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Metabolism of Monoterpenes[1, 2]

EVIDENCE FOR COMPARTMENTATION OF *l*-MENTHONE METABOLISM IN PEPPERMINT (*MENTHA PIPERITA*) LEAVES

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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 leaf discs 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 (Croteau, Martinkus 1979 Plant Physiol 64: 169-175). The metabolic disposition of the epimeric reduction products of the ketone, which is a major constituent of peppermint oil, is highly specific, in that little neomenthyl acetate and little menthyl glucoside are formed. However, when *l*-[3-^{3}H]menthol and *d*-[3-^{3}H]neomenthol are separately administered to leaf discs, both menthyl and neomenthyl acetates and menthyl and neomenthyl glucosides are formed with nearly equal facility, suggesting that the metabolic specificity observed with the ketone precursor was not a function of the specificity of the transglucosylase or transacetylase but rather a result of compartmentation of each stereospecific dehydrogenase with the appropriate transferase. A UDP-glucose:monoterpenol glucosyltransferse, which utilized *d*-neomenthol or *l*-menthol as glucose acceptor, was demonstrated in the 105,000*g* supernatant of a peppermint leaf homogenate, and the enzyme was partially purified and characterized. Co-purification of the acceptor-mediated activities, and differential activation and inhibition studies, provided strong evidence that the same UDP-glucose-dependent enzyme could transfer glucose to either *l*-menthol or *d*-neomenthol. Determination of K_m and V for the epimeric monoterpenols provided nearly identical values. The acetylcoenzyme A:monoterpenol acetyltransferase previously isolated from peppermint extracts (Croteau, Hooper 1978 Plant Physiol 61: 737-742) was re-examined using *l*-[3-^{3}H]menthol and *d*-[3-^{3}H]neomenthol as acetyl acceptors, and the K_m and V for both epimers were, again, very similar. These results demonstrate that the specific *in vivo* conversion of *l*-menthone to *l*-menthyl acetate and *d*-neomenthyl-β-D-glucoside cannot be attributed to the selectivity of the transferases, and they clearly indicate that the metabolic specificity observed is a result of compartmentation effects.

Evidence for monoterpene turnover in plants has come from a variety of sources, including tracer studies and analyses of both short term and long term variation in monoterpene content (23, 24). Rapid and permanent turnover of monoterpenes has been shown to occur in flowering peppermint plants (*Mentha piperita* L.), and during this period of apparent catabolism, at least some of the otherwise undamaged leaf oil glands are emptied of their contents (3, 9). Coincident with the 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 (4). Similar metabolic processes occur in other *Mentha* species (14). Detailed studies 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 furthermore revealed that a significant proportion of the *l*-menthone was transformed to *d*-neomenthyl-β-D-glucoside (7, 9) (Fig. 1). Little neomenthyl acetate or menthyl-β-D-glucoside was formed from [G-^{3}H]menthone, indicating a high degree of specificity in the metabolic disposition of the epimeric reduction products of the ketone (i.e. menthol and menthyl acetate accumulate in the volatile oil, whereas neomenthol is specifically converted to the water-soluble glucoside). Further analytical studies and *in vivo* tracer studies indicated that roughly half of the *l*-menthone metabolized was converted to *l*-menthol (of which approximately 20% was acetylated), while the remaining half was transformed to *d*-neomenthol (of which nearly all was glucosylated) (9). 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 roots, and was shown to undergo subsequent conversion to unidentified polar products at this location (9, 18). While a number of non-iridoid monoterpenyl glycosides have been reported in plants (2, 5, 12, 19, 26, 28, 30, 32, 33), including a recent report on the occurrence of *l*-menthyl-β-D-glucoside in the rhizomes of Japanese peppermint (*Mentha arvensis* Mal. × *M. piperita* L.) (27), and the suggestion made that such glycosides are transport derivatives (11, 15), studies with peppermint were the first to implicate glycosylation of monoterpenols directly as a prelude to monoterpene metabolism at sites quite distant from the presumed site of synthesis, the epidermal oil glands (6).

Studies on the metabolism of *l*-menthone in peppermint have thus provided a number of interesting findings, not the least of which is the observation that this ketone is reduced to significant quantities of both possible epimeric alcohols, each of which is subsequently conjugated in a highly selective manner. While the rationale for menthone metabolism is not yet clear, two possible explanations for the specific formation of *l*-menthyl acetate and *d*-neomenthyl-β-D-glucoside seemed evident. Two stereospecific dehydrogenases may be present and each compartmentized with the appropriate auxiliary transferase. Thus, the dehydrogenase specific for neomenthol production could be accessible to the

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[2] Although the systematic name for *l*-menthone is (5*R*,2*S*)-*trans*-5-methyl-2-(l-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*.

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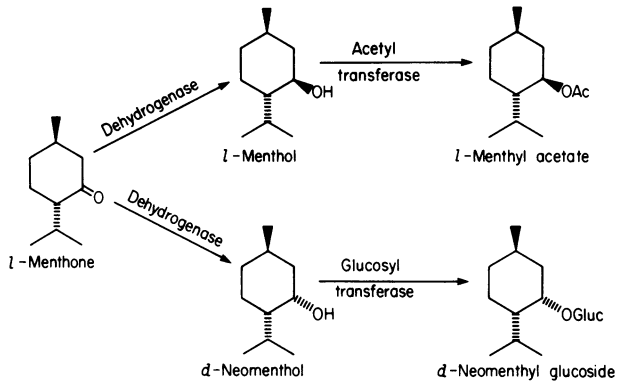

FIG. 1. Metabolic transformations of *l*-menthone in peppermint.

glycosylating system, while the dehydrogenase specific for menthol production could be accessible only to the transacetylase (which preliminary studies have indicated to be relatively nonspecific with regard to the alcohol co-substrate [7]). As an alternate explanation to physical compartmentation, 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 both the accumulation of neomenthol and the formation of neomenthyl acetate. In the present communication, we describe experiments designed to distinguish between these two possibilities.

