The Stereochemistry of Hexahydroprenol, Ubiquinone and Ergosterol Biosynthesis in the Mycelium of *Aspergillus fumigatus* Fresenius

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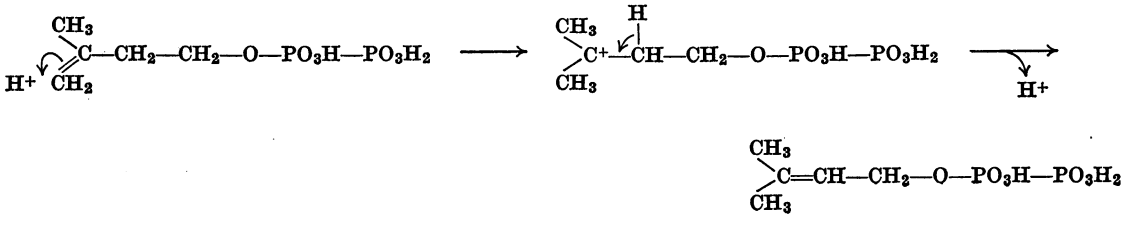
1. The mycelium of *Aspergillus fumigatus* has been shown to incorporate mevalonate into squalene, ubiquinone, ergosterol and hexahydroprenol. 2. The $^3H/^{14}C$ ratio in ubiquinone, biosynthesized from [2-^{14}C-(4*R*)-4-$^3H_1$]mevalonate, is the same as in the squalene; essentially no ^3H was incorporated from [2-^{14}C-(4*S*)-4-$^3H_1$]-mevalonate, indicating the biosynthesis of biogenetically *trans*-isoprene units. 3. The $^3H/^{14}C$ ratio for ergosterol (from '4*R*-mevalonate') was 3:5, showing that the proton at C-24 is not lost during alkylation of the side chain; it probably migrates to C-25. 4. As ^3H from both mevalonates was incorporated into the hexahydroprenols the biosynthesis of both *cis*- and *trans*-isoprene units must occur. 5. The saturated ω- and ψ-isoprene units are shown to be biogenetically *trans*, as are two of the unsaturated residues. 6. The saturated α- and unsaturated β-isoprene residues are both biogenetically *cis*. 7. An inexplicable loss of approximately half of the olefinic protons from the *cis*-portion of hexahydroprenol occurs; possible reasons for this loss are discussed. 8. Increase in chain length of the hexahydroprenols is by a *cis* addition. 9. A biosynthesis of hexahydroprenols by addition of *cis*-isoprene units to all-*trans*-geranylgeranyl pyrophosphate, or a dihydro or tetrahydro derivative thereof, is suggested.

The use of MVA* as a substrate in polyisoprenoid-synthetase systems is now a well-established procedure for studying the mechanisms of isoprenoid biosynthesis. Ever since Tavormina, Gibbs & Huff (1956) showed that cell-free extracts of liver were able to incorporate radioactivity labelled MVA into cholesterol the conversion of MVA into practically every known type of isoprenoid compound has been shown.

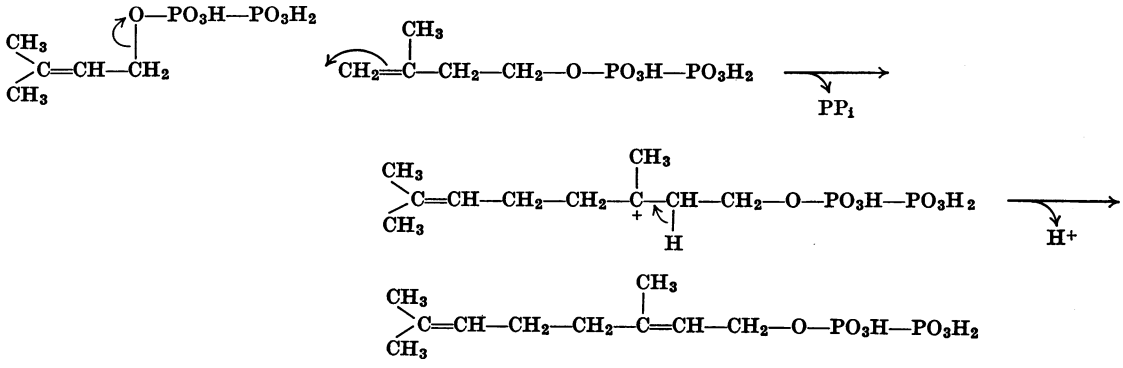
Elucidation of the role of MVA has led to the identification of all the major intermediates from acetate to the triterpenes and tetraterpenes (see e.g. Richards & Hendrickson, 1964). Important reactions after the MVA stage involve the isomerization of isopentenyl pyrophosphate to 3,3-dimethylallyl pyrophosphate (Scheme 1) and the condensation of isopentenyl (3-methylbut-3-enyl) pyrophosphate with either dimethylallyl pyrophosphate or its higher isoprenologues (Scheme 2). In each of these steps the formation of a carbon-to-carbon bond is accompanied by the elimination of a proton from C-2 of the isopentenyl pyrophosphate. The hydrogen atom lost is one of the two originally attached to C-4 of mevalonic acid.

Recently the study of biological terpene synthesis

* Abbreviation: MVA, mevalonic acid.



Scheme 1.



Scheme 2.

has entered a new phase with the development of MVA labelled stereospecifically with tritium (Cornforth, Cornforth, Donninger & Popják, 1966). Thus the stereochemistry of the proton elimination from C-2 of the isopentenyl pyrophosphate has been studied with [2-^{14}C-(4*R*)-4-^{3}H$_1$]MVA and [2-^{14}C-(4*S*)-4-^{3}H$_1$]MVA as substrates in polyisoprenoid-synthetase systems. The work reported by Cornforth *et al.* (1966) and Popják (1965) on the biosynthesis of *trans–trans*-farnesyl pyrophosphate and all-*trans*-squalene by a rat-liver preparation shows that all of the 4*S*-hydrogen is lost and all of the 4*R*-hydrogen is retained. The biosynthesis of squalene and phytoene by carrot-root preparations (Goodwin & Williams, 1966) and of squalene and phytol by the leaves of *Ficus elastica* and *Aesculus hippocastaneum* (Wellburn, Stone & Hemming, 1966) have also been shown to involve the loss of 4*S*-hydrogen and retention of 4*R*-hydrogen. By contrast, the biosynthesis of poly-*cis*-rubber in the latex of *Hevea brasiliensis* involves the removal of the 4*R*-hydrogen and the retention of 4*S*-hydrogen, showing the rubber to be biogenetically *cis* (Archer *et al.* 1966).

In a brief communication (Stone & Hemming, 1965) the incorporation of the stereospecifically labelled substrates into squalene, ubiquinone and ergosterol of *Aspergillus fumigatus* Fresenius was described. The mycelium of this mould also contains a complex of hexahydroprenols-18 to -24. Each of these alcohols contains a saturated 'hydroxy-terminal' isoprene residue, a saturated ω-terminal isoprene residue and a saturated ψ-isoprene residue (adjacent to the ω-residue). The remaining unsaturated residues have the *cis* configuration except for two which have the *trans* configuration. The precise position of the *trans*-isoprene residues in each molecule is not known (Stone, Butterworth & Hemming, 1967*b*).

The present paper describes the utilization of the stereospecifically labelled MVA to study the biosynthesis of *cis*-, *trans*- and saturated isoprene residues in each hexahydroprenol molecule.

MATERIALS AND METHODS

Materials. 3*R*-[2-^{14}C-(4*R*)-4-^{3}H$_1$],3*S*-[2-^{14}C-(4*S*)-4-^{3}H$_1$]-MVA (4*R*-MVA), containing 0·852 μc of ^{14}C and 4·961 μc of ^{3}H/μmole, and 3*R*-[2-^{14}C-(4*S*)-4-^{3}H$_1$],3*S*-[2-^{14}C-(4*R*)-4-^{3}H$_1$]-MVA (4*S*-MVA), containing 0·772 μc of ^{14}C and 2·805 μc of ^{3}H/μmole, together with the corresponding benzhydrylamide derivatives, were a gift from Professor G. J. Popják. 3*R*,*S*-[2-^{14}C]Mevalonic acid lactone was obtained from The Radiochemical Centre, Amersham, Bucks. Squalene biosynthesized from 4*R*-MVA by the leaves of *Ficus elastica* was isolated and purified as reported by Wellburn *et al.* (1966). Diethyl ether was dried over sodium wire and distilled over reduced iron. Light petroleum was dried over sodium wire and distilled (b.p. 40–60°). Acetone was dried over Hi-Drite (Hi-Drite Ltd., London, W. 1) and distilled (b.p. 56·5°). Chloroform was a general analytical reagent containing ethanol (1%, v/v) as stabilizer. Di-isopropyl ether was technical grade containing quinol (0·01%, v/v) to prevent peroxide formation. Liquid paraffin was obtained from A. Gallenkamp and Co., Widnes, Lancs. All other reagents unless otherwise specified were of A.R. purity.

