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FINAL TECHNICAL REPORT

Project DE-FG06-88ER13869*
Regulation of Terpene Metabolism*
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Progress Report

Objective 1. Biosynthesis and Catabolism of Monoterpenes. Research under DOE support during the period 1988-1991 established the pathways for the metabolism of menthone in peppermint and camphor in sage through a series of *in vivo* feeding experiments and *in vitro* studies. Each enzyme of each pathway was partially purified from leaf extracts and characterized. This work has been described in detail (7,27). A recent review (30) correlates genetic data from classical crossing experiments with a biochemical evaluation of the monoterpene biosynthetic pathways in mint and proposes genetic associations for monoterpene biosynthetic enzymes.

In the commercially propagated, clonal mints (peppermint, spearmint, scotchmint) monoterpene biosynthesis begins with the cyclization of geranyl pyrophosphate to (-)-limonene, which subsequently undergoes allylic hydroxylation, oxidation to the conjugated carbonyl compound, and then stereospecific reduction of the α,β -unsaturated bonds and, ultimately, the carbonyl function. This scheme (Fig. 1) represents a general metabolic strategy for the conversion of monoterpene olefins to oxygenated products (7,31) and we recently demonstrated the biosynthetic enzyme complement of all the commercial mints to be very similar (32). The essential difference is in the exclusive presence of a regiospecific cytochrome P450 limonene-3-hydroxylase in peppermint which directs the formation of menthone and its congeners, and in the exclusive presence of a regiospecific limonene-6-hydroxylase in spearmint and scotchmint which directs the formation of the C6-oxygenated carvone family (30,32). The pathway to (+)-camphor in sage is much shorter, involving the cyclization of geranyl pyrophosphate to bornyl pyrophosphate, sequential hydrolysis to borneol, and subsequent oxidation (Fig. 2). We have lacked the manpower to pursue the camphor

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pathway in detail and have concentrated our efforts on monoterpene biosynthesis in peppermint which is the more representative of the two in being far more complex and a better model for extended biosynthetic pathways involving a range of enzyme types (cyclization, hydroxylation, isomerization, redox transformations, conjugations). Nevertheless, the bornyl pyrophosphate synthase has been the focus of much of our research on the mechanism of monoterpene cyclization reactions (31) and a cloning effort has been justified on our NIH grant. Both antibody and DNA probes should be available in the future for confirming regulatory studies with sage.

Catabolism of both menthone and camphor is initiated in apparently senescing oil glands on mature leaves and leads to oil yield decline that is not a result of evaporative losses (28,29,33). In peppermint, a portion of the menthone is converted to menthol and the corresponding acetate (which accumulate), but much of the ketone (>50%), by reduction and conjugation, is converted to (+)-neomethyl- β -D-glucoside, which is subsequently transported to the rhizome (34). At this site, a series of reactions ensue (Fig. 3) whereby menthone is ultimately degraded to acetyl CoA by modified β -oxidation sequence, and the carbon utilized for acyl lipid and phytosterol synthesis. Camphor catabolism in sage is conceptually similar in leading to carbon utilization in the root, but the pathway is more complex since it involves cleavage of a bicyclic skeleton. Studies on camphor degradation with root tissue were difficult (35) and we resorted to undifferentiated suspension cell cultures which possess little camphor biosynthetic activity (36), but are adept at catabolism (37). The degradative pathway was traced in six steps to isoketocamphoric acid as the last defined metabolite that contains all 10 carbons of the original bicyclic nucleus (37) (Fig. 4). The initial step of the major catabolic pathway involves P450-dependent conversion to 6-*exo*-hydroxycamphor, conjugation to the glucoside and transport from leaf to root for subsequent degradation (38).

