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Monoterpene and sesquiterpene biosynthesis in glandular trichomes of peppermint (*Mentha × piperita*) rely exclusively on plastid-derived isopentenyl diphosphate

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Abstract. The subcellular compartmentation of isopentenyl diphosphate (IPP) synthesis was examined in secretory cells isolated from glandular trichomes of peppermint (*Mentha × piperita* L. cv. Black Mitcham). As a consequence of their anatomy and the conditions of their isolation, the isolated secretory cells are non-specifically permeable to low-molecular-weight water-soluble metabolites. Thus, the cytoplasm is readily accessible to the exogenous buffer whereas the selective permeability of subcellular organelles is maintained. With the appropriate choice of exogenous substrates, this feature allows the assessment of cytoplasmic and organellar (e.g. plastidic) metabolism in situ. Glycolytic substrates such as [^{14}C]glucose-6-phosphate and [^{14}C]pyruvic acid are incorporated into both monoterpenes and sesquiterpenes with a monoterpene:sesquiterpene ratio that closely mimics that observed in vivo, indicating that the correct subcellular partitioning of these substrates is maintained in this model system. Additionally, exogenous [^{14}C]mevalonic acid and [^{14}C]IPP, which are both intitially metabolized in the cytoplasm, produce an abnormally high proportion of sesquiterpenes. In contrast, incubation with either [^{14}C]citrate or [^{14}C]acetyl-CoA results in the accumulation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) with no detectable isoprenoids formed. Taken together, these results indicate that the cytoplasmic mevalonic acid pathway is blocked at HMG-CoA reductase and that the IPP utilized for both monoterpene and sesquiterpene biosynthesis is synthesized exclusively in the plastids.

Key words: Isopentenyl diphosphate – Isoprenoid biosynthesis – Leucoplast – *Mentha* – Mevalonic acid pathway

Abbreviations: DMAPP = dimethylallyl diphosphate; FPP = farnesyl diphosphate; GLC = gas liquid chromatography; GPP = geranyl diphosphate; HMG-CoA = 3-hydroxy-3-methylglutaryl-CoA; IPP = isopentenyl diphosphate; PEP = phosphoenolpyruvic acid

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Introduction

Isopentenyl diphosphate (IPP) is the immediate, essential precursor leading to the formation of over 22 000 known isoprenoids (Connolly and Hill 1992). The subcellular location(s) of the synthesis of IPP in plants, and of the allylic prenyl diphosphates utilized for isoprenoid biosynthesis, has been the subject of considerable study (reviewed in Liedvogel 1986; Gray 1987; Kleinig 1989). The two subcellular compartments responsible for the bulk of isoprenoid biosynthesis in plants are the cytoplasm/endoplasmic reticulum involved in the synthesis of farnesyl diphosphate (FPP)-derived isoprenoids such as sterols, and the plastids involved in the formation of geranylgeranyl diphosphate (GGPP)-derived isoprenoids such as phytol and carotenoids (the mitochondrial synthesis of ubiquinones will not be considered here). However, in addition to ubiquitous isoprenoids such as sterols and carotenoids, plants often accumulate large amounts of a wide variety of defense-related compounds including mono-, sesqui-, di- and triterpenes (see e.g. Gershenzon and Croteau 1991). The subcellular compartmentation of IPP utilization is well established. Geranyl diphosphate (GPP) and GGPP are synthesized from IPP, and subsequently converted into monoterpenes and diterpenes in the plastid, whereas FPP is synthesized and subseqently converted into sesquiterpenes in the cytoplasm/endoplasmic reticulum (reviewed in Gershenzon and Croteau 1993).

Despite this general agreement regarding the localization of steps utilizing IPP, the subcellular locations of IPP synthesis is not completely resolved. In addition, the regulation of partitioning of IPP between the different families of isoprenoids in the plant cell is currently only poorly understood. There are two competing models of subcellular compartmentation of IPP synthesis: the first involves the parallel synthesis of IPP in each compartment in which it is utilized; the second involves the exclusive synthesis of IPP in the cytoplasm, followed by distribution of the IPP between the different locations at which it is utilized (reviewed in Gray 1987). However, more recent studies with developing chloroplasts in barley leaves have

indicated that the subcellular localization of IPP synthesis can change during development (Heintze et al. 1990). In very young barley leaf tissue, plastidic isoprenoids are synthesized from plastid-derived IPP, whereas chloroplasts in mature leaf tissue utilize IPP imported from the cytoplasm for isoprenoid biosynthesis. Studies demonstrating developmental changes in plastid envelope permeability towards acetate, citrate and mevalonate (Wellburn and Hampp 1976; Schneider et al. 1977) and IPP (Heintze et al. 1990) reinforce the view that the subcellular localization of IPP synthesis depends on the type of tissue examined and may change during development of the tissue in response to different metabolic requirements.

Glandular trichomes on peppermint (*Mentha* × *piperita*) leaves synthesize and store an essential oil composed primarily of monoterpenes with smaller amounts of sesquiterpenes (Amelunxen et al. 1969). The secretory cells of these glandular trichomes are not photosynthetic and contain only leucoplasts (Werker et al. 1985), thus these cells must rely on the import of carbon from the underlying tissue to support isoprenoid synthesis that leads to oil accumulation. Secretory cells of these glandular trichomes can be mechanically isolated in high yield as a disc of eight cells each (Gershenzon et al. 1992), reflecting the original anatomy of the gland. Several features of these isolated cells make them an ideal system for examining the subcellular compartmentation of isoprenoid biosynthesis. The glandular trichomes from which the secretory cells are isolated are at a developmental stage where cell growth and division have ceased (Amelunxen 1965) and rapid accumulation of essential oil occurs (Croteau and Martinkus 1979). For this reason, the rates of sterol and carotenoid formation should be insignificant relative to the massive levels of monoterpene and sesquiterpene biosynthesis leading to essential-oil accumulation, simplifying analysis and interpretation of the results. The isolated cells are capable of the de-novo synthesis of monoterpenes from basic precursors such as [^{14}C]sucrose (McCaskill et al. 1992), indicating that they contain all of the enzymes necessary for the synthesis of the essential-oil terpenes characteristic of peppermint. In addition, the isolated cells are non-specifically permeable towards low-molecular-weight water-soluble metabolites as a consequence of the anatomy of the trichome and the conditions of their isolation. The molecular-weight exclusion limit for this permeability is less than 1800 based on the exclusion of fluorescein-dextran (McCaskill et al. 1992) and is believed to be due to the exposure of a large number of plasmodesmata between the secretory cells and the underlying stalk cell to the external buffer as the secretory cells are isolated. As a result, endogenous low-molecular-weight constituents are washed out of the cytoplasm during isolation of the cells. This feature allows exogenous manipulation of the cytoplasmic composition of cofactors and substrates.

