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Metabolism of Monoterpenes

DEMONSTRATION OF (+)-NEOMENTHYL-β-D-GLUCOSIDE AS A MAJOR METABOLITE OF (−)-MENTHONE IN PEPPERMINT (*MENTHA PIPERITA*)[1]

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ABSTRACT

(−)-Menthone, the major monoterpene component of the essential oil of maturing peppermint (*Mentha piperita* L.) leaves (6 micromoles per leaf) is rapidly metabolized at the onset of flowering with a concomitant rise in the level of (−)-menthol (to about 2 micromoles per leaf). Exogenous (−)-[G-^{3}H]menthone is converted into (−)-[^{3}H]menthol as the major steam-volatile product in leaf discs in flowering peppermint (10% of incorporated tracer); however, the major portion of the incorporated tracer (86%) resided in the nonvolatile metabolites of (−)-[G-^{3}H]menthone. Acid hydrolysis of the nonvolatile material released over half of the radioactivity to the steam-volatile fraction, and the major component of this fraction was identified as (+)-neomenthol by radiochromatographic analysis and by synthesis of crystalline derivatives, thus suggesting the presence of a neomenthyl glycoside. Thin layer chromatography, ion exchange chromatography, and gel permeation chromatography on Bio-Gel P-2 allowed the purification of the putative neomenthyl glycoside, and these results suggested that the glycoside contained a single, neutral sugar residue. Hydrolysis of the purified glycoside, followed by reduction of the resulting sugar moiety with NaB^3H_4, generated a single labeled product that was subsequently identified as glucitol by radio gas-liquid chromatography of both the hexatrimethylsilyl ether and hexaacetate derivative, and by crystallization to constant specific radioactivity of both the alditol and the corresponding hexabenzoate. These results, along with studies on the hydrolysis of the glycoside by specific glycosidases, strongly suggest that (+)-neomenthyl-β-D-glucoside is a major metabolite of (−)-menthone in flowering peppermint. This is the first report on the occurrence of a neomenthyl glycoside, and the first evidence implicating glycosylation as an early step in monoterpene catabolism.

While monoterpenes were once regarded as metabolically inert "secondary products" (17), considerable evidence, based on both short term tracer studies and long term periodic analyses, now indicates that monoterpenes are, in fact, metabolically active and subject to rapid turnover in plants (15). The physiological rationale for such catabolism is not presently known, although it has been suggested that monoterpenes may be utilized as a carbon or energy source after other stored substrates are depleted (7). Plants are capable of converting exogenous ^{14}C-labeled monoterpenes into primary metabolites, such as amino acids and sugars (2), supporting such a possibility; however, little is known about the pathways or mechanisms of such catabolism.

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Evidence for monoterpene turnover in peppermint (*Mentha piperita* L.) has been obtained by periodic analysis of plants grown under controlled conditions (7). In these studies, midstem leaves accumulated high levels of (−)-menthone until the time of floral initiation, and then lost much of the menthone. Previous evidence has indicated that (−)-menthone can be converted to (−)-menthol (4, 5, 18) and (−)-menthyl acetate (9) in mint and, during the period of menthone catabolism, a portion of the menthone appeared to be converted to these metabolites. However, the quantity of menthol and menthyl acetate formed was not sufficient to account for the amount of menthone lost, and no other metabolites of menthone have been identified. Here we describe, for the first time, the identification of (+)-neomenthyl-β-D-glucoside as a major metabolite of (−)-menthone in flowering peppermint.

MATERIALS AND METHODS

Plants, Substrates and Reagents. Peppermint (*M. piperita* L. cv. Black Mitcham) plants were grown from stolons in sand-Perlite-peat moss (1:1:1, v/v) in a growth chamber maintained at 30 C day temperature and 15 C night temperature during a regular 24-h cycle with 16-h days under 15,000-lux light intensity. Plants were fertilized with Osmocote (14:14:14, N:P:K) controlled release fertilizer. By the end of 6 weeks most plants bore flowers, and, unless otherwise specified, leaves from the midstem (leaf pairs number 7–10 from the bottom) of 5- to 7-week-old plants were used for experiments with leaf discs.

NaB^3H_4 (333 Ci/mol) was purchased from Amersham/Searle. (−)-[G-^{3}H]Menthone (122 Ci/mol) was prepared by two-phase CrO_3 oxidation (6) of (−)-[G-^{3}H]menthol obtained as described previously (9). The chromic acid reagent (50 μl) was added to an excess of (−)-[G-^{3}H]menthol dissolved in 250 μl of diethyl ether and the mixture was shaken vigorously for 30 min. Isopropyl alcohol was added to clear the solution, and (−)-[G-^{3}H]menthone was isolated directly from the reaction products by TLC (Silica Gel G, with hexane-ethyl acetate [4:1, v/v] as developing solvent). Negligible amounts of (+)-isomenthone were formed if the oxidation was not allowed to go to completion (*i.e.* excess menthol), and the purity of the isolated (−)-[G-^{3}H]menthone (99%) was verified by radio GLC. For use as a substrate, the menthone was dispersed in water with the aid of Tween-20 and sonication.

Glucitol and other carbohydrate standards were gifts from Drs. F. Loewus and P. E. Kolattukudy of this department. (−)-Menthone, (−)-menthol, (+)-neomenthol, and other monoterpene standards (all 99+%) were provided through the generosity of Dr. K. Bauer of Haarmann and Reimer GmbH, Holzminden, West Germany. (−)-Menthyl-β-D-glucoside and (+)-neomenthyl-β-D-glucoside were prepared by coupling the monoterpenols to acetobromo-α-D-glucose (Sigma Chemical Co.) in anhydrous ether in the presence of Ag_2O (16). The monoterpenyl-tetra-*O*-acetyl-β-D-glucoside intermediates were treated with sodium methoxide,

and the resulting monoterpenyl glucosides were purified by TLC (Silica Gel G with ethanol-ethyl acetate [1:6, v/v] as developing solvent, R_F = 0.42 to 0.45). *p*-Mentha-1,2,3,4-tetraol was prepared by treating α-terpinene with OsO_4 in diethyl ether-pyridine (9:1, v/v). The solvent was removed under vacuum, and the osmate esters dissolved in methanol and decomposed with saturated aqueous Na_2SO_3. The tetraol was isolated from the reaction mixture by TLC (Silica Gel G with ethanol-ethyl acetate [3:7, v/v] as solvent, R_F = 0.28). α-Glucosidase (yeast, type 1), β-glucosidase (almonds), α-galactosidase (*Aspergillus niger*), β-galactosidase (*A. niger*, grade V), and β-glucuronidase (*Escherichia coli*, type VII) were obtained from Sigma Chemical Co., as were other biochemicals and reagents, unless otherwise specified.

