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BIOSYNTHESIS OF MONO- AND SESQUITERPENES IN PEPPERMINT FROM MEVALONATE-2-^{14}C*

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Abstract—The volatile oil from peppermint (*Mentha piperita* L.) is composed primarily of monoterpenes with less than 2% sesquiterpenes. However, radioactivity from mevalonate-2-^{14}C is incorporated into caryophyllene and other sesquiterpene hydrocarbons much more extensively than into monoterpenes by peppermint cuttings. Both mono- and sesquiterpenes show maximum incorporation of label after 6 hr (0·03% vs. 0·33% of the physiological isomer) and lose 75% of the incorporated label after an additional 6 hr. Caryophyllene derived from mevalonate-2-^{14}C after 6 hr of incorporation was chemically degraded. The isoprenoid origin of caryophyllene was confirmed, and preferential labelling of the isopentenyl pyrophosphate derived portions of the molecule was noted. On the basis of such evidence it appears that separate sites may exist for the biosynthesis of mono- and sesquiterpenes and that an endogenous dimethylallyl pyrophosphate pool may participate in the biosynthesis of sesquiterpenes in peppermint.

INTRODUCTION†

COMPARTMENTALIZATION of terpenoid metabolism is now well established, and it is regarded as one probable factor in the regulation of higher terpenoid biosynthesis in plants.[1] The main aspects of this regulatory mechanism appear to be the segregation of enzymes, and the relative impermeability of intracellular membranes (e.g. chloroplast membranes) to terpenoids and their immediate precursors, including mevalonic acid. Thus the site of sterol and triterpene biosynthesis in leaves is extrachloroplastic and is relatively accessible to exogenous MVA–^{14}C, while the biosynthesis of carotenoids occurs only within chloroplasts, preferentially utilizing endogenous MVA derived from photosynthetically-fixed CO_2. Whether analogous regulatory mechanisms apply in the biosynthesis of the lower (hemi-, mono- and sesqui-) terpenes is uncertain.

The biosynthesis of monoterpenes from exogenous MVA has been firmly established in certain flower tissues.[2,3] However, plants with distinct oil glands, such as peppermint, generally appear unable to effectively utilize exogenous MVA for the *in vivo* biosynthesis of monoterpenes. Numerous investigators have reported very low levels (0·01–0·1%) of MVA incorporation into monoterpenes,[4] and in some cases label appeared to be assimilated only after prior degradation to CO_2.[5] In fact, CO_2 was shown to be the most efficient precursor of monoterpenes in peppermint.[4] Low levels of mevalonate incorporation might sometimes

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† Abbreviations used throughout the text: MVA = mevalonic acid, IPP = isopentenyl pyrophosphate, DMAPP = dimethylallyl pyrophosphate.

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[2] M. J. O. FRANCIS, D. V. BANTHORPE and G. N. J. LE PATOUREL, *Nature, Lond.* **228**, 1005 (1970).

[3] P. J. GODIN, H. S. INGLIS, M. SNAREY and E. M. THAIN, *J. Chem. Soc.* 5878 (1963).

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[5] R. G. BATTU and H. W. YOUNGKEN, *Lloydia* **29**, 360 (1966).

be attributed to poor uptake of this material. In peppermint, for example, MVA is not readily translocated when administered through a 'leaf flap' (phloem system) although transport is rapid when MVA is administered through the cut stem (primarily the xylem system).[6] Similar results have been noted by Waller and co-workers in studies with other plants.[7,8] Low incorporations of exogenous MVA cannot then be attributed to poor uptake when the xylem system is employed, as in the present work, but would appear to reflect the inability of exogenous MVA to penetrate from the xylem to the site of monoterpene biosynthesis, possibly the secretory cells of oil glands. As xylem transport of MVA is probably not a normal physiological phenomenon, it is not surprising that there should exist a barrier to translocation of exogenous MVA from leaf xylem elements to the specific leaf cells that synthesize monoterpenes. Such transport, like phloem transport, would be via the protoplasm of living cells.

Few observations have been made concerning the *in vivo* biosynthesis of sesquiterpenes in higher plants. MVA is incorporated into abscisic acid in intact avocado and banana fruit,[9] and into the picrotoxane sesquiterpenes coriamyrtin and tutin in *Coriaria japonica*.[10,11] Both MVA and farnesol are incorporated into the furanoid sesquiterpene ipomeamarone in sweet potato root infected with black rot.[12,13] In none of these cases did incorporation of label exceed a few tenths of a per cent.

Mono- and sesquiterpenes have traditionally been found together in essential oil extracts or distillates, and recent work has demonstrated that oil glands from several plants, including peppermint, actually contain the mono- and sesquiterpenes characteristic of the plant.[14–19] There is a natural tendency then to regard mono- and sesquiterpenes as closely related, both in regard to biochemical precursors and to site of synthesis. This association, however, has not been documented, and while mono- and sesquiterpenes may be biosynthetically related in a broad sense, their isolation together does not necessarily imply a common site or pathway of origin.

