Relationship of Camphor Biosynthesis to Leaf Development in Sage (*Salvia officinalis*)[1, 2]

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ABSTRACT

The camphor content of sage (*Salvia officinalis* L.) leaves increases as the leaves expand, and the increase is roughly proportional to the number of filled peltate oil glands which appear on the leaf surface during the expansion process. $^{14}CO_2$ is more rapidly incorporated into camphor and its direct progenitors in expanding leaves than in mature leaves, and direct *in vitro* measurement of the key enzymes involved in the conversion of geranyl pyrophosphate to camphor indicates that these enzymes, including the probable rate-limiting cyclization step, are at the highest levels during the period of maximum leaf expansion. These results clearly demonstrate that immature sage leaves synthesize and accumulate camphor most rapidly.

A key question about monoterpene formation concerns the stage of plant development at which monoterpene biosynthesis occurs. The oil glands are presumed to be the site of synthesis of monoterpenes (14, 28, 30), and microscopic examination of peppermint has indicated that the extracellular secretory space of the oil glands may fill with terpenes at a very early stage, well before the leaves have fully expanded (1, 2, 25). Experiments with peppermint have also indicated that there is little *de novo* synthesis of monoterpenes from $^{14}CO_2$ in mature leaves, but only in immature leaves that are still expanding (8, 21, 28). A direct periodic analysis of peppermint oil from plants grown under rigidly controlled optimal conditions showed that monoterpenes continue to accumulate in mature leaves until the plants flower (9, 21). In contrast to the $^{14}CO_2$ incorporation studies, these results suggest that monoterpenes may be synthesized in mature tissue only from endogenous stored substrates (9). Related studies have demonstrated that label from [U-^{14}C]sucrose is incorporated into monoterpenes most rapidly in *Majorana hortensis* leaf discs during the stage of leaf expansion (14). However, as previous studies have suggested that the number of oil glands is fixed at the time of leaf emergence (10, 11, 23, 24, 36), lower rates of incorporation by discs from fully expanded leaves may simply represent a lower density of oil glands and, thus, fewer biosynthetic sites per unit area or mass of tissue. Although the summary of the evidence above suggests that monoterpene biosynthesis may be most rapid in expanding leaves, recent studies with *Tanacetum vulgare* have indicated that incorporation of exogenous precursors, such as

mevalonic acid, into monoterpenes was most efficient during periods of dormancy or very slow growth (6, 7). These apparently conflicting results may be attributed to differences in the transport of exogenous precursors (28, 30), to variation in internal substrate pools (12, 28), or to developmental changes in the levels of competing side reactions (3–5). To resolve the question, it seemed necessary to obtain a direct measurement of biosynthetic capacity at the enzyme level that was independent of the difficulties associated with *in vivo* experiments.

The essential oil of sage (*Salvia officinalis*) contains camphor as a major component [*i.e.* up to 20% of the oil (16, 26)], and the biosynthesis of this monoterpene ketone has been shown to involve, as the first committed step, the cyclization of geranyl pyrophosphate to bornyl pyrophosphate (18). Bornyl pyrophosphate then is hydrolyzed to borneol (18), which is subsequently oxidized to camphor by a specific dehydrogenase (17) (Fig. 1). The bornyl pyrophosphate synthetase (19) and the borneol dehydrogenase (15) have been partially purified and characterized, as have two distinct types of phosphohydrolases capable of cleaving bornyl pyrophosphate to the alcohol (20). The enzymes of this biosynthetic sequence are sufficiently separated by chromatography on Sephadex G-150 to allow direct measurement of all relevant activities after this single fractionation step (15, 19, 20). Using this technique to measure biosynthetic capacity at the enzyme level, we report here that the ability to synthesize camphor from its immediate acyclic precursor is highest in expanding leaves.

