The Stereochemistry of Betulaprenol Biosynthesis

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(Received 1 December 1969)

By using stereospecifically double-labelled radioactive mevalonates it was shown that betulaprenols-6 to -9, found in the woody tissue of *Betula verrucosa*, each contain three biogenetically *trans*-isoprene residues and that the remaining residues are biogenetically *cis*. The results obtained with these radioactive mevalonates also indicated that the activity of isopentenyl pyrophosphate isomerase is low relative to the activity of prenyltransferase in this woody tissue. The incorporation of radioactive farnesyl pyrophosphate and radioactive geranylnerol and the lack of incorporation of radioactive geranylgeraniol into betulaprenols-6 to -9 demonstrated that they are formed by the *cis*-additions of isoprene residues to all-*trans*-farnesyl pyrophosphate.

The woody tissue of *Betula verrucosa* (silver birch) contains betulaprenols-6, -7, -8 and -9. It has been shown that in each polyprenol two of the internal isoprene residues are chemically *trans*, and the other internal residues and the α -residue are chemically *cis* (Lindgren, 1965; Wellburn & Hemming, 1966). These polyprenols are present in woody tissue mainly as their fatty acid esters at concentrations of up to 1.5 g/kg of tissue (Lindgren, 1965). They are also found in the non-esterified free alcohol form in the leaf tissue of *B. verrucosa*, where they reach concentrations of 10 mg/kg of tissue (Wellburn & Hemming, 1966).

The leaf tissue also contains *cis-trans*-polyprenols with chain lengths of ten to 13 isoprene residues. In each of these betulaprenols-10 to -13 it has been shown that three internal residues are chemically *trans* and the other internal residues and the α -residue are chemically *cis*. These are present in their free alcohol form at concentrations of up to 134 mg/kg of leaf tissue, and have also been shown to be present, to a lesser extent, as the free alcohols in the woody tissue at concentrations reaching 19 mg/kg of tissue (Wellburn & Hemming, 1966).

It has been suggested that the betulaprenols-6 to -9 are probably formed from *trans-trans*-farnesyl pyrophosphate by *cis*-additions of isoprene residues, and that the leaf polyprenols-10 to -13 are probably formed from all-*trans*-geranylgeranyl pyrophosphate in a similar manner (Wellburn & Hemming, 1966). Such a mechanism would require the internal chemically *trans* residues and the ω -residue to be

biogenetically *trans*, and the chemically *cis* residues to be biogenetically *cis*. It would also require the internal *trans* residues to be at the ω -end of the molecule.

The biosynthesis of polyprenols involves the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate and the condensation of isopentenyl pyrophosphate with dimethylallyl pyrophosphate or its higher isoprenologues. In each of these steps a C–C bond is formed, accompanied by the loss of a hydrogen atom from C-2 of isopentenyl pyrophosphate. The hydrogen atom lost is one of two originally attached to C-4 of mevalonic acid. By using [2-¹⁴C,(4*R*)-4-³H₁]- and [2-¹⁴C,(4*S*)-4-³H₁]-mevalonate as substrates, the stereochemistry of this hydrogen elimination has been studied in several systems. Work on the biosynthesis of *trans-trans*-farnesyl pyrophosphate and all-*trans*-squalene with a rat liver preparation (Cornforth, Cornforth, Donninger & Popják, 1965), of squalene and phytoene with carrot-root preparations (Goodwin & Williams, 1965), of squalene and ubiquinone in *Aspergillus fumigatus* Fresenius (Stone & Hemming, 1967) and of squalene and phytol in green leaves (Wellburn, Stone & Hemming, 1966) shows that all of the 4*S*-tritium is lost and all of the 4*R*-tritium is retained. Hence, in the biogenesis of all-*trans*-polyisoprenoid compounds the 4-*pro-R*-hydrogen of mevalonic acid is retained and the 4-*pro-S*-hydrogen is eliminated. In the biosynthesis of poly-*cis*-rubber, the 4*R*-tritium is lost and the 4*S*-tritium retained (Archer *et al.* 1965). Hence, although the evidence is limited, it seems that in the biogenesis of all-*cis*-polyisoprenoid compounds the 4-*pro-R*-hydrogen of mevalonic acid is

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lost and the 4-*pro-S*-hydrogen is retained. The generalization can thus be made that in the biogenesis of *trans*-isoprene units the 4-*pro-R*-hydrogen of mevalonic acid is retained and the 4-*pro-S*-hydrogen eliminated, and in the biogenesis of *cis*-isoprene units the 4-*pro-R*-hydrogen is eliminated and the 4-*pro-S*-hydrogen is retained.

