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METABOLIC POOLS ASSOCIATED WITH MONOTERPENE BIOSYNTHESIS IN HIGHER PLANTS*

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Key Word Index—*Tanacetum vulgare*; Compositae; *Pelargonium graveolens*; Geraniaceae; *Mentha pulegium*; Labiatae; monoterpene biosynthesis; labelling patterns; metabolic pools.

Abstract—Partial degradations of (+)-isothujone biosynthesised in *Tanacetum vulgare* after feeding IPP-[4-^{14}C], DMAPP-[4-^{14}C] or 3,3-dimethylacrylate-[Me-^{14}C], and of geraniol and (+)-pulegone formed in *Pelargonium graveolens* and *Mentha pulegium* respectively after uptake of 3,3-dimethylacrylate-[Me-^{14}C], indicated that none of these metabolites was a direct source of the part of the monoterpene skeleton derived hypothetically from DMAPP. Uptake of glucose-[U^{14}C] into *P. graveolens* led, in contrast, to both IPP and DMAPP-derived moieties of geraniol being extensively labelled. Feeding of L-valine-[U-^{14}C] and L-leucine-[U-^{14}C] to all three plants resulted in negligible incorporation of tracer into monoterpenes. A soluble enzyme system prepared from foliage of *T. vulgare* that had been exposed to CO_2-[^{14}C] for 20 days converted isotopically-normal IPP into GPP with the DMAPP-derived portion containing essentially all (>98%) of the radioactivity present. These observations and those previously obtained from feeding experiments with other [^{14}C]-labelled precursors on the same plant species are consistent with the occurrence of two metabolic pools of intermediates for monoterpene biosynthesis, one of which is probably protein-bonded.

INTRODUCTION

The phenomenon of asymmetric labelling of monoterpenes biosynthesised in higher plants is well established. The I-unit (from IPP) is typically predominently labelled (70–100% of incorporated tracer) after uptake of [^{14}C]-labelled acetate, MVA or of CO_2 supplied at physiological concentrations. This pattern persists in a variety of plant species over a range of feeding conditions and the few exceptions can be attributed to special circumstances [1; and references therein]. Various explanations for these observations have been proposed [2] but hitherto there has been no direct evidence for the origin of the D-unit (from DMAPP) of monoterpenes under these conditions. We now describe experiments directed to these problems, that are complementary to those reported in the previous paper [3].

RESULTS

Incorporation of C_5 and C_6 precursors into monoterpenes. (+)-Isothujone (**1**; *trans*-thujan-3-one), biosynthesised in *Tanacetum vulgare* L. (Compositae) after feeding IPP-[4-^{14}C], DMAPP-[4-^{14}C], and 3,3-dimethyl-acrylate-[Me-^{14}C], was partially degraded (Scheme 1) to give the results shown in Table 1. These and later experiments were carried out in May–September and all products were purified to constant specific radioactivity; standard deviations of these activities were usually ± 2%, and always less than ± 5%. For comparison, incorporation of CO_2-[^{14}C] in a typical experiment carried out at the same period is included; this is an index of terpene biosynthesis under unperturbed conditions.

The origin of prop-1-ene (**5**) was checked by degradation of a synthetic sample of specifically-labelled α-terpineol-[9,10-^{14}C] (**6**; *p*-menth-1-en-8-ol) as described in the Experimental. **6** (5625 dpm mmol^{-1}) yielded prop-1-ene (**5**; 5500 dpm mmol^{-1}).

(+)-Pulegone (**7**; *p*-menth-4(8)-en-3-one) and geraniol (**8**: 3,7-dimethylocta-*trans* 2,6-dien-1-ol) formed in *Mentha pulegium* L. (Labiatae) and *Pelargonium graveolens Ait.* (Geraniaceae) respectively after feeding 3,3-dimethyl-acrylate-[Me-^{14}C] and, in one instance, glucose-[U-^{14}C] were degraded as in Schemes 2 and 3 yielding the results given in Table 2.

Leucine-[U-^{14}C] and valine-[U-^{14}C] were fed to specimens of the three plants in several sets of independent experiments over the same summer period and tracer in isothujone, geraniol and pulegone was monitored at 5, 20, 40, 72, 96, 138 and 185 hr after feeding.