Previous work has demonstrated that administered MVA-2-^{14}C is not appreciably incorporated into monoterpenes of peppermint leaf.[4,5] It was noted in this laboratory, however, that other higher molecular weight compounds, tentatively identified as sesquiterpene hydrocarbons, became significantly labelled when mint cuttings were administered MVA-2-^{14}C through the cut stems.

The present study was directed toward determining the origin, identity, and metabolic fate of these higher molecular weight compounds in an attempt to clarify lower terpenoid biosynthesis in this plant.

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RESULTS AND DISCUSSION

Essential Oil Composition

The essential oil from *Mentha piperita* L. has been well characterized with respect to the major components and contains 60–85% menthone and menthol, 20–30% mixed oxygenated monoterpenes, and 6–12% mixed mono- and sesquiterpene hydrocarbons.[20–24] This plant yields roughly 0·1 per cent steam–volatile oil on a fresh-weight basis;[25] however, environmental factors have a profound influence on both the quantity and composition of the oil.[26]

When mevalonate-2-^{14}C was tested as a precursor for the biosynthesis of terpenes in mint cuttings, radioactivity was not appreciably incorporated into the monoterpenes (a maximum of 0·03% incorporation of the L-isomer). Gas radiochromatography of hexane extracts of the cuttings did indicate, however, that higher molecular weight compounds, eluting after the monoterpenes on both polar and non-polar columns, did become labelled. Treatment of extracts with silicic acid to remove oxygenated terpenes, followed by gas radiochromatography of the hydrocarbon fraction, revealed that the radioactive components were hydrocarbon in nature.

Similar unlabelled hydrocarbon fractions from peppermint were subjected to GLC–MS, and the following sesquiterpenes were identified by comparison of gas chromatographic retention behavior and mass spectra to authentic compounds and to the literature:[27–29] β-elemene, β-bourbonene, caryophyllene, α-humulene, γ-muurolene and δ-cadinene (or possibly ω-cadinene as indicated by Connell *et al.*[30]). Components tentatively identified were α-cubebene, α-bourbonene, α-muurolene and γ-cadinene. Several components with retention times in the sesquiterpene hydrocarbon range remained unidentified, but in all cases the mass spectra indicated a molecular ion at *m/e* 204, consistent with the assignments of empirical formula $C_{15}H_{24}$. Caryophyllene and γ-muurolene were the major sesquiterpenes, and under the growth conditions employed in this study, caryophyllene comprised over 50% of the sesquiterpene fraction and up to 0·8% of the essential oil. These analytical results are in general agreement with other more detailed analyses of the sesquiterpene hydrocarbons in *M. piperita* oil.[23,24]

Mevalonic Acid-2-^{14}C as a Terpenoid Precursor

Aqueous solutions of DL-mevalonic acid-2-^{14}C (5 μc, 1·06 μmoles) were administered to single peppermint cuttings through the cut stem. After appropriate time intervals the essential oil was extracted from each cutting. The hydrocarbons were separated from the oil by treatment with silicic acid, and both the hydrocarbon and oxygenated terpene fractions were analyzed by gas radiochromatography. The incorporation of radioactivity from MVA-2-^{14}C into mono- and sesquiterpenes varied with time as shown in Fig. 1. Caryophyllene incorporated label to the greatest extent, followed by γ-muurolene which acquired maximum

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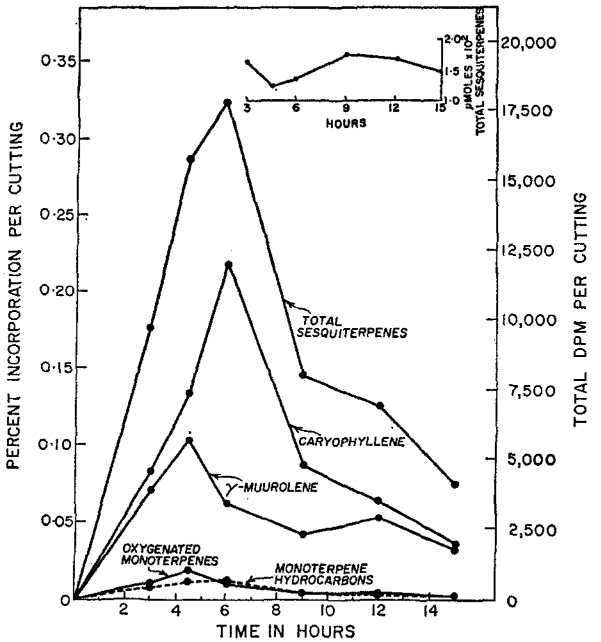

Fig. 1. Time course of labelling of peppermint mono- and sesquiterpenes from mevalonate-2-^{14}C.

Data are from a single set of matched cuttings but three replicates revealed curves essentially identical. Percent incorporation is calculated assuming that only L-MVA-2-^{14}C is physiologically active.

label somewhat earlier than caryophyllene. All of the other sesquiterpenes incorporated some label, and often with a higher specific activity than caryophyllene. Under the conditions of GLC analysis the individual identities of some of the minor components were difficult to assess because of poor resolution, and these activites are included only in the 'total sesquiterpene' plot. Since the oxygenated monoterpenes and monoterpene hydrocarbons acquired very little label they are plotted as classes rather than as individual components.