Micro-organism and culture methods. *Aspergillus fumigatus* Fresenius (L.S.H.T.M. A.46; C.M.I. 89353) was maintained by subculture on slopes of potato–dextrose agar (Oxoid) every 6–8 weeks. Each slope provided sufficient spores, as a suspension in water, to inoculate ten Roux bottles each containing 200ml. of Raulin–Thom medium (Anslow & Raistrick, 1938). The bottles containing medium were sterilized, before inoculation, by autoclaving at 15lb./in.^{2} for 15min. On incubation at 30° the surface of the medium became covered with a pad of mycelium.

Incorporation of mevalonate. Expt. A. To discover the stage in the growth period most favourable to prenol biosynthesis 3*R*,*S*-[2-^{14}C]MVA was incorporated into groups of five cultures and a group of cultures was harvested every 48hr. throughout the growth period. 3*R*,*S*-[2-^{14}C]MVA (0·4 μc, 0·08 μmole) was injected, as a solution in phosphate buffer, pH7·2, through each mycelium 48hr. before harvesting.

Expt. B. This experiment differed from Expt. A only in that the 3*R*,*S*-[2-^{14}C]MVA, as a solution in phosphate buffer, pH 7·2, was added to each bottle of medium at the same time as inoculation with spore suspension.

Three experiments (C, D and E) were also carried out in which doubly labelled 4*R*-MVA and 4*S*-MVA were incorporated into the mould. Each sample of doubly labelled MVA was injected, through the mycelium, into the medium of five Roux bottles at the time shown to be most favourable to prenol biosynthesis. The two groups of five cultures were harvested separately on the ninth day of growth, by which time the yield of isoprenoid compounds is maximal (K. J. Stone & F. W. Hemming, unpublished work).

It was found necessary to repeat the incorporation more than once, as it proved technically difficult to re-isolate either the prenols or their acetate derivatives from scintillation fluid in a pure condition after ^{3}H and ^{14}C assay. Decomposition particularly during thin-layer chromatography has proved a serious problem generally when handling polyprenols, especially those related to the dolichols, i.e. those in which the 2,3-double bond is saturated (Stone *et al.* 1967*b*). Precautions were thus taken throughout each experiment to reduce decomposition to a minimum.

Expt. C. On the fourth day of growth a total of either 2·73 μmoles of 4*R*-MVA or 2·41 μmoles of 4*S*-MVA was injected into the growth medium of the five cultures. The complex of hexahydroprenols was rigorously purified before assay of radioactivity.

Expt. D. On the fifth day of growth a total of either 3·69 μmoles of 4*R*-MVA or 1·205 μmoles of 4*S*-MVA was injected into the growth medium of the five cultures. The complex of hexahydroprenols, after purification, was separated into its individual components, each of which was assayed for ^{3}H and ^{14}C content.

Expt. E. On the fifth day of growth a total of either 2·46 μmoles of 4*R*-MVA or 0·802 μmole of 4*S*-MVA was injected into the growth medium of the five cultures. After purification the complex of hexahydroprenols was degraded by ozonolysis. The saturated and unsaturated portions of the prenols were separated and assayed for ^{3}H and ^{14}C content.

In each experiment, ubiquinone, squalene and ergosterol were extensively purified before assay of radioactivity. Great care was taken to subject the material labelled with 4*R*-MVA and that labelled with 4*S*-MVA to exactly the same treatment. Similarly, all of the cultures in any one experiment were inoculated with spores from the same subculture of *Aspergillus*.

Extraction of the thallus. The medium from each group of five cultures was decanted from the mycelia, which were dried between filter papers. The 'damp-dry' weight thus obtained and the pH of the medium provided a reliable guide to the stage of growth of the fungus. In Expts. C, D and E, the mycelia from five cultures grown in the absence of any labelled substrate were added to those grown in the presence of labelled MVA to provide carrier amounts of isoprenoid material. The washed and dried mycelia were blended with methanol (2 vol.) containing pyrogallol (2·5%, w/v) by using an Ultra-Turrax homogenizer. Then 1 vol. of aq. 60% (w/v) KOH was added and the mixture was boiled under reflux for 45 min. This procedure enabled all the ubiquinone to be recovered without destruction (Packter, 1962). The hot saponification mixture was filtered through fluted filter paper and diluted with 3 vol. of cold water. The filtrate, when cool, was extracted four times with diethyl ether. The combined ethereal extracts were washed free of alkali, dried over anhydrous Na_2SO_4 and evaporated, and the residue was blown to dryness under N_2.

Two-dimensional chromatography. Portions of the resulting unsaponifiable lipid, from Expts. A and B, were dissolved in chloroform, and accurately measured samples (approx. 2·0 mg.) were subjected to two-dimensional thin-layer chromatography (Kieselgel G, 275 μ thick) with the system devised for tocopherols by Pennock, Hemming & Kerr (1964). This system gave good separation of all the isoprenoid compounds examined. Duplicate chromatograms of each sample were developed in the first dimension by chloroform, followed by 25% (v/v) di-isopropyl ether in light petroleum in the second dimension. Spraying one of the plates with 20% (w/v) phosphomolybdic acid in ethanol and heating made the lipids visible as blue spots (Fig. 1). This plate was subjected to X-ray radioautography (Kodak Kodirex X-ray film). The second plate was stained non-destructively with 0·01% (w/v) fluorescein in ethanol (Dunphy, Whittle & Pennock, 1965) and the spots corresponding in position to ubiquinone, ergosterol and the prenol complex were eluted with diethyl ether and evaporated to dryness under N_2. Each eluted fraction was assayed for ^{14}C content.

Isolation of squalene, ergosterol, ubiquinone and prenol (Expts. B, C, D and E). The unsaponifiable lipid (approx. 700 mg.) was dissolved in 2% (v/v) diethyl ether in light petroleum and chromatographed on a 50 g. column of alumina [Woelm; acid-washed and weakened to Brockmann grade III with 5% (w/w) of water] with increasing proportions of diethyl ether in successive 500 ml. portions of eluent. Thus squalene and other non-polar hydrocarbons were eluted with 2% (v/v) diethyl ether in light petroleum, ubiquinone and the prenol complex were eluted with 12% (v/v) diethyl ether in light petroleum and ergosterol was eluted with diethyl ether.

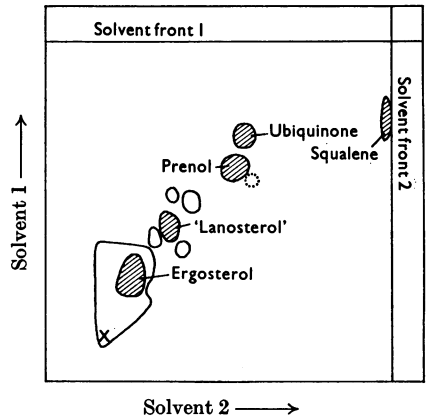


Fig. 1. Tracing of a typical two-dimensional thin-layer chromatogram of unsaponifiable lipid from *Aspergillus fumigatus* Fresenius. Solvent 1, chloroform; solvent 2, 25% (v/v) di-isopropyl ether in light petroleum. A shaded area indicates a compound significantly labelled with ^{14}C from [2-^{14}C]MVA.

The fraction containing prenols and ubiquinone was acetylated and the ubiquinone separated from prenyl acetates by alumina column chromatography (for details see Stone *et al.* 1967*b*). However, before acetylation of the fractions from Expt. E, 7·0mg. of unlabelled pure hexahydroprenol-22 was added as diluent to the prenol labelled from 4*R*-MVA. Similarly, 2·0mg. each of pure unlabelled hexahydroprenols-22 and -23 were added to the prenol labelled from 4*S*-MVA.

Rigorous purification of squalene. Squalene present in the column-chromatography fraction eluted with 2% (v/v) diethyl ether in light petroleum was further purified by preparative thin-layer chromatography (Kieselgel G, 275μ thick) with light petroleum as developing solvent. The position of the squalene band moving with a marker spot of authentic squalene (R_F 0·3) was indicated by spraying with 0·01% (w/v) fluorescein in ethanol (Dunphy *et al.* 1965). The squalene, which was eluted with diethyl ether, now chromatographed as a single stainable spot [with 20% (w/v) phosphomolybdic acid in ethanol] on the chromatography system above. A sample was assayed for ^3H and ^{14}C content. In Expt. C the remaining squalene (0·85mg.) was further purified by the preparation of a squalene–thiourea adduct, squalene hexahydrochloride and squalane.

(1) Squalene–thiourea adduct preparation. The squalene–thiourea adduct was prepared essentially as described by Dicker & Whiting (1958). Thus 0·28mg. of the labelled squalene was diluted with 3·0mg. of unlabelled squalene and dissolved in 0·04ml. of benzene. The resulting solution was transferred, with a capillary tube, to 0·24ml. of a saturated solution of thiourea in methanol. Crystallization of the clathrate began immediately at room temperature and was completed overnight at −15°. The white needle-shaped crystals were centrifuged and the supernatant was removed by pipette. Addition of water (1·0ml.) decomposed the clathrate and the mixture was extracted with light petroleum. After washing to remove traces of thiourea and drying with anhydrous Na_2SO_4 the petroleum solution was evaporated to dryness and assayed for ^3H and ^{14}C content.

