesis in midvein sections labeled as described reached a level of about 50% of maximum at approximately 24 h (G. J. Wagner *et al.*, unpublished data) so, in the above experiments, active biosynthetic pools which may exist would be expected to have contained label at the time of analysis.

The above results are consistent with the contention that glandular heads of T.I. 1068 are the sole site of DVT biosynthesis and accumulation. Another possibility, however unlikely, would explain these results. It is possible that α and βDVT are primarily formed in trichome stalk cells, epidermal or subepidermal cells, and then transported to the glandular head for secretion. Removal of heads might remove the sink and inhibit biosynthesis or damage biosynthetic sites. We have not made any observations in the course of our studies which would substantiate this unlikely possibility. Direct proof of DVT biosynthesis in glandular heads would cast further doubt on this possibility.

To directly test the ability of glandular heads to biosynthesize DVT we developed a method (see "Materials and Methods") for isolating intact, functional glandular heads free from trichome

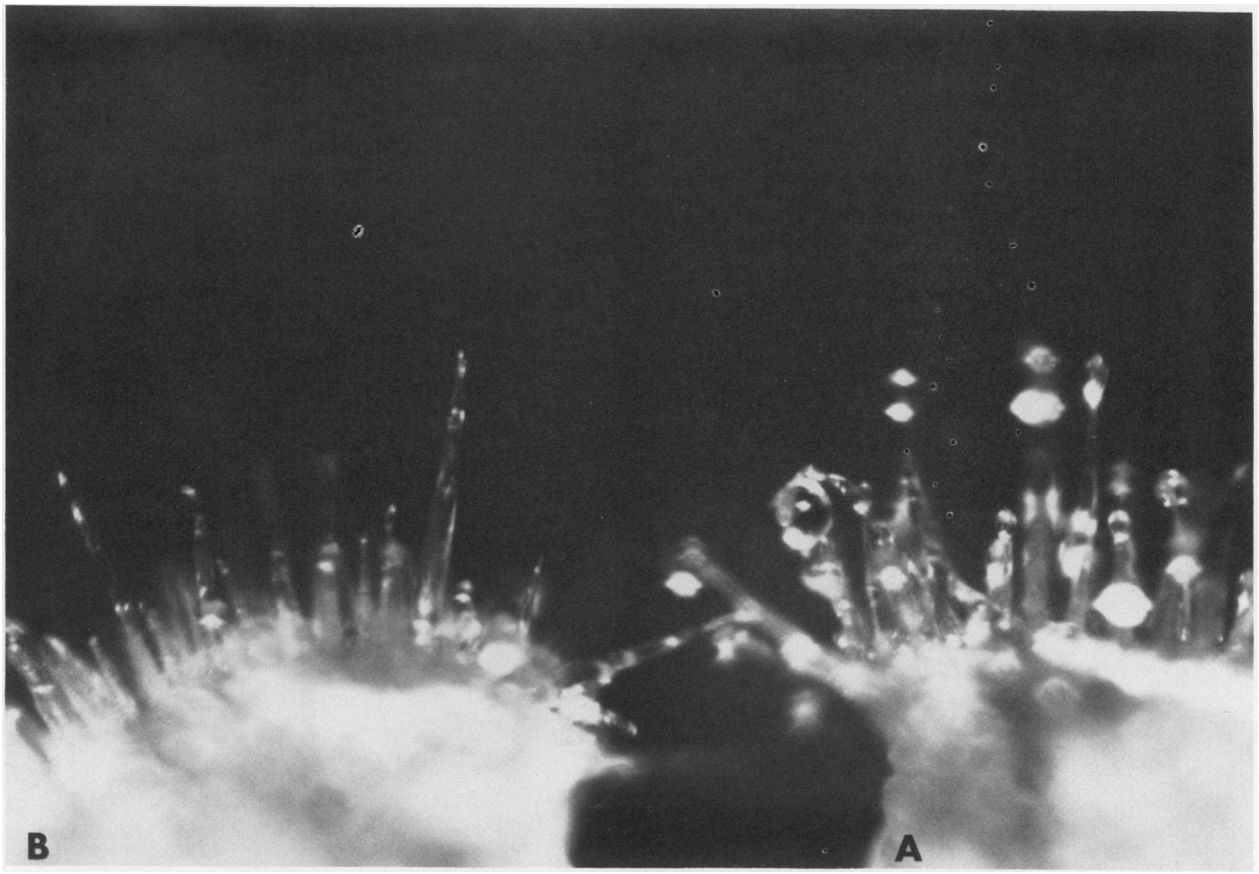

FIG. 3. Midvein section of T.I. 1068: A, with exudate and trichome glandular heads intact and B, with these removed by brushing (see "Results and Discussion").