The sesquiterpenes acquired label rapidly for approximately 6 hr and then lost label again. At the end of 9 hr approximately 60% of maximal label had been lost, while after 15 hr approximately 80% of the label had been turned over. A rapid turnover was thus established for sesquiterpenes in peppermint, as has been previously demonstrated for peppermint monoterpenes derived from $^{14}CO_2$.[31] This evidence appears to be the first demonstration of a rapid turnover of sesquiterpenes in plants and adds to the growing body of evidence indicating that lower terpenoids and other 'secondary plant products' are metabolically active and are not biosynthetic 'dead ends'. Figure 1 also shows the sesquiterpene content per cutting throughout the time course experiment. The cutting-to-cutting variation in μmoles exceeds the amount of MVA incorporated, and may represent differences in essential oil storage pools of the cuttings. Within the rather narrow limits observed in this experiment the apparent pool size bore little relation to the ability of the cutting to utilize exogenous MVA. Storage pools, such as the oil contained in glandular secretory spaces, probably turn over quite slowly, and the turnover of sesquiterpenes observed in this experiment most likely represents the turnover of a more active 'metabolic'

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pool. However, present techniques cannot distinguish between these pools, so Fig. 1 necessarily represents the overall changes occurring within the cuttings. The change in slope beginning at about 9 hr in Fig. 1 may then represent secretion of terpene to a less active storage pool rather than change in activity of a metabolic pool.

The most striking aspect of the time course study is the fact that the sesquiterpenes, which comprised but a small fraction of the essential oil, incorporated label from DL-MVA-2-^{14}C much more extensively than did the monoterpenes (0·33% vs. 0·03% incorporation of L-MVA in 6 hr). This contrasts with recent work demonstrating the incorporation of $^{14}CO_2$ and glucose-U-^{14}C into mono- and sesquiterpenes of peppermint cuttings.[32] In the case of glucose-U-^{14}C both mono- and sesquiterpenes again acquired maximum label at 6 hr and showed rapid turnover. However, the monoterpenes incorporated considerably more label than the sesquiterpenes, in a ratio roughly approximating the natural proportion of these materials in the plant (1·17% vs. 0·06%). Similar results were obtained when $^{14}CO_2$ was administered to peppermint cuttings in the light. Although specific inhibition of monoterpene biosynthesis under the conditions of MVA incorporation is a possibility that cannot be overlooked, the evidence suggests the presence of separate sites for monoterpene and sesquiterpene biosynthesis, the sesquiterpene site being the more accessible to exogenous MVA-2-^{14}C, while the monoterpene site is more accessible (or at least as accessible) to exogenous glucose-U-^{14}C and photosynthetically-fixed $^{14}CO_2$.

The extent of incorporation of MVA into sesquiterpenes is considerably greater than the incorporation of MVA into monoterpenes in mint, but is considerably less than, for example, the incorporation of MVA into monoterpenes in rose petals under similar conditions (up to 21·6% of the L-isomer in 1 hr[33]). Rose petals also show a very much faster monoterpene turnover rate than does mint (over 50% turnover in less than 2 hr[33]) and rose petals lack the ability of mint to accumulate monoterpenes. These comparisons could be taken to mean that mono- and sesquiterpene biosynthesis in mint occurs at sites relatively isolated from the rest of the plant, and seem to be related to the fact that mint leaves bear distinct oil glands,[34] while rose petals have no such glands.[2] In fact it has been previously suggested[4] that monoterpene biosynthesis in mint may be compartmentalized in the secretory cells of oil glands, in a site not readily accessible to exogenous MVA. If sesquiterpene biosynthesis is now assumed to be compartmentalized in these secretory cells, then an even greater degree of isolation of the site of monoterpene biosynthesis must be invoked to explain the difference in the degree of labelling observed. An alternative explanation might be that only monoterpene biosynthesis is compartmentalized within the secretory cells, while sesquiterpene biosynthesis is associated, in some way, with higher terpenoid synthesis taking place elsewhere in the plant. Presumably such a 'non-glandular' site is more accessible to MVA but lacks the ability to biosynthesize monoterpenes. In support of this latter possibility is recent indirect evidence suggesting that sesquiterpenes in *Pogostemon cablin* may be formed at other locations in the leaf in addition to the external oil glands.[19]

At present there is insufficient evidence to allow a choice between these, or other alternatives, but it does appear that some type of compartmentalization of biosynthetic sites is involved in the biosynthesis of lower terpenoids in mint. The oil glands may be of fundamental importance in such compartmentalization as significant differences are observed in the ability to utilize exogenous MVA between tissues that have oil glands (e.g. peppermint)

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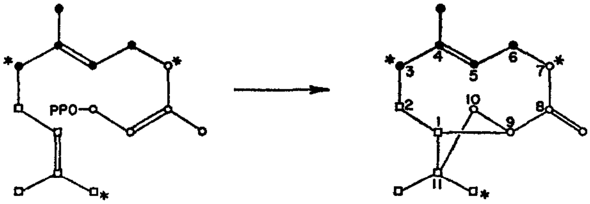
and those that do not (e.g. rose petals). Amelunxen[35] has observed unique osmium-staining bodies in cells of peppermint leaves, both in the oil glands and in mesophyll cells, and he has suggested that these bodies may function as sites of essential oil synthesis. If this interpretation is correct, it strongly suggests the possibility of compartmentalization between cells, or even within a cell, and it could provide a structural basis for our experimental results.

