AN ABSTRACT OF THE THESIS OF

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Title: NON-RANDOM LABELLING OF GERANIOL BIOSYNTHESIZED FROM $^{14}CO_2$ IN PELARGONIUM GRAVEOLENS

Abstract approved: Redacted for Privacy
Thomas C. Moore

The acyclic monoterpene geraniol was biosynthesized from $^{14}CO_2$ in Pelargonium graveolens (rose geranium) and isolated from the hexane-extractable fraction in five experiments. Experiments 1 and 2 were preliminary experiments in which the total incorporation of label into geranium oil was determined. In experiments 3 and 4a and 4b both incorporation and distribution of ^{14}C in geraniol were determined.

Radioactive geraniol from experiments 3 and 4a and 4b was degraded by ozonolysis. Radioactivity of the terminal three carbons (C-7, 8 and 10), which presumably represents 3/5 of that portion of the geraniol molecule derived from 3, 3-dimethylallyl pyrophosphate (DMAPP), was determined. In early samples (two hours) there was some apparent preferential labelling in the DMAPP moiety of geraniol. In longer time, as the level of label in geraniol increased,

the isopentenyl pyrophosphate (IPP) portion became preferentially labelled. This trend was observed consistently.

The presence of a metabolic pool of DMAPP in the plant is suggested as one factor in explaining these results. Further possible factors are proposed, based on the results, which include compartmentation of IPP and DMAPP metabolism, alternative pathways to geraniol, and multiple sites of geraniol biosynthesis.

Non-Random Labelling of Geraniol Biosynthesized from $^{14}CO_2$ in *Pelargonium graveolens*

by

Ta-Yun Wuu

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Typed by Mary Jo Stratton for Ta-Yun Wuu

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Sincere gratitude is expressed to my dearest parents, for their love, patience, and encouragement throughout my life. This thesis is dedicated to them.

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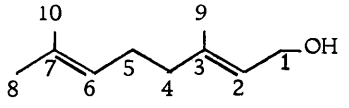
NON-RANDOM LABELLING OF GERANIOL BIOSYNTHESIZED FROM $^{14}CO_2$ IN PELARGONIUM GARVEOLENS

INTRODUCTION

Oil of geranium usually refers to the essential oil derived from Pelargonium graveolens L'Her (rose geranium). This is because it is the parent plant of all Pelargonium varieties used today for the commercial production of geranium oil. Oil of geranium is of economic importance because it is one of the most important ingredients in perfume. The oil is of considerable interest to chemists and physiologists also because of the variety of its constituents and the significant positions of those constituents, especially geraniol, in biosynthetic pathways. In the geranium plant, the essential oil is associated with small glandular hairs which are distributed over the green parts of the plant, particularly on the leaf surface.

One of the main constituents of many essential oils, including oil of geranium, is geraniol, an acyclic monoterpene (ten-carbon isoprenoid compound):



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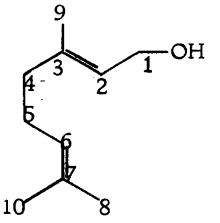


It occurs as the free alcohol or in the form of esters or glycosides (Francis and Allcock, 1969). In oil of P. graveolens, the geraniol

content varies from 35 to 50% of the total oil (Guenther, 1950). In the form of its pyrophosphate derivative, geraniol is the first product of condensation of 2 five-carbon "active isoprene" units and is an established intermediate in the biosynthesis of sterols and other isoprenoids (Loomis, 1967). Monoterpenes also are often assumed to be synthesized from dephosphorylations of Ger-PP[1/] or its isomer Ner-PP (Francis, 1970).

Some common steps are involved in the early stages of isoprenoid synthesis, which have been well established (Figure 1). Among various precursors related to isoprenoid synthesis, MVA has been found to be efficiently incorporated into certain isoprenoid compounds with loss of C-1. The incorporation of MVA into cholesterol was near 80% (Tavormina, Gebbs and Huff, 1956). To date, MVA has been demonstrated to be the best precursor in cell-free systems for cholesterol and squalene biosynthesis (Cornforth et al., 1958; Rilling and Bloch, 1959) and is now considered to be a key intermediate in isoprenoid biosynthesis in general.

[1/] Ger-PP and the following abbreviations will be used:
Ger-PP, Geranyl pyrophosphate
Ner-PP, Nerol pyrophosphate
MVA-2-^{14}C, Mevalonate-2-^{14}C
MVA, Mevalonic acid
DMAPP, 3, 3-Dimethylallyl pyrophosphate
IPP, Isopentenyl pyrophosphate
Acetone-^{14}C-DNP, Acetone-^{14}C-2, 4-dinitrophenyl hydrazone

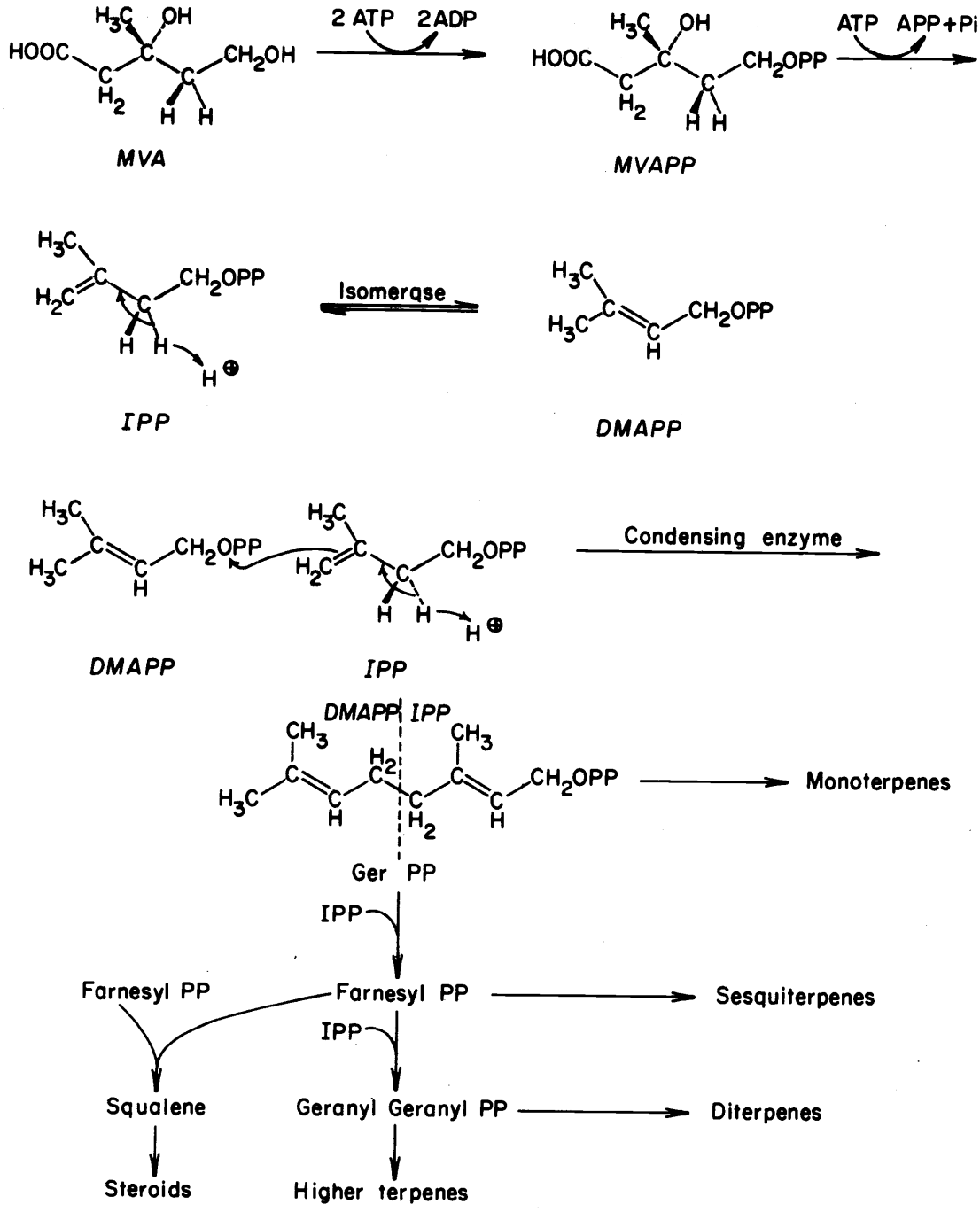
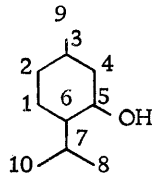
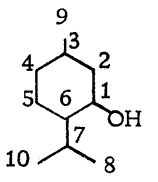


Figure 1. Some common steps involved in isoprenoid synthesis.

It is postulated that monoterpenes are formed biosynthetically as shown in Figure 1. One mole of DMAPP condenses with one mole of IPP in head to tail fashion without further rearrangement. However, when a cyclic monoterpene is formed, there are two ways to arrange the two isoprene units of the molecule. For example, retaining the geraniol numbering system, menthol could be either I or II.

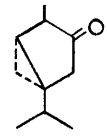


I



II

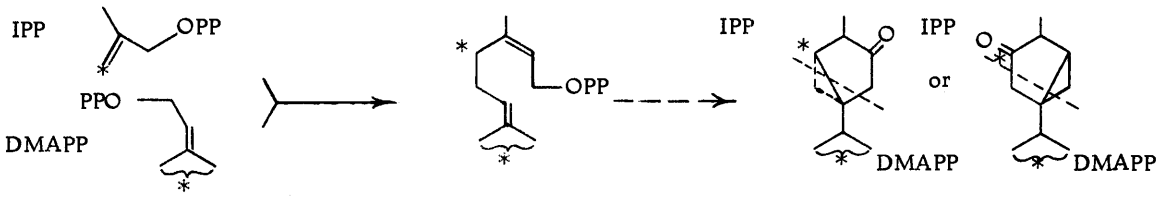
The incorporation of MVA-2-^{14}C into free monoterpenes is usually poor, and for a long time there was no conclusive evidence for the participation of MVA in monoterpene biosynthesis, although a low level of incorporation (< 0.01%) was detectable (Banthorpe and Turnbull, 1966; Banthorpe and Baxendale, 1968). Recently, Francis and O'Connell (1969) successfully incorporated MVA-2-^{14}C into geraniol, nerol and their β-D-glucosides in the petals of a hybrid tea rose, var. "Lady Seton". Up to 10.8% of the supplied label was found to be incorporated within one hour. Later, in degradation studies (Francis et al., 1970), it was found that label from MVA-2-^{14}C was distributed equally on C-4 (from IPP) and C-8, 10 (from DMAPP).

Banthorpe and Turnbull (1966) determined the labelling pattern of the monoterpene thujone 

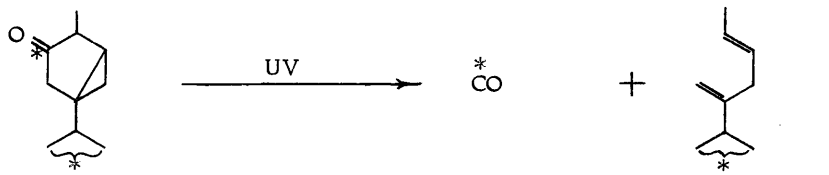
. In their experiments,

radioactive MVA-2-^{14}C was administered through cut stems to twigs and leaves of Thuja occidentalis. Plants were kept in nutrient solution for eight days before harvesting. The isolated thujone was cleaved by irradiation with ultraviolet light. By assaying the specific activities of the products, it was found that the carbon atom that presumably arises from the methylene carbon of the IPP unit was highly labelled, whereas the part of the molecule coming from DMAPP had much less label.

