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Demonstration of the Intercellular Compartmentation of *l*-Menthone Metabolism in Peppermint (*Mentha piperita*) Leaves[1]

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ABSTRACT

The metabolism of *l*-menthone, which is synthesized in the epidermal oil glands of peppermint (*Mentha piperita* L. cv. Black Mitcham) leaves, is compartmented; on leaf maturity, this ketone is converted to *l*-menthol and *l*-menthyl acetate in one compartment, and to *d*-neomenthol and *d*-neomenthyl glucoside in a separate compartment. All of the enzymes involved in these reactions are soluble when prepared from whole-leaf homogenates. Mechanical separation of epidermal fragments from the mesophyll, followed by preparation of the soluble enzyme fraction from each tissue, revealed that the neomenthol dehydrogenase and the glucosyl transferase resided specifically in the mesophyll layer, whereas the menthol dehydrogenase and substantial amounts of the acetyl transferase were located in the epidermis, presumably within the epidermal oil glands. These results suggest that the compartmentation of menthone metabolism in peppermint leaves is intercellular, not intracellular.

Rapid turnover of monoterpenes in the epidermal oil glands of maturing peppermint leaves is accompanied by the reduction of *l*-menthone, the major constituent of the essential oil, to *l*-menthol and *d*-neomenthol (3, 4, 7). Of the two diastereomeric alcohols, only *l*-menthol is partially converted to the corresponding acetate, whereas *d*-neomenthol is nearly all glucosylated and the resulting β-D-glucoside is transported to the rhizome (Refs. 7 and 8; Fig. 1). *In vivo* and *in vitro* studies (8, 9, 15) have shown that the observed specificity of the pathways of *l*-menthone metabolism is a result of compartmentation of a menthol-specific dehydrogenase with a relatively nonselective acetyltransferase, and of a neomenthol-specific dehydrogenase with a relatively nonselective glucosyltransferase. All of these dehydrogenases (menthone reductases) and transferases have been isolated from whole-leaf homogenates and characterized (6, 9, 15), and all are 'operationally soluble' (i.e. located almost exclusively in the 105,000*g* supernatant). Thus, the physical basis for the compartmentation of pathways that must be operative in peppermint was not readily apparent from the previous studies with whole leaves. In this communication, we provide evidence that the enzymes of these two distinct pathways (Fig. 1) are located *in situ* in different types of leaf tissue.

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MATERIALS AND METHODS

Peppermint (*Mentha piperita* L. cv. Black Mitcham) plants were grown from stolons as described previously (7), and leaves from the midstem of flowering plants were washed with 1.5 mM EDTA before use. Whole-leaf extracts were prepared (all operations carried out at 0–4°C) by grinding 2 g (fresh weight) of this tissue in a Ten-Broeck homogenizer with 0.1 M Na-phosphate buffer (pH 7.0) containing 0.25 M sucrose, 25 mM $Na_2S_2O_5$, 5 mM Na-ascorbate, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithioerythritol, and 2 g of insoluble PVP (Polyclar AT; GAF Corp., New York, NY) (12). The homogenate was slurried briefly with 2 g of Amberlite XAD-4 polystyrene resin (Rohm and Haas Corp., Philadelphia, PA) (14), filtered through cheesecloth, and centrifuged at 105,000*g* (90 min) to provide a supernatant fraction containing the soluble enzymes. Epidermis extracts were prepared by submerging leaves (2 g) in the extracting medium described above and gently brushing the upper and lower surface with a soft bristle toothbrush. The removal of the epidermis is evidenced by the sloughing of transparent fragments and the bright green appearance of the underlying tissue. The buffer containing the epidermal fragments was then homogenized and treated as above to obtain a 105,000*g* supernatant fraction. A comparable mesophyll supernatant fraction was obtained from the leaves remaining after removal of the epidermis. Each of the soluble preparations (whole leaf, epidermis, mesophyll) was brought to assay conditions by dialysis against 50 mM Na-phosphate buffer (pH 7.0) containing 12 mM mercaptoethanol, 6 mM $MgCl_2$, 1 mM $CaCl_2$, and 4 g/L each of insoluble PVP and XAD-4 resin.

Particulate fractions were prepared with the same extraction buffer, but soluble PVP (Plasdone K-90; GAF Corp.) was substituted for the insoluble polymer, and the XAD-4 treatment and filtration were omitted. The membranes separated by standard differential centrifugation and sucrose density-gradient centrifugation techniques (10) did not contain significant levels of the relevant dehydrogenase or transferase activities, and these fractions were not examined further.