In 1991, we developed a method for the large-scale isolation oil glands from leaf surfaces and demonstrated these structures to be the exclusive sites of monoterpene (and sesquiterpene) metabolism (39,40, and see below). Isolated gland cells are metabolically active and represent a very highly enriched source of the enzymes and mRNAs associated with monoterpene metabolism. We have relied heavily on this unique experimental system ever since for metabolic studies, enzyme isolation and cDNA library construction. We first focused on the limonene synthase of mint, since this enzyme catalyzes the first committed pathway reaction, and *in vivo* evidence had indicated it to be an important rate controlling step. We also pursued the cytochrome P450 hydroxylases, since these enzymes determine the oxygenation pattern of the terpenes formed (30,41). For operational reasons, we worked first with spearmint glands from which the limonene synthase was purified and characterized (42,43). Polyclonal antibodies were prepared (44) which, along with amino acid sequence information for the design of oligonucleotide probes, allowed the cDNA to be isolated (45). An identical reverse genetic approach was used to provide antibodies and sequence information to isolate a cDNA clone encoding the spearmint limonene-6-hydroxylase (46). These tools were then utilized to isolate, from a peppermint gland cDNA expression library, the corresponding limonene synthase cDNA from peppermint (essentially identical to the spearmint clone) and the peppermint hydroxylase cDNA (the sequence similar, but regiospecific, variant that catalyzes, exclusively, limonene-3-hydroxylation) (46). Antibodies and DNA probes now make it possible to assess the regulation of limonene synthase and hydroxylase in developing mint glands.

Recent "*in vivo*" studies with isolated peppermint glands and developmental studies involving *in vitro* measurement of enzyme activities (see below) have also implicated geranyl diphosphate synthase, the prenyltransferase that condenses IPP and DMAPP, in controlling the rate of menthone formation. Enzyme purification and a PCR-based cloning effort are under way. The two stereospecific menthone reductases responsible for the selective conversion to, and accumulation of, menthol, and

for the selective reduction and glucosylation to neomenthyl glucoside for transport and catabolism (47), represent obvious targets for enzyme purification, antibody production and cDNA cloning. These efforts are described in the proposal section.

Objective 2. Organization of Monoterpene Metabolism. The production and accumulation of significant quantities of C₁₀, C₁₅ and C₂₀ terpenoids are almost always associated with the presence of distinct, highly specialized secretory structures (6) which, in the case of mint and sage, are peltate glandular trichomes consisting of a basal cell, stalk cell and 8 secretory head cells that share a common subcuticular space for oil storage (Fig. 5). Isolation of these non-photosynthetic structures from leaf surfaces provides an intact disc (~60 µm diam.) of the 8 secretory cells in which the interconnecting plasmodesmata remain open (39,40). These isolated structures are capable of monoterpene (and sesquiterpene) biosynthesis from ¹⁴C-sucrose (48), while the remaining leaf (minus glands) lacks this capability (49) thus indicating the glands to be the principal site of monoterpene metabolism. The molecular weight exclusion limit for the non-specific permeability of gland cell clusters was shown to be less than 1800 by the differential uptake or exclusion of a series of fluorescein conjugated dextrans as determined by laser scanning confocal microscopy (48). Staining with diaminophenylindole has been employed to localize plastids within the secretory cells and Nile red to selectively stain profuse lipid droplets throughout the cytoplasm that are thought to represent intracellular terpene pools based on chemical analysis and staining characteristics of Nile red (50); the cytoplasm of the secretory cells is rapidly and uniformly stained with a thiol-reactive derivative of Lucifer yellow (48).

At the ultrastructural level, classical fixation and observation techniques (glutaraldehyde/OsO₄-lead citrate/uranyl acetate (51)) were employed to examine the glands on peppermint leaves at different stages of development. Five stages of gland development were identified and correlated with changes in enzyme activity levels and oil chemistry as the glands passed from emerging initials, to gland filling and then into the catabolic phase. At the stage of maximum monoterpene biosynthesis, secretory cells were observed to be spherical and multivacuolate with a dense cytoplasm and a full complement of organelles, including mitochondria, smooth ER, ribosomes and a prominent nucleus. Especially abundant were ovoid leucoplasts, colorless plastids with an electron-dense, granular stroma and few internal membranes. Prominent electron dense secretions (monoterpenes?) are associated with both ER and the numerous leucoplasts and, at this stage, a periplastidial sheath of multiple layers of ER is often found in close association with the leucoplasts. The final two stages of peltate gland development are associated with mature (senescing?) glands from fully expanded leaves that are undergoing a net loss of monoterpenes. The ultrastructural observations are suggestive of the cessation of normal metabolic activity with cellular degeneration and collapse of the subcuticular oil storage cavity (47). At the extreme, the cell walls distort, the cytoplasm becomes devoid of organelles and numerous densely staining vesicles appear. However, the fragility and high oil content of the mature glands caused concern that our classical fixation methods were generating artifacts, even when we varied the osmoticum and included *p*-phenylenediamine during dehydration to improve lipid retention. We have withheld publication of the structural correlative studies until we can re-evaluate mature gland ultrastructure using newer fixation techniques designed for tissues of this type.

