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Metabolism of Monoterpenes[1]

EARLY STEPS IN THE METABOLISM OF *d*-NEOMENTHYL-β-D-GLUCOSIDE IN PEPPERMINT (*MENTHA PIPERITA*) RHIZOMES

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ABSTRACT

Previous studies have shown that the monoterpene ketone *l*-[G-^{3}H] menthone is reduced to the epimeric alcohols *l*-menthol and *d*-neomenthol in leaves of flowering peppermint (*Mentha piperita* L.), and that a portion of the menthol is converted to menthyl acetate while the bulk of the neomenthol is transformed to neomenthyl-β-D-glucoside which is then transported to the rhizome (Croteau, Martinkus 1979 Plant Physiol 64: 169–175). Analysis of the disposition of *l*-[G-^{3}H]menthone applied to midstem leaves of intact flowering plants allowed the kinetics of synthesis and transport of the monoterpenyl glucoside to be determined, and gave strong indication that the glucoside was subsequently metabolized in the rhizome. Studies with *d*-[G-^{3}H]neomenthyl-β-D-glucoside as substrate, using excised rhizomes or rhizome segments, confirmed the hydrolysis of the glucoside as an early step in metabolism at this site, and revealed that the terpenoid moiety was further converted to a series of ether-soluble, methanol-soluble, and water-soluble products. Studies with *d*-[G-^{3}H]neomenthol as the substrate, using excised rhizomes, showed the subsequent metabolic steps to involve oxidation of the alcohol back to menthone, followed by an unusual lactonization reaction in which oxygen is inserted between the carbonyl carbon and the carbon bearing the isopropyl group, to afford 3,4-menthone lactone. The conversion of menthone to the lactone, and of the lactone to more polar products, were confirmed *in vivo* using *l*-[G-^{3}H]menthone and *l*-[G-^{3}H]-3,4-menthone lactone as substrates. Additional oxidation products were formed *in vivo* via the desaturation of labeled neomenthol and/or menthone, but none of these transformations appeared to lead to ring opening of the *p*-menthane skeleton. Each step in the main reaction sequence, from hydrolysis of neomenthyl glucoside to lactonization of menthone, was demonstrated in cell-free extracts from the rhizomes of flowering mint plants. The lactonization step is of particular significance in providing a means of cleaving the *p*-menthane ring to afford an acyclic carbon skeleton that can be further degraded by modifications of the well-known β-oxidation sequence.

Rapid turnover of monoterpenes has been shown to occur in mature leaves of flowering peppermint plants (*Mentha piperita* L.) (4, 17). During this period of active metabolism, at least some of the leaf oil glands are emptied of their terpenoid contents. Coincident with the overall decrease in monoterpene content of peppermint leaves is the conversion of the major monoterpene constituent, *l*-menthone, to *l*-menthol and to lesser quantities of *l*-menthyl acetate and *d*-neomenthol[3] (5). Detailed examination of the metabolism of *l*-[G-^{3}H]menthone in peppermint leaf discs confirmed earlier observations that menthone was converted to menthol and menthyl acetate, and additionally revealed that up to half of the *l*-menthone metabolized was transformed via neomenthol to *d*-neomenthol-β-D-glucoside (14, 17) (Fig. 1). The high degree of selectivity in the metabolic disposition of the epimeric reduction products of the ketone was subsequently shown to result from compartmentation (27). Examination of the specificity and location of each enzyme of the sequence revealed the NADPH-dependent, menthol-specific dehydrogenase and the relatively nonspecific acetyl CoA-dependent acetyl transferase to reside primarily in the epidermis (presumably the epidermal oil glands), whereas the NADPH-dependent, neomenthol-specific dehydrogenase and associated UDP-glucose:monoterpenol glucosyl transferase were located almost exclusively in the mesophyll (14, 18, 23, 27).

With the pathway of menthone metabolism in leaves well established, and with tentative evidence that menthol and menthyl acetate were apparently refractory to further transformation *in situ*, our attention turned to the possible fate and function of the glucoside. When *l*-[G-^{3}H]menthone was applied to leaves of intact mint plants, the resulting [^{3}H]neomenthyl glucoside could be detected in the rhizome/roots, and was found to undergo subsequent conversion to unidentified polar products at this location (17, 22). These results confirmed earlier suggestions that such terpenyl glycosides were transport derivatives (20, 21), and were the first to directly implicate glycosylation of monoterpenols as a prelude to monoterpene metabolism at sites quite distant from the presumed site of synthesis, *i.e.* the epidermal oil glands (10).

Studies on the metabolism of *l*-menthone in peppermint have thus provided a number of interesting findings, not the least of which is the strong indication that the rhizome is the ultimate site of monoterpene catabolism. In this communication we describe *in vivo* studies with *d*-[G-^{3}H]neomenthyl-β-D-glucoside and its derivatives as substrates in peppermint rhizomes, which establish the early catabolic transformations in this tissue. The pathway, which involves hydrolysis of the glucoside, oxidation

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[3] Although the systematic name for *d*-neomenthol is (5*R*,2*S*,1*S*)-*trans*-5-methyl-2-(1-methylethyl)cyclohexanol, we have utilized here the more common nomenclature based on numbering of the *p*-menthane system (*i.e.* neomenthol = *p*-menthan-3-ol) in which the methyl-substituted carbon is 1*R*, the carbinol carbon is 3*S*, and the isopropyl-substituted carbon is 4*S*.

of the resulting neomenthol to menthone and ring opening via an unusual keto-lactonization reaction, was confirmed by demonstration of the relevant enzymic steps in cell-free extracts. This pathway is of particular significance in providing an acyclic skeleton which can be further degraded by modifications of the β-oxidation sequence having ample precedent in other biological systems (6, 36–38).

