Biochem. J. (1972) **130**, 1045–1054
Printed in Great Britain

Biosynthesis of Geraniol and Nerol and their β-D-Glucosides in *Pelargonium graveolens* and *Rosa dilecta*

By DEREK V. BANTHORPE and GEOFFREY N. J. LE PATOUREL
Christopher Ingold Laboratories, University College London, 20 Gordon Street, London WC1H 0AJ, U.K.
and MARTIN J. O. FRANCIS
Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford, U.K.

(*Received* 25 *July* 1972)

1. 3*R*-[2-^{14}C]Mevalonate was incorporated into geranyl and neryl *β*-D-glucosides in petals of *Rosa dilecta* in up to 10.6% yield, and the terpenoid part was specifically and equivalently labelled in the moieties derived from isopentenyl pyrophosphate and 3,3-dimethylallyl pyrophosphate. A similar labelling pattern, with incorporations of 0.06–0.1% was found for geraniol or nerol formed in leaves of *Pelargonium graveolens* The former results provide the best available evidence for the mevalonoid route to regular monoterpenes in higher plants. 2. Incorporation studies with 3*RS*-[2-^{14}C,(4*R*)-4-$^{3}H_1$]-mevalonate and its (4*S*)-isomer showed that the *pro*-4*R* hydrogen atom of the precursor was retained and the *pro*-4*S* hydrogen atom was eliminated in both alcohols and both glucosides. These results suggest that the correlation of retention of the *pro*-4*S* hydrogen atom of mevalonate with formation of a *cis*-substituted double bond, such as has been found in certain higher terpenoids, does not apply to the biosynthesis of monoterpenes. It is proposed that either nerol is derived from isomerization of geraniol or the two alcohols are directly formed by different prenyltransferases. Possible mechanisms for these processes are discussed. 3. The experiments with [^{14}C,^{3}H]mevalonate also show that in these higher plants, as has been previously found in animal tissue and yeast, the *pro*-4*S* hydrogen atom of mevalonate was lost in the conversion of isopentenyl pyrophosphate into 3,3-dimethylallyl pyrophosphate.

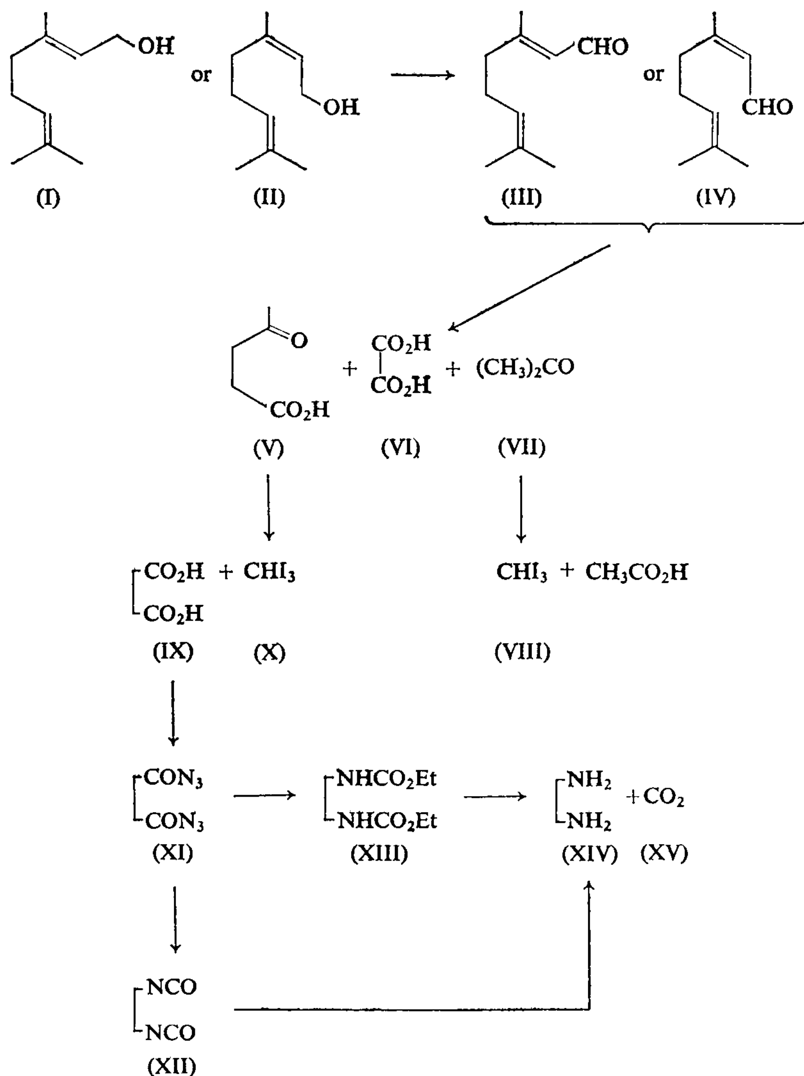
Monoterpene biosynthesis is believed to involve the conversion of mevalonate into isopentenyl pyrophosphate and 3,3-dimethylallyl pyrophosphate, which in turn condense to form geranyl pyrophosphate (Francis, 1971). However, incorporations of mevalonate into the monoterpenes of higher plants are usually extremely low, often below 0.01% (Banthorpe *et al.*, 1972*a*), and these values would not qualify the compound to be an obligate precursor without: (*a*) its well-authenticated role in the biosynthesis of higher terpenes (Clayton, 1965*a*,*b*); (*b*) the specific labelling patterns observed in several monoterpenes after incorporation of appropriately labelled material (Banthorpe *et al.*, 1970; Banthorpe & Baxendale, 1970; Banthorpe & Charlwood, 1971; Banthorpe *et al.*, 1972*b*); (*c*) the lack of a reasonable alternative pathway to these compounds.