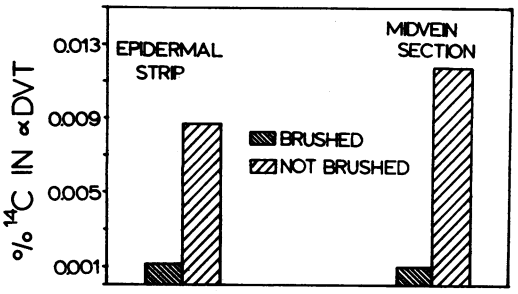

FIG. 4. Incorporation of [2-^{14}C]acetate into αDVT of brushed/non-brushed midvein sections and epidermal strips of a midvein section of T.I. 1068. Incubation with label for 24 h and incorporation is as % of initial [^{14}C] in αDVT.

stalk cells, epidermal and subepidermal tissue (Fig. 5). Staining experiments using Neutral red and Evan's Blue in H_2O or 0.6 M mannitol showed that these stains were concentrated in or excluded from, respectively, detached heads which were not completely occluded with exudate. In unoccluded heads, Neutral Red appeared first at the point of detachment of heads from the trichome stalk and then proceeded towards cells at the tip of the head (not shown). Incubation *in vitro* of isolated glandular heads in buffered, aerated sucrose containing sodium [2-^{14}C]acetate in the light for 24 h resulted in formation of labeled DVT. The acetonitrile-soluble radiolabeled products had elution profiles which were virtually identical to those obtained with capillary-collected-exudate and acetonitrile dip collected material from labeled midrib sections (Fig. 6). Radiolabel in unknown components eluting at about 2 min and 12 min was observed to vary from experiment to experiment for both detached heads and midvein sections. As yet we have not been fully successful in standardizing the method for preparing detached heads because the stickiness of tissue can vary greatly with growth conditions and the method of collection can influence the number of unoccluded and therefore potentially functional heads recovered. However, based on quantitation of α and βDVT, one can obtain preparations which vary by about 15% in total exudate collected per cover glass. Rate of conversion of sodium [2-^{14}C]acetate to α and βDVT by detached heads was similar to that observed with midvein sections after 24 h (not shown). This result suggests that the distance between the label and the biosynthetic site is not principally responsible for the low level of conversion of early pathway precursors to these terpenoids.

Our results conclusively show that glandular heads of T.I. 1068 are capable of DVT biosynthesis and together with results described in Table II and Figure 4 strongly suggest that glandular heads are the primary and perhaps only site of DVT biosynthesis in this tissue.

Glandular head cells of T.I. 1068 are visibly green and appear to be densely packed with chloroplasts. We tested the role of head cell chloroplasts in DVT biosynthesis by examining the effects of incubation in the dark and incubation in the presence of the inhibitors DCMU, $HgCl_2$, and antimycin A. As shown in Table III, the absence of light prevented DVT formation. Attached midvein sections are also incapable of producing DVT in