MATERIALS AND METHODS

Plant Material, Substrates, and Reagents. Peppermint (*M. piperita* L. cv. Black Mitcham) plants were grown from stolons under controlled conditions described previously (9). Unless otherwise specified, leaves from the midstem (leaf pairs number 7–10 from the bottom) of flowering plants were used for all experiments.

l-[G-^{3}H]Menthone (112 Ci/mol) was prepared by CrO_3 oxidation of *l*-[G-^{3}H]menthol as described in detail elsewhere (9). *l*-[3-^{3}H]Menthol and *d*-[3-^{3}H]neomenthol (both 77.5 Ci/mol) were prepared by NaB^3H_4 (310 Ci/mol, from Amersham/Searle Corp.) reduction of *l*-menthone (99.5% chemical and optical purity). The ketone, dissolved in methanol, was allowed to react with the hydride for 48 h, after which the mixture was acidified. *l*-[3-^{3}H]Menthol (44% yield) and *d*-[3-^{3}H]neomenthol (56% yield) were isolated directly from the reaction mixture by TLC (Silica Gel G, with hexane:ethyl acetate (4:1 v/v) as developing solvent [System A]). The purity of each epimer was verified as 99+% by radio-GLC. *d*-Neoisomenthol and *d*-isomenthol were similarly prepared by NaB^3H_4 reduction of *d*-isomenthone, and the epimers were purified (99+%) by TLC (System A) and verified by radio-GLC as before. For use as substrates, the monoterpenes were dispersed in water with the aid of Tween 20 (10 μg/μmol) and sonication (Biosonik III).

The β-D-glucosides of *l*-menthol, *d*-neomenthol, *d*-isomenthol, and d-neoisomenthol were prepared from acetobromo-α-D-glucose (Sigma Chemical Co.) and the corresponding monoterpenol by the Ag_2O-coupling procedure described previously (9). Monoterpene standards (all 99+%) were provided through the generosity of K. Bauer, Haarmann and Reimer GmbH, Holzminden, West Germany, and R. Carrington, I. P. Callison & Sons, Chehalis, WA. β-Glucosidase (almonds) was obtained from Sigma Chemical Co., as were other biochemicals and reagents, unless otherwise noted. Polyvinylpyrrolidone (GAF Corp.) and Amberlite XAD-4 resin (Rohm and Haas Corp.) were purified by standard procedures for use as adsorbents (22, 25).

Experiments with Leaf Discs. Leaves from the midstem of flowering peppermint plants were cut into 8-mm discs with a cork borer and batches of 50 discs were incubated with 0.5 ml an aqueous solution containing either 30 μCi (0.26 μmol) *l*-[G-^{3}H]menthone, 20 μCi (0.26 μmol) *l*-[3-^{3}H]menthol, or 20 μCi (0.26 μmol) *d*-[3-^{3}H]neomenthol, plus 10 μg Tween 20 for 12 h at 30 C in a covered Erlenmeyer flask. At the end of incubation, each set of discs was washed thoroughly with distilled H_2O and then ground in a mortar and pestle with 15 ml pentane. Internal standards (20 mg each *l*-menthone, *l*-menthol [and the corresponding acetate], and *d*-neomenthol [and the corresponding acetate]) were added to the pentane extract which was concentrated to 2 ml and transferred to a micro steam distillation apparatus (8) containing 5 ml H_2O. Distillation was carried out until 3 ml H_2O had passed over, and an aliquot of the still pot residue was taken for determination of radioactivity. The aqueous phase of the distillate was saturated with NaCl, and after thorough mixing, the pentane layer was removed, concentrated under a stream of N_2 at 0 C, and subjected to TLC (System A) after removal of an aliquot for tritium determination. An alcohol fraction (containing *l*-menthol and *d*-neomenthol; R_F = 0.22–0.32) and a fraction containing *l*-menthone and the acetates of *l*-menthol and d-neomenthol (R_F = 0.37–0.47) were isolated. In the experiment with *l*-[G-^{3}H]menthone as substrate, the mixed fraction containing the ketone and acetates was refluxed briefly in ethanol:pyridine (25:1, v/v) containing excess hydroxylamine hydrochloride. This material was then again submitted to TLC (System A), at which time the acetate fraction was separated from *l*-menthone oxime (representing the residual substrate). In preliminary experiments, *d*-pulegone and *d*-piperitone were added as internal standards to the pentane extract obtained with *l*-[G-^{3}H]menthone as substrate. After the described work-up, the oximes of *d*-pulegone and *d*-piperitone were shown (via radio-GLC) to contain no detectable tritium, indicating that *l*-menthone was not desaturated to these derivatives in leaf discs. Because of the position of the label in *l*-menthol and *d*-neomenthol (^{3}H at C3), the *in vivo* oxidation of these substrates to ketones was not determinable. Related experiments suggest, however, that the *in vivo* dehydrogenation of *l*-menthol and its stereoisomers is negligible (7). Each of the fractions isolated by TLC of the steam-distilled pentane extract was examined for tritium content and further analyzed by radio-GLC under conditions described in the text.

The leaf residue remaining after the above pentane extraction was transferred to a TenBroeck homogenizer with 10 ml hot 0.1 M $(NH_4)_2CO_3$ (pH 9.0) and, after thorough homogenization, centrifuged at 27,000*g* for 15 min. The pellet was suspended in water and an aliquot taken for determination of tritium content. Internal standards (10 mg each of the β-D-glucosides of *l*-menthol and *d*-neomenthol) were added to the supernatant, which was then lyophilized after removal of an aliquot for tritium determination. The lyophilized residue was taken up in 5 ml ethanol, 50 mM NH_4OH (2:3, v/v) and applied to a 2 × 6 cm column 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 eluant, which contained over 95% of the tritium applied to the column, was concentrated under vacuum and subjected to TLC on Silica Gel G with ethyl acetate:ethanol (6:1, v/v) as developing solvent (System B). Standards were located on the plate by fluorescein spray, and radioactive components by radioscanning. The appropriate materials were eluted from the gel with methanol. Aliquots were taken to dryness under N_2 and incubated with 10 units β-glucosidase (almonds) in 0.5 ml 50 mM Na-phosphate (pH 5.0) for 3 h at 30 C, after which the ether-soluble components liberated were analyzed by radio-TLC and radio-GLC as described above. Alternatively, aliquots were transferred to the micro steam distillation apparatus with 5 ml 3 N HCl and subjected to the acid hydrolysis-steam distillation procedure previously described (9). As before, the ether-soluble components liberated were analyzed by radio-TLC and radio-GLC.