3*R*-[2-^{14}C]Mevalonate was recently found to be incorporated very efficiently (approx. 22%) into the monoterpenes formed in the petals of *Rosa dilecta* cv. Lady Seaton (Francis & O'Connell, 1969; Francis & Allcock, 1969). We now report the labelling pattern obtained in geraniol (I; 3,7-dimethylocta-*trans*-2,6-dien-1-ol), nerol (II; 3,7-dimethylocta-*cis*-2,6-dien-1-ol) and their *β*-D-glucosides formed in similar experiments. We have also determined the stereochemistry of proton loss in the formation of the *trans*- and *cis*-substituted double bonds in these compounds. Part of this work has been presented as a preliminary communication (Francis *et al.*, 1970).

Results

Studies with ^{14}C

Geraniol and nerol occurring free in the leaves of *Pelargonium graveolens* Ait. (Family Geraniaceae) and obtained by enzymic hydrolysis of the corresponding *β*-D-glucosides that were extracted from the petals of *Rosa dilecta* cv. Lady Seaton (Family Rosaceae) were isolated after administration of [2-^{14}C]mevalonate to the two plant species. The isolated monoterpene alcohols were rigorously purified to constant specific radioactivity and were then degraded as shown in Scheme 1. The free alcohols from the petals of *R. dilecta* were labelled to a relatively small extent (approx. 0.9% of administered [2-^{14}C]mevalonate in these experiments compared



Scheme 1. *Degradation schemes for geraniol and nerol*

(I) Geraniol; (II) nerol; (V) laevulinic acid; (VI) oxalic acid; (VII) acetone; (VIII) iodoform; (IX) succinic acid; (X) iodoform; (XIV) ethylenediamine; (XV) carbon dioxide.

with the uptakes into the corresponding glucosides (see Table 1), and were not further investigated. Dilution factors are not recorded, as these have little meaning unless they refer to true metabolic pools, and there is reason to believe (Banthorpe *et al.*, 1972*a*) that compartmentation effects and the isolated situation of biosynthetic sites can lead to large, variable and isolated pools of many terpenoids.

In no case did the sugar moiety of the glucosides contain tracer. The specific radioactivities of monoterpenols after addition of carrier and degradation of products are recorded in Table 1. These activities had standard deviations in different experiments of ± 2 to $\pm 5\%$ of the quoted values.

Double-labelling experiments

The $^{3}H/^{14}C$ ratios in free terpenols and their β-glucosides isolated after the plant tissue had been given specifically labelled [^{14}C, ^{3}H]mevalonate are

Table 1. *Radioactive tracer pattern in monoterpenes after uptake of* 3*RS*-[2-^{14}*C*]*mevalonate*

For experimental details see the text. The time of harvesting after initial administration of tracers is shown. A refers to specific radioactivities of initial degradation products, and B to specific radioactivities after addition of compound (V) as carrier and further degradation. For identity of (I)–(XV) see Scheme 1.

Compound	Source	Time (h)	3*R*-[2-^{14}C]Mevalonate incorporated (%)	Specific radioactivity (d.p.m./mmol)
Geraniol (from glucoside)	*R. dilecta* petals	1	10.6	A: (I) 86620; (V) 44680; (VII) 43940; (VI) 0; (VIII) 21500 B: (X) 0; (IX) 5244; (XIV) 5190; (XV) 0
Nerol (from glucoside)	*R. dilecta* petals	1	0.25–0.5	A: (II) 17580; (V) 8098; (VII) 9743; (VI) 0; (X) 0 B: (IX) 2809; (XIV) 2562; (XV) 0
Geraniol	P. *graveolens* leaves	24	0.1	(V) 3779; (VII) 3372
Nerol	*P. graveolens* leaves	24	0.06	(II) 5684; (V) 2606; (VII) 2580

Table 2. *Radioactive tracer in products after feeding* [^{14}*C*,^{3}*H*]*mevalonate to plants*

For experimental details see the text. Errors in all initial ratios are ±0.04, and for products (monoterpene) are ±0.04 for experiments on *R. dilecta* and ±0.03 for others. Counts in each isotope channel were measured in d.p.m.: typically the ^{14}C radioactivity was 500–5000 d.p.m.

Labelled 3*RS*-mevalonate	Product	Source	Initial ^{3}H/^{14}C ratio in mevalonate	^{3}H/^{14}C ratio in product
[2-^{14}C,(4*S*)-4-^{3}H$_1$]	Geraniol	*R. dilecta* petals	1.25	0.08
[2-^{14}C,(4*R*)-4-^{3}H$_1$]	Geraniol	*R. dilecta* petals	1.99	1.91
[2-^{14}C,(4*R*)-4-^{3}H$_1$]	Nerol	*R. dilecta* petals	1.99	2.20
[2-^{14}C,(4*S*)-4-^{3}H$_1$]	Geranyl glucoside	*R. dilecta* petals	1.25	0.06
[2-^{14}C,(4*R*)-4-^{3}H$_1$]	Geranyl glucoside	*R. dilecta* petals	1.99	1.96
[2-^{14}C,(4*S*)-4-^{3}H$_1$]	Neryl glucoside	*R. dilecta* petals	1.25	0.09
[2-^{14}C,(4*R*)-4-^{3}H$_1$]	Neryl glucoside	*R. dilecta* petals	1.99	2.31
[2-^{14}C,(4*S*)-4-^{3}H$_1$]	Geraniol	*P. graveolens* leaves	0.57	0.13
[2-^{14}C,(4*R*)-4-^{3}H$_1$]	Geraniol	*P. graveolens* leaves	0.80	0.75
[2-^{14}C,(4*S*)-4-^{3}H$_1$]	Nerol	*P. graveolens* leaves	0.57	0.08
[2-^{14}C,(4*R*)-4-^{3}H$_1$]	Nerol	*P. graveolens* leaves	0.80	0.79

recorded in Table 2. In the experiments with *R. dilecta*, 3*R*-[2-^{14}C]mevalonate was incorporated into glucosides and free alcohols to the extent of 8.2 and 3.0% respectively. Incorporations into the free alcohols in this set of experiments were considerably higher than those listed in the previous section. The reasons for these differences are not known but may be related to the fact that the flowers used in the experiments with [^{14}C]mevalonate were produced in greenhouse conditions, whereas the experiments with [^{14}C, ^{3}H]mevalonate used flowers grown under natural conditions. Incorporations of 3*R*-mevalonate into free alcohols in *P. graveolens* were almost identical with those found in the previous set. Both sets of experiments on this species used flowers grown under natural conditions.