Periodic Analysis of Peppermint Leaf Oil. Twelve midstem leaves from the plants described above were harvested at weekly intervals and the oil was extracted from the pooled sample by grinding in a mortar with hexane in the presence of anhydrous Na_2SO_4. The hexane extract was transferred to a glass steam distillation apparatus, and the extract was concentrated to about 2 ml under a stream of N_2 at 0 C. Water (5 ml) was added to the distilling flask and distillation was carried out until 1.5 ml of water had passed over (calibration of the system with [^{3}H]menthol indicated that steam distillation of the oil was nearly quantitative). The aqueous phase of the distillate was saturated with NaCl, and after thorough mixing, the hexane layer was removed and an aliquot analyzed by GLC under conditions described previously (8). The chromatograph was calibrated externally with (−)-menthol, and the quantity of each component was determined by triangulation. Oil components were identified by comparison of retention times to authentic standards and by combined GLC-MS.

Experiments with Leaf Discs. Leaves from the midstem of flowering peppermint plants were cut into 8-mm-discs with a cork borer, and 100 discs were incubated with 1 ml of an aqueous solution containing 30 μCi of (−)-[G-^{3}H]menthone and 5 μg of Tween-20 for 7 h at 30 C in an Erlenmeyer flask. At the end of incubation, the discs were transferred to a filter and washed thoroughly with water, and the oil was extracted from fifty discs as described above. Standards (25 mg each of (−)-piperitone, (+)-pulegone, (−)-menthone, (+)-neomenthol, and (−)-menthol) were added to the hexane extract, which was concentrated and steam-distilled as outlined above. Aliquots of the distillate were taken for determination of total radioactivity and for radio GLC.

The remaining discs were transferred to a TenBroeck homogenizer with 10 ml of 0.1 M $(NH_4)_2CO_3$, and heated on a steam bath for several minutes to inactivate enzymes. The discs were then homogenized and the homogenate was centrifuged at 27,000*g* for 20 min. The resulting pellet, which contained a negligible amount of tritium, was discarded, and the radioactive supernatant was first extracted with portions of hexane (3 × 5 ml) and then lyophilized. The lyophilized residue was taken up in about 1 ml of 0.1 M $(NH_4)_2CO_3$ and filtered (dilute $(NH_4)_2CO_3$ was used as solvent in these early steps in order to minimize interference by phenolic materials in the subsequent TLC purification). All of the radioactivity was contained in the filtrate, and the residual solid was discarded. An aliquot of the filtrate was diluted into 5 ml of 3 N HCl, 1 ml of hexane containing the appropriate standards was added, and the mixture was steam-distilled as above. Volatile products were recovered in the hexane phase of the distillate as before, and an aliquot was taken for determination of total radioactivity and for radio GLC analysis. Control experiments identical to those above were run with discs that had been held on the steam bath for 10 min before incubation with substrate.

Purification of Neomenthyl Glycoside. The remaining filtrate from the water-soluble products described above was streaked on a Silica Gel G plate that was developed with ethyl acetate-ethanol-concentrated NH_4OH (7:3:1, v/v/v). Radioactive components were located as described below, eluted from the gel with methanol, and an aliquot of each was subjected to acid hydrolysis and steam distillation as before. The appropriate product was further purified by TLC on Silica Gel G with ethyl acetate-ethanol (6:1, v/v) as developing solvent. Radioactive products were located and eluted as before, and the eluant was concentrated to dryness under vacuum. The residue was dissolved in a minimum quantity of ethanol-0.05 M NH_4OH (2:3, v/v) and applied to a column (1.5 × 10 cm) of Dowex 1-anion exchange resin (Bio-Rad AG 1-X8 [formate]) which was eluted with 2 bed volumes of ethanol-water (2:3, v/v) (the ethanol-containing solvent was required to eliminate hydrophobic interaction with the polystyrene gel matrix). The eluant contained over 95% of the tritium applied to the column. This material was concentrated under vacuum and lyophilized from water to remove any traces of NH_3. The residue was next dissolved in a minimum quantity of ethanol-0.05 M HCl (2:3, v/v) and applied to a column (1.5 × 11 cm) of Dowex 1-anion exchange resin (Bio-Rad AG 1-X8 [formate]) which was eluted with 2 bed volumes of ethanol-water (2:3, v/v) (the ethanol-containing solvent was required to eliminate hydrophobic interaction with the polystyrene gel matrix). The eluant contained over 95% of the tritium applied to the column. This material was concentrated under vacuum and lyophilized from water to remove any traces of NH_3. The residue was next dissolved in a minimum quantity of ethanol-0.05 M HCl (2:3, v/v) and applied to a column (1.5 × 11 cm) of Dowex 50W-cation exchange resin (Bio-Rad AG 50W-X8 [H^+]) that was eluted with 2 bed volumes of ethanol-water (2:3, v/v). The eluant contained essentially all of the tritium applied to the column. This material was concentrated to dryness and lyophilized as before. The residue was dissolved in 1 ml of 0.01 M acetate buffer (pH 6.0) and applied to a previously calibrated column (1.3 × 80 cm) of Bio-Gel P-2 (Bio-Rad P-2, -400) that was equilibrated and eluted with the same buffer. Aliquots of the 1-ml column fractions were taken for determination of tritium content, and to locate carbohydrate with the phenol-H_2SO_4 reagent (11).

