

# Metabolism of Monoterpenes<sup>1</sup>

## EVIDENCE FOR THE FUNCTION OF MONOTERPENE CATABOLISM IN PEPPERMINT (*MENTHA PIPERITA*) RHIZOMES

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### ABSTRACT

*l*-Menthone of peppermint leaves is reduced to *d*-neomenthol which is glucosylated and transported to the rhizome, whereupon the  $\beta$ -D-glucoside is hydrolyzed, the aglycone oxidized back to *l*-menthone, and this ketone converted to *l*-3,4-menthone lactone. *l*-[G-<sup>3</sup>H]-3,4-Menthone lactone and its labeled progenitors, when incubated with excised mint rhizomes, gave rise to nonvolatile lipids as well as polar metabolites. The lipids thus generated consisted of labeled squalene and phytosterols in the nonsaponifiable fraction and C<sub>14</sub>-C<sub>26</sub> fatty acids in the saponifiable fraction. These results imply degradation of the terpenoid to acetyl-coenzyme A and reduced pyridine nucleotide, and reincorporation of label via these products. Starch and soluble carbohydrates were also found to be labeled; however, chemical degradation of the [<sup>3</sup>H]glucose obtained on hydrolysis of starch indicated the presence of tritium only on interior carbons, suggesting that labeling had occurred via reduced pyridine nucleotides. Analysis of the labeled organic acids revealed the presence of several hydroxy methylacyl intermediates suggesting the operation of a modified  $\beta$ -oxidation pathway in the degradation of the acyclic terpenoid skeleton. The results indicate that monoterpenes transported to the rhizome are oxidized to yield acetyl-coenzyme A and reduced pyridine nucleotides, and suggest that metabolic turnover of monoterpenes in mint represents a mechanism for recycling carbon and energy from foliar terpenes into other metabolites of the rhizome.

Monoterpenes accumulated in mature leaves of flowering peppermint plants (*Mentha piperita* L.) undergo metabolic turnover by a mechanism involving, as an early step, the reduction of the major monoterpene component, *l*-menthone<sup>2</sup>, to the epimeric alcohols *l*-menthol and *d*-neomenthol (7, 23). The latter alcohol is preferentially converted to the  $\beta$ -D-glucoside and transported to the rhizome (Fig. 1) (15). The kinetics of *d*-neomenthol- $\beta$ -D-glucoside synthesis and transport have been determined (16), and the location and properties of each enzyme of the metabolic sequence in leaves have been examined (13, 17, 21, 23).

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<sup>2</sup> Although the systematic name for *l*-menthone is (5*R*,2*S*)-*trans*-5-methyl-2-(1-methylethyl)cyclohexanone, we have utilized here the more common nomenclature based on numbering of the *p*-menthane system (*i.e.* menthone = *p*-menthan-3-one) in which the methyl-substituted carbon is 1*R* and the isopropyl-substituted carbon is 4*S*.

On reaching the rhizome, the glucoside is hydrolyzed and the aglycone oxidized back to menthone, which undergoes oxygenation to yield *l*-3,4-menthone lactone (*i.e.*, the biological equivalent of the Baeyer-Villiger reaction) (Fig. 1) (16). The lactone, in turn, is metabolized to several unidentified nonvolatile polar and nonpolar products. Each enzyme of the metabolic sequence from neomenthyl glucoside to menthone lactone has been demonstrated in cell-free preparations from mint rhizomes, and tentative evidence has also been obtained for the enzymic activation of the lactone to the corresponding hydroxy dimethylacyl CoA ester (16).

A lactonization reaction has also been demonstrated recently as a step in the metabolism of the monoterpene ketone camphor in sage (*Salvia officinalis*) (12), and the reaction type is well-known in microbial systems (18 and references therein). In fact, the lactonization of such monoterpenes as camphor and fenchone, by microorganisms which can grow on cyclic monoterpenes as the sole source of carbon, accomplishes the crucial ring opening step as a prelude to the catabolism of these compounds (9, 10). In the present instance, the oxygenation of menthone to the lactone accomplishes the conversion of the cyclic *p*-menthane skeleton to the acyclic 3,7-dimethyloctane skeleton. The subsequent degradation of this skeletal type, by a modification of the  $\beta$ -oxidation sequence involving carboxylation and subsequent removal of the  $\beta$ -methyl groups as acetic acid, also has considerable precedent in microbial systems (8, 28). An alternate route for the degradation of the acyl chain might involve an  $\alpha$ -oxidation followed by  $\beta$ -oxidation, a catabolic sequence known to occur in higher plants (29) and which would afford in this instance CO<sub>2</sub>, and the CoA esters of acetate, propionate, and isobutyrate as cleavage products. On the basis of the circumstantial evidence and the analogy to microbial systems, it is tempting to suggest that the observed conversion of the lactone (and its immediate precursors) to nonvolatile polar and nonpolar products in mint rhizome represents the oxidative degradation of the terpenoid to simple precursors, which are subsequently incorporated into other cellular constituents. Until the ultimate fate of the terpenes in mint rhizomes is known, however, the proposal remains speculation.