With polyisoprenoid compounds of mixed stereochemistry the evidence is not quite so conclusive. By using $[2-^{14}\text{C}, (4R)-4-^3\text{H}_1]$ mevalonate, the presence of four biogenetically *trans* residues in the hexahydriopolyisoprenols of *A. fumigatus* has been shown. However, the $^3\text{H}/^{14}\text{C}$ ratios in the hexahydriopolyisoprenols biosynthesized from $[2-^{14}\text{C}, (4S)-4-^3\text{H}_1]$ mevalonate were about 40% lower than expected (Stone & Hemming, 1967). This loss of 4*S*-tritium is probably accounted for by the presence of a highly active isopentenyl pyrophosphate isomerase. The isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate is reversible (Agranoff, Eggerer, Henning & Lynen, 1960; Shah, Cleland & Porter, 1965; Holloway &

Popják, 1968). The ω -residue of polyisoprenoid chains always appears to be biogenetically *trans*. Thus 4*S*-tritiated mevalonic acid gives rise to 2*R*-tritiated isopentenyl pyrophosphate, which can be isomerized to dimethylallyl pyrophosphate with the stereospecific removal of tritium. This dimethylallyl pyrophosphate is the source of the biogenetically *trans* ω -residues (Scheme 1a). If step (1) is readily reversible, then isopentenyl pyrophosphate is formed that is devoid of tritium. Hence, if step (1) is much more rapid than step (2), the $^3\text{H}/^{14}\text{C}$ ratios from incubations with $[2-^{14}\text{C}, (4S)-4-^3\text{H}_1]$ mevalonate will be lower than expected. It is notable that in the latex of *Hevea brasiliensis*, where the $^3\text{H}/^{14}\text{C}$ ratios obtained by the incorporation of $[2-^{14}\text{C}, (4S)-4-^3\text{H}_1]$ mevalonate into poly-*cis*-rubber were as expected (Archer *et al.* 1965), the activity of isopentenyl pyrophosphate isomerase has been reported to be very low (Archer, Audley, Cockbain & McSweeney, 1963; Archer & Audley, 1967). This would explain why there was no loss of tritium from 4*S*-tritiated mevalonate in this system. The pres-

Scheme 1. (a) Incorporation of ^3H from $[(4S)-4-^3\text{H}]$ -mevalonic acid (MVA) into isoprenoid residues. Abbreviations: DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate. Enzymes: 1, IPP isomerase; 2, prenyltransferase.

Scheme 1. (b) Incorporation of ^3H from [(4*R*)-4- ^3H]-mevalonic acid (MVA) into isoprenoid residues.

ence of a highly active isopentenyl pyrophosphate isomerase does not affect the $^3\text{H}/^{14}\text{C}$ ratios obtained from incubations with [2- ^{14}C , (4*R*)-4- $^3\text{H}_1$]mevalonate. 4*R*-Tritiated mevalonic acid gives rise to 2*S*-tritiated isopentenyl pyrophosphate, which can then be isomerized to dimethylallyl pyrophosphate with the stereospecific retention of tritium (Scheme 1b). Thus ω -residues formed from 4*R*-tritiated mevalonic acid will possess tritium, showing them to be biogenetically *trans*. Isomerization of this tritiated dimethylallyl pyrophosphate back to isopentenyl pyrophosphate results in the re-formation of 2*S*-tritiated isopentenyl pyrophosphate. Hence it seems that the $^3\text{H}/^{14}\text{C}$ ratios obtained from incubations with these double-labelled mevalonates can provide information about the relative activities of isopentenyl pyrophosphate isomerase (EC 5.3.3.2) and prenyltransferase (EC 2.5.1.1), in addition to providing information about the stereochemistry of biosynthesis of isoprene residues.

The general availability of [2- ^{14}C , (4*R*)-4- $^3\text{H}_1$] and [2- ^{14}C , (4*S*)-4- $^3\text{H}_1$]mevalonate provided an opportunity to determine whether the chemically *trans*-isoprene residues and the ω -residues of the

betulaprenols are, in fact, biogenetically *trans* and the chemically *cis* residues in these polyprenols are biogenetically *cis*, and whether or not the activity of isopentenyl pyrophosphate isomerase relative to the activity of prenyltransferase is high in *Betula verrucosa*. In addition, the incorporation of [^{14}C]farnesyl pyrophosphate and of [^{14}C]geranylgeraniol and [^{14}C]geranylnerol into the betulaprenols has also been studied. The results are reported in this paper. Some of the results have been reported previously (Gough & Hemming, 1967).

MATERIALS AND METHODS

Incorporation of the radioactive mevalonates. [2- ^{14}C , (4*R*)-4- $^3\text{H}_1$]mevalonate and [2- ^{14}C , (4*S*)-4- $^3\text{H}_1$]mevalonate were prepared as described by Wellburn *et al.* (1966) from mixtures of original specific radioactivities as follows: 3*R*-[(4*R*)-4- $^3\text{H}_1$]- and 3*S*-[(4*S*)-4- $^3\text{H}_1$]-mevalonic acid lactone, 134 mCi/mmol; 3*R*-[(4*S*)-4- $^3\text{H}_1$]- and 3*S*-[(4*R*)-4- $^3\text{H}_1$]-mevalonic acid lactone 115 mCi/mmol; 3*R,S*-[2- ^{14}C]mevalonic acid lactone, 5.03 mCi/mmol.