MATERIALS AND METHODS

Plant Material, Substrates, and Reagents. Peppermint (*Mentha piperita* L. cv Black Mitcham) plants were grown from stolons under controlled conditions described previously (14, 17). Unless otherwise specified, tissues of flowering plants were used for all experiments.

d-[G-^{3}H]Neomenthol was prepared by $^3H_2$ exposure (New England Nuclear) according to Wilzbach's method (41), and was purified by TLC (silica gel G, developed with hexane-ethyl acetate [5:1, v/v] [system A]) and diluted to a specific activity of 50 or 20 Ci/mol with authentic material. *l*-[G-^{3}H]Menthone (at 50 or 20 Ci/mol) was prepared by CrO_3 oxidation of *d*-[G-^{3}H] neomenthol by literature procedures (2) described in detail elsewhere (17). Peracetic acid oxidation of *l*-[G-^{3}H]menthone, using the buffered system of Sauers (35) with an extended reaction time, afforded *l*-3,4-menthone lactone, and both ketone and lactone were purified by TLC as above. Chemical and radiochemical purity of the products (99+%) was verified by radio GLC, and for use as a substrate each was dispersed in water with the aid of Tween-20 (15 μg/μmol) and sonication.

d-[G-^{3}H]Neomenthyl-β-D-glucoside was prepared by the procedure of Noller and Rockwell (28) in which the alcohol and acetobromo-α-D-glucose (Sigma Chemical Co.) are coupled in anhydrous ether in the presence of Ag_2O. The resulting tetra-*O*-acetyl derivative was purified by TLC (silica gel G with hexane:acetone (3:2, v/v), R_F = 0.5) and was treated with excess sodium methoxide in methanol to yield the monoterpenyl-β-D-glucoside (31) which was purified by TLC (silica gel G with ethyl acetate-ethanol [6:1, v/v] as developing solvent (system B), R_F = 0.45). Synthesis of the glucoside was carried out at the 10 Ci/mol level (10% yield), since the method used and several other variations of the Koenigs-Knorr coupling procedure (24, 31) were less successful at higher specific activities.

l-Menthone, *d*-neomenthol, *d*-piperitone, and other monoterpene standards (all 99+%) were provided through the generosity of R. Hopp, of Haarmann and Reimer GmbH, Holzminden, West Germany, and R. Carrington, of I. P. Callison and Sons, Chehalis, WA. An authentic sample of *l*-3,4-menthone lactone (mp 34°C; $[\alpha]_D^{25}$ −18.8°) was obtained from PCR Research Chemicals, Inc. β-Glucosidase (almonds) was obtained from Sigma Chemical Co., as were other biochemicals and reagents, unless otherwise noted. Polyvinylpolypyrrolidone (GAF Corp.) and Amberlite XAD-4 resin (Rohm and Haas Corp.) were purified by standard procedures for use as adsorbents (25, 26).

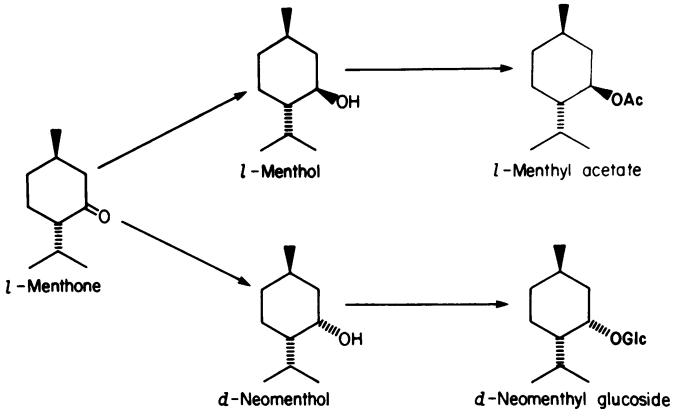

FIG. 1. Scheme for the metabolism of *l*-menthone in peppermint leaves.

***In Vivo* Experiments.** For *in vivo* studies with intact peppermint, flowering plants (9-leaf stage, ~30 cm in height) were transplanted into sand culture containers equipped with an XAD-4 polystyrene resin trap essentially as described by Tang and Young (39). The root system was irrigated with 0.1-strength Hoagland solution-1, and 5 d after establishing the plants under a day/night temperature cycle of 30/19°C, with a 16 h photoperiod (10,000 lux; mixture of wide spectrum Gro-lux and cool white), an aqueous suspension of *l*-[G-^{3}H]menthone (20 μCi at 50 Ci/mol; 0.4 μmol in 0.1 ml) was spread onto the upper surface of a mechanically supported midstem leaf (5th or 6th from the bottom), and the leaf was covered with a microscope slide to minimize evaporative losses. After an additional 12, 24, or 48 h, during which the same nutrient solution was aerated and recirculated every 6 h, the source leaf which had been administered the [G-^{3}H]menthone was excised and washed thoroughly with water to remove unincorporated substrate. The rest of the plant was then divided into tissue above the source leaf (stem, leaves, and flowers), leaves and stem below the source leaf, and the root/rhizome system, and each tissue was analyzed for incorporated products as described below. An aliquot of the aqueous nutrient solution was taken for determination of tritium content, and the XAD-4 resin column was eluted with methanol to remove trapped hydrophobic substances, which were also examined by aliquot counting.