* Part 13 of the series "Terpene Biosynthesis". For Part 12 see previous paper D. V. Banthorpe, Bucknall, G. A., Doonan, H. J., Doonan, S. and Rowan, M. G. (1976) *Phytochemistry*, **15**, 91.

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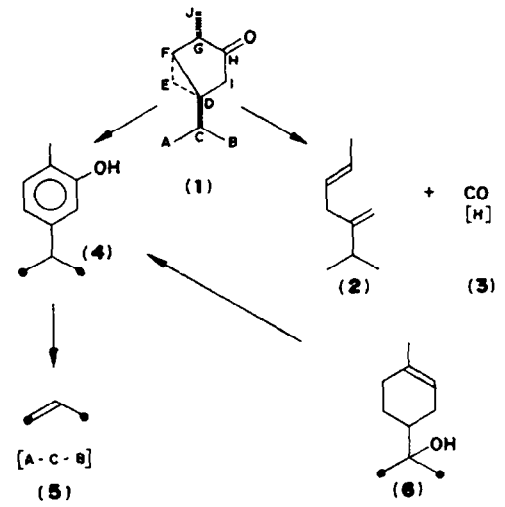
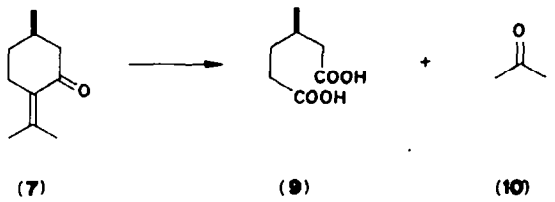
Abbreviations used: *IPP*, isopentenyl pyrophosphate; *DMAPP*, 3,3-dimethylallyl pyrophosphate; *MVA*, mevalonate; *GPP*, geranyl pyrophosphate; *I-unit*, IPP-derived moiety of monoterpene skeleton (see reference 1); *D-unit*, DMAPP-derived moiety of monoterpene skeleton; *HMG-CoA*, 3-hydroxy-3-methylglutaryl Coenzyme A.



Scheme 1. Degradation of (+)-isothujone. Carbons in (+)-isothujone (1) and certain of its degradation products are lettered in correspondence.



Scheme 2. Partial degradation of (+)-pulegone.

In no experiments was the percentage incorporation, when tracer levels were significantly above background, greater than 0·0005%. These assays involved TLC and GLC fractions that had not been purified to constant specific radioactivity, and so any incorporations were upper limits, probably considerably greater than the true values.

Attempts to characterise a C_5-pool. Homogenates of foliage (100g) of *T. vulgare* or *P. graveolens* contained no detectable C_5 alcohol, either free or as phosphate esters. Controls using isopentenol (3-methylbut-3-ene-1-ol) 3,3-dimethylallyl alcohol (3-methylbut-2-ene-1-ol) and dimethylvinylcarbinol (3-methylbut-1-ene-3-ol) indicated that minute quantities (*ca.* 1 μg) would have been detected. Specimens of *T. vulgare* and *P. graveolens* were then stem-fed with glucose-[U-^{14}C] or exposed to CO_2-[^{14}C] and after a period of metabolism (8–10 days) were assayed by isotope dilution using geraniol, isothujone, the three C_5-alcohols, isoprene, mevalonate, mevalonic acid lactone, tiglate and 3,3-dimethylacrylate as carriers in both the hexane-ether extracts of leaf homogenates and such extracts after any endogenous phosphate esters had been cleaved. Aliquots that contained the monoterpenes labelled at *ca* 10^4–10^5 dpm also yielded chromatographic fractions containing the C_5 carriers labelled up to 10^2 dpm, but these latter values fell to that of the backgound on derivatisation and recrystallisation. In parallel experiments, proteins from the leaves of both plant species (after the 10-day exposure to tracer) were separated by gel filtration, and fractions containing radioactivity were incubated with appropriate reagents

Table 1. Tracer patterns in (+)-isothujone biosynthesised by *T. vulgare* from various (^{14}C)-precursors