In this communication we provide evidence that the terpene is degraded to acetyl-CoA and reduced pyridine nucleotide, either of which may be subsequently utilized in the biosynthesis of other isoprenoid lipids as well as acyl lipids of the rhizome. We additionally provide tentative evidence for the catabolic pathway by which the terpenoid is degraded. The results presented here give a strong indication that metabolic turnover of monoterpenes represents a mechanism by which a carbon and energy supply of the leaves (as accumulated monoterpene) is recycled in the developing rhizome.

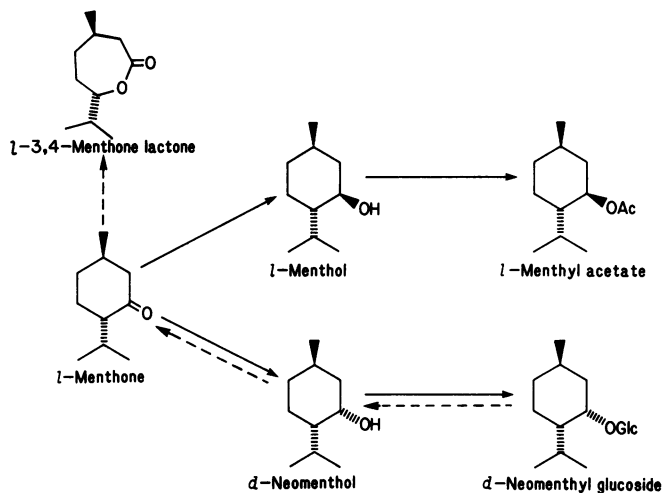


FIG. 1. Pathways of *l*-menthone metabolism in peppermint leaves (—) and of *d*-neomenthyl-β-D-glucoside metabolism in peppermint rhizomes (---).

## MATERIALS AND METHODS

**Plant Material, Substrates, and Reagents.** Peppermint (*Mentha piperita* L. cv Black Mitcham) plants were grown to maturity from stolons under controlled conditions described previously (13, 15). *d*-[G-<sup>3</sup>H]Neomenthol was prepared by <sup>3</sup>H<sub>2</sub> exposure (New England Nuclear), purified by TLC as described previously (16), and diluted to a specific activity of 20 Ci/mol with authentic material. *l*-[G-<sup>3</sup>H]Menthone (20 Ci/mol) was prepared from *d*-[G-<sup>3</sup>H]neomenthol as before (16) and purified by TLC. Chemical and radiochemical purity of the products (99+%) was verified by radio GLC. For use as substrates, the [<sup>3</sup>H]neomenthol and [<sup>3</sup>H]menthone were dispersed in water with the aid of Tween-20 (15 μg/μmol) and sonication. Sodium [2-<sup>14</sup>C]acetate (51 Ci/mol, radiochemical purity >98%) was obtained from ICN Pharmaceuticals, Inc.

Squalene, sitosterol, and stigmasterol were obtained from Sigma Chemical Co. 2-Hydroxy-3-methylbutyric acid and most other organic acids were from Aldrich Chemical Co. A mixture of geranic acid and nerolic acid was obtained by oxidation of citral with Tollens reagent (1). Butyl 6-hydroxy-3,7-dimethyloctanoate and butyl citronellate (as a minor side product) were obtained by treating *l*-3,4-menthone lactone (PCR Research Chemicals) with excess 14% BF<sub>3</sub> in butanol at 90°C for 2 h. The reaction mixture was decomposed with water and the esters were extracted with pentane and concentrated under vacuum to remove residual butanol. Both 14% BF<sub>3</sub> in butanol and 14% BF<sub>3</sub> in methanol were obtained from Applied Science Laboratories. Carbohydrate standards and fatty acid standards were gifts from F. A. Loewus and P. E. Kolattukudy, respectively, of this Institute. Pronase (*Streptomyces griseus*) was obtained from Calbiochem, and amyloglucosidase (*Rhizopus*), pectinase (*Aspergillus niger*), and cellulase (*Penicillium funiculosum*) were obtained from Sigma Chemical Co., as were most other biochemicals.

**In Vivo Experiments.** All *in vivo* experiments were carried out with excised rhizomes (2–3 g) from mature flowering plants. The tissue was washed free of soil with water and placed in a 25-ml Erlenmeyer flask with 1 ml of an aqueous solution of *d*-[G-<sup>3</sup>H]neomenthol (20 μCi, 1 μmol), *l*-[G-<sup>3</sup>H]menthone (20 μCi, 1 μmol) or Na-[2-<sup>14</sup>C]acetate (20 μCi, 0.4 μmol). The flask was closed with aluminum foil containing a sufficient number of pinholes to permit exchange with the atmosphere, and was maintained at room temperature under laboratory lighting for 24 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 unabsorbed substrate. Control experiments to evaluate the possible influence of microbial contamination were run with steam-inactivated tissue, and, as in previous experiments of this type, only unutilized substrate was recovered (16).