Phosphorylated derivatives of mevalonate

Chromatography of extracts of leaves of *P. graveolens* at 5.5h after uptake of [2-^{14}C]mevalonate showed that mevalonic acid 5-phosphate, mevalonic acid 5-pyrophosphate and isopentenyl pyrophosphate were present, with incorporations of 0.8, 0.6

and 0.2% tracer respectively from the 3*R*-mevalonate precursor.

Discussion

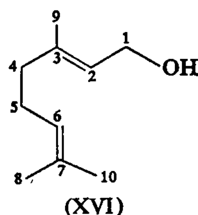
Pattern of ^{14}C labelling

Degradation of geraniol and nerol isolated from the β-D-glucosides biosynthesized in petals of *Rosa dilecta* cv. Lady Seaton revealed that the tracer incorporated from [2-^{14}C]mevalonate was distributed equally (within the experimental error) between (C-4+C-5) and (C-8+C-10) (see formula XVI). On biogenetic grounds the locations can be almost certainly assigned to (C-4+C-10). The incorporations observed for the terpene glucosides (Table 1) represent an increase by a factor of 10^2–10^3 over those typically obtained for the monoterpenes formed in the foliage of higher plants (Banthorpe *et al.*, 1972*a*), and together with the position specificity of labelling provide the best evidence to date for the mevalonoid route to monoterpenes of the conventional type, although high and position-specific incorporations have been recorded for certain irregular monoterpenes and those of the cyclopentanoid group (Banthorpe *et al.*, 1972*a*). The incorporations of tracer into the free monoterpenes in petals was also some 10–10^2-fold greater than those generally found in leaf tissue.

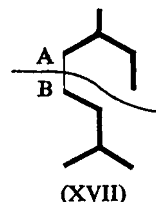
Several rationalizations have been proposed (Banthorpe *et al.*, 1970) for the generally found low incorporations of mevalonate into the leaf monoterpenes, and two of these in particular (the occurrence of compartmentation effects and the shunting of precursor into carotenoids and steroids) may be much less important in petals than in leaf tissue.

A comparison with the biosynthesis of unconjugated geraniol and nerol in leaves is provided by the work with *Pelargonium graveolens*. Here, uptake of [2-^{14}C]mevalonate again resulted in equivalent labelling at C-4 and C-10, but the incorporations into geraniol (although high for leaf monoterpenes) were some 100-fold less than in the rose petals. The time of maximum incorporation of tracer from administered [2-^{14}C]mevalonate was about 1 h for the terpene glucosides of *R. dilecta* (Francis & O'Connell, 1969). We found the corresponding time for geraniol and nerol in *P. graveolens* to be approx. 24 h; this is considerably less than that (approx. 4–5 days) for mono- and bi-cyclic monoterpenes further along the presumed biosynthetic pathway (Banthorpe *et al.*, 1970; Banthorpe & Baxendale, 1970; Banthorpe & Charlwood, 1971; Banthorpe *et al.*, 1972*b*).

An important difference between both these sets of labelling patterns and those obtained for thujane derivatives (Banthorpe *et al.*, 1970), camphor (Banthorpe & Baxendale, 1970), artemisia ketone (Banthorpe & Charlwood, 1971), pulegone (Banthorpe *et al.*, 1972*b*), (possibly) citral (Neethling *et*



(XVI)



(XVII)

al., 1963) and for certain sesquiterpenes (Biollaz & Arigoni, 1969; Croteau & Loomis, 1972) is that in the present work equal labelling of the moieties derived from isopentenyl pyrophosphate and 3,3-dimethylallyl pyrophosphate was observed, whereas preferential labelling (usually greater than 95%) into that part derived from isopentenyl pyrophosphate (formula XVII, A) was found previously. *A priori*, there is no reason to suppose that the specific radioactivities of the moieties A and B in the skeleton (XVII) should be the same, as isopentenyl pyrophosphate and 3,3-dimethylallyl pyrophosphate could be diluted to differing extents by inactive precursors in metabolic pools of various sizes, but the asymmetrical labelling pattern was contrary to expectation (Clayton, 1965*a*; Waller, 1969). Our presently reported examples of symmetrical labelling and a similar recent report for linalool formed in *Cinnamonum camphora* (Suga *et al.*, 1971) suggest that such patterns may occur both in petals (where high incorporations of precursor occur) and in leaves (with low incorporations) and both in free terpenols and in those conjugated as glucosides.

It may be significant that symmetrical labelling occurred for the first-formed monoterpenes (geraniol, nerol and linalool) whereas the alternative pattern was found for derived monoterpenes. This may indicate that these three monoterpenes (which may be readily interconvertible *in vivo*; see below) may exist in more than one metabolic pool. Mevalonate may be rapidly incorporated into the constituents of one such pool to give products with symmetrical labelling patterns that accumulate or are used as precursors for higher terpenoids, whereas the same precursor may be incorporated into the components of another pool to a much lesser extent to give asymmetrically labelled products that can act as precursors of other monoterpenes. The asymmetry of labelling could result from the presence of endogenous 3,3-dimethylallyl pyrophosphate (Banthorpe *et al.*, 1970). The idea of different metabolic pools is consistent with the occurrence of synthesis of monoterpenes and higher terpenes at different sites in the plant (Waller, 1969; Croteau & Loomis, 1972) and with limited results (Clayton, 1965*a*,*b*; Waller, 1969) suggesting that, in contrast to the pattern of asymmetric labelling found in monoterpenes, phytosterols

and carotenoids formed in plants from [2-^{14}C]-mevalonate show a symmetrical labelling pattern.

Incorporations of 0.8, 0.6 and 0.2% of 3*R*-[2-^{14}C]-mevalonate into mevalonic acid 5-phosphate, mevalonic acid 5-pyrophosphate and isopentenyl pyrophosphate in leaves of *P. graveolens* occurred within 5h of feeding, a time shown by control experiments to allow maximum incorporation into these intermediates. These incorporations were considerably greater than those into the monoterpenes formed in the same tissue.