Chemical Degradation of Caryophyllene and Distribution of Label

To prove conclusively that labelled sesquiterpenes in peppermint were derived directly from MVA-2-^{14}C it was necessary to chemically degrade one of these compounds to determine the labelling pattern. Caryophyllene was chosen for this purpose because it was the major sesquiterpene component and acquired the greatest amount of radioactive label. Hypothetical biogenesis of caryophyllene (4,11,11-trimethyl-8-methylene-bicyclo[7:2:0] undec-4-ene) via *cis, trans*-farnesyl pyrophosphate[36] predicts that equivalent amounts of radioactivity from MVA-2-^{14}C will be incorporated into carbons 3,7 and 11-methyl of the caryophyllene molecule if the isoprenoid pathway is operative (Fig. 2). As is shown in Fig. 2, ^{14}C should be incorporated into C-11 methyl via DMAPP, while C-3 label should come from the first IPP incorporated, and C-7 label should come from the second, or terminal, IPP. The degree of labelling in these positions was determined using two well-established degradative routes previously employed in the structural elucidation of caryophyllene.[37,38]



cis, trans-FARNESYL PYROPHOSPHATE (I) CARYOPHYLLENE

FIG. 2. HYPOTHETICAL BIOGENESIS OF CARYOPHYLLENE VIA *cis,trans*-FARNESYL PYROPHOSPHATE DERIVED FROM MEVALONATE-2-^{14}C.
Labelled carbon atoms theoretically derived from MVA-2-^{14}C are identified by an asterisk. The cyclization to caryophyllene is probably stereospecific such that only one C-11 methyl is labelled. Open squares represent carbon atoms derived from dimethylallyl pyrophosphate. Closed circles represent carbon atoms from the first isopentenyl pyrophosphate added to yield geranyl pyrophosphate; open circles represent carbon atoms from the terminal isopentenyl pyrophosphate.

The hexane extract from eight MVA-2-^{14}C fed cuttings (6 hr incorporation) was treated with silicic acid, and the labelled caryophyllene was isolated from the hydrocarbon fraction by preparative GLC after the addition of carrier. Further carrier was added to yield 30 mg of caryophyllene with specific activity of 163 μc/mole. Degradation of caryophyllene (I) was carried out on two aliquots, as shown in Fig. 3. One aliquot of caryophyllene was ozonized, and formaldehyde derived from the C-8 *exo*-methylene was trapped as the dimedone derivative and radioassayed. The ether soluble ozonolysis products, primarily ketoacids II and III, on treatment with nitric acid yielded a mixture of norcaryophyllenic

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[35] F. AMELUNXEN, *Planta Med.* **15**, 32 (1967).
[36] J. B. HENDRICKSON, *Tetrahedron* 7, 82 (1959).
[37] P. DE MAYO, *The Chemistry of Natural Products*, Vol. II, p. 286, Interscience, New York (1959).
[38] A. R. PINDER, *The Chemistry of the Terpenes*, p. 138, John Wiley, New York (1960).

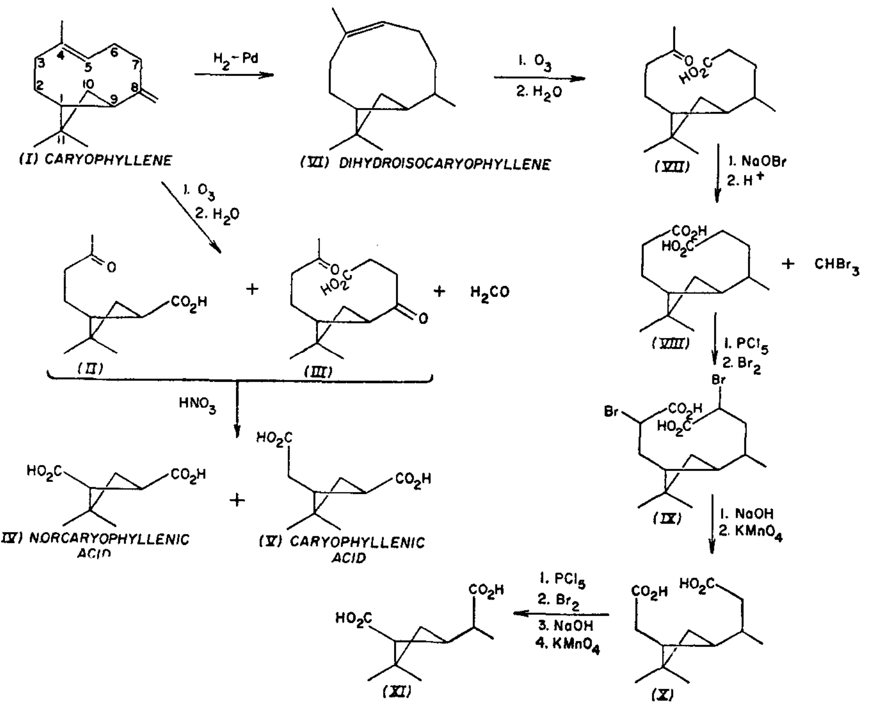

FIG. 3. CHEMICAL DEGRADATION OF CARYOPHYLLENE.