The judicious choice of radiolabeled substrates and cofactors added to the isolated cells, followed by quantification of the radiolabeled monoterpenes and sesquiterpenes produced, permits delineation of the subcellular partitioning of the different substrates in the cells. This in-situ approach avoids the difficulties associated with attempting to quantify the relative contributions of parallel pathways for IPP synthesis in different subcellular compartments using cell-free preparations. This report describes the use of different radiolabeled substrates to assess the relative contributions of cytoplasmic and plastidic IPP synthesis for both monoterpene and sesquiterpene biosynthesis.

Materials and methods

Plant material, chemicals and substrates. Peppermint (*Mentha* × *piperita* L. cv. Black Mitcham) plants were propagated and grown under greenhouse conditions as previously described (Gershenzon et al. 1992). Unless otherwise indicated, all chemicals and enzymes were purchased from either Sigma Chemical Co. (St. Louis, Mo., USA) or Research Organics (Cleveland, Ohio, USA). Flo-Scint III scintillation fluid for radio-high performance liquid chromatography (HPLC) was from Radiomatic Instruments and Chemical Co. (Meriden, Conn., USA). Mevinolin was a generous gift of Merck, Sharpe and Dohme Research Laboratories (Rahway, N.J., USA). Monoterpene and sesquiterpene standards were from our own collection.

[2-^{14}C]Pyruvic acid (0.59 GBq · mmol^{-1}), [1,5-^{14}C]citric acid (3.0 GBq · mmol^{-1}), *RS*-[2-^{14}C]mevalonic acid (2.12 GBq · mmol^{-1}), [1, 2-^{14}C]acetic acid (sodium salt, 2.08 GBq · mmol^{-1}), and *n*-[1-^{14}C]butyryl-CoA (0.15 GBq · mmol^{-1}) were all purchased from DuPont Co. (Boston, Mass, USA). [1-^{14}C]Acetyl-coA (2.18 GBq · mmol^{-1}), phosphoenol-[1-^{14}C]pyruvate (PEP, cyclohexylammonium salt, 0.96 GBq · mmol^{-1}) and [1-^{4}C]isopentenyl diphosphate (1.93 GBq · mmol^{-1}) were purchased from Amersham Corp., (Arlington Heights, Il., USA). D-[U-^{14}C]glucose-6-phosphate (11.0 GBq · mmol^{-1}) was purchased from American Radiolabeled Chemicals (St. Louis, Mo., USA). [1-^{3}H]Farnesyl diphosphate (3.71 GBq · mmol^{-1}; Munck and Croteau 1990) and [8-^{3}H]geranyl diphosphate (2.30 GBq · mmol^{-1}; Coates et al. 1987) were synthesized as previously described.

Isolation and incubation of secretory cells. Apical leaves (10–15 g, < 10 mm length) were harvested from vegetative stems of peppermint. Secretory cells were isolated from the leaves by mechanical abrasion with glass beads and washed essentially as described in McCaskill et al. (1992). The buffer used for washing the isolated cells consisted of 200 mM sorbitol, 10 mM sucrose, 50 mM KCl, 5 mM succinic acid, 5 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA), 0.5 mM Na_2HPO_4, 0.1 mM $Na_4P_2O_7$ and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), adjusted to pH 7.3 with KOH.

Incubation of the isolated cells with radiolabeled substrates was carried out essentially as described previously by McCaskill et al. (1992). After isolation, the cells were washed into a cofactor-rich buffer consisting of the above wash buffer supplemented with 5 mM ATP and 0.1 mM each of NAD^+, NADPH, CoA, FAD and thiamine pyrophosphate. The cell density was then adjusted to give approx. $2.5 \cdot 10^6$ cells ($3.1 \cdot 10^5$ cell disks) per 1 ml assay. Aliquots of the cells were distributed into tubes and the cells were aerated (approx. 3 ml air/min) at room temperature for 15 min using a fused silica capillary inserted into each tube. Any inhibitors or other additions to the assays were also added at this time. At the end of this preincubation period, the cells were pelleted by brief centrifugation (approx. 30 s at 100 · *g*) and then resuspended in 1 ml fresh cofactor-rich buffer per assay. At this point, the radiolabeled substrates were added along with any inhibitors or unlabeled substrates used, the cells were overlaid with 2 ml of pentane to trap the volatile isoprenoids formed, and the cells were aerated as before for 1 h at room temperature. When indicated, $^{14}CO_2$ produced from ^{14}C-labeled substrates was trapped by passing the air from the assay tubes into KOH traps as previously described (McCaskill et al. 1992).

At the end of the incubation period, the cells were extracted with the pentane overlay, followed by 3 × 1 ml of diethyl ether. After combining, the organic extracts were backwashed twice with 1 ml of

1 M Na_2CO_3, and then passed through a short column of silica gel overlaid with Mg_2SO_4 in a Pasteur pipette. An aliquot of the resulting eluate was removed for scintillation counting and the remainder was stored at −20°C until analysis by radio-gas liquid chromatography (GLC).