Analysis of Sugar Residue of Glycoside. A portion of the purified glycoside obtained from Bio-Gel P-2 chromatography was added to 2 ml of 1 N HCl and steam distilled until all of the labeled aglycone passed over into the distillate. The material remaining in the still pot was lyophilized repeatedly from water to remove any traces of HCl. The residue, dissolved in 0.5 ml water, was treated with 30 μmol of NaB^3H_4 (10 mCi) for 10 h at room temperature. The reaction mixture was acidified and lyophilized repeatedly to remove labile tritium, and the labeled residue was dissolved in water and desalted on a Bio-Rad AG 501-X8 mixed resin column (1.5 × 15 cm) (Dowex 50W-X8 [H^+] plus Dowex 1-X8 [OH^-]). The labeled material was quantitatively eluted with water. The eluant was lyophilized and the residue dissolved in a minimum quantity of 0.1 M NH_4OH and loaded on a Dowex 1-anion exchange column (1.5 × 11 cm) (Bio-Rad AG 1-X8 [formate]). The labeled material was quantitatively eluted with 1 bed volume of water and the eluant was lyophilized. The residue was dissolved in a minimum quantity of 0.1 M HCl and loaded on a Dowex 50W-cation exchange column (1.5 × 11 cm) (Bio-Rad AG 50W-X8 [H^+]). The labeled material was quantitatively eluted with 1 bed volume of water and the eluant was lyophilized. The labeled material from above was further purified by TLC on Silica Gel G with ethyl acetate-pyridine-water (5:5:2, v/v/v). The isolated product (R_F = 0.6) was eluted from the gel with water. The solvent was removed under vacuum and the concentrate applied to a cellulose plate which was developed with ethyl acetate-pyridine-water (1:1:1, v/v/v). The labeled product (R_F = 0.4) was eluted as before and the water removed under vacuum.

Chemical Conversions and Preparation of Derivatives. Trimethylsilyl ether derivatives of alditols were prepared by treatment with Tri-Sil (Pierce Chem. Co.). The reaction mixture was held at 120 C until the alditol dissolved (several hours in the case of galactitol). Excess reagent was evaporated off under a stream of N_2 and the products dissolved in benzene for GLC. Acetates of alditols were prepared by treating with an excess of a 2:1 mixture

of acetic anhydride-pyridine. The sealed reaction vessel was heated on a steam bath until the solid dissolved and then held at room temperature overnight. Ethyl acetate (1 ml) was added to the reaction mixture, and the organic layer was washed with several portions of 0.1 M HCl followed by aqueous saturated $NaHCO_3$ and water. The organic phase was dried and concentrated to small volume for GLC analysis. Benzoates of alditols were prepared by treating with an excess of benzoyl chloride in pyridine at room temperature for 48 h. The reaction mixture was diluted with ethyl acetate and, after backwashing with water, an excess of methanol was added and the mixture was refluxed briefly. Solvents were removed under vacuum, and the residue was taken up in hot methanol and filtered. The alditol hexabenzoate, which crystallized from the filtrate on standing, was recrystallized to constant specific activity from hot methanol. Alditols were also recrystallized directly from hot methanol. The tritium-labeled alditol was converted to the corresponding saccharic acid by treatment with 0.5 ml of HNO_3 (specific gravity = 1.42) at room temperature overnight. The reaction mixture was cooled in ice and transferred to a lyophilizer tube with 0.5 ml of H_2O and lyophilized. An aliquot of the water recovered was taken for determination of tritium content. Chromic acid oxidation of monoterpenols and the synthesis of monoterpenol phenylurethane derivatives were carried out by standard procedures (6, 10). The semicarbazone of [^{3}H]menthone was prepared by dissolving 0.5 g of semicarbazide-HCl and 0.75 g of Na-acetate in 8 ml of water, followed by the addition of the ketone (0.5 g) and sufficient ethanol to make one phase. The mixture was heated on a steam bath for 1 h and then held at 0 C overnight. The semicarbazone was recovered by filtration and crystallized to constant specific activity from hot ethanol. Hydrogenolysis of glycosides was carried out with $LiAlH_4$ in refluxing dioxane. Excess reagent was destroyed with water and the mixture was neutralized and steam-distilled to recover volatile products. Alkaline hydrolysis of glycosides was carried out by refluxing in aqueous 10% KOH. Volatile products were recovered by steam distillation of the neutralized hydrolysate as above.

Chromatography and Determination of Radioactivity. TLC was carried out on both activated Silica Gel G (EM laboratories) and MN-cellulose 300 (Macherey, Nagel, and Co.) plates (0.1-mm and 0.05-mm thickness, respectively). Radioactive components were located by scanning the plates with a Berthold TLC radio scanner, and a portion of the plates was also sprayed with 2,7-dichlorofluorescein (UV visualization) (9), or KIO_4-benzidine reagent (13). Phenol-H_2SO_4 reagent (11) was used for detection of carbohydrates in ion exchange or Bio-Gel P-2 column effluents. Radio GLC analyses were conducted with a Varian gas chromatograph attached to a Nuclear-Chicago model 7357 radioactivity monitor. The instrument was calibrated externally with [^{3}H]toluene or [^{14}C]toluene. Columns used were stainless steel (3 m × 3 mm o.d.) packed with 12% Carbowax 4000 on 80/100 mesh Gas-chrom Q, and stainless steel (2 m × 3 mm o.d.) packed with 20% SE-30 on 80/100 mesh Gas-chrom Q. Analytical conditions are described elsewhere.

Radioactivity in TLC fractions was determined either by eluting the material from the gel, or by scraping the gel directly into a counting vial followed by the addition of 1 ml of water and 15 ml of Scinta-Verse (Fisher Scientific). A Packard liquid scintillation spectrometer was utilized (efficiency for ^{3}H = 34%). Radiation in liquid samples was determined directly in Scinta-Verse (efficiency for ^{3}H = 45%). All assays were conducted with a standard deviation of less than 3%.

