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BIOSYNTHESIS OF MONO- AND SESQUI-TERPENES IN PEPPERMINT FROM GLUCOSE-^{14}C AND $^{14}CO_2$*

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Key Word Index—*Mentha piperita*; Labiatae; peppermint; monoterpenes; sesquiterpenes; biosynthesis; non-random labeling of pulegone; dimethylallyl pyrophosphate pool.

Abstract—Labeled glucose and CO_2 are more efficient precursors of monoterpenes in peppermint (*Mentha piperita* L.) cuttings than is mevalonate, which is the best precursor of sesquiterpenes in this plant. Metabolic turnover of the labeled monoterpenes was observed, in agreement with previous observations. Pulegone derived from $^{14}CO_2$ after 1, 3, 6, 9 and 12 hr of incubation was chemically degraded, and in every case at least 90% of the ^{14}C-label was found in the seven-carbon fragment containing the isopentenyl pyrophosphate-derived portion of the molecule. The isopropylidene side chain, containing three carbons hypothetically derived from dimethylallyl pyrophosphate, was found to be essentially unlabeled. The results suggest that an endogenous dimethylallyl pyrophosphate pool participates in monoterpene biosynthesis, much as earlier work had suggested that a similar pool participates in sesquiterpene biosynthesis in this plant. These findings are of particular interest because it appears, based on the differential utilization of labeled precursors, that monoterpenes and sesquiterpenes are produced at separate sites in the plant.

INTRODUCTION†

THE MONO- and sesqui-terpenes are thought to originate via the conventional isoprenoid pathway, involving condensation of a DMAPP 'starter unit' with, respectively, one or two IPP units. As both IPP and DMAPP are formed *in vivo* from MVA, and IPP is the immediate precursor of DMAPP, the biosynthesis of mono- and sesqui-terpenes from exogenous ^{14}C-labeled precursors, such as MVA-2-^{14}C or $^{14}CO_2$, would be expected to result in equivalent amounts of ^{14}C-tracer being incorporated into IPP- and DMAPP-derived moieties of the terpene molecules. Although such equivalence of labeling was observed in the biosynthesis of certain monoterpenes in flowers,[1,2] the biosynthesis of mono- and sesqui-terpenes in other tissues from several plant species has generally yielded preferentially

* A preliminary account of this work was presented at the Annual Meeting of the American Society of Plant Physiologists, Pacific Grove, California, August 1971. *Plant Physiol.* **41** (Suppl.), 124 Abs. (1971).

† Abbreviations used: MVA, mevalonic acid; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate.

[1] M. J. O. FRANCIS, D. V. BANTHORPE and G. N. J. LE PATOUREL, *Nature, Lond.* **228**, 1005 (1970).
[2] P. J. GODIN, H. S. INGLIS, M. SNAREY and E. M. THAIN, *J. Chem. Soc.* 5878 (1963).

labeled terpenes,* containing the bulk of incorporated tracer in the IPP-derived portion of the molecule.[4–10]

Banthorpe *et al.*[4] found that MVA-2-^{14}C was specifically incorporated into thujone, isothujone and sabinene in cuttings from *Thuja*, *Tanacetum* and *Juniperus* but that from 90 to over 99% of the incorporated label was located in the IPP-derived portions of the molecules. Camphor,[5] α-pinene,[6] pulegone[6] and artemisia ketone[7] derived from MVA-2-^{14}C in several species were also shown to be preferentially labeled in the IPP-derived moiety. In these experiments, the MVA-2-^{14}C was stem-fed over a 4–5 day period with incorporations of about 0·02%. That this type of preferential labeling is not restricted to the monoterpenes was shown[8] by degradation of the picrotoxane sesquiterpenes, coriamyrtin and tutin, derived from MVA-2-^{14}C in *Coriaria japonica*, in which 80% of the incorporated radioactivity of both compounds was found in the IPP portion of the molecule, as compared to the theoretical 67%. We have found[9] that MVA-2-^{14}C is readily incorporated into sesquiterpenes in peppermint but is a very poor precursor of peppermint monoterpenes. In the biosynthesis of the sesquiterpene caryophyllene from MVA-2-^{14}C in peppermint cuttings, the IPP-derived portion of the molecule contained 88% of the incorporated label after a 6-hr incorporation period.[9]