acid (IV) and caryophyllenic acid (V), which were methylated and analyzed by gas radiochromatography. Reduction of the second portion of caryophyllene over palladium yielded dihydroisocaryophyllene (VI), which was ozonized to ketoacid VII. Treatment of VII with alkaline sodium hypobromite afforded dibasic acid VIII and bromoform from the C-4 methyl, which was radioassayed. Treatment of VIII with PCl_5, then Br_2 (Hell–Volhard–Zelinsky reaction), gave dibromoacid IX, which on hydrolysis and oxidation afforded dibasic acid X. X was brominated, and the reaction sequence was repeated to give dibasic acid XI, which was methylated and analyzed by gas radiochromatography.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN CARYOPHYLLENE DERIVED FROM MEVALONATE-2-^{14}C IN PEPPERMINT AFTER AN INCORPORATION PERIOD OF 6 hr

Component isolated	Specific activity (μc/mole)	Radio-activity (%)	Carbon atoms*
Caryophyllene (I)	163	100†	All
Formaldehyde	8	5	C-8 methylene
Caryophyllenic acid (V)	77	47	1,2,3,8,9,10,11 and 11,11-dimethyl
Norcaryophyllenic acid (IV)	20	12	1,2,8,9,10,11 and 11,11-dimethyl
Bromoform	11	7	C-4 methyl
Dibasic acid XI	94	58	1,2,7,8,9,10,11 and 11,11-dimethyl and C-8 methyl (from methylene)

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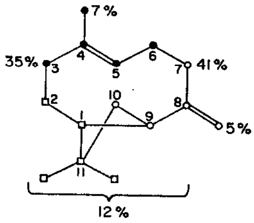
* Numbering system refers to Figs. 2 and 3, which also show pertinent structural formulae.
† 0·22% of administered L-MVA-2-^{14}C.

Results presented in Table 1 summarize the distribution of label in caryophyllene derived from MVA-2-^{14}C. The difference between the radioactivity found in caryophyllenic acid (47%) and in norcaryophyllenic acid (12%) yields the radioactivity present in C-3 (35%). Radioactivity in dibasic acid XI (58%) minus the sum of activities in norcaryophyllenic acid (12%) plus formaldehyde (5%) yields the radioactivity attributable to C-7 (41%). No significant activity can be attributed to carbons 4, 5 and 6. The distribution of label in caryophyllene derived from MVA-2-^{14}C may then be represented as shown in Fig. 4, thus confirming the isoprenoid origin of this molecule.



(I) CARYOPHYLLENE

FIG. 4. EXPERIMENTALLY DETERMINED DISTRIBUTION OF LABEL IN CARYOPHYLLENE DERIVED FROM MEVALONATE-2-^{14}C.

Meanings of squares and circles are the same as in Fig. 2. Bracket indicates the portion of the caryophyllene molecule yielding norcaryophyllenic acid (carbons 1,2,8,9,10,11 and 11,11-dimethyl).

Norcaryophyllenic acid (IV) was not degraded further, so it is uncertain whether the activity of this compound (12%) can be attributed to the predicted DMAPP-derived methyl (i.e. C-11 methyl in Fig. 2) or whether it is distributed in other portions of the molecule derived either from DMAPP or the terminal IPP. However, carbons 4, 5 and 6 of caryophyllene were unlabelled. As these carbons are derived from the first IPP it seems likely that the corresponding carbons of the terminal IPP (C-8, 9 and 10) are also unlabelled (see Fig. 4). The evidence therefore suggests that the activity of norcaryophyllenic acid (IV) is due to the DMAPP-derived portion of the molecule.

It is clear that the IPP-derived portions of caryophyllene incorporate label much more extensively than does the portion of the molecule derived from DMAPP. Preferential labelling of IPP moieties has been noted previously in the biosynthesis of the monoterpenes thujone,[39] camphor,[40] pulegone[41] and artemisia ketone[41] from MVA-2-^{14}C and in the biosynthesis of geraniol from $^{14}CO_2$.[42] Biollaz and Arigoni[10] have also noted a similar preferential labelling pattern in the biosynthesis of the sesquiterpenes coriamyrtin and tutin from MVA-2-^{14}C in *Coriaria japonica*. However, Jommi and co-workers[11] studied the same system and noted that, in the fall, *C. japonica* contains tutin almost to the exclusion of coriamyrtin and that, at this stage of growth, radioactive tutin derived from administered MVA-2-^{14}C showed no preferential labelling pattern. This discrepancy may be explained by differences in the developmental stages of the plants employed, or may be a result of the

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[40] D. V. BANTHORPE and D. BAXENDALE, *Chem. Commun.* 1553 (1968); *J. Chem. Soc.* (C), 2694 (1970).