Preparation of the Glycosyltransferase. Leaves (5 g) from the

midstem of flowering peppermint plants were washed with 1 mM EDTA and then ground in a TenBroeck homogenizer with 2 g insoluble PVPP (Polyclar AT, GAF Corp.) in cold 0.1 M Na-phosphate buffer (pH 6.5), containing 0.25 M sucrose, 50 mM $Na_2S_2O_5$, 50 mM Na-ascorbate, 1 mM dithioerythritol, and 5 mM $MgCl_2$. The homogenate was then slurried with an additional 3 g insoluble PVPP and 5 g Amberlite XAD-4 polystyrene resin for 5 min at 4 C. The slurry was filtered through several layers of cheesecloth and the filtrate centrifuged at 27,000*g* for 15 min (pellet discarded), followed by centrifugation at 105,000*g* for 60 min to provide the soluble supernatant used as the enzyme source. Finely powdered $(NH_4)_2SO_4$ was added to the soluble enzyme preparation (at 0–4 C) until 70% saturation (0.472 g/ml) was obtained. After stirring for an additional 30 min, the precipitated protein was collected by centrifugation at 10,000*g* for 10 min. The precipitate was suspended in 50 mM Na-phosphate buffer (pH 7.7) containing 5 mM $MgCl_2$, 5 mM mercaptoethanol, and 0.25 mM dithioerythritol, dialyzed against this same buffer for several hours, and then applied to a Sephadex G-100 column (2.5 × 120 cm) previously equilibrated with the same buffer. Proteins were eluted (~0.34 ml/min, 5.6-ml fractions) while monitoring the column effluent at 280 nm. Fractions containing glucosyltransferase activity were pooled, concentrated by ultrafiltration (Amicon PM-30), and, when necessary, adjusted to appropriate assay conditions by dialysis. Protein in partially purified preparations was determined by the standard Lowry method.

For preparing particulate fractions, the same extraction buffer was used, except that soluble PVP (Plasdone K-90, mol wt = 400,000, GAF Corp.) was substituted for the insoluble polymer, and that the XAD-4 treatment and preliminary filtration were omitted. Particulate fractions, prepared by differential centrifugation, were washed before the assay (by suspension and recentrifugation) with 50 mM Na-phosphate buffer (pH 7.7), containing 5 mM $MgCl_2$, 5 mM mercaptoethanol, and 0.25 mM dithioerythritol.

Assay for Glucosyltransferase. The reaction mixture, containing 40 to 80 μg protein, 0.1 mM *l*-[3-^{3}H]menthol or *d*-[3-^{3}H]neomenthol (or other menthol stereoisomer), and 1.0 mM UDP-glucose, in a total volume of 1 ml 50 mM Na-phosphate buffer (pH 7.7, containing $MgCl_2$, mercaptoethanol, and dithioerythritol as indicated above) was incubated in a sealed tube at 30 C for up to 2 h. The reaction was stopped by heating for 2 min at 90 C, and the chilled samples were extracted with ethyl acetate (2 × 2 ml) to remove residual substrate. The aqueous phase was lyophilized after the addition of internal standard (10 mg appropriate β-D-glucoside), and the residue obtained was taken up in 0.3 ml ethanol:water (2:3, v/v). In assays of crude preparations (105,000*g* supernatants, $(NH_4)_2SO_4$ precipitates, particulate fractions), the suspended residue was filtered through a small glass wool-plugged column containing a 0.5 × 1 cm layer of Dowex 1 (Bio-Rad AG 1-X8 [formate]). This procedure, which was unnecessary with the Sephadex G-100 purified preparation, served to remove denatured protein and phenolic substances from these cruder samples before the isolation of the biosynthetic glucoside product by TLC (System B: neomenthol glucoside R_F = 0.45; menthol glucoside R_F = 0.41). Appropriate boiled controls were included in each experiment, with individual controls in those experiments in which different control values were possible (pH optimum, response to cations, etc.). In all instances, nonenzymic glucosylation of menthol and its stereoisomers was negligible.

Preparation of, and Assay for, Acetyltransferase. The soluble acetyl-CoA:monoterpenol acetyltransferase from peppermint leaves was prepared and purified by combination of Sephadex G-100 gel filtration and chromatography on *O*-diethylaminoethyl-cellulose as described previously (7). Assays were run as before (7), with saturating levels of acetyl-CoA (0.5 mM) and the appropriate concentrations of the tritium-labeled menthol stereoisomers, and the acetylated biosynthetic products were isolated by TLC after the addition of the corresponding internal standard.

Chromatography and Determination of Radioactivity. TLC was carried on activated (4 h at 110 C) Silica Gel G (EM Laboratories) plates of 1-mm thickness. Developing solvents are indicated elsewhere in the text. After development, the chromatograms were sprayed with an ethanolic solution of 2,7-dichlorofluorescein (0.2%, w/v) and viewed under UV light to locate the appropriate product. Radioactive monoterpenyl glucosides were also located by scanning the plate with a Berthold TLC radioscanner. Radioactivity in TLC fractions was determined either by eluting the material from the gel (ether or methanol), or by scraping the gel directly into a counting vial followed by either the addition of 15 ml Permablend III cocktail described below (for monoterpenes or monoterpenyl acetates) or the addition of 1 ml water and 15 ml Fisher Scientific ScintiVerse (for monoterpenyl glucosides). Radioactivity in aqueous samples was determined directly in ScintiVerse (34% efficiency for ^{3}H). Organic liquid samples were assayed in a counting solution consisting of 0.55% (w/v) Permablend III (Packard) dissolved in 30% ethanol in toluene (37% efficiency for ^{3}H). All assays were done with a standard deviation of less than 3%.