Several investigators[1,4,6,9–11] have suggested that such preferential labeling may result from the condensation of IPP derived from exogenous ^{14}C-precursor with DMAPP that is mainly present in a metabolic pool. Other explanations that have been suggested, but which seem less likely, include a non-mevalonoid origin of DMAPP, and selective membrane permeabilities with respect to DMAPP and IPP. We have previously suggested[3,9] that compartmentalization of mono- and sesqui-terpene biosynthesis, perhaps within the oil glands, and the apparent participation of a DMAPP pool, may be related phenomena.

The poor incorporation of MVA-^{14}C and acetate-^{14}C into lower terpenoids, especially monoterpenes, has made degradative studies difficult. At the same time, it was taken for granted that $^{14}CO_2$ or ^{14}C-sugars, which were incorporated much more effectively into the monoterpenes,[3,12] would yield randomly labeled products. The studies just described suggested that this might not be so, and prompted degradative studies of monoterpenes biosynthesized from $^{14}CO_2$. Fang and Baisted[10] degraded the acyclic monoterpene geraniol synthesized from $^{14}CO_2$ in *Pelargonium graveolens* and found preferential labeling of the IPP-derived portion of the molecule, followed in time by a preferential decrease in IPP-derived label, resulting in a *relative increase* in DMAPP-derived label.

* Equivalent labeling of mono- and sesqui-terpenes in leaf and stem tissue has been reported in a few instances, but the conditions of these experiments were such that preferential labeling might easily have gone undetected. See Refs 3 and 6 for further discussion.

[3] W. D. LOOMIS and R. CROTEAU, in *Recent Advances in Phytochemistry*, (edited by V. C. RUNECKLES), Vol. 6, Academic Press, New York (1972).
[4] D. V. BANTHORPE and K. W. TURNBULL, *Chem. Commun.* 177 (1966); D. V. BANTHORPE, J. MANN and K. W. TURNBULL, *J. Chem. Soc.* C, 2689 (1970).
[5] D. V. BANTHORPE and D. BAXENDALE, *Chem. Commun.* 1553 (1968); *J. Chem. Soc.* C, 2694 (1970).
[6] D. V. BANTHORPE, B. V. CHARLWOOD and M. J. O. FRANCIS, *Chem. Rev.* **72**, 115 (1972).
[7] D. V. BANTHORPE and B. V. CHARLWOOD, *Nature, New Biol.* **231**, 285 (1971).
[8] M. BIOLLAZ and D. ARIGONI, *Chem. Commun.* 633 (1969).
[9] R. CROTEAU and W. D. LOOMIS, *Phytochem.* **11**, 1055 (1972).
[10] T. Y. (WUU) FANG and D. J. BAISTED; in preparation for *Phytochemistry*; T. Y. WUU, Non-Random Labeling of Geraniol Biosynthesized from $^{14}CO_2$ in *Pelargonium graveolens*, *M. S. Thesis*, Oregon State University, Corvallis (1971).
[11] M. J. O. FRANCIS, in *Aspects of Terpenoid Chemistry and Biochemistry* (edited by T. W. GOODWIN), p. 29, Academic Press, London (1971).
[12] W. D. LOOMIS, in *Terpenoids in Plants* (edited by J. B. PRIDHAM), p. 59, Academic Press, London (1967).