MATERIALS AND METHODS

Plant Material and Enzyme Preparation. Sage (*S. officinalis* L.) plants were grown from seed (Burpee Co.) under controlled conditions described previously (18), and they were fertilized with Osmocote (14:14:14) controlled release fertilizer. For preparation of the soluble enzyme extract, 2 g leaf pairs were collected 2 h after the start of the light cycle and they were washed with 0.5 mM EDTA solution followed by distilled H_2O. The leaves, along with 2 g (dry) washed PVP (Polyclar AT, GAF Corp.), were ground to a fine powder in liquid N_2 in a mortar, and the powder was homogenized in a Ten-Broeck homogenizer with 25 ml of cold 100 mM Na-phosphate buffer (pH 6.5) containing 150 mM sucrose,
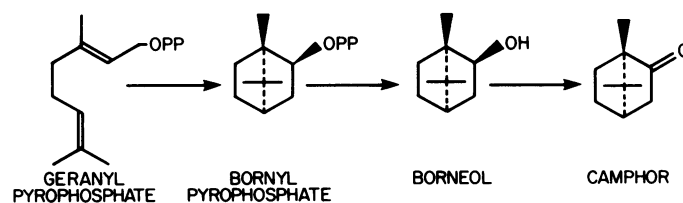

Fig. 1. Pathway for the biosynthesis of *d*-camphor from geranyl pyrophosphate.

GERANYL PYROPHOSPHATE   BORNYL PYROPHOSPHATE   BORNEOL   CAMPHOR

10 mM sodium metabisulfite, 10 mM sodium ascorbate, 10 mM MgCl$_2$, and 5 mM dithioerythritol. The homogenate then was slurried with 2 g washed and hydrated XAD-4 polystyrene resin (Rohm and Haas Corp.) for 5 min at 0 C [The preparation and use of both PVP and polystyrene resin in enzyme extraction is described in the literature (29, 31).] The slurry was filtered through cheesecloth and the filtrate centrifuged at 100,000g for 90 min to provide the soluble supernatant. This soluble enzyme fraction was concentrated to 3 ml by dialysis against the above extraction buffer (minus sucrose) that was saturated with PEG 6,000 (Fisher Scientific). The protein concentrate then was loaded onto a 2.5 × 130-cm column of Sephadex G-150 that was equilibrated and eluted (30 ml/h, 5-ml fractions) with 5 mM Na-phosphate buffer (pH 6.2) containing 1 mM sodium ascorbate, 2 mM MgCl$_2$, 0.5 mM dithioerythritol, and 10% glycerol. Aliquots of column fractions were assayed directly for bornyl pyrophosphate synthetase activity as described previously, using geranyl pyrophosphate as substrate (19). For measurement of the various bornyl pyrophosphate and bornyl phosphate hydrolases, aliquots were adjusted to the appropriate pH and ionic strength by dilution with make-up buffer before the assays [which are described in detail elsewhere (20)]. Similarly, aliquots were adjusted to the appropriate conditions before the standard assay for borneol dehydrogenase activity (15). Boiled controls were run for each set of experiments and, in all cases, nonenzymic activity was negligible. By loading a relatively small amount of protein on the large capacity Sephadex column, excellent resolution of activities were obtained: elution volume for bornyl pyrophosphate synthetase was 214 ml; for borneol dehydrogenase, it was 222 ml; for bornyl pyrophosphate hydrolase (acid pH optimum), it was 241 ml; for bornyl phosphate hydrolase (acid pH optimum), it was 287 ml; and for the low-molecular-weight hydrolase possessing both bornyl pyrophosphate and bornyl phosphate hydrolase activity, it was 317 ml. Total enzyme activity per leaf pair was determined by summing the area under the elution curve, determined by the standard assay of each activity (15, 19, 20), and then by multiplying by the aliquot-dilution factor and dividing by the number leaf pairs in the 2-g preparation.

Analysis of Sage Leaf Oil. The d-camphor content and d-borneol content (negligible) of the steam distilled oil from 20 sage leaf pairs was determined by GLC under conditions described before (13). Recovery of product by the microsteam distillation procedure (16) was essentially quantitative for all analyses as determined by calibration with d-[G-^{3}H]camphor. The gas-liquid chromatograph was calibrated externally with authentic d-camphor.