RESULTS

Periodic Analysis of Peppermint Leaves. The (−)-menthone content of midstem peppermint leaves has been shown to increase until the time of floral initiation, and to decrease rapidly thereafter with a concomitant rise in (−)-menthol content (7). While these trends appear to be general in peppermint, some variation in this behavior has been noted in response to growth conditions (7). In order to examine menthone metabolism in peppermint raised under the present experimental conditions, and to determine when this tissue would be most suitable for further biosynthetic experiments, periodic analysis of the volatile oil from midstem leaves was carried out (Fig. 1). Consistent with previous observation (7), menthone turned over rapidly at the onset of flowering. The decline in (−)-menthone content was accompanied by a significant increase in (−)-menthol content and lesser increases in (−)-menthyl acetate and (+)-neomenthol, all products that appear to be derived from (−)-menthone. On the basis of these studies, midstem leaves from 5- to 7-week-old flowering plants were judged to be the most active in menthone metabolism, and this tissue was used in subsequent experiments.

Incorporation of [^{3}H]Menthone into Leaf Discs and Analysis of Products. To examine the metabolism of menthone in greater detail, discs from midstem leaves of 6-week-old peppermint plants were incubated with 30 μCi of (−)-[G-^{3}H]menthone. The hexane-soluble lipids were isolated and subjected to steam distillation. Essentially all of the radioactivity obtained in the hexane extract (1.5% of applied tracer, 10% of incorporated tracer) was recovered in the steam distillate. Radio GLC of the distillate indicated the presence of some residual substrate and two other components coincident with neomenthol and menthol (major component), respectively (Fig. 2a). The epimeric alcohols were isolated by TLC (R_F menthol = 0.18, R_F neomenthol = 0.25) and their identities confirmed by synthesis of the corresponding phenylurethanes which were crystallized to constant specific activity (for menthol, 30 μCi/mol, m.p. = 111 − 112 C; for neomenthol, 9.1 μCi/mol, m.p. = 106 − 107 C; literature m.p. = 112 C and 107–108 C, respectively [3]). Thus, exogenous [G-^{3}H]menthone was reduced to menthol and neomenthol in leaf discs, and the ratio of the labeled alcohols formed (menthol to neomenthol = ~15:1) was similar to the epimer ratio observed on analysis of the volatile oil from comparable tissue (Fig. 1).

Examination of the water-soluble products derived from [^{3}H]-menthone (after lyophilization to remove volatile substances)

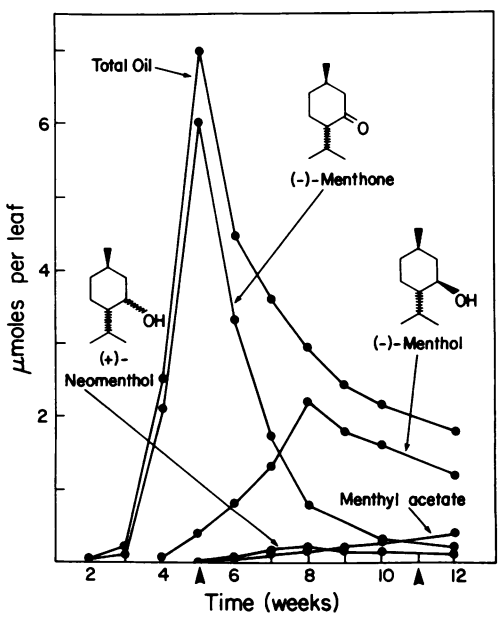

FIG. 1. Volatile oil composition of midstem peppermint leaves as a function of growth. Oil was extracted and steam-distilled as described under "Materials and Methods" and was analyzed by GLC under conditions described previously (8). Results are averages of analyses run in triplicate. Menthofuran and pulegone are not plotted, although low levels of these compounds were detected from 3 to 6 weeks. The first arrow indicates the approximate time of floral initiation, and the second arrow indicates the approximate time of full bloom.

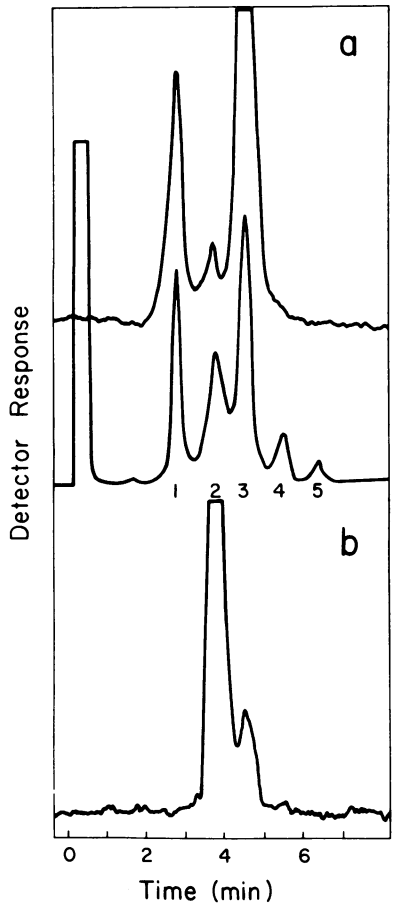

FIG. 2. (a) Radio gas-liquid chromatogram of the hexane-soluble steam-volatile products obtained from peppermint leaf discs that had been incubated with (−)-[G-^{3}H]menthone (30 μCi) for 7 h. Upper tracing is response of radioactivity monitor attached to gas-liquid chromatograph; smooth middle tracing is flame ionization detector response obtained from internal standards of (−)-menthone [1]; (+)-neomenthol [2]; (−)-menthol [3]; (+)-pulegone [4]; and (−)-piperitone [5]. Chromatographic column (Carbowax 4000, described under "Materials and Methods") was held at 165 C with an argon flow rate of 160 cm^3/min. (b) Radio gas-liquid chromatogram of the steam-distilled acid hydrolysate of the water-soluble products obtained from peppermint leaf discs that had been incubated with [G-^{3}H]menthone as described above. Chromatographic conditions and internal standards were identical to those described in (a) above.

indicated that approximately 15% of the applied menthone (~85% of incorporated tracer) had been converted into this fraction. Examination of both the solid residue remaining after extraction of water-soluble material, and the water removed on lyophilization, revealed only low levels of radioactivity in these fractions (<5% of incorporated tracer). Thus, the bulk of the tracer derived from exogenous [^{3}H]menthone in leaf discs resided in the water-soluble fraction. When boiled leaf discs were incubated with [^{3}H]menthone in the control experiment, neither the water-soluble fraction nor the hexane-soluble volatile fraction acquired significant label (less than 0.1% and 1%, respectively, of applied tracer).