Radio-GLC was performed on a Varian chromatograph attached to a model 7356 Nuclear Chicago radioactivity monitor which was calibrated with [^{3}H]toluene. The stainless steel column used (3 m × 3 mm o.d.) was packed with 12% Carbowax 4000 on 80 to 100 mesh Gas-chrom Q. Analytical conditions are described elsewhere in the text or in the appropriate figure legends.

RESULTS AND DISCUSSION

Experiments with Peppermint Leaf Discs. The two most likely explanations for the selective formation of *l*-menthyl acetate and *d*-neomenthyl-β-D-glucoside from *l*-menthone in peppermint are compartmentation of pathways and/or specificity of the respective transferase enzymes. To examine these possibilities, studies with peppermint leaf discs were carried out using *l*-[3-^{3}H]menthol and *d*-[3-^{3}H]neomenthol as exogenous precursors in an attempt to overcome any existing compartmentation effects and to test the specificity of the transferases directly. Three sets of leaf discs, obtained from midstem leaves of flowering plants, were incubated for 12 h with equivalent amounts of *l*-[G-^{3}H]menthone, *l*-[3-^{3}H]menthol, and *d*-[3-^{3}H]neomenthol, respectively, and the pentane-soluble, steam-volatile metabolites and the water-soluble, nonvolatile metabolites were isolated. The steam-volatile metabolites were separated by TLC into a monoterpenol fraction and a mixed monoterpene ketone-monoterpenyl acetate fraction (from which the ketone was subsequently removed and determined as the oxime), and each fraction was analyzed by radio-GLC. The water-soluble metabolites were treated with Dowex 1 anion exchange resin to remove phenolic substances, and then subjected to TLC (System B) to allow separation of the monoterpenyl glucoside fraction (R_F = 0.41–0.45) from the previously noted (9) unidentified metabolite (R_F = 0.32–0.35). Acid hydrolysis of the glucoside, or β-glucosidase treatment, liberated the terpenol aglycone, which was isolated by steam distillation and subsequently analyzed by radio-GLC.

Analysis of the aglycone from the glucoside fraction derived from *l*-[G-^{3}H]menthone (Fig. 2a) revealed the presence of *d*-neomenthol with a very minor amount of *l*-menthol, confirming the preferential formation of *d*-neomenthyl-β-D-glucoside from the ketone precursor as previously described (9). Radio-GLC examination of the terpenyl acetate fraction derived from *l*-[G-^{3}H]menthone (Fig. 2d) indicated the presence of *l*-menthyl acetate only, demonstrating the specific synthesis of this ester. Analysis of the derived aglycone and monoterpenyl acetate fraction obtained from incubation of leaves with *l*-[3-^{3}H]menthol (Fig. 2, b and e), provided readily detectable levels of *l*-menthyl-β-D-glucoside and *l*-menthyl acetate, respectively, while similar analysis of the prod-

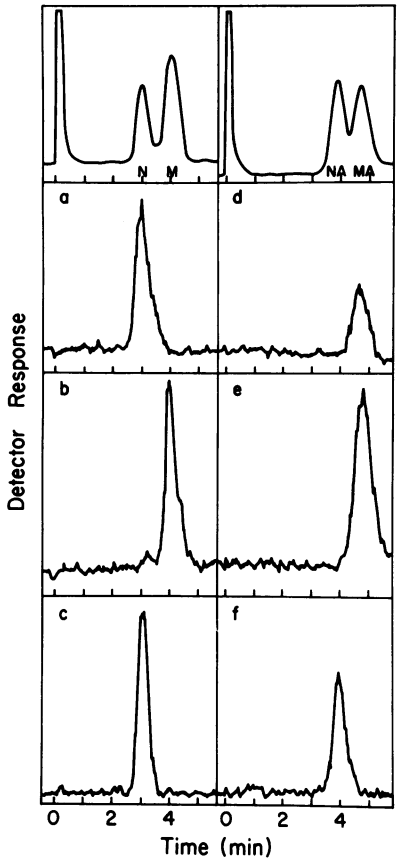

FIG. 2. Radio gas-liquid chromatograms of the steam-distilled acid hydrolysate of the water-soluble products obtained from peppermint leaf discs that had been incubated with *l*-[G-^{3}H]menthone (a), *l*-[3-^{3}H]menthol (b), and *d*-[3-^{3}H]neomenthol (c), and of the monoterpenyl acetate fraction of the steam-distilled oil obtained from the same discs that had been incubated with *l*-[G-^{3}H]methone (d), *l*-[3-^{3}H]menthol (e), and *d*-[3-^{3}H]-neomenthol (f). The smooth upper tracings represent the response of the flame ionization detector to coinjected standards of *d*-neomenthol (N), *l*-menthol (M), *d*-neomenthyl acetate (NA), and *l*-menthyl acetate (MA). The gas-liquid chromatographic column (Carbowax 4000) was maintained at 132 C for separation of the alcohols and at 165 C for separation of the acetates. The argon flow rate was held at 160 cm^3/min.

