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PROGRESS REPORT

Research over the last seven months has progressed very closely along the lines proposed, and the current status of each of the original research objectives is outlined below.

Objecrive 1. Biosynthesis and Catabolism of Monoterpenes. Based on compositional analysis it is clear that geranyl pyrophosphate:(-)-limonene cyclase catalyzes the rate-limiting step of monoterpene biosynthesis in both peppermint and spearmint (*M. piperita* and *M. spicata*). Difficulties encountered with variable extraction efficiency and stability of this enzyme were traced to aggregation of the protein. This problem was overcome by including a low level of detergent in the extraction buffer and assay buffer, and this procedure has permitted characterization of this enzyme in preparation for developmental studies. In most properties, this enzyme is typical of monoterpene cyclases; however, it is very hydrophobic which no doubt is responsible for the tendency of this enzyme to aggregate.

Regarding the onset of catabolism in peppermint leaves, the NADH-dependent reduction of (-)-menthone to (+)-neomenthol and the UDP-glucose-dependent glycosylation of the latter are the two key enzymatic steps in the production of the transport derivative. Both enzymes have been isolated from whole leaf extracts and are well-characterized. In preparation for developmental studies, we have converted the reductase assay from a radiochemical basis to capillary GLC analysis. The latter is less sensitive, but also far less prone to interferences which is an important consideration for developmental studies in which tissues of vastly different ages must be analyzed. The glucosyl transferase assay has also been simplified and is now based on simple reversed-phase separation of the labeled glucoside following pentane extraction of

residual labeled neomenthol from the reaction mixture. With the above preliminary studies completed we have initiated research on the developmental regulation of monoterpene biosynthesis and catabolism in peppermint leaves as a function of leaf maturity (see Objective 4 below).

Another area of recent emphasis is the pathway for the catabolism of camphor, via 1,2-campholide, in sage roots. This pathway has been difficult to elucidate because detectible levels of metabolites between the lactone and acetate are not observed from the labeled precursor. Rather, label appears very rapidly in the end-products, acyl lipids and phytosterols. Two catabolic routes, having precedent in microbial systems, seem likely. The first is patterned on that of a soil diphtheroid and involves C6-oxygenation, ultimately giving rise to 3,4,4-trimethyl-5-oxo-*trans*-2-hexenoic acid as the last confirmed metabolite. The second resembles that of *Pseudomonas* and involves C5-oxygenation, ultimately giving rise to 5-hydroxy-3,4,4-trimethyl-Δ^2-pimelic acid as the last well-defined metabolite. To examine the pathway in sage we will employ isotope dilution analysis using these late-stage metabolites, both of which we have just recently prepared by synthesis. Moreover, we have devised a means of labeling these metabolites by cleavage of the carboxymethylene group and resynthesis from the bisnoraldehyde using Wittig techniques via the phosphorane prepared from [^{14}C]bromoacetate. In this way, we will be able to follow up the isotope dilution experiment with more detailed studies using the appropriate labeled metabolite. For the isotope dilution experiment, we will apply a 5-to 10-fold excess of either advanced metabolite to sage roots, followed by a pulse of (+)-[U-^{14}C]1,2-campholide, and analyze the trapped label by standard radioanalytical procedures. The 1,2-campholide required will be prepared from (+)-[U-^{14}C]camphor obtained biosynthetically in sage leaves via $^{14}CO_2$. Roughly half of the needed camphor has been prepared.