For studies with *d*-[G-^{3}H]neomenthyl-β-D-glucoside as substrate, plants established in sand culture were also employed, but in this instance the above ground portion of the plant was removed 3 cm above the soil line. An 18-gauge syringe needle was inserted into the center of the remaining stem and 0.1 ml of an aqueous solution of *d*-[G-^{3}H]neomenthyl-β-D-glucoside (20 μCi; 0.4 μmol) administered. After 12 h, the stem was separated from the root system and both tissues washed thoroughly with water prior to analysis of incorporated products. Two other incorporation systems were also employed with the glucoside as substrate; in both cases the rhizome and adventitious roots obtained from flowering plants grown from stolons in the soil mix under conditions previously described (14, 17) were used. In one case, the rhizome/root system (1–2 g) was severed from the remaining plant, washed free of soil with water, and then incubated as such in a 25-ml Erlenmeyer flask with 0.5 ml of an aqueous solution of *d*-[G-^{3}H]neomenthyl-β-D-glucoside (20 μCi; 0.4 μmol). The flask was closed with aluminum foil containing a sufficient number of pinholes to permit free exchange with the atmosphere, and was maintained at room temperature for 12 h with frequent shaking to improve contact of the substrate solution with the root system. Following incubation, and before analysis of products, the tissue was washed thoroughly with water to remove unincorporated substrate. In the second type of experiment, the rhizome (with attached root system) was cut with a razor blade into 1- to 2-cm segments before incubation with substrate as above. A number of attempts were made to carry out these incorporation experiments with labeled glucoside prepared at a specific activity of 10 Ci/mol, but at the count levels required (20 μCi; 2 μmol) the substrate was apparently toxic (necrosis), presumably a result of the excellent detergent properties of this material.

For studies using [G-^{3}H]-labeled *d*-neomenthol, *l*-menthone, and *l*-3,4-menthone lactone as substrates (administered at the 20 μCi [1 μmol] level in 1 ml water), excised rhizomes (2–3 g) were used, and were incubated at room temperature as above for 24 h. As before, the tissue was washed free of residual substrate before product analysis. Control experiments with steam-inacti-

vated tissue were also run with this system to evaluate the possible influence of microbial contamination. For this purpose, the rhizome was first washed free of soil by the standard procedure. The rhizome was then incubated with 0.5 ml of water for 1 h. This aqueous extract was withdrawn and the rhizome treated with steam for 15 min. Following cooling to room temperature, the aqueous extract (presumed to be an appropriate microbial inoculum) and the substrate were added to the tissue and the mixture was incubated and then washed free of residual substrate as above.

Product Analysis of *In Vivo* Experiments. Each tissue, after washing with water to remove unincorporated substrate, was frozen in liquid nitrogen and powdered with a mortar and pestle. The powder was transferred to a 15-ml Ten-Broeck homogenizer and extracted sequentially with diethyl ether (10 ml/g tissue) and with methanol (10 ml/g tissue). Boiling water (20 ml/g tissue) was then added to the previously extracted solid, followed by the addition of an equal tissue weight of insoluble polyvinylpolypyrrolidone, and the mixture thoroughly homogenized and then centrifuged at 17,000*g* to separate the water-soluble products from residual solid. Aliquots of each extract were assayed for radioactivity, as was a portion of the remaining solid. The ether-soluble products were subjected to microscale vacuum steam distillation (16) in order to separate the volatile and nonvolatile fractions. The appropriate carrier standards (2 mg each) were added to the volatile fraction before further analysis by either TLC (silica gel G, system A) or radio-GLC.

Preparation and Assay of Cell-Free Systems. Peppermint rhizomes including the adventitious roots (1–3 g) were washed thoroughly with distilled H_2O to remove adhering soil, and then frozen in liquid N_2 and powdered with a mortar and pestle. The powder was then thawed in 100 mM Na-phosphate buffer (pH 6.5; 10 ml/g tissue) containing 250 mM sucrose, 50 mM Na-ascorbate, 50 mM $Na_2S_2O_5$, 5 mM $MgCl_2$, and 1 mM DTE, and then ground with an equal tissue weight of insoluble polyvinylpolypyrrolidone (Polyclar AT) (25) in a Ten-Broeck homogenizer. The homogenate was next slurried with an equal tissue weight of hydrated Amberlite XAD-4 polystyrene resin (26) for several minutes at 0 to 4°C, and centrifuged at 10,000*g* for 20 min (pellet discarded) to provide the supernatant used as the enzyme source. The preparation was then dialyzed to the appropriate conditions for each enzyme assay. Boiled controls and controls run without cofactor (where appropriate) were included in each experiment.

For the assay of β-glucosidase activity, the preparation was dialyzed against 0.1 M Na-acetate buffer (pH 5.0) containing 2 mM $MgCl_2$, centrifuged (10,000*g* for 10 min) to remove denatured material, and aliquots incubated in screw-capped vials for 90 min at 30°C in the presence of 0.1 mM *d*-[G-^{3}H]neomenthyl-β-D-glucoside. At the end of incubation, the vial was chilled in ice and extracted with ether (2 × 1 ml), and, following addition of 2 mg of carrier *d*-neomenthol to the extract, the aglycone was isolated by TLC (silica gel G, system A) and its identity verified by radio-GLC.

For the assay of *d*-neomenthol dehydrogenase activity, the preparation was dialyzed against 50 mM Na-phosphate buffer (pH 8.0) containing 100 mM sucrose, 5 mM $MgCl_2$, 1 mM Na-ascorbate, and 1 mM DTE, and aliquots incubated in screw-capped vials for 1 h at 30°C in the presence of 0.1 mM *d*-[G-^{3}H] neomenthol and 1 mM of oxidized pyridine nucleotide. The products were extracted with ether as before, carrier was added (2 mg *l*-menthone), and the ketone isolated by TLC (silica gel G, system A) and its identity confirmed by radio-GLC.