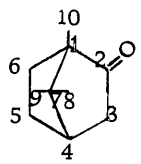
Postulated biosynthesis:



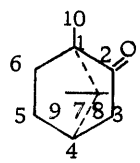
Degradation: UV irradiation



In subsequent experiments (Banthorpe and Baxendall, 1968), the labelling pattern of (±) camphor (mixture of



and



) was determined. MVA-2-^{14}C solution was stem-fed into leaves of Artemisia californica L., Salvia leucophylla L., and Chrysanthemum balsamita L. for four to

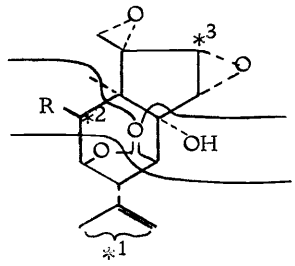
five days before harvesting. Label from MVA-2-^{14}C is expected to be found on C-8 or C-9 and on C-2 or C-6. Labelled (±) camphor was degraded by four different methods. In all the cases, 78-83% of label was found on the C-5:C-6 fragment, presumably localized on C-6, which indicates that C-1, 2, 3, 6 and 10 are from the IPP unit and that the IPP unit is preferentially labelled. Labelled IPP apparently was not appreciably converted into DMAPP, which is consistent with the earlier result (Banthorpe and Turnbull, 1966) that IPP condensed with unlabelled DMAPP which possibly came from a pre-existing metabolic pool.

In addition, unequal labelling of two sesquiterpenes recently has been found. Biollaz and Arigoni (1969) administered (±) Na-2-^{14}C-MVA to cut twigs of Coriaria japonica. Coriamyrtin and tutin were isolated and degraded. One expected to find label distributed equally

2nd IPP

1st IPP

DMAPP



Coriamyrtin R = H

Tutin R = OH

among $*^1$, $*^2$ and $*^3$ if DMAPP is formed by the isomerization of labelled IPP and subsequently condenses with additional IPP. But the results showed 20% of the label on each of $*^1$ and $*^2$ and 60% of the label on $*^3$, which arises from the second IPP unit added to the

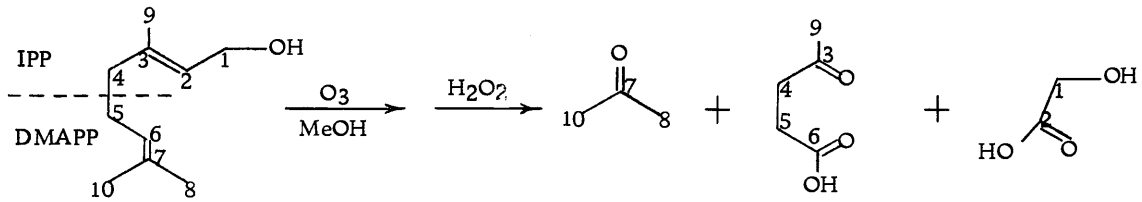
molecule in both cases.

Purpose of Study

Uneven incorporation of MVA-2-^{14}C into mono- and sesquiterpenes was clearly shown by the work of Banthorpe and co-workers (1966, 1968) and Biollaz and Arigoni (1969). But exogenous MVA has been found to be a poor precursor of monoterpenes in most plant tissues tested (Loomis, 1967). Up to now, $^{14}CO_2$ has been found to be the best exogenous precursor of monoterpenes in all tissues except rose petals (Battaile and Loomis, 1961; Loomis, 1967; Francis and O'Connell, 1969). In peppermint leaves, the incorporation of label from $^{14}CO_2$ is not only rapid but also attains a significant level (0.2-0.4%). It has been suggested that the site of monoterpene synthesis is isolated so that exogenous MVA cannot reach it, and that MVA for monoterpene synthesis is normally synthesized *in situ* from translocatable carbon compounds such as sucrose.

If geraniol synthesis from MVA occurs by the condensation of one molecule of DMAPP which comes directly from IPP with one molecule of IPP, then, using $^{14}CO_2$ as the precursor, one should observe uniform distribution of the radioactivity in the geraniol molecule. On the other hand, if a DMAPP pool exists in geranium tissue such as that postulated by Banthorpe and his co-workers (1966, 1968), this metabolic pool should be detected using $^{14}CO_2$ as the precursor.

Radioactive geraniol biosynthesized from $^{14}CO_2$ in Pelargonium graveolens was chosen as the monoterpene with which to examine this possibility in this study not only because it is a metabolically significant product, but also because of the convenience of obtaining the material. Over the past several years, the biochemistry of this monoterpene has received considerable attention in several laboratories (Allison, 1963; Baisted, 1967; Madyastha and Loomis, 1969; Francis and O'Connell, 1969). Thus source material and techniques for the isolation and purification of the compound were available. Furthermore, geraniol contains an isopropylidene group which may be readily cleaved with ozone under specific conditions to produce acetone (Milas



and Nolan, 1959). This relatively simple degradation procedure provides a method for examining the three terminal carbons (C-7, 8 and 10) of the five carbon unit presumably derived from DMAPP.

Geraniol isolated from geranium cuttings at different time intervals after pulse labelling with $^{14}CO_2$ was degraded. If label from $^{14}CO_2$ is incorporated randomly into geraniol, then 30% of the geraniol label should be found in the acetone fragment after degradation.

Deviation from 30% incorporation of the total geraniol label in this C_3 unit would indicate either that geraniol is not synthesized via the known pathway from MVA, or that IPP and DMAPP are not in equilibrium. The existence of a metabolic DMAPP pool was proposed for Thuja occidentalis (Banthorpe and Turnbull, 1966), Artemisia californica, Salvia leucophylla, Chrysanthemum balsamita (Banthorpe and Baxendale, 1968); compartmentation between DMAPP and IPP was suggested by Francis (1970). A pathway of monoterpene biosynthesis in which only one of the C_5 units comes from MVA was suggested by Francis et al. (1970).

MATERIALS AND METHODS

Materials

Pelargonium graveolens L'Her plants were grown in the greenhouse with a photoperiod of at least 15 hours and a day-night temperature cycle of 24°C and 18°C. Plants were grown in Perlite (Supreme Perlite Company, Portland, Oregon) and were irrigated three times a week with Hoagland and Arnon nutrient solution No. 2 (Hoagland and Arnon, 1950).

Standard geraniol was obtained from Aldrich Chemicals, Milwaukee, Wisconsin and found to be >99% pure by gas chromatography. A trace of nerol was found as an impurity.

$^{14}CO_2$ used for shoot tip incorporation was obtained in the form of $Ba^{14}CO_3$, specific activity 62.2 mc/mmole, from New England Nuclear Corporation. It was converted to sodium carbonate by decomposing it in an excess of perchloric acid in an evacuated system in which the released $^{14}CO_2$ was trapped in NaOH solution. Stock solutions were prepared at a concentration of 2.5 mc/ml. The adsorbent for thin-layer chromatography (TLC) was Silica Gel G obtained from E. Merck AG., Darmstadt, Germany.

Gas chromatographic analyses were done on a 28 foot x 1/8 inch aluminum column packed with 100/120 mesh Chromosorb G with a coating of 1.5% sucrose acetate isobutyrate (SAIB) and 1% phenyl diethanolamine succinate (PDEAS). All three were obtained from

Varian Aerograph, Walnut Creek, California. The column, packed according to the method of Horning, VandenHeuval and Creech (1963), was operated isothermally at 140°C with helium carrier gas at a flow rate of 12 ml/min.

Hexane used in the experiment was Skellysolve B, which is essentially n-hexane, with a boiling range of 60-68°C.

Rhodamine 6G, obtained from National Aniline Division, Allied Chemical Corporation, New York, New York, in a 0.05% (w/v) aqueous solution was used as a spray to detect compounds on TLC plates.

Methods

Measurements of Radioactivity

Three types of instruments were used in the entire work. A liquid scintillation spectrometer was used in counting the ^{14}C in hexane extracts and geraniol. A radiochromatography detector coupled with a gas-liquid chromatograph was used to determine the specific activity of labelled geraniol. Also an α, β, γ-proportional counter was used for the planchet counting used in the specific activity determination of acetone ^{14}C-DNP. Radioactivity of all samples throughout this work is expressed in disintegrations per minute (dpm) because the various instruments have different counting efficiencies.

Liquid Scintillation Spectrometer, Packard 3003. The efficiency

of the instrument was determined every time it was used and was found to be 85% by using a ^{14}C-toluene solution. Ten ml of the counting solution were used in each sample vial. The counting solution used for non-aqueous samples contained 40 mg PPO (2, 5-diphenyloxazole) and 0.3 mg POPOP [1, 4-bis-2'-(5'-phenyloxazolyl)-benzene] per liter of toluene. The counting solution used for aqueous samples was Bray's solution (Bray, 1960), which consists of 0.6g naphthalene, 40 mg PPO, 2 mg POPOP, 1 ml absolute methanol and 0.2 ml ethylene glycol in 1 liter of purified p-dioxane. When Bray's solution was used, any quenching was corrected for by "spiking" the sample with a known amount of ^{14}C-toluene.

Alpha, Beta, Gamma-Proportional Counting. Radioactivity determinations of acetone-^{14}C-DNP were carried out with a NMC Nuclear Measurements instrument, Model PC 3A, using argon-methane (9:1) counting gas. It was necessary to use this instrument because the quenching of the acetone-^{14}C-DNP derivative was almost 100% on the liquid scintillation counter. Although the efficiency of the α, β, γ - proportional counter was less than that of the liquid scintillation counter, statistically significant counting was obtained by accumulating over 10,000 counts for each sample. The efficiency of the proportional counter was determined by counting an aliquot of a ^{14}C-cholesterol sample of known radioactivity. This efficiency was found to be about 58%.

A preliminary determination was made on a thin-window, low-background counter, the efficiency of which was only approximately 10%. Such counting efficiency led to the use of the α, β, γ-proportional counter, which was used for most of the work.

Gas-Liquid Chromatography-Radiochromatography Detector System. A Beckman GC-4 gas chromatograph equipped with a flame ionization detector was used. A 10:1 splitter at the end of the column allowed 90% of the effluent to pass through a radioactivity detector (Nuclear Chicago, Model 4998). The radiochromatography detector system is designed to assay low energy β-rays emitted by the radioactive material emerging from the gas chromatograph. By this technique the only mass peak from geraniol-^{14}C purified by TLC corresponded to standard geraniol and was coincident with the only radioactivity peak. Both the radioactivity and mass of eluate were recorded on a Sargent dual recorder, Model DSRG, fitted with dual disc integrators.

The instruments were first calibrated with standard solutions of non-radioactive geraniol and ^{14}C-toluene. The corresponding radioactivity peak as well as the mass peak were measured by comparing the area or the sweeps with standards. The specific activity of geraniol-^{14}C was then determined.