Two terminal twigs of *B. verrucosa*, about 9 in long and carrying 8–12 leaves, were each allowed to take up either [2- ^{14}C , (4*R*)-4- $^3\text{H}_1$]- or [2- ^{14}C , (4*S*)-4- $^3\text{H}_1$]-mevalonate

(5 μ Ci of 14 C in approx. 0.5 ml of 60 mM- K_2HPO_4 - KH_2PO_4 buffer, pH 7.4) by transpiration over 2–4 h. By this time all of the solution had been taken up by the twigs. Unlabelled mevalonate (1 mg) was then taken up in a similar way over 24 h. The twigs were then left in water for a further 24 h at approx. 20°C.

Preparation and incorporation of [14 C]farnesyl pyrophosphate. [14 C]Farnesyl pyrophosphate was prepared from [14 C]mevalonate (5 μ Ci, 1 μ mol) with a rat liver enzyme system as described by Goodman & Popják (1960).

The preparation was purified from any contaminating radioactive mevalonate by preparative t.l.c. (silica gel H) with propan-2-ol-aq. NH_3 (sp.gr. 0.88)-water (6:3:1, by vol.) as the developing solvent, with authentic samples of farnesyl monophosphate and farnesyl pyrophosphate as markers. In this system farnesyl pyrophosphate has R_F 0.30, farnesyl monophosphate has R_F 0.60 and mevalonate has R_F 0.66. Radioactivity was located with a Panax model RTLS-1A thin-layer scanner, and the authentic markers were located by spraying with anisaldehyde reagent (McSweeney, 1965). Radioactivity was detected corresponding only to farnesyl pyrophosphate and mevalonate. [14 C]Farnesyl pyrophosphate was extracted with ethanol-aq. NH_3 (sp. gr. 0.88)-water (7:2:1, by vol.) and the solution was evaporated to dryness at reduced pressure in a vacuum desiccator fitted with a 'cold-trap' (solid CO_2 -acetone) connected to a secondary drying unit. The dry residue was dissolved in 10 ml of 0.01 M- $NaHCO_3$. Two samples (0.01 ml) of this solution were assayed for 14 C with a Geiger-Müller proportional counter. The total extract contained 5.36×10^5 d.p.m. of 14 C, representing an incorporation of approx. 9.6% of the available [14 C]-mevalonate. A sample (0.1 ml) of this extract was checked for the presence of any 14 C-labelled compounds other than [14 C]farnesyl pyrophosphate by mild acid hydrolysis. A portion (10%) of the ether-soluble material was assayed for 14 C with the Geiger-Müller proportional counter, and it was found that a 100% conversion of 14 C-labelled aqueous soluble material into 14 C-labelled ether-soluble material had occurred. The remaining portion of the ether extract was subjected to reversed-phase t.l.c. [kieselguhr; 5% paraffin in light petroleum (b.p. 40–60°C)] with acetone-water (13:7, v/v) as the mobile phase. Authentic samples of farnesol and nerolidol were used as markers. Radioactivity was located by t.l.c. scanning, and farnesol and nerolidol were located by spraying with anisaldehyde reagent. Radioactivity was found corresponding only to farnesol (R_F 0.45) and nerolidol (R_F 0.37), in approximately equal amounts. Hence the [14 C]farnesyl pyrophosphate preparation appeared to be free from any other 14 C-labelled material.

A sample (8 ml) of the $NaHCO_3$ solution of [14 C]farnesyl pyrophosphate (4.29×10^5 d.p.m. of 14 C) was freeze-dried and the residue was dissolved in water (2 ml). This solution was taken up by transpiration over 12 h into a terminal twig of *B. verrucosa* about 9 in long and carrying 12 leaves. The twig was then left in water for a further 40 h at approx. 20°C.

Preparation and incorporation of [14 C]geranylgeraniol and [14 C]geranylnerol. *trans-trans*-Farnesol was purified from a commercial mixture of *trans-trans*- and *cis-trans*-farnesol by chromatography on a column of alumina

(Woelm, Eschwege, Germany; Brockmann grade III, acid-washed) with 5–15% (v/v) E/P* as eluent. The purification was followed by t.l.c. (silica gel G) with 5% (v/v) ethyl acetate in benzene as the developing solvent and ethanolic phosphomolybdate as staining reagent. *trans-trans*-Farnesol had R_F 0.17 in this system and *cis-trans*-farnesol R_F 0.22. *cis-trans*-Farnesol was eluted in the early fractions and *trans-trans*-farnesol in the later fractions. Those fractions containing *trans-trans*-farnesol alone were pooled. I.r. spectroscopy of the *trans-trans*-farnesol so isolated confirmed its purity.

trans-trans-Farnesol was converted into *trans-trans*-farnesyl bromide and then allowed to react with ethyl acetoacetate to form *trans-trans*-farnesylacetone. The method used was essentially that of Isler, Rüegg, Chopard-dit-Jean, Winterstein & Wiss (1956). *trans-trans*-Farnesylacetone was purified by chromatography on a column of alumina (Woelm; Brockmann grade III, acid-washed) with light petroleum (b.p. 40–60°C) followed by 0.5–8% (w/v) E/P as the eluting solvents. T.l.c. on silica gel G with benzene as the developing solvent, with authentic *trans-trans*-farnesylacetone (a gift from Dr O. Isler of Hoffmann-La Roche, Basle, Switzerland) as a marker (R_F 0.24), and ethanolic phosphomolybdate as staining reagent, showed that most of the farnesylacetone was eluted by 0.5% and 1% (v/v) E/P. *trans-trans*-Farnesylacetone was further purified by column chromatography on silicic acid (Mallinckrodt, St Louis, Mo., U.S.A.) with the same eluent mixtures as used for the alumina column chromatography. *trans-trans*-Farnesylacetone was eluted by 2% (v/v) E/P. The i.r. spectrum of the purified preparation was identical with that of a sample of pure *trans-trans*-farnesylacetone.