**Lipid Extraction and Analysis.** The rhizome tissue, following incubation and washing, was frozen in liquid N<sub>2</sub> and powdered with a mortar and pestle. The powder was transferred to a 15 ml Ten-Broeck homogenizer and extracted with CHCl<sub>3</sub>:methanol (2:1, 10 ml/g tissue) to remove total lipids, and the extract cleared by centrifugation at 10,000g for 10 min. An equal volume of water was added to the extract and, following shaking and separation of phases, the lower CHCl<sub>3</sub>-containing layer was removed and concentrated under vacuum at 50°C, a procedure which also served to remove residual substrate and other volatile products derived therefrom. The aqueous layer from above, and the solid remaining after lipid extraction, were retained as such, whereas the lipid extract was saponified (1 N KOH, 1 h, steam bath) and the nonsaponifiable lipids were extracted from the alkaline reaction mixture with ether. The ether extract was concentrated under a N<sub>2</sub> stream at 0°C before further analysis by TLC (silica gel G with hexane:ethyl acetate [4:1, v/v]) and radio-GLC. The aqueous reaction mixture was then acidified and the saponifiable lipids were removed by ether extraction. The extract was concentrated as before and the residual lipids were methylated with excess 14% BF<sub>3</sub> in methanol (90°C, 2 h). The excess BF<sub>3</sub> was decomposed in water, the methyl esters extracted with ether:hexane (1:1, v/v), and the extract was concentrated in preparation for TLC (silica gel G with hexane:ether [4:1, v/v]) and radio-GLC analysis.

**Extraction and Analysis of Water-Soluble Products.** Boiling water (20 ml/g tissue) was added to the solid residue remaining from the lipid extraction, followed by the addition of an equal tissue weight of insoluble polyvinyl-poly pyrrolidone. This mixture was thoroughly homogenized and then centrifuged at 10,000g to separate the water-soluble products from residual solid (which previous experiments had shown to contain little radioactivity [16]). This aqueous extract and the aqueous phase obtained from the above CHCl<sub>3</sub>-methanol extraction procedure were combined and lyophilized, and the residue dissolved in the minimum quantity of water and dialyzed (Spectrapor 3, mol wt cutoff of 3,500) against 50 mM Na-acetate buffer, pH 5. The resulting precipitate (nonradioactive) was removed by centrifugation (10,000g) and an aliquot of the radioactive supernatant was placed in a 10-ml Amicon ultrafiltration cell containing a YM2 membrane (mol wt cutoff of 1,000). The unit was pressurized and an aliquot of the filtrate taken for determination of <sup>3</sup>H content. Amyloglucosidase (120 units) was then added to the chamber and ultrafiltration and aliquot counting continued. The combined filtrate was lyophilized and the material dissolved in the minimum quantity of methanol:water (1:1, v/v). Carrier D-glucose (2 mg) was added, the sample separated by TLC (acetone:H<sub>2</sub>O [19:1, v/v]), and glucose was eluted from the plate with H<sub>2</sub>O. A portion of the glucose sample was counted directly, while another was converted to the trimethylsilyl ether derivative (Pierce Tri-Sil, 80°C, 2 h, with concentration under N<sub>2</sub> and solution in benzene) in preparation for radio-GLC. The glucose in another aliquot was oxidized to the saccharic acid with 12 N HNO<sub>3</sub>, and the H<sub>2</sub>O recovered from the lyophilized reaction mixture was examined for tritium content (15).

In addition to amyloglucosidase hydrolysis, aliquots of the above described water-soluble products were examined by ultrafiltration and treatment with cellulase (20 units in 50 mM Na-acetate, pH 5), pectinase (32 units in 50 mM Na-acetate, pH 5) and pronase (50 units in 50 mM Na-phosphate, pH 7.5).

**Extraction and Analysis of Organic Acids.** The rhizome tissue, following incubation and washing, was frozen in liquid N<sub>2</sub> and

powdered as before. The powder was transferred to a Ten-Broeck homogenizer and homogenized with 0.1 N KOH (10 ml/g tissue), a procedure which, in addition to extracting organic acids, also hydrolyzed any CoA esters present. Solid material was removed by centrifugation, and the alkaline solution was repeatedly extracted with ether to remove neutral species. The solution was then acidified with 5 N HCl, saturated with NaCl, and then extracted with ether to remove the organic acids. The latter extract was dried over  $\text{Na}_2\text{SO}_4$  and concentrated to a small volume under  $\text{N}_2$ , and the products contained therein were converted to the corresponding butyl esters by treatment with excess 14%  $\text{BF}_3$  in butanol (90°C, 2 h). The excess  $\text{BF}_3$  was decomposed in water, the butyl esters were extracted with pentane:ether (1:1, v/v) and this extract was repeatedly washed with water to remove butanol in preparation for subsequent TLC (silica gel G with hexane:ether [4:1, v/v]) and radio-GLC analysis.

**Chromatography and Determination of Radioactivity.** TLC was done on 1.0 mm layers of silica gel G activated at 110°C for 3 h. Developing solvents are indicated elsewhere in the text. 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, methanol, or  $\text{H}_2\text{O}$ .