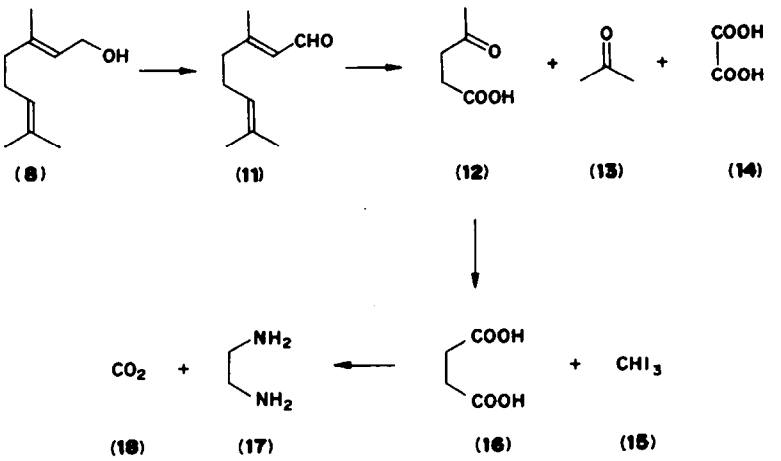
Precursor	*t*(hr)*	% I†	Sp. Act‡ dpm μmol^{-1}	Product (%)§
IPP-[4-^{14}C]	24	0·5	25·8	**2** (13); **3** (88); **5** (10)
DMAPP-[4-^{14}C]	24	0·04	3·0	**3** (86); **5** (14)
DMAPP-[4-^{14}C]	48	0·05	·2·8	**2** (16); **3** (80); **5** (13)
3,3-Dimethylacrylate-[Me-^{14}C]	50	0·001	1·3	**2** (80); **3** (18); **5** (13)
3,3-Dimethylacrylate-[Me-^{14}C]	180	0·009	1·4	**2** (91); **3** (12); **5** (10)
CO_2-[^{14}C]	30	0·02	—	—

* Metabolic period after uptake of tracer. For feeding conditions see Experimental. † % Incorporation of ^{14}C into isothujone. ‡ Values cannot be compared in different experiments as different quantities of carrier and/or tracer were used. § Degradation products (cf. Scheme 1) and, in brackets, % of tracer in these. Each value is an independent determination.

Table 2. Tracer patterns in (+)-pulegone and geraniol biosynthesised in *M. pulegium* and *P. graveolens* from various precursors

Plant	Precursor*	*t*(hr)†	% I‡	Sp. act§	Product (%)‖
M. pulegium	*MMA*	24	0·03	4·10	**9** (91); **10** (11)
M. pulegium	$^{14}CO_2$	24	0·23	—	—
P. graveolens	MMA	20	0.03	5·23	**12** (70); **13** (13); **14** (15)
P. graveolens	glucose [U-^{14}C]	24	0·001	2·35	**12** (51); **13** (30); **14** (20)
P. graveolens	$^{14}CO_2$	24	0·33	—	—

* MMA = 3,3-dimethylacrylate-[Me-^{14}C]. † Metabolic period after uptake of tracer. For feeding conditions see Experimental. ‡ % Incorporation of ^{14}C into pulegone (*M. pulegium*) or geraniol (*P. graveolens*). § Sp. act of pulegone or geraniol (both $\times 10^{-3}$; dpm mmol^{-1}). Values cannot be compared (see Table 1). ‖ Degradation products (cf. Schemes 2 and 3), and, in brackets, % of tracer in these. Each value is an independent determination.



Scheme 3. Partial degradation of geraniol.

(see Experimental) expected to cleave off any bonded hemi- or mono-terpenol. In some cases tracer was released from protein into hexane-ether or aqueous extracts but isotope dilution analysis revealed no significant amounts in geraniol, nerol, C_5 alcohols or MVA. Controls showed that the potential biosynthetic intermediates were stable under conditions of extraction and analysis and provided correction factors for losses during work-up.

In a final experiment, a soluble enzyme system that converted IPP into geraniol was prepared from foliage of *T. vulgare* that had been exposed to CO_2-[^{14}C] at the physiological concentration of the gas for 20 days, and was incubated under carefully defined [3] conditions with isotopically-normal IPP. The geraniol that was produced (endogenous geraniol-[^{14}C] having been removed by column chromatography during preparation of the cell free system) was purified to constant specific radioactivity and was partially degraded as in Scheme 3. Geraniol (8230 dpm mmol^{-1}) yielded laevulinic acid (**12**; 3050 dpm mmol^{-1}); acetone (**13**; 5075 dpm mmol^{-1}) and oxalic acid (**14**; 0 dpm mmol^{-1}). Further degradation of **12** yielded iodoform (**15**; 10 dpm mmol^{-1}); succinic acid (**16**; 3120 dpm mmol^{-1}); ethylenediamine (**17**; 1490 dpm mmol^{-1}) and CO_2 (**18**, 2 mols per mol of **16**; 775 dpm mmol^{-1}). When foliage exposed to tracer for 3 days was used, the resultant geraniol contained little (200 dpm mmol^{-1}) radioactivity.