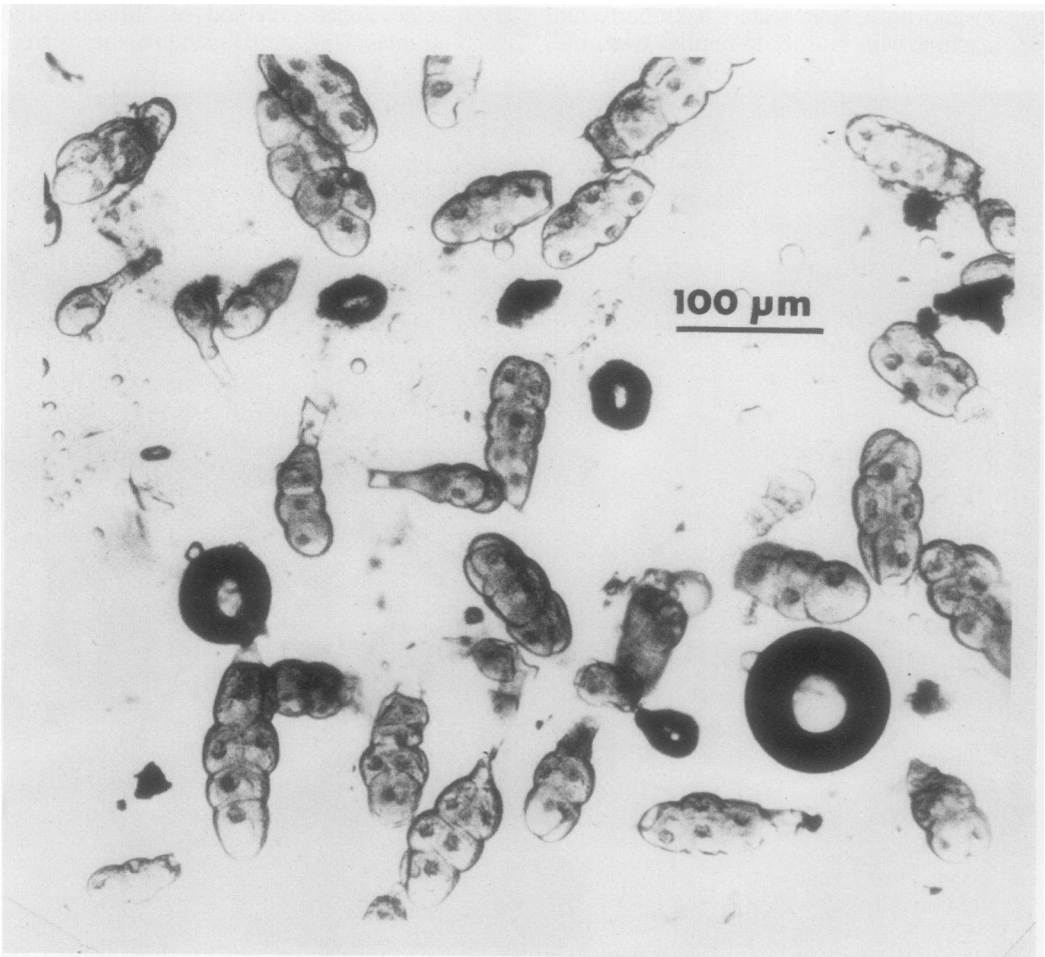

FIG. 5. Light micrograph of detached/intact glandular heads of T.I. 1068 isolated from leaf midvein trichomes. Exudate was removed with acetonitrile.

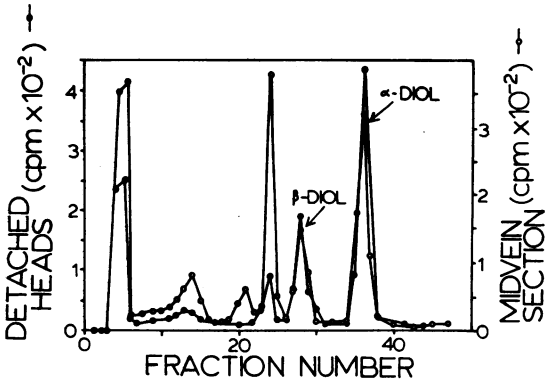

FIG. 6. Liquid chromatograms of acetonitrile extracts of sodium [2-^{14}C]acetate labeled midvein section and detached glandular heads of T.I. 1068.

Table III. *Relative Effects of Light, Inhibitors and their Treatments on α DVT Biosynthesis in Detached, Intact Trichome Heads of TI 1068*

Except where indicated, incubations contained 20 mM Tris/Mes (pH 5.0), 0.1 M sucrose. In a typical light control experiment 600 to 900 dpm and 230 to 380 dpm were found in α and βDVT, respectively.

Treatment	Relative Inhibition[a,b]
	%
Light	0
Dark	
Experiment 1	97
Experiment 2	91
Light—DCMU[c]	
1 μM	85
5 μM	94
10 μM	95
50 μM	95
Light—$HgCl_2$	
1 μM	87
10 μM	87
50 μM	96
Light—Antimycin A[c]	
0.5 μg/ml	0
5 μg/ml	64
Light—minus sucrose	34
Light—minus aeration	0

[a] Experimental variation was ≤15%. [b] Similar results obtained for βDVT. [c] Values relative to appropriate solvent control.

the dark (data not shown). DCMU at levels (1–5 μM) used to inhibit photosynthetic electron transport in isolated chloroplasts (24) abolished synthesis while antimycin A at levels (0.5 μg/ml) used to inhibit mitochondrial electron transport (22) had little effect. These results suggest that chloroplasts but not mitochondria are directly involved in DVT biosynthesis. Mercuric chloride, a general metabolic inhibitor, was also effective in greatly reducing DVT formation. Biosynthesis was greater when sucrose was present in the incubation medium, but we do not as yet know if this represents an osmotic, or carbon source effect. Aeration was apparently not necessary under the conditions used, presumably because the surface area of the incubation solution was adequate for oxygenation. Consistent with the above results, radiolabeled midvein sections of T.I. 1406, a tobacco which has nongreen glandular heads and produces no sticky exudate did not form measurable labeled DVT in the light. Similarly, tobacco T.I. 1112, a simple trichome bearing plant which lacks glandular heads and is nonexudated, produced no measurable labeled DVT (data not shown).

The above results are consistent with chloroplasts of glandular heads providing ATP, reducing equivalents or another crucial factor for DVT biosynthesis. Further studies are required to clarify the role(s) of light and chloroplasts in DVT formation in this system. The *in vitro* labeling method using detached heads provides a means for such studies. We note that [2-^{14}C]mevalonate lactone was an equally or more efficient precursor to α and βDVT in both midvein sections and detached heads. Qualitatively, radiolabeled products of both precursors were similar (data not shown).

CONCLUSIONS

Our results show conclusively that glandular heads of tobacco T.I. 1068 are capable of converting sodium [2-^{14}C]acetate (and [2-^{14}C]mevalonic acid lactone) to [^{14}C]DVT of trichome exudate. We believe this represents the first direct demonstration of the biosynthesis of terpenes by isolated trichome glandular heads. Our results also strongly suggest that glandular heads are the only site of biosynthesis of these compounds in T.I. 1068. Chloroplasts and light appear to play crucial, but as yet undefined, role(s) in DVT biosynthesis in glandular heads. Studies using detached glandular heads of T.I. 1068 designed to compare conversion of various radiolabeled, potential precursors to α and βDVT and other exudate constituents are planned, as are efforts to define the role of glandular head chloroplasts and other organelles in terpene formation.

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