Objective 2. Ultrastructure of Oil Gland Senescence. The bulk of our recent effort has been dedicated to completing the ultrastructural investigation of oil gland development and senescence, and this work is now being prepared for publication. Developmental aspects were included in this work when we discovered that newer fixation techniques did a far better job of preserving hydrophobic structures than did the methods used in the 1960s when mint glands were last examined in detail. A full description of secretory cell function will be provided which differs from earlier work largely in the fact that preservation of plastids and vesicular structures has allowed a more detailed evaluation of the roles of these organelles in terpene synthesis and secretion to the extracellular cavity. The glandular stalk cell has been largely ignored in earlier studies. We have found this cell to be exceptionally active in lipid metabolism during oil gland filling. Several lines of evidence strongly suggest that this cell does not participate in terpene synthesis, but rather is responsible for the synthesis of the cuticle, the lipid polymer that surrounds the gland. During development, the basal cell is quiescent, serving only as a supporting structure and

conduit for photosynthate en route to the stalk and secretory cells. As the extracellular cavity fills, terpene synthesis appears to cease and the stalk cell becomes quiescent. Later in development (several weeks after gland filling) the secretory cells undergo rapid degeneration. Degradation of both cell wall and cytoplasmic constituents is pronounced, with nucleus, mitochondria and other organelles becoming typically senescent. The stalk cell, on the other hand, appears to be reanimated. It stains very heavily for lipid, through which fully functional organelles are visible and around which a fully intact wall is observed. The basal cell also seems to remain functional and lipid bodies and microbodies appear. There does not appear to be movement of lipid (terpenoids) from the stalk cell to the basal cell, only from the degenerate secretory cells to the stalk cell. It is tempting to suggest that terpenoids from the secretory cells and cavity pass through and around these cells to the stalk cell, where they undergo conversion to the glucoside transport derivative. Only the water-soluble conjugate would then pass from stalk to basal cell and thence to the vascular system with which the latter is generally associated. Whether this model is valid will depend upon the outcome of the enzyme localization studies.

Objective 3. Enzyme Localization. Spearmint was chosen for initial enzyme localization studies because of the simplicity of the biosynthetic pathway (i.e., the primary monoterpene is the ketone (-)-carvone, formed by cyclization of geranyl pyrophosphate to (-)-limonene, hydroxylation to (-)-*trans*-carveol and dehydrogenation). Selective extraction of the contents of the glandular trichomes indicated that essentially all of the cyclase and hydroxylase activities resided in these structures, whereas only about 30% of the carveol dehydrogenase was located here with the remainder located in the rest of the leaf. This distribution of carveol dehydrogenase activity was confirmed by histochemical methods. Electrophoretic analysis of the partially purified carveol dehydrogenase from extracts of both the glands and the leaves following gland removal indicated the presence of a unique carveol dehydrogenase species in the glandular trichomes, suggesting that the other dehydrogenase found throughout the leaf probably utilizes carveol only as an adventiticus substrate. These results demonstrate that carvone biosynthesis takes place exclusively in the glandular trichomes in which this natural product accumulates. This work has been submitted for publication (preprint appended).

Scanning electron microscopy indicates that the selective extraction procedure removes all of the secretory cells of the gland, but it is not yet clear whether the stalk cell is removed or remains attached to the leaf surface. The very heavily cutinized walls of the stalk cell suggest that this cell remains attached to the epidermal layer and we hope to confirm this by transmission electron microscopy. Should this occur, it should be possible to take leaves from which the secretory gland cells have been removed and treat them with cellulase and pectinase to digest the mesophyll, thus leaving the epidermis containing the stalk and basal cell. In this way it should be possible

to distinguish enzyme activities residing in the secretory cells from those in the stalk and basal cell, and the activities residing in the latter two cells from those residing in the rest of the leaf.