Analysis of isoprenoids by GLC. Quantification of the monoterpene and sesquiterpene products derived from radiolabeled substrates was carried out using radio-GLC. Mass standards of the major monoterpenes and sesquiterpenes (5 µg each) present in peppermint were added to each sample. Each extract was slowly concentrated on ice under a gentle stream of N_2 to approx. 250 µl, and then allowed to concentrate by evaporation at −20°C in a conical vial to a final volume of approx. 5 µl. Radio-GLC of the resulting sample was carried out essentially according to Croteau and Satterwhite (1990) using a Gow-Mac 550P gas chromatograph (injector temperature 200°C, thermal conductivity detector 220°C, 140 mA) attached to a Packard 894 gas proportional counter with 10–12 ml · min^{-1} propane quench gas for radio detection. The column was a Superox FA wide-bore capillary column (1.2 µm film; 0.53 mm diameter, 30 m long; 9 ml · min^{-1} He carrier gas with 90 ml · min^{-1} He makeup gas; Alltech Associates, Deerfield, Il., USA) with a temperature program of 60°C (10 min hold) followed by 5°C · min^{-1} to a final temperature of 220°C (30 min hold). The signals from the thermal conductivity detector and the radio detector were processed with a dual-channel integrator, and the data were quantified as previously described (McCaskill et al. 1992). Minor radiolabeled peaks which did not correspond to available mass standards were characterized as either monoterpenes or sesquiterpenes based on their presence in or absence from samples of cells incubated with [^{3}H]GPP or [^{3}H]FPP, respectively. The amount of radioactivity present in each resolved component was calculated from the fractional area of each peak relative to the total peak area and to the total radioactivity present in the extract. The dpm present in each resolved component was converted to nmol of isoprenoid product based on the specific activity of the radiolabeled substrate and the stoichiometry of incorporation into either monoterpenes (C_{10}) or sesquiterpenes (C_{15}). The composition of endogenous mono- and sesquiterpenes present in isolated secretory cells was determined by capillary GLC as previously described (McCaskill et al. 1992).

Detection and identification of mevalonate-pathway intermediates. Metabolism of different exogenous substrates was assessed by radio-HPLC of extracts derived from incubation of isolated cells with radiolabeled substrates as previously described (McCaskill and Croteau 1993). Briefly, cells were incubated with radiolabeled substrates (without a pentane overlay) and the incubations were quenched after 45 min by adding 4 ml of 9 M urea to the assay followed immediately by freezing in liquid N_2. After thawing on ice, the cell debris was removed by centrifugation, in the indicated assays an internal standard of *n*-[^{14}C]butyryl-CoA was added, and the radiolabeled intermediates were partially purified by adsorption on an AG 1-X8 anion-exchange membrane (acetate form; BioRad Laboratories, Hercules, Calif., USA) followed by elution with 4 ml of 1.5 M KCl in 25% (v/v) ethanol. The KCl was subsequently removed by precipitation with ethanol, followed by lyophilization. This precipitation of KCl was repeated twice more in successively smaller volumes to yield a salt-free sample in a final volume of 100 µl. The radiolabeled intermediates present in this partially purified extract were subsequently separated and quantified by reversed-phase ion-pair HPLC using an Adsorbosphere HS C18 column (5 µm particle size; 4.6 mm diam, 250 mm long, with a guard column cartridge of the same material; Alltech Associates) using a ternary solvent system of water and methanol, with tetrabutyl ammonium sulfate (10 mM, pH 6.0) as the ion-pair reagent. The eluate was passed through a Radiomatic Flo-One A120 HPLC radiodetector (Radiomatic Instruments & Chemical Co.) using a 4:1 ratio of scintillation fluid:eluate. Except where indicated, the elution conditions are identical to those previously described (McCaskill and Croteau 1993). The internal standard of *n*-[^{14}C]butyryl-CoA was used primarily as a retention time marker to aid in the identification of any closely resolved radiolabeled acyl-CoA esters detected.

Radiolabeled standards of [^{14}C]malic acid and [^{14}C]oxaloacetic acid were prepared enzymatically from [^{14}C]PEP and [^{14}C]citric acid. [^{14}C]Citric acid was incubated with 0.01 units of citrate lyase (EC 4.1.3.6 from *Enterobacter aerogenes*) to generate [^{14}C]oxaloacetate. [^{14}C]Malic acid was generated by incubating [^{14}C]citrate with 0.01 units of citrate lyase, 30 units of malic dehydrogenase (EC 1.1.1.37 from porcine heart) and 2 mM NADH. [^{14}C]Oxaloacetic acid was also generated by incubating [^{14}C]PEP with 0.1 units of PEP carboxylase (EC 4.1.1.31 from corn) in 10 mM Na_2CO_3. In each case, the radiolabeled substrates were diluted with unlabeled substrate to give a final concentration of 5 mM and the assays were carried out in 100 mM Hepes adjusted to pH 7.5 with Tris in a total volume of 100 µl with a total incubation time of 45–60 min. The assays were quenched with 300 µl of acetone and, after chilling at −20°C for 30 min, the precipitated protein was removed by centrifugation. After removal of the acetone in vacuo with a Speed-Vac concentrator (Savant Instruments Inc. Farmingdale, N.Y., USA) the retention times on HPLC of the radiolabeled products were determined.