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  $^3\text{H}$ toluene). Chromatography columns were 4 ft  $\times$  0.125 inch o.d. stainless steel containing 5% OV-101 on 80/100 mesh Chromosorb P-AW-DCMS and 11 ft  $\times$  0.25 inch o.d. glass containing 5% OV-17 on 80/100 mesh Chromosorb W-HP. Chromatographic conditions are described under the appropriate figures. Analytical chromatography and GLC-MS (at 70 eV) 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% Omnifluor (New England Nuclear) dissolved in 30% ethanol in toluene. Aqueous samples were counted in 15-ml ScintiVerse (Fisher Scientific Co.). Samples were quench corrected by internal standardization ( $^3\text{H}$ toluene) and counted to <1% probable error.

## RESULTS

**Conversion of  $d$ -[G- $^3\text{H}$ ]Neomenthol to Acyl and Isoprenoid Lipids of Mint Rhizomes.** Previous studies (16) had indicated that excised rhizomes incorporated tritium-labeled neomenthol, menthone, and menthone lactone into ether-soluble products, about two-thirds of which were steam-volatile and comprised the residual substrate and its immediate derivatives, and about one-third of which were nonvolatile lipids. To examine in greater detail the nature of the nonvolatile lipids, excised rhizomes were incubated with  $d$ -[G- $^3\text{H}$ ]neomenthol (20  $\mu\text{Ci}$ , 1  $\mu\text{mol}$ ) and the total lipids (11  $\mu\text{Ci}$ ) extracted with  $\text{CHCl}_3$ :methanol (2:1, v/v). Concentration of the extract under vacuum at 50°C removed the volatile lipids (comprised primarily of neomenthol, 3,4-menthone lactone, and menthone and related ketones) and yielded a nonvolatile fraction (3.2  $\mu\text{Ci}$ ) which was saponified. The nonsaponifiable material (2.7  $\mu\text{Ci}$ ) afforded on TLC analysis (silica gel G with hexane:ethyl acetate [4:1, v/v]) two regions of radioactivity, one at  $R_f \sim 0.7$  corresponding to hydrocarbons, and another at  $R_f \sim 0.15$  corresponding to phytosterols. Appropriate carrier standards were added to the nonsaponifiable fraction which was subjected to radio-GLC analysis (Fig. 2) and shown to be comprised of one major radioactive component, coincident with squalene, and three minor labeled components, two of which were coincident with authentic standards of stigmasterol and sitosterol, respectively. The third labeled component, which cor-

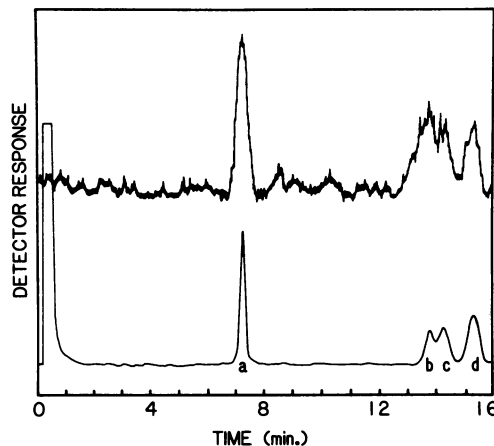


FIG. 2. Radio gas-liquid chromatogram of the nonsaponifiable lipids isolated from peppermint rhizomes that had been incubated with 20  $\mu\text{Ci}$  (1  $\mu\text{mol}$ )  $d$ -[G- $^3\text{H}$ ]neomenthol for 24 h. The smooth lower tracing is the thermal conductivity detector response obtained from coinjected authentic standards of squalene (a), campesterol (b), stigmasterol (c), and sitosterol (d). The upper tracing is the response of the radioactivity monitor attached to the gas-liquid chromatograph. The chromatographic column (OV-17, described in "Materials and Methods") was held at 250°C for 9 min and then programmed at 5°C/min to 300°C at a He flow rate of 65  $\text{cm}^3/\text{min}$ .

responded to a contaminant in the sitosterol preparation, was identified as campesterol based on relative chromatographic retention and spectra (3, 20, 22). Thus, the monoterpene precursor had been converted to higher isoprenoid lipids, presumably by preliminary degradation to acetyl CoA and conventional transformation via mevalonic acid, the key intermediate in the synthesis of all isoprenoids. Earlier studies had indicated that the above-ground portion of peppermint plants contains squalene, sitosterol, and other sterols, and also demonstrated that mevalonate was a very efficient precursor of these isoprenoids, especially squalene (14).

If  $^3\text{H}$ neomenthol is degraded, via menthone and menthone lactone, to  $^3\text{H}$ acetyl CoA, as seems probable, and if this product is available for general lipid metabolism in the rhizome, then any acyl lipids synthesized *de novo* should also be labeled. Accordingly, the saponifiable fraction from above (0.5  $\mu\text{Ci}$ ) was methylated and the methyl esters, after dilution with appropriate carriers, were separated by radio-GLC (Fig. 3). Not surprisingly, the typical plant-derived fatty acids from  $\text{C}_{14}$  to  $\text{C}_{26}$  were radioactive. Thus, supporting evidence was provided that the  $^3\text{H}$ -labeled monoterpene was degraded to acetate and that this metabolite was utilized in the synthesis of both higher isoprenoid lipids and acyl lipids of the rhizome.