Objective 4. Developmental Regulation of Metabolism. Preliminary analytical evidence suggests that, while substrate availability probably influences short-term production of monoterpenes, the overall accumulation of these products is most directly dependent on the relative rates of biosynthesis and catabolism as determined by the developmental control of the time-course of production and the absolute levels of the relevant enzymes. The activity of limonene cyclase, which catalyzes the rate limiting step of monoterpene biosynthesis in peppermint, was shown to increase rapidly during leaf expansion, level off at full expansion, and decline precipitously during the next two weeks coincident with the degeneration of the secretory cells of the oil glands. The glucosyl transferase which catalyzes the formation of the monoterpenyl transport derivative exhibits a level of activity 3-to 4-fold that of the cyclase from the onset of leaf emergence, increasing to roughly ten times that of the maximum cyclase activity. The latter increase in glucosyl transferase activity roughly coincides with oil gland senescence; however, transferase activity exceeds cyclase activity throughout. Since catabolism does not occur until gland degeneration, it is clear that compartmentation of the biosynthetic and catabolic pathways must occur in early development. We have yet to examine other relevant enzyme activities of the biosynthetic and catabolic pathways, yet the data thus far obtained for two enzymes can be nicely fitted to the time-course of development based on oil analysis (yield and composition) and ultrastructural changes. The correlation of enzyme activity changes with oil gland senescence and yield decline is striking.

Objective 5. Tissue Culture Systems. As has been experienced by many other investigators, we have been unsuccessful in obtaining cell cultures of either sage or mint which accumulate significant quantities of monoterpenes (i.e., >10 μg product/g tissue). The general failure of tissue culture systems (callus) to accumulate monoterpenes has often been attributed to the loss of such products by volatilization (a problem now overcome by trapping techniques) or to the lack of differentiation required for the expression of the relevant genes or for the extracellular sequestering of the lipophilic, cytotoxic terpenoid products. The relatively few examples of monoterpene accumulation in callus tissues and cell suspensions would appear to argue against the aforementioned considerations as universal barriers to terpene production in culture. A third rationale, often stated but never tested, posits that monoterpenes are produced in culture but are as rapidly degraded. Such a circumstance would obviously provide an exceptionally useful alternative to the intact plant for studies on the regulation of terpene metabolism.

We have examined the conversion of [1-^{3}H]geraniol to camphor, and the transformation of [G-^{3}H]camphor to water-soluble metabolites (the

glucoside-glucose ester of 1,2-campholide) by sage cells harvested at different stages of the culture cycle. The ability to metabolize camphor was present at all stages of the cycle, but the ability to biosynthesize camphor was detectable only at the late log to early stationary phase transition. We have also recently analyzed sage cells for the presence of the first committed step of camphor biosynthesis catalyzed by geranyl pyrophosphate:(+)-bornyl pyrophosphate cyclase. Activity as measured in cell-free systems is absent in lag and log phase, and exhibits a transient appearance at late log/early stationary phase. Thus, activity measurement in cell-free preparations correlates well with the results obtained using exogenous [^{3}H]geraniol as a probe. Although we have not yet examined the time-course of appearance of the key catabolic enzyme (the camphor lactonase), the results thus far suggest that the sage cell system will be a good model for studies on the regulation of terpene metabolism.

PUBLICATIONS FROM PROJECT

Croteau, R., El-Bialy, H. and Dehal, S.S. (1987) Metabolism of Monoterpenes: Metabolic Fate of (+)-Camphor in Sage (*Salvia officinalis*), Plant Physiol., 84, 649-653.

Croteau, R. (1987) Catabolism of Monoterpenes in Essential Oil Plants *in* "Flavors and Fragrances: A World Perspective" (Lawrence, B.M., Mookherjee, B.D. and Willis, B., eds.), Elsevier Press, Amsterdam, p. 65-84.

Croteau, R. (1987) Biosynthesis and Catabolism of Monoterpenoids. Chem. Rev., 87, 929-954.

Croteau, R. (1988) Metabolism of Plant Monoterpenes *in* "ISI Atlas of Science: Biochemistry " Vol. 1, ISI, Philadelphia, PA, p. 182-188.

Satterwhite, D.M. and Croteau, R. (1988) Applications of Gas Chromatography to the Study of Terpenoid Metabolism. J. Chromatogr., in press.

Gershenzon, J., Maffei, M. and Croteau, R. (1988) Biochemical and Histochemical Localization of Monoterpene Biosynthesis in the Glandular Trichomes of Spearmint (*Mentha spicata*). Plant Physiol., submitted.

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