Experiments with ^{14}CO$_2$. ^{14}CO$_2$ incorporation studies with sage leaves were carried out as described previously (32) in a 250-ml glass chamber in which 0.25 mCi ^{14}CO$_2$ was generated. After incorporation of label, the monoterpenes were isolated by extraction followed by microsteam distillation (16). Internal standards (15 mg each) of d-borneol and d-camphor were added to the ethereal distillate and the labeled products were isolated by TLC [Silica Gel G with benzene-ethyl acetate (9:1, v/v)]. Identities of the labeled products as d-borneol and d-camphor were confirmed by radio GLC (17, 18). To examine the possible presence of [^{14}C]bornyl pyrophosphate, the tissue, after ^{14}CO$_2$ incorporation, was plunged into hot 50 mM (NH$_4$)$_2$CO$_3$ to inactivate enzymes and then homogenized in this medium. The soluble material remaining after centrifugation at 100,000g for 1 h was lyophilized and the residue was dissolved in 20 mM (NH$_4$)$_2$CO$_3$ and loaded on a 1 × 10-cm column of DEAE-cellulose for the purification of bornyl pyrophosphate. After continuous elution with 10 void volumes of 20 mM (NH$_4$)$_2$CO$_3$, the putative bornyl pyrophosphate was eluted with 2 void volumes of 50 mM (NH$_4$)$_2$CO$_3$ (18). This eluant was lyophilized, and the residue was dissolved in 0.5 ml 100 mM Na-acetate buffer (pH 5.5) containing 10 mM MgCl$_2$ and treated for

2 h with two units each of wheat-germ acid phosphatase and potato apyrase (both from Sigma). Any borneol liberated by this procedure (19) was extracted with ether (containing 15 mg unlabeled borneol) and the product was isolated by TLC as above.

Microscopy. Fresh leaf material was utilized for direct determination of oil gland (filled peltate gland) numbers by low magnification (× 50) light microscopy. For more detailed microscopy, specimens were fixed in formaldehyde-glacial acetic acid-ethanol (18:1:1, v/v), dehydrated with a chloroform series, embedded in hard paraffin, sectioned (10 μm), and stained with safranin and fast green. For scanning electron microscopy (Electron Microscope Center, Washington State University) the tissue was fixed in glutaraldehyde-osmium tetraoxide and dehydrated with a Freon series.

RESULTS AND DISCUSSION

The camphor content of the steam distilled oil from the first foliage leaves of 2-week-old sage plants was determined by GLC, and the analysis was repeated at weekly intervals with a similar batch of plants until this first leaf pair had fully expanded. During the 5-week interval over which the periodic analysis was carried out, the initial leaf pair had become the fourth leaf pair below the shoot apex, and this tissue had expanded from approximately 0.5 to 40 cm^2 (full expansion). A plot of leaf pair surface area and camphor content as a function of time clearly indicated that the increase in camphor content closely paralleled leaf expansion (Fig. 2a). Examination of the second and third leaf pairs as they expanded provided similar results, although the levels of camphor were generally higher from beginning to end, reaching approximately 0.7 mg/leaf pair on full expansion.