ucts derived from *d*-neomenthol (Fig. 2, c and f) also gave evidence for the presence of the corresponding β-D-glucoside and acetate. Determination of radioactivity in all fractions, coupled to the verification of each product by radio-GLC, provided the quantitative data in Table I. The incorporation of labeled substrate was over 50% in each case, and in each case over 60% of the incorporated label could be accounted for in the metabolites noted. The rest was largely contained in the previously noted, but unidentified, water-soluble metabolite more polar than the glucosides (9), with lesser amounts in hexane-soluble, nonvolatile substances and insoluble material. The preferential synthesis of *d*-neomenthyl-β-D-glucoside and *l*-menthyl acetate from *l*-menthone is, again, apparent, and while a minor amount of *l*-menthol-β-D-glucoside was formed (as was noted previously [9]), the level of *d*-neomenthyl acetate formed was below the detectable level. Conversely, when the alcohol substrates *l*-menthol and *d*-neomenthol were employed as substrates, *l*-menthyl glucoside and *d*-neomenthyl glucoside were formed with nearly equal efficiency, as were the epimeric acetates. Thus, the specificity of product formation observed from *l*-menthone was not reflected on direct administration of the derived epimeric alcohols. These results strongly suggest that the glucosyltransferase and acetyltransferase (7) are not highly specific enzymes, and that the specificity observed with the ketone substrate results from compartmentation of the menthol-specific dehydrogenase with the acetyltransferase, and of the neomenthol-specific dehydrogenase with the glucosyltransferase (a compartmentation effect clearly overcome by direct administration of the reduction products of menthone).

Based on earlier experiments, we had suggested that the site of glucosylation was more accessible to exogenous [^{3}H]menthone than to endogenous menthone generated from $^{14}CO_2$ (9). The ready formation of the glucoside from each exogenous monoterpene (Table I) appears to support the suggestion of such differentially accessible sites.

Isolation of UDP-Glucose:Monoterpenol Glucosyltransferase. To confirm that the glucosyltransferase involved in the synthesis of monoterpenyl-β-D-glucosides in peppermint was, in fact, relatively nonspecific with regard to the terpenol moiety, it was necessary to examine the enzyme(s) directly. A UDP-glucose-dependent glucosyltransferase activity (*l*-[3-^{3}H]menthol or *d*-[3-^{3}H]neomenthol as glucose acceptor) was readily demonstrated in the 105,000*g* supernatant prepared from a homogenate of midstem peppermint leaves (products verified by TLC, β-glucosidase hydrolysis, and subsequent radio-GLC of the liberated aglycone [9]). Terpenyl glucosides were not formed in the absence of UDP-glucose, or when UDP-glucose and either ^{3}H-labeled terpenol were incubated with boiled enzyme. Less than 5% of the total transglucosylase activity was present in particulate fractions prepared from the homogenate by differential centrifugation, and this activity was not examined further.

The soluble preparation was concentrated by $(NH_4)_2SO_4$ precipitation (0–70%), and the concentrate separated by chromatography of Sephadex G-100 (Fig. 3). Glucosyltransferase activity was again measured with both *l*-[3-^{3}H]menthol and *d*-[3-^{3}H]neomenthol as co-substrate, and activities for the transfer of glucose from UDP-glucose to menthol and neomenthol were coincident. Additionally, under identical assay conditions the rates of synthesis of menthyl glucoside and neomenthyl glucoside were nearly the same. To assure that the results observed were not biased by the possible presence of a β-glucosidase, a mixture of *d*-[3-^{3}H]neomenthyl-β-D-glucoside and *l*-[3-^{3}H]menthyl-β-D-glucoside was prepared (biosynthetically) and incubated with the Sephadex G-100 column fractions (fractions 13–24) containing the transferase activity (Fig. 3). The release of the ether-soluble aglycone was then monitored, but neither free [^{3}H]neomenthol nor [^{3}H]menthol could be detected, indicating the absence of β-glucosidase activity in the glucosyltransferase region. Column fractions containing the glucosyltransferase activity were combined and concentrated by ultrafiltration (Amicon PM-30), and this partially purified preparation was used in all subsequent experiments.

Properties of UDP-Glucose:Monoterpenol Glucosyltransferase. The apparent mol wt of the glucosyltransferase from peppermint was determined to be about 46,000 by gel permeation chromatography on a calibrated Sephadex G-100 column, the activity eluting slightly before ovalbumin (mol wt 43,500). A variety of plant-derived glucosyltransferases, which transfer glucose to both hydrophobic and hydrophilic alcohols, exhibit mol wt in the 40,000 to 60,000 range (17, 31).

The rate of *l*-menthol and *d*-neomenthol glucosylation increased linearly with protein concentration up to about 100 μg/ml for the described 1 h assay, beyond which the rate was no longer proportional to protein concentration. The rate of glucosylation under the standard assay conditions was linear up to 2 h at a protein concentration of 45 μg/ml. All further measurements were made within these ranges of protein concentration and time.

The glucosyltransferase exhibited a sharp pH optimum at 7.7 (half-maximal activities at pH 7.25 and 8.25) when examined in 70 mM Na-phosphate buffer (Fig. 4). The pH curves with either *d*-neomenthol or *l*-menthol as glucose acceptor were nearly identical.

Table I. *Metabolism of l-Menthone, l-Menthol, and d-Neomenthol in Peppermint Leaf Discs*

Batches of 50 leaf discs (8 mm) were incubated with 0.26 μmol of either *l*-[G-^{3}H]menthone, *l*-[3-^{3}H]menthol, or *d*-[3-^{3}H]neomenthol for 12 h at 30 C. The pentane-soluble, steam-volatile metabolites and the water-soluble, nonvolatile metabolites were isolated and analyzed by radio-TLC and radio-GLC.

Substrate	Total incorporation	Distribution of Incorporated Label						
		Neomenthyl glucoside	Menthyl glucoside	Neomenthyl acetate	Menthyl acetate	Neomenthol	Menthol	Menthone
	%	%	%	%	%	%	%	%
l-Menthone	54.8	43.0	3.4	nd[a]	2.5	0.3	7.4	4.6
l-Menthol	50.6	nd	44.7	nd	4.4	nd	14.3	
d-Neomenthol	53.8	49.4	nd	3.6	nd	14.1	nd	

[a] not detected.