Traditional subcellular fractionation techniques cannot be applied to gland secretory cells due to the rigorous cell disruption technique (sonic shear) required to breach the highly modified, encrusted cell wall of these structures (52). Preliminary protoplasting is not possible either because of the very thick cuticle (53); the use of cutinase for this purpose proved unsuitable in practice because of its sharp optimum at pH 9, needed to effect efficient digestion, was incompatible with cellulase and

pectinase, and this pH was also detrimental to gland cell function. Immunocytochemical localization with anti-limonene synthase and protein A gold labeling recently showed limonene synthase to be confined exclusively to the leucoplasts, with label distributed throughout the stroma. These results and those of others (54-56) signal an emerging role for plastids in monoterpene biosynthesis.

Objective 3. Developmental Regulation of Monoterpene Metabolism. Our first goal was to evaluate the relative importance of biosynthesis, catabolism and volatilization in controlling monoterpene content during peppermint leaf ontogeny. The monoterpene content of peppermint leaves rises rapidly during the first two weeks of development, but levels off as full expansion is reached (3-5 weeks) and then declines. Over this same period, monoterpene biosynthesis, measured *in vivo* from a pulse of $^{14}\text{CO}_2$, was found to rise rapidly to a peak between the first and second weeks of development and then to decline (Fig. 6). In contrast, the rates of monoterpene volatilization (measured by dynamic headspace sampling) and monoterpene turnover (determined by $^{14}\text{CO}_2$ pulse-chase experiments) were low and unchanging during the course of early development (29). These results indicated that monoterpene accumulation in young peppermint leaves (0-5 weeks) is controlled chiefly by the rate of biosynthesis; ultimately, the balance between biosynthesis and catabolism determines yield which is critically dependent on harvest timing. To determine which enzymatic step(s) might limit the rate of monoterpene biosynthesis, the time-course of monoterpene formation was compared with changes in the activities of the 7 enzymes involved in the biosynthesis of menthone, the principal monoterpene constituent of expanding peppermint leaves (Fig. 6). The two menthone reductases, the pulegone-8-hydroxylase involved in menthofuran formation, and the glucosyltransferase involved in synthesis of the transport derivative were also evaluated. Enzyme activities were measured *in vitro* in extracts of isolated gland cells. Since the number of gland initials is fixed at leaf emergence both analytical data and activity levels can be equivalently expressed for comparison on a per gland or per leaf basis. The rate of menthone formation was found to correlate closely with the activities of three enzymes, geranyl diphosphate synthase, limonene synthase, and limonene hydroxylase, reaching a maximum at 1 week post leaf emergence, with subsequent decline to a level paralleling oil accumulation until late in gland maturity and yield decline. The activities of the menthone reductases appeared later (as did pulegone hydroxylase), reaching maximum at two weeks post-emergence and declining more slowly through leaf expansion (Fig. 6). Glucosyltransferase activity (utilizing neomenthol as substrate) was always present in excess and showed little developmental variation. Compartmentation effects clearly play a role in determining the fates of menthone in the selective conversion to menthol and its acetate ester or to neomenthol and the transport glucoside (57). Very preliminary evidence based on Western immunoblotting (two time points) suggests that limonene synthase activity correlates with synthase protein levels; no evidence has been found for the control of synthase activity *per se* (e.g., by allosterism).