As the oil glands are the primary site of monoterpene accumulation and are considered to be the major site of monoterpene biosynthesis (14, 28, 30), the number of oil glands present on the sage leaf surface was determined as a function of leaf expansion. This procedure was complicated by the fact that *S. officinalis* possesses five distinct types of oil glands, including stalked capitate glands bearing a single terminal secretory cell, small sessile capitate glands containing one, two, or four secretory cells surmounting a basal and stem (stipe) cell, and large peltate glands containing eight secretory cells surmounting a basal and stem cell and entirely surrounded by an enlarged extracellular oil-filled cavity (35). Studies with related essential oil-producing species indicate that the sessile capitate glands with one, two, and four secretory cells are developmental stages in the formation of the large peltate glands (22–24, 33). Light microscope and scanning electron microscope examination (Fig. 3) indicated an increase in the proportion of peltate glands and a corresponding decrease in juvenile and intermediate gland types as the sage leaves expanded, suggesting such an ontogenic sequence in this species. However, a significant proportion of single secretory-celled capitate glands did not undergo cell division but, rather, initiated secretory activity at this stage of development with the formation of an enlarged oil-filled head (Fig. 3). Although the number of oil gland initials is generally considered to be fixed at the time of leaf emergence (10, 11, 24, 36), an increase in total number of oil glands (all types included) was noted during development of sage leaves. Thus, either a fixed number of gland initials was present, but they were not readily visible until further development, or glands were continuously initiated throughout leaf expansion, as has recently been observed for glandular hairs of *Cannabis sativa* (34). Similar trends in both gland numbers and distribution were observed on both dorsal and ventral leaf surfaces, and all stages of gland development were noted on every type of leaf. Although Lemli (27) observed that the oil glands of peppermint did not all fill with oil simultaneously, few peltate glands with unfilled or partially filled subcuticular cavities were noted on sage, suggesting rapid filling with terpenes once cell division ceases at the eight secretory cell stage. Because
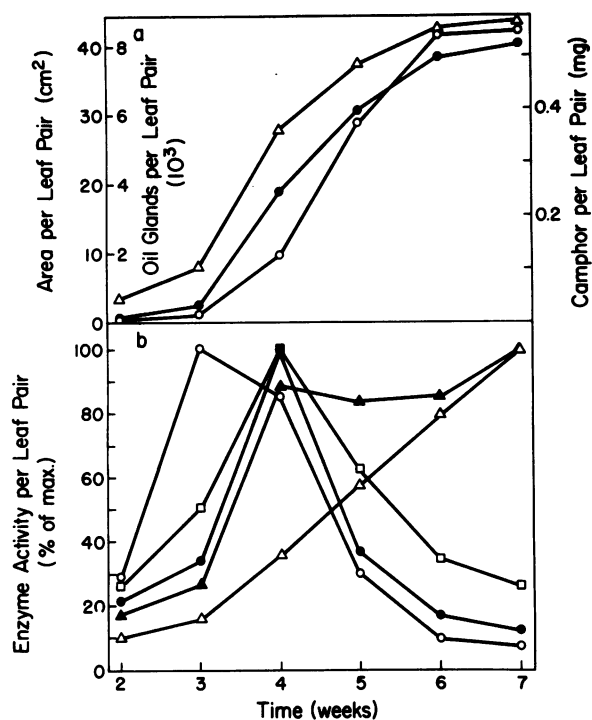
FIG. 2. a), surface area (O——O), camphor content (●——●), and the number of filled peltate oil glands (△——△) per sage leaf pair as a function of leaf development. b), relative activities of the enzymes of camphor biosynthesis as a function of the development of a leaf pair. Activities of bornyl pyrophosphate synthetase (O——O), borneol dehydrogenase (●——●), bornyl pyrophosphate phosphohydrolase (acid pH optimum) (△——△), bornyl phosphate phosphohydrolase (acid pH optimum) (▲——▲), and a low-molecular-weight neutral phosphohydrolase capable of hydrolyzing both bornyl pyrophosphate and bornyl phosphate (□——□) are plotted. If the activity for bornyl pyrophosphate synthetase at its maximum (on a mol/h·leaf pair basis) is taken as unity, then the levels of the other activities at maximum are: borneol dehydrogenase, 2.5; bornyl pyrophosphate phosphohydrolase, 35; bornyl phosphate hydrolase, 25; and the low-molecular-weight phosphohydrolase, 5.0 (for bornyl pyrophosphate) and 7.6 (for bornyl phosphate).
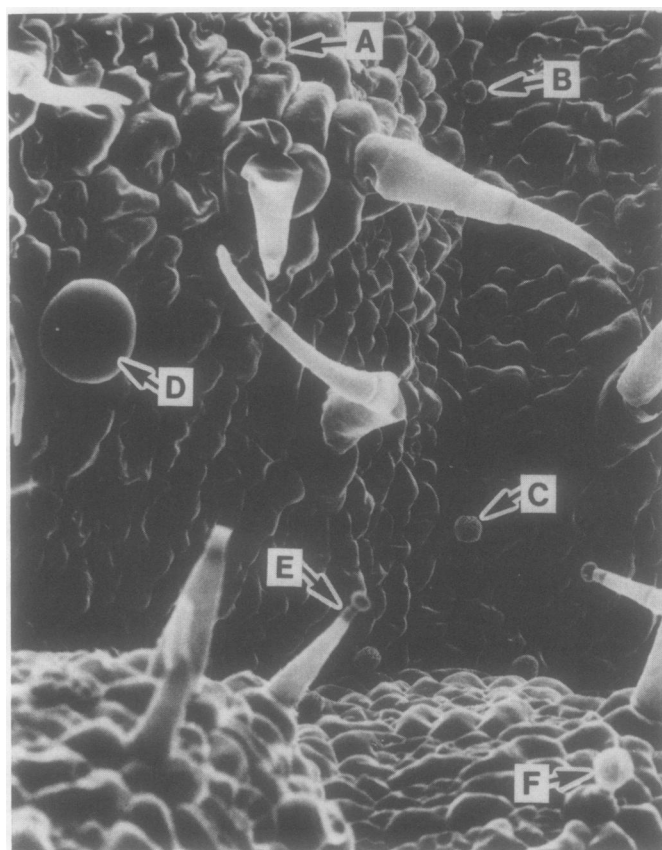