[41] B. V. CHARLWOOD, Ph.D. Thesis, University College, London (1970), as cited in Refs. 2 and 39.

[42] T. WUU, Non-Random labelling of geraniol biosynthesized from $^{14}CO_2$ in *Pelargonium graveolens*, M.S. Thesis, Oregon State University, Corvallis (1971).

extended feeding period presumably required for the cotton-wick technique employed by the latter investigators[11] (i.e. a dilution effect).

In all cases where preferential labelling was noted, the IPP portions of the terpene molecule incorporated a significantly greater proportion of label than would be expected on the basis of theoretical considerations. The evidence appears to support the existence of an endogenous DMAPP pool in the plant, a concept consistent with the equilibrium ratio of IPP to DMAPP conversion (about 1:10) as established by *in vitro* studies of IPP-isomerase from several sources.[43–46] The change in slope beginning at about 9 hr in the time course curves for sesquiterpenes (Fig. 1) might then be interpreted in terms of the rapid loss of label from incoming IPP superimposed on the slower turnover of a DMAPP pool. If this is the case, then at longer times the newly synthesized caryophyllene should be preferentially labelled in the DMAPP portion. However, a storage pool of labelled caryophyllene might well hide this effect.

In contrast to these previous examples in which preferential labelling was observed, rose petals readily incorporate MVA-2-^{14}C into monoterpenes with an equal distribution of label between IPP and DMAPP moieties.[2] Similarly, the monoterpenoid chrysanthemum acids produced by chrysanthemum ovules show an equal distribution of MVA label between the two C_5 units.[3,47] This evidence suggests that an endogenous DMAPP pool may participate in lower terpenoid biosynthesis only in those tissues in which discrete oil glands are present. It is also possible, however, that the presence of DMAPP pools is widespread, and that the participation of such a pool in terpene biosynthesis is only evident when the amount of incorporation of labelled precursor is low enough or the pool is large enough to allow a detectible proportion of the DMAPP moieties to remain unlabelled.

Figure 4 also shows that the amount of label in the first IPP moiety (C-3 plus C-4 methyl) is less than the amount of label in the terminal IPP (C-7 plus C-8 methylene). Although the difference in degree of labelling of these IPP moieties is slight (46% vs. 42%) it does seem to indicate that a somewhat higher proportion of terminal IPP moieties are labelled, which suggests the presence of a small geranyl pyrophosphate pool.

Preferential labelling has been interpreted in terms of the participation of an endogenous DMAPP pool in terpene biosynthesis, although other considerations, such as a non-mevalonoid origin of DMAPP, or certain compartmentalization effects,[2,39] could also explain the observed results. Such explanations need not be mutually exclusive, and, as previously suggested, compartmentalization of the biosynthetic site and the apparent DMAPP pool may be related phenomena.

Another observation from the present work is the apparent exchange of some MVA-2-^{14}C label into the C-4 methyl and C-8 methylene of caryophyllene. A degree of randomization might be anticipated from the degradation of MVA-2-^{14}C into other products followed by resynthesis. However, no significant label was noted in other IPP-derived carbons such as C-4, 5 and 6, while the proportion of label in C-4 methyl and C-8 methylene is roughly equivalent. The evidence suggests that some exchange of label between the IPP methyl and methylene groups can occur at the IPP or DMAPP level. Similar exchange of label has been

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noted in the literature[7] although it has not been adequately explained. The established mechanism for isomerization of IPP to DMAPP via IPP isomerase (E.C.5.3.3.2) is stereospecific such that the 2-R hydrogen of IPP is eliminated and the IPP-methylene group (carrying label from MVA-2-^{14}C) is converted to the DMAPP *trans*-methyl.[48,49] In the reverse reaction the ^{14}C-labelled *trans*-methyl of DMAPP reverts to the methylene carbon of IPP. Considerable interconversion of IPP and DMAPP might be expected to take place in the presence of a DMAPP pool. The exchange of label observed could then be explained by the intervention, in either direction, of an IPP-isomerase of opposite stereospecificity that eliminates the 2-S hydrogen of IPP with consequent formation of DMAPP in which the labelled methyl is *cis*. Although such an enzyme is, as yet, unknown it is quite possible that such a *cis*-IPP isomerase exists, analogous to the *cis*-prenyl transferase of rubber synthesizing systems.[49,50] Two IPP isomerases have recently been isolated from pumpkin fruit, but the stereospecificity of only one of them (the established 2-R elimination) has been determined.[51]