The composition of geranium oil is complex. It includes primarily geraniol, citronellol, menthol, linalool, citral, citronellal

and menthone. Most of these components have been identified by comparing the retention times of the peaks with those of known monoterpenes through gas chromatography (Allison, 1963). A typical strip chart scan of a chromatogram of a hexane extract from experiment 2 is shown on Figure 2.

Incorporation of $^{14}CO_2$ into Shoots of Pelargonium graveolens

Rose geranium cuttings with approximately 2 cm stems and having no more than three developing leaves and the growing tip were used. Their average weight was approximately 340 mg. The cuttings were severed from larger shoots under water in order to prevent air bubbles forming in the basal ends of the cuttings and inhibiting the subsequent uptake of water. Two shoot cuttings were maintained in a small vial or a 5 ml beaker containing water. All the experiments were started in the morning and shoots were obtained from the greenhouse immediately prior to the start of experiments.

Each $^{14}CO_2$ fixation was carried out in glass vessels as shown in Figure 3. The vessels were kept as small as possible to prevent dilution of the $^{14}CO_2$. The Erlenmeyer flasks were 125 ml capacity and the bell jars were either 325 ml or 1000 ml, depending on the number of cuttings used in the experiment. In every experiment, a central vial contained a solution of $Na_2{}^{14}CO_3$. The fixation vessel was sealed and flushed with CO_2-free air. A slight negative pressure

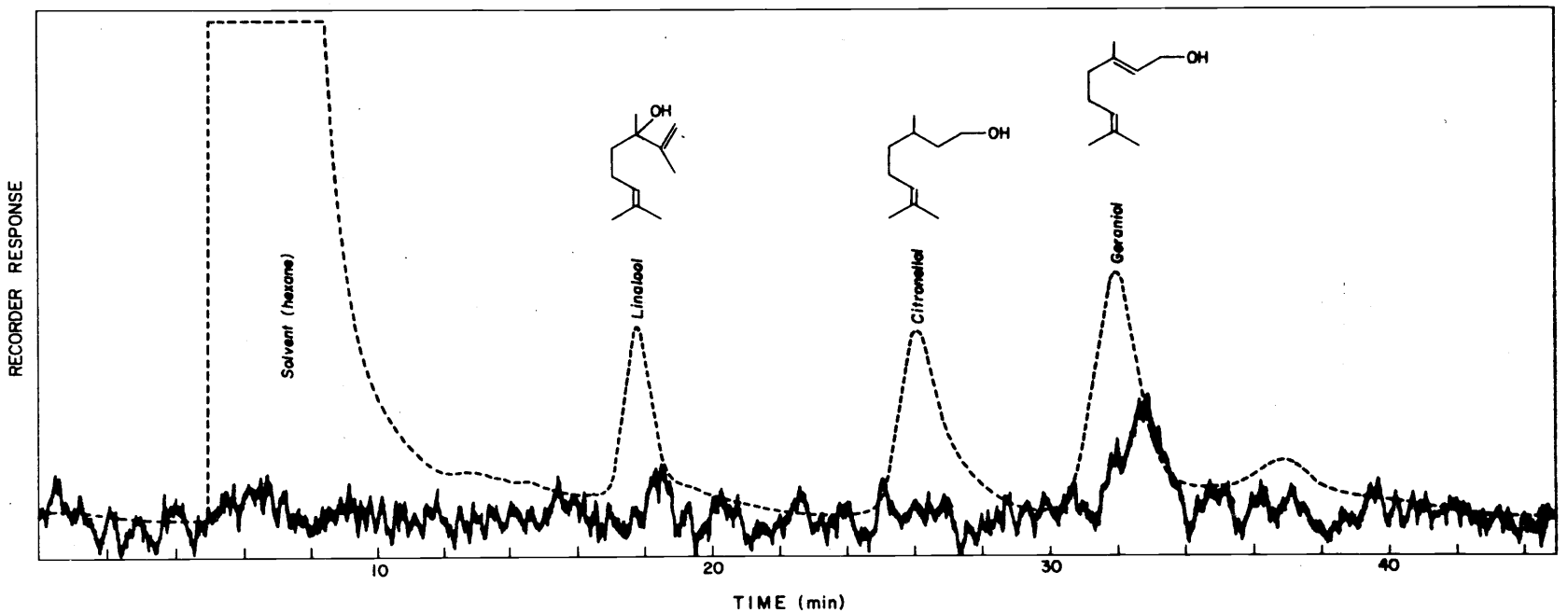


Figure 2. Radiochromatogram of hexane extract from experiment 2, seven hours sample.
- - - - mass radioactivity

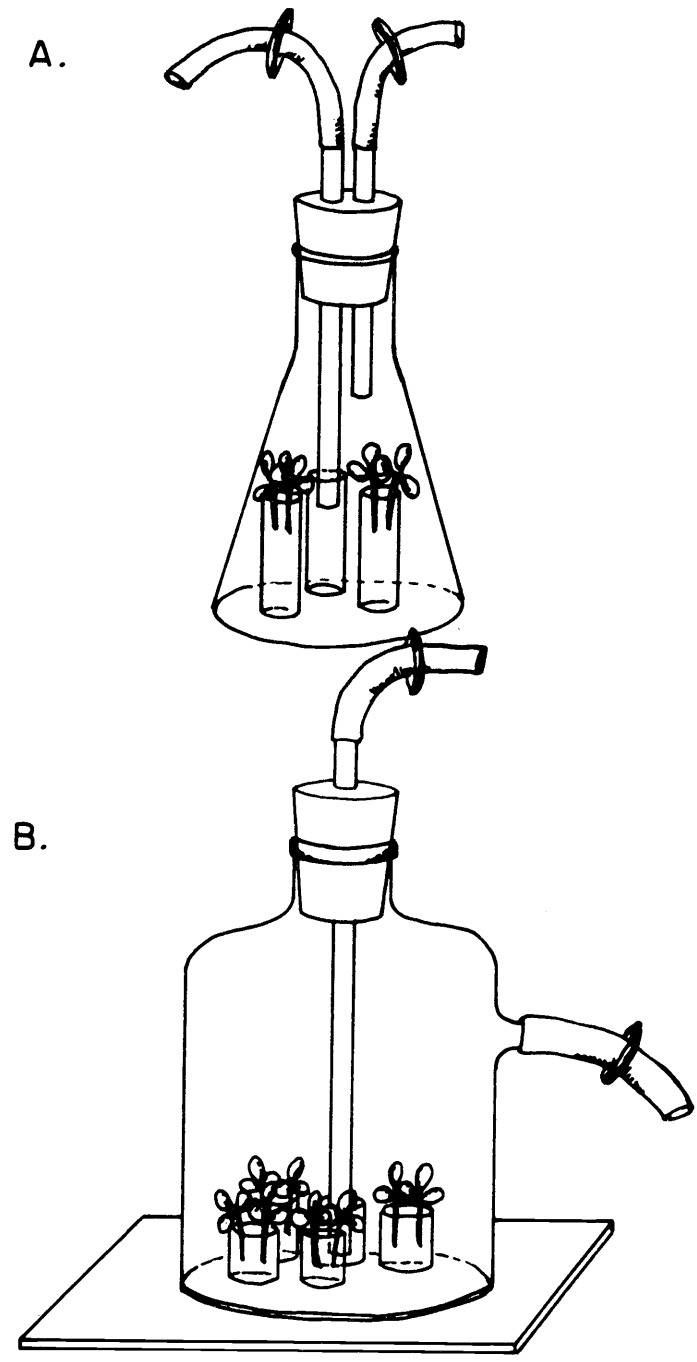


Figure 3. Reaction vessels: $^{14}CO_2$ fixation by geranium cuttings.
A. For small scale experiments.
B. For larger scale experiments.

was then applied to the vessel and the $^{14}CO_2$ was released by injecting an excess of 10% perchloric acid into the central vial. The fixation vessel was placed in a growth chamber with controlled light conditions (1000 foot-candle intensity, as determined with a General Electric No. 213 light meter). Illumination was provided continuously by either Sylvania VHO Gro-Lux lamps supplemented with incandescent bulbs or an equal mixture of VHO Gro-Lux and VHO Gro-Lux wide spectrum lamps. A constant temperature (24°C) was maintained during the experiment.

At the end of the fixation period, any remaining $^{14}CO_2$ was flushed into 4N KOH solution and the radioactivity was measured by liquid scintillation counting.

Isolation of ^{14}C-labelled Geranium Oil

Isolation of geranium oil was carried out according to the procedure of Allison (1963). The shoots were ground in a mortar, and geranium oil was extracted with hexane. Anhydrous sodium sulfate, as a drying agent, and sand, as an abrasive were used in the grinding procedure. Carrier geraniol (20 mg) was added to prevent evaporative loss of labelled geraniol during the extraction. Extractions were repeated until the last fraction of extract was colorless. The combined hexane extracts were decolorized with activated charcoal (Norit A). After centrifugation, the volume was carefully reduced under a

stream of nitrogen at room temperature.

Isolation and Purification of Geraniol-^{14}C

Isolation of the geraniol was accomplished by taking advantage of the fact that upon TLC on silica gel G, geraniol and citronellol are separated from the other constituents of geranium oil. The separation of geraniol from citronellol is achieved on silver nitrate-impregnated silica gel plates because of the greater affinity of geraniol, which contains two carbon-carbon double bonds, than citronellol, with only one double bond, for Ag^+. In practice, the two chromatographic separations were carried out in the reverse order to avoid the chance of contamination of the isolated geraniol by oxidative products formed in the presence of $AgNO_3$ (Baisted, unpublished results).

The preparative thin-layer chromatographic plates had a thickness of 0.25 mm. The adsorbents were spread as slurries of silica gel G in H_2O or in 4% aqueous $AgNO_3$ (1:2, w/v) on 20 x 20 cm glass plates. Plates were activated in the oven at 100°C for 25 minutes before use. Since $AgNO_3$ is readily reduced in light and air, the plates containing this substance were usually made shortly before use. Spreading, drying, activating, application of sample and development of the plate were all carried out in dim light to decrease light-catalyzed degradation of geraniol.

The solvent system used for $AgNO_3$ impregnanted plates was ethyl acetate:hexane (1:2, v/v) and for silica gel G plates was ethyl acetate:hexane (12:88, v/v). Standard geraniol, as a marker, was run alongside the geranium oil. After development, reference samples of geraniol were located by spraying the area of the chromatogram which contained the marker with Rhodamine-6G, while keeping the remainder of the plate covered. Reference compounds were also detected under ultraviolet light (254 nm). The portion of the radioactive sample which migrated identically with authentic, non-radioactive geraniol was scraped off, and the silica gel was eluted with ether. During the elution an additional 20 mg of carrier geraniol was added. The ether solution of the partially purified geraniol from the silver nitrate-impregnated plate was again carefully evaporated to a small volume under a stream of nitrogen. Rechromatography of this sample on a silica gel plate in the second solvent system was again performed using geraniol as a marker. The identification procedure and recovery from the silica gel adsorbent was the same as for the silver nitrate plate.

The final eluate was concentrated to 10 ml by evaporation in order to determine its ^{14}C content. A 100 µl aliquot was taken out of 10 ml of ether extract. The purity and specific activity of the geraniol-^{14}C sample were determined by gas chromatography coupled to a radioactive counting system, as described before, together with

the liquid scintillation counting system.