FIG. 3. Scanning electron micrograph of the dorsal surface of a sage leaf (3-week stage) illustrating the ontogenic sequence from capitate sessile glands with one (A), two (B), and four (C) secretory cells to the peltate gland with eight secretory cells and greatly enlarged oil-filled cavity (D). The diameter of the mature peltate gland is about 0.06 mm. Also visible in the field is a stalked capitate gland with a single terminal secretory cell (E), and a mature capitate sessile gland bearing a single secretory cell and enlarged oil-filled head (F). The latter (F) appears to represent the terminal stage of development of a sessile gland in which the secretory cell does not divide.

of the relative size and number of the peltate glands, these organs would be expected to contain the bulk of the oil, and, within experimental limitations, the apparent increase in stored oil, as evidenced by the appearance of the filled peltate glands, did correlate well with the increase in camphor content and leaf area (Fig. 2a). Similar correlation of oil gland number with both monoterpene and sesquiterpene content of leaves has been observed in other species (23, 24, 36).

To examine directly the capacity for camphor biosynthesis as a function of leaf expansion, soluble enzyme preparations, containing the requisite activities (17), were prepared from leaves harvested at different stages of expansion. The biosynthesis of camphor from [1-³H]geranyl pyrophosphate could not be measured directly in these soluble preparations because of the presence of competing phosphohydrolases that rapidly destroyed the substrate (20). Therefore, the preparations were concentrated and the individual enzymes of the biosynthetic sequence were separated by chromatography on Sephadex G-150. Column fractions were assayed for *d*-bornyl pyrophosphate synthetase (geranyl pyrophosphate as substrate) (19), NAD-dependent *d*-borneol dehydrogenase (15), *d*-bornyl pyrophosphate phosphohydrolase activity [an acid pH optimum hydrolase that cleaves the phosphoanhydride linkage to yield bornyl phosphate (20)], *d*-bornyl phosphate phosphohydrolase activity [an acid phosphatase (20)], and a relatively specific low-molecular-weight neutral phosphohydro-

lase capable of hydrolyzing *d*-bornyl pyrophosphate to *d*-bornyl phosphate and *d*-bornyl phosphate to *d*-borneol (20). The acid pH optimum pyrophosphate hydrolase, although capable of hydrolyzing bornyl pyrophosphate to bornyl phosphate, was far more active in hydrolyzing geranyl pyrophosphate to the corresponding monophosphate, and this enzyme, as indicated above, was the main source of interference in the crude soluble preparations.