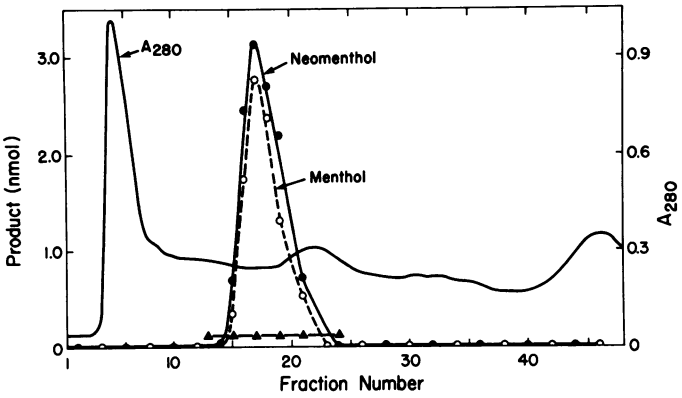

FIG. 3. Sephadex G-100 gel filtration of the 105,000*g* supernatant preparation from peppermint leaves. *A* at 280 nm, UDP-glucose:monoterpenol glucosyltransferase activity with *d*-neomenthol (●——●) and *l*-menthol (○- - -○) as acceptors, and β-glucosidase activity (▲——▲) are plotted. Chromatography and assay procedures are described under "Materials and Methods." V_0 was at fraction 4.

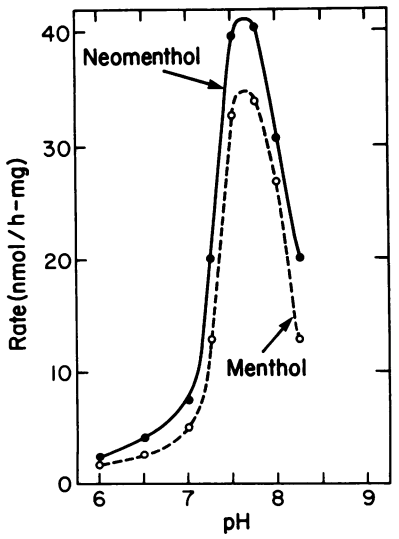

FIG. 4. Effect of pH on the transfer of glucose from UDP-glucose to *d*-neomenthol (●——●) and to *l*-menthol (○- - -○) by the glucosyltransferase from peppermint. Each reaction mixture, containing 76 μg protein, 1.0 mM UDP-glucose, 0.1 mM *d*-[3-^{3}H]neomenthol or *l*-[3-^{3}H]menthol, 5 mM mercaptoethanol, and 5 mM $MgCl_2$ in a total volume of 1.0 ml of appropriate 0.07 M Na-phosphate buffer, was incubated at 30 C for 1 h.

In 70 mM Tris-sodium maleate buffer, the pH curves with either neomenthol or menthol as co-substrate were again very similar, but considerably broadened. The pH optimum in both instances was about 7.3 with half-maximal activities at pH 6.0 and 8.5. While no other UDP-glucose:monoterpenol glucosyltransferases have been described thus far; a variety of other glucosyltransferases, that transfer glucose to hydrophobic acceptors, exhibit optima in the pH 7.0 to 8.0 range (16, 31, 34). A similar decrease in pH optimum in Tris buffer (relative to Na-phosphate) has also been observed with other glucosyltransferases (17).

The effect of metal ions on glucosyltransferase activity is described in Table II. Magnesium ion stimulated activity, with a maximum increase in activity (at 20 mM) of about 30% above the control level. Certain transglucosylases of plant origin have been shown to be stimulated by Mg^{2+} (31), although for other enzymes of this type, the cation is without significant effect (16, 17). The manganese cation exhibited a consistent but very slight degree of stimulation, while the other cations tested (Co^{2+}, Ni^{2+}, Ca^{2+}, Zn^{2+}) were significantly inhibitory at the 5 mM level. Unlike the operationally soluble enzyme described here, membranous glucosyltransferases are generally stimulated by Ca^{2+} (1). As indicated in Table II, the effect of metal ions on the rates of glucosylation of *d*-neomenthol and *l*-menthol was nearly identical.

The effect of thiol-directed reagents on the glucosyltransferase

Table II. *Effect of Metal Ions on UDP-Glucose:Monoterpenol Transglucosylase Activity*

The preparation was dialyzed against 50 mM Na-phosphate buffer (pH 7.7) containing 1 mM EDTA and 5 mM mercaptoethanol, followed by dialysis against the same buffer minus the EDTA. One-ml aliquots (containing 72 μg protein) were then incubated with the indicated concentration of cation (the Cl^- anion was shown to be without effect), UDP-glucose (1 mM), and [3-^{3}H]alcohol (0.1 mM) for 1 h at 30 C. Base rates for the controls without metal ion were 37 nmol/h·mg protein with *d*-[3-^{3}H]neomenthol as co-substrate, and 32 nmol/h·mg protein with *d*-[3-^{3}H]menthol as co-substrate.

Addition	Concentration	Relative Rate of Product Formation	
		Neomenthyl glucoside	Menthyl glucoside
	mM		
None		100	100
$MgCl_2$	5	110	112
$MgCl_2$	20	132	134
$MnCl_2$	5	103	105
$CoCl_2$	5	25	33
$NiCl_2$	5	22	27
$CaCl_2$	5	14	17
$ZnCl_2$	5	12	15

activity was examined after slowly removing the mercaptoethanol (and residual dithioerythritol) from the preparation by dialysis (Table III); a procedure which resulted in a significant (75%) decrease in activity that was only partially restored by readdition of the thiol-protecting reagent (controls dialyzed against 5 mM mercaptoethanol for an equal length of time lost <10% of the activity). The residual activity remaining after removal of mercaptoethanol was very sensitive to low levels of *p*-hydroxymercuribenzoate (10 μM) and heavy metals (0.1 mM, in the presence of 5 mM $MgCl_2$). Thus, activity was severely diminished by the removal of thiol-protecting reagents and by the presence of thiol-directed reagents, suggesting that a thiol function(s) is essential for activity, as is the case with other transglucosylases (16, 17, 31). Again, inhibition of activity was nearly the same with either *d*-neomenthol or *l*-menthol as co-substrate.