Ozonolysis

In order to study the labelling in the three terminal carbons (C-7, 8, 10) of the geraniol molecule, ozonolysis was used to break the double bond between C-6 and C-7. The Welsbach ozonator Model T-23 was used in this procedure. Because ozonolysis of substances such as cinnamyl alcohol, α-methylallyl ethyl ether, crotonaldehyde and geraniol has been shown to lead to rearrangements, low temperature conditions and the use of methanol as a solvent were chosen (Milas and Nolan, 1959) to avoid accumulating rearrangement products. The reaction tube and adapter were specially made so that the inlet tubing of the adapter is about 0.5 mm away from the tube bottom. A fine opening at the end of the inlet tube permits ozone to disperse into the reaction solution as small bubbles at a flow rate of 17 ml per minute. Under these conditions, the oxidation is accomplished efficiently. In a reaction mixture that contains 0.5 m mole of material, the reaction was completed in 12 minutes.

A standard solution of authentic geraniol in methanol was made and a measured volume of this solution (containing 20-50 mg geraniol) was added as carrier to each radioactive geraniol sample. The combined solutions, in a 5 ml volumetric flask, were then diluted to 5 ml with methanol. The specific activity of the diluted geraniol-^{14}C

was determined by counting an aliquot with liquid scintillation counter. The geraniol-^{14}C was transferred to the reaction vessel and cooled to -70°C in a dry ice-acetone bath.

A 2% ozone-oxygen mixture was flushed through the reaction mixture and then through an acidic potassium iodide trap to detect unreacted ozone which would indicate the completion of ozonolysis.

Following the completion of the reaction the ozonide was decomposed by the addition of acetic acid (1 ml) to the ice-cold reaction tube. The mixture was then brought to room temperature during which time 1 ml of 50% H_2O_2 was added. The tube was shaken at room temperature for one hour and an additional 2 ml of 25% H_2O_2 was added while allowing the tube to stand at room temperature for a further hour. Then the mixture was heated in a hot water bath at 65-70°C under a reflux condenser. After one hour water (2 ml) was added to the solution. The mixture which contained acetone, levulinic acid and glyoxylic acid was finally brought to pH 5 with 2 N NaOH (Cornforth and Popjak, 1954).

Acetone was isolated from this mixture by distillation directly into 2, 4-dinitrophenyl hydrazine solution (Shriner, Fuson and Curtin, 1964). The distillation was carried out at a temperature that never exceeded 65°C (boiling point of methanol), so that the 2, 4-dinitrophenyl hydrazone obtained was essentially that of acetone.

The crude crystals were filtered under reduced pressure and

recrystallized from hot ethanol. The melting point of the sample was 123-125°C [literature values range from 123 to 126°C (Shriner et al., 1964)] and was not depressed when mixed with an authentic sample.

Acetone in the free form is very volatile; therefore, it was necessary to prepare a non-volatile derivative to prevent uncontrolled losses of material. The 2, 4-dinitrophenyl hydrazone is relatively stable in air even after several months. Although quenching is almost 100% when using a liquid scintillation counter, the low background counter and α, β, γ-proportional counter provided good results.

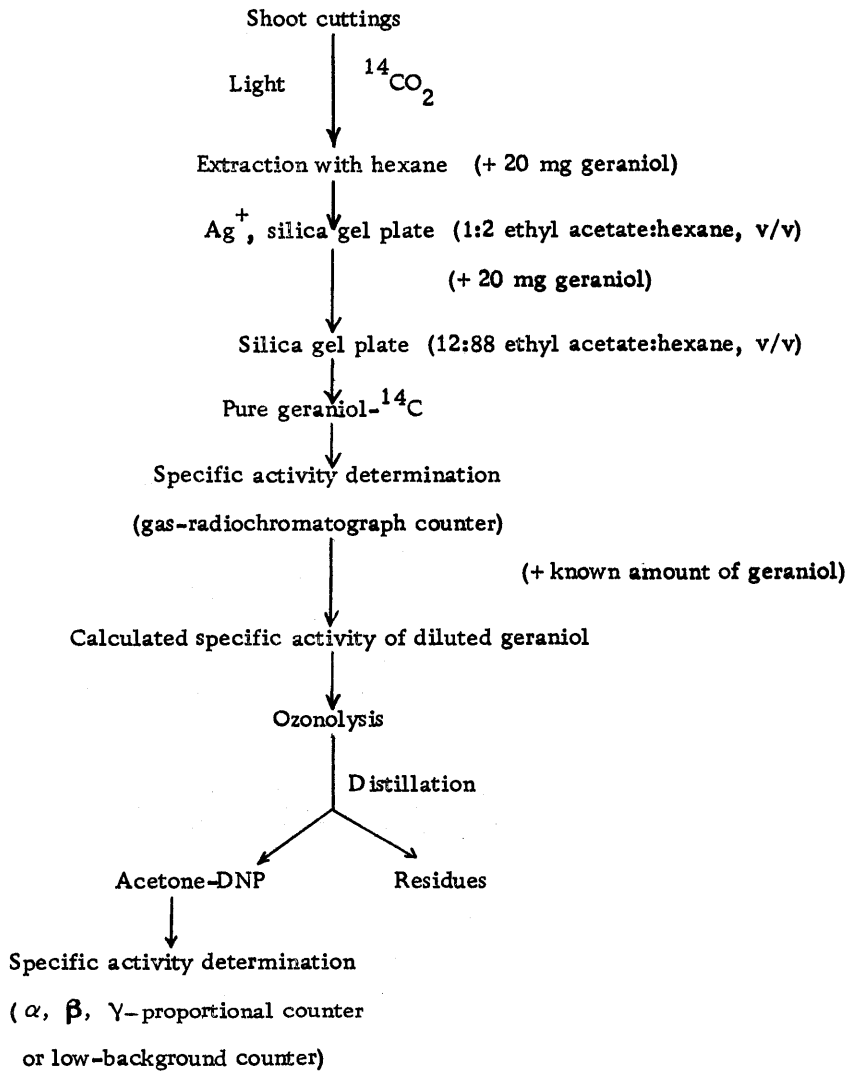
The specific activity determination was made by weighing 10 mg from each crystallization and dissolving it in chloroform in a 1 ml volumetric flask. A 100 µl aliquot was applied to a 3 cm diameter planchet. When three successive crystallizations had essentially the same specific radioactivity, the counts from the last recrystallization were used in calculating the specific activity of acetone-^{14}C-DNP.

The flow sheet which follows summarizes the procedure of extraction, separation, purification and degradation of biosynthesized geraniol-^{14}C.

Statistical Treatment of Results

The standard deviation of the radioactivity counts was calculated as described by Wang and Willis (1963). The standard deviation ($\hat{\sigma}_{"C_3"}$) is calculated as

Flow sheet showing the experimental procedure



$$\sigma_{"C_3"} = \sqrt{\frac{r_g}{t_g} + \frac{r_b}{t_b}}$$

where r_g is gross sample counting rate and r_b is background counting rate, t_g and t_b are sample and background counting time. The approximate standard deviation of percent of the geraniol label found in the "C_3" portion was calculated by

$$\hat{\sigma}_{"C_3"} = \frac{\mu^2_{ger}\,\hat{\sigma}^2_{"C_3"} + \mu^2_{"C_3"}\,\hat{\sigma}^2_{ger}}{\mu^4_{ger}}$$

percent standard deviation of DMAPP in total geraniol is 5/3 $\hat{\sigma}_{"C_3"\%}$ and that percent standard deviation of IPP in total geraniol is calculated by $\hat{\sigma}^2_{IPP\%} = \hat{\sigma}^2_{ger\%} + \hat{\sigma}^2_{DMAPP\%}$. The μ's stand for approximate mean disintegrations per minute and $\hat{\sigma}$'s represent the approximate standard deviations (Mullooly, J.P., personal communication). All results are expressed as the approximate means $\pm$ 2.575 $\hat{\sigma}$, i.e., 99% confidence limits.

RESULTS AND DISCUSSION

In order to determine if a metabolic DMAPP pool exists in P. graveolens, five $^{14}CO_2$ fixation experiments were carried out to follow the incorporation of ^{14}C into hexane-extractable compounds and to determine the proportion of label incorporated into the two halves of the geraniol molecule. These experiments were: (Experiment 1) continuous fixation of $^{14}CO_2$ in sealed chambers for periods of 20 minutes to 2 hours; (Experiment 2) a one-hour fixation of $^{14}CO_2$ followed by continued metabolism for 23 hours in the sealed chamber, which was flushed with air each time a sample was removed (pulse-labelling); (Experiment 3) 4 hours of fixation followed by 8 hours of metabolism in a sealed chamber; and 2 hours of fixation followed by 22 hours (Experiment 4a) or 20 hours (Experiment 4b) of metabolism in continuously circulating air. Experiments 1 and 2 were preliminary experiments which determined the total incorporation of label. Experiments 3 and 4 determined both incorporation of label into the hexane-extractable material and the incorporation and distribution of ^{14}C in geraniol.

Time Course Studies of $^{14}CO_2$ Incorporation into Geraniol (Experiment 1)

In Experiment 1, four pairs of cuttings were placed in four

individual chambers (Figure 3A), and 50μc of $^{14}CO_2$ were supplied to each chamber. Exposure times of 20, 40, 60 and 120 minutes were used. Although the results (Table 1) showed a progressive increase in labelling with longer times of $^{14}CO_2$ fixation, the total ^{14}C content was too low in all but the 120-minute sample to permit further separation, purification and degradation of the isolated geraniol.

A similar type of result was indicated by Battaile's (1960) investigations with peppermint. In his work the total incorporation of label and the number of terpenes which incorporated ^{14}C from $^{14}CO_2$ increased as time progressed.

Table 1. Fixation of $^{14}CO_2$ by P. graveolens (Experiment 1)

Time of $^{14}CO_2$ incorporation (min.)	No. of cuttings	Radioactivity in hexane extract (DPM)	Percent incorporation
20	2	2710	0.0025
40	2	6650	0.0060
60	2	17620	0.0160
120	2	95880	0.0871

Pulse Labelling in P. graveolens with $^{14}CO_2$ (Experiment 2)

The experiment was designed to determine whether, when geranium cuttings are exposed to $^{14}CO_2$ atmosphere for a short period,

the ^{14}C that was incorporated into other constituents of the plant would be turned over and incorporated into monoterpenes. Fourteen geranium cuttings were exposed to 1.0 mc of $^{14}CO_2$ under continuous light (Figure 3B). After exposure to the $^{14}CO_2$ for one hour, the chamber was flushed with air, and the first sample was taken. The chamber was resealed and illuminated further. Additional samples were taken at intervals up to 24 hours from the start of the experiment. The time course of labelling in total hexane-extractable material is shown in Figure 4.

Results of these preliminary $^{14}CO_2$ fixation studies indicated that incorporation of ^{14}C from $^{14}CO_2$ into the hexane extractable fraction increased with the time of fixation (Experiment 1) and with time following pulse labelling (Experiment 2). However, this represented the total incorporation into the mixture or hexane extract from geranium shoots.