(2) Squalene hexahydrochloride preparation. The hexahydrochloride derivative of squalene was prepared by a modification of the procedure described by Heilbron, Kamm & Owens (1926). The labelled squalene (0·28mg.) was diluted with 42·0mg. of unlabelled squalene in a 10·0ml. conical centrifuge tube. Dry HCl gas was bubbled through dry acetone for 2hr. with the solution maintained at −5°. Approx. 8·0ml. of the saturated acetone solution was added to the squalene and bubbling of the HCl continued at −5° for a further 2hr. The solution was left overnight at −15°. Then N_2 was bubbled through the solution for 30min. to remove excess of HCl and the white crystals were centrifuged and washed twice with dry diethyl ether at −5° to remove coloured impurity. The acetone supernatant solution yielded a further crop of crystals (approx. 7% of the total) after bubbling dry HCl through the solution for a further 2hr. at −5°. These were washed with dry diethyl ether as before. The combined crystals were recrystallized from acetone three times before being assayed for ^3H and ^{14}C content.

(3) Squalane preparation. Squalane was prepared by hydrogenation of 0·28mg. of the labelled squalene [diluted with 3·1mg. of unlabelled squalene and dissolved in 10·0ml. of ethanol–cyclohexane (1:1, v/v)] at room temperature and pressure in a Towers micro-hydrogenation apparatus with platinum oxide as catalyst. Hydrogenation was quantitative and the squalane thus prepared was purified by preparative thin-layer chromatography (Kieselgel G, 275μ thick) with light petroleum as developing solvent (R_F 0·69). The eluted squalane was assayed for ^3H and ^{14}C as above.

Rigorous purification of ergosterol. (1) Recrystallization. Ergosterol present in the column-chromatography fraction eluted with diethyl ether was purified, to constant specific activity, by four crystallizations from ethanol containing a little water. After the second crystallization the $E^{1\%}_{1cm.}$ at 282mμ was close to the theoretical value and remained constant with further recrystallizations.

(2) Preparative thin-layer chromatography. A second sample of ergosterol from the column-chromatography fraction eluted with diethyl ether was subjected to chromatography on thin layers of Kieselgel G (275μ thick) with ethyl acetate–benzene (9:41, v/v) as developing solvent. In this system ergosterol moved with R_F 0·25 and was located on the chromatogram with ultraviolet light.

Rigorous purification of ubiquinone. The ubiquinone (5·5mg.) separated from prenyl acetates by column chromatography was further purified by preparative thin-layer chromatography and reversed-phase paper chromatography. Perhydroubiquinone was also prepared.

(1) Thin-layer chromatography. A 3·0mg. sample of the labelled ubiquinone was purified by preparative thin-layer chromatography (Kieselgel G, 275μ thick) with methanol–benzene (1:60, v/v) as developing solvent. The ubiquinone, which was visible as a yellow band at R_F 0·52, was eluted with diethyl ether. One-third of this was assayed for ^3H and ^{14}C content after being reduced (decolorized) in ethanolic solution to ubiquinol with a few grains of $NaBH_4$.

The remaining two-thirds of the purified ubiquinone was hydrogenated, as above, and further purified on a continuous-adsorption thin-layer-chromatographic system (Bennett & Heftmann, 1963) with Kieselgel G (275μ thick) and benzene as developing solvent. After 2hr. the perhydroubiquinone had moved approximately half-way up the plate and was eluted with diethyl ether and assayed for radioactivity (after reduction with $NaBH_4$).

(2) Reversed-phase paper chromatography. A 2·5mg. sample of the labelled ubiquinone was run as a line on Whatman no. 3MM paper treated with 5% (v/v) liquid paraffin in light petroleum and allowed to dry. *NN*-Dimethylformamide–water (19:1, v/v) saturated with paraffin was used as the mobile phase and in this system ubiquinone moved to R_F 0·3 after 4hr. Ubiquinone was located on the chromatogram with ultraviolet light and eluted with diethyl ether. Paraffin was removed from this eluted fraction by chromatography on a 5g. column of alumina (Woelm; acid-washed, Brockmann grade III). Paraffin was eluted with 2% (v/v) diethyl ether in light petroleum and discarded, and ubiquinone was eluted with diethyl ether, reduced with $NaBH_4$ and assayed for ^3H and ^{14}C.

Purification of the prenol complex. The complex of prenyl acetates separated by column chromatography from ubiquinone was further purified by preparative thin-layer chromatography (Kieselgel G, 500μ thick) with light petroleum–benzene (1:1, v/v) as developing solvent. The position of the prenyl acetate band moving with the same R_F (0·52) as a marker spot of authentic prenyl acetate was indicated by spraying with 0·01% (w/v) fluorescein in ethanol (Dunphy *et al.* 1965) and making visible under ultraviolet light. Infrared spectroscopy of the eluted

acetate, examined as an oily smear between NaCl disks in a Perkin–Elmer model 137 Infracord spectrometer, indicated that this preparation, which now chromatographed as a single stainable spot on adsorption thin-layer chromatography, was pure. Support for this is shown by that isolated in Expt. C, which had unchanged $^3H/^{14}C$ ratios after subjection to further purification techniques.

Methods used to check the purity of the prenol complex (Expt. C). (1) Reversed-phase paper chromatography. A 0·8mg. sample of the labelled prenyl acetate complex was diluted with 3·5mg. of unlabelled acetate and chromatographed as a line on Whatman no. 3MM paper impregnated with 5% (v/v) liquid paraffin in light petroleum. Dry acetone saturated with paraffin was used as the ascending mobile phase. By exposing marker spots of authentic prenyl acetate to iodine vapour the position of the labelled prenyl acetate on the chromatogram was detected. Paraffin was removed from the eluted prenyl acetate fraction on a 5g. column of alumina (Woelm; acid-washed, Brockmann grade III) with light petroleum. The prenyl acetate, eluted from the column with 5% (v/v) diethyl ether in light petroleum, was assayed for ^3H and ^{14}C.

(2) Perhydroprenyl acetate preparation. A 2·0mg. sample of the prenyl acetate complex was diluted with 1·5mg. of unlabelled prenyl acetate and hydrogenated as in the preparation of squalane from squalene. The perhydroprenyl acetates were purified by preparative thin-layer chromatography (Kieselgel G, 275μ thick) with light petroleum–benzene (2:3, v/v) as developing solvent. Pure perhydroprenyl acetate (R_F 0·49) was eluted and assayed for ^3H and ^{14}C.

(3) Hydrolysis to free prenol. The remaining 2·7mg. of prenyl acetate complex was diluted with 5·0mg. of unlabelled prenyl acetate and hydrolysed (for details see Stone *et al.* 1967*b*). After preparative thin-layer chromatography (Kieselgel G, 275μ thick) with methanol–benzene (1:99, v/v) as developing solvent, pure prenol complex was eluted from the chromatogram (R_F 0·4) and assayed for radioactivity.

Isolation of individual hexahydroprenols (Expts. B and D). Each prenyl acetate fraction was hydrolysed as above and the resulting prenol fractions were subjected to reversed-phase partition chromatography on thin-layers of kieselguhr (275μ thick) impregnated with 3·5% (v/v) liquid paraffin in light petroleum. Dry acetone, saturated with paraffin, was used as mobile phase (Dunphy, Kerr, Pennock, Whittle & Feeney, 1967) to separate the complex (1·0mg./thin-layer plate) into its seven individual components (Stone *et al.* 1967*b*). Each individual hexahydroprenol that was located on the chromatogram by staining with 0·01% (w/v) fluorescein in ethanol and made visible under ultraviolet light (Whittle, Dunphy & Pennock, 1966) was eluted and separated from contaminating paraffin by chromatography on a column of alumina (see Wellburn, Stevenson, Hemming & Morton, 1967). Chromatography of a sample of each resulting prenol fraction on the above reversed-phase system showed that, although each chromatographed almost entirely as a single component, traces (less than 5% of the total fraction) of prenols one isoprene unit larger and one isoprene unit smaller than the main one were also present. However, because of the low weight of each prenol and the risk of losses during further handling, reversed-phase chromatography was not repeated. The hexahydroprenols were assayed for ^3H and ^{14}C content. The presence of traces of other prenol components almost certainly had a negligible effect on the $^3H/^{14}C$ ratio of the main component of each chromatographic fraction.

Ozonolytic degradation (Expt. E). Ozonolytic degradation of the prenyl acetate from Expt. E was carried out in exactly the same manner as described by Stone *et al.* (1967*b*). Thus 11·9mg. of hexahydroprenyl acetate labelled from 4*R*-MVA was ozonized and reduced with $LiAlH_4$, and the resulting alcohols were acetylated to yield 20·7mg. of a light-brown oil. Similarly 7·8mg. of hexahydroprenyl acetate labelled from 4*S*-MVA yielded 14·6mg. of a mixture of acetates.