Double-labelling studies

A fundamental question in monoterpene biosynthesis is the nature of the routes to geranyl pyrophosphate and neryl pyrophosphate, the presumed precursors of acyclic and higher terpenoids or cyclic monoterpenes respectively. It is possible that a pathway to the last class of compounds exists in plants that is absent from animals, where such compounds are rarely found.

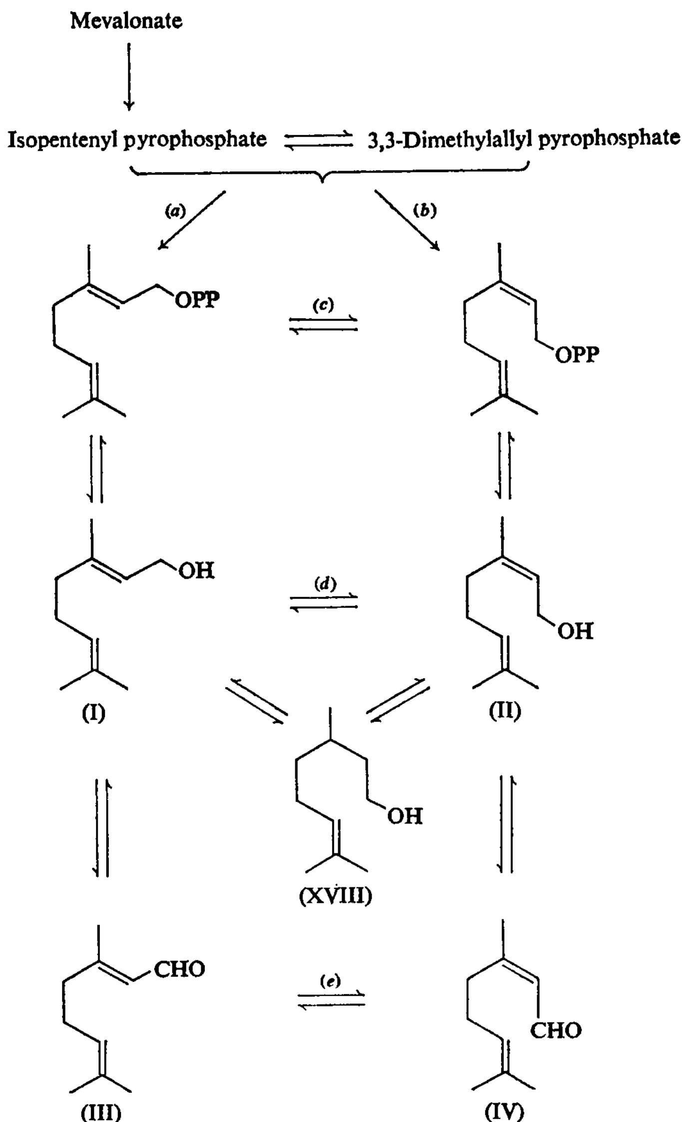
The results obtained from feeding with [^{14}C, ^{3}H]-mevalonate (Table 2) indicate that the *pro*-4*R* hydrogen atom of the precursor was almost entirely retained whereas the *pro*-4*S* hydrogen atom was very largely eliminated in the formation of geraniol and nerol, both free and bonded as glucosides. The incorporation of tracer from [2-^{14}C,(4*S*)-4-$^{3}H_1$]-mevalonate was usually significantly greater than zero, and this residual value, which is believed not to represent contamination by radioactive impurities, may result from some process that randomizes the locations of ^{3}H labelling (see the penultimate paragraph of this Discussion). The experiments with *P. graveolens* involved a ^{3}H/^{14}C ratio in precursor that was lower than that used in the experiments with *R. dilecta*, but the value was adequate for the purpose. Incorporations of mevalonate into the monoterpenes of *P. graveolens* were low relative to those in *R. dilecta*, and to obtain both an adequate ^{14}C radioactivity count and a high ^{3}H/^{14}C ratio in products, larger samples of mevalonate had to be used with *P. graveolens*. However, preliminary studies using 10-fold larger doses than those recorded in the Experimental section gave erratic results, owing perhaps to the operation of processes that randomize the position of tracer.

Our results were unexpected, for although the stereochemistry of hydrogen loss had not been demonstrated for monoterpenes, the biosynthesis of *trans*-olefinic linkages in several sesquiterpenoids and higher terpenoids had been found to involve elimination of the *pro*-4*S* hydrogen atom of mevalonate (see, e.g., Stone & Hemming, 1965; Goodwin & Williams, 1965, 1966; Archer *et al.*, 1966; Dada *et al.*, 1968), whereas construction of *cis*-olefinic linkages in rubber (Archer *et al.*, 1966) and in certain polyisoprenols Stone & Hemming, 1967; Gough & Hemming, 1967, 1970) led to the loss of the *pro*-4*R* hydrogen atom. The correlation between geometry of olefinic linkage and stereospecificity of hydrogen loss had seemed well established (Stone & Hemming, 1967).

One explanation of our results is that neryl pyrophosphate (or nerol) is derived by isomerization of geranyl pyrophosphate (or geraniol) (route *c* or *d*, Scheme 2). Such a direct isomerization of a double bond has few biochemical analogies (cf. Parke & Williams, 1951; Hubbard, 1955; Lack, 1961), but a possible indirect route in this case could be the enzyme-catalysed *cis*-addition of the elements of X-H to the double bond, followed by a *trans*-elimination. An attractive special case of this mechanism involves Michael addition of water to geranial (III): the latter could be formed from geraniol by enzyme systems similar to those characterized from several sources (Varma & Chichester, 1962; Donninger & Ryback, 1964; Potty & Bruemmer, 1970). Preliminary double-labelling experiments on the formation of geranial and neral (IV) in *Cymbopogon martinii* (lemongrass) suggest that these compounds are rapidly interconverted *in vivo* (M. Mort & G. N. J. Le Patourel, unpublished work), and further, the entire redox-isomerization route from geraniol to nerol (I→III→IV→II) has recently been characterized by using acetone-dried powders from rose petals (P. Dunphy, Unilever Research, personal communication). A geraniol–nerol reductase that converts either substrate into citronellol (XVIII) has been isolated from rose petals (Dunphy & Allcock, 1972); coupling of this with an appropriate dehydrogenase could constitute a system capable of interconverting geraniol and nerol, although the details of the suggested dehydrogenase step are obscure.