DISCUSSION

Metabolic and degradation products were all rigorously purified to constant specific radioactivity using previously-described criteria [1]. Complete degradation of the labelled monoterpenes was not attempted, but the routes employed were sufficient to define position-specific incorporation into the D-units, given the particular labelled potential precursors. Thus carbon monoxide and prop-1-ene cleaved from isothujone (Scheme 1) contained all tracer originating without randomisation from IPP-[4-^{14}C] and its derived DMAPP (the carbonyl carbon of isothujone is well-established [4] to be derived from C-2 of MVA and hence from C-4 of IPP), whereas prop-1-ene derived from isothujone, and acetone derived from geraniol of pulegone would contain tracer from 3,3-dimethylacrylate-[Me-^{14}C] directly incorporated into the D-unit. Thus the schemes allowed a decision as to whether the [^{14}C]-labelled intermediates were *directly* incorporated into the D-unit or tracer passed into this unit by degradation to C_1 or C_2 units followed by incorporation of these.

To ensure the validity of this procedure, the origin of prop-1-ene (Scheme 1) had to be checked. The remarkable cleavage of carvacrol (**4**) on treatment with phosphorus pentoxide has only once been recorded [5] and in the present work the counter-fragment (*o*-cresol?) was involved in tar formation and could not be isolated in order to achieve an isotopic balance. However, the control scheme starting from α-terpineol-[9,10-^{14}C] proved the source of the olefin to be the isopropyl group of carvacrol and ultimately of isothujone.

Incorporation of [^{14}C]*-labelled IPP and DMAPP.* Our degradations of isothujone formed in *T. vulgare* after uptake of IPP-[4-^{14}C] and DMAPP-[4-^{14}C] showed that the bulk (86-90%) of incorporated tracer resided at the carbon cleaved out as carbon monoxide corresponding to the position of the monoterpene skeleton derived from C-4 of either precursor. As the residue was eventually located in the portion cleaved as prop-1-ene, there could have been little randomisation of tracer and both IPP and DMAPP were preferentially incorporated into the I-unit in a position-specific manner. Analogous patterns have been proved by complete degradation of artemisia ketone biosynthesised in *Artemisia annua* and *Santolina chamaecyparissus* after uptake of the same precursors [6].

Factors causing asymmetric labelling patterns. The above results and those previously reported [1, 4] show that preferential labelling of the I-unit of isothujone occurred on feeding *T. vulgare* with (^{14}C)-labelled CO_2, acetate, MVA, IPP and DMAPP over a wide range of experimental and seasonal conditions; and less extensive studies have revealed the same phenomenon for the formation of geraniol and pulegone in *P. graveolens* and *M. pulegium* respectively after uptake of the first three [^{14}C]-precursors [1] and also for a variety of other monoterpenes biosynthesised in a position-specific fashion from MVA[2]. Several explanations have been proposed [2] for this apparently general phenomenon.

One explanation is that feeding of unphysiological quantities of precursors, or in the case of MVA of the

unnatural 3S isomer present in the commercially-available racemate, could inhibit a key enzyme (e.g. IPP-isomerase, EC 5.3.3.2) and result in unequal labelling of the I and D moieties. However, the one precursor that can be applied at physiological concentration, CO_2-[^{14}C], is incorporated *a fortiori* in an asymmetric manner [2]. It is noteworthy that unphysiological concentrations of CO_2 can alter the enzyme levels in carbohydrate metabolism [7].