Before subjecting the ^3H- and ^{14}C-labelled prenols to ozonolysis a sample of squalene biosynthesized from 4*R*-MVA, by the leaves of *Ficus elastica* (Wellburn *et al.* 1966), was used to check that ozonolytic degradation does not result in a partial or complete loss of hydrogen from C-1 of a degradation product isolated. During ozonolysis of squalene the same solvents were used as were employed later during the degradations of the hexahydroprenol. Similarly the reaction conditions were identical.

Gas–liquid chromatography of the products of ozonolytic degradation (Expt. E). The system used for gas–liquid chromatography consisted of silane-treated Celite (Gas Chrom CLH, 100–120 mesh) that had been coated with ethylene glycol succinate polyester (15%, w/w) and packed in a 9ft.-long stainless-steel tube of internal diam. $\frac{3}{32}$in. An Aerograph Autoprep 700 Gas Chromatograph (Wilkens Instrument and Research Inc.) fitted with a flame ionization detector was used isothermally at a temperature of 150°. The carrier gas was N_2 at a flow rate of 90ml./min. The splitting ratio was 4·7:1 in analytical work and 23:1 when the instrument was used preparatively.

Before adding carrier amounts of synthetic material 1% of each of the mixtures of biogenetic acetates was chromatographed analytically on the gas–liquid chromatographic system; the chromatograms produced were essentially as described by Stone *et al.* (1967*b*).

The mixture of acetates labelled from 4*R*-MVA was diluted with 1·7mg. of 6,10-dimethyl-*n*-undecan-2-yl acetate and 30mg. of 3-methyl-*n*-hexane-1,6-diyl diacetate. Similarly the mixture of acetates labelled from 4*S*-MVA was diluted with 3·5mg. of 6,10-dimethyl-*n*-undecan-2-yl acetate, 6·3mg. of *n*-pentane-1,4-diyl diacetate and 30mg. of 3-methyl-*n*-hexane-1,6-diyl diacetate. These synthetic acetates were prepared as described by Stone *et al.* (1967*b*). Each mixture of acetates was made up to 0·1ml. with benzene and successive 10·0μl. amounts were manually injected directly on to the column.

The individual acetates that emerged from the end of the column were adsorbed on to glass wool loosely packed in the upper compartment of 10·0ml. collection bottles partly immersed in ice-cold water. The acetates were recovered by rinsing the glass wool (four times) with diethyl ether and centrifuging the solvent to the lower compartment, where it was removed by pipette. All samples collected from the column were assayed for ^3H and ^{14}C content.

Ergosterol estimation. Ergosterol was assayed from its absorption spectrum in ethanol by a modification of the Morton & Stubbs correction procedure (Mercer, 1960). Estimations were carried out with a Unicam SP.800 spectrophotometer.

Radioactive assay. All samples were assayed for ^3H and ^{14}C content in a Packard Tri-Carb liquid-scintillation

spectrometer with toluene containing 2,5-diphenyloxazole (0·5%, w/v) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene (0·03%, w/v) as the scintillation fluid. Counting was by the screening method and was checked by the discriminator-ratio method (Stein & Stein, 1962; Okita, Kabara, Richardson & LeRoy, 1957). Samples were checked for quenching by the addition of separate standard amounts of ^{14}C- and ^{3}H-labelled toluene as internal standards (Okita *et al.* 1957).

RESULTS AND DISCUSSION

Radioactive assay. The results indicated in Tables 1–4 and 6–8 are those obtained by the screening method. Counting by means of the discriminator-ratio method produced results essentially the same.

Samples were counted several times and in no case was the deviation more than 1·3%. Samples that were checked for quenching showed that the amount of quenching of each isotope was identical and never constituted more than 1·2% of the total counts. This amount of quenching had a negligible effect on the resulting 'corrected ratios'.

Efficiencies of counting the radioactive isotopes (by the screening method) were 25·5% for ^{3}H and 46·7% for ^{14}C; less than 0·01% of the ^{3}H counts appeared in the ^{14}C channel but 11·3% of the ^{14}C counts was found in the ^{3}H channel. The disintegrations/min. in each sample are recorded in Tables 1–4 and 6–8. In all calculations it has been assumed that 3*S*-MVA does not react enzymically (Cornforth, Cornforth & Popják, 1962). Each $^{3}H/^{14}C$ ratio was divided by the $^{3}H/^{14}C$ ratio of the original MVA (counted as the benzhydrylamide derivative) to give those ratios that would have been obtained had the $^{3}H/^{14}C$ ratio in the original MVA been 1:1. Before insertion into Tables 1–4 and 6–8 these ratios were multiplied by the number of original C-2 atoms of MVA that are incorporated into each molecule to give the 'corrected ratio'.

The actual $^{3}H/^{14}C$ ratio of 4*R*-MVA was 5·83:1 and the actual $^{3}H/^{14}C$ ratio of 4*S*-MVA was 3·63:1. The specimens of stereospecifically labelled MVA were from the same batch as described and used by Cornforth *et al.* (1966). The $^{3}H/^{14}C$ ratios given here are those determined in our Laboratory and differ significantly from those given by Cornforth *et al.* (1966). The difference is most likely due to a difference in the ^{3}H standards used in the two Laboratories. This circumstance has no effect on the results.

Gas–liquid chromatography. Gas–liquid chromatography was conducted so that each peak shown in Fig. 2 was collected separately. In no case did the amount of ^{14}C in any one of peaks 1, 2, 4 and 6 exceed 3% of the ^{14}C put on the column and the total ^{14}C of these fractions was less than 15% of the ^{14}C put on the column. The most likely explanation of the low total recovery of ^{14}C (about 55% of that put on the column) was inefficient trapping of the fractions in collecting vessels. Recoveries of this

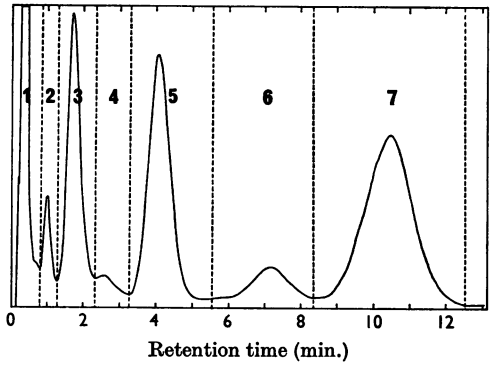
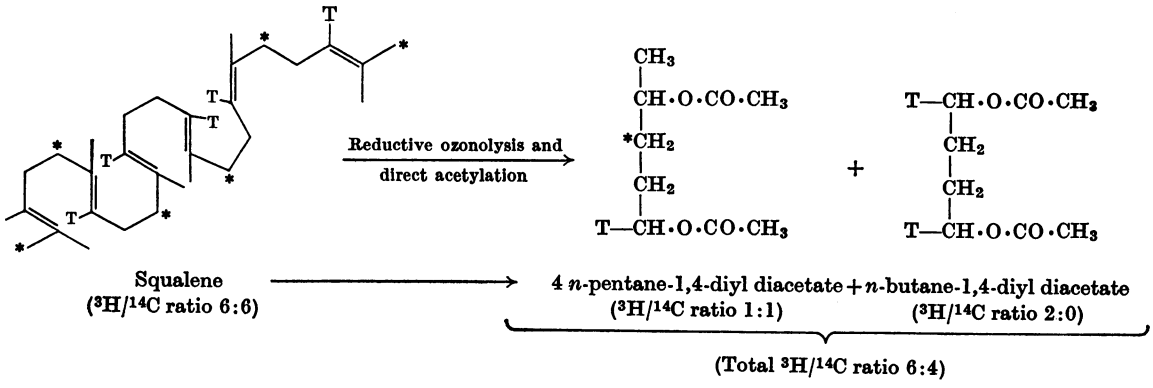


Fig. 2. Gas–liquid chromatogram of the ozonolytic degradation products of *Aspergillus* hexahydroprenol after supplementation with synthetic acetates (see the text).



Scheme 3. * Indicates ^{14}C; T indicates ^{3}H. The isopropyl acetate that was also produced was not recovered.

Table 1. *Radioactive content of the ozonolytic degradation products of squalene biosynthesized from 4R-mevalonate*

The values given for ^{3}H and ^{14}C are the actual disintegrations/min. recorded. The corrected and theoretical $^{3}H/^{14}C$ ratios are given assuming that the $^{3}H/^{14}C$ ratio in the original MVA was 1:1.