Our earlier work suggested, based on differential utilization of labeled precursors, that mono- and sesqui-terpenes in peppermint are produced at different sites.[9,12] Both types of sites appear to be compartmentalized, but not to the same degree. As peppermint sesquiterpenes synthesized from MVA-2-^{14}C were preferentially labeled in the IPP-derived moieties it was of great interest to determine the pattern of labeling in peppermint monoterpenes synthesized from labeled substrates. Pulegone (Scheme 1) was the obvious choice for this study. It is a component of central importance in monoterpene metabolism in peppermint,[13] occurs in reasonable concentration in the oil from young tissue, acquires a good share of label from incorporated $^{14}CO_2$ or glucose-^{14}C, and is easily degraded. $^{14}CO_2$ was chosen as the radioactive precursor as it is obtainable in very high specific activity.

A priori, the biogenesis of pulegone from $^{14}CO_2$ would be expected to result in random labeling, with all carbon atoms acquiring equivalent amounts of radioactivity. However, if a DMAPP pool participates in pulegone biosynthesis then the DMAPP-derived portion of the molecule should acquire less label than the portion of the molecule derived from IPP, although random labeling should still apply within each individual IPP- or DMAPP-derived unit. Such a distribution pattern may be readily detected by assaying any cleavage fragment of pulegone that contains (or has lost), exclusively, DMAPP- or IPP-derived carbons.

RESULTS

Essential Oil Composition

The oil extracted from small vegetative tips (250–300 mg fr. wt) taken from plants grown under controlled long-day, cool-night conditions (see Experimental) contains about 50% menthones, 10% menthols, 10% pulegone, 20% other oxygenated monoterpenes, 6% monoterpene hydrocarbons and only 2% sesquiterpene hydrocarbons.[9] The menthol content is considerably lower in the oil from the growing tip than in the oil from mature leaves (10 vs. 40–50%), while the pulegone content is considerably higher (10 vs. 1–2%).

Glucose-^{14}C and $^{14}CO_2$ as Terpenoid Precursors

Previous studies have shown that MVA-2-^{14}C, although a relatively good precursor of sesquiterpenes, is a poor precursor of monoterpenes in both peppermint[9] and hops.[14] However, earlier work had also shown glucose-^{14}C and $^{14}CO_2$ to be among the best monoterpene precursors in these plants,[3,12,14,15] probably due to preferential transport of sugars, and so the utilization of these radioactive substrates was further tested with the peppermint system. Aqueous solutions of D-glucose-U-^{14}C (3 μCi, 1 μmol, in 0·1 ml) were administered to single peppermint cuttings through the cut stems. After appropriate time intervals the essential oil was extracted from each cutting and analyzed by gas radiochromatography. The incorporation of radioactivity from glucose-U-^{14}C into pulegone, and into total mono- and sesqui-terpenes, as a function of time is shown in Fig. 1. At the 6-hr maximum, pulegone and menthone had acquired the most label, in roughly equal amounts, while menthofuran and 1,8-cineole had each acquired about one-third as much label as pulegone. All of the other monoterpenes that could be analyzed by gas radiochromatography acquired some label, except for menthol which contained little or no ^{14}C-tracer even at the later sampling

[13] J. BATTAILE, A. J. BURBOTT and W. D. LOOMIS, *Phytochem.* **7**, 1159 (1968).
[14] A. J. BURBOTT, R. CROTEAU and W. D. LOOMIS, unpublished work.
[15] A. J. BURBOTT and W. D. LOOMIS, *Plant Physiol.* **44**, 173 (1969).

times. The sesquiterpenes contained less than 5% of the total essential oil label at this time, roughly in proportion to the concentration of sesquiterpenes in the oil. Both mono- and sesqui-terpenes incorporated label for about 6–8 hr and then lost label again. Similar turnover has been noted previously in mono- and sesqui-terpenes derived from MVA-2-^{14}C and in monoterpenes derived from $^{14}CO_2$,[3,12,15] and appears to represent primarily the turnover of active metabolic pools.