For the assay of *l*-menthone monooxygenase (keto-lactonization), the preparation which had been dialyzed to the above Na-phosphate buffer system (pH 8.0) was employed, and aliquots were incubated in the presence of 0.1 mM *l*-[G-^{3}H]menthone and 1 mM NADPH. Extraction of products as before was followed by the addition of 2 mg 3,4-menthone lactone as carrier and isolation of this product by TLC (silica gel G, system A). The identity of the lactone product was confirmed by radio-GLC.

For the assay of menthone lactone thiokinase activity, the preparation was dialyzed against 50 mM Na-phosphate buffer (pH 7.0) containing 50 mM sucrose, 5 mM $MgCl_2$, 1 mM Na-ascorbate, and 1 mM DTE, and aliquots incubated for 90 min at 30°C in the presence of 0.1 mM *l*-[G-^{3}H]-3,4-menthone lactone, 0.5 mM ATP, and 0.5 mM CoA. At the end of incubation, the reaction mixture was adjusted to pH 6.0 with 0.3 M phosphoric acid and extracted with ethyl acetate (2 × 1 ml) to remove residual substrate. The remaining aqueous phase was then adjusted to pH 9.0 with 3 N NaOH and the mixture heated at 50°C for 10 min to hydrolyze CoA esters. Re-acidification with concentrated HCl, followed by ether extraction (2 × 1 ml), allowed recovery of the liberated product as the lactone, which was isolated by TLC as above following the addition of carrier. Product confirmation was again performed by radio-GLC.

Enzymatic and Chemical Conversions. Hydrolysis of samples with β-glucosidase was carried out in screw-capped vials containing 10 units of enzyme in 1 ml of 0.1 M Na-acetate buffer (pH 5.0). Following 1 h incubation at room temperature, aglycones were extracted with ether and, after addition of internal standards (2 mg), were separated by TLC (system A). Samples containing phenolic material or other substances likely to inhibit glucosidase were also subjected to acid hydrolysis (3 N HCl at 30°C overnight) and the aglycones released were isolated and analyzed as above.

Chromatography and Determination of Radioactivity. TLC was done on 1.0-mm layers of silica gel G activated at 110°C for 3 h. The developing solvents were system A, hexane:ethyl acetate (5:1, v/v) and system B, ethyl acetate:ethanol (6:1, v/v). The developed chromatograms were sprayed with a 0.2% ethanolic solution of 2,7-dichlorofluorescein to locate (under UV light) the appropriate components, which were eluted from the gel with diethyl ether or methanol.

Radio-GLC was performed on a Gow-Mac 550P thermal conductivity gas chromatograph attached to a model 7357 Nuclear Chicago radioactivity monitor (calibrated externally with [^{3}H]toluene). Chromatography columns were 12 ft × 0.125 in o.d. stainless steel containing either 15% Carbowax 20M or 15% SE-30 on 80/100 mesh Chromosorb W-HP. Chromatographic conditions are described under the appropriate figures. Analytical chromatography and GLC-MS were performed on a 25-m fused silica capillary column coated with SE-30.

Radioactivity in organic solvent samples and TLC isolates was determined in a counting solution (15 ml) consisting of 0.3% (w/v) Omnifluor (New England Nuclear) dissolved in 30% ethanol in toluene. Aqueous samples were counted in 15 ml of ScintiVerse (Fisher Scientific Co.). Samples were quench corrected by internal standardization ([^{3}H]toluene) and counted to <1% probable error.

RESULTS

Administration of *l*-[G-^{3}H]Menthone to Intact Plants. Preliminary experiments in which *l*-[G-^{3}H]menthone was applied to the surface of midstem leaves of intact, flowering peppermint plants revealed that, within 8 h of leaf application of the ketone, labeled *d*-neomenthyl-β-D-glucoside could be detected in the roots (17, 22). In an attempt to examine in greater detail the kinetics of metabolism and transport of the glucoside, and to examine the possible formation of root exudates, these experiments were repeated with the modification that the plants were maintained in sand (rather than soil mix) and with the provision of a recirculating system such that the nutrient medium (0.1-strength Hoagland solution) could be periodically pumped through a cartridge of XAD-4 polystyrene resin to trap hydro-

phobic substances (39). At intervals of 12, 24, and 48 h after application of 20 μCi of [G-^{3}H]menthone to a midstem leaf, plants were removed, divided into their various parts, and each sequentially extracted with ether, methanol, and water. The summation of radioactivity in each extract, plus that in residual solid and in the medium used to irrigate the root system, was taken as total incorporation and represented from 24% (12 h) to 53% (48 h) of the applied menthone. The data reported below are given as per cent of incorporated substrate. The limits of incorporation provided are the extremes of three experiments (for each incorporation period), and the range in values is most likely a result of limitations of the experimental design, including variation in the amount of menthone lost by evaporation from the leaf surface and the inability to precisely match plants with regard to developmental status and physiological condition. Since the tissue above the source leaf (leaves, stems and flowers), the leaves below the source leaf, the nutrient medium, and the XAD-4 trap contained negligible levels of radioactivity in all cases (<1%), no attempt was made to define the nature of the labeled products contained therein. The stem below the source leaf contained appreciable amounts of incorporated activity in all cases (1–3%), which resided specifically in the methanol-soluble fraction (neither ether-soluble nor water-soluble materials were appreciably labeled). Treatment of the methanol-soluble product with β-glucosidase released more than 85% of the radioactivity in the form of labeled neomenthol in all cases, indicating the presence of *d*-neomenthol-β-D-glucoside in the stem tissue.