	^{3}H (disintegrations/min.)	^{14}C (disintegrations/min.)	Corrected $^{3}H/^{14}C$ ratio	Theoretical $^{3}H/^{14}C$ ratio
Squalene	4587	790	5·97:6	6:6
n-Pentane+*n*-butane derivatives	1472	165	6·12:4	6:4

Table 2. *Effect of hydrogenation of isoprenoid residues labelled with* ^{3}H *from* C-4 *of mevalonate*

The values given for ^{3}H and ^{14}C are the actual disintegrations/min. recorded. The corrected $^{3}H/^{14}C$ ratios are given assuming that the $^{3}H/^{14}C$ ratio in the original MVA was 1:1.

	^{3}H (disintegrations/min.)	^{14}C (disintegrations/min.)	Corrected $^{3}H/^{14}C$ ratio
Squalene	26100	4525	5·94:6
Squalane	9645	2022	4·90:6
Ubiquinone	1932	332	9·98:10
Perhydroubiquinone	1361	264	8·85:10
Prenyl acetate	3774	3691	3·68:21
Perhydroprenyl acetate	2218	5042	1·61:21

order were experienced with trial runs on the ozonolytic degradation products of squalene biosynthesized from [2-^{14}C]MVA.

Ozonolytic degradation. The pentane-1,4-diyl diacetate and butane-1,4-diyl diacetate produced by ozonolytic degradation of squalene (Scheme 3) biosynthesized from 4*R*-MVA had a combined $^{3}H/^{14}C$ ratio in good agreement with the theoretical value (Table 1). No loss of ^{3}H was thus shown to occur as a result of chemical degradation.

Hydrogenation. Chemical hydrogenation of isoprenoid material labelled with ^{3}H from C-4 of MVA resulted in a 10–60% loss of tritium (Table 2). Hydrogenation as a means of purification was therefore unreliable. This effect differs from enzymic hydrogenation of isoprenoid residues, which results in no loss of the original olefinic proton (Wellburn *et al.* 1966).

Biosynthesis of ubiquinone and squalene. Ubiquinone from *Aspergillus fumigatus* contains ten isoprene units in its side chain (Burgos, Butterworth, Hemming & Morton, 1964; Lavate & Bentley, 1964). In ubiquinone-10 from other natural sources, the stereochemistry of the isoprene unit next to the quinone nucleus is uncertain; the terminal unit is *cis–trans* and the remaining eight are known to be chemically *trans* (Bates & Gale, 1960; Bates, Carnighan, Rakutis & Schauble, 1962). The $^{3}H/^{14}C$ ratios (Table 3) for ubiquinone indicate that all ten isoprene units are biogenetically *trans*. The ratios rule out the possibility that the isoprene units are incorporated in the *cis* configuration followed by isomerization to the *trans* configuration.

Samples from the same batch of 4*S*-MVA have been shown to contain a small amount of impurity labelled with ^{3}H but not with ^{14}C (Archer *et al.* 1966). This impurity, easily separated from squalene but not from rubber, may be the cause of the small amount of ^{3}H apparently incorporated into the ubiquinone from 4*S*-MVA (Table 3). The nature of this impurity is unknown.

Squalene derived from 4*R*-MVA gave $^{3}H/^{14}C$ ratios very close to 6:6, showing that each isoprene unit contained a ^{3}H atom that was originally in the 4*R*-configuration in the mevalonate. Essentially no ^{3}H was incorporated from 4*S*-MVA. Thus, as expected from work with other organisms (Cornforth *et al.* 1966; Popják, 1965; Goodwin & Williams, 1966; Rees, Mercer & Goodwin, 1966), all of the isoprene units in squalene are biogenetically *trans*.

Biosynthesis of ergosterol. Squalene has been shown to be a precursor of ergosterol in yeast (Corwin, Schroeder & McCullough, 1956; Alexander, Gold & Schwenk, 1957, 1958) and thus, as expected from the ratios obtained for squalene (Table 3), a negligible amount of ^{3}H from 4*S*-MVA was incorporated into ergosterol (Table 4). However, the $^{3}H/^{14}C$ ratio for ergosterol derived from 4*R*-MVA has an important bearing on the mode of introduction of the extra methyl group at C-24 of this sterol. Cholesterol formed from 4*R*-MVA by liver preparations retains ^{3}H in positions 17, 20 and 24, and ^{14}C in positions 1, 7, 15, 22 and 26 (or 27), giving a corrected $^{3}H/^{14}C$ ratio 3:5 (Popják, 1965). If one assumes that, in principle, the cyclization of squalene

Table 3. *Incorporation of* 4R-*mevalonate and* 4S-*mevalonate into squalene and ubiquinone*

The values given for ^3H and ^{14}C are the actual disintegrations/min. recorded. The corrected $^3H/^{14}C$ ratios are given assuming that the $^3H/^{14}C$ ratio in the original MVA was 1:1.

	From 4*R*-MVA			From 4*S*-MVA		
	^3H (disintegrations/min.)	^{14}C (disintegrations/min.)	Corrected $^3H/^{14}C$ ratio	^3H (disintegrations/min.)	^{14}C (disintegrations/min.)	Corrected $^3H/^{14}C$ ratio
Expt. C						
Squalene after thin-layer chromatography	26100	4525	5·94:6	298	6510	0·08:6
Squalene–thiourea clathrate	12750	2290	5·73:6			
Squalene hexahydrochloride	3523	632	5·74:6			
Ubiquinone after thin-layer chromatography	1932	332	9·98:10	162	997	0·45:10
Ubiquinone after paper chromatography	5850	963	10·43:10	157	1008	0·43:10
Expt. D						
Squalene after thin-layer chromatography	17100	2975	5·91:6	40	956	0·07:6
Ubiquinone after thin-layer chromatography	4391	772	9·76:10	18	159	0·32:10
Expt. E						
Squalene after thin-layer chromatography	12250	2116	5·96:6	39	709	0·09:6
Ubiquinone after thin-layer chromatography	5442	941	9·93:10	38	312	0·34:10

Table 4. *Incorporation of* 4R-*mevalonate and* 4S-*mevalonate into ergosterol*

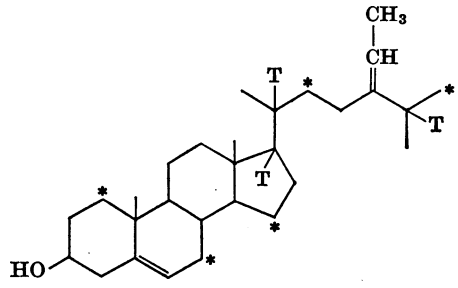
The values given for ^3H and ^{14}C are the actual disintegrations/min. recorded. The corrected $^3H/^{14}C$ ratios are given assuming that the $^3H/^{14}C$ ratio in the original MVA was 1:1.

	From 4*R*-MVA			From 4*S*-MVA		
	^3H (disintegrations/min.)	^{14}C (disintegrations/min.)	Corrected $^3H/^{14}C$ ratio	^3H (disintegrations/min.)	^{14}C (disintegrations/min.)	Corrected $^3H/^{14}C$ ratio
Expt. C						
Crystalline	6980	2003	2·99:5			
After thin-layer chromatography	11500	3265	3·02:5	408	5156	0·11:5
Expt. D						
Crystalline	13510	3787	3·06:5	232	4578	0·07:5
Expt. E						
Crystalline	9510	2632	3·10:5	102	1768	0·08:5

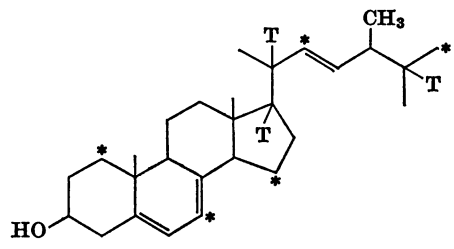
to phytosterols (see e.g. Battersby & Parry, 1964) is essentially the same as in the formation of cholesterol in animal tissues, it follows that the 3:5 ratio obtained for ergosterol (Table 4) indicates the presence of ^3H in the side chain.

In a previous communication (Stone & Hemming, 1965) it was assumed that the ^3H in the side chain remained on C-24 during alkylation. However, evidence for the migration of tritium from C-24 to C-25 during alkylation has been provided by the biosynthesis of fucosterol from 4*R*-MVA by *Fucus spiralis* (Goad & Goodwin, 1965). The corrected $^3H/^{14}C$ ratio of the fucosterol (I) was the same as that obtained from ergosterol (II). As no tritium can be present in fucosterol at C-24, a migration to C-25, or less likely to C-23, must have occurred. The methyl group at C-24 of ergosterol is therefore considered to be introduced by the mechanism shown in Scheme 4 (Goad, Hamman, Dennis & Goodwin, 1966).

Biosynthesis of the hexahydroprenols (III). The rate of biosynthesis of the hexahydroprenols (and ubiquinone) increases rapidly after the fifth day in the growth period and reaches a maximum at the

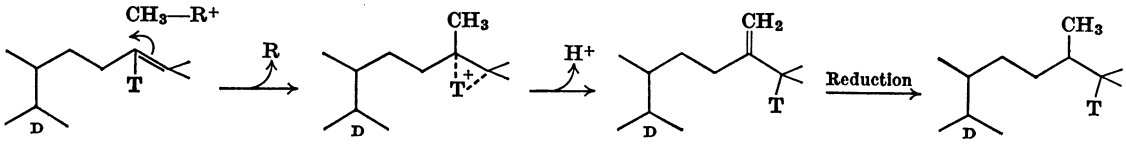


(I)



(II)

* indicates ^{14}C; T indicates ^{3}H



Scheme 4. T indicates ^{3}H.