A second proposal is that DMAPP utilised in monoterpene biosynthesis is of non-mevalonoid origin. The most probable precursor is 3,3-dimethylacrylyl-Coenzyme A [2] derived either from amino acid metabolism or by degradation of the existing monoterpene pool. The latter pool in *T. vulgare* is known [8,9] to be in metabolic flux and 3,3-dimethylacrylyl-Coenzyme A and DMAPP could possibly result from a sequence of degradative reactions similar to those that have been identified in certain microorganisms [10]. The role of 3,3-dimethylacrylate in terpenoid biosynthesis has never been defined: reports of its direct incorporation into cholesterol by rat liver [11] and into HMG-CoA in plants [12] are balanced by those of negligible or nonspecific incorporation into other terpenoids [13–15], and of its occurring on a shunt regenerating HMG-CoA from MVA [16,17]. Our present results (Tables 1 and 2) indicate that 3,3-dimethylacrylate-[Me-^{14}C] is not incorporated intact into isothujone, geraniol or pulegone under conditions where terpene synthesis (as monitored by incorporation of CO_2-[^{14}C]) is at the seasonal norm. The pattern obtained for all three monoterpenes suggests degradation of the acid to C_1 or C_2 units and incorporation of these to give the typical "asymmetric" labelling. Degradation of pulegone biosynthesised in *M. pulegium* after feeding 3,3-dimethylacrylate-[1-^{14}C] had led to a similar conclusion [13].

We also found negligible (< 0·0005%) incorporation of tracer into monoterpenes after feeding valine-[U-^{14}C] and leucine-[U-^{14}C] for protracted periods to our three species, although in each case autoradiography revealed that radioactivity rapidly became distributed throughout the foliage. Similar results have been reported for *Humulus lupulus* [10]. Various suggestions have been made that leucine is a precursor of MVA [18], carotenoids [19], and steroids [20–22]. Recently leucine-[^{14}C] was shown to be incorporated (0·004%) into linalool in *Cinnamomum camphora* such that 80% of the tracer occurred in the D-unit and this was held to prove (*sic*) the non-mevalonoid origin of this moiety, and 3,3-dimethyl-acrylate was proposed to be the direct precursor of DMAPP [23]. These results are totally inconclusive: position-specific transfer of ^{14}C from leucine to the D-unit was not demonstrated and thus the observations may be the consequence of degradation of the (massive doses of) amino acid to C_2 units and uptake of these into MVA under conditions (see next section) of compartmentation of acetate pools. Consequently, no conclusive evidence is available to date for the non-mevalonoid origin of the D-unit.

The third explanation for asymmetric labelling invokes condensation of exogenous IPP with a metabolic pool of DMAPP or its biosynthetic equivalent. The maintenance of such a pool by selective membrane permeability to potential intermediates [cf. 2] such as has been established in other contexts in the biosynthesis of certain carotenoids [24–5] is ruled out for *T. vulgare* by our finding [3] that asymmetric labelling (65–100% in I-unit) occurs in geraniol synthesis in soluble enzyme systems. Thus a free or a protein-bonded pool is likely and the latter is favoured on general theoretical grounds [26]. Hemiterpene alcohols occur in trace (< 0·1%) amounts in several plant oils [27–34] and have been tentatively identified in *T. vulgare* [35] but in the present work no detectable amounts of these or their phosphate esters (i.e. < 1 μg per 100 g foliage) have been found in *T. vulgare* or *P. graveolens* although estimates of the extent of monoterpene synthesis based on uptake of CO_2-[^{14}C] indicate that pool sizes of several μg in this quantity of tissue would be necessary to produce the observed asymmetry of labelling. Carrier techniques involving addition of the hemiterpenols, MVA, MVA-lactone, tiglate, isoprene or 3,3-dimethylacrylate to homogenates prepared from leaf tissue that had been exposed to (variously) CO_2-[^{14}C] at physiological conditions, glucose-[U-^{14}C] and acetate-[1-^{14}C] for 8 to 10 days also failed to provide any evidence for a pool of free C_5 or C_6 intermediates, although previous degradative studies [1] indicated that in *P. graveolens* at least any pool contributing to the synthesis of geraniol should have been significantly labelled in this period. However, significant (0·1% of applied tracer) radioactivity was associated with the protein fractions obtained in these experiments. Others have reported that tracer from MVA is bonded to protein and have speculated, without further investigation, that bonded intermediates of terpene synthesis are involved [36–38]. Attempts to remove such intermediates from protein failed in our hands (the rationale for the use of sucrose, see Experimental, is that it effectively removes presqualene alcohol pyrophosphate from a protein-bonded complex [39]).

Any bonded intermediate is thus probably covalently and specifically linked to protein, and if this material is to provide the D-unit of monoterpene the best trap is presumably the I-unit in the presence of prenyltransferase [EC 2.5.1.1]. In the event, a cell-free system prepared from *T. vulgare* that had been exposed to CO_2-[^{14}C] for 20 days yielded, after incubation for 2 hr with isotopically normal IPP, geraniol which partial degradation indicated was almost exclusively labelled in the D-unit. When cell-free systems that had been exposed to tracer for only 3 days were used, the geraniol produced was only labeled to the extent of 2% of that in the former experiments: presumably the bonded C_5-pool had not been appreciably labelled during this short exposure.