The source leaf which had been administered the [^{3}H]menthone contained the bulk of radioactivity in all cases. After 12 h of incorporation, this leaf contained ~95% of the incorporated activity (a minimum of 20% of applied tracer), whereas after 48 h, this leaf contained ~75% of the incorporated activity (and a maximum of 41% of applied tracer). Most of the radioactivity associated with the source leaf (75–90%) resided in the methanol-soluble fraction, and β-glucosidase hydrolysis, followed by radio-GLC and TLC of the aglycone, confirmed the major product to be neomenthyl-β-D-glucoside as expected on the basis of earlier results (17). The remaining activity associated with the source leaf (10–20%) was found to reside in the ether-soluble fraction, and was shown by radio-chromatographic analysis to consist of residual substrate along with lesser amounts of menthol and neomenthol, consistent with previous studies (17). The level of radioactivity in water-soluble products was negligible.

The levels of radioactivity incorporated into the rhizome/root system increased with time from 3 to 5% of incorporated tracer at 12 h, to 9 to 11% of incorporated tracer at 24 h, to 19 to 26% of incorporated tracer at 48 h (Table I). At the earliest time period, about half of the radioactivity located in the rhizome/root system was present as neomenthol-glucoside (as demonstrated by hydrolysis and radio-GLC analysis of the resulting neomenthol). The remaining activity was about equally distributed between other methanol-soluble products and ether-soluble products (in which neomenthol was detected by radio-GLC). Water-soluble products contained relatively little of the incorporated label at this time (<1%). By 24 h, up to 11% of the incorporated radioactivity was present in the rhizome/root extracts, but the relative quantity of neomenthyl-β-D-glucoside present was somewhat less than that observed after 12 h. The remaining activity was about equally distributed between ether-soluble products (neomenthol was again detected), methanol-soluble products refractory to β-glucosidase treatment, and water-soluble materials. After 48 h, some 19 to 26% of the incorporated tracer resided in water-soluble products, a substantial fraction was methanol-soluble (~40%, almost none of which was the glucoside), and the remainder (~10%) was present in ether-soluble materials. Analysis of the latter by radio-GLC revealed the presence of neomenthol, and a minor component chromatographically coincident with menthone. In no instance did the residual solid, remaining after the extraction sequence, contain appreciable activity, nor was ^3H_2O produced in significant amounts (*i.e.* only 5–10% of the total activity contained in methanol extracts was lost during lyophilization). Finally, it should again be noted that, even after 48 h, the levels of radioactivity contained in the irrigation medium and XAD-4 trap were less than 1% of the total tracer incorporated.

Table I. *Incorporation of Radioactivity into Rhizomes from [^{3}H] Methone Applied to Midstem Leaves of Flowering Peppermint Plants*

Results for total incorporation represent the range of three experiments at each time interval in which 20 μCi of *l*-[G-^{3}H]menthone was administered to a midstem leaf of an intact plant. Data for distribution of incorporated activity are the averages of the three experiments, and the figure in parentheses under the methanol-soluble column is the per cent of incorporated label in neomenthyl glucoside as determined by β-glucosidase hydrolysis. Further details of the experiments are provided under "Materials and Methods."

Time	Total Incorporation	Distribution of Incorporated Activity			
		Ether soluble	Methanol soluble	Water soluble	Residue
h	%	%			
12	3–5	23	76 (52)	<1	<1
24	9–11	21	63 (44)	16	<1
48	19–26	11	41 (2)	46	1

Although this type of *in vivo* experiment has a number of shortcomings, several conclusions seem justified. First, most of the menthone incorporated into the midstem leaf is converted to neomenthol-β-D-glucoside, a significant proportion of which is specifically transported to the rhizome/root system (*i.e.* some 20–25% of the tracer incorporated as menthone was shown to reside in the root system after 48 h). The glucoside undergoes subsequent metabolism in the rhizome, giving rise to ether-soluble, methanol-soluble, and water-soluble products, very little of which is exuded from the roots. Finally, since little of the intact glucoside could be recovered from the rhizome/root, it seems likely that hydrolysis of the glucoside is an early step in the metabolism of this transport derivative at the rhizome site, a conclusion further strengthened by the detection of labeled neomenthol in the ether-soluble products.

Administration of *d*-[G-^{3}H]Neomenthyl-β-D-Glucoside to Rhizomes. *d*-[G-^{3}H]Neomenthyl-β-D-glucoside, prepared at a specific radioactivity of 10 Ci/mol, was tested as a substrate by stem feeding of intact mint rhizomes from flowering plants, but was found to be toxic when administered at the 20 μCi level, presumably as a result of the excellent detergent properties of this material. The glucoside, therefore, was prepared at a specific radioactivity of 50 Ci/mol (albeit in a very low yield), and aqueous solutions of this product (0.5 ml containing 20 μCi) were administered separately to an excised rhizome (1.1 g), a rhizome cut into segments (1.0 g), and an intact rhizome (by stem feeding a root system weighing ~1.3 g). After a 12-h incorporation period, the excised rhizome and the segments were washed free of unincorporated substrate, and the feed-stem removed from the intact rhizome system (subsequent analysis revealed the stem to contain the glucoside as the major labeled product). Each tissue was then subjected to sequential extraction with ether, methanol, and water.