A representation of the gas radiochromatographic scan of the seven hours sample of Experiment 2 is shown in Figure 2. It is evident that not only is geraniol the major volatile component present but that it is always labelled by $^{14}CO_2$. Previous experiments had shown that geraniol and citronellol were the first terpenes to be labelled in rose geranium (Allison, 1963).

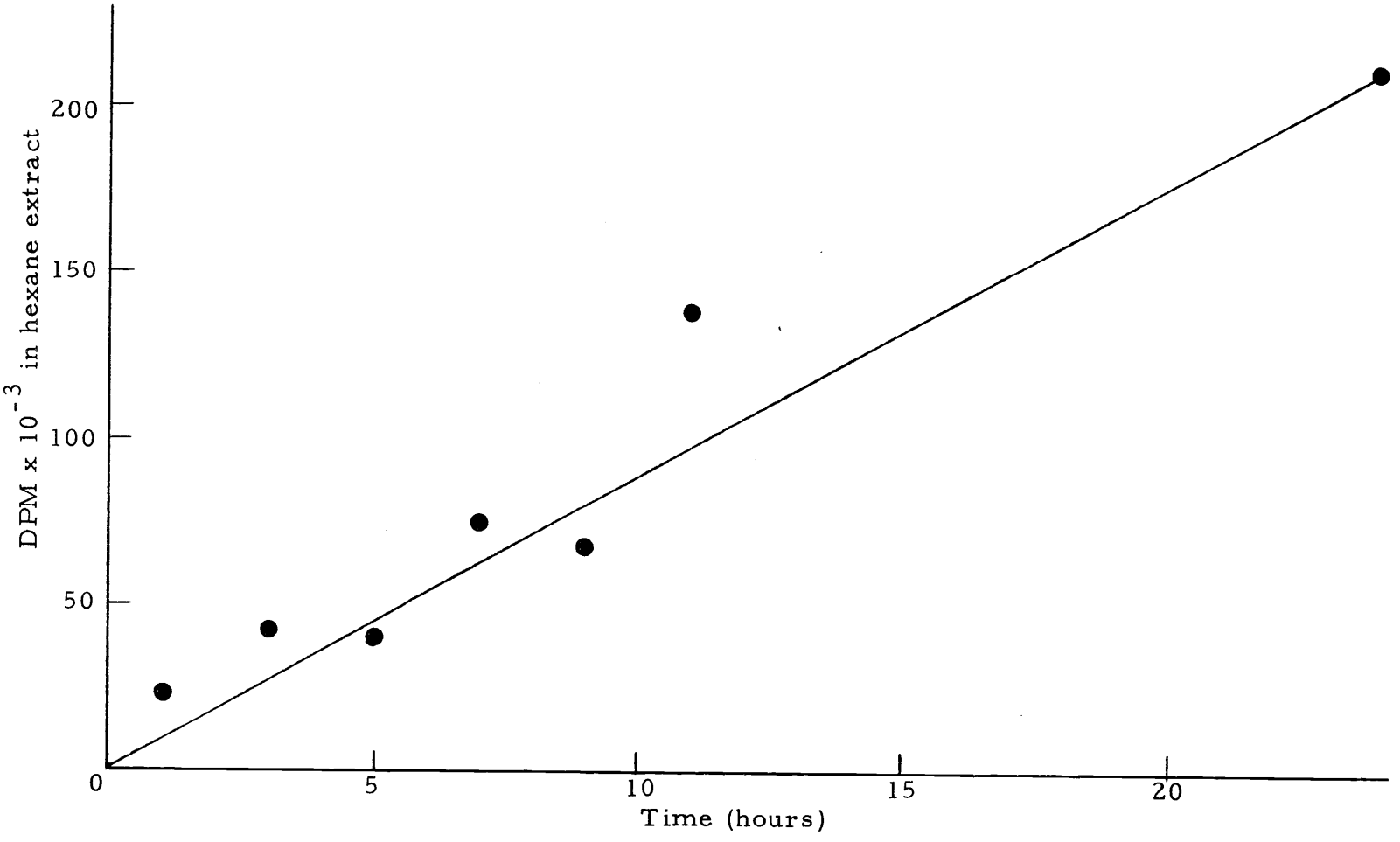


Figure 4. Pulse labelling study of P. graveolens with $^{14}CO_2$ (expt. 2).

Determination of the Labelling Pattern of Geraniol with Four Hours Fixation of $^{14}CO_2$ and Eight Hours Metabolism in a Sealed Chamber (Experiment 3)

In order to obtain a higher incorporation of $^{14}CO_2$ into geraniol, a longer period of $^{14}CO_2$ fixation was utilized. Eight geranium shoots were exposed to 1 mc of $^{14}CO_2$ in a 325 ml bell jar for four hours (Figure 3B). Excess $^{14}CO_2$ was flushed out of the chamber and was determined to be 8.5% of the $^{14}CO_2$ administered; that is, 91.5% of the $^{14}CO_2$ was fixed by the shoots. After four shoots were removed at the end of the four hour fixation period, the chamber was resealed and the remaining cuttings were maintained in the light for an additional eight hours. The 4- and 12-hour samples were ground and extracted with Skellysolve B and the geraniol was separated and purified by TLC. The specific activities were determined as described above. The geraniol-^{14}C of known specific activity was then subjected to ozonolysis. The acetone produced by ozonolysis was distilled and obtained in the form of its 2, 4-dinitrophenyl hydrazone derivative. The acetone fraction, C-7, 8, 10 of the geraniol, will hereafter be referred to as the "C_3" unit.

The specific activities of geraniol-^{14}C and "C_3", and the calculated distributions of radioactivity in the hypothetical IPP and DMAPP moieties are listed in Table 2. The radioactivity of the "C_3" unit after each crystallization is shown in Table 3. It was found that

Table 2. Time course study of geraniol-^{14}C with four hours exposure to $^{14}CO_2$ and further metabolized in a sealed chamber (expt. 3).

Time (hrs)	Radioactivity (dpm)		Specific activity (dpm/μ mole)*			Percent of total geraniol label*		
	Hexane ext.	Geraniol-^{14}C	Geraniol-^{14}C	Diluted geraniol-^{14}C	"C_3"	"C_3" (obs.)	DMAPP** (calc.)	IPP** (calc.)
4	26×10^6	7.3×10^6	$47 \pm 4 \times 10^3$	950 ± 80	197 ± 4	20.8 ± 2.4	34.6 ± 3.9	65.8 ± 11.7
12	83×10^6	26×10^6	$110 \pm 11 \times 10^3$	1135 ± 113	236 ± 4	20.8 ± 2.7	34.8 ± 4.5	65.2 ± 13.5

*Approximately 99% confidence limits.

**Calculated values based on "C_3" by assuming geraniol came from condensation of two isporene units, each randomly labelled.

Table 3. Radioactivities of three successive crystallizations of "C_3" samples (expt. 3).

Time (hrs)	Number of crystallizations	dpm/mg	dpm/μ mole	Percent of total geraniol label
4	1	820	196	20.5 ± 2.4
	2	800	190	20.0 ± 2.3
	3	829	197	20.8 ± 2.4
12	1	1031	245	21.7 ± 2.8
	2	1020	243	21.4 ± 2.8
	3	994	237	20.8 ± 2.7

at the end of four hours, 1.2% of ^{14}C was present in the hexane-extractable fraction and 0.33% in geraniol. After the shoots were allowed to metabolize in the sealed chamber for eight more hours, 3.8% of the radioactivity was found in the hexane extract and 1.2% was in the geraniol fraction. The percent of incorporation was calculated based on the assumption that ^{14}C entered into each geraniol cutting with equal probability. Both after four hours and after 12 hours the "C_3" unit contained only 20% of the total geraniol label rather than the expected 30%. This suggests, as shown in Table 2, a preferential labelling of the IPP-derived portion of the geraniol molecule.

Determination of the Labelling Pattern of Geraniol After 2 Hours $^{14}CO_2$ Fixation Followed by 22 Hours (Experiment 4a) and 20 Hours (Experiment 4b) Metabolism in Circulating Air

Experiments 4a and 4b were designed to observe the change of the labelling pattern in geraniol after $^{14}CO_2$ feeding ceased when a normal atmospheric CO_2 concentration was maintained. Geranium cuttings were allowed to fix $^{14}CO_2$ photosynthetically for two hours; then the cuttings were maintained under illumination while the chamber was continuously flushed with air, and samples were taken at different time intervals. Under these conditions, refixation of $^{14}CO_2$, liberated by respiration, into geraniol should be minimized.

In experiment 4a, 14 cuttings were allowed to fix 1.5 mc $^{14}CO_2$ in the sealed chamber (Figure 3B). After two hours, the chamber was flushed and the first sample was taken. The air drawn through the chamber was continuously flushed into 4N KOH. The second sample was taken at four hours and others were taken at four-hour intervals for a total of 24 hours. In the second experiment (4b), 2 mc of $^{14}CO_2$ was used for ten cuttings. The samples were taken every five hours after the two hours of $^{14}CO_2$ fixation. The last sample was taken at 22 hours from the start of the experiment.

The percentages of incorporation of $^{14}CO_2$ into the hexane extractable fraction and geraniol fraction in experiment 4a are listed in Table 4. There is an increase in incorporation up to 20 hours. The marked drop at 24 hours may be due to depletion of radioactive intermediates combined with metabolic turnover of geraniol (Burbott and Loomis, 1969).

After the samples were ozonized, radioactivity in "C_3" was compared with that in the total geraniol. Table 5 shows the percentage of radioactivity of "C_3" in total geraniol as well as the values of DMAPP and IPP moieties calculated from "C_3". Percent of total geraniol label in the observed "C_3" and calculated DMAPP and IPP moieties is also plotted against the time of sampling as shown in Figure 5. If, as postulated, geraniol is synthesized by the condensation of a DMAPP unit with an IPP unit, the results of these experiments

Table 4. Time course experiment with two hours $^{14}CO_2$ fixation and 22 hours metabolism under circulating air--percent incorporation (expt. 4a).

Time (hrs)	No. of cuttings	Radioactivity (dpm)		% Incorporation of ^{14}C	
		Hexane ext.	Geraniol-^{14}C	Hexane ext.	Geraniol-^{14}C
2	2	1.63×10^6	0.13×10^6	0.34	0.03
4	2	1.71×10^6	0.24×10^6	0.36	0.05
8	2	0.74×10^6	0.21×10^6	0.15	0.05
12	2	2.51×10^6	1.13×10^6	0.53	0.24
16	2	2.41×10^6	1.01×10^6	0.51	0.21
20	2	3.41×10^6	1.31×10^6	0.72	0.28
24	2	1.15×10^6	0.29×10^6	0.25	0.06

Table 5. Time course experiment with two hours $^{14}CO_2$ fixation and 22 hours metabolism under circulating air--labelling patterns (expt. 4a).