Experiments, similar to those above, were also carried out with  $l$ -[G- $^3\text{H}$ ]menthone as substrate. The distribution of label among the nonvolatile lipids was virtually identical, but the overall incorporation was somewhat higher consistent with the earlier, preliminary observation (16).

**Conversion of  $d$ -[G- $^3\text{H}$ ]Neomenthol to Water-Soluble Products.** The earlier work (16) had indicated that the monoterpene precursors, when administered to mint rhizomes, gave rise to radioactive products (1–10% incorporation) which were water-soluble, but not soluble in 100% methanol, and thus suggestive of a labeled carbohydrate polymer. To investigate the properties of the labeled water-soluble material in greater detail, the plant residue remaining after lipid extraction was re-extracted with water and this extract was combined with the aqueous phase obtained on washing the  $\text{CHCl}_3$ :methanol lipid extract. This combined material (0.4  $\mu\text{Ci}$ ) was lyophilized with  $\sim 10\%$  loss of radioactivity (primarily  $^3\text{H}_2\text{O}$ ) and the residue dissolved in a

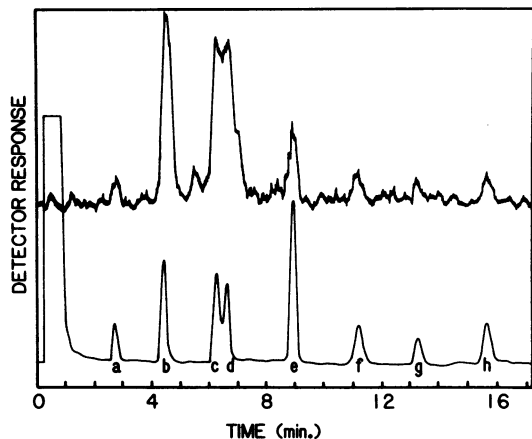


FIG. 3. Radio gas-liquid chromatogram of the methyl esters of the saponifiable lipids isolated from peppermint rhizomes that had been incubated with 20  $\mu\text{Ci}$  (1  $\mu\text{mol}$ ) *d*-[ $^3\text{H}$ ]neomenthol for 24 h. The smooth lower tracing is the thermal conductivity detector response obtained from coinjected authentic standards of the methyl esters of tetradecanoic (a), hexadecanoic (b), octadec-9-enoic (c), octadecanoic (d), eicosanoic (e), docosanoic (f), tetracosanoic (g), and hexacosanoic acid (h). The upper tracing is the response of the radioactivity monitor attached to the gas-liquid chromatograph. The chromatographic column (OV-101, described in "Materials and Methods") was programmed from 175°C to 275°C at 5°C/min at a He flow rate of 80  $\text{cm}^3/\text{min}$ .

minimum quantity of 50 mM Na-acetate, pH 5, and dialyzed (3,500 mol wt cutoff membrane) against this buffer with additional loss of only 20% of the initial radioactivity. These results confirm the polymeric nature of the labeled product(s). This material when subjected to ultrafiltration (Amicon YM2, 1000 mol wt cutoff) was largely retained until amyloglucosidase was added to the filtration chamber, whereby some 50 to 70% of the  $^3\text{H}$  passed through the membrane within several hours. These results indicate that labeled starch was present and had been hydrolyzed to glucose. Similar treatment of the retained material with cellulase, pectinase, and pronase yielded considerably lower levels of filterable  $^3\text{H}$ , and the resulting hydrolysis products were not examined further. The filtrate obtained from the amyloglucosidase treatment of the water-soluble products was lyophilized, and following the addition of carrier D-glucose (2 mg), this material was subjected to TLC (silica gel G with acetone:water [19:1, v/v]) and the labeled glucose isolated (0.1  $\mu\text{Ci}$ ). An aliquot of this material was converted to the trimethylsilyl ether derivative and the identity of the product confirmed as glucose by coincidence of radioactivity with the mixture of authentic anomers on GLC analysis.

If the glucose had been labeled directly via [ $^3\text{H}$ ]acetate (e.g. by way of the glyoxylate cycle [2]),  $^3\text{H}$  would be present only at C1 and C6. If, on the other hand, labeling had occurred via glyceraldehyde-3-P dehydrogenase using [ $^3\text{H}$ ]pyridine nucleotides (produced on oxidation of the terpenyl chain), then only C3 and C4 would bear label. Accordingly, the [ $^3\text{H}$ ]glucose (0.08  $\mu\text{Ci}$ ) was oxidized to the corresponding saccharic acid with  $\text{HNO}_3$  and the reaction mixture then lyophilized. The water thus trapped was not detectably radioactive, indicating that the original glucose was labeled only on the interior carbons. Thus, it was concluded that the starch from which the [ $^3\text{H}$ ]glucose was obtained could only have been labeled indirectly via reduced pyridine nucleotides generated on oxidation of the monoterpenol substrate. Although some  $^3\text{H}_2\text{O}$  was produced from [ $^3\text{H}$ ]neomenthol *in vivo*, dilution with bulk  $\text{H}_2\text{O}$  would be far too great for enolase-catalyzed incorporation of  $^3\text{H}_2\text{O}$  into glucose to be of significance.