As monoterpenyl glycosides have been found in several essential oil-bearing plants (1, 12, 14, 19–21), it was appropriate to determine if such glycosides might be present in the labeled water-soluble fraction derived from [^{3}H]menthone. An aliquot of the water-soluble material was therefore hydrolyzed with HCl and the hydrolysate was steam-distilled. Approximately 75% of the original water-soluble nonvolatile radioactivity was recovered in the steam distillate. Radio GLC of this volatile material revealed the presence of one major radioactive component coincident with neomenthol, and a minor component coincident with menthol (Fig. 2b). Calibration of the radio gas-liquid chromatograph indicated that over 80% of the injected radioactivity was contained in these constituents, the remaining activity did not elute from the chromatograph under these experimental conditions. Unlike acid hydrolysis, hydrogenolysis ($LiAlH_4$ in dioxane) or alkaline hydrolysis (10% aqueous KOH) of the water-soluble products, followed by steam distillation, produced little volatile material, and radio GLC confirmed that negligible quantities of neomenthol or menthol were released by these treatments. The apparent specific release of neomenthol and lesser quantities of menthol by acid hydrolysis, but not by hydrogenolysis or alkaline hydrolysis, suggested the presence of glycosides in the water-soluble products.

Identification of Aglycones. TLC of the volatile products released by acid hydrolysis allowed the separation of neomenthol and menthol (in a ratio of about 15:1). A portion of the neomenthol was oxidized with CrO_3 (6). Radio TLC and radio GLC of the products indicated the presence of a single radioactive product coincident with authentic (−)-menthone, and the (−)-menthone was converted to the semicarbazone which was crystallized to constant specific radioactivity (33.6 μCi/mol, m.p. = 192–193 C; literature m.p. = 193 C [3]). A portion of the menthol was also oxidized to (−)-menthone and the semicarbazone was also crystallized to constant specific activity (6.4 μCi/mol, m.p. = 192–193 C). The major aglycone was therefore tentatively identified as (+)-neomenthol, and the minor aglycone as (−)-menthol.

Although acid hydrolysis of a *p*-menthanyl glycoside should proceed with fission of the glycosyl-oxygen bond with retention of configuration of the resulting *p*-menthanol, the identification of neomenthol as the major aglycone prompted us to investigate the possibility that this compound was generated by inversion of the aglycone upon acid hydrolysis of a menthyl glycoside. An authentic sample of (−)-menthyl-β-D-glucoside was therefore subjected to the acid hydrolysis and steam distillation procedure, and the volatile products were analyzed by GLC. Only menthol was found. Similarly, (+)-neomenthyl-β-D-glucoside yielded only neomenthol on acid hydrolysis and steam distillation. Even under more drastic hydrolysis conditions (6 N HCl, 100 C sealed tube), the menthyl glycoside yielded only menthol. In the case of the neomenthyl glycoside, however, the more vigorous hydrolysis conditions led to the formation of considerable elimination products (60% of the steam volatile fraction), as might be expected from the more favorable transdiaxial orientation of the C3 hydroxyl and the C2 and C4 hydrogens of this epimer. When the labeled water-soluble products were subjected to the more drastic hydrolysis procedures and the volatile fraction analyzed by radio GLC, a very similar pattern of elimination products was observed, thus supporting the identification of the major aglycone as neomenthol.

From the combined analysis of the hexane-soluble and water-soluble products we concluded that exogenous (−)-[G-^{3}H]menthone is reduced to both (−)-menthol (~2% of the applied tracer) and (+)-neomenthol (~9% of the applied tracer) in peppermint leaf discs, and that, while most of the menthol is found in the volatile oil, most of the neomenthol occurs as nonvolatile glycoside.

Purification of Neomenthyl Glycoside. To examine the putative neomenthyl glycoside in greater detail, the water-soluble biosynthetic products were fractionated by TLC (Silica Gel G, with ethyl acetate-ethanol-concentrated NH_4OH [7:3:1, v/v/v] as developing solvent) into two major radioactive components (Fig. 3). Each labeled component was eluted from the gel, and an aliquot was subjected to acid hydrolysis followed by steam distillation and radio GLC analysis of the distillate. The component with the highest R_F value (0.48) was almost quantitatively converted to a mixture of neomenthol (94%) and menthol (6%) by such treatment, suggesting that this product was the glycoside (representing ~9% incorporation of the precursor). Furthermore, this product was chromatographically identical to authentic (+)-neomenthyl-β-D-

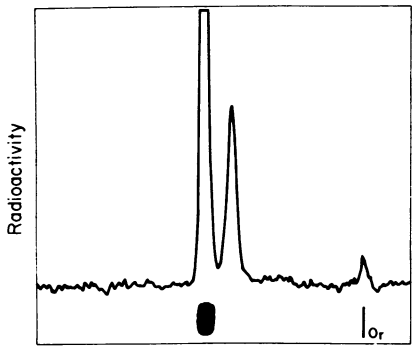

FIG. 3. Radio thin layer chromatogram of water-soluble, nonvolatile products isolated from peppermint leaf discs that had been incubated with (−)-[G-^{3}H]menthone. TLC was done on Silica Gel G with ethyl acetate-ethanol-concentrated NH_4OH (7:3:1, v/v/v) as developing solvent. Standard indicated is (+)-neomenthyl-β-D-glucoside. Or is the origin.

glucoside (Fig. 3), and it gave a weak, but positive, reaction with KIO_4-benzidine spray (13), suggesting the presence of carbohydrate.