The measurement of glucosyltransferase activity was based on the UDP-glucose-dependent glucosylation of *d*-[G-^{3}H]neomenthol with TLC isolation of the labeled product as described in detail elsewhere (15). The radiochemical assay for acetyltransferase was based on the acetyl CoA-dependent acetylation of *l*-[G-^{3}H]menthol and TLC isolation of the product (6). Assay for *l*-menthone reduction was based on the conversion of the tritium-labeled ketone to *d*-neomenthol and *l*-menthol in the presence of NADPH and a regenerating system, with TLC isolation of the diastereomeric products (9). All assays were conducted with linear kinetics and saturating levels of substrates and cofactors. Boiled controls were run for each experiment, and the identification of products confirmed by radio-GLC analysis and by preparation of crystalline derivatives using techniques described before (6, 9, 15). Activ-

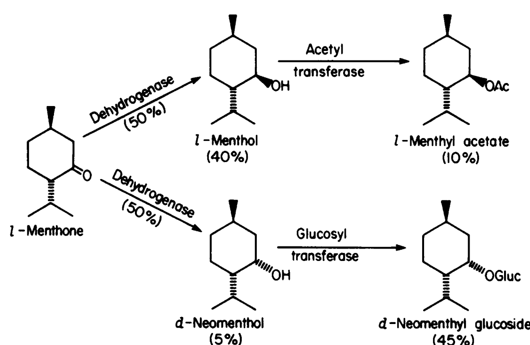

FIG. 1. Pathways of *l*-menthone metabolism in peppermint. The percentages indicate the approximate distribution of the products derived from menthone in mature leaf tissue.

Table I. *Distribution of the Enzymes of Menthone Metabolism in Peppermint Leaf Tissue*

A unit equals 1 nmol of product/h·g of original leaf tissue from which the soluble extract was prepared. Results are the mean ± SE of eight experiments, and the assays are described under "Materials and Methods."

Tissue	Glucosyl Transferase	Acetyl Transferase	Menthone Reductase	
			Menthol	Neomenthol
		units		
Whole leaf	918 ± 38	227 ± 16	393 ± 24	285 ± 18
Epidermis	10 ± 1.2	54 ± 4.5	390 ± 23	28 ± 2.4
Mesophyll	875 ± 35	162 ± 12	30 ± 2.7	229 ± 15

ities are reported in units of nmol/h·g of leaf tissue used to prepare the enzyme extract.

RESULTS AND DISCUSSION

The epidermal oil glands are considered to be the primary sites of monoterpene biosynthesis (5, 11, 13), and we initially assumed the sites of menthone metabolism to occur within these structures. Inasmuch as we wished to use, as an enzyme source, a tissue enriched in oil glands, we mechanically separated epidermal fragments from the underlying mesophyll tissue. Assay of the soluble enzyme fraction of the epidermis preparation revealed a distribution of activities radically different than that derived from whole-leaf homogenates (Table I). The level of menthol-specific reductase was far greater than that of neomenthol-specific reductase, compared to the more nearly equal activities observed in the whole-leaf system. Additionally, the acetyl CoA:monoterpenol acetyltransferase activity was higher than the UDP-glucose: monoterpenol glucosyltransferase activity in this epidermal preparation, in contrast to the reverse situation observed in whole-leaf preparations. These results suggested a possible segregation of enzymic activities such that menthone could be preferentially converted to menthol and to lesser amounts of menthyl acetate in the leaf epidermis (presumably within the epidermal oil glands), whereas menthone may be preferentially transformed to neomenthol and neomenthyl-β-D-glucoside in mesophyll tissue. This latter possibility was strongly supported by assay of the relevant activities in soluble enzyme preparations from the mesophyll remaining after removal of the epidermis (Table I). The distribution of activities varied somewhat from preparation to preparation, and some cross-contamination of tissues was unavoidable (*e.g.* the removal of all adhering epidermis from the mesophyll tissue was particularly difficult). However, the differential segregation of activities between epidermis and mesophyll is sufficient to account for the selectivity of metabolic pathways observed *in vivo*. Thus, the menthol-specific reductase is located almost exclusively in the epidermis, and, while the distribution of acetyltransferase clearly favors the mesophyll, a sufficient level is probably present in epidermis (relative to the glucosyltransferase) to allow for the preferential acetylation of significant quantities of the menthol formed (6, 15). Conversely, the neomenthol-specific reductase is located almost entirely in the mesophyll, and the level of glucosyltransferase activity (relative to both acetyltransferase and menthone reductase) is likely to be sufficient to account for the nearly quantitative glucosylation of the neomenthol formed (7, 15). Support for the conclusion that all relevant activities were measured in the separated fractions was provided by comparison of the favorable overall recoveries to the total activities in soluble enzyme preparations from comparable quantities of whole leaves (Table I). It should be reemphasized here that enzyme levels were measured under standardized assay conditions, and that caution is required in extrapolating these results to the *in vivo* situation where many factors, such as the availability of cofactors in each compartment, can influence relative rates.

The high level of acetyltransferase present in the mesophyll was somewhat surprising, as the primary involvement of this enzyme in menthone metabolism appears to be the acetylation of menthol in the epidermis. However, because menthol acetylation represents a relatively minor activity, and the enzyme is not specific for menthol (*n*-alcanols are preferred substrates [6]), the overall significance of this enzyme in monoterpene metabolism is questionable. In fact, it is possible that monoterpene acetylation may be incidental to the functioning of this enzyme in other pathways such as sterol or acyl lipid metabolism.