The present work has shown that although sesquiterpenes constitute a very small proportion of peppermint oil (less than 2 %) they incorporate label from MVA-2-^{14}C much more efficiently than do the monoterpenes which constitute the bulk of the oil. In view of this fact it seems pertinent to review some earlier conclusions based on the incorporation of MVA into volatile terpenoids of peppermint. Battu and Youngken[5] fed MVA-2-^{14}C to peppermint plants and after periods of 2–14 days isolated the oil by steam distillation. The oil was separated by TLC into several oxygenated monoterpenes and a hydrocarbon fraction which was not further characterized and which, presumably, contained both mono- and sesquiterpene hydrocarbons. Although the levels of incorporation were low and the incorporation times very long, these investigators noted that the hydrocarbon fraction reached high specific activity before any appreciable change occurred in the oxygenated fractions. On the basis of these findings, Battu and Youngken proposed that terpene hydrocarbons are the primary products from which oxygenated monoterpenes are derived. From the present work it would appear that the bulk of the label in the undifferentiated hydrocarbon fraction may well have resided in the sesquiterpenes, and not the monoterpenes as tacitly assumed by Battu and Youngken. Furthermore, Figure 1 shows that in short term experiments the oxygenated monoterpenes appear to label somewhat before the monoterpene hydrocarbons.

Similarly, Hefendehl *et al.*[52] found that MVA-2-^{14}C was incorporated into the steam distilled 'volatile oil' of peppermint to the extent of 0·8 % in 24 hr and considered this finding as evidence for monoterpene biosynthesis from MVA. The present work, again, suggests that the bulk of radioactive tracer may have resided in sesquiterpenes rather then monoterpenes.

We have also noted a similar pattern of differential labelling between mono- and sesquiterpenes in preliminary work with hop (*Humulus lupulus* L.) cuttings fed MVA-2-^{14}C and glucose-U-^{14}C, which suggests that this phenomenon may be widespread in plants. It seems reasonable, therefore, to advise cautious interpretation of results based on MVA

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incorporation into 'monoterpene fractions' in which even very small amounts of sesquiterpene may be present.

Work is now underway in an attempt to clarify the question of differing biosynthetic sites for production of terpenoids in mint and to determine if a DMAPP pool may participate in the biosynthesis of other terpenoids in this plant.

EXPERIMENTAL

Plant Material

Peppermint plants were the Black Mitcham cultivar of *Mentha piperita* L., propagated vegetatively from the clone used previously,[26] and grown in a growth chamber maintained at 24° day temperature and 10° night temperature during a regular 24-hr cycle with 16-hr day under 10,500–11,000 lx light intensity, as determined with a Se photocell.[26,53] Illumination was from Sylvania VHO Gro-Lux and Wide-Spectrum Gro-Lux lights in equal numbers. Cuttings, consisting of the tuft of youngest leaves at the growing tip plus the next two leaf pairs, were taken in the morning 2·5 hr after the beginning of the light period. Stems were cut under water and the cuttings were tested before feeding began to determine that they were able to take up water actively. Fresh weights of cuttings were between 250 and 300 mg and cuttings were matched visually as closely as possible.

Administration of Mevalonate-2-^{14}C

For all experiments DL-mevalonate-2-^{14}C (*N*,*N*-dibenzylethylenediamine salt) obtained from New England Nuclear Corp., Boston, Massachusetts was employed. This substance was reported by the manufacturer to possess a specific activity of 4·7 μc/μmole and a radiochemical purity greater than 99% as determined by paper chromatography.

Cuttings were placed in vials in a small growth chamber under day conditions as previously described and given an aqueous solution of DL-MVA-2-^{14}C (5 μc in 0·1 ml) through the cut stems. The optimum dose level of MVA administered by this technique was about 1 μmole per cutting. After the uptake of labelled material (1–2 hr) the vials were kept filled with distilled water. At appropriate time intervals cuttings (four replicates per time period) were removed and the essential oil was immediately extracted.

Isolation and Fractionation of Essential Oil

Cuttings were extracted with hexane in the presence of anhydrous Na_2SO_4, and the extracts were decolorized with activated charcoal as previously described.[26] Hydrocarbons were separated from the essential oil extracts by slurrying with 1 g of silicic acid (Baker and Adamson, reagent grade) per cutting extract. After centrifugation, the hydrocarbons were removed in the supernatant hexane. The silicic acid was then slurried in EtOAc, and centrifuged, and oxygenated terpenes were removed in the supernatant. The hydrocarbon fractions obtained by this technique were free of oxygenated terpenes as shown by IR analysis (fractions showed no absorption in the 1700–1750, 3300–3600 or 1160–1250 cm^{-1} regions, confirming the absence of carbonyl, hydroxyl or ring oxygen-containing compounds).

Chromatographic Analysis and Identification of Essential Oil Components and Caryophyllene Degradation Products

Essential oil extracts were analyzed by GLC using a Beckman Thermotrac temperature programmer fitted with a Carle Micro-Detector (thermal conductivity). The column employed was 3·8 m × 3 mm stainless steel with 3% SF-96 (methyl silicone) coated on 100/120 mesh chromosorb G and operated at 150° for oxygenated fractions and 170° for hydrocarbon fractions. The dibasic acids IV, V and XI, from the chemical degradation of caryophyllene were analyzed as their dimethyl esters on this column at 210°. Peak areas were measured with a Disc integrator and quantitative analysis was made by comparison with standard curves obtained with pure compounds. A 1% phenyl diethanolamine succinate–1·5% sucrose acetate isobutyrate (PDEAS–SAIB) column described previously[53] was also employed in examining essential oii extracts and in assessing the purity of radioactive caryophyllene.