Results

Incubation conditions. The non-specific permeability of the isolated secretory cells requires that the buffers used for isolation and incubation of the cells mimic the native composition of the cytoplasm as closely as possible. The conditions used for isolation of the secretory cells are essentially the same as previously described (McCaskill et al. 1992) with minor modifications. Succinic acid (5 mM) is included in the buffers because of its demonstrated ability to restore respiratory control in isolated mitochondria (Huang and Romani 1991); additionally succinic acid is unlikely to significantly dilute incorporation of any of the administered radiolabeled substrates. Any Ca^{2+} released during isolation due to damage to the underlying leaf tissue is removed by the inclusion of EGTA (1 mM). The cofactor supplements included in the assays were determined by examining the cofactors that would be required for incorporation of metabolic precursors into isoprenoids. For example, ATP and pyridine-nucleotide cofactors are required throughout metabolism including both the mevalonic acid pathway and the monoterpene biosynthetic pathway leading to menthone in peppermint (Croteau 1987). Thiamine pyrophosphate and CoA are included because of their roles as cofactors for pyruvate dehydrogenase (see e.g. Randall et al. 1989) and because of the role of CoA as a substrate for cytoplasmic ATP citrate lyase (Kaethner and ap Rees 1985). FAD is included because of its role as a cytoplasmic electron acceptor for cytochrome P450 reductase (Peterson and Prough 1986) which is involved in the formation of oxygenated monoterpenes from the initial cyclic olefin formed by cyclization of GPP (Croteau 1987). There are relatively few estimates available for the molar concentrations of cofactors and intermediates in subcellular compartments. For the adenine- and pyridine-nucleotide cofactors, the concentrations included in the incubation buffer were chosen to be in the range previously reported for cytoplasmic cofactor pools (reviewed in Srivastava and Bernhard 1987; Wagner and Backer 1992). Because of the paucity of information available on the subcellular concentrations of the other cofactors used, their concentrations were arbitrarily set to 100 µM.

Table 1. Rates of synthesis of monoterpenes and sesquiterpenes from radiolabeled substrates by isolated secretory cells of pepermint

Substrate	Concentration μM (MBq)	Monoterpenes nmol $(10^6$ cells$)^{-1}\cdot h^{-1} \pm$ SE	Sesquiterpenes nmol $(10^6$ cells$)^{-1}\cdot h^{-1} \pm$ SE
[2-^{14}C]Pyruvic acid ($n = 4$)	625-1200 (0.35–0.7)	0.67 ± 0.18	0.068 ± 0.022
[1-^{14}C]PEP ($n = 1$)	770 (0.7)	ND[a]	ND
[1, 5-^{14}C]Citric acid ($n = 2$)	240 (0.7)	ND	ND
[1-^{14}C]Acetyl-CoA ($n = 2$)	340 (0.7)	ND	ND
[1, 2-^{14}C]Acetate ($n = 3$)	350 (0.7)	ND	ND
R-[2-^{14}C]Mevalonic acid ($n = 4$)	22–43 (0.1–0.2)	0.24 ± 0.058	0.23 ± 0.025
[1-^{14}C]IPP ($n = 3$)	19 (0.035)	0.074 ± 0.006	0.042 ± 0.008
[8-^{3}H]GPP ($n = 1$)	8 (0.018)	4.05	NA[b]
[1-^{3}H]FPP ($n = 2$)	10 (0.035)	NA	1.37 ± 0.19

[a] ND = not detectable
[b] NA = not applicable

Table 2. Comparison of isoprenoids in isolated secretory cells of peppermint with those synthesized in situ

Component	% of Total composition ± SE ($n = 4$)	
	Endogenous isoprenoids	Synthesized from [^{14}C]pyruvic acid
Limonene (C_{10})	31 ± 3.6	13 ± 2.6
Menthone (C_{10})	49 ± 4.4[a]	53 ± 4.6
Pulegone (C_{10})	2 ± 0.5	11 ± 2.6
Caryophyllene (C_{15})	5 ± 1.1	4 ± 1.5
Humulene (C_{15})	2 ± 0.4	2 ± 1.2
Germacrene D (C_{15})	9 ± 1.6	4 ± 0.7
Other	2 ± 0.5[b]	13 ± 3.1[c]

[a] For the purpose of comparison with the radiolabeled products formed, both isomenthone and menthofuran were included in the values for menthone since these monoterpenes were not completely resolved from menthone by the radio detector
[b] Minor isoprenoids comprising < 1% of the total each
[c] Minor, unidentified radiolabeled monoterpenes and sesquiterpenes

Incorporation of radiolabeled substrates into isoprenoids. Incorporation of [2-^{14}C]pyruvic acid into both monoterpenes and sesquiterpenes by isolated secretory cells could be readily demonstrated (Table 1), although relatively high levels (0.6–1.2 mM) of undiluted radiolabeled substrate are required in order to reliably quantify the low levels of sesquiterpenes formed. The distribution of isoprenoid products derived from [^{14}C]pyruvate closely resembles the composition of endogenous monoterpenes and sesquiterpenes observed in the isolated cells (Table 2), indicating that the isolated secretory cells are able to mimic the in vivo biosynthesis of essential oil using [^{14}C]pyruvate and that limonene, the initial monoterpene olefin formed by the cyclization of GPP (Croteau 1987), is metabolized to the oxygenated monoterpenes characteristic of the essential oil in peppermint. In addition, [^{14}C]pyruvate gives varying levels of three other non-isoprenoid, ether-soluble products that are detected by radio-GLC (Fig. 1, trace b). These products are characterized as non-isoprenoid in origin because they are not detected in the assay products derived from either [^{14}C]mevalonic acid, [^{3}H]GPP, [^{3}H]FPP (Fig. 1) or [^{14}C]IPP (not shown). No further attempt was made to identify these non-isoprenoid products, although the two early-eluting products are undoubtedly low molecular weight (due to their elution behavior on radio-GLC) and the third product is likely derived from metabolism of cytoplasmic [^{14}C]acetyl-CoA derived from [^{14}C]pyruvate (see below).