As the concentration of UDP-glucose in the assay medium was increased, the rate of formation of *d*-neomenthyl glucoside and *l*-menthyl glucoside from the corresponding epimeric alcohol (at a saturating level of 0.1 mM) also increased, giving rise in each case to a typical Michaelis-Menten hyperbolic saturation curve. The double reciprocal plots were linear from which an apparent K_m of 200 μM and V of 51 nmol/h·mg protein for UDP-glucose were calculated with *d*-neomenthol as co-substrate. With *l*-menthol as co-substrate, the K_m and V for UDP-glucose were 250 μM and 48 nmol/h·mg protein, respectively (*i.e.* essentially identical with the kinetic constants for UDP-glucose with *d*-neomenthol as co-substrate). Increasing the concentration of either *l*-menthol or *d*-neomenthol in the presence of saturating levels of UDP-glucose (1 mM) provided a pair of similar hyperbolic saturation curves, and the double reciprocal plots were linear (Fig. 5). The K_m and V for *d*-neomenthol were determined to be 48 μM and 52 nmol/h·mg protein, respectively; while for *l*-menthol, the corresponding values were 59 μM and 50 nmol/h·mg protein, illustrating the lack of specificity with regard to the epimeric glucose acceptors. Since the monoterpenol substrates were dispersed with Tween 20 and were not in a true solution, these values should be taken with the usual precautions. It should be noted, however, that Tween 20 had no apparent effect on transglucosylase activity when present at 10-fold the concentration present in the substrate. As was observed for the present transferase, the K_m for UDP-glucose is often many times higher than that for the glucose acceptor (16, 17).

Table III. *Effect of Inhibitors on UDP-Glucose:Monoterpenol Transglucosylase Activity*

The preparation was dialyzed against 50 mM Na-phosphate buffer (pH 7.7) containing 5 mM $MgCl_2$ to remove mercaptoethanol and residual dithioerythritol, and 1-ml aliquots (containing 68 μg protein) were preincubated for 5 min with the addition indicated, followed by the addition of UDP-glucose (1 mM) and [3-^{3}H]alcohol (0.1 mM), and incubation for 1 h at 30 C. Base rates for the undialyzed controls with mercaptoethanol were 35 nmol/h·mg protein with *d*-[3-^{3}H]neomenthol as co-substrate, and 31 nmol/h·mg protein with *l*-[3-^{3}H]menthol as co-substrate.

Addition	Concentration	Relative Rate of Product Formation	
		Neomenthyl glucoside	Menthyl glucoside
	mM		
Mercaptoethanol (undialyzed)	5.000	100.0	100.0
None (after dialysis)		23.2	27.0
p-Hydroxymercuribenzoate	0.005	6.3	6.0
p-Hydroxymercuribenzoate	0.010	3.2	2.9
$CdCl_2$	0.100	5.5	4.9
$HgCl_2$	0.100	2.7	2.3
Mercaptoethanol (readded)	5.000	67.0	63.3

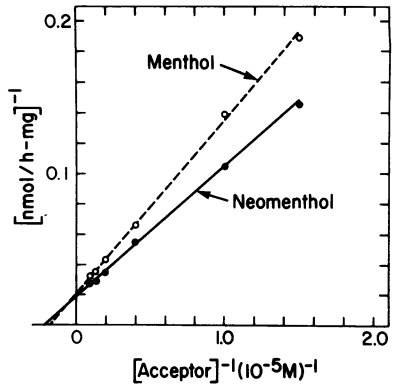

FIG. 5. Plot of reciprocal of reaction velocity *versus* reciprocal of concentration of glucose acceptor. Each reaction mixture, containing 71 μg protein, 1.0 mM UDP-glucose, 5 mM mercaptoethanol, 5 mM $MgCl_2$, 0.25 mM dithioerythritol, and the appropriate amount of *d*-[3-^{3}H]neomenthol (●——●) or *l*-[3-^{3}H]menthol (○- - -○) in a total volume of 1.0 ml 0.05 M Na-phosphate buffer (pH 7.7), was incubated at 30 C for 1 h.

The ketone *d*-isomenthone (1*R*:4*R*, epimeric with *l*-menthone at the isopropyl-substituted carbon) is also a component of peppermint oil (3.5%, compared to ~25% menthone in a commercial oil sample [21]). The reduction products of *d*-isomenthone are *d*-isomenthol ([1*R*:3*S*:4*R*], epimeric with *d*-neomenthol [1*R*:3*S*:4*S*] at the isopropyl-substituted carbon) and *d*-neoisomenthol ([1*R*:3*R*:4*R*], epimeric with *l*-menthol [1*R*:3*R*:4*S*] at the isopropyl-substituted carbon) (Fig. 1), which are minor constituents of peppermint oil (10, 21, 29). *d*-[3-^{3}H]Isomenthol and *d*-[3-^{3}H]neoisomenthol were prepared for use as substrates, and the appropriate kinetic constants again determined. As expected, neither isomenthol nor neoisomenthol at saturating concentrations had an appreciable effect on K_m or V for UDP-glucose (~240 μM and ~50 nmol/h·mg protein, respectively). In the presence of saturating concentrations of UDP-glucose, the K_m and V for *d*-neoisomenthol were determined to be 52 μM and 48 nmol/h·mg protein, respectively, while for *d*-isomenthol, a K_m of 61 μM and V of 46 nmol/h·mg protein were obtained, again demonstrating the relative lack of specificity of the enzyme with regard to the menthol diastereoisomer utilized as the glucose acceptor. The effect of unlabeled *l*-menthol and *d*-neoisomenthol on the transglucosylation of *d*-[3-^{3}H]neomenthol, and the effect of unlabeled *d*-neomenthol and *d*-isomenthol on the transglucosylation of *l*-[3-^{3}H]menthol were determined. Although all the possible permutations were not examined, these limited results indicated that the menthol stereoisomers are mutually competitive inhibitors.