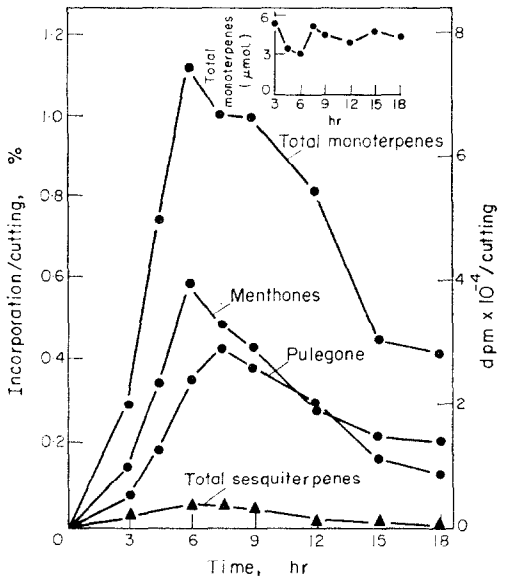


FIG. 1. TIME-COURSE OF LABELING OF PEPPERMINT MONO- AND SESQUI-TERPENES FROM D-GLUCOSE-U-^{14}C.

Data presented are from a single set of visually matched cuttings fed 1 μmol glucose-^{14}C per cutting. Several replications of the experiment gave similar time-courses. Data points for total mono- and sesqui-terpenes represent the sums of activities in detectable gas radiochromatographic peaks. Time is from start of glucose-U-^{14}C feeding.

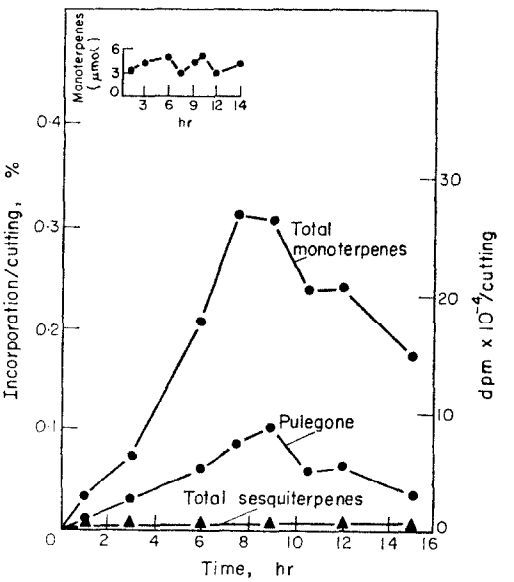


FIG. 2. TIME-COURSE OF LABELING OF PEPPERMINT MONO- AND SESQUI-TERPENES AFTER 1 hr EXPOSURE TO $^{14}CO_2$ IN CONTINUOUS LIGHT.

Data presented are from a single set of visually matched cuttings fed 0·71 μmol $^{14}CO_2$ per cutting. Two replications of the experiment gave similar time-courses. Data points for total mono- and sesqui-terpenes represent the sums of activities in detectable gas radiochromatographic peaks. Time is from start of exposure to $^{14}CO_2$.

A study of the incorporation of several specifically labeled ^{14}C-glucoses was next undertaken. Cuttings were fed glucose-^{14}C (labeled in position 1, 2, 3–4 or 6) through the cut stem, and maintained in the light for 6 hr. The monoterpenes were then extracted and analyzed, and the results are shown in Table 1. For ease of comparison, the data are also expressed on a relative basis, with glucose-6-^{14}C incorporation taken as unity. The 'theoretical' values shown are calculated based on the assumption that glucose is assimilated entirely via glycolysis, and that the triose phosphates are in complete equilibrium. The data suggest that, in fact, the labeled glucose was converted to acetyl-CoA (and thence to MVA) by simultaneous action of the glycolytic pathway and the pentose phosphate pathway.[16] The relatively poor utilization of glucose-1-^{14}C compared to glucose-6-^{14}C also argues that the

[16] T. AP REES and H. BEEVERS, *Plant Physiol.* **35**, 830 (1960).

main pathway of incorporation involves direct conversion of glucose carbons to acetyl–CoA and MVA, and is not via CO_2.