Mitochondrial and plastidic pyruvate dehydrogenase represent the two subcellular sites for the formation of acetyl-CoA from pyruvic acid (Randall et al. 1989). Cytoplasmic acetyl-CoA utilized for isoprenoid biosynthesis is most likely derived from mitochondrial acetyl-CoA by the export of mitochondrial citrate to the cytoplasm (reviewed in Hanson 1985), with the subsequent cleavage of the citrate to acetyl-CoA and oxaloacetate by cytoplasmic ATP citrate lyase (Kaethner and ap Rees 1985; Liedvogel 1986). For this reason, both [^{14}C]citric acid and [^{14}C]acetyl-CoA were assessed as substrates for isoprenoid formation. In contrast to the efficient incorporation of [2-^{14}C]pyruvic acid, at similar levels neither [1, 5-^{14}C]citric acid nor [1-^{14}C]acetyl-CoA give any detectable incorporation into isoprenoids (Table 1). Both [^{14}C]acetyl-CoA and [^{14}C]citrate readily equilibrate with cytoplasm due to the permeability of the isolated cells (McCaskill et al. 1992); since the plastid envelope is impermeable to acyl-CoA esters (Ohlrogge et al. 1993) both of these substrates would therefore be restricted to providing acetyl-CoA for isoprenoid biosynthesis via the cytoplasmic mevalonic acid pathway. Indeed, both [^{14}C]acetyl-CoA (Fig. 1, trace a) and [^{14}C]citrate (not shown) do produce one of the same non-isoprenoid products that are also derived from [^{14}C]pyruvate, indicating that these two substrates are metabolized by the isolated cells. The lack of incorporation of either [^{14}C]citrate or [^{14}C]acetyl-CoA into isoprenoids indicates that [^{14}C]pyruvic acid is probably not incorporated into isoprenoids via the cytoplasmic mevalonic acid pathway.

The possible involvement of the cytoplasmic mevalonic acid pathway was also assessed by measuring the effect of mevinolin on the incorporation of [^{14}C]pyruvic acid into isoprenoids. Mevinolin is a potent, specific inhibitor of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, and has been used to distinguish between cytoplasmic and plastidic isoprenoid biosynthetic pathways (reviewed in

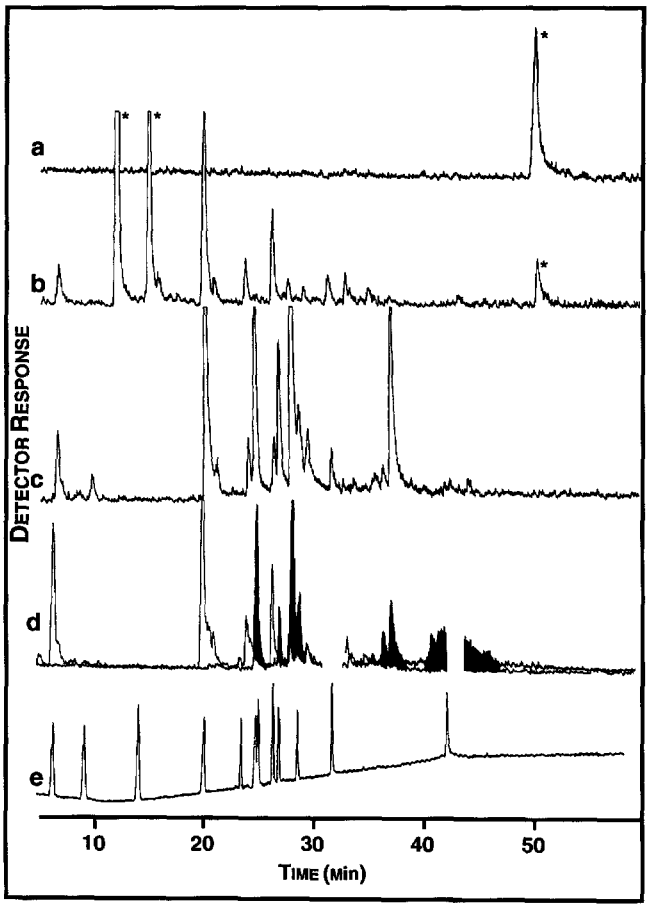

Fig. 1. Representative radio-GLC traces of radiolabeled ether-soluble products synthesized by isolated secretory cells of peppermint from [^{14}C]acetyl-CoA (*trace a*), [^{14}C]pyruvic acid (*trace b*), [^{14}C]mevalonic acid (*trace c*), and [^{3}H]GPP and [^{3}H]FPP (*trace d*). The radio-chromatograms derived from assays with [^{3}H]GPP and [^{3}H]FPP as substrates were overlaid to facilitate comparison of the monoterpene and sesquiterpene products formed. Radiolabeled monoterpenes derived from [^{3}H]GPP are shown in white, whereas sesquiterpenes derived from [^{3}H]FPP are shown in black. For clarity, the radiolabeled geraniol and farnesol, derived from hydrolysis of the corresponding substrates, were omitted from the chromatograms. The internal standards (*trace e*), in order of their elution are; limonene, isopentenol, dimethylallyl alcohol, menthone, linalool, caryophyllene, neomenthol, pulegone, humulene, piperitone, geraniol and farnesol. The marked peaks (*) in (*a*) and (*b*) represent unknown, non-isoprenoid products

Burden et al. 1989). Cytoplasmic sterol biosynthesis can be inhibited by mevinolin at concentrations in the low-nanomolar range, whereas it has little effect on plastidic carotenoid biosynthesis even at elevated concentrations. Preincubation of secretory cells with mevinolin (1 μM) for 20 min before the addition of [^{14}C]pyruvate had no effect on either the rate of monoterpene and sesquiterpene formation, or the overall ratio of monoterpenes to sesquiterpenes (C_{10}:C_{15}) formed from [^{14}C]pyruvic acid. Three independent experiments gave a C_{10}:C_{15} ratio of 6.0 ± 1.6 without mevinolin and 5.9 ± 0.4 with mevinolin, indicating that it is unlikely that the cytoplasmic mevalonic acid pathway contributes significantly to either mono- or sesquiterpene biosynthesis from [^{14}C]pyruvic acid.

[1, 2-^{14}C]Acetate also gives no detectable incorporation into isoprenoids (Table 1). Although McCaskill et al (1992) previously demonstrated low levels of incorporation of [^{14}C]acetate into monoterpenes by isolated secretory cells, that study used a considerably larger number of cells per assay (4–8·10^{6} vs. 2.5·10^{6} cells/assay in this study) and extended incubation periods of up to 4 h. The lack of observed incorporation of [^{14}C]acetate into isoprenoids in this study indicates that export of free acetate from the mitochondria with subsequent conversion to acetyl-CoA by plastidic acetyl-CoA synthetase, as may occur in acyl lipid biosynthesis (reviewed in Ohlrogge et al. 1993), contributes little, if any, acetyl-CoA for isoprenoid biosynthesis in secretory cells relative to that provided by metabolism of pyruvate.