The more polar component isolated by TLC (Fig. 3) gave rise to little volatile material on acid hydrolysis and steam distillation (25% of the radioactivity), and negligible amounts of neomenthol and menthol were detected on radio GLC analysis of this steam distillate. Furthermore, neither $LiAlH_4$ treatment nor alkaline hydrolysis released significant volatile radioactivity from this material. Although this product has not yet been identified, preliminary evidence (Croteau and Martinkus, unpublished) suggests that it is not a glycoside, but rather a highly oxygenated metabolite of neomenthol, menthol, or menthone (*i.e.* it is more polar than *p*-menthan-1,2,3,4-tetraol on TLC and elutes between monosaccharide and disaccharide on Bio-Gel P-2 column chromatography). Thus, preliminary TLC fractionation of the water-soluble products derived from [G-^{3}H]menthone indicated the presence of two labeled components and confirmed that the major radioactive product was the neomenthyl glycoside.

Further purification of the glycoside by TLC (Silica Gel G with ethyl acetate-ethanol [6:1, v/v]) provided a single, KIO_4-benzidine positive, radioactive component that was coincident with authentic (+)-neomenthyl-β-D-glucoside (R_F = 0.45). Acid hydrolysis of an aliquot of this material, followed by steam distillation, provided quantitative yield of the neomenthol-menthol mixture (15:1). The tritium-labeled glycoside was not retained by either Dowex-1 (formate) anion exchange resin or Dowex-50W (hydrogen) cation exchange resin, and radioactivity was quantitatively eluted with authentic neomenthyl glucoside with 1 bed volume of ethanol-water (2:3, v/v) as the eluant. Thus, we concluded that the glycoside contained only neutral sugar(s). The labeled glycoside obtained from the ion exchange step was dissolved in a minimum amount of 0.01 M acetate buffer (pH 6.0) and loaded on a Bio-Gel P-2 column that was equilibrated and eluted with the same buffer. Virtually all of the radioactivity eluted as a single component coincident with an authentic standard of (+)-neomenthyl-β-D-glucoside (Fig. 4). The elution of the glycoside after monosaccharide probably results from the hydrophobic interaction of the aglycone with the polyacrylamide gel matrix, and this property would appear to be characteristic of a *p*-menthanyl glycoside containing a single sugar moiety. From the results of TLC, ion exchange chromatography and gel permeation chromatography, as well as radio GLC of the volatile products obtained on acid hydrolysis, we concluded that the glycoside contained mainly neomenthol as the aglycone (~95%) and that the glycoside contained only one neutral sugar residue. Approximately 5% of the purified glycoside fraction contained menthol as the aglycone.

Identification of Sugar Moiety. To examine the sugar moiety of the glycoside, the purified glycoside (after gel filtration) was first hydrolyzed and the volatile aglycone was removed by steam distillation. The aqueous material remaining was lyophilized, and the residue dissolved in water and treated with NaB^3H_4. Labile tritium was removed, and the radioactive product (3.6×10^7 dpm) was desalted on a mixed resin column. TLC of the labeled product on both Silica Gel G and cellulose revealed a single radioactive component coincident with authentic alditol standards (Fig. 5a)

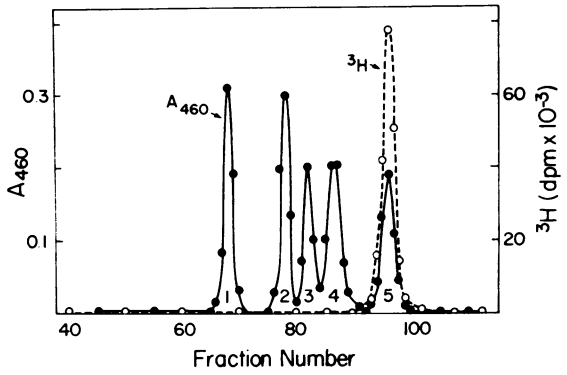

FIG. 4. Bio-Gel P-2 gel filtration of the [^{3}H]neomenthyl glycoside obtained from peppermint. Column effluent was monitored for carbohydrate standards with the phenol-H_2SO_4 reagent (*A* at 460 nm) and for the labeled glycoside (^{3}H dpm). Standards indicated are stachyose [1]; maltotriose [2]; maltose [3]; glucose [4]; and (+)-neomenthyl-β-D-glucoside [5]. V_0 is at fraction 40 (40 ml).

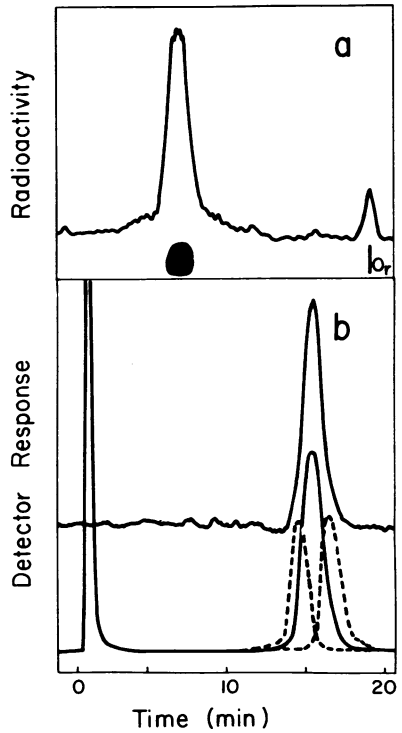

FIG. 5. (a) Radio thin layer chromatogram of the NaB^3H_4-treated acid hydrolysate (nonvolatile fraction) of the neomenthyl glycoside obtained from mint. TLC was done on Silica Gel G with ethyl acetate-pyridine-water (5:5:2, v/v/v) as developing solvent. Standard indicated is glucitol. Or is the origin. (b) Radio gas-liquid chromatogram of the trimethylsilyl ether derivative of the labeled product described in (a) above. Upper tracing is response of radioactivity monitor attached to the gas-liquid chromatograph. Smooth lower tracing (——) is flame ionization detector response obtained from a coinjected authentic standard of the trimethylsilyl ether derivative of glucitol. (– – –): Positions of authentic trimethylsilyl ether derivatives of mannitol (preceding glucitol) and galactitol (following glucitol). Chromatographic column (SE-30, described under "Materials and Methods") was held at 180 C with an argon flow rate of 150 cm^3/min.