The only reasonable alternative explanation of our results requires either different prenyltransferases or a single prenyltransferase with two different active sites that catalyse the direct couplings of isopentenyl pyrophosphate with 3,3-dimethylallyl pyrophosphate to form either neryl pyrophosphate or geranyl pyrophosphate (steps *a* and *b*, Scheme 2). If the relative orientations of the reactants at the active sites were those shown in formulae (XIX) and (XX), coupled (S_N2+E2) processes involving *trans*-elimination would lead to loss of the *pro*-4*S* hydrogen atom of mevalonate irrespective of whether geranyl pyrophosphate or neryl pyrophosphate was formed. A similar route has been suggested (Professor O. Cori, University of Chile, personal communication) to account for the failure to detect interconversions of geranyl pyrophosphate and neryl pyrophosphate in cell-free systems from orange or from *Pinus radiata* (George-Nascimento & Cori, 1971; Jacob *et al.*, 1972).

Our results do not allow a choice to be made at



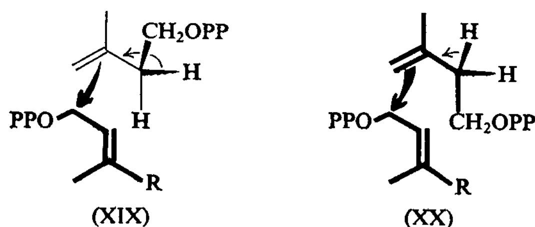
Scheme 2. *Proposed schemes for the interconversion of geranyl pyrophosphate and neryl pyrophosphate*

OPP, Pyrophosphate.

present between these alternatives, and further work is required to elucidate this fundamental point in monoterpene biosynthesis. The previously observed correlation of the loss of the *pro*-4*R* hydrogen atom with formation of a *cis*-olefinic linkage rests almost entirely on the results involving rubber biosynthesis in latex, and this stereochemical result may not apply to terpene synthesis in general. No detailed studies have been made on prenyltransferases from higher plants, but the enzyme from pig liver (EC 2.5.1.1) has been shown to catalyse the formation of both geranyl pyrophosphate and farnesyl pyrophosphate, although the stereochemistry of proton loss in the coupling is unknown (Holloway & Popják, 1967; Popják *et al.*, 1969*a*,*b*).

Our results do, however, exclude a mechanism wherein the reactants are held in a specific orientation on the enzyme surface, such as in formula (XIX), and

in which *trans*-elimination from the single binding site would lead to the loss of the *pro*-4*S* or *pro*-4*R*-hydrogen atom of mevalonate when geranyl pyrophosphate or neryl pyrophosphate is formed respectively. This situation (XIX, R = C_5H_9 etc.) could account for the results with rubber and the concomitantly formed *trans*–*trans*-farnesol. A variant of this process is an 'X-group' mechanism such as has been proposed to be general for chain extension of terpenoids (Cornforth, 1968, 1969): here an intermediate (formula XXII; Scheme 4) (enzyme-bonded?) can either break down with *trans*-elimination of H-X to give geranyl pyrophosphate or neryl pyrophosphate (with loss of the appropriate epimeric hydrogen atom) or be modified to form linalool (XXIII). Again this particular mechanism is excluded by our results, although it is compatible with either of two different prenyltransferases or one enzyme with two different active sites. Two additional points arise from the results in Table 2. First, nerol and its glucoside formed in petals of *R. dilecta* from [2-^{14}C,(4*R*)-4-^{3}H$_1$]mevalonate both had ^{3}H/^{14}C ratios significantly higher than the values in the precursor or in geraniol and its glucoside that was concomitantly formed. Contamination will have been eliminated by the rigorous purification to constant specific radioactivity and it is difficult to see how isotope fractionation could have occurred. The results suggest the



Scheme 3. *Stereochemistry of loss of pro-4S hydrogen atom of mevalonate in formation of geranyl pyrophosphate or neryl pyrophosphate*

OPP, Pyrophosphate.

incorporation of extraneous ^{3}H, and a very tentative explanation is that tracer derived from stereospecific oxidative degradation of excess of the precursor (or of physiologically inactive 3*S*-mevalonate) at the biosynthetic site was transferred to C-1 of neral in the redox process (Scheme 2: I→III→IV→II). Whatever the details of the mechanism, the occurrence of apparently extraneous tracer in nerol but not in geraniol is consistent with the formation of the former from the latter.

Secondly, the situation in the two higher plants studied was confirmed to be similar to that in animal tissue and in yeast (Cornforth, 1968, 1969) in that the *pro*-4*S* hydrogen atom of mevalonate was lost in the conversion of isopentenyl pyrophosphate into 3,3-dimethylallyl pyrophosphate.

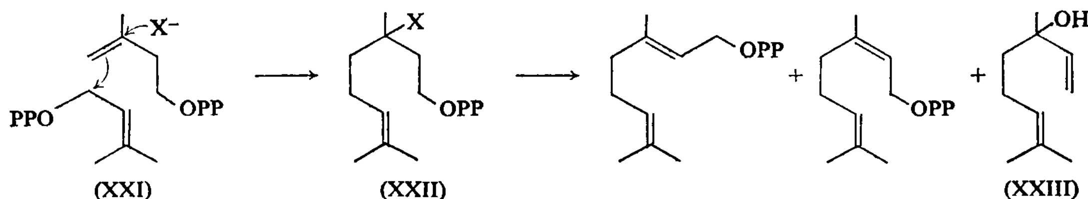
Experimental

Materials

R. dilecta and *P. graveolens* were obtained from the Unilever Research Laboratory, Sharnbrook, Bedford, U.K. The former specimens were grown either outdoors or in greenhouses; the latter were all grown outdoors (see the Results section).