The dialysate obtained from the labeled water-soluble products

was lyophilized, and the radioactive products contained therein were converted to the trimethylsilyl ethers. Radio-GLC analysis of this material indicated the presence of five radioactive components chromatographically coincident with the trimethylsilyl ethers of authentic xylose, arabinose (minor), galactose (minor), glucose (minor), and sucrose, respectively. Thus, [ $^3\text{H}$ ]neomenthol had also labeled soluble carbohydrates of the rhizome.

Similar experiments to those in this section above were again carried out with *l*-[ $^3\text{H}$ ]menthone as substrate. The results were essentially identical with the exception that the overall incorporation into water-soluble products was somewhat higher.

**Control Experiments with Sodium [ $^{14}\text{C}$ ]Acetate.** Since the foregoing experiments provided suggestive evidence that [ $^3\text{H}$ ]neomenthol had undergone degradation to [ $^3\text{H}$ ]acetyl-CoA which was reincorporated into acyl and isoprenoid lipids, but that carbohydrates had been labeled most likely via reduced [ $^3\text{H}$ ]pyridine nucleotides generated in the oxidation of the terpenoid substrate, it was appropriate to examine the disposition of [ $^{14}\text{C}$ ]acetate in peppermint rhizomes. Therefore, sodium [2- $^{14}\text{C}$ ]acetate (20  $\mu\text{Ci}$ , 0.4  $\mu\text{mol}$ ) was incubated with excised rhizomes as before and the saponifiable lipids (0.6  $\mu\text{Ci}$ ) and nonsaponifiable lipids (4.6  $\mu\text{Ci}$ ) were isolated. Analysis of the nonsaponifiable fraction by radio-GLC revealed a distribution of label in squalene, campesterol, stigmasterol, and sitosterol nearly indistinguishable from that observed with [ $^3\text{H}$ ]neomenthol as precursor. Similarly, methylation of the saponifiable lipids and radio-GLC analysis of the resulting methyl esters as before indicated a fatty acid labeling pattern very similar to that afforded by [ $^3\text{H}$ ]neomenthol as precursor (with [ $^{14}\text{C}$ ]acetate, the proportion of label in  $\text{C}_{18}$  and  $\text{C}_{18:1}$  was somewhat higher, and that in acids longer than  $\text{C}_{20}$  was somewhat lower, than observed with [ $^3\text{H}$ ]neomenthol). Thus, the mixture of acyl and isoprenoid lipids generated by the metabolism of [ $^3\text{H}$ ]neomenthol was quite similar to that generated by direct administration of [ $^{14}\text{C}$ ]acetate itself. Since the metabolic disposition of exogenous [2- $^{14}\text{C}$ ]acetate is more likely to resemble that of [ $^3\text{H}$ ]acetate generated *in situ* from [ $^3\text{H}$ ]neomenthol than that of [ $^3\text{H}$ ]pyridine nucleotide generated *in situ* from [ $^3\text{H}$ ]neomenthol, the results support the suggestion that [ $^3\text{H}$ ]neomenthol was converted to acyl and isoprenoid lipids by way of [ $^3\text{H}$ ]acetate, but they do not rule out the alternative participation of [ $^3\text{H}$ ]pyridine nucleotides in lipid biosynthesis.

A small quantity of nonvolatile, water-soluble material (0.4  $\mu\text{Ci}$ ) was isolated from the rhizomes after incubation with [ $^{14}\text{C}$ ]acetate. This material was subjected to dialysis, ultrafiltration, and amyloglucosidase hydrolysis as before. As expected, no evidence for labeled starch or soluble carbohydrate was obtained.

As a sidelight to the acetate incorporation experiment, the volatile lipids removed during concentration of the  $\text{CHCl}_3$  extract were examined via radio-GLC following the addition of peppermint oil as a carrier. No evidence for the conversion of [ $^{14}\text{C}$ ]acetate to monoterpenes was obtained, suggesting either that rhizomes are incapable of *de novo* monoterpene biosynthesis, or that acetate does not penetrate to the site of synthesis because of compartmentation effects. Since mint rhizomes are not known to accumulate monoterpenes, the former possibility seems most likely.

**Conversion of *d*-[ $^3\text{H}$ ]Neomenthol to Organic Acids.** Earlier experiments established the conversion of *d*-neomenthol to menthone and 3,4-menthone lactone, and provided presumptive evidence for the activation of the lactone to the corresponding  $\beta$ -methyl- $\epsilon$ -hydroxyacyl CoA ester in mint rhizomes (16), while the present results suggested the ultimate degradation product of the terpenol to be acetate. Such degradation of the acyclic terpenyl chain would likely involve either  $\alpha$ -oxidation and subsequent  $\beta$ -oxidation (29) (yielding  $\text{CO}_2$ , and the CoA esters of propionate, acetate, and isobutyrate as cleavage products), or carboxylation of the  $\beta$ -methyl(s) and removal as the two-carbon

