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Dolichols, Ubiquinones, Geranylgeraniol and Farnesol as the Major Metabolites of Mevalonate in *Phytophthora cactorum*

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Farnesol, geranylgeraniol, dolichols and ubiquinones were the main radioactive components of the unsaponifiable lipid recovered from *Phytophthora cactorum* grown in aerated cultures containing [2-^{14}C]mevalonate. The ^{14}C recovered in each of these components was in the approximate proportion 2:4:3:5. When the culture was not aerated no radioactive ubiquinone was recovered. Most of the ^{14}C recovered in the dolichols was found in dolichol-15 (37%), with decreasing amounts in dolichol-14 (30%) and -13 (14%) and only a little (5%) in dolichol-16, whereas the major components, by weight, of the mixture (13μg/g of damp-dry tissue) were dolichol-14, -15 and -16 in the approximate proportion of 1:3:1. Radioautography of appropriate chromatograms indicated the presence also of traces of radioactivity in dolichol-9, -10, -11, -12 and -17. Most (80%) of the ^{14}C recovered in the ubiquinones was associated with ubiquinone-9, the rest being in ubiquinone-8. Most (80%) of the weight of ubiquinones (19μg/g of damp-dry tissue) was also ubiquinone-9. The identification of these compounds was by chromatographic methods and, for the ubiquinones and dolichols, was confirmed by mass spectrometry. In addition, the incorporation of 4*R*- and/or 4*S*-^{3}H from [4-^{3}H]-mevalonates showed the expected stereochemistry of biosynthesis, namely that farnesol, geranylgeraniol and ubiquinones were biogenetically all *trans* and the dolichols each contained three biogenetically *trans* isoprene residues, the remaining residues being biogenetically *cis*. The distribution of ^{14}C in the components of the whole lipid of the fungus was consistent with 97% of both the farnesol and geranylgeraniol being present as the fatty acid ester. The corresponding value for dolichols was 37%. The observation by other workers, that this fungus does not form either squalene or sterol, was confirmed.

The results of several workers have established the absence of sterols in *Phytophthora* when grown on a sterol-free medium. The evidence has been obtained from experiments involving digitonin precipitation (Elliot *et al.*, 1964; Hendrix, 1966), radioactive tracers (Hendrix, 1966), g.l.c. and t.l.c. (McCorkindale *et al.*, 1969) and the Lieberman–Burchard colour reaction (Ocana Guardia, 1967; cited by Hendrix, 1970). No studies on metabolism of mevalonic acid by *Phytophthora* have been reported, but it has been shown that this group of fungi will incorporate [2-^{14}C]acetate into unsaponifiable lipids (Hendrix, 1966). However, none of this ^{14}C was associated with sterols, and no studies were made to determine the nature of the radioactive lipids formed. Some of the difficulties in studying the biochemistry of polyprenols in other fungi stem from the presence of sterols (Stone *et al.*, 1967; Stone & Hemming, 1967; Barr & Hemming, 1972). It was therefore decided to investigate the nature of the unsaponifiable lipids of *Phytophthora cactorum*, paying particular attention to any metabolites of mevalonic acid that might be present.

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Materials and Methods

Growth conditions

Phytophthora cactorum (I.M.I. 21168; Commonwealth Mycological Institute, Kew, U.K.) was grown on slopes of Difco Bacto agar (Difco Laboratories, East Molesey, Surrey, U.K.) containing basal medium plus cholesterol (10mg/l; Elliot *et al.*, 1966) at 27°C. The presence of cholesterol was essential for the production of oospores (Elliot *et al.*, 1964, 1966; Hendrix, 1970; Leal *et al.*, 1964). These were usually apparent within 3–5 days and at this stage the spores were transferred to liquid medium (Elliot *et al.*, 1966) containing neither sterol nor agar. Liquid cultures were grown for 3–5 weeks both with and without agitation. Agitation (and aeration) was effected by bubbling air gently through the medium. Volumes of cultures varied from 200ml to 10 litres, depending on the nature of the experiment. Large-scale isolation of

mycelium involved agitated 10-litre cultures, giving a yield of 10g damp weight/litre. The mycelium was harvested by filtering on a Buchner funnel.

Incorporation of radioactive mevalonates

Solutions of 3*RS*-[2-^{14}C]mevalonate (5μCi, 0.96μmol) were injected into 1-litre cultures through a sterile Millipore filter. The highest incorporation of ^{14}C into the unsaponifiable lipid of the mycelium (8.5–10%) was observed when the mevalonate was administered during the fourth day of growth of an agitated culture and the mycelium harvested after a further 28 days. This method of incorporation was adopted as standard procedure.