On the basis of these results we propose the tentative model for monoterpene biosynthesis in Fig. 1, in which many details are adjustable and in which not all the biosynthetic intermediates are shown. Two metabolic pools are envisaged in the unperturbed plant: One (A) being free but sparsely filled, the other (B) being essentially irreversibly bonded to protein, well-filled, and larger than pool A. At normal rates of monoterpene synthesis, ^{14}C-labelled precursor (CO_2 at natural concentrations; or MVA etc. at conditions that necessarily introduce perturbations) is channelled into pool A and IPP from this condenses with bonded DMAPP from pool B to form GPP and thence derived monoterpenes in which the I-unit is preferentially labelled. When terpene synthesis is more rapid or metabolism times are longer, both pools fill with tracer and symmetrically-labelled products result. The present and previous studies on *T. vulgare*, *P. graveolens*, *M. pulegium* [1,4,10,40] and

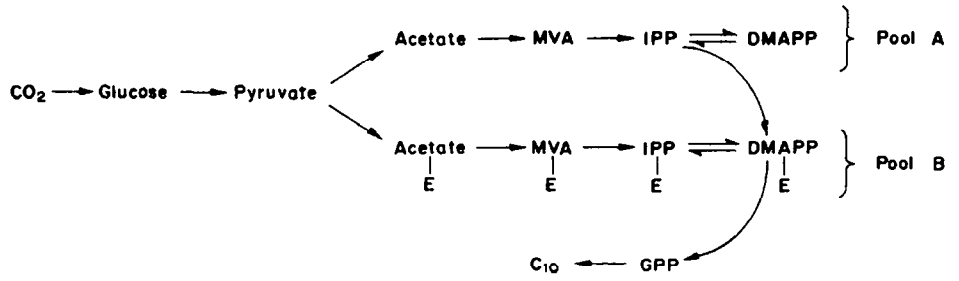


Fig. 1. Hypothetical scheme for metabolic pools in monoterpene biosynthesis. E in Pool B represents protein. Not all biosynthetic intermediates are shown.

other species [2] are consistent with this gradual infilling of pool B as the metabolism period increases.

In the unperturbed plant the 2 metabolic pools could be supplied by different pathways: there is evidence for compartmented acetate pools in terpenoid biosynthesis [1,41–42] and the hexose and glyoxylate routes [25] to acetyl-Coenzyme A could be respectively employed or pool B could be fed by degradation of endogenous terpenoids to C_5 units [9,43] or by the operation of ATP-citrate lyase [44]. The much-discussed 'malonate' pathway to MVA [45] actually involves protein-bonded intermediates and though this has been discounted for the biosynthesis of certain higher terpenoids formed at high turnover rates [46–47] the situation for monoterpenes is open.

In the perturbed plant (fed with exogenous tracer) the typical result is that the D-unit contains usually appreciable quantities (up to 20%) of the incorporated radioactivity. This may indicate either the depletion of endogenous pool B over the metabolism period (this effect is clearly shown by the time-dependence of passage of tracer into the D and I units of geraniol biosynthesised from CO_2-[^{14}C] in *P. graveolens* [1]) or the operation of degradative processes to give C_1 or C_2 units that can enter the pool. MVA is degraded thus in several types of tissue during terpenoid biosynthesis [4,48–50]. In the present work glucose-[U-^{14}C] was the only precursor whose tracer appeared to enter both pools to result in equivalent labelling of the D and I units of geraniol formed in *P. graveolens*.

Similar pools presumably occur for the biosynthesis of higher terpenes but are often obscured by the relatively high (compared to monoterpene) rates of synthesis that are observed both *in vitro* and *in vivo*. Thus recent studies of phytosterols in plants indicated that all the I and D-units were equivalently labelled after uptake of C^{14}-precursors [51–52]. Sesquiterpenes may be biosynthesised at sites compartmented from those of monoterpene formation [53–54] but in two cases asymmetric labelling patterns (I-units preferentially labelled) have been observed [53, 55]. In a recent study dendrobine was formed from MVA-[2-^{14}C] in *Dendrobium nobile* with a symmetric pattern of labelling, but the incorporations (*ca* 1·4%) were unprecedentedly high for this type of experiment [56].