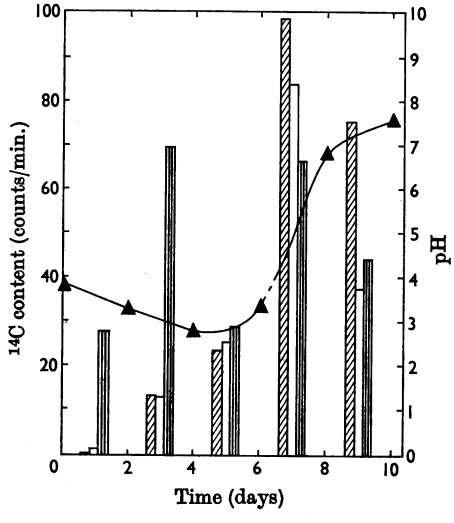


Fig. 3. Variation of incorporation of [2-^{14}C]MVA into isoprenoid compounds with time; the label was added to the medium 48hr. before each harvesting (Expt. A). □, $10^{-2} \times {}^{14}C$ in ubiquinone; ▨, $10^{-2} \times {}^{14}C$ in hexahydroprenol; ▥, $2 \times 10^{-4} \times {}^{14}C$ in ergosterol. ▲—▲, pH of medium.

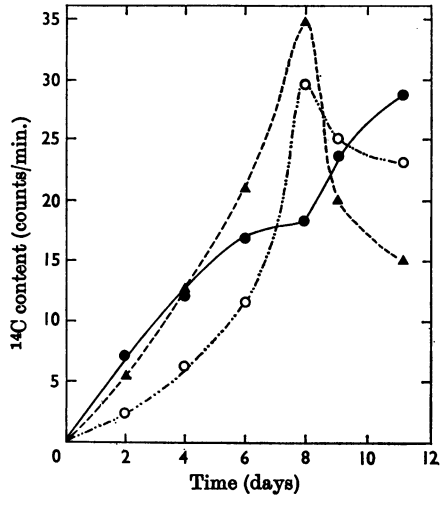
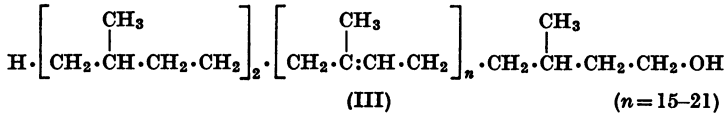


Fig. 4. Variation of incorporation of [2-^{14}C]MVA into isoprenoid compounds with time; the label was added to the medium at 0 days (Expt. B). ▲, $10^{-3} \times {}^{14}C$ in ubiquinone; ○, $10^{-3} \times {}^{14}C$ in hexahydroprenol; ●, $5 \times 10^{-5} \times {}^{14}C$ in ergosterol.

seventh day (Figs. 3 and 4). This increase in biosynthetic rate is reflected in an increase of concentration of prenol (and ubiquinone) in the organism during the same period (K. J. Stone & F. W. Hemming, unpublished work). It is noteworthy that at this same stage in the growth period there appears to be a temporary decrease in the rate of sterol biosynthesis (Figs. 3 and 4). Thus after the fifth day a change in the regulation of isoprenoid metabolism is apparent and this has been of practical use in allowing a greater incorporation of



(III) (n = 15–21)

Table 5. *Variation of incorporation of ^{14}C into each prenol component, expressed as a percentage of the incorporation into the prenol complex, with time (Expt. B)*

	Incorporation of ^{14}C (% of incorporation into complex)					
Time after inoculation ...	... 2 days	4 days	6 days	8 days	9 days	11 days
Prenol component						
Hexahydroprenol-18	7·8	5·5	6·2	7·3	6·3	6·6
Hexahydroprenol-19	10·8	10·1	10·7	12·7	9·9	10·5
Hexahydroprenol-20	18·2	18·3	18·1	20·3	21·3	21·4
Hexahydroprenol-21	29·4	29·9	30·9	29·2	31·1	29·9
Hexahydroprenol-22	22·6	24·5	24·7	20·8	22·5	22·2
Hexahydroprenol-23	7·5	8·8	7·4	6·8	7·1	7·3
Hexahydroprenol-24	3·8	2·9	2·0	2·9	1·7	2·2
Total radioactivity per Roux bottle (counts/min.)	490	1280	2320	5590	4970	4590

the doubly labelled MVA into the hexahydroprenols.

The proportion of the ^{14}C present in the polyprenol complex (Expt. B) recovered in the individual polyprenols remained constant throughout the growth period (see Table 5). Thus over the time-interval of 2–11 days there was no evidence of the shorter polyprenols, or closely related compounds, acting as precursors of the longer polyprenols.

The hexahydroprenols present in this organism have been shown to contain both *cis*- and *trans*-isoprene residues (Stone *et al.* 1967*b*). When either 4*R*-MVA or 4*S*-MVA is incorporated into the prenols, a considerable amount of ^{3}H is incorporated. This is unequivocal evidence for direct synthesis of *cis*- and *trans*-isoprene units.

The percentage composition of the prenol complex indicates that the mean chain length of the mixture can be taken as C_{105} (Stone *et al.* 1967*b*). Further, the percentage distribution of ^{14}C incorporated from [2-^{14}C]MVA among the individual prenols (Table 5) shows that it is valid to use this value in interpreting $^{3}H/^{14}C$ ratios for the prenol complex. The $^{3}H/^{14}C$ ratio for the prenol complex biosynthesized from 4*R*-MVA indicates a direct synthesis of four *trans*-isoprene residues for every 21 isoprene residues present (Table 6). Each hexahydroprenol is known, from nuclear-magnetic-resonance evidence, to contain only two isoprene units in the *trans* configuration (Stone *et al.* 1967*b*; J. Feeney & F. W. Hemming, unpublished work). It therefore follows that two of the three saturated isoprene residues known to be present (Stone *et al.* 1967*b*) must be biogenetically *trans*.

For a hexahydroprenol of chain length C_{105}, it is known that 16 of the 21 isoprene units are in the *cis* configuration (Stone *et al.* 1967*b*). Assuming that the biosynthesis of *cis*-isoprene units in *Aspergillus* is identical with that in the biosynthesis of rubber in the latex of *Hevea brasiliensis*, a $^{3}H/^{14}C$ ratio at least 16:21 was expected for prenol complex biosynthesized from 4*S*-MVA. In fact $^{3}H/^{14}C$ ratios between 10·8:21 and 12·5:21 were obtained (Table 6), indicating a very significant but unaccountable loss of some 28% of the ^{3}H. The prenol isolated in Expt. C appears to contain 12 isoprene units directly synthesized in the *cis* configuration, whereas that from Expts. D and E appears to contain only 11. The reason for the discrepancy (between Expts. C and D or E) is not understood; whether or not it is significant is uncertain.

The individual members of the hexahydroprenol series have previously been shown to differ in chain length by the number of isoprene units in the *cis* configuration (Stone *et al.* 1967*b*). This is in accord with the $^{3}H/^{14}C$ ratios shown in Table 7. Incorporation of 4*R*-MVA into each hexahydroprenol gave consistent $^{3}H/^{14}C$ ratios, indicating the biosynthesis of four *trans*-isoprene residues per prenol molecule. The incorporation of 4*S*-MVA shows that the increase in chain length of the members of the series is due to an increase in the number of biosynthetic *cis*-isoprene units.

Ozonolytic degradation of hexahydroprenol enables the separation of the saturated portions of

Table 6. *Incorporation of* 4R-*mevalonate and* 4S-*mevalonate into the prenol complex*

The values given for ^3H and ^{14}C are the actual disintegrations/min. recorded. The corrected $^3H/^{14}C$ ratios are given assuming that the $^3H/^{14}C$ ratio in the original MVA was 1:1.