TABLE 1. INCORPORATION OF LABEL FROM SPECIFICALLY ^{14}C-LABELED GLUCOSES INTO PEPPERMINT MONOTERPENES*

^{14}C-substrate	% incorporation	Incorporation relative to glucose-6-^{14}C Obs.	Theor.†
Glucose-1-	0·09	0·24	1·00
Glucose-2-	0·16	0·42	0·83
Glucose-3,4-	0·14	0·37	0·00
Glucose-6-	0·38	1·00	1·00

* Each substrate was fed at a 3-μmol dose level and for a 6-hr incorporation period.

† Theoretical ratio assuming that acetyl–CoA for MVA synthesis arises entirely via glycolysis and that the triose phosphates are equivalent.

The ability of $^{14}CO_2$ to function as a terpene precursor was next examined. Peppermint cuttings were exposed to $^{14}CO_2$ for 1 hr in the light and then flushed continuously with air, still in the light. After appropriate time intervals the mono- and sesqui-terpenes were extracted and analyzed. The time-course of incorporation of $^{14}CO_2$ label into mono- and sesqui-terpenes is shown in Fig. 2. As expected, the $^{14}CO_2$ time-course is quite similar to the glucose-U-^{14}C time-course (Fig. 1), showing a similar (although somewhat delayed) maximum and turnover period. The distribution of radioactivity in individual mono- and sesqui-terpenes was similar to that found on feeding glucose-U-^{14}C.

Chemical Degradation of Pulegone and Distribution of Label

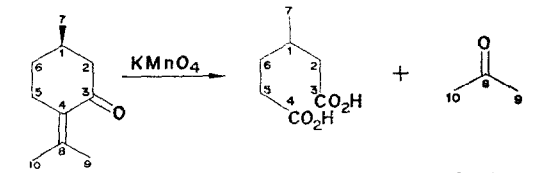
There are two ways of fitting the two isoprene groups into pulegone, going either in a clockwise or in a counterclockwise direction around the ring, so that the DMAPP-derived portion of the molecule may comprise carbons 4, 5, 8, 9 and 10 (as suggested by biogenetic considerations[12]) or carbons 3, 4, 8, 9 and 10 (see Scheme 1). In either case, however, the isopropylidene group (carbons 8, 9 and 10) represents a portion of the molecule entirely derived from DMAPP. The isopropylidene group is readily cleaved off by oxidation of pulegone with neutral $KMnO_4$,[17] as shown in Scheme 1, whereby the isopropylidene group is converted to acetone (containing three DMAPP carbons and a theoretical 30% of the incorporated label), and the ring is opened between C-3 and C-4 to yield 3-methyladipic acid (containing the five IPP carbons plus two remaining DMAPP carbons and a theoretical 70% of the incorporated label).

Peppermint cuttings were exposed to $^{14}CO_2$ under conditions identical to those used for the time-course shown in Fig. 2. At time periods of 1, 3, 6, 9 and 12 hr, cuttings were removed, and the essential oil was extracted. After addition of carrier pulegone, an aliquot of the extract was analyzed by gas radiochromatography and the specific activity of pulegone was determined. Pulegone was then isolated from the extract by TLC,[18] and treated with

[17] F. W. SEMMLER, *Chem. Ber.* **25**, 3519 (1892).

[18] J. BATTAILE, R. L. DUNNING and W. D. LOOMIS, *Biochim. Biophys. Acta* **51**, 538 (1961).

$KMnO_4$ to yield 3-methyladipic acid, which was methylated and then assayed by gas radiochromatography. The results of this study, shown in Table 2, clearly demonstrate that at each time period examined essentially all of the incorporated ^{14}C was in 3-methyladipic acid, presumably in the IPP-derived carbon atoms. Although 3-methyladipic acid was not



SCHEME 1. DEGRADATION OF PULEGONE DERIVED FROM $^{14}CO_2$.