The overall C_{10}:C_{15} ratio formed from both [^{14}C]glucose-6-phosphate and [^{14}C]pyruvate is similar to that observed in the endogenous essential oil (Table 3), indicating that these two glycolytic intermediates are partitioned between the two families of isoprenoids in a way that mimics that observed in vivo. Although both [^{14}C]mevalonic acid and [^{14}C]IPP give readily detectable incorporation into both monoterpenes and sesquiterpenes (Table 1, Fig. 1), the product distribution is strongly biased in favor of sesquiterpene formation, in contrast to that observed with the two glycolytic intermediates (Table 3). This bias reflects both a lower rate of monoterpene biosynthesis and, in the case of mevalonic acid, a higher rate of sesquiterpene biosynthesis relative to that observed from [^{14}C]pyruvate (Table 1). The formation of monoterpenes from exogenous [^{14}C]IPP indicates that the plastids are permeable towards cytoplasmic IPP, although the bias towards sesquiterpene biosynthesis indicates that exogenous [^{14}C]IPP is utilized preferentially by cytoplasmic FPP synthase rather than plastidic GPP synthase. The observation that exogenous [^{14}C]mevalonic acid produces a similar bias towards sesquiterpene formation indicates that exogenous mevalonic acid is also metabolized principally in the cytoplasm, producing cytoplasmic IPP which is preferentially utilized by FPP synthase.

Recent reports of the presence of a novel pathway for IPP synthesis in bacteria (see e.g. Rohmer et al. 1993), involving condensation of an activated-two carbon intermediate derived from pyruvate decarboxylation with a three-carbon phosphorylated intermediate derived from glycolysis, prompted the examination of [1-^{14}C] PEP as a potential substrate for isoprenoid biosynthesis. Assuming that there is enough gluconeogenic flux for PEP to

Table 3. Partitioning of radiolabeled substrates between monoterpenes and sesquiterpenes by isolated secretory cells of peppermint

Substrate	C_{10}:C_{15} ± SE
Endogenous composition (n = 4)[a]	7.4 ± 2.6
[^{14}C]Glucose-6-phosphate (n = 1)	8.9
[^{14}C]Pyruvic acid (n = 4)	10.8 ± 1.7
[^{14}C]Mevalonic acid (n = 4)	1.04 ± 0.23
[^{14}C]IPP (n = 3)	1.86 ± 0.33

[a] Composition of oil in isolated secretory cells

form the presumptive three-carbon phosphorylated intermediate, [1-^{14}C]PEP should allow for the incorporation of ^{14}C into IPP, whereas incorporation via the classical mevalonic acid pathway would result in complete loss of label from [1-^{14}C]PEP as $^{14}CO_2$, from decarboxylation of the [1-^{14}C]pyruvic acid formed. Incubation of isolated secretory cells with [1-^{14}C]PEP gives no detectable incorporation into isoprenoids (Table 1), although up to 30% of the administered ^{14}C is converted to $^{14}CO_2$, indicating that the PEP is metabolized extensively via pyruvate dehydrogenase. The very efficient incorporation of [2-^{14}C]pyruvic acid into isoprenoids, coupled with the complete lack of detectable incorporation from [1-^{14}C]PEP, indicate that the incorporation observed from [^{14}C]pyruvic acid is not due to this novel pathway present in bacteria.

Detection of radiolabeled intermediates of the mevalonic acid pathway. The lack of detectable incorporation of either [^{14}C]citrate or [^{14}C]acetyl-CoA into isoprenoids, coupled to the efficient incorporation of [^{14}C]mevalonic acid into both monoterpenes and sesquiterpenes, albeit with an abnormal product distribution, prompted an examination of the metabolism of these different substrates via radio-HPLC. Cells were incubated with the corresponding substrates as well as with [^{14}C]pyruvic acid for 45 min, and the radiolabeled water-soluble intermediates were extracted and analyzed as described in *Materials and methods*. Metabolism of [^{14}C]pyruvic acid produces readily detectable levels of acetoacetyl-CoA, HMG-CoA, mevalonic acid and mevalonate phosphate (Fig. 2, trace a). A substantial amount of HMG-CoA is produced from [^{14}C]acetyl-CoA, although only low levels of HMG-CoA are formed from [^{14}C]citric acid (Fig. 2, trace b and c). However, neither [^{14}C]citrate nor [^{14}C]acetyl-CoA produce any detectable intermediates of the mevalonic acid pathway beyond HMG-CoA. Metabolism of [^{14}C]mevalonic acid (Fig. 2, trace d) produces readily detectable levels of mevalonate phosphate, mevalonate diphosphate, IPP, dimethylallyl diphosphate (DMAPP) and FPP. The lack of detectable GPP is not surprising considering the bias towards sesquiterpene biosynthesis exhibited with [^{14}C]mevalonic acid as substrate (Fig. 1; Tables 1, 3). Although no attempt was made to determine whether the intermediates observed in Fig. 2 represent steady-state levels, the lack of detectable intermediates beyond HMG-CoA derived from either [^{14}C]citrate or [^{14}C]acetyl-CoA, taken together with the lack of observed incorporation into either monoterpenes or sesquiterpenes from these substrates and the readily observable intermediates derived from exogenous mevalonic acid, indicate that the cytoplasmic mevalonic acid pathway is impaired at the level of HMG-CoA reductase.