In the case of the intact rhizome, about 10% of the label administered as [^{3}H]neomenthyl glucoside was recovered in the ether-soluble fraction, some two-thirds of which were volatile on steam distillation. Radio-GLC analysis of this volatile material revealed the presence of neomenthol (major component), men-

thone, and several other labeled compounds which were not identified until subsequent experiments (see below). Nearly 40% of the radioactivity administered was obtained in the methanol-soluble fraction. Aliquots of this fraction were subjected to acid hydrolysis or β-glucosidase treatment and the [^{3}H]neomenthol liberated was determined. Surprisingly, these results indicated that only 10 to 15% of the label administered as [^{3}H]neomenthyl glucoside was recoverable as such from the tissue after the incubation. Lyophilization of the methanol-soluble fraction resulted in little loss of radioactivity, setting an upper limit of ~2% for the biological conversion of the administered substrate to ^3H_2O. Some 6% of the administered radioactivity was recovered in the H_2O-soluble products, and analysis of this material, as above, indicated that this fraction contained neither [^{3}H]neomenthyl glucoside nor ^3H_2O.

Overall incorporation of the [^{3}H]neomenthyl glucoside was somewhat higher in the case of the excised rhizome ([^{3}H]neomenthol was again detected in the ether-soluble volatile fraction, and [^{3}H]neomenthol glucoside in methanol-soluble products) and considerably lower in the case of rhizome segments (with only 4% of the administered label recovered in ether-soluble products, 12% in methanol-soluble products, and 2% in H_2O-soluble products).

These and the earlier results with intact plants strongly suggest that neomenthyl glucoside is not stored in the rhizome, but rather that this transport derivative is rapidly hydrolyzed by a glucosidase on arrival from the leaves, a conclusion supported by later studies with cell-free systems. Similarly, the immediate hydrolysis product *d*-neomenthol does not accumulate but rather appears to be further metabolized. Subsequent *in vivo* studies with excised rhizomes, therefore, were carried out with *d*-[^{3}H] neomenthol as substrate.

Administration of *d*-[G-^{3}H]Neomenthol and Its Derivatives to Rhizomes. To examine the fate of the aglycone, an aqueous suspension of *d*-[G-^{3}H]neomenthol (20 μCi; 1 μmol in 1 ml) was administered to excised rhizomes and, following 24 h incubation at 20°C and washing of the tissue to remove residual substrate, the ether-soluble, methanol-soluble, and water-soluble products were extracted. Typically, 40 to 50% of the applied tracer was located in the ether-soluble fraction (about two-thirds of which were steam volatile), some 10 to 15% was recovered in methanol-soluble products, and from 1 to 2% was present in water-soluble materials. The residual solid, remaining after the extraction sequence, contained negligible levels of tritium. Lyophilization of the methanol-soluble products revealed that this material contained little ^3H_2O (<2%), while acid hydrolysis followed by recovery of [^{3}H]neomenthol indicated the presence of relatively little neomenthyl glucoside (<1%). The latter observation is in sharp contrast to comparable experiments with peppermint leaves in which the glucoside is the major product of neomenthol metabolism (27). Preliminary examination by TLC of the methanol-soluble fraction (silica gel G with ether:methanol [1:1, v/v]) and of the nonvolatile, ether-solubles (silica gel G with hexane:ethyl acetate [4:1, v/v]) indicated the presence in both cases of several products of diverse polarity. These fractions, as well as the water-soluble products, were not examined further.

Radio-GLC analysis of the ether-soluble, steam-distilled products on a Carbowax column indicated the presence of several products (Fig. 2) of which the major component was found to be residual substrate, *d*-[^{3}H]neomenthol. The oxidation product of neomenthol, menthone, was also present, as were several unsaturated derivatives of this ketone including pulegone, piperitone, piperitenone, and isopiperitenone. The most polar component of the product series, eluting last on the Carbowax column, initially proved difficult to identify. Since a lactone was recently identified as a major metabolite of the bicyclic monoterpene ketone camphor in sage leaves (12), the possibility of a

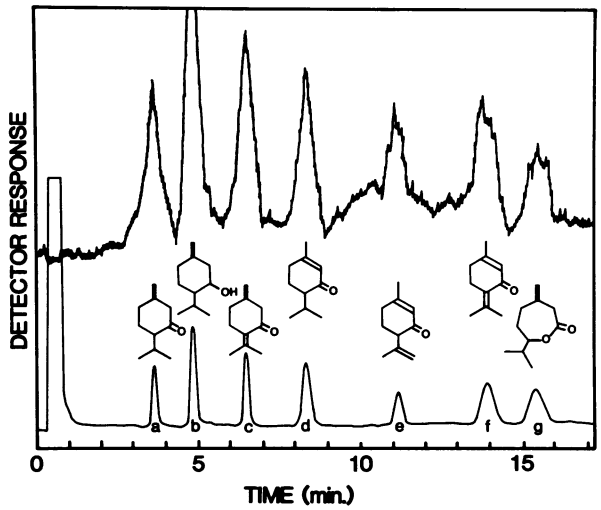

FIG. 2. Radio gas-liquid chromatogram of the ether-soluble, steam-distilled products isolated from peppermint rhizomes that had been incubated with *d*-[G-^{3}H]neomenthol. The smooth lower tracing is the thermal conductivity detector response obtained from coinjected authentic standards of menthone (a), neomenthol (b), pulegone (c), piperitone (d), isopiperitenone (e), piperitenone (f), and 3,4-menthone lactone (g). The upper tracing is the response of the radioactivity monitor attached to the gas-liquid chromatograph. The chromatographic column (Carbowax 20M, described under "Materials and Methods") was programmed from 160 to 180°C at 2°C/min following injection at a He flow rate of 65 cm^3/min.

lactone was explored. The polar component was thus identified as 3,4-menthone lactone, a ring-cleavage product almost certainly derived by oxygenation of menthone. The identity of each of the above products was confirmed by coincidence of radioactivity with the authentic standard on TLC (silica gel G and 8% $AgNO_3$-silica gel G with system A), and by isolation of each product by this means and co-chromatography with authentic standard on an SE-30 GLC column. Control incubations were run with heat-inactivated tissue inoculated with a surface extract to test the possibility that the metabolites observed were of microbial origin. Analysis of the volatile products of these incubations revealed the presence of primarily the unreacted substrate.