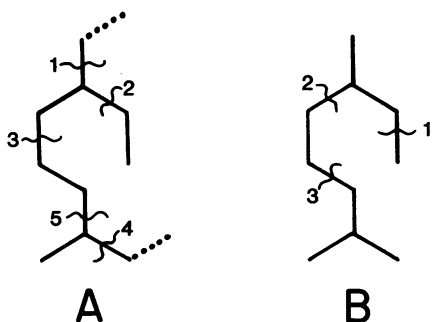


FIG. 4. Two possible routes for the oxidative cleavage of the 3,7-dimethyloctanoate skeleton. Scheme A involves carboxylation of the  $\beta$ -methyl groups and their removal as acetic acid. The dotted lines indicate the carboxylation sites on the acyl chain. Scheme B involves an  $\alpha$ -oxidation followed by  $\beta$ -oxidation to afford one, two, three, and four carbon-containing fragments. The numbers indicate the cleavage sequence.

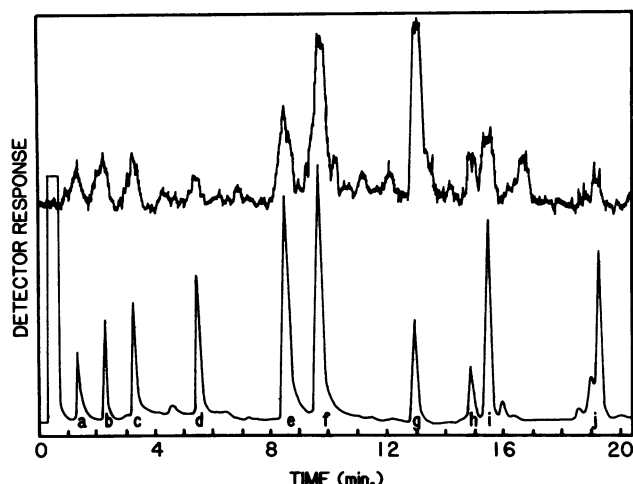


FIG. 5. Radio gas-liquid chromatogram of the *n*-butyl esters of the organic acids isolated from peppermint rhizomes that had been incubated with 20  $\mu$ Ci (1  $\mu$ mol) *d*-[G- $^3$ H]neomenthol for 24 h. The smooth lower tracing is the thermal conductivity detector response obtained from coinjected authentic standards of the butyl esters of acetic (a), propionic (b), isobutyric (c), isovaleric (3-methylbutanoic) (d), 3-methyl-2-butenic (e), 2-hydroxy-3-methylbutanoic (f), 4-hydroxy-5-methylhexanoic (g), citronellic (3,7-dimethyloct-6-enoic) (h), geranic (3,7-dimethylocta-2,6-dienoic) (i), and 6-hydroxy-3,7-dimethyloctanoic acid (j). The upper tracing is the response of the radioactivity monitor attached to the gas-liquid chromatograph. The chromatographic column (OV-101, described in "Materials and Methods") was held at 70°C for 6 min and then programmed at 10°C/min to 220°C at a He flow rate of 80 cm<sup>3</sup>/min.

fragment, a well-known microbial pathway allowing complete degradation of the 3,7-dimethyloctane skeleton to acetic acid and acetyl CoA (8, 28) (Fig. 4). To confirm that oxidative degradation of the acyclic terpenyl chain did occur in the rhizome, and to distinguish between the possible modes of oxidation, organic acids generated *in vivo* from *d*-[G- $^3$ H]neomenthol were examined. For this purpose, rhizomes which had been incubated with *d*-[G- $^3$ H]neomenthol (20  $\mu$ Ci) were extracted with 0.1 N KOH and neutral substances were removed from the extract by extensive washing with ether. Acidification of the alkaline extract, followed by ether extraction, afforded the organic acids (0.55  $\mu$ Ci) which were butylated with BF<sub>3</sub> in butanol. The butyl esters were examined by radio-GLC and several radioactive constituents were thus shown to be present, one of which was coincident with an endogenous, extracted component, pres-

ent in sufficient quantity to permit combined GLC-MS. From the mass spectrum ( $M^+$  = 202, with intense ions at *m/e* 43, 57, and 73, and major  $\alpha$ -cleavage ions at *m/e* 129 and 159 [4]), this compound was identified as the butyl ester of 4-hydroxy-5-methylhexanoic acid (*i.e.* the acyl chain produced after the first two cleavage steps of the oxidation scheme shown in Fig. 4A). Additional carrier standards appropriate for both oxidation schemes were then added and the material reanalyzed to produce the chromatogram illustrated in Figure 5. The bulk of the radioactivity of this fraction was associated with three components, the butyl esters of 3-methyl-2-butenic acid, 2-hydroxy-3-methylbutanoic acid, and the aforementioned 4-hydroxy-5-methylhexanoic acid, each of which is a predicted intermediate in the oxidation scheme whereby the  $\beta$ -methyl of the original 6-hydroxy-3,7-dimethyloctanoate is removed (by carboxylation and cleavage to acetic acid) to afford a suitable intermediate for subsequent  $\beta$ -oxidation.