Ontogenic differences in gland population on a growing leaf might be anticipated to complicate this developmental analysis. However, enzyme activity differences from leaf tip to base and from side to middle are within 15% on a gland or tissue basis, and scanning electron microscopic observation indicated that at least 80% of the glands on a leaf are indistinguishable in size and general morphology. These results suggest that the glands develop more or less in synchrony, and that meaningful measurements of activities, protein and message abundances, etc., can be made and correlated with the timeframe of observed changes in gland ultrastructure of representative samples.

Objective 4. Flux Control of Precursor Supply. This objective focuses on developing secretory cells isolated from glandular trichomes of peppermint as a model system for studying the regulation of precursor supply and the subcellular compartmentation of isoprenoid metabolism. As indicated,

isolated secretory cells derived by surface abrasion of peppermint leaves consist of flattened disks of eight cells each, derived by rupture of the stalk (supporting) cells of the peltate glandular trichomes (40). Several lines of evidence established that these isolated cells are non-specifically permeable toward low molecular weight, water-soluble metabolites as a consequence of the presence of a large number of undamaged plasmodesmata between the secretory cells and the underlying (now broken and exposed) stalk cell (48) (such plasmodesmata are a common feature of many types of glandular trichomes (58,59)). Biochemical experiments demonstrated efficient incorporation of labeled IPP and GPP into monoterpenes (exogenous FPP is converted to sesquiterpenes), and added pyridine nucleotides and redox substrates (glucose-6-phosphate) promote the reductive metabolism of limonene to menthone. The non-physiological pyridine nucleotide antagonist Cibacron blue decreases menthone production from [^3H]GPP.

As a consequence of the non-specific permeability, endogenous low molecular weight cofactors are washed out of the secretory cell cytoplasm during isolation. The incorporation of [$\text{U-}^{14}\text{C}$]sucrose into both monoterpenes and $^{14}\text{CO}_2$ therefore requires the addition of exogenous cofactors. The type and concentration of each cofactor required for maximum incorporation of [$\text{U-}^{14}\text{C}$]sucrose at optimum were determined empirically, and the kinetics of incorporation evaluated (48). Under these conditions (and others established empirically for pH, ionic strength, etc.), the very efficient incorporation (20-30%) of mevalonate and pyruvate into glandular terpenoids was also demonstrated (48). The efficient *de novo* biosynthesis of monoterpenes from a basic, imported carbon source such as sucrose, coupled to the ability to manipulate cytoplasmic composition at will and to isolate secretory cells at any desired stage of development, provide an ideal and powerful system for examining isoprenoid biosynthesis.

In order to examine the flux of intermediates into isoprenoids using isolated cells, methods were developed for the isolation and quantification of the intermediates of the MVA pathway (60). After preincubation with radiolabeled substrates, this procedure utilizes extraction of the cells under neutral conditions designed to minimize degradation of the labile water-soluble intermediates of the MVA pathway. Partial purification of the extracted intermediates with an anion-exchange membrane is followed by baseline resolution and quantification of the intermediates by ion-pair reversed-phase HPLC with on-line radio-detection. The use of internal radiolabeled standards throughout the extraction and purification procedure allows rigorous quantification of the radiolabeled intermediates observed by HPLC. All eleven intermediates of the MVA pathway, from acetyl-CoA through GGPP, can be extracted and quantified with a single tissue extraction and a single HPLC analysis using this method (60). This is the first method reported for any experimental system which allows the intermediates throughout the entire MVA pathway to be measured, and will allow measurement of the flux and steady state levels of intermediates on either side of HMGR.