A plot of the various enzyme activities (as per cent of maximum level per leaf pair) as a function of leaf expansion indicated that bornyl pyrophosphate synthetase was most active between 3 and 4 weeks, whereas both borneol dehydrogenase and the low-molecular-weight phosphohydrolase (which possessed both phosphatase and pyrophosphatase activity) reached maximum activity at the fourth week (Fig. 2b). Each of these activites declined to a low, but readily detected, level at full expansion. The acid pH optimum phosphohydrolases, which were capable of hydrolyzing bornyl esters but which preferred phosphoesters of allylic alcohols (20), did not reach maximum activity until full expansion (Fig. 2b). Thus, these phosphohydrolases exhibited a developmental pattern quite unlike that of the other enzymes described, suggesting that these hydrolases are not involved in camphor biosynthesis. On the basis of substrate specificity studies (20), we had previously suggested that the low-molecular-weight neutral pH optimum phosphohydrolase was probably involved in the hydrolysis of *d*-bornyl pyrophosphate *in vivo*, and the similar pattern of development of

bornyl pyrophosphate synthetase, borneol dehydrogenase, and the low-molecular-weight hydrolase supports this suggestion. Although caution is needed in extrapolating *in vitro* enzyme measurements to *in vivo* biosynthesis, the key enzymes of camphor biosynthesis were most active in expanding leaves during the period of most rapid camphor accumulation, suggesting a very close relationship between the measured capacity for camphor biosynthesis and the *in vivo* accumulation of camphor. When these enzyme studies were repeated with the second and third leaf pairs to emerge, a very similar pattern of enzyme activity *versus* expansion was observed. All enzyme levels were somewhat elevated in these tissues, and this observation was reflected in the higher camphor levels previously noted.

$^{14}CO_2$ is one of the most efficient exogenous precursors of monoterpenes (30) and, to obtain further information on the dynamics of camphor biosynthesis in the expanding leaves, $^{14}CO_2$ incorporation studies were carried out at the 3-, 5-, and 7-week time periods. After 6 h continuous exposure to $^{14}CO_2$ in the light, the 3-week-old leaf pairs had accumulated 0.080% of the label into bornane monoterpenes (*i.e.* borneol and camphor) of the steam-volatile fraction, compared to 0.028 and 0.007% incorporation, respectively, for the same number of leaf pairs from 5- and 7-week-old plants. The ratios of the quantity of label incorporated rather closely reflect the relative observed levels of bornyl pyrophosphate synthetase present in the tissue. Bornyl pyrophosphate synthetase catalyzes the first committed step of camphor biosynthesis and probably represents the rate-limiting step of the sequence.

Borneol does not accumulate to a significant level in sage oil (<1%) (16, 26) and in the 5- and 7-week-old leaf pairs; the ratio of [^{14}C]camphor to [^{14}C]borneol observed after $^{14}CO_2$ exposure was about 20:1. In the 3-week-old leaves, however, the ratio of [^{14}C]camphor to [^{14}C]borneol was only 8:1, probably reflecting the higher synthetase to dehydrogenase ratio of this young tissue. In any case, when the 3-week-old leaves exposed to $^{14}CO_2$ were transferred to normal air, the labeled borneol present was essentially all converted to [^{14}C]camphor within 2 h, indicative of this dynamic process.

The relatively high level of bornyl pyrophosphate synthetase relative to the other enzymes at 3 weeks prompted us to examine the possible occurrence of [^{14}C]bornyl pyrophosphate in this tissue after continuous $^{14}CO_2$ exposure. [^{14}C]Bornyl pyrophosphate could be detected in the aqueous extract of such leaves, but the level was very low (~0.002% incorporation of precursor), indicating that this intermediate did not accumulate to an appreciable degree and thus supporting the suggestion that the formation of bornyl pyrophosphate was the rate-limiting step in camphor biosynthesis even in this immature tissue. No [^{14}C]bornyl pyrophosphate was detected after $^{14}CO_2$ labeling of the older leaves.

The *in vivo* rate of accumulation of monoterpenes is probably influenced by many factors which can modulate the inherent capacity (as measured *in vitro*) to synthesize these compounds. In the present instance, very good correlation was obtained between camphor content, essential oil accumulation (as evidenced by the number of filled oil glands), $^{14}CO_2$ incorporation, and direct *in vitro* measurement of the biosynthetic enzymes. On the basis of all of these criteria, the biosynthesis of camphor is most rapid during leaf expansion, although camphor biosynthesis can, and apparently does, continue to a limited extent in mature leaves. It is equally clear that camphor biosynthesis is under rather strict physiological control and that the enzymes of this process (or their activities) are regulated in a coordinated manner.

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