Feeding with radioactive material

Flowerheads of *R. dilecta* in the pre-perfect stage (Francis & Allcock, 1969), 8 days after initial splitting of the calyx, were fed (Francis & Allcock, 1969; Francis & O'Connell, 1969) under sterile conditions with either the dibenzylethylenediamine salt of [2-^{14}C]mevalonate (5μCi; 0.23mg per flowerhead) or with the potassium salt of the [4-^{3}H$_1$]-mevalonate isomers (1.0μCi) mixed with [2-^{14}C]-mevalonate (0.5μCi) as marker. 3*RS*-[2-^{14}C]- and 3*RS*-[4*R*(*S*)-4-^{3}H$_1$]-mevalonate (from The Radiochemical Centre, Amersham, Bucks., U.K.) were used at specific radioactivities of 6.4 and 116mCi/mmol respectively. After harvesting the plant material, nerol and geraniol, both free and as the



Scheme 4. *'X-group' mechanism for the formation of geranyl pyrophosphate and neryl pyrophosphate*

OPP, Pyrophosphate.

β-D-glucosides, were extracted and purified (Francis & Allcock, 1969; Francis & O'Connell, 1969) and the glucosides were cleaved with β-glucosidase [emulsin; from Sigma (London) Chemical Co., London S.W.6, U.K.] and the monoterpenols were recovered for degradation experiments.

Young leaves (1–2 weeks after budding; average weight 0.07 g) of *P. graveolens* were fed under sterile conditions and with forced transpiration (Banthorpe *et al.*, 1970) with tracer at six to ten times the amount of radioactivity used for *R. dilecta*. The isotope ratios were as shown in Table 2. Monitoring of the leaves revealed that radioactivity in the laminae became constant within 20 min of completion of uptake of tracer and remained so for 24 h. At the end of the metabolism period, the leaves were ground in liquid N_2 and the resulting powder was mixed (1:1, w/w) with anhydrous Na_2SO_4 and extracted with ether (20 ml). The extract was concentrated under reduced pressure (to 2 ml) and was chromatographed on a column of MgO (15 cm × 1 cm external diam.), with hexane as eluent, to give a mixture of nerol and geraniol. Carrier (50 mg of geraniol or nerol) was added and the alcohols were purified by preparative-scale g.l.c. on Carbowax 20 M (6 m × 1 cm external diam.; 20%, w/w) at 150°C with a flow rate of 5 litres of N_2/h. A detachable section (30 cm) at the inlet end of the g.l.c. column trapped non-volatile impurities and with continuous high-temperature purging enabled the background radioactivity to be decreased to about 6 d.p.m. above background. Samples were collected in a U-tube (20 ml capacity) that was half-filled with glass beads (1 mm diam.), which in turn were covered with hexane containing carrier nerol or geraniol (50 mg); the whole was cooled at −70°C. With this device, geraniol could be collected with 90% recovery at flow rates up to 18 litres/h.

Radiochemical methods

Solids or solid derivatives of liquids were recrystallized or resublimed, normally at least three times, to constant specific radioactivity.

Radiochemical assays were made by using a Beckman three-channel scintillation system, generally with 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD) (CIBA Ltd.) in sodium-dried toluene (8 g/litre) as scintillant. Counting efficiencies were typically approx. 96 and 55% for ^{14}C and ^{3}H respectively and were unchanged by redistillation of the toluene or removal of O_2 from the scintillation solution in a stream of N_2. Butyl-PBD (5 g) in toluene (500 ml) and methanol (500 ml), or Bray's (1960) solution, were used for samples that were insoluble in toluene. Usually about 20 mg samples of labelled materials were used; these samples sometimes consisted of combined fractions from several feedings carried out at the same time.

The methodology of counting samples followed that in a definitive text (Turner, 1967). ^{14}C and ^{3}H radioactivities were usually determined from counts of radioactivity in the appropriate channels after corrections had been made for quenching and counting efficiency. These corrections were obtained from calibration curves connecting the quenching properties of various amounts of chloroform on standard solutions of [^{14}C]- and [^{3}H]-hexadecane with counting efficiencies that were automatically computed by the counter, with ^{137}Cs as an external standard in the channels-ratio mode of operation. For a few highly quenched or feebly radioactive samples the internal-standard method was used to determine counting efficiency by using the appropriately labelled hexadecane.

Each set of feeding, isolation and degradation experiments was done in duplicate, and individual radioactive assays were repeated two or three times to accumulate (in each counting period) approx. 4×10^4 scintillations; this ensured that 2σ was ±1%. No corrections were made for ^{3}H decay, as the [$^{14}C,^{3}H_1$]mevalonate was assayed directly after the derived products. Combined errors in weighing, counting procedures etc. were estimated at approx. ±2% and reproducibility between different experiments was approx. ±3%.

Degradation schemes

General. All compounds had melting points (uncorrected) in agreement (±1°C) with literature values and i.r., proton-magnetic-resonance and mass spectra were consistent with the accepted structures. The following degradation schemes were variously performed on 100–500 mg of starting material. Quantities are given for one set of degradations.

Purification and oxidation of geraniol and nerol. Geraniol (98% pure, by g.l.c.) for use as carrier was purified from commercial material by means of its $CaCl_2$ complex (Simonsen, 1947); nerol for similar use was purchased (Fluka A.G., Buchs, Switzerland; 98% pure). The specific radioactivities of geraniol and nerol, purified as the 3,5-dinitrobenzoates, could not be accurately measured because of extensive quenching; consequently the alcohols were oxidized to geranial and neral (III, IV) and derivatives of these were prepared. In a few experiments geraniol was purified through its $CaCl_2$ complex. The alcohol (450 mg) in light petroleum (b.p. 40–60°C; 30 ml) was treated with MnO_2 (3 g) under N_2 at 20°C for 15 h. The oxidant was freshly prepared and was stored in a sealed tin: storage in a desiccator gave material of lower activity. Geranial and neral were separated on a column of MgO with hexane as

eluent and were converted into semicarbazones (69% yield), melting points 162°C and 183°C (after recrystallization from aq. alcohol).