The above results suggested that neomenthol had undergone extensive metabolism in peppermint rhizomes to give rise to polar products soluble in methanol and water. Such a transformation would almost certainly be oxidative in nature and likely to involve cleavage of the cyclohexanoid ring. A reaction sequence involving the oxidation of neomenthol to menthone, and oxygenation of the ketone to the lactone, seemed likely, with the unsaturated ketones as apparent side-products having no obvious role in ring cleavage processes. To examine this putative reaction sequence, isotopic dilution experiments were carried out in which 1 μmol (20 μCi) of [^{3}H]neomenthol was administered to excised rhizomes in the presence of 5 μmol unlabeled *l*-menthone or *l*-3,4-menthone lactone. *l*-Menthone did decrease labeling of menthone lactone, and both diluents decreased incorporation into polar products (by 20 and 40%, respectively). More definitive results were provided when *l*-[G-^{3}H]menthone was employed as the substrate with excised rhizomes (20 μCi, 1 μmol in 1 ml). The amount of label incorporated into methanol-soluble and water-soluble products increased to 32% and 8%, respectively (compare 10 to 15% and 1 to 2%, respectively, for [G-^{3}H] neomenthol), whereas in the case of [G-^{3}H]3,4-menthone lactone as substrate the amount of label in these respective fractions increased to 39% and 15%. The summary of these studies

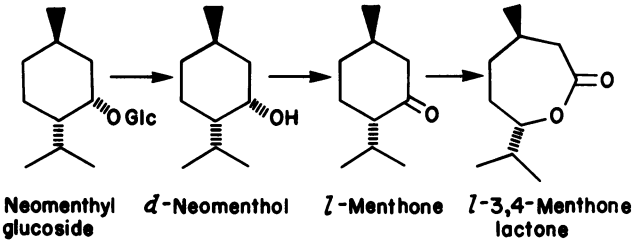

FIG. 3. Scheme for the metabolism of *d*-neomenthyl-β-D-glucoside in peppermint rhizomes.

provided strong evidence that the conversion of the terpenoid transport derivative to polar, nonvolatile products did involve the preliminary hydrolysis of the glucoside to the aglycone, oxidation to *l*-menthone, and ring cleavage to the corresponding lactone (Fig. 3).

Demonstration of the Pathway in Cell-Free Systems. To confirm the proposed pathway, an attempt was made to demonstrate the relevant enzymic activities in cell-free preparations from peppermint rhizomes. Incubation of a rhizome homogenate at pH 5.0 in the presence of *d*-[G-^{3}H]neomenthyl-β-D-glucoside did afford an ether-soluble product identified by radio TLC and GLC as neomenthol, thus confirming the presence of a β-glucosidase. Although the system was not optimized for either enzyme extraction or assay parameters, under the conditions of the experiment nearly 50% of the substrate (at the 0.1 mM level) was hydrolyzed in a 90-min period. There is considerable interest in the occurrence and probable function of monoterpenyl glycosides as transport derivatives (11, 12, 20, 21, 33 and citations therein), yet relatively little information is available on the hydrolysis of these conjugates at the transport destination. Recently, Reznikova and associates (3, 30) have examined the transport and metabolism of monoterpenyl glucosides in rose flowers, and have provided convincing evidence for a dramatic increase in β-glucosidase activity in the blossoms concomitant with the most active period of oil secretion and monoterpenyl glucoside mobilization in this tissue. Developmental alteration in the levels of the relevant β-glucosidase in mint rhizomes has not yet been examined; however, similar increase in glucosidase activity in rhizomes at the onset on menthone metabolism in leaves might be anticipated.

When rhizome homogenates were incubated at pH 8.0 in the presence of *d*-[G-^{3}H]neomenthol and $NADP^+$, *l*-[^{3}H]menthone was produced at rates (10–15 nmol/h·g tissue equivalent) comparable to those previously observed for a number of monoterpenol dehydrogenases isolated from leaf tissue of other plant species (13, 15). Substitution of NAD^+ for $NADP^+$ in the reaction mixture reduced enzymic menthone formation by 30% as determined by TLC isolation and radio-GLC of the product.

The next step in the proposed reaction sequence is the lactonization of *l*-menthone, and when *l*-[G-^{3}H]menthone was incubated with the rhizome homogenate in the presence of NADPH, 3,4-menthone lactone was produced (~10 nmol/h·g tissue equivalent). The lactone product was isolated by TLC and verified by radio-GLC. In this instance, the boiled control gave a high background after TLC separation of the putative product, but on radio-GLC analysis, labeled lactone was not detected and the radioactivity present was associated with unidentified materials even less polar than the substrate. A similar lactonization step has recently been demonstrated to be involved in the metabolism of the bicyclic monoterpene ketone, camphor, in sage (*Salvia officinalis*) leaves (12); however, detailed information on this unusual monooxygenase reaction is available thus far only from studies with microbial enzyme systems (1, 9, 19).