We have recently exploited the feature of cell semipermeability to manipulate cytoplasmic substrates and cofactors, while maintaining the selective permeability of organelles such as the plastids, to examine the subcellular compartmentation of IPP synthesis (61). In this instance, we employed secretory cells isolated at the developmental stage where cell division and growth have ceased and at which the most rapid accumulation of essential oil occurs (rates of sterol synthesis are insignificant at this point relative to the massive levels of monoterpene and sesquiterpene accumulation, thus simplifying the analysis and interpretation). Glycolytic substrates such as [^{14}C]glucose-6-phosphate and [^{14}C]pyruvic acid are incorporated into both monoterpenes and sesquiterpenes in a ratio that closely mimics that of the endogenous essential oil, indicating that the IPP derived from these substrates is partitioned correctly between C_{10} and C_{15} pathways. Exogenous [^{14}C]MVA and [^{14}C]IPP are also efficiently incorporated into both monoterpenes and sesquiterpenes, albeit with a

strong bias toward the formation of cytoplasmic-derived sesquiterpenes. In contrast, cytoplasmic metabolism of both [^{14}C]citric acid and [^{14}C]acetyl-CoA both result in the accumulation of HMG-CoA, with no detectable MVA pathway intermediates beyond HMG-CoA, nor any detectable isoprenoids. The lack of observed incorporation from exogenous [^{14}C]acetate precluded the export of mitochondrial acetate with subsequent uptake and conversion to acetyl-CoA by the plastids. Thus, incorporation of pyruvic acid probably occurs through the formation of acetyl-CoA by plastidic pyruvate dehydrogenase. Taken together, these results established that the cytoplasmic MVA pathway is latent (i.e., blocked at HMGR), and that both plastidic monoterpene and cytoplasmic sesquiterpene biosynthesis during this period of rapid accumulation rely exclusively on plastid-derived IPP synthesized via the plastidic MVA pathway. This approach of using semi-permeable cells to assess the relative contributions of parallel biosynthetic pathways *in situ* has allowed us to gain new insights into the compartmentation of IPP synthesis that would otherwise be virtually impossible to achieve using conventional methods of subcellular fractionation.

Objective 5. Integration of Monoterpene and Higher Terpenoid Metabolism. We have only recently started work on this objective, the goal of which is to explore the integration of cytoplasmic phytosterol and sesquiterpene metabolism via FPP, and of plastidial mono- di- and tetra-terpene biosynthesis. In peppermint, glandular phytosterol (principally sitosterol) biosynthesis essentially ceases (by analytical determination, per gland basis) within 4 days of leaf emergence, presumably when cell division and development (membrane formation) ceases and the gland cells devote to essential oil biosynthesis and filling of the extracellular cavity. Sesquiterpene formation from cytoplasmically-generated IPP should peak prior to this point, if cytosolic HMGR is functional in early development (see above), but we have not yet explored this possibility. Peppermint glands produce little diterpenoid material, but carotenoids are detectable and their formation parallels monoterpene production (by monitoring β -carotene λ_{max} in gland extracts). Sage, like most *Salvia* species (62), produces appreciable diterpenoid material in the oil glands (by GC-MS survey of gland extracts). Sage glands appear to contain a more complex array of carotenoids than peppermint glands by HPLC analysis.

Publications Resulting from this Project

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Research Personnel

	Period of Service	Effort on Project	DOE Support
Rodney Croteau, Principal Investigator	3/91-present	17%	0%
Jonathan Gershenzon, Postdoctoral Associate	3/91-present	100%	50%
David McCaskill, Postdoctoral Associate	3/91-present	100%	100%
Kurt C. Wagschal, Graduate Student	3/91-3/92	50%	50%
Jean Rajaonarivony, Graduate Student (AFGRAD Fellow)	3/91-12/91	50%	0%
Christoph Funk, Postdoctoral Fellow (Swiss NSF Fellow)	3/91-2/93	100%	0%
G. John Murtagh, Visiting Scientist (North Coast Agricultural Institute, Australia)	8/92-8/93	100%	0%
Glenn Turner, Sabbatical Visitor (Middle Tennessee State University)	6/95-8/95	100%	0%
Marie Rufener, Graduate Student	8/94-present	50%	25%
Gary Rushing, Graduate Student (PBRTC Graduate Trainee)	6/95-present	50%	0%

Estimate of Unobligated Funds

It is estimated that no unobligated funds will remain by the March 14, 1996, termination date of Grant DE-FG06-88ER13869.