Methyl[14 C]geranylgeranoate and methyl[14 C]geranylnerolate were synthesized from all-*trans*-farnesylacetone (75 mg) by a modified Wittig reaction with methyl bromo[14 C]acetate (Upper & West, 1967). The products were purified by silicic acid (Mallinckrodt) column chromatography, with 0.5%, 1.0%, 1.5% and 2% (v/v) E/P, as eluting solvents. Samples of each fraction were subjected to t.l.c. (silica gel G; benzene) and the compounds located by spraying with ethanolic phosphomolybdate. Both methyl geranylgeranoate (R_F 0.50) and methyl geranylnerolate (R_F 0.61) were eluted from the column by 1% E/P. Unchanged *trans-trans*-farnesylacetone was present in the later fractions. Methyl[14 C]geranylgeranoate and methyl[14 C]geranylnerolate were separated from each other by preparative t.l.c. (silica gel G; benzene). Radioactivity was located with a Panax thin-layer scanner and the compounds were extracted with ether. About 75% of the radioactivity corresponded to the all-*trans*-isomer of methyl[14 C]geranylgeranoate. The purity of these compounds was checked by t.l.c. (silica gel G; benzene) of a sample of each isomer. Each sample was a single pure compound as judged by scanning for radioactive areas and by staining with ethanolic phosphomolybdate.

Reduction of the 14 C-labelled methyl esters with $LiAlH_4$ (Upper & West, 1967), yielded [14 C]geranylgeraniol and [14 C]geranylnerol. Each of these was purified by chromatography on a column of alumina (Woelm, Brockmann grade III, acid-washed) with light petroleum (b.p. 40–60°C), 2% and 20% (v/v) E/P as the eluting solvents. A sample of each fraction was subjected

* Abbreviation: E/P, solution of diethyl ether in light petroleum (b.p. 40–60°C).

to t.l.c. (silica gel G) with 5% (v/v) ethyl acetate in benzene as solvent. Radioactivity was located by scanning and the chromatogram was sprayed with ethanolic phosphomolybdate. [2-¹⁴C]Geranylgeraniol (R_F 0.20) and [2-¹⁴C]geranylnerol (R_F 0.25) were present in the 20% E/P fractions, and unreduced 2-¹⁴C-labelled methyl esters in the 2% E/P fractions. These alcohols had specific radioactivity 1.22×10^6 d.p.m./mg.

Two terminal twigs of *B. verrucosa*, about 9 in long and carrying 8–12 leaves, were each allowed to take up either [2-¹⁴C]geranylgeraniol [0.3 mg suspended in 2 ml of aq. 2.5% (v/v) ethanol] or [2-¹⁴C]geranylnerol (0.1 mg suspended in 2 ml of aq. 2.5% ethanol) by transpiration over 10–14 h. The twigs were then left in water for a further 40 h at approx. 20°C.

Extraction and chromatography of lipid. A sample (xg) of tissue was macerated in 4x ml of ethanol-ether (2:1, v/v) with an Ultra-Turrax homogenizer. The suspension was filtered through fluted filter paper. The precipitate was washed with 2x ml of ether and 2x ml of light petroleum (b.p. 40–60°C). The pooled filtrates were added to water (2x ml) in a separating funnel. After being shaken, the mixture was left to separate. The lower layer was re-extracted with 2x ml of 50% (v/v) E/P. The E/P extracts were pooled and washed several times with water, dried over anhydrous Na₂SO₄ and evaporated to dryness finally under N₂.

The weighed lipid was chromatographed on a column of alumina (Woelm, Brockmann grade III, acid-washed) with 0.1 g of alumina and x ml of eluent/x mg of lipid. Polyprenyl esters and squalene were eluted by 2% E/P, polyprenols by 20% E/P and chlorophyll by 10% (v/v) methanol in ether. Samples (2 mg) of a mixture of betulaprenols-6 to -9 and of a mixture of ficaprenols-9 to -13 were added to the 2% E/P fractions and the lipid was saponified (see below). The unsaponifiable lipid was recovered and the alumina column chromatography was repeated. Any squalene present was again eluted by 2% E/P and free polyprenols were eluted by 20% E/P. Phytol (2 mg) was added to the fraction eluted with 10% methanol in ether and the lipid was saponified. Unsaponifiable lipid was chromatographed on alumina as described above and phytol was eluted by 20% E/P.