Time (hrs)	Geraniol*	"C_3"*	Percent of total geraniol label*		
			"C_3"	DMAPP**	IPP**
	(dpm/μ mole)	(dpm/μ mole)	(obs.)	(calc.)	(calc.)
2	187 ± 6	66.0 ± 2.0	35.3 ± 2.0	58.8 ± 3.4	41.2 ± 5.4
4	313 ± 12	49.7 ± 2.0	15.9 ± 1.2	26.5 ± 1.9	73.5 ± 5.4
8	299 ± 13	44.8 ± 2.0	15.0 ± 1.2	24.9 ± 2.0	75.1 ± 6.1
12	1760 ± 23	228 ± 3	13.0 ± 0.3	21.7 ± 0.4	78.3 ± 1.8
16	1356 ± 12	209 ± 2	15.5 ± 0.3	25.8 ± 0.4	74.2 ± 1.2
20	1790 ± 16	330 ± 3	18.4 ± 0.3	30.8 ± 0.4	69.2 ± 1.3
24	403 ± 8	97.9 ± 1.8	24.3 ± 0.9	40.5 ± 1.4	59.5 ± 2.9

*Approximately 99% confidence limits.

**Calculated percent activity based on the assumption that "C_3" came from the three terminal carbons of DMAPP, and that geraniol was made by condensation of these two isoprene units.

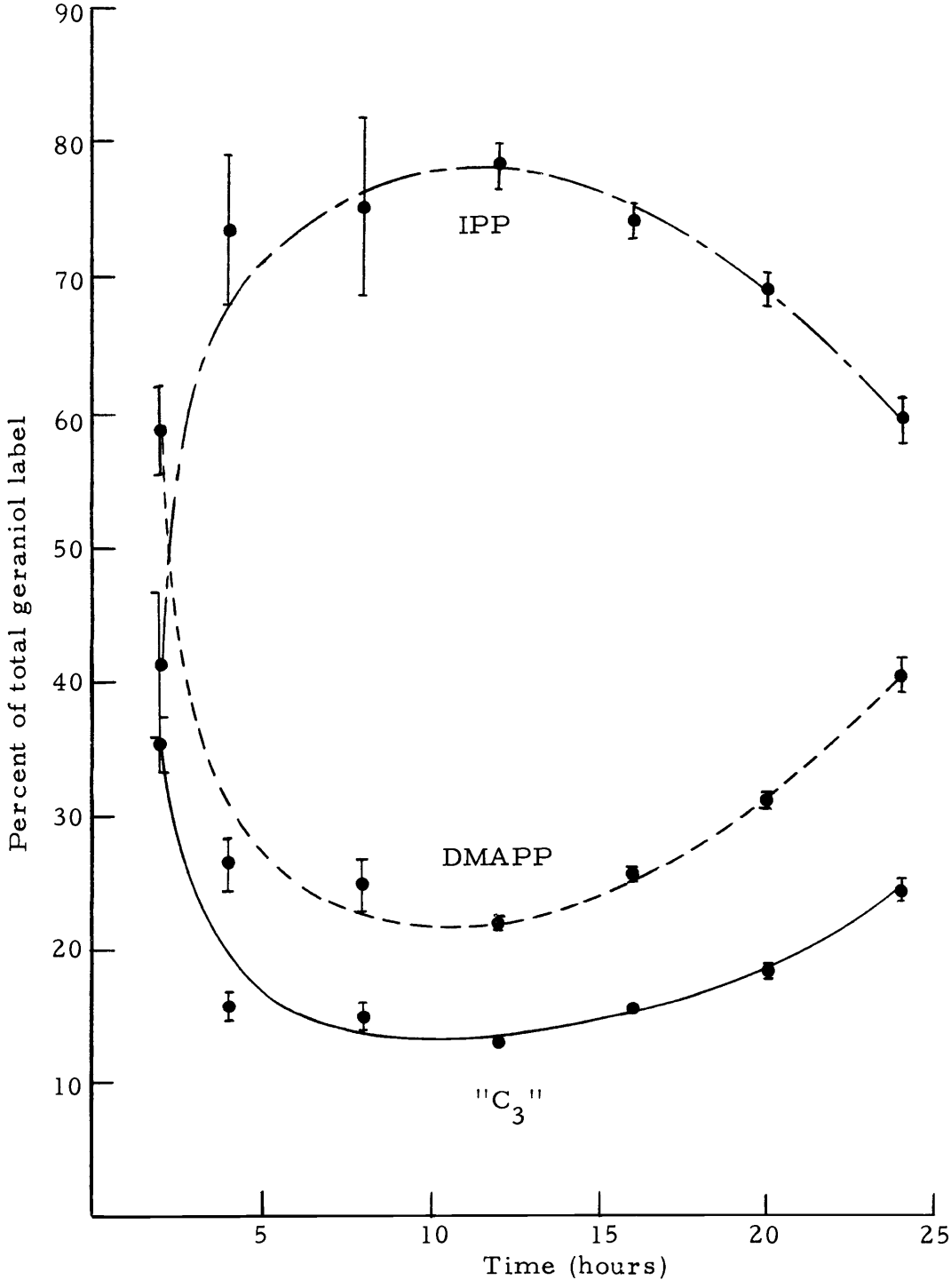


Figure 5. Percent of total geraniol label in time course experiment with two hours $^{14}CO_2$ fixation and 22 hours metabolism in circulating air (expt. 4a).

show that rarely did equivalent labelling occur in the two isoprene units. At two hours there appeared to be a slight preferential labelling of the DMAPP moiety, but in later samples the IPP moiety was clearly labelled preferentially. The same phenomenon is shown in Figure 6, in which the total label of IPP and DMAPP are calculated and compared with the total label of geraniol and "C_3". In DMAPP the total label remains unchanged for six hours after exposure to $^{14}CO_2$ ceases although the total label in geraniol and IPP shows a slight increase. A large increase in IPP label is observed from 8 to 12 hours, followed by an apparent plateau from 12 to 20 hours, and a sharp decrease from 20 to 24 hours. DMAPP gains label slowly and steadily from 8 to 20 hours, and then loses label from 20 to 24 hours.

In experiment 4b, similar data were obtained except the incorporation seems poorer than before, as shown in Table 6 for the total radioactivity obtained and the percent of incorporation. Percent of label of the observed value of "C_3" and calculated values of DMAPP and IPP in total geraniol are listed in Table 7 and plotted in Figure 7. Equivalent labelling was found in the DMAPP and IPP units only at seven hours. In both experiments 4a and 4b, DMAPP had the higher percentage of label in the early samples and IPP in the later samples.

Continuous increases in the total label in geraniol, and IPP and DMAPP moieties with lag periods between two to seven and 12 to 17

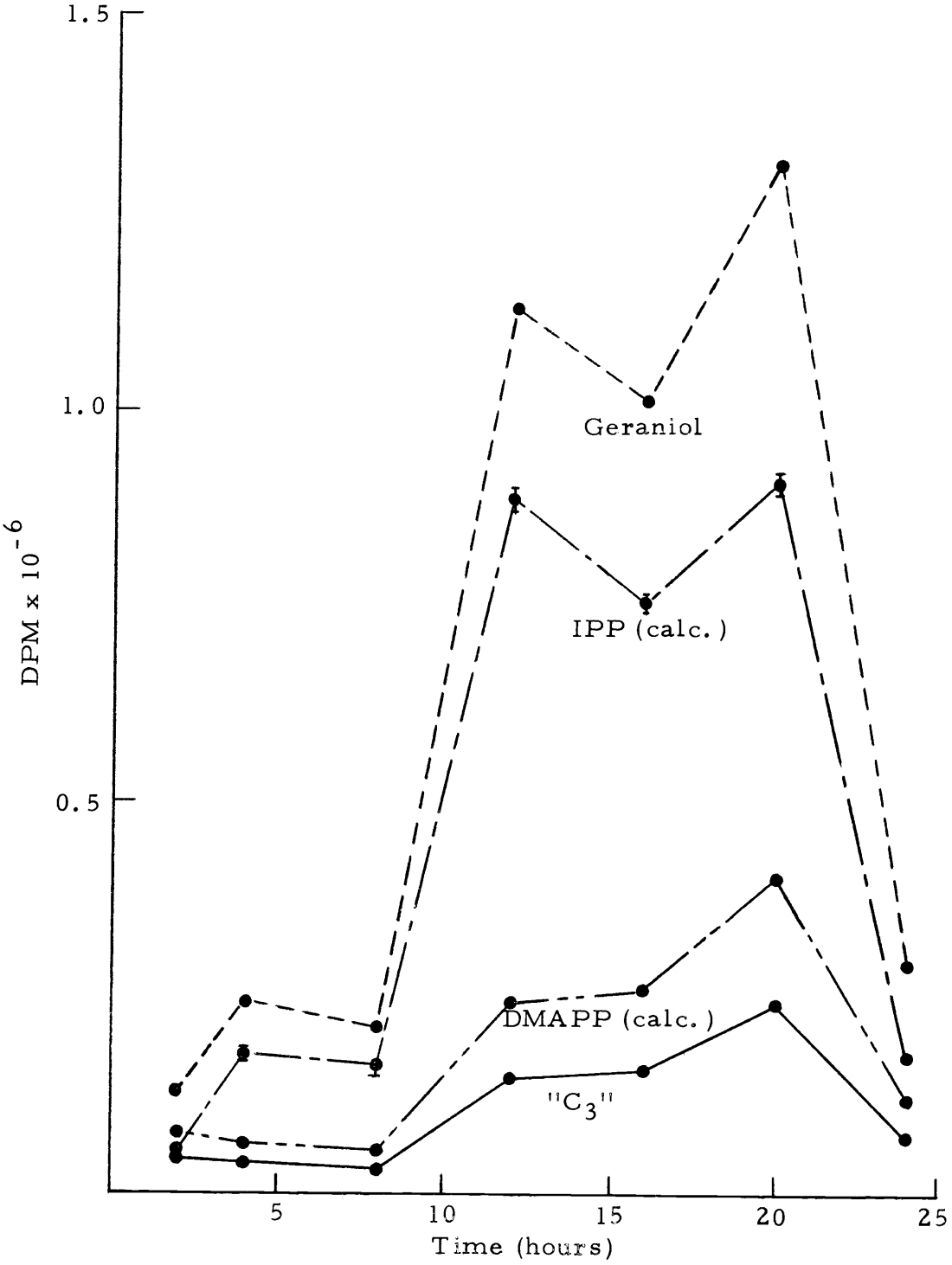


Figure 6. Distributions of radioactivity of geraniol, IPP, DMAPP and "C_3" in experiment 4a (two hours $^{14}CO_2$ fixation followed by 22 hours metabolism in circulating air). Some deviations are small and covered by the original points.

Table 6. Time course experiment with two hours $^{14}CO_2$ fixation and 20 hours metabolism under circulating air--percent incorporation (expt. 4b).

Time (hrs)	No. of cuttings	Radioactivity (dpm)		% Incorporation	
		Hexane ext.	Geraniol-^{14}C	Hexane ext.	Geraniol-^{14}C
2	2	238×10^3	16.9×10^3	0.025	0.0019
7	2	143×10^3	20.0×10^3	0.020	0.0023
12	2	537×10^3	87.7×10^3	0.061	0.010
17	2	286×10^3	77.8×10^3	0.033	0.0089
22	2	1856×10^3	394×10^3	0.21	0.043

Table 7. Time course experiment with two hours $^{14}CO_2$ fixation and 20 hours metabolism under circulating air--labelling patterns (expt. 4b).