If subsequent steps in the metabolism of the lactone are oxidative, it seems likely that the lactone would initially undergo activation to the corresponding hydroxy acyl CoA ester. This possibility was examined by incubation of the rhizome homogenate at pH 7.0 in the presence of 0.1 mM *l*-[^{3}H]3,4-menthone lactone, 0.5 mM ATP, and 0.5 mM CoA. Following incubation, the residual substrate was removed by thorough extraction of the reaction mixture with ethyl acetate, and CoA esters present in the remaining aqueous phase were subjected to alkaline hydrolysis. The lactone recovered on acidification was readily detected by radio-GLC, and although the activity was low (~1 nmol/h·g tissue equivalent), product formation was shown to be ATP- and CoA-dependent, suggestive of the presence of the relevant thiokinase.

DISCUSSION

Results described in this and earlier communications (17, 18, 22, 27) provide strong evidence that a significant fraction of the *l*-menthone produced in the leaves of flowering peppermint plants is converted to *d*-neomenthyl-β-D-glucoside, which is transported to the rhizomes and further metabolized at this site. Relatively little of the glucoside reaching the rhizome remains intact, but rather is transformed to a mixture of ether-soluble, methanol-soluble, and water-soluble metabolites. Analysis of the labeled ether-soluble metabolites in the rhizome, derived from [^{3}H]-menthone applied to leaves or from the labeled glucoside applied directly to the rhizome, indicated that the glucoside was hydrolyzed to neomenthol as an early step in metabolism, while *in vivo* studies with labeled neomenthol and its derivatives indicated that the subsequent steps in the metabolism of neomenthol in the rhizome were dehydrogenation to menthone and then oxidation of the ketone to 3,4-menthone lactone. Additional support for this proposal was provided by the demonstration of the relevant activities in cell-free systems obtained from rhizome extracts (*i.e.* a *d*-neomenthyl-β-D-glucoside hydrolase, a neomenthol dehydrogenase, and the enzymic oxidation of menthone to the lactone by what is probably an NADPH-dependent mixed-function oxidase).

The present research on the metabolic fate of *l*-menthone in peppermint represents one of very few detailed studies on monoterpene catabolism in plants, and is the first work to demonstrate the long-range transport of monoterpenyl glucosides and to document the involvement of the rhizome in catabolic processes. Evidence for the transport of monoterpenyl glucosides from leaves to blossoms, and the subsequent hydrolysis of the transport derivative at this site, has been described in the essential oil rose (3, 29, 30); however, the ultimate fate of the monoterpenols in this instance appears to be release as such from the flowers, rather than catabolism as observed in the present case. In studies very closely related to the present work, Sakata and associates (32, 34) have carried out detailed analysis of the monoterpene content of *Mentha arvensis*, a species which also produces *l*-menthone as a major metabolite. Unlike the situation with *M. piperita*, the menthone produced by *M. arvensis* appears to be converted almost exclusively to *l*-menthol, and *l*-menthyl-β-D-glucoside has been demonstrated in both the leaves and rhizomes of this species. Most recently, these workers (33) have examined seasonal variations in menthyl glucoside, menthol, and menthone content in developing *M. arvensis*, and presented evidence strongly implicating synthesis and turnover of menthyl glucoside during the flowering stage when the rhizome is rapidly developing. Evidence also suggested that the monoterpenyl glucosides played but a minor role in monoterpene metabolism in *M. arvensis* flowers. Although the metabolism of the glucoside *per se* was not examined in *M. arvensis* (only changes in the levels present), the analogy to *M. piperita* is obvious, and an identical scheme for the metabolism of the epimeric glucoside and alcohol to menthone and menthone lactone can be envisioned. In a related study on the metabolism of *d*-camphor in

sage (*S. officinalis*) leaves, a very similar keto-lactonization step has been demonstrated (12). Interestingly, the lactonization of camphor to 1,2-campholide precedes conjugation of the corresponding hydroxy acid to the glucoside-glucose ester in sage leaves; the bis-glucose conjugate presumably being the transport derivative in this system.

The lactonization of cyclic monoterpene ketones is an unusual biological reaction having little precedent in higher plants or animals. The reaction type is well known, however, in the microbial metabolism of saturated monoterpene ketones such as camphor and fenchone (7–9, 40), accomplishing the crucial ring opening step as a prelude to further degradation of the terpene skeleton by such organisms which can grow on monoterpenes as the sole carbon source. By way of further analogy to microbial metabolism, the lactonization of menthone in the present case not only accomplishes ring-opening of the *p*-menthane nucleus, but affords conversion to the acyclic 3,7-dimethyloctane skeleton which could be subsequently degraded to acetyl CoA by established biological means involving a well known microbial variant of the β-oxidation pathway (6, 36, 37). The degradation of *d*-camphor in *Salvia* (presumably via 1,2-campholide [12]) also has direct microbial precedent (8, 9, 40).

On the basis of present evidence from *Mentha* and *Salvia*, and by analogy to microbial systems, it is tempting to speculate that monoterpenes transported from the leaf play a role in carbon and/or energy metabolism in the rhizome/root system, and that the phenomenon may be widespread in essential oil plants of the Lamiacae family. However, the ultimate fate of monoterpenes in the rhizome is still conjectural and, until more information is available, the function of the process will remain uncertain. Studies on the subsequent steps in the metabolism of 3,4-menthone lactone, and on the nature of the methanol-soluble and water-soluble products derived from the lactone and related metabolites, are now underway.

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