(glucitol, galactitol, and mannitol were not readily resolved under these conditions). If the alditol was derived by NaB^3H_4 reduction of an aldose, then the ^3H should be specifically located at C1. A portion of the [^{3}H]alditol was therefore oxidized to the corresponding saccharic acid with HNO_3, and the reaction products were lyophilized. The bulk of the ^3H was recovered as ^3H_2O, indicating the presence of a [1-^{3}H]alditol. Radio GLC of the trimethylsilyl ether derivative of the labeled alditol showed that all of the radioactivity was coincident with the coinjected trimethylsilyl ether derivative of authentic glucitol (Fig. 5b). Radio GLC of the acetate-derivative of the labeled product provided similar results: a single radioactive component coincident with authentic glucitol hexaacetate. Portions of the labeled alditol were next diluted with mannitol, galactitol or glucitol, and each was crystallized from hot methanol. The mannitol and galactitol contained negligible radioactivity, whereas the glucitol was radioactive, and was brought to a constant specific radioactivity of 210 μCi/mol by the second crystallization. Treatment of the glucitol with benzoyl chloride yielded a hexabenzoate which was also crystallized to constant specific radioactivity (206 μCi/mol). On the basis of these results we concluded that the labeled neutral carbohydrate was [1-^{3}H]-glucitol, which must have been derived by NaB^3H_4 reduction of glucose obtained by hydrolysis of a glucoside.

To examine the nature of the glycosidic linkage, aliquots of the purified glucoside (after P-2 chromatography) were incubated with various glycohydrolases, and the release of [^{3}H]neomenthol was monitored. Only β-glucosidase readily catalyzed the hydrolysis of the glucoside (Table I). The slower but significant rate of hydrolysis observed with β-galactosidase was shown to result from contaminating β-glucosidase activity of this preparation (measured with *p*-nitrophenyl-β-D-glucoside). It was therefore inferred that the unknown glycoside was (+)-neomenthyl-β-D-glucoside. To substantiate this conclusion, the labeled glycoside was diluted about 100-fold with authentic (+)-neomenthyl-β-D-glucoside and the mixture was incubated with β-glucosidase. The rate of hydrolysis was identical when measured by the release of neomenthol (GLC assay) or [^{3}H]neomenthol (Fig. 6), confirming the identity of the biosynthetic product as (+)-neomenthyl-β-D-glucoside.

Detection of the Glucoside in Intact Peppermint Leaves and Experiments with $^{14}CO_2$. If (+)-neomenthyl-β-D-glucoside is a significant metabolite of (−)-menthone *in vivo*, then this glycoside would be expected to be present in leaves from flowering peppermint plants. To test this possibility, the volatile oil and the water-soluble, nonvolatile materials were isolated from 25 g of midstem leaves of 6-week-old plants. GLC of the volatile oil revealed the approximate composition shown in Figure 1 (at the 6th week).

Table I. *Enzymic Hydrolysis of the (+)-Neomenthyl Glycoside Obtained from Peppermint*

Each reaction mixture, containing 0.25 unit of the hydrolase (based on *p*-nitrophenyl glycoside hydrolysis at the optimum pH), was incubated with 8.0×10^5 dpm of the labeled neomenthyl glycoside in a total volume of 1.0 ml of 0.05 M sodium phosphate or sodium acetate buffer for 40 min at 30 C. The labeled neomenthol released was isolated by ether extraction followed by TLC as described under "Materials and Methods."

Glycosidase	% Hydrolysis
β-Glucosidase (almonds, sodium phosphate pH 5.0)	78
α-Glucosidase (yeast, sodium phosphate pH 6.0)	3
β-Galactosidase (*A. niger*, sodium acetate pH 4.0)	15
α-Galactosidase (*A. niger*, sodium acetate pH 4.0)	<1
β-Glucuronidase (*E. coli*, sodium phosphate pH 7.0)	<1

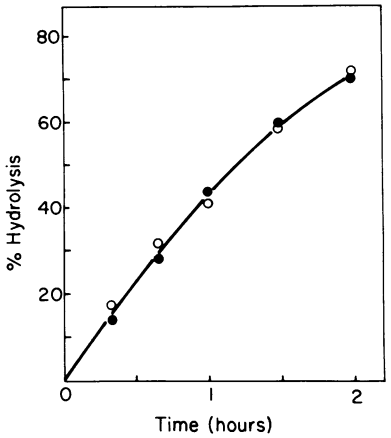

FIG. 6. Time course of enzymic hydrolysis of (+)-neomenthyl glucoside. Reaction mixtures, containing 5×10^5 dpm of the labeled glycoside and 10 μmol of authentic (+)-neomenthyl-β-D-glucoside, were incubated with 5 units of β-glucosidase (almonds) in a total volume of 1 ml of 0.05 M Na-phosphate (pH 5.0) at 30 C. At the times indicated, reaction mixtures were extracted with hexane (1 ml) and aliquots were taken for the determination of mass (neomenthol via GLC, ●——●) and radioactivity ([H^3]neomenthol via scintillation spectrometry, ○——○).