Based on the assumption that pulegone derived from $^{14}CO_2$ is randomly labeled, 3-methyladipic acid should contain 70% and acetone 30% of the incorporated label. As the labeling pattern of pulegone derived from MVA has not yet been determined, the DMAPP-derived portion of the molecule may represent either carbons 4, 5, 8, 9 and 10 or carbons 3, 4, 8, 9 and 10, depending on whether the DMAPP moiety is fitted clockwise or counterclockwise into the ring.

degraded further, the fact that acetone (derived from three DMAPP carbons) was necessarily unlabeled, or nearly unlabeled, gave very strong indication that the other two DMAPP carbons (C-3 and 4 or C-4 and 5) also carried no label. Repetition of this experiment gave almost identical results, with the repeated 3-hr sample showing 99% of the incorporated label in 3-methyladipic acid.

TABLE 2. EXPERIMENTALLY DETERMINED PER CENT OF ^{14}C-LABEL IN 3-METHYLADIPIC ACID AFTER EXPOSURE OF PEPPERMINT CUTTINGS TO $^{14}CO_2$ FOR 1 hr*

Time (hr)†	Specific activity‡ Pulegone	Specific activity‡ 3-Methyladipic acid	% of label in 3-methyladipic acid§
1	1000	980	98
3	2530	2190	87
6	1960	1970	101
9	2800	2820	101
12	1610	1670	104

* Conditions of the experiment were identical to those used for the time-course shown in Fig. 2.

† Time from start of exposure to $^{14}CO_2$.

‡ Specific activity in dpm/μmol.

§ Hypothetically, pulegone derived from $^{14}CO_2$ should be randomly labeled, and 3-methyladipic acid should therefore contain 70% of the label.

DISCUSSION

The results described here provide an additional example of physiological non-equivalence of C_5 units in the biosynthesis of lower terpenoids in plants. Since preferential labeling of the IPP-derived portion of pulegone was observed even from such a non-specific substrate as $^{14}CO_2$, it must be concluded that the part of the molecule hypothetically derived from

DMAPP is preferentially synthesized from endogenous precursor. It still is not clear whether the endogenous precursor is in fact DMAPP, or is even mevalonoid in origin, but a non-mevalonoid origin would not in itself explain the present results. It does seem clear that there is an endogenous pool of C_5-precursor, and it seems quite likely that this precursor is DMAPP.

The evidence that CO_2 and glucose are better precursors of mono- than of sesqui-terpenes, coupled with the previous demonstration that mevalonate is a better precursor of sesquiterpenes than of monoterpenes in peppermint,[9] strongly suggests that the sites of synthesis of these two groups of compounds are different: the sesquiterpene biosynthetic site being the more accessible to exogenous MVA while the monoterpene site is at least as accessible as the sesquiterpene site to exogenous glucose and photosynthetically-fixed CO_2. If mono- and sesqui-terpenes are synthesized at separate sites, they are presumably synthesized from separate DMAPP pools as well. In fact, since the synthetic sites for mono- and sesqui-terpenes appear to be compartmentalized in oil glands, there are perhaps several thousand discrete DMAPP pools in a single leaf.[3] Accumulation of a DMAPP pool at such sites implies metabolic control at the C_5-pyrophosphate level and is consistent with the equilibrium ratio of the IPP $\rightleftharpoons$ DMAPP isomerization (about 1:10) as measured *in vitro* (see further discussion in Ref. 3).