Squalene, phytol and polyprenols were purified by t.l.c. with several systems. In each case, the isoprenoid compounds were located by spraying with ethanolic fluorescein (Dunphy, Whittle & Pennock, 1965), extracted with ether, and evaporated to dryness under N₂. Squalene was purified by preparative t.l.c. (silica gel G) with chloroform (R_F 0.80) followed by repeated preparative t.l.c. (silica gel G) with light petroleum (b.p. 40–60°C) (R_F 0.30). The squalene isolated was purified from contaminating fluorescein (which may cause quenching in the scintillation spectrometer) by dissolving in petroleum (in which fluorescein is insoluble), decanting and evaporating to dryness under N₂. Phytol was twice subjected to preparative t.l.c. (silica gel G) with chloroform (R_F 0.48) followed by repeated preparative reversed-phase t.l.c. on kieselguhr impregnated with 5% (v/v) paraffin in light petroleum (b.p. 40–60°C) as the stationary phase, and acetone-water (3:1, v/v) as the mobile phase (R_F 0.40). Contaminating paraffin and fluorescein were removed from the phytol by alumina column chromatography. Paraffin was eluted by 2% E/P and phytol by 20% E/P.

Fluorescein remained adsorbed on the alumina. Betulaprenols-6 to -9 were twice subjected to preparative t.l.c. (silica gel G) with CHCl₃ (R_F 0.55), followed by preparative reversed-phase t.l.c. [kieselguhr; 5% (v/v) paraffin in light petroleum (b.p. 40–60°C)], with acetone-water (17:3, v/v) as the mobile phase. In this system, betulaprenols-6, -7, -8 and -9 had R_F values of approx. 0.75, 0.62, 0.45 and 0.32 respectively. Those portions of the chromatogram (hereafter termed 'gaps') between the individual betulaprenol bands were transferred directly to scintillation vials. The individual betulaprenols-6 to -9 were each rechromatographed in the reversed-phase system. Paraffin and fluorescein were removed from each betulaprenol by alumina column chromatography as above, the betulaprenols being eluted by 20% E/P. Betulaprenols-10 to -13 (with carrier ficaprenols-9 to -13) were partially purified by repeated preparative t.l.c. (silica gel G) with chloroform (R_F 0.64). Contaminating fluorescein was removed by dissolving these polyprenols in light petroleum (b.p. 40–60°C).

Saponification of lipids. This was achieved by dissolving in benzene (final concn. 3 mg/ml), adding an equal volume of 15% (w/v) KOH in aq. 85% (v/v) ethanol and refluxing for 20 min. The solution was then cooled, water was added (3 vol.) and the mixture was extracted several times with ether. The pooled ether extracts were washed free of alkali, dried over anhydrous Na₂SO₄ and finally evaporated to dryness under N₂.

Determination of radioactivity. The ³H and ¹⁴C content of samples was assayed as described by Stone & Hemming (1967) with the Packard Tri-Carb liquid-scintillation spectrometer. The radioactivity of the samples was counted several times, and, when checked for quenching, showed that the amount of quenching was very small (<1% of the total counts) and about the same for each radioisotope. This amount of quenching had a negligible effect on the resulting 'corrected ratios'. The efficiencies of counting were 18.04% for ³H and 31.35% for ¹⁴C; less than 0.01% of the ³H counts appeared in the ¹⁴C channel, but 14.61% of the ¹⁴C counts was found in the ³H channel. In all calculations it has been assumed that 3S-mevalonic acid has no biological activity (Cornforth, Cornforth & Popják, 1962). The 'corrected ratio' is the ratio (a) assuming the ³H/¹⁴C ratio of the original mevalonic acid was 1:1 (the actual ³H/¹⁴C ratio for [2-¹⁴C,₄(4R)-4-³H₁]-mevalonic acid was 10.18:1, and that for [2-¹⁴C,₄(4S)-4-³H₁]-mevalonic acid 9.95:1, counted at the same time as the samples to allow for ³H decay), and (b) having multiplied the answer by the number of C-2 atoms from mevalonic acid expected to be incorporated into each molecule of the corresponding compound.

When samples were known not to contain ³H the instrument settings were altered to give a counting efficiency for ¹⁴C of 62%.

RESULTS

Incorporation of radioactive mevalonic acid. The corrected ³H/¹⁴C ratios for phytol and squalene from the woody tissue (Table 1) were as expected, being consistent with their being biogenetically all-*trans*. The percentage incorporation of the available [2-¹⁴C]mevalonate (3*R*-isomer) into the esterified

Table 1. *Total incorporation of radioactivity into phytol and squalene of woody tissue*

Abbreviation: MVA, mevalonic acid.				
	³ H (d.p.m.)	¹⁴ C (d.p.m.)	Corrected ³ H/ ¹⁴ C ratio	Incorporation of available [2- ¹⁴ C]MVA (%)
(a) From [2- ¹⁴ C,(4 <i>R</i>)-4- ³ H ₁]MVA				
Phytol	10526	986	4.19:4	0.0172
Squalene	4020	401	5.91:6	0.0072
(b) From [2- ¹⁴ C,(4 <i>S</i>)-4- ³ H ₁]MVA				
Phytol	58	522	0.04:4	0.0092
Squalene	39	233	0.10:6	0.0041