While purification of the transglucosylase to homogeneity would provide the only conclusive proof that the same, relatively nonspecific, enzyme utilizes all the menthol isomers, the coelution of activities on Sephadex G-100, the response to pH and differential stimulation and inhibition, and the kinetic data all strongly suggest this possibility. This is the first report on the isolation and properties of an enzyme which is capable of synthesizing monoterpenyl glucosides and which participates in a well defined metabolic sequence. The glucosyltransferase from peppermint is unremarkable in its properties, and probably typical of the class of enzymes responsible for the occurrence of monoterpenyl glucosides in numerous other species (2, 5, 12, 19, 26, 28, 30, 32, 33).

Substrate Specificity of Acetyltransferase. The acetyl-CoA: monoterpenol acetyltransferase involved in the synthesis of *l*-menthyl acetate in peppermint has been partially purified and characterized (7). As the selectivity of product formation during the metabolism of *l*-menthone was not recognized at the time of this earlier work, there was no compelling reason to examine the substrate specificity of this enzyme in any detail. However, with the findings that *l*-menthone was selectively converted to *l*-men-

thyl acetate and *d*-neomenthyl-β-D-glucoside, and that the glucosyltransferase was not very specific with regard to the monoterpenol cosubstrate, it seemed pertinent to re-examine the question of acetyltransferase specificity. The K_m and V for the four relevant menthol stereoisomers were therefore determined (typical hyperbolic saturation curves and linear double reciprocal plots were observed in all cases in the presence of saturating levels of acetyl-CoA [0.5 mM]), and shown to be 0.32 mM and 18 nmol/h·mg protein for *l*-menthol, 0.35 mM and 16 nmol/h·mg protein for *d*-neomenthol, 0.24 mM and 37 nmol/h·mg protein for *d*-neoisomenthol, and 0.31 mM and 19 nmol/h·mg protein for *d*-isomenthol. The higher V and somewhat lower K_m for *d*-neoisomenthol cannot readily be explained, but these and the other kinetic constants are consistent with the earlier studies (7) in which relative rates were compared at a single acceptor concentration of 0.8 mM. Although the kinetic constants should, again, be taken with the usual precautions, it is obvious that the enzyme is not highly selective, and it clearly does not readily distinguish between *l*-menthol and *d*-neomenthol (which were also shown to be mutually competitive inhibitors).

d-Neoisomenthol (derived from *d*-isomenthone) and *l*-menthol (derived from *l*-menthone), while epimeric at the isopropyl-substituted carbon, both exhibit the same 3*R* configuration of the hydroxyl-substituted carbon, suggesting derivation from the same stereospecific dehydrogenase (a suggestion supported by genetic studies [20]). In the same way, *d*-isomenthol (1*R*:3*S*:4*R*, from *d*-isomenthone) is related to *d*-neomenthol (1*R*:3*S*:4*S*, from *l*-menthone). One might therefore expect the metabolic fate of *d*-neoisomenthol to parallel that of *l*-menthol, while that of *d*-isomenthol would parallel that of *d*-neomenthol. Interestingly, *d*-neoisomenthyl acetate was reported as a constituent of peppermint oil in which *l*-menthyl acetate, *l*-menthol, and *d*-neomenthol were present, but *d*-neomenthyl acetate and *d*-neoisomenthol were not (21). Subsequent workers (10, 29) have reported the presence of all four menthol stereoisomers and their corresponding acetates in peppermint oil, although, with the exception of *l*-menthol, *l*-menthyl acetate, and *d*-neomenthol, all are rather minor constituents.

It is clear that the selectivity of product formation from *l*-menthone cannot be attributed to the specificity of the transferase enzymes, but must be ascribed to compartmentation effects. Because the relative activities of the two transferases appear to be sufficiently different to suggest that, in the presence of both enzymes, glucoside formation would predominate, it is necessary to postulate that the dehydrogenase specific for *l*-menthol synthesis is accessible primarily to the transacetylase, whereas the dehydrogenase specific for *d*-neomenthol synthesis is accessible to the transglucosylase (and perhaps the transacetylase as well). The system is not absolutely specific, in that exogenous *l*-menthone is converted to low, but significant, levels of *l*-menthyl-β-D-glucoside (Table I), and in that *d*-neomenthyl acetate has been identified as a minor constituent of peppermint oil (29) (although it was not detected as a metabolite of exogenous *l*-[^{3}H]menthone [Table I]). In any case, the *in vitro* and *in vivo* studies, as well as analytical studies, are all consistent with the presence of two distinct sites for the selective conversion of *l*-menthone to *l*-menthol (and the corresponding acetate) on the one hand, and to *d*-neomenthol (and the corresponding glucoside) on the other. Although preliminary evidence suggests that the sites are differentially accessible to exogenous precursors and to precursors generated endogenously (9), the nature of the postulated compartments is not known.

The rationale for two distinctive pathways for menthone metabolism is also not clear. The fact that neomenthyl glucoside formed in leaves can be later isolated from the roots (9, 18) has provided the first direct evidence supporting the hypothesis that monoterpenyl glucosides are transport derivatives (11, 15), while the recent finding that neomenthyl glucoside is subsequently converted to other metabolites (unidentified) in the roots (18) suggests that this pathway may have a specific physiological function. Alternatively, it is also possible that the glucosylation pathway in peppermint (which is probably operational in other mint species as well [27]) may be a manifestation of a more general process such as that responsible for the metabolic conjugation of xenobiotic substances in plants (13). Further studies are underway in an attempt to define in greater detail this novel metabolic system.

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