Time (hrs)	Geraniol*	"C_3"*	Percent of total geraniol label*		
			"C_3"	DMAPP**	IPP**
	(dpm/μ mole)	(dpm/μ mole)	(obs.)	(calc.)	(calc.)
2	59.4 ± 2.0	23.8 ± 0.9	40.7 ± 2.7	67.8 ± 4.5	32.2 ± 6.3
7	65.9 ± 2.0	19.7 ± 0.8	30.3 ± 2.0	50.5 ± 2.9	49.5 ± 5.7
12	453 ± 27	103 ± 4	22.7 ± 2.1	37.8 ± 3.7	62.2 ± 8.7
17	284 ± 2	52.0 ± 2.7	18.3 ± 1.3	30.5 ± 2.1	69.5 ± 2.3
22	1246 ± 10	249 ± 4	20.0 ± 0.5	33.3 ± 0.9	66.6 ± 1.3

*Approximately 99% confidence limits.

**Calculated percent activity based on the assumption that "C_3" came from three terminal carbons of DMAPP, and that geraniol was made by condensation of these two isoprene units.

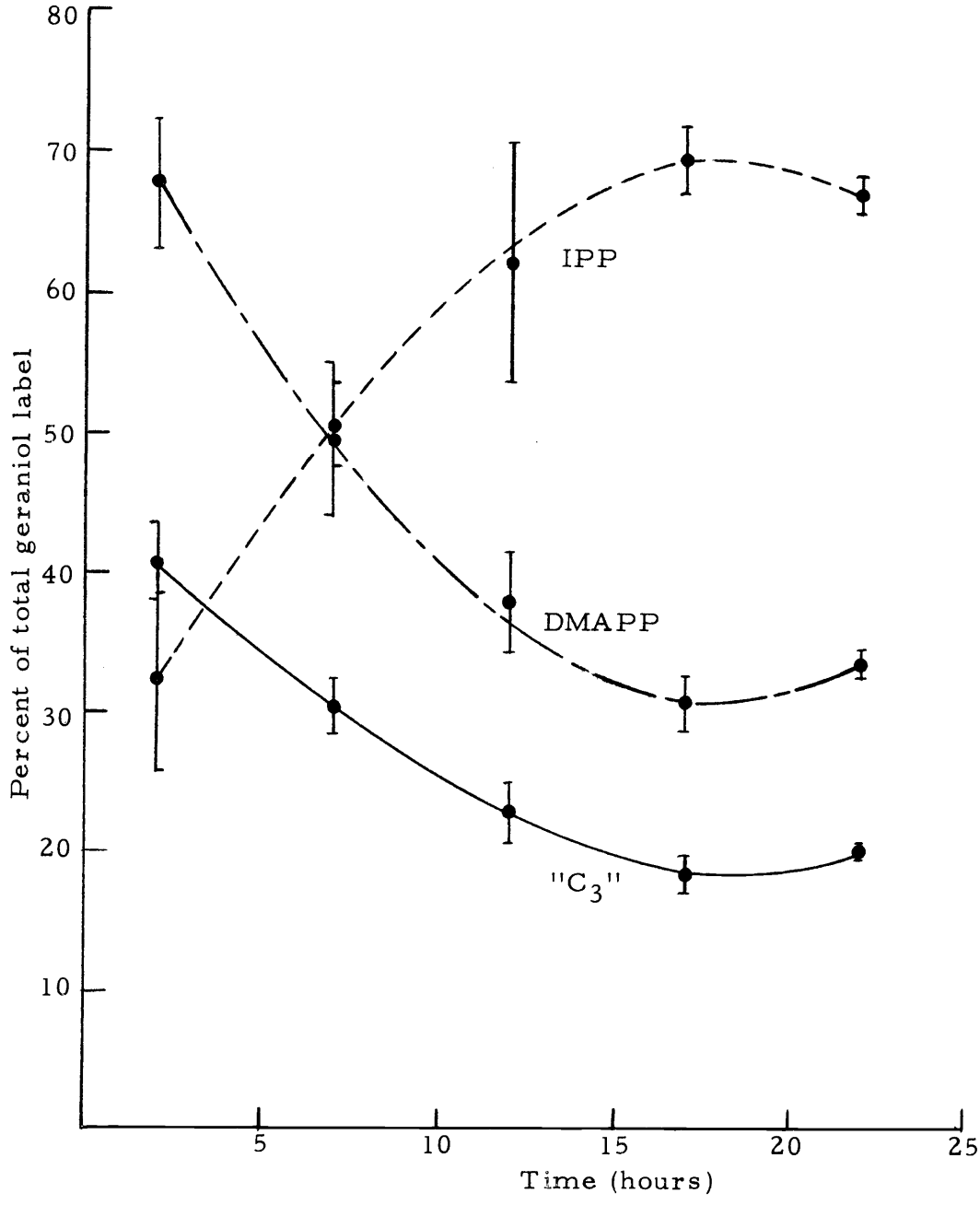
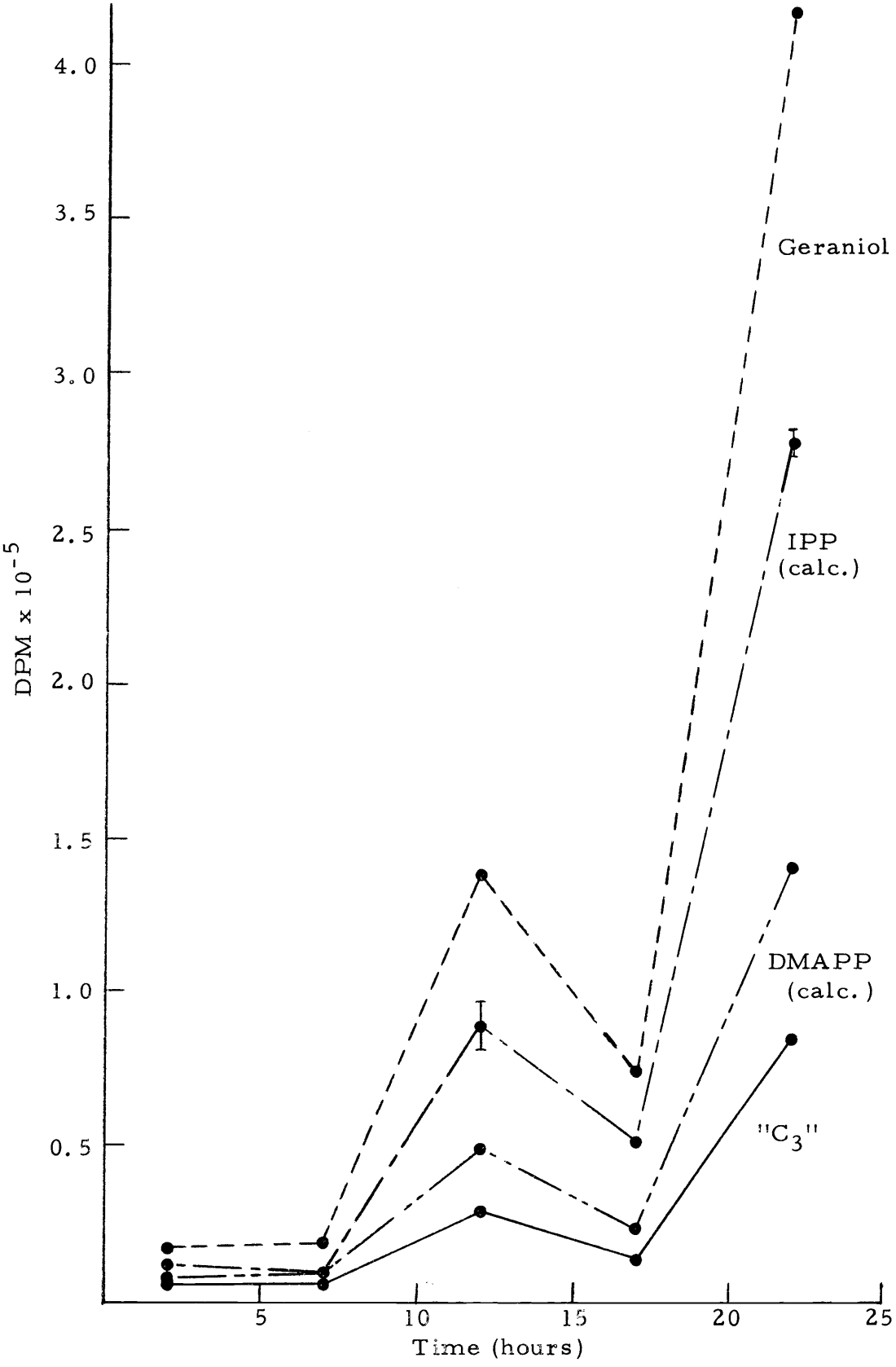


Figure 7. Percent of total geraniol label in time course experiment with two hours $^{14}CO_2$ fixation and 20 hours metabolism in circulating air (expt. 4b).

hours are also observed (Figure 8). Since this experiment was not carried further it did not confirm the marked drop in label observed in the last sample of experiment 4a.

Figure 8. Distributions of radioactivity of geraniol, IPP, DMAPP and "C_3" in experiment 4b (two hours $^{14}CO_2$ fixation followed by 20 hours metabolism in circulating air). Some deviations are small and covered by the original points.


4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
DPM x 10^{-5}
Geraniol
IPP
(calc.)
DMAPP
(calc.)
"C_3"
5
10
15
20
25
Time (hours)

GENERAL DISCUSSION

Throughout all these studies, the incorporation of label from $^{14}CO_2$ into geraniol varied from 0.002 to 0.28%, with the exception of experiment 3, in which 1 mc $^{14}CO_2$ was used for four hours fixation and eight hours metabolism in a sealed chamber, which gave a remarkably high incorporation of 1.5%. The reason for such variation has not been determined. However, as shown in experiment 1, ^{14}C incorporation increased with increasingly longer fixation times, and the high percentage of incorporation in this experiment may have been due to the long exposure to $^{14}CO_2$ in a sealed chamber, with unlabelled CO_2 excluded.

In the pulse labelling experiment (expt. 2) radioactivity in the hexane extracts increased linearly with time. Since labelled CO_2 was removed at the end of the first hour, the increase in label must have come from the transformation of compounds not extractable by hexane. Plant constituents such as sugars, proteins, and fatty acids synthesized during the fixation period were degraded upon subsequent metabolism. Hence, refixation of respiratory CO_2 may also have contributed to the incorporation of label after the initial fixation period. In experiments 3 and 4a and 4b, where the plants metabolized with or without the presence of additional $^{14}CO_2$, similar increases of label in the hexane-extractable and geraniol fractions were also observed except at the end of one experiment (4a). This suggests that once the

external $^{14}CO_2$ had been fixed, the label from turnover of other plant constituents contributed to the synthesis of geraniol to a significant extent.

The data are consistent with the existence of a large metabolic pool of dimethylallyl pyrophosphate (DMAPP) in the shoots of Pelargonium graveolens, just as Banthorpe and Turnbull (1966) suggested for Thuja occidentalis. However, in their experiments, the incorporation of MVA-2-^{14}C was measured over a period of several days whereas in this experiment only two to four hours were used for $^{14}CO_2$ fixation, followed by varying periods of metabolism, up to a maximum of 24 hours total time.