Table 2. *Total incorporation of radioactivity into betulaprenol esters of woody tissue.*

Abbreviation: MVA, mevalonic acid.				
	³ H (d.p.m.)	¹⁴ C (d.p.m.)	Corrected ³ H/ ¹⁴ C ratio	Incorporation of available [2- ¹⁴ C]MVA (%)
(a) From [2- ¹⁴ C,(4 <i>R</i>)-4- ³ H ₁]MVA				
Betulaprenol-6	14000	1544	5.35:6	0.0272
Gap 6/7	5516	737	—	—
Betulaprenol-7	23980	5170	3.19:7	0.0911
Gap 7/8	1285	258	—	—
Betulaprenol-8	7345	2092	2.76:8	0.0371
Gap 8/9	1082	193	—	—
Betulaprenol-9	175	51	3.03:9	0.0009
			Total	0.1563
(b) From [2- ¹⁴ C,(4 <i>S</i>)-4- ³ H ₁]MVA				
Betulaprenol-6	1262	1257	0.59:6	0.0222
Gap 6/7	451	277	—	—
Betulaprenol-7	19250	3233	4.15:7	0.0573
Gap 7/8	980	193	—	—
Betulaprenol-8	5483	826	5.29:8	0.0147
Gap 8/9	1087	180	—	—
Betulaprenol-9	41	6	5.78:9	0.0001
			Total	0.0943

betulaprenols-6 to -9 was in each case greater than that into the free betulaprenols-6 to -9 from the woody tissue (Tables 2 and 3 respectively). In each case, betulaprenol-7 contained most of this radioactivity.

Samples (freed from fluorescein by dissolving in petroleum) of mixtures of free betulaprenols-6 to -9, free betulaprenols-10 to -13 from the leaf tissue, and free betulaprenols-10 to -13 from the woody tissue [as partially purified by t.l.c. (silica gel G; chloroform)] were assayed for ³H and ¹⁴C (Table 4). These showed relatively little incorporation of radioactivity and were contaminated with all-*trans* material.

The incorporation of [¹⁴C]farnesyl pyrophosphate. The results obtained for the esterified betulaprenols-6 to -9 are shown in Table 5. In each case the percentage incorporation of ¹⁴C into each prenol was greater than that in the experiment with the radio-

active mevalonates. A sample of the mixture of free betulaprenols-6 to -9, as partially purified by t.l.c. (silica gel G; chloroform) was assayed for ¹⁴C content (Table 6) and since the amount of radioactivity was found to be small the mixture was not separated into its individual betulaprenol components. The incorporation of ¹⁴C into phytol and squalene is also shown in Table 6. The low activity of squalene is probably due to the rapid conversion of squalene into sterols. An examination of the 20% E/P fraction from the alumina column chromatography of the total lipid from woody tissue showed the presence of radioactive material (total, approx. 1000 d.p.m. ¹⁴C) with the same *R_F* value (0.27) as authentic β-sitosterol on t.l.c. (silica gel G; chloroform).

Incorporation of [2-¹⁴C]geranylgeraniol and [2-¹⁴C]geranylnerol. The incorporations of ¹⁴C (in the two experiments) into phytol and squalene are

Table 3. *Total incorporation of radioactivity into unesterified betulaprenols of woody tissue*

Abbreviation: MVA, mevalonic acid.

	³ H (d.p.m.)	¹⁴ C (d.p.m.)	Corrected ³ H/ ¹⁴ C ratio	Incorporation of available [^{2-¹⁴C}]MVA (%)
(a) From [^{2-¹⁴C} , (4 <i>R</i>)-4- ³ H ₁]MVA				
Betulaprenol-6	36	7	2.95:6	0.0001
Gap 6/7	759	92	—	—
Betulaprenol-7	757	202	2.58:7	0.0032
Gap 7/8	138	24	—	—
Betulaprenol-8	80	30	2.11:8	0.0005
Gap 8/9	203	39	—	—
Betulaprenol-9	61	25	2.13:9	0.0004
			Total	0.0042
(b) From [^{2-¹⁴C} , (4 <i>S</i>)-4- ³ H ₁]MVA				
Betulaprenol-6	128	27	2.86:6	0.0005
Gap 6/7	35	168	—	—
Betulaprenol-7	833	146	4.09:7	0.0026
Gap 7/8	10	41	—	—
Betulaprenol-8	735	115	5.14:8	0.0020
Gap 8/9	27	57	—	—
Betulaprenol-9	76	10	6.68:9	0.0002
			Total	0.0053

Table 4. *Total incorporation of radioactivity into partially purified free betulaprenols-6 to -9 and -10 to -13 of leaf tissue and free betulaprenols-10 to -13 of woody tissue*