	From 4*R*-MVA			From 4*S*-MVA		
	^3H (disintegrations/min.)	^{14}C (disintegrations/min.)	Corrected $^3H/^{14}C$ ratio	^3H (disintegrations/min.)	^{14}C (disintegrations/min.)	Corrected $^3H/^{14}C$ ratio
Expt. C						
Prenyl acetate after thin-layer chromatography	3774	3691	3·68:21	2167	1005	12·46:21
Prenyl acetate after paper chromatography	3436	3325	3·73:21	2853	1334	12·38:21
Prenol after thin-layer chromatography	7839	7065	4·00:21	4166	2010	11·98:21
Expt. D						
Prenyl acetate after thin-layer chromatography	895	844	3·82:21	339	182	10·77:21
Prenol complex	1175	1119	3·78:21	528	283	10·79:21
Expt. E						
Prenyl acetate after thin-layer chromatography	666	614	3·91:21	331	177	10·80:21

Table 7. *Incorporation of* 4R-*mevalonate and* 4S-*mevalonate into the individual hexahydroprenols*

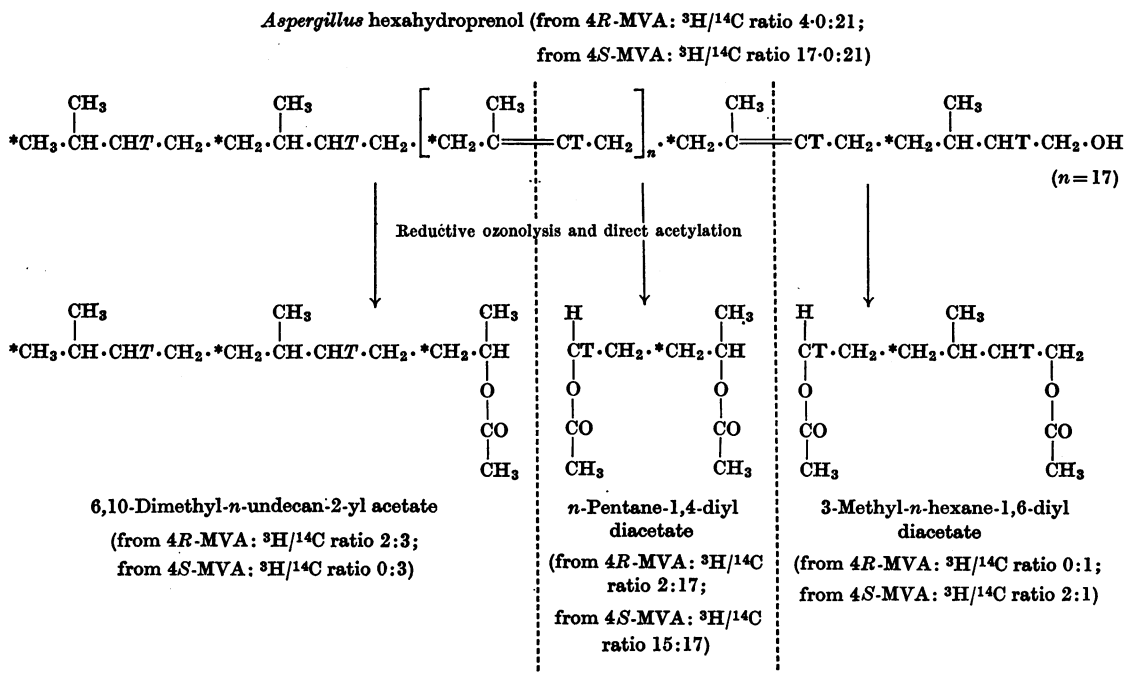
The values given for ^3H and ^{14}C are the actual disintegrations/min. recorded. The corrected $^3H/^{14}C$ ratios are given assuming that the $^3H/^{14}C$ ratio in the original MVA was 1:1.

	From 4*R*-MVA			From 4*S*-MVA		
	^3H (disintegrations/min.)	^{14}C (disintegrations/min.)	Corrected $^3H/^{14}C$ ratio	^3H (disintegrations/min.)	^{14}C (disintegrations/min.)	Corrected $^3H/^{14}C$ ratio
Prenol complex	1175	1119	3·78:21	528	283	10·79:21
Hexahydroprenol-18		N.D.*		550	357	7·64:18
Hexahydroprenol-19	1944	1685	3·76:19	1008	691	8·24:19
Hexahydroprenol-20	4310	3973	3·72:20	2068	1207	9·44:20
Hexahydroprenol-21	4823	4810	3·61:21	2974	1421	11·37:21
Hexahydroprenol-22	3260	3484	3·53:22	1842	868	12·85:22
Hexahydroprenol-23	941	1014	3·63:23	710	312	14·42:23
Hexahydroprenol-24		N.D.*			N.D.*	

* Not determined, as insufficient radioactivity was recovered.

the hexahydroprenol from the unsaturated ones to be made, as shown in Scheme 5. The $^3H/^{14}C$ ratios (Table 8) for the *n*-undecane derivative (peak 3) indicate that both the saturated ω- and ψ-residues are biogenetically *trans*.

Nuclear-magnetic-resonance spectra of individual prenols (Stone *et al.* 1967*b*; J. Feeney & F. W. Hemming, unpublished work) indicate no more than two unsaturated *trans*-isoprene residues. Incorporation of 4*R*-MVA into the undegraded prenol (Tables 6 and 7) allows four biogenetically *trans*-residues in each prenol. Since two of these residues are accounted for in the saturated ω- and ψ-residues, it follows that two of the unsaturated isoprene residues are biogenetically *trans*. Both of these pieces of evidence favour a ratio not more than 2:17 for the *n*-pentane derivative (peak 5). The 4*R* ratio for peak 5 (Table 8) appears to be in error by one isoprene residue in 17 (6%). Undegraded prenol complex biosynthesized from 4*S*-MVA had a $^3H/^{14}C$ ratio 10·8:21. Assuming a 0:3 ratio for the *n*-undecane derivative and a 2:1 ratio for the *n*-hexane derivative (peak 7), the ratio should become 8·8:17 for the *n*-pentane derivative. The actual ratio obtained, namely 8·58:17 (Table 8), is thus in good agreement and indicates that the assumption of a 2:1 ratio for the *n*-hexane derivative was valid (see below).



Scheme 5. Expected labelling pattern assuming that two unsaturated isoprene residues and the saturated ω- and ψ-isoprene residues are biogenetically *trans* and the remainder are biogenetically *cis*. * Indicates ^{14}C; *T* indicates ^3H from 4*R*-MVA; T indicates ^3H from 4*S*-MVA; T indicates ^3H from 4*R*-MVA or 4*S*-MVA.

Table 8. *Incorporation of* 4R-*mevalonate and* 4S-*mevalonate into the ozonolytic degradation products of the hexahydroprenol complex*

The values given for ^3H and ^{14}C are the actual disintegrations/min. recorded. The corrected $^3H/^{14}C$ ratios are given assuming that the $^3H/^{14}C$ ratio in the original MVA was 1:1.

	^3H (disintegrations/min.)	^{14}C (disintegrations/min.)	^{14}C recovery from gas–liquid chromatography (% of that applied to the column)		Corrected $^3H/^{14}C$ ratio	
			Actual	Theoretical*	Actual	Theoretical†
From 4*R*-MVA						
Prenyl acetate complex	666	614			3·91:21	4·00:21
n-Undecane derivative (peak 3)	1180	330	4·2	14·3	1·84:3	2·00:3
n-Pentane derivative (peak 5)	2384	2293	29·4	80·9	3·03:17	2·00:17
n-Hexane derivative (peak 7)	281	345	4·4	4·8	0·14:1	0·00:1
From 4*S*-MVA						
Prenyl acetate complex	328	177			10·70:21	17·00:21
n-Undecane derivative (peak 3)	15	82	6·4	14·3	0·15:3	0·00:3
n-Pentane derivative (peak 5)	636	347	27·8	80·9	8·58:17	15·00:17
n-Hexane derivative (peak 7)	241	92	7·4	4·8	0·72:1	2·00:1

* Calculated assuming that the mean chain length for the prenol mixture is C_{105} and that all of each fraction had been collected.

† Calculated assuming that each polyprenol contains saturated ω- and ψ-isoprene residues of *trans* biogenetic origin, an α-saturated residue of *cis* biogenetic origin with two of the unsaturated internal residues being biogenetically *trans*.

The $^3H/^{14}C$ ratios obtained for the *n*-hexane derivative (Table 8, peak 7) indicate the retention of 4*S*-tritium and the complete eliminaton of 4*R*-tritium. The results with 4*R*-MVA thus offer evidence that both the α- (saturated) and β- (unsaturated) residues are biogenetically *cis*. There is, however, a discrepancy between the observed $^3H/^{14}C$ ratio and the theoretical value in the 4*S*-MVA experiment. One possible interpretation is that peak 7 contains an impurity labelled with ^{14}C but not with ^3H, thus making the $^3H/^{14}C$ ratio spuriously low. Such an explanation is supported by the fact that this fraction contained over 150% (7·4 as against 4·8) of the theoretical proportion of the ^{14}C present in the mixture of acetates before gas–liquid chromatography. If one assumes that the recovery of the *n*-hexane derivative after gas–liquid chromatography was similar to the recovery of the other fractions, it follows that only one-third of the ^{14}C in peak 7 was associated with the *n*-hexane derivative. If all or most of the ^3H in peak 7 was also associated with this derivative, the $^3H/^{14}C$ ratio becomes close to the theoretical value 2:1 previously predicted. It thus seems likely that the mixture of acetates contained some 3–5% of its ^{14}C in the form of an impurity that on gas–liquid chromatography was collected in peak 7.