The results described here support the intercellular compartmentation of *l*-menthone metabolism in peppermint leaves whereby *l*-menthol and lesser quantities of *l*-menthyl acetate are synthesized in the epidermis (probably within the oil glands where these compounds are known to reside [1]), and *d*-neomenthol and *d*-neomenthyl-β-D-glucoside are synthesized in the mesophyll (from which the glucoside is transported to the roots [7, 8]). With such a system, the distribution of end-products necessarily depends on the relative amounts of menthone transferred from the extracellular gland cavity to each compartment (which *in vivo* seems to be about equal [7, 8]). Previous studies (7, 15) with leaf discs showed that the sites of synthesis of neomenthol/neomenthyl glucoside and of menthol/menthyl acetate were differentially accessible to exogenous [^{3}H]menthone and to endogenous menthone generated from $^{14}CO_2$ (*i.e.* the glucoside pathway was more accessible to exogenous precursor). Menthone is presumably synthesized within the secretory cells of the oil glands (11, 13), and the bulk of this material initially synthesized appears to be stored in the extracellular gland cavity (1). Thus, with the present demonstration of the extraglandular location of the neomenthyl glucoside pathway (Fig. 1), the difference in accessibility noted previously can now be readily rationalized (*i.e.* in a leaf disc experiment, the mesophyll is very accessible to exogenous menthone, whereas movement of this precursor into the epidermal glands requires penetration through the cuticular barrier or passage through the single-celled glandular stalk).

An additional observation from the present study was the finding that an enzymic activity described previously (9) as being responsible for the synthesis from menthone of a high mol wt unidentified product (comigrating with menthol on TLC and requiring the necessary correction in reductase assays) was located specifically in mesophyll tissue. Epidermis preparations thus provided a simple means for examining the menthol-specific reductase without interference from the competing activity.

A rationale for the compartmentation of menthone metabolism in peppermint is not yet apparent because, while the extraglandular location of neomenthyl glucoside synthesis and the transport of this derivative to the roots are obviously related, the role of the

terpenyl glycoside at the transport destination is not yet clear.

As a final note, it should be added that the demonstrated segregation of metabolic enzymes in different cell types may be a consideration in studies on the biotransformation of monoterpenes by *Mentha* callus cultures in which only a limited range of activities are observed (2).

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LITERATURE CITED

1. Amelunxen F, T Wahlig, H Arbeiter 1969 Über den Nachweis des ätherischen Öls in isolierten Drüsenhaaren und Drüsenschuppen on *Mentha piperita* L. Z Pflanzenphysiol 61: 68–72
2. Aviv D, E Krochmal, A Dantes, E Galun 1981 Biotransformation of monoterpenes by *Mentha* cell lines: conversion of menthone to neomenthol. Planta Med 42: 236–243
3. Burbott AJ, WD Loomis 1967 Effects of light and temperature on the monoterpenes of peppermint. Plant Physiol 42: 20–28
4. Burbott AJ, WD Loomis 1969 Evidence for metabolic turnover of monoterpenes in peppermint. Plant Physiol 44: 173–179
5. Croteau R 1977 Site of monoterpene biosynthesis in *Majorana hortensis* leaves. Plant Physiol 59: 519–520
6. Croteau R, CL Hooper 1978 Metabolism of monoterpenes: acetylation of (−)-menthol by a soluble enzyme preparation from peppermint (*Mentha piperita*) leaves. Plant Physiol 61: 737–742
7. Croteau R, C Martinkus 1979 Metabolism of monoterpenes: demonstration of (+)-neomenthyl-β-D-glucoside as a major metabolite of (−)-menthone in peppermint (*Mentha piperita*). Plant Physiol 64: 169–175
8. Kjonaas R, C Martinkus, R Croteau 1980 Metabolism of *l*-menthone in peppermint. Plant Physiol 65: S-96
9. Kjonaas R, C Martinkus-Taylor, R Croteau 1982 Metabolism of monoterpenes: conversion of *l*-menthone to *l*-menthol and *d*-neomenthol by stereospecific dehydrogenases from peppermint (*Mentha piperita*) leaves. Plant Physiol. In press
10. Leech RM 1977 Subcellular fractionation techniques in enzyme distribution studies. *In* H Smith, ed, Regulation of Enzyme Synthesis and Activity in Higher Plants. Academic Press, New York, pp 289–327
11. Loomis WD 1967 Biosynthesis and metabolism of monoterpenes. *In* JB Pridham, ed, Terpenoids in Plants. Academic Press, New York, pp 59–82
12. Loomis WD 1974 Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. Methods Enzymol 31: 528–544
13. Loomis WD, R Croteau 1973 Biochemistry and physiology of lower terpenoids. Recent Adv Phytochem 6: 147–185
14. Loomis WD, JD Lile, RP Sandstrom, AJ Burbott 1979 Adsorbent polystyrene as an aid to plant enzyme isolation. Phytochemistry 18:1049–1054
15. Martinkus C, R Croteau 1981 Metabolism of monoterpenes: evidence for compartmentation of *l*-menthone metabolism in peppermint (*Mentha piperita*) leaves. Plant Physiol 68: 99–106