Abbreviation: MVA, mevalonic acid

	From [^{2-¹⁴C} , (4 <i>R</i>)-4- ³ H ₁]MVA		From [^{2-¹⁴C} , (4 <i>S</i>)-4- ³ H ₁]MVA	
	³ H (d.p.m.)	¹⁴ C (d.p.m.)	³ H (d.p.m.)	¹⁴ C (d.p.m.)
Leaf betulaprenols-6 to -9	4176	200	343	120
Leaf betulaprenols-10 to -13	6771	320	61	100
Wood betulaprenols-10 to -13	12246	580	639	420

Table 5. *Total incorporation of [¹⁴C]farnesyl pyrophosphate into esterified betulaprenols-6 to -9 of woody tissue*

	¹⁴ C (d.p.m.)	Incorporation of [¹⁴ C]farnesyl pyrophosphate (%)
Betulaprenol-6	755	0.1759
Gap 6/7	42	—
Betulaprenol-7	1535	0.3570
Gap 7/8	53	—
Betulaprenol-8	275	0.0640
Gap 8/9	58	—
Betulaprenol-9	42	0.0098
	Total	0.6067

Table 6. *Total incorporation of [¹⁴C]farnesyl pyrophosphate into free betulaprenols-6 to -9, phytol and squalene of woody tissue*

	¹⁴ C (d.p.m.)	Incorporation of [¹⁴ C]farnesyl pyrophosphate (%)
Betulaprenols-6 to -9	600	0.1495
Phytol	100	0.0233
Squalene	7	0.0016

shown in Table 7. There was no detectable incorporation of ¹⁴C from either [^{2-¹⁴C}]geranylgeraniol or [^{2-¹⁴C}]geranylnerol into squalene. The indi-

vidual betulaprenols-6 to -9 were isolated but since the amount of radioactivity incorporated into phytol was small, it was anticipated that the radioactivity associated with the betulaprenols-6 to -9 would also be small and the individual prenols (initially free plus esterified) were assayed for ¹⁴C as a reconstituted mixture of betulaprenols-6, -7, -8

Table 7. Total incorporation of ^{14}C into squalene, phytol and betulaprenols of woody tissue

Since the amount of radioactivity incorporated was low, all samples were counted several times for periods of 30 min each and an average value was obtained. N.D., None detectable (<4 d.p.m.)

	From [2- ^{14}C]-geranylgeraniol		From [2- ^{14}C]-geranylnerol	
	^{14}C (d.p.m.)	Incor- poration (%)	^{14}C (d.p.m.)	Incor- poration (%)
Phytol	217	0.0593	N.D.	—
Squalene	N.D.	—	N.D.	—
Betulaprenols-6 to -9	N.D.	—	41	0.0315
Betulaprenols-10 to -13	45	0.0123	N.D.	—

and -9. The results are shown in Table 7. The radioactivity associated with betulaprenols-10 to -13 as partially purified by repeated preparative t.l.c. (silica gel G; chloroform) is also recorded in Table 7.

DISCUSSION

The corrected $^3\text{H}/^{14}\text{C}$ ratios for esterified betulaprenol-7 and free betulaprenol-7 (Tables 2 and 3) show this polyprenol to possess three biogenetically *trans*-isoprene units and the remaining isoprene units to be biogenetically *cis*. Hence, the two internal chemically *trans* units and the ω -unit are biogenetically *trans*. Since the sum of the corrected ratio from [2- ^{14}C , (4*R*)-4- $^3\text{H}_1$]mevalonic acid plus the corrected ratio from [2- ^{14}C , (4*S*)-4- $^3\text{H}_1$]mevalonic acid for betulaprenol-7 (both free and esterified) is approx. 7, the activity of isopentenyl pyrophosphate isomerase in the woody tissue of *B. verrucosa* must be low relative to the activity of prenyl-transferase. The corrected ratios for esterified betulaprenol-6 show that this compound was contaminated with all-*trans* material; however, the corrected ratios obtained for free betulaprenol-6 are in agreement with this polyprenol possessing three biogenetically *trans* units. The corrected ratios for esterified betulaprenol-8 and esterified betulaprenol-9 are in agreement with each of these polyprenols possessing three biogenetically *trans* units and the remaining units being biogenetically *cis*. For both the free and esterified betulaprenols, the radioactivity found in the 'gaps' between each betulaprenol-6 to -9 as purified by reversed-phase t.l.c. indicate the presence of all-*trans* material. It is probably contamination with some of this material that explains the discrepancies in the ratios of free betulaprenols-8 and -9 (Table 3).

The $^3\text{H}/^{14}\text{C}$ ratios for betulaprenols -6 to -9 from the woody tissue (free and esterified), showing that these polyprenols have three biogenetically *trans* units and that the remaining isoprene units are biogenetically *cis*, are consistent with the theory that each polyprenol is probably formed from all-*trans*-farnesyl pyrophosphate by *cis*-additions of isoprene units. However, these $^3\text{H}/^{14}\text{C}$ ratios give no information as to the positions of the two internal *trans* residues within the polyprenol molecules.