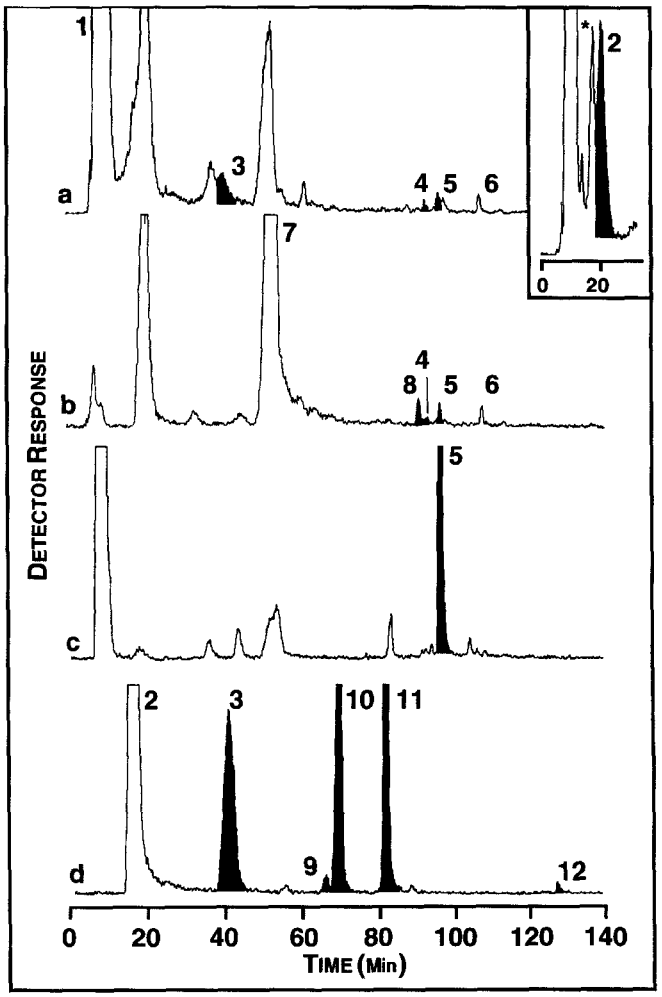

Fig. 2. Analysis by radio-HPLC of water-soluble metabolites derived from [^{14}C]pyruvic acid (*trace a*), [^{14}C]citric acid (*trace b*) [^{14}C]acetyl CoA (*trace c*) and [^{14}C]mevalonic acid (*trace d*). The inset in *trace a* shows improved resolution of [^{14}C]mevalonic acid from other organic acids derived from [^{14}C]pyruvic acid that was achieved by an initial isocratic elution with 20 mM (rather than 10 mM) tetra-*n*-butylammonium sulfate. The indicated peak in the inset (*) was tentatively identified as [^{14}C]malic acid. Intermediates of the mevalonic acid pathway derived from each substrate are indicated in black. The numbered peaks correspond to: *1*, [^{14}C]pyruvic acid; *2*, [^{14}C]mevalonic acid; *3*, [^{14}C]mevalonate phosphate; *4*, [^{14}C]acetoacetyl-CoA; *5*, [^{14}C]HMG-CoA; *6*, *n*-[^{14}C]butyryl-CoA (internal standard); *7*, [^{14}C]citric acid; *8*, acetyl-CoA; *9*, [^{14}C]DMAPP; *10*, [^{14}C]mevalonate diphosphate; *11*, [^{14}C]IPP; *12*, [^{14}C]FPP

Discussion

This study demonstrates that both monoterpene and sesquiterpene synthesis leading to essential-oil formation in the glandular trichomes of peppermint rely exclusively on plastid-derived IPP. Figure 3 summarizes these results with a model for subcellular compartmentation of IPP synthesis in the non-photosynthetic secretory cells of glandular trichomes. The major features of this model are: the cytoplasmic mevalonic acid pathway is blocked at HMG-CoA reductase; the acetyl-CoA utilized for IPP synthesis is derived from plastidic pyruvate dehydrogenase; and the IPP utilized for both monoterpene and sesquiterpene biosynthesis is derived exclusively from the plastidic mevalonic acid pathway.

The efficient incorporation of [^{14}C]pyruvic acid into both monoterpenes and sesquiterpenes, with a product distribution that closely mimics that observed in vivo indicates that exogenous pyruvic acid is partitioned

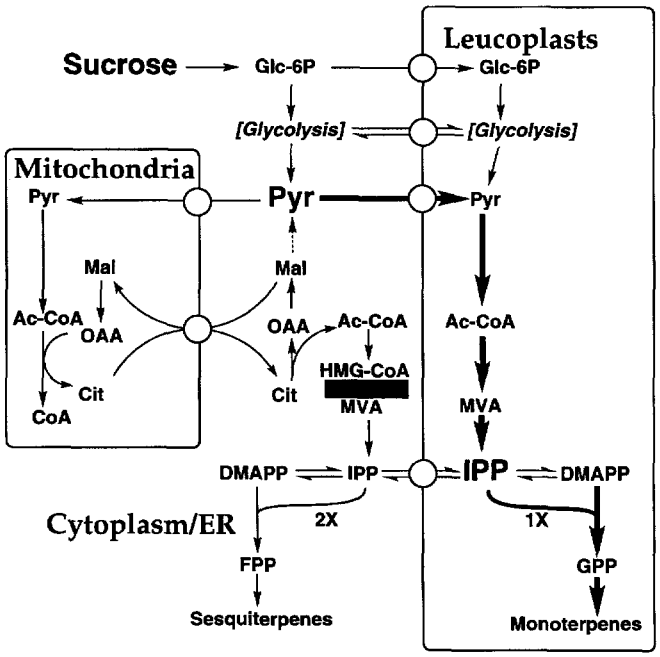

Fig. 3. Proposed model for subcellular compartmentation of isoprenoid biosynthesis in the secretory cells of peppermint glandular trichomes. The abbreviations correspond to: *Cit*, citric acid; *Glc-6P*, glucose-6-phosphate; *Mal*, malic acid; *MVA*, mevalonic acid; *OAA*, oxaloacetic acid; *Pyr*, pyruvic acid. The *bold arrows* indicate the route of pyruvic acid incorporation into isoprenoids exclusively via the plastidic mevalonic acid pathway; the major products formed are monoterpenes. The cytoplasmic mevalonic acid pathway is blocked at HMG-CoA reductase