In earlier studies,[9] degradation of the sesquiterpene caryophyllene derived from MVA-2-^{14}C after a 6-hr incorporation period indicated that some 12% of the incorporated label was in the DMAPP-derived portion of the molecule, thus suggesting that exogenous MVA was not totally excluded from the apparent DMAPP pool. In the pulegone study one might have expected to find similar incorporation of $^{14}CO_2$ label into the endogenous DMAPP pool, particularly after the longer time periods. However, it would appear that the DMAPP-derived portion of pulegone contained little or no $^{14}CO_2$ label, even after 12 hr, when approx. 25% of labeled pulegone had turned over (judging from the data of Fig. 2). This finding might be explained by the fact that $^{14}CO_2$ of high specific activity was employed, which could have resulted in the production of essentially pulse-labeled pulegone of high specific activity, with relatively little net synthesis, and thus with relatively little turnover of the DMAPP pool. Furthermore, since our specific activities were subject to errors of up to $\pm 10\%$ (see Experimental), and since the label in DMAPP-derived carbons was calculated by difference, as much as 16–17% of label in this portion of the molecule could conceivably have gone undetected.

The results described here appear to be typical of vegetative tissues but are quite different from observations on flowers.[1,2,19] In these studies, flower tissues utilized MVA-2-^{14}C readily for monoterpene synthesis, without the apparent participation of a DMAPP pool. In the vegetative tissues that have been studied, the sites of mono- and sesqui-terpene synthesis appear to be highly compartmentalized, largely dependent on fermentative metabolism for energy and for acetyl-CoA production, and energy deficient due to a lack of sugars.[3,20] In contrast, the floral biosynthetic sites appear to be energy-rich and in good communication with adjacent cells and the atmosphere.[3] The physiological uniqueness of floral secretion is probably related to the presence of unique glandular structures in flowers, which have been referred to as osmophors.[3,21]

[19] M. J. O. Francis and M. O'Connell, *Phytochem.* **8**, 1705 (1969).
[20] R. Croteau, A. J. Burbott and W. D. Loomis, *Phytochem.* **11**, 1055 (1972).
[21] S. Vogel, *Akad. Wiss. Lit., Mainz, Abh. Math.-Naturwiss, Kl.*, No. 10, pp. 599–763 (pp. also numbered separately: 1–165), (1962).

EXPERIMENTAL

Plant material. Peppermint plants were the Black Mitcham cultivar of *Mentha piperita* L., propagated vegetatively from the clone used previously,[22] in a growth chamber maintained at 24° day temp. and 10° night temp. during a regular 24-hr cycle with 16-hr day under 10 500–11 000 lx light intensity, as determined with a Se photocell.[13,22] 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 carefully tested before feeding began to insure 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 ^{14}C-labeled precursors. D-Glucose-1-^{14}C (3·1 μCi/μmol) and D-glucose-3,4-^{14}C (0·6 μCi/μmol) were obtained from New England Nuclear Corp., Boston, Massachusetts. D-glucose-2-^{14}C (2·0 μCi/μmol) and D-glucose-6-^{14}C (1·6 μCi/μmol) were obtained from Volk Radiochemical Co., Chicago, Illinois, while D-glucose-U-^{14}C (3·0 μCi/μmol) was obtained from Amersham/Searle, Arlington Heights, Illinois. All of the above compounds possessed radiochemical purities of 99% or higher as reported by the manufacturer. For the glucose-U-^{14}C time-course study, cuttings were placed in vials in a small growth chamber under daylight conditions as described above and given an aqueous solution of D-glucose-U-^{14}C (3 μCi, 1 μmol in 0·1 ml) through the cut stem. After the uptake of labeled material (1–2 hr) the vials were kept filled with distilled water. At appropriate time intervals cuttings (in triplicate) were removed, and the essential oil was immediately extracted. To study the relative incorporation of the various specifically labeled ^{14}C-glucoses, cuttings were prepared as described above, and each sugar was administered at a dose level of 3 μmol in 0·05 ml H_2O. At the end of 6 hr the cuttings were immediately extracted. $Ba^{14}CO_3$ (58·8 μCi/μmol) was obtained from New England Nuclear Corp., and quantitatively converted to $Na_2{}^{14}CO_3$ solution by standard techniques. For exposure to $^{14}CO_2$ (in both time-course and degradation studies) 24 cuttings were placed in small vials of water in a sealed Plexiglas chamber (20 l.) around a central vial containing $Na_2{}^{14}CO_3$ solution (1 mCi), and $^{14}CO_2$ (0·71 μmol per cutting; initial CO_2 concentration, 0·22%) was generated by injecting a solution of perchloric acid into the carbonate solution through a rubber-capped inlet tube. The exposure chamber was then placed inside the small growth chamber under daylight conditions for 1 hr. At the end of this period the exposure chamber was flushed with air through several NaOH-traps and opened for the remainder of the incorporation period. At appropriate time intervals cuttings were removed and immediately extracted. For the pulegone degradation study cuttings were pooled according to the following schedule: 1-hr, 8 cuttings; 3-hr, 6 cuttings; 6- and 9-hr, 3 cuttings each; 12-hr, 4 cuttings.