GLC–MS was performed on a coupled Varian 1200 GC-Atlas CH7 MS system utilizing the columns and GC parameters described above. Spectra were taken at 70 eV, with accelerating voltage of 3 kV and ion source temp. of 175°.

Isotope Analysis

Components separated by GLC were assayed for radioactivity with a Nuclear Chicago Biospan 4998 continuous gas flow counter. The instrument was calibrated with toluene-^{14}C and peak areas were determined with a Disc integrator. Bromoform, the dimedone derivative of formaldehyde, and aliquots of the

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dibasic acid fractions from the degradation of caryophyllene were counted directly in a Packard Tri-Carb liquid scintillation spectrometer using a scintillation solvent composed of 0·4% 2,5-diphenyloxazole (PPO) and 0·003% *p*-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in toluene. The system had an efficiency of 86% with a background count of 23 DPM. Labelled compounds exhibited no quenching at the concentrations employed. Thirty minute counts were taken, and well over 25,000 counts were accumulated for each radioactive fraction. Scintillation counting errors were about ±3% but additional errors due to weighing, volatility losses, etc. gave specific activities accurate to about ±10%, which was also the range of error in determining specific activity by gas radiochromatography.

Chemical Degradation of Caryophyllene[37,38]

Preparation of labelled caryophyllene. Eight cuttings were allowed to incorporate 5 μc of DL-MVA-2-^{14}C each for a period of 6 hr. The essential oil was extracted and the hydrocarbon fraction isolated. Five mg of caryophyllene carrier were added to the hydrocarbon fraction which was then separated gas chromatographically. Radioactive caryophyllene was repeatedly trapped off the exit port of the GLC in glass tubes filled with quartz wool, with an efficiency of about 60%. This material possessed a radiochemical purity of 97% as determined by gas radiochromatography on both the PDEAS-SAIB and SF-96 columns. Further dilution with carrier to 30 mg yielded caryophyllene with a specific activity of 163 μc/mole (corrected for 3% impurity).

Ozonolysis of caryophyllene.[54] Fifteen mg of caryophyllene (I) were oxonized in dry alcohol-free EtOAc at 0°. After removal of the solvent the ozonides were decomposed in boiling water and the products separated into ether-soluble and water-soluble portions. Aldehydes in the water-soluble portion were removed by formation of the dimedone derivatives with alcoholic dimethyldihydrorescorcinol. Formaldehyde–dimedone was separated from other reaction products by TLC on silica gel G (Merck) with 3% methanol in benzene as solvent. The derivative was located by spraying with 0·005% aq. rhodamine 6G (R_f 0·35), scraped from the plate, extracted from silica gel and rhodamine with dry Et$_2$O, and radioassayed.

Nitric acid oxidation of caryophyllene ozonolysis products.[55] The ether-soluble fraction from the ozonolysis of caryophyllene was extracted with dil. Na$_2$CO$_3$. The Na$_2$CO$_3$ solution was acidified and extracted with ether to yield, primarily, ketoacids II and III,[38] which were oxidized by addition to hot 50% HNO$_3$ followed by refluxing for several hours. Cooling and dilution with water, followed by extraction with ether, afforded norcaryophyllenic acid (IV) and caryophyllenic acid (V),[38] the identities of which were confirmed by GLC–MS (M.W. 200 and 214) after methylation with CH$_2$N$_2$. The dimethyl esters of IV and V were subjected to gas radiochromatography using the dimethyl ester of azelaic acid as an external mass standard.

Reduction of caryophyllene to dihydroisocaryophyllene and further degradation to dibasic acid XI.[56] Caryophyllene (15 mg) in abs. EtOH was shaken overnight under H$_2$ at 3 atm in the presence of 10% Pd/C to yield dihydroisocaryophyllene (VI).[57] Ozonolysis and degradation of the ozonides, as described above, provided ketoacid VII, which was treated with 1 ml 10% NaOH followed by dropwise addition of excess 10% Br$_2$–20% KBr with shaking. After the addition of ice, bromoform was extracted in ether, washed with dil. Na$_2$S$_2$O$_3$, dried and radioassayed. The reaction mixture was acidified with dil. HCl and the dibasic acid VIII taken up in ether then dried over Na$_2$SO$_4$. To the dibasic acid VIII fraction was added 1 mg PCl$_5$, followed by 15 mg Br$_2$ after warming. After the addition of ice, the dibromodibasic acid (IX) was recovered in ether. Hydrolysis in dil. NaOH, followed by oxidation with KMnO$_4$, acidification, and extraction in ether, yielded dibasic acid X. X was brominated, as above, and the above reaction sequence repeated to give dibasic acid XI, which was methylated (identity confirmed by GLC–MS; MW 228) and subjected to gas radiochromatography using the dimethyl ester of azelaic acid as an external standard.

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