Oxidation of geranial and neral. The appropriate aldehyde (100mg) in purified methanol (10ml) or carbon tetrachloride (12ml) was ozonized (Ozonizer model 15-69K; Nu-aire Ltd., Kings Langley, Herts., U.K.) at −70°C or −15°C respectively and the crude ozonides were recovered as viscous oils and were decomposed by treatment for 3 days at 20°C with a mixture of formic acid (98%, w/w; 1.5ml) and hydrogen peroxide (30vol.; 1.1ml), after which the peroxides were destroyed by treatment with MnO_2 (2mg) for 3h at 0°C. This experiment was carried out several times with radioactive geranial and neral and the overall yields were: laevulinic acid (V) 55%; acetone 72%; oxalic acid 5%. No formaldehyde (cf. Knights & Waight, 1955) was found.

Acetone was distilled out of the reaction mixture and was purified as the 2,4-dinitrophenylhydrazone, m.p. 128°C. The ketone was regenerated from this by refluxing (5h) with a tenfold excess of aq. sodium pyruvate, and was subjected to the iodoform reaction (Hickinbottom, 1957) to give a poor (10%) yield. In later experiments, acetone was purified as the 4-phenylcarbazone (m.p. 160°C), thiosemicarbazone (m.p. 179°C) or tosylhydrazone (m.p. 146°C) (all after recrystallization from aq. alcohol); these derivatives were superior to the 2,4-dinitrophenylhydrazone for scintillation counting.

The residue of laevulinic acid and oxalic acid (total approx. 180mg) was acidified (with 10% HCl) and continuously extracted (5h) with ether (50ml) to remove the laevulinic acid. The ether extract was evaporated to dryness, and re-extracted with chloroform and the latter extract (approx. 0.5ml) was added to silicic acid (2g; type CC7, 100–200 mesh; Mallinckrodt Inc., St. Louis, Mo., U.S.A.) and shaken to form a powder. This was added to the top of a column (20cm × 1cm ext. diam.) that had been made up in benzene with silicic acid (10g), which in turn had been shaken with 0.025M-H_2SO_4 (6ml), and the column was eluted with benzene–ether (1:1, v/v), which had been similarly equilibrated with H_2SO_4, to give laevulinic acid (155mg), purified as the 4-phenylsemicarbazone (yield 70%; m.p. 185°C, after recrystallization from aq. alcohol) or the tosylhydrazone (yield 80%; m.p. 159°C, after recrystallization from chloroform). The residue from the ether extraction was similarly chromatographed and eluted with ether to give oxalic acid (yield 14mg; m.p. 189°C, after recrystallization from ethanol–benzene).

Degradation of laevulinic acid. The laevulinic acid obtained as described above was typically diluted fourfold with carrier and was further degraded. An iodoform reaction on the acid (600mg) gave succinic acid (IX) (yield 85%; m.p. 185°C), which was purified by chromatography on silicic acid with benzene–ether (2:1, v/v; 60ml), benzene–ether (1:2, v/v; 30ml) and finally ether (30ml) as eluents. This acid (500mg) was converted into its dimethyl ester with diazomethane (195mg) in ether: the product was not purified but was shaken with hydrazine hydrate (300mg) for a few minutes, when the dihydrazide (yield 95%; m.p. 173°C, from alcohol) precipitated. Treatment of the dihydrazide (400mg) in water (4ml) with the stoicheiometric quantity of nitrous acid gave the diazide (XI), which was extracted into ether (15ml) but was not further purified because of its instability.

The diazide underwent Curtius rearrangement to form the urethane (XIII), m.p. 111°C (from carbon tetrachloride), in overall yield (from the dihydrazide) of approx. 40%, when the ethereal solution obtained above and ethanol (5ml) were refluxed for 1h. This product (160mg) was in turn decarboxylated with HBr (constant-boiling mixture; 3ml), on heating to 130°C to form ethylenediamine (XIV), which was purified by sublimation of its dihydrobromide (decomposes above 180°C); the latter was formed in 83% yield, based on the urethane. The CO_2 was concomitantly collected (88% yield based on the urethane) as $BaCO_3$. An alternative procedure for effecting the rearrangement was to reflux the diazide (100mg) in benzene (5ml) for 1h: the isocyanate (XII) that was formed was not isolated but was decomposed by HCl (12M, 0.5ml) at room temperature to give the dihydrochloride of ethylenediamine (43% yield based on the diazide) and CO_2 (87% yield). The CO_2 was regenerated from $BaCO_3$ and was passed into ethanolamine–2-methoxyethanol (2:1, v/v; 10ml); a portion (5ml) of this was added to the scintillation fluid (10ml) for radioactivity counting.

Phosphorylated intermediates from mevalonate. Small leaves (approx. 0.5g) of *P. graveolens* (4-week-old plants) were placed in vials containing [2-^{14}C]-mevalonate (2μCi; 0.1ml) and were illuminated with natural sunlight augmented with a 100W tungsten lamp at a distance of 1m. After 5½h the foliage was ground with sodium phosphate buffer (0.1M, pH7.0; 2.0ml) and the filtrate was heated at 100°C for 1min, cooled and chromatographed on a column (15cm × 1.5cm external diam.) of Dowex 1 (X8; formate form; 200–300 mesh) and subjected to gradient elution with aq. formic acid–ammonium formate (Bloch *et al.*, 1959). Fractions (10ml) were collected and the radioactivity of samples (1ml) of each was measured in portions (20ml) of a scintillation medium made up from naphthalene (16g) and butyl-PBD (1.4g) in toluene (120ml) and 2-methoxyethanol (80ml). Peaks of radioactivity occurred in fractions 18–26, 37–45 and 68–73, and were assigned to mevalonic acid 5-phosphate, mevalonic acid 5-pyrophosphate and isopentenyl pyrophosphate by comparison with standards by using paper chromatography with four

solvent systems (Tchen, 1958; Benedict *et al.*, 1965; Chesterton & Kekwick, 1968) and by t.l.c. with a range of benzene–ether–ethyl acetate mixtures.