The short chain acids, acetic, propionic, isobutyric, and isovaleric (as the butyl esters) were also detectably labeled. Since the CoA esters of propionate and isobutyrate are predicted products of the degradation scheme involving an  $\alpha$ -oxidation followed by  $\beta$ -oxidation (Fig. 4B), this result would suggest the operation of such a pathway, although perhaps at a slower rate. The butyl esters corresponding to citronellic and geranic acid were also labeled. These products could have originated from either the corresponding enoic and dienoic acid intermediates, or as elimination products of the corresponding hydroxy and dihydroxy acyl derivatives on butylation. The last product to elute from the column was identified as the butyl ester of 6-hydroxy-3,7-dimethyloctanoic acid, which could have been derived from the corresponding hydroxy dimethylacyl CoA ester or from 3,4-menthone lactone itself. The above investigation of the organic acids derived from [ $^3$ H]neomenthol gave clear evidence for the operation of a  $\beta$ -oxidation sequence in the degradation of the terpenol following its conversion to 3,4-menthone lactone.

## DISCUSSION

Research presented in this and the earlier communication (16) provides convincing evidence that neomenthol administered to mint rhizomes is converted, via menthone lactone, to acyl lipids and higher isoprenoid lipids. These observations, coupled to the demonstration of several probable intermediates in the  $\beta$ -oxidation of the terpenoid skeleton and the observed similarity of the ultimate product distribution to that generated from exogenous [ $^{14}$ C]acetate, suggest that the terpenoid is degraded to acetyl CoA followed by the incorporation of this common precursor into the other lipids. It is possible that the labeled acyl and isoprenoid lipids derived from [ $^3$ H]neomenthol in this study were generated, at least in part, by way of [ $^3$ H]pyridine nucleotides. It is also conceivable that 6-hydroxy-3,7-dimethyloctanoate (corresponding to the lactone) might, through several steps, give rise to geranyl pyrophosphate, and that the partial oxidation product 3-methyl-2-butenic acid could, via reduction and phosphorylation, give rise to dimethylallyl pyrophosphate, both of which are more direct precursors on the isoprenoid pathway. The present evidence does not allow adequate evaluation of such alternate pathways for the formation of higher isoprenoid lipids from neomenthol; however, the incorporation of label into typical acyl lipids does suggest that at least a portion of the terpenoid precursor is degraded to, and incorporated as, acetyl CoA.

It is important to note that the terpenoid precursor employed in these studies was G- $^3$ H-labeled and, because of the metabolic lability of tritium on  $\beta$ -oxidation (and lipid synthesis), the incorporations based on radioactivity are likely to underestimate total carbon flow through these pathways. In this context, it is also worthy of note that in these *in vivo* studies with G- $^3$ H-labeled monoterpene precursors, relatively little  $^3$ H<sub>2</sub>O was produced (16).

This would suggest that  $^3\text{H}$ -labeled nucleotides generated in the oxidation of the terpenoid chain were employed primarily in reductive carbon metabolism, a suggestion well supported by the observation that labeled hexose generated from the terpenoid precursor *in vivo* bore tritium only on the interior carbons. As indicated above, the possible involvement of such labeled nucleotides in the synthesis of acyl and isoprenoid lipids cannot be readily determined from the present results.

Several hypothetical functions for monoterpene turnover in higher plants have been previously proposed, including a possible role in carbon and energy metabolism in rhizomes (11). On the basis of the present evidence it now seems likely that the foliar glycosylation of terpenols followed by transport to and subsequent oxidation of the terpenoid moiety in the rhizome does, in fact, represent a means of recycling a mobile carbon and energy supply accumulated in the leaves during development. Since the essential oil content of peppermint plants may approach 1% (fresh weight) before the post-bloom decline (19, 30), the potential contribution of the monoterpenes to rhizome metabolism is not insignificant. The general strategy for monoterpene catabolism in mint, including the lactonization of the *p*-menthane nucleus to afford an acyclic carbon skeleton and the oxidation of the resulting 3,7-dimethyloctane chain, has ample precedent in microorganisms which can utilize monoterpenes as the sole source of carbon and energy (8–10, 28).

Sakata and co-workers (26) have recently presented evidence implicating synthesis and turnover of monoterpenyl glucosides in *Mentha arvensis* during the flowering stage when the rhizome is rapidly developing. These studies also suggested that monoterpenyl glucosides of the leaves play but a minor role in monoterpene metabolism in *M. arvensis* flowers, as is also the case in *M. piperita* (16). Conversely, evidence for the transport of monoterpenyl glucosides from leaves to flowers, and the subsequent hydrolysis of the transport derivative at this site, has been described in the essential oil rose (5, 24, 25). Bugorskii and Zaprometov (6) have recently demonstrated that the hydrolysis of [ $^3\text{H}$ ] geranyl glucoside in rose petals is accompanied not only by release of geraniol from the flower but also by conversion of this terpenol (*i.e.* 3,7-dimethylocta-2,6-dienol) into waxes, organic acids, and other water-soluble products. Thus, monoterpene catabolism also appears to occur in flower tissue, probably by a means similar to that in rhizomes.

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