between the monoterpene and sesquiterpene biosynthetic pathways properly. The absence of observable isoprenoid formation from either exogenous [^{14}C]citrate or [^{14}C]acetyl-CoA in secretory cells, coupled to the efficient incorporation of exogenous [^{14}C]mevalonic acid into isoprenoids, indicate that one or more steps of the cytoplasmic mevalonic acid pathway is impaired. The observation that HMG-CoA derived from exogenous [^{14}C]acetyl-CoA accumulates, whereas no intermediates of the mevalonic acid pathway beyond HMG-CoA are detectable (Fig. 2, trace c), indicates that cytoplasmic HMG-CoA reductase is inoperative, thus explaining the lack of incorporation into isoprenoids from either cytoplasmic acetyl-CoA or citrate. Taken together, along with the lack of observed incorporation of exogenous [^{14}C]acetate into isoprenoids, these results argue against the involvement of acetyl-CoA derived from mitochondrial pyruvate dehydrogenase in either monoterpene or sesquiterpene formation. Therefore, both monoterpene and sesquiterpene biosynthesis observed from administered glycolytic intermediates, such as [^{14}C]glucose-6-phosphate and [^{14}C]pyruvate, must be supported exclusively by plastidic formation of acetyl-CoA, and thus plastid-derived IPP.

Sesquiterpenes typically comprise approx. 2% of the essential oil accumulated by peppermint (Lawrence et al. 1972). However, a comparison of the rates of monoterpene biosynthesis from [^{3}H]GPP with sesquiterpene biosynthesis from [^{3}H]FPP (Table 1) indicates that the capacity for sesquiterpene formation is much higher than is reflected in oil composition. Additionally, the disproportionate amount of sesquiterpenes formed from exogenous [^{14}C]IPP (Tables 1, 3) indicates that the relatively low level of sesquiterpenes accumulated in the oil is not due to limited capacity for cytoplasmic FPP synthesis relative to the capacity for plastidic GPP synthesis. The ability of IPP to partition in either direction across the plastid envelope is demonstrated by the incorporation of [^{14}C]pyruvic acid (and thus plastid-derived IPP) into sesquiterpenes via cytoplasmic FPP synthase and the incorporation of exogenous (cytoplasmic) [^{14}C]IPP into monoterpenes via plastidic GPP synthase. Since both monoterpene and sesquiterpene biosynthesis in secretory cells must rely on plastid-derived IPP, the relatively small amount of sesquiterpenes found in peppermint oil is probably determined by competition for the pool of plastidic IPP between plastidic GPP synthase and cytoplasmic FPP synthase.

The demonstration that essential-oil formation in secretory cells from glandular trichomes of peppermint relies exclusively on plastidic (i.e. leucoplast) synthesis of IPP raises several questions regarding the manner in which essential-oil formation is sustained in these specialized cells. The relative contributions of cytoplasmic and plastidic glycolysis to the production of plastidic pyruvic acid are unknown and, although it is not shown in Fig. 3, it is likely that considerable flux through the pentose-phosphate pathway is required in order to provide the NADPH required for synthesis of IPP inside the leucoplast (i.e., for HMG-CoA reductase). The observation that the cytoplasmic mevalonic acid pathway appears to be latent (i.e., blocked only at HMG-CoA reductase) suggests that earlier in development the secretory cells probably could utilize IPP synthesized in the cytoplasm for the synthesis of sterols required for membrane biogenesis during cell growth. It is unlikely that this impaired cytoplasmic HMG-CoA reductase activity is an artifact due to the isolation conditions of the cells since the incorporation of [^{14}C]pyruvic acid through an exclusively plastidic pathway produces a C_{10}:C_{15} ratio that closely mimics the composition of the endogenous oil. Thus, significant flux of intermediates through the cytoplasmic pathway in vivo would be expected to lead to a much higher proportion of sesquiterpenes than that observed. There are numerous regulatory controls of HMG-CoA reductase activity (reviewed in Stermer et al. 1994); the manner and timing in which cytoplasmic HMG-CoA reductase in secretory cells has been down-regulated during development remains to be determined.

The glandular trichomes of a wide variety of species synthesize and store essential oils, and undergo similar ontogeny to that observed in peppermint (Amelunxen 1965). The presence of leucoplasts with a well-defined morphology in secretory cells present in glandular trichomes, resin ducts, secretory cavities and idioblasts has been closely correlated with monoterpene formation (Cheniclet and Carde 1985). This study also demonstrated a quantitative relationship between leucoplast volume and the relative amount of monoterpenes present in the essential oil. However no correlation was found between leucoplast morphology and sesquiterpene content in the essential oil. These morphological observations, taken together with the biochemical evidence in the current study,

suggest that the synthesis of IPP exclusively in the leucoplast to support monoterpene biosynthesis may be a common feature among tissues accumulating essential oils.

An important advantage of utilizing semi-permeable cells for this type of study is that the cytoplasm is readily accessible to the exogenous buffer, whereas the selective permeability of intracellular membranes, such as the plastid envelope, is retained. This study demonstrates that with the careful selection of administered radiolabeled substrates, this feature allows discrimination between parallel cytoplasmic and organellar (e.g. plastidic) metabolic pathways, without the complications associated with preparing cell-free extracts. Previous models for IPP synthesis have focused either on the exclusive synthesis of IPP in the cytoplasm, with subsequent distribution of IPP to the different subcellular sites of isoprenoid biosynthesis, or the parallel synthesis of IPP in separate, independant compartments in which isoprenoid biosynthesis occurs (reviewed in Gray 1987). This study illustrates a third alternative, in which IPP is synthesized exclusively in the plastid, with subsequent partitioning between plastidic monoterpene biosynthesis and cytoplasmic sesquiterpene biosynthesis.

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