Isolation of essential oil. Cuttings were extracted with hexane in the presence of anhydrous Na_2SO_4, and the extracts were decolorized with Norit A activated charcoal as previously described.[22] When the addition of carrier was required in the degradation studies, the extracts were concentrated to 3 ml, and from 10 to 15 mg of (+)-pulegone were added to yield a specific activity of about 2000 dpm/μmol.

Chromatographic analysis of essential oil, pulegone and 3-methyladipic acid dimethyl ester. Essential oil extracts were analyzed by gas chromatography using a Beckman Thermotrac temperature programmer fitted with a Carle Micro Detector (thermal conductivity). The column employed was 6·1 m × 3·2 mm stainless steel with 1% phenyl diethanolamine succinate (PDEAS) and 1·5% sucrose acetate isobutyrate (SAIB) coated on 100–120 mesh Chromosorb G and was programmed from 125 to 165° at 1°/min with a helium flow rate of 25 ml/min. To assay pulegone in those fractions to which carrier had been added, this same column was run isothermally at 170°. Under these conditions, pulegone was completely resolved from all other detectable essential oil components. 3-Methyladipic acid dimethyl ester, from the degradation of pulegone, was also assayed on the PDEAS-SAIB column, which in this instance was run isothermally at 175°. Peak areas were measured with a Disc integrator, and quantitative analysis was made by comparison to standard curves obtained with pure compounds. A 3% SF-96 column, described previously,[9] was also employed in determining the purity of carrier (+)-pulegone and the 3-methyladipic acid standard (both compounds analyzed 99% pure on both PDEAS-SAIB and SF-96). The SF-96 column was also used, along with the PDEAS-SAIB column, in confirming the identity of 3-methyladipic acid by GLC and combined GLC–MS.

Isotope analysis. Components separated by GLC were assayed for radioactivity directly with an attached Nuclear Chicago Biospan 4998 continuous gas flow counter. The instrument was calibrated for each assay with toluene-^{14}C, and peak areas were determined with a Disc integrator. Analysis of standards by gas radiochromatography showed that specific activity could be routinely determined to within ±10% of the true value.

Chemical degradation of pulegone.[17] After the addition of carrier pulegone and the gas radiochromatographic determination of specific activity, the hexane extract was further concentrated and was separated by TLC on silica gel (20 cm × 30 cm × 0·5 mm) in EtOAc–hexane (9:91, v/v).[18] The pulegone band was located under short wave UV light and, while still wet with solvent, was scraped into a test tube. Pulegone was not

[22] A. J. BURBOTT and W. D. LOOMIS, *Plant Physiol.* **42,** 20 (1967).

eluted from the silica gel but was treated directly with 2·5 ml of 4% aqueous $KMnO_4$ to yield 3-methyladipic acid, which was extracted from the acidified reaction mixture with Et_2O and taken to dryness. The 3-methyladipic acid was methylated in ethereal CH_2N_2 (identity confirmed by comparison of MS and GLC retention to the authentic compound) and then assayed by gas radiochromatography. Specific activities of pulegone and 3-methyladipic acid were determined in aliquots containing 2000–8000 dpm.

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