In experiments designed to study stereochemical aspects of the biosynthesis of the polyprenols 3*RS*-[2-^{14}C]mevalonate (5μCi, 0.96μmol) was mixed with either a 1:1 mixture of 3*R*-[(4*R*)-4-$^{3}H_1$]- and 3*S*-[(4*S*)-4-$^{3}H_1$]-mevalonate (134mCi/mmol, approx. 47μCi) or a 1:1 mixture of 3*R*-[(4*S*)-4-$^{3}H_1$]- and 3*S*-[(4*R*)-4-$^{3}H_1$]-mevalonate (115mCi/mmol, approx. 37μCi) as described by Wellburn *et al.* (1966). The two mixtures were injected into two separate 1-litre cultures.

Isolation of lipids

Unsaponifiable lipid was extracted from the mycelium by the method described by Stone & Hemming (1967). The lipid was separated into its components by preparative t.l.c. of fractions obtained by an initial chromatography on a column of alumina (Barr & Hemming, 1972). This column was eluted sequentially with 1:49 (fraction *a*) and 1:9 (fraction *b*) (v/v) mixtures of diethyl ether and light petroleum (b.p. 40–60°C), followed by diethyl ether (fraction *c*).

Total lipid was extracted from the mycelium by homogenizing with butanol (saturated with water). The solution was filtered and the solvent evaporated at reduced pressure. The butanol-soluble lipid was subjected to a preliminary chromatography on a column of silicic acid by the method of Scher *et al.* (1968). The column was eluted sequentially with chloroform, acetone and chloroform–methanol (1:1, v/v). The neutral lipid eluted by chloroform was separated into fractions (*a*), (*b*) and (*c*) by the methods used for the unsaponifiable lipid.

Lipid fractions were saponified by dissolving them in benzene (3mg/ml) and refluxing them with an equal volume of KOH (15%, w/v) dissolved in ethanol–water (17:3, v/v). The unsaponifiable lipid was then extracted with ether as before.

Thin-layer chromatography

Fraction (*a*) from the alumina column was subjected to preparative and analytical absorption chromatography on thin layers (0.25mm thick for analytical work, 0.5mm thick for preparative work) of Kieselgel G, with light petroleum (b.p. 40–60°C) as solvent (unless otherwise stated) (system A). Similar chromatography of fractions (*b*) and (*c*) employed chloroform as solvent (unless otherwise stated) (system B). Lipids on preparative chromatograms were located by viewing under u.v. light after the chromatogram had been sprayed with fluorescein (Dunphy *et al.*, 1965). Lipids were extracted from the adsorbent with diethyl ether. Analytical chromatograms were stained by spraying with anisaldehyde reagent (McSweeney, 1965).

Fractions from preparative adsorption t.l.c. suspected of containing ubiquinone were subjected to analytical reversed-phase partition t.l.c. (see, e.g., Barr & Hemming, 1972) in acetone–water (47:3, v/v) as solvent (system C). The corresponding solvent mixture for fractions suspected of containing farnesol and geranylgeraniol was acetone–water (13:7, v/v) (system D). Polyprenols were chromatographed in a 49:1 (v/v) mixture of the same solvents (system E). Lipids were located on reversed-phase chromatograms by spraying with fluorescein and viewing under u.v. light, or by using the anisaldehyde reagent. Preparative reversed-phase partition t.l.c. utilized the same systems, and after extraction of the lipids from the chromatograms with diethyl ether, paraffin and fluorescein these were removed by chromatography on alumina (Wellburn *et al.*, 1967).

Assay of radioactivity

^{14}C and ^{3}H were assayed by liquid-scintillation techniques by using the Beckman LS-100 or LS-200 instruments. The scintillation fluid used was made up of 5g of 2,5-diphenyloxazole and 0.3g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene dissolved in 1 litre of AnalaR toluene. All results were corrected for any quenching (usually less than 1%) that occurred and were counted to a counting error of not greater than 1%.

Detection of radioactivity on chromatograms

Scanning. A Panax RTLS-1A thin-layer chromatogram scanner (Panax Equipment Ltd., Redhill, Surrey, U.K.) was used.

Radioautography. Kodirex X-ray film (Kodak Ltd., London W.C.2, U.K.) was used. The film was developed in PQ Universal developer and fixed in Ilfofix (both Ilford Ltd., Ilford, Essex, U.K.) according to the manufacturer's instructions.

Spectroscopy. N.m.r. spectra were obtained with a Varian HA 100 MHz spectrometer by Dr. R. J. Abraham and Mrs. A. Spencer of the Department of Organic Chemistry, University of Liverpool. The solvent was carbon tetrachloride, with tetramethylsilane as internal standard.

Mass spectra were determined by Mrs. A. Ball and Mrs. J. A. Holcroft on the Departmental MS12 instrument (AEI, Manchester, U.K.).