We acknowledge much help from Dr. G. Ayrey (Isotope Unit, Queen Elizabeth College, London) with counting techniques and we thank Unilever Ltd. (London) for a studentship to G. N. J. Le P. and for permission for M. J. O. F. to undertake and publish this work. We are also very grateful to Dr. P. Dunphy (Unilever Research, Sharnbrook) and Professor O. Cori (Santiago) for making available to us results before publication.

References

Archer, B. L., Barnard, D., Cockbain, E. G., Cornforth, J. W., Cornforth, R. H. & Popják, G. (1966) *Proc. Roy. Soc. Ser. B* **163**, 519–523

Banthorpe, D. V. & Baxendale, D. (1970) *J. Chem. Soc. C* 2694–2696

Banthorpe, D. V. & Charlwood, B. V. (1971) *Nature (London) New Biol.* **231**, 285–286

Banthorpe, D. V., Mann, J. & Turnbull, K. W. (1970) *J. Chem. Soc. C* 2689–2693

Banthorpe, D. V., Charlwood, B. V. & Francis, M. J. O. (1972*a*) *Chem. Rev.* **72**, 115–155

Banthorpe, D. V., Charlwood, B. V. & Young, M. R. (1972*b*) *J. Chem. Soc. Perkin Trans. 1*, 1532–1534

Benedict, C., Kett, J. & Porter, J. W. (1965) *Arch. Biochem. Biophys.* **110**, 611–621

Biollaz, M. & Arigoni, D. (1969) *Chem. Commun.* 633–634

Bloch, K., Chaykin, S., Phillips, A. H. & de Waard, A. (1959) *J. Biol. Chem.* **234**, 2595–2604

Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–286

Chesterton, C. J. & Kekwick, R. G. O. (1968) *Arch. Biochem. Biophys.* **125**, 76–85

Clayton, R. B. (1965*a*) *Quart. Rev. Chem. Soc.* **19**, 168–200

Clayton, R. B. (1965*b*) *Quart. Rev. Chem. Soc.* **19**, 201–230

Cornforth, J. W. (1968) *Angew. Chem. Int. Ed. Engl.* **7**, 903–911

Cornforth, J. W. (1969) *Quart. Rev. Chem. Soc.* **23**, 125–140

Croteau, R. & Loomis, W. D. (1972) *Phytochemistry* **11**, 1055–1066

Dada, O. A., Threlfall, D. R. & Whistance, G. R. (1968) *Eur. J. Biochem.* **4**, 329–333

Donninger, C. & Ryback, R. (1964) *Biochem. J.* **91**, 11P

Dunphy, P. J. & Allcock, C. (1972) *Phytochemistry* **11**, 1887–1892

Francis, M. J. O. (1971) in *Aspects of Terpenoid Chemistry and Biochemistry* (Goodwin, T. W., ed.), pp. 29–51, Academic Press, London

Francis, M. J. O. & Allcock, C. (1969) *Phytochemistry* **8**, 1339–1347

Francis, M. J. O. & O'Connell, M. (1969) *Phytochemistry* **8**, 1705–1708

Francis, M. J. O., Banthorpe, D. V. & Le Patourel, G. N. J. (1970) *Nature (London)* **228**, 1005–1006

George-Nascimento, C. & Cori, O. (1971) *Phytochemistry* **10**, 1803–1810

Goodwin, T. W. & Williams, R. J. H. (1965) *Biochem. J.* **94**, 5C–7C

Goodwin, T. W. & Williams, R. J. H. (1966) *Proc. Roy. Soc. Ser. B* **163**, 515–518

Gough, D. P. & Hemming, F. W. (1967) *Biochem. J.* **105**, 10C–12C

Gough, D. P. & Hemming, F. W. (1970) *Biochem. J.* **117**, 309–317

Hickinbottom, W. J. (1957) *Reactions of Organic Compounds*, 3rd. edn., p. 257, Longmans, London

Holloway, P. W. & Popják, G. (1967) *Biochem. J.* **104**, 57–70

Hubbard, R. (1955) *Fed. Proc. Amer. Soc. Exp. Biol.* **14**, 229

Jacob, G., Cardemil, E., Chayet, L., Tellez, R., Pont-Lezica, R. & Cori, O. (1972) *Phytochemistry* **11**, 1683–1688

Knights, J. & Waight, E. S. (1955) *J. Chem. Soc. London* 2830–2834

Lack, L. (1961) *J. Biol. Chem.* **236**, 2835–2840

Neethling, L. P., Reiber, H. G. & Chichester, C. O. (1963) *Nat. Conf. Nucl. Energy Appl. Isotop. Radiat., Proc.* 451–460

Parke, D. V. & Williams, R. T. (1951) *Biochem. J.* **49**, lii

Popják, G., Holloway, P. W., Bond, R. P. M. & Roberts, M. (1969*a*) *Biochem. J.* **111**, 333–343

Popják, G., Rabinowitz, J. L. & Baron, J. M. (1969*b*) *Biochem. J.* **113**, 861–868

Potty, V. H. & Bruemmer, J. H. (1970) *Phytochemistry* **9**, 1003–1007

Simonsen, J. L. (1947) *The Terpenes*, 2nd. edn., vol. 1 p. 41, Cambridge University Press, London

Stone, K. J. & Hemming, F. W. (1965) *Biochem. J.* **96**, 14C–17C

Stone, K. J. & Hemming, F. W. (1967) *Biochem. J.* **104**, 43–56

Suga, T., Shishibori, T. & Bukeo, M. (1971) *Phytochemistry* **10**, 2725–2726

Tchen, T. T. (1958) *J. Biol. Chem.* **233**, 1100–1103

Turner, J. C. (1967) *Sample Preparation for Liquid Scintillation Counting*, The Radiochemical Centre, Amersham

Varma, T. N. R. & Chichester, C. O. (1962) *Arch. Biochem. Biophys.* **96**, 419–422

Waller, G. R. (1969) *Progr. Chem. Fats Other Lipids* **10**, 151–238