The incorporation of [^{14}C]farnesyl pyrophosphate into betulaprenols-6 to -9 indicates that these polyprenols are formed from all-*trans*-farnesyl pyrophosphate by *cis*-additions of isoprene residues, and that the two internal biogenetically *trans*-isoprene residues in each polyprenol molecule must be adjacent to the biogenetically *trans* ω -residue. If this is the biosynthetic pathway for the formation of betulaprenols-6 to -9, then geranylneryl pyrophosphate, but not geranylgeranyl pyrophosphate, should serve as a precursor for these *cis-trans*-polyprenols.

Since there are reports of the pyrophosphorylation of short-chain prenols by plant systems (Loomis & Battaile, 1966; van Aller & Nes, 1968), and in particular radioactive geranylgeraniol has been shown to be incorporated into phytol and into carotenoids in maize (Costes, 1966), the radioactive C_{20} alcohols geranylgeraniol and geranylnerol were incorporated into the woody tissue of *B. verrucosa* to determine whether this proposed scheme of biosynthesis of betulaprenols-6 to -9 is in fact correct. If betulaprenols-6 to -9 are biosynthesized from *trans-trans*-farnesyl pyrophosphate by *cis*-additions of isoprene units, then [^{14}C]geranylnerol, but not [^{14}C]geranylgeraniol, should be incorporated into these polyprenols.

The lack of incorporation of radioactivity from either of the C_{20} alcohols into squalene served as an internal control, showing that there was no detectable randomization of the ^{14}C label (Table 7). As expected [2- ^{14}C]geranylgeraniol was incorporated into phytol, but [2- ^{14}C]geranylnerol was not. This is consistent with previous experiments where phytol has been found to be biogenetically all-*trans* (Costes, 1966; Wellburn *et al.* 1966).

The incorporation of ^{14}C into both betulaprenols-6 to -9 and betulaprenols-10 to -13 was low. This was thought to be due, in all probability, to the use of the free 2- ^{14}C -labelled C_{20} alcohols rather than their pyrophosphate esters as the incubation materials in this experiment. These free C_{20} alcohols almost certainly have to be pyrophosphorylated before the addition of further isoprene residues. The percentage incorporation of ^{14}C into phytol was higher than that into betulaprenols-6 to -9. This is contrary to the results obtained with [2- ^{14}C]mevalonate and [^{14}C]farnesyl pyrophosphate. Even though the

values for the percentage incorporations of [2-¹⁴C]-geranylgeraniol into phytol and of [2-¹⁴C]geranylnerol into betulaprenols-6 to -9 were from different experiments, the relatively higher value obtained for phytol may indicate that the pyrophosphate ester of geranylgeraniol is not necessary for the formation of chlorophyll, and that the free alcohol itself can serve as an effective precursor. Further examination of the 20% E/P fractions from the alumina column chromatography of the total lipid revealed radioactivity corresponding to [2-¹⁴C]-geranylgeraniol (total, 4400 d.p.m.) and to [2-¹⁴C]-geranylnerol (total, 2630 d.p.m.) indicating that the rate of pyrophosphorylation of these C₂₀ alcohols in the woody tissue is low. No activity could be detected corresponding to free phytol.

The lack of detectable incorporation of [2-¹⁴C]-geranylgeraniol into the betulaprenols-6 to -9 and the incorporation of [2-¹⁴C]geranylnerol into these polyprenols, compared with the incorporation of [2-¹⁴C]geranylgeraniol into phytol and the lack of detectable incorporation of [2-¹⁴C]geranylnerol into phytol, is consistent with these betulaprenols-6 to -9 being formed from *trans-trans*-farnesyl pyrophosphate by the *cis*-additions of isoprene residues.

The lack of detectable incorporation of [2-¹⁴C]-geranylnerol and the incorporation of [2-¹⁴C]-geranylgeraniol into the betulaprenols-10 to -13 compared with the results for phytol could be interpreted as indicating that these polyprenols, which are known to contain three internal chemically *trans*-isoprene residues, are formed from all-*trans*-geranylgeranyl pyrophosphate by the *cis*-additions of isoprene residues. However, the betulaprenols-10 to -13, partially purified in exactly the same way, were shown to be contaminated by highly radioactive all-*trans* material (possibly all-*trans*-prenols formed extraplastidically) in the experiments with the radioactive mevalonates (Table 4). Hence, most of the radioactivity incorporated from [2-¹⁴C]geranylgeraniol, which appeared to be associated with betulaprenols-10 to -13, was probably associated with all-*trans* material.

From the results obtained for the incorporation of ¹⁴C into the woody-tissue betulaprenols-6 to -9 from the radioactive mevalonates, from [¹⁴C]-farnesyl pyrophosphate and from [2-¹⁴C]geranylnerol it seems that each of these polyprenols, whether free or esterified, possesses a biogenetically *trans* ω -residue, and two biogenetically *trans* in-

ternal residues, directly adjacent to the ω -residue, and the other isoprene residues are biogenetically *cis*. The results are in agreement with these betulaprenols-6 to -9 being formed from all-*trans*-farnesyl pyrophosphate by *cis*-additions of isoprene residues.

The authors are grateful to the Science Research Council for a Research Grant to F.W.H. and a Research Studentship to D.P.G.

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