Preferential labelling in the IPP unit is shown in all samples except for the two-hour samples of the two-hour $^{14}CO_2$ fixation time course studies (4a, b) and seven-hour sample of 4b. In both of these experiments, the proportion of label in DMAPP increased after 16 hours. Although the percent of label in the DMAPP moiety reached a minimum at 8-16 hours, the total label in DMAPP was increasing. A metabolic pool may therefore have existed to cause the dilution effect.

Four possible explanations are suggested for the initial favorable labelling on the DMAPP moiety and the subsequent shift to preferential labelling of the IPP moiety of the geraniol. First, under $^{14}CO_2$, IPP had been actively synthesized and isomerized into DMAPP; therefore,

a highly labelled DMAPP pool was built up. It might also be postulated that, during fixation, the high CO_2 concentration (0.1%, several times the normal atmospheric concentration) may have stimulated an increase in the size of the DMAPP pool and possibly in the synthesis of geraniol. If such a DMAPP pool existed, the initial peak of label in IPP was passed before the first sample was taken. One, therefore, observed higher label in the DMAPP moiety because the geraniol was synthesized from labelled DMAPP, plus less highly labelled IPP. Some of this IPP was then isomerized and fed into the DMAPP pool while newly formed IPP had an increasingly larger quantity of label from the turnover of metabolites which were labelled during the $^{14}CO_2$ fixation period. From there on, most label went to the IPP moiety at the normal biosynthetic rate. The DMAPP moiety used in synthesis during this time came from the gradually diluted pool. And the isomerization of the newly synthesized IPP to DMAPP may have started after the DMAPP pool drops back to its normal size while label in both units increased simultaneously from there on.

The second explanation includes the possibility of compartmentation of the two isoprene units (IPP and DMAPP), as suggested by Francis (1970). Francis postulated that if monoterpenes are synthesized at an intracellular surface, the two halves of the monoterpene molecule could be formed from two MVA molecules situated on each side of an impermeable membrane, only one side of which is

accessible to added MVA-2-^{14}C. This suggestion was based on labelling in several monoterpenes biosynthesized specifically from MVA-2-^{14}C. When $^{14}CO_2$ is used as the precursor in geraniol biosynthesis, it is possible that the rates of synthesis in these two compartments are different; therefore, one observed different labelling in these two moieties at different time intervals.

A third theoretically possible explanation, but one for which there is no evidence, is that DMAPP can be synthesized in plants via pathways other than the MVA pathway, and that there is a more direct route from $CO_2 \longrightarrow$ DMAPP than from $CO_2 \longrightarrow$ MVA $\longrightarrow$ IPP $\longrightarrow$ DMAPP. Hence, more ^{14}C was found on DMAPP moiety after the first two hours of fixation. Again this theoretically more direct route might be stimulated by the high concentration of CO_2 when fixation began. After $^{14}CO_2$ was depleted, a delay of increase in total label and a rapid drop in the percentage of label in the DMAPP half of geraniol were observed. From there on, contributions of MVA or other pathways became more significant than the more direct pathway to DMAPP synthesis because the labelled plant constituents started to turn over. The radioactivity of the DMAPP pool increased not as fast as the radioactivity of IPP as one can see in the change of percent of label in total geraniol. If the more direct route for DMAPP synthesis exists, unlabelled CO_2 went into DMAPP at the same time as the labelled IPP was isomerized into DMAPP; therefore,

more ^{14}C was observed in the IPP moiety.

Fourth, from another point of view that geraniol can be synthesized at more than one site, the preferential labelling of IPP at most time intervals, and the shift of labelling pattern in experiment 4a and 4b theoretically might be explained as follows: DMAPP is more heavily labelled or equally labelled in comparison with IPP only at the beginning when the level of label is very low. At this time, the ^{14}C has not yet penetrated to the DMAPP pool, which is perhaps hidden away in oil glands. Therefore, small amounts of radioactive geraniol are synthesized at this time in an easily accessible compartment, where $CO_2 \longrightarrow$ sugar-P $\longrightarrow$ MVA $\longrightarrow$ IPP $\rightleftharpoons$ DMAPP with the mechanism closely related to that in rose petals (Francis and Allcock, 1969). At this site, no large pool of DMAPP exists, and since Keq of the isomerase favors DMAPP it would not be surprising to observe a slight preferential labelling of the DMAPP portion of geraniol, which the data suggest. At a later stage of metabolism after $^{14}CO_2$ incorporation (experiment 3 and 4a and 4b), label gets to a second site where geraniol is synthesized in larger quantity from condensation of newly formed IPP and pooled DMAPP. During this period, the DMAPP pool is becoming more and more labelled due to the turnover of plant constituents. A scheme for geraniol biosynthesis which includes all these possibilities is presented in Figure 9. One should not ignore the possibility that the geraniol is not synthesized via the

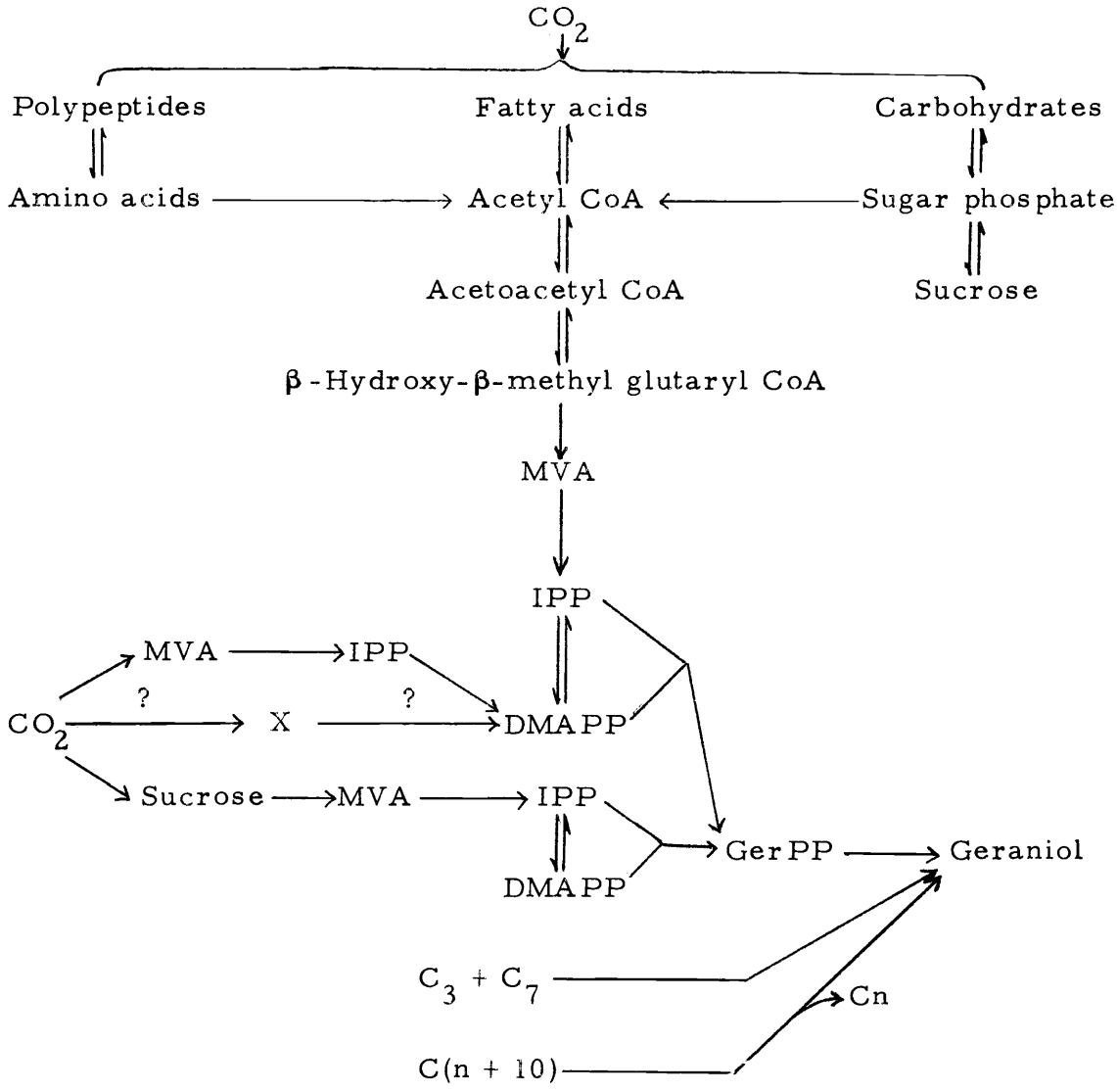


Figure 9. Some possible intermediates in forming MVA and the proposed alternative pathways of geraniol biosynthesis.

MVA route at all. The suggested routes are also included in Figure 9.

In the experiment where cuttings had four hours exposure to $^{14}CO_2$ and subsequently metabolized in a sealed chamber (experiment 3), predominant labelling on the IPP moiety was observed at the end of four hours fixation. Presumably the early stage of apparent preferential labelling of DMAPP was already past at the end of four hours in this experiment. This suggestion is consistent with the unusually high incorporation of ^{14}C in this experiment. This high incorporation may be related to the fact that the cuttings were kept in a sealed chamber between the sampling times and had little access to normal CO_2.

A common interesting feature is observed in both time course experiments with two hours $^{14}CO_2$ fixation and subsequent metabolism under continuously circulating air (experiment 4a and b). In Figures 6 and 8, it is obvious that label on geraniol remained at a fairly steady low level from two to eight hours and then increased significantly after eight hours. Geranyl glucosides have been found in P. graveolens (Bourquelot and Bridel, 1913); therefore, labelled geraniol may not be found in the hexane extract until it has been released from the sugar. This may be the reason that the label of geraniol increased after eight hours. Variability between cuttings is unavoidable (Burbott and Loomis, 1969) and undoubtedly contributes to the fact that no two experiments gave exactly the same result.

However, the trends of both total geraniol label, and percent of label in "C_3" at different time intervals were observed consistently in all experiments.

CONCLUSION

One would expect, a priori, that geraniol biosynthesized from $^{14}CO_2$ would be randomly labelled. The results reported here show clearly that it is not. After two hours exposure to $^{14}CO_2$, the "C_3" unit contains somewhat more than 30% of the total label in geraniol (C_{10}). After longer times the ratio shifts, and "C_3" contains much less than 30% of the geraniol label.

The results clearly indicate non-random incorporation of label from $^{14}CO_2$ into geraniol. It seems most likely that geraniol is biosynthesized from randomly labelled IPP and randomly labelled DMAPP, but that IPP and DMAPP are, for some reason, not in equilibrium in the plant. Not excluded by the results presented here is the possibility that DMAPP may not be uniformly labelled, or geraniol is not formed by direct condensation of DMAPP and IPP.

This uneven labelling and the shift in the "C_3" unit:geraniol labelling ratio suggest that a DMAPP pool, such as that postulated by Banthorpe and his coworkers (1966, 1968), exists in P. graveolens shoots. These results can be contrasted with a later study of rose petals which shows equal labelling of the two isporene units (Francis, Banthorpe and Le Patourel, 1970). Geraniol leaves have specialized oil glands, while rose petals do not. Non-random labelling of terpenoid compounds may be associated especially with secertory glands,

perhaps due to a large pool of DMAPP compartmentalized within the glandular cells.

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