The apparently low recovery of 4*S*-tritium in the prenol complex was maintained consistently through numerous purification procedures (Expt. C). Loss of 4*S*-tritium to the same extent was evident from the results for the individual hexahydroprenols (Expt. D) and their ozonolytic degradation products (Expt. E). The low $^3H/^{14}C$ ratios obtained with 4*S*-MVA must therefore be accepted as a real result. Further, as the $^3H/^{14}C$ ratios for the prenols biosynthesized from 4*R*-MVA may be accounted for on the basis of the known chemical structure, and, as the $^3H/^{14}C$ ratios for all-*trans*-squalene and all-*trans*-ubiquinone were as expected, it is clear that the loss of ^3H is specific to the biosynthesis of the *cis*-isoprenoid portion of the prenols.

It would be possible to explain the ratios obtained with 4*R*-MVA and 4*S*-MVA by assuming: (i) that the degradation of poly-*cis*-isoprenoid compounds is significantly less than that of poly-*trans*-isoprenoid compounds: (ii) that the resynthesis of *trans*-isoprene residues from the '*trans*'-degradation products involves retention of the olefinic hydrogen (or tritium); (iii) that the synthesis of *cis*-isoprene residues from the '*trans*'-degradation products involves loss of the olefinic hydrogen (or tritium).

Thus a synthesis of isoprene residues from *trans*-degradation products would result in the tritium, incorporated into *cis*-residues from 4*S*-MVA, being diluted out relative to the ^{14}C. The $^3H/^{14}C$ ratios for *trans*-isoprene units, originally synthesized from 4*R*-MVA, would be unaltered. However, retention of the olefinic proton in the same position and configuration during catabolism of an isoprene residue to an isoprenoid precursor is most unlikely and can probably be discounted.

It is possible that some *cis*-residues of hexahydroprenol may be formed by an isomerization of *trans*-residues involving exchange of the olefinic hydrogen (tritium). This would mean that some of the *cis*-isoprene units were biogenetically *cis* and some biogenetically *trans*, but in view of the results with poly-*cis*-rubber (Archer *et al.* 1966) this seems unlikely.

Other possible interpretations of the apparent loss of ^3H have been considered but rejected, as none appears to be in accord with all the known facts. The observation that each consecutive prenol in the series increases in ^3H content by approx. 5·2% per *cis*-isoprene residue (Fig. 5) is particularly difficult to explain. It implies either that the shorter prenols are not precursors of the longer ones or else that the addition of *cis*-residues having a $^3H/^{14}C$ ratio greater than 1:1 occurs.

Probably the main hindrance to a correct interpretation of all the results is due to the labelled substrates' being incubated with the complete organism for several days. It is therefore likely that the problem will only be solved by biosynthetic studies at the enzymic level.

The evidence presented in this paper clearly indicates the biosynthetic origin of the saturated ω- and ψ-isoprene residues to be *trans*, two of the unsaturated isoprene residues to be biogenetically *trans* and increase in chain length to occur by a *cis* addition. The precise position of the *trans*-unsaturated residues is not known (nuclear-magnetic-resonance and mass spectra give no

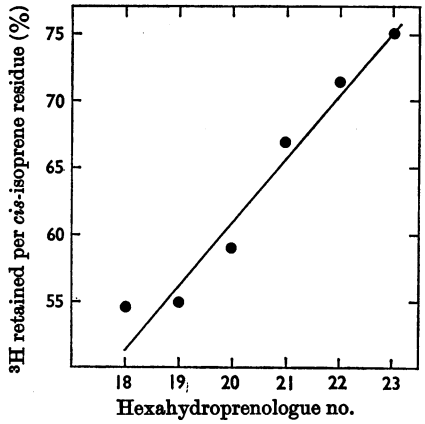


Fig. 5. Variation in retention of tritium, from 4*S*-MVA, with chain length of hexahydroprenol (100% is equivalent to 1 $^3H/^{14}C$ in *cis*-residues).

information on this), but it is tempting to assume that they are immediately adjacent to the saturated ψ-isoprene residue. It would then follow that the *Aspergillus* hexahydroprenols could have been formed by addition of *cis*-isoprene units to all-*trans*-geranylgeranyl pyrophosphate or a dihydro or tetrahydro derivative thereof.

It is noteworthy that the *cis–trans*-polyprenols isolated from green leaves (Wellburn *et al.* 1967; Stone, Wellburn, Hemming & Pennock, 1967*a*) contain three internal *trans*-isoprene residues. As no saturated isoprene residues occur in the leaf prenols, the ω-isoprene residue is chemically *cis–trans*; nuclear-magnetic-resonance evidence shows the 'hydroxy-terminal' residue to be *cis*. It is thus possible to envisage these prenols also to be formed by a *cis* addition to the precursor geranylgeranyl pyrophosphate. The biosynthetic pathways probably differ only in that the *cis* addition in leaves, which is not followed by saturation of any residues, is terminated at shorter chain lengths.

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REFERENCES

Alexander, G. J., Gold, A. M. & Schwenk, E. (1957). *J. Amer. chem. Soc.* **79**, 2967.

Alexander, G. J., Gold, A. M. & Schwenk, E. (1958). *J. biol. Chem.* **232**, 599.

Anslow, W. K. & Raistrick, H. (1938). *Biochem. J.* **32**, 687.

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

Bates, R. B., Carnighan, R. H., Rakutis, R. O. & Schauble, J. H. (1962). *Chem. & Ind.* p. 1020.

Bates, R. B. & Gale, D. M. (1960). *J. Amer. chem. Soc.* **82**, 5749.

Battersby, A. R. & Parry, G. A. (1964). *Tetrahedron Lett.* **14**, 787.

Bennett, R. D. & Heftmann, E. (1963). *J. Chromat.* **12**, 245.

Burgos, J., Butterworth, P. H. W., Hemming, F. W. & Morton, R. A. (1964). *Biochem. J.* **91**, 22P.

Cornforth, J. W., Cornforth, R. H., Donninger, C. & Popják, G. (1966). *Proc. Roy. Soc.* B, **163**, 492.

Cornforth, R. H., Cornforth, J. W. & Popják, G. (1962). *Tetrahedron*, **18**, 1351.

Corwin, L. M., Schroeder, L. J. & McCullough, W. G. (1956). *J. Amer. chem. Soc.* **78**, 1372.

Dicker, D. W. & Whiting, M. C. (1958). *J. chem. Soc.* p. 1994.

Dunphy, P. J., Kerr, J. D., Pennock, J. F., Whittle, K. J. & Feeney, J. (1967). *Biochim. biophys. Acta*, **136**, 136.

Dunphy, P. J., Whittle, K. J. & Pennock, J. F. (1965). *Chem. & Ind.* p. 1217.

Goad, L. J. & Goodwin, T. W. (1965). *Biochem. J.* **96**, 79P.

Goad, L. J., Hamman, A. S. A., Dennis, A. & Goodwin, T. W. (1966). *Nature, Lond.*, **210**, 1322.

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

Heilbron, I. M., Kamm, E. D. & Owens, W. M. (1926). *J. chem. Soc.* p. 1630.

Lavate, W. V. & Bentley, R. (1964). *Arch. Biochem. Biophys.* **108**, 287.

Mercer, E. I. (1960). Ph.D. Thesis: University of Liverpool.

Okita, G. T., Kabara, J. J., Richardson, F. & LeRoy, G. V. (1957). *Nucleonics*, **15**, 111.

Packter, N. M. (1962). Ph.D. Thesis: University of Liverpool.

Pennock, J. F., Hemming, F. W. & Kerr, J. D. (1964). *Biochem. biophys. Res. Commun.* **17**, 542.

Popják, G. (1965). *Biochem. J.* **96**, 1P.

Rees, H. H., Mercer, E. I. & Goodwin, T. W. (1966). *Biochem. J.* **99**, 726.

Richards, J. H. & Hendrickson, J. B. (1964). In *Biosynthesis of Terpenes, Steroids and Acetogenins*, p. 173. New York: W. A. Benjamin Inc.

Stein, Y. & Stein, O. (1962). *Biochim. biophys. Acta*, **60**, 58.

Stone, K. J., Butterworth, P. H. W. & Hemming, F. W. (1967*b*). *Biochem. J.* **102**, 443.

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

Stone, K. J., Wellburn, A. R., Hemming, F. W. & Pennock, J. F. (1967*a*). *Biochem. J.* **102**, 325.

Tavormina, P. A., Gibbs, M. H. & Huff, J. W. (1956). *J. Amer. chem. Soc.* **78**, 4498.

Wellburn, A. R., Stevenson, J., Hemming, F. W. & Morton, R. A. (1967). *Biochem. J.* **102**, 313.

Wellburn, A. R., Stone, K. J. & Hemming, F. W. (1966). *Biochem. J.* **100**, 23C.

Whittle, K. J., Dunphy, P. J. & Pennock, J. F. (1966). *Chem. & Ind.* p. 1303.