GLC of the volatile products obtained by acid hydrolysis of the lyophilized water-soluble material revealed the presence of neomenthol, but the quantity of neomenthol obtained was only about 20% of the amount of menthol observed in the volatile oil. Thus, the ratio of neomenthyl glucoside to menthol formed from exogenous (−)-[G-^{3}H]menthone in leaf discs was far greater than that observed in intact leaves. Several explanations for these apparently divergent observations are possible: (*a*) the present results may represent artifactual "induced metabolism" resulting from administration of exogenous [^{3}H]menthone to leaf discs; (*b*) menthol and neomenthyl glucoside may arise in different compartments, with the latter being more accessible to exogenous menthone; and (*c*) while the menthol may accumulate in leaves in relatively inactive pools, the glucoside may not accumulate in leaves, but rather may undergo further metabolism or be transported elsewhere in the plant (the latter possibility being precluded in a leaf disc experiment).

To examine these questions, midstem leaves (petiole plus leaf) were exposed to $^{14}CO_2$ continuously for 12 h in the light. Incorporation of $^{14}CO_2$ into the monoterpenoids of these mature peppermint leaves was low, confirming earlier observations (5). Yet, sufficient count levels were obtained in the volatile oil to allow radio GLC analysis, and it was shown that both menthone and menthol were labeled (in a ratio of about 6:1). Thus, under these experimental conditions both the appropriate precursor and the volatile product were being synthesized. In addition to low incorporation rates, the analysis of the volatile products obtained on acid hydrolysis of the water-soluble material was further complicated because of the indiscriminate labeling of many compounds with $^{14}CO_2$. To obtain reliable data, the [^{14}C]neomenthol generated on acid hydrolysis was purified by TLC and chemically oxidized to menthone which was analyzed as the crystalline semicarbazone. In spite of these experimental limitations, and the fact that only the 12-h incorporation period was examined, it was evident that neomenthyl glucoside (as measured by neomenthol release on acid hydrolysis) and menthol were labeled to roughly equivalent degrees. These results suggest that the sites of synthesis of menthol and neomenthyl glucoside may be differentially accessible to exogenous [^{3}H]menthone and to endogenous menthone generated from $^{14}CO_2$, and they argue strongly against the possibility that neomenthyl glucoside is an artifact.

To examine the possibility that neomenthyl glucoside may be further metabolized, a portion of the biosynthetically labeled

glucoside was administered to leaf discs. After 6 h, the products were analyzed, but only the starting material was detected (recovered in ~60% yield). Similarly, the unknown water-soluble product was isolated and administered to leaf discs, and analysis of products indicated only starting material (recovered in ~70% yield). These observations, along with the fact that little ^3H_2O was produced in any of the leaf disc experiments, indicate that extensive catabolism does not occur under these experimental conditions. To examine the possibility that the glucoside may be transported out of the leaves, (−)-[G-^{3}H]menthone was applied to the surface of the midstem leaves of an intact plant. After 8 h in the light, the leaves, central stem, roots, and flowering tip were analyzed as before. Because the volatile substrate readily evaporates from the leaf surface under these experimental conditions, the rate of incorporation was low. However, neomenthyl glucoside was readily demonstrated in the source leaves, and it could be detected (by [^{3}H]neomenthol release on acid hydrolysis) both in the mainstem below the source leaves and in the roots. Radioactivity was also detected in the mainstem above the source leaves and in the flowering tip, but the level of radioactivity was too low to permit further study.

DISCUSSION

From the results presented, (+)-neomenthyl glucoside would appear to be a major metabolite of (−)-menthone in flowering peppermint. While both acyclic and cyclohexanoid monoterpenyl glucosides (1, 12, 19, 21) and cyclohexanoid monoterpenyl galactosides (14, 20) have previously been reported in essential oil plants, this appears to be the first report of the occurrence of a neomenthyl glycoside. More importantly, the present study provides the first direct evidence for the involvement of a glycoside in monoterpene catabolism. The role and fate of neomenthyl glucoside are not presently known. However, if, during turnover, the monoterpenes are utilized at sites other than the epidermal oil glands, then a means of transporting these lipophilic materials would be required. Monoterpenyl glycosides may represent such a transport form, and tentative evidence obtained in this study supports such a possibility.

The present results further indicate that during menthone catabolism in flowering peppermint, this ketone is reduced to both neomenthol and menthol. While the relative quantities of these epimeric alcohols formed *in vivo* are difficult to estimate from the present work because of the experimental limitations noted, it seems likely that the quantities formed are roughly similar. Consistent with this suggestion is the recent finding that crude cell-free preparations from the leaves of flowering peppermint catalyze the NADPH-dependent reduction of (−)-[G-^{3}H]menthone to both (+)-neomenthol and (−)-menthol, with neomenthol formation slightly favored (Croteau and Martinkus, unpublished). While the enzymic reduction of (−)-menthone to significant quantities of both possible epimeric alcohols is in itself curious, the present *in vivo* results further indicate that the bulk of the menthol formed appears in the volatile oil (as such, or as the acetate ester) whereas the bulk of the neomenthol formed is converted to a glycoside. Although almost nothing is presently known about the enzymes involved in either menthone reduction or neomenthol glycosylation, two possible explanations for these unusual results seem evident. Two stereospecific dehydrogenases may be present, but compartmentalized. Thus, the dehydrogenase specific for neomenthol production could be accessible to a glycosylating system, while the dehydrogenase specific for menthol production is not. The present *in vivo* studies do suggest the presence of two compartments differentially accessible to exogenous [^{3}H]menthone. Also consistent with compartmentation is the observation that the transacetylase responsible for the synthesis of (−)-menthyl acetate readily utilizes (+)-neomenthol as a substrate *in vitro* (74% as efficient as (−)-menthol), but not, apparently, *in vivo* (9). Thus, only trace levels of neomenthyl acetate are present in mint oil compared to menthyl acetate, implying that the only epimer available to the transacetylase is menthol. As an alternate explanation to physical compartmentation of specific dehydrogenases with their accessory enzymes, menthone reduction to menthol and neomenthol may be carried out in the presence of a glycosylating system that is highly specific for neomenthol, and that operates at sufficient rates to prevent either the accumulation of neomenthol or the formation of neomenthyl acetate. Experiments to test these possibilities and to examine further the roles and fates of menthol and neomenthyl glucoside are now in progress.

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