EXPERIMENTAL

Materials. Plants were clonal material from specimens whose origin and cultivation have been described [1]. Stem-feeding expts. used shoots (10–15 cm; 8–12 week-old plants) that had been excised under sterile H_2O. Potted specimens of the same age were used for CO_2-feeding. (+)-Isothujone, (+)-pulegone and geraniol were purified as previously reported [1]. IPP-[4-^{14}C] and DMAPP-[4-^{14}C], 17 mCi mmol^{-1} each, were prepared enzymatically [57–8] from 3RS-[2-^{14}C]-MVA. The ethyl ester of 3,3-dimethylacrylic acid-[Me-^{14}C] was prepared by the Wittig reaction [59] of freshly prepared [60–61] triethylphosphonoacetate (140–1°/10 mm; 555 mg) and EtONa (183 mg) in DMF (2 ml) with Me_2CO-[1,3-^{14}C] (100 μCi; 27 mg) at 25°/30 h. After reflux (2½ h) with a four-fold excess of NaOH in EtOH, the acid was recovered, mp 71° (from H_2O; petrol (bp 60–80°) 30 mg; 30·8 μCi). $BaCO_3$-[^{14}C] 53 mCi mmol^{-1}; Glc-[U-^{14}C] 230 mCi mmol^{-1}; Val-[U-^{14}C] 225 mCi mmol^{-1}; Leu-[U-^{14}C] 270 mCi mmol^{-1} and NaOAc-[1-^{14}C] 60 mCi mmol^{-1} were obtained from the Radiochemical Centre, Amersham, England.

Administration of tracer. CO_2-[^{14}C] was generated from $BaCO_3$-[^{14}C] with H_2SO_4 (6N) or lactic acid to give the natural concentration (*ca* 0·031% v/v) of the gas in a chamber (200 l.) equipped with a fan and a device for introducing nutrients, etc. The chamber was sealed during the metabolism period but controls showed that venting and replacing CO_2-[^{14}C] at three-day intervals made little difference to the levels or nature of the incorporation of tracer over the maximum period (20 days) of the expts.

^{14}C-Labelled IPP, DMAPP, NaOAc and Glc (50 to 100 μCi variously; undiluted with carrier) in H_2O (2 ml) containing ATP (0·2 mg) were stem fed to foliage [50–80 g] under illumination and forced transpiration [1]. 3,3-Dimethylacrylate-[Me-^{14}C] (30 μCi; 30 mg) in $NaHCO_3$ (sat. aq.; 5·0 ml) and ATP (7 mg) was fed, for solubility reasons, to a larger quantity (*ca* 150 g) of plants. Val-[^{14}C] and Leu-[^{14}C] (2 μCi aliquots) were fed to series of matched shoots (5–10 g), each providing one point on the time-incorporation profile.

Degradation schemes. The extraction and purification of geraniol, pulegone, isothujone, and the degradation schemes for the first two and the photolytic cleavage of CO from isothujone (**1** → **2** + **3**) have been fully documented [1]. Isothujone was aromatised by heating with a four-fold excess of 2,3-dichlor-5,6-dicyanobenzoquinone (DDQ) in dioxan at 130°/14 days under N_2. The Et_2O extract of the reaction mixture was added to petrol (bp 60–80°) to precipitate unreacted DDQ and the residue was chromatographed on plates of Si gel H with petrol (bp 60–80°)-Et_2O (17 : 1) to give carvacrol (**4**; 45%) which was converted into its sodium salt (dec. > 300°) and recrystallised from H_2O and aq Me_2CO. The free phenol on heating with P_2O_5 [5] yielded prop-1-ene (50%) which was collected as the dibromide and purified by TLC on Si gel H and cellulose with Et_2O–EtOAc, C_6H_6–CCl_4 and petrol (bp 60–80°)-Et_2O mixtures as eluants. α-Terpineol-[9,10-^{14}C] (**6**; 5625 dpm mmol^{-1}) prepared as previously described [8] was converted into limonene and the *cis* and *trans* oxides of the latter were prepared by standard procedures [62–3]. The

mixture of oxides was reduced with $LiAlH_4$ to give dehydrocarvone which was converted into carvacrol (4) by treatment with $FeCl_3$–HOAc [64]. The overall yield was 14%, and a specimen of the product was derivatised and purified to constant sp act (see above).

Search for C_5 pool. Foliage (100–150 g) was ground with solid CO_2 and all subsequent operations were designed to avoid volatility losses. After filtration through glass wool and extraction of terpenols with Et_2O (2 × 5 ml) or hexane (2 × 5 ml), the residue was suspended in buffer (MES pH 7·0 1M; 75 ml) and assayed for phosphate or pyrophosphate esters by standard methods [65–66]. Foliage (200 g) was also stem fed with Glc-[U-^{14}C] (125 μCi) or NaOAc-[1-^{14}C] 100 μCi or exposed to CO_2-[^{14}C] (1 mCi) for 8–10 days before being similarly processed and treated with alkaline phosphatase and apyrase [67] in turn. Carrier (50 mg of each) was added to the hexane–Et_2O extract and was recovered by GLC on Carbowax 20M or FFAP (3 m × 5 mm; 20% on 80–100 mesh G-Cel; N_2 4 1 hr^{-1} FID) programmed from 80–150°. The effluent was passed directly into counting vials and was assayed for tracer; if significant quantities were present the separated fractions were further purified by TLC on Si gel H with Et_2O–EtOAc and were converted (in the case of the hemiterpenols) into *p*-nitrobenzoates which were recrystallised (usually thrice) to constant sp act. The *p*-nitrobenzoates of isopentenol, 3,3-dimethylallylalcohol and dimethylvinylcarbinol had mp 55, 67 and 115° respectively (from MeOH–H_2O or HOAc–H_2O). Recovery of carrier varied from 40% (isopentenol) to 75% (hemiterpenol). 3,3-Dimethylacrylate and tiglate were more soluble in the Et_2O–hexane extract than in the aq. solution and MVA was considerably soluble in both: after addition of carrier labelled samples of these were sought by TLC of the former extract on Si gel H using *iso* PrOAc–0·88 NH_3–MeOH (3 : 1 : 1) as solvent: R_f's 0·63, 0·27 and 0·50. MVA and MVA-lactone were extracted from the acidified (pH 4·0) aq fraction with $CHCl_3$ and were chromatographed on Si gel H with EtOAc–C_6H_6 (15 : 85): R_f 0·41 and 0·23. After exposure of a second set of foliage to CO_2-[^{14}C], the aq extract prepared as above was centrifuged (5000 *g*; 30 min) and protein was precipitated in the presence of bovine serum albumin as carrier by saturation of the supernatant with $(NH_4)_2SO_4$. The ppt was dissolved in H_2O (20 ml) and passed through Sephadex G-10 (30 cm × 1 cm) with a flow rate of 100 ml hr^{-1}. Effluent was collected in aliquots (10 ml) and was assayed for protein (280 nm absorption) and for ^{14}C. Radioactivity essentially all occurred in the 60–160 ml fraction (the same distribution was observed when Glc-[U-^{14}C] was fed in a parallel experiment) which contained 76 μg protein ml^{-1} (modified Folin assay [68]). Aliquots were then incubated with/at (a) pH 1·0, 7·0, or 13·0 in aq soln 37°/2 hr, (b) KOH (1·0N) in EtOH, 80°/1 hr, (c) 50% sucrose soln [39] or sodium metabisulphite (0·1N), 50°/3 hr, (d) alkaline phosphatase at pH 8·0, (e) acid phosphatase at pH 5·0, (f) apyrase at pH 6·5. After denaturation and removal (TCA, 10%) of protein, hexane extracts were assayed for ^{14}C. Significant tracer was only released by the incubation at pH 1·0; about 0·3% of the bonded radioactivity was extractable but none of this was detected in the carriers (hemiterpenols; MVA; MVA-lactone; 3,3-dimethylacrylate; tiglate).

Cell free system. Foliage (15 g) of *T. vulgare* was exposed to CO_2-[^{14}C] (2 mCi; 0·003% v/v concentration) in the growth chamber for 14 days and then a cell-free system was prepared and incubated with isotopically normal IPP as previously described [3].

Radiochemical techniques. Criteria for radiochemical purity for solids and liquids, assay methods and counting statistics used have been fully described [1], as too have autoradiographic techniques [8]. Rough assays were sometimes made of TLC chromatograms using a radiochromatography scanner.

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