Results and Discussion

Incorporation of [2-^{14}*C*]*mevalonate*

Recovery of ^{14}C from the chromatography of the radioactive unsaponifiable lipid was 80–95% of that added (10% of the dose) to the alumina column, and of this 70–80% was usually present in fraction (*b*), 10–20% in fraction (*c*) and 5–10% in fraction (*a*).

These three fractions were each subjected to t.l.c. Fraction (*b*) gave a chromatogram (system B) containing three areas of radioactivity. One area (R_F 0.38) corresponded to marker spots of geranylgeraniol (R_F 0.39) and farnesol (R_F 0.36), and the second (termed the 'polyprenol' area) had R_F 0.59, midway between that of marker pig liver dolichols (R_F 0.63) and ficaprenols (R_F 0.55). The third area had R_F 0.77, identical with that of a marker spot of ubiquinone-10. The two slower-moving areas contained approximately equal quantities of ^{14}C, whereas the third area (ubiquinone) contained approx. two-thirds of the quantity present in each of the other areas. In fact the proportion of ^{14}C in this part of the chromatogram varied with changes in aeration of the growing culture. When the mycelium was grown in a still culture ^{14}C was absent from this part of the chromatogram. It seems probable that ubiquinone synthesis depends on the O_2 concentration of the medium during growth, a phenomenon observed previously in yeast (Lester & Crane, 1959; Sugimura & Rudney, 1960).

The lipids of the three areas of radioactivity were recovered by preparative adsorption t.l.c. (system B) and were then subjected to reversed-phase partition chromatography with appropriate markers alongside.

The fraction corresponding in R_F value to ubiquinone showed in system C a major peak of radioactivity (on the scanner) corresponding to ubiquinone-9 (R_F 0.42) and a minor peak corresponding to ubiquinone-8 (R_F 0.54). Both areas absorbed u.v. light.

The fraction corresponding in R_F value to geranylgeraniol and farnesol showed two areas of radioactivity in the reversed-phase system (system D). The major peak corresponded to marker geranylgeraniol and a smaller peak corresponded to marker farnesol (see Fig. 1). Both areas stained grey with the anisaldehyde stain in exactly the same way as the marker spots of authentic geranylgeraniol and farnesol.

The radioautogram of the reversed-phase chromatogram (system E) of the 'polyprenol' fraction showed the presence of three bands of radioactive material (R_F values 0.57, 0.51 and 0.44) corresponding in position to dolichol-13, -14 and -15 respectively, the last containing most of the radioactivity. The anisaldehyde stain revealed three compounds with R_F values of 0.51, 0.44 and 0.38, corresponding to dolichol-14, -15 and -16 respectively, the middle one being the most intensely stained (grey–green). This result suggests the presence of a family of polyprenols with at least four isoprenologues, the three shorter ones being radioactive and the shortest being present in insufficient quantity to give a detectable stain.

In general, 15–25% of the radioactivity present in fraction (*b*) was associated with ubiquinone, 25–40% with polyprenol, 25–35% with geranylgeraniol and 15–20% with farnesol.

Most of the ^{14}C in fraction (*a*) was attributed to the presence of prenol esters that had escaped saponification (see below). There was no evidence of the incorporation of ^{14}C into squalene, for t.l.c. (system A) showed the complete absence of ^{14}C corresponding to marker squalene (R_F 0.46).

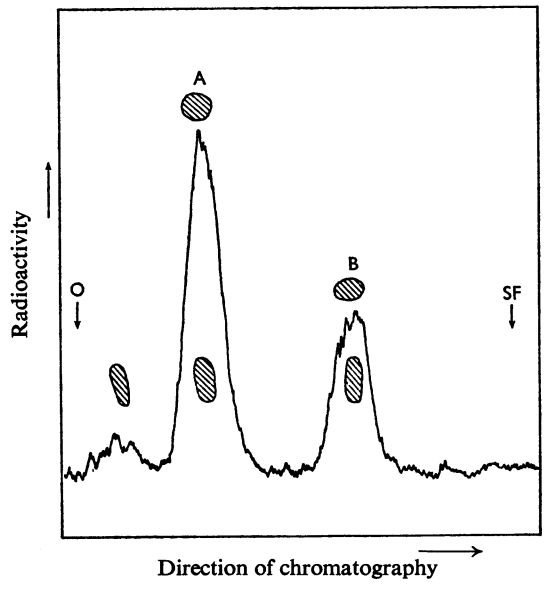


Fig. 1. *Distribution of radioactivity along a chromatogram after reversed-phase t.l.c. of a subfraction of fraction (b) derived from the unsaponifiable lipid of mycelium from a culture grown in the presence of* [2-^{14}*C*]*mevalonate*

The mobile phase was acetone–water (47:3, v/v). The subfraction was prepared from fraction (*b*) (see the text) by preparative adsorption t.l.c. (chloroform as solvent), in which system it corresponded in R_F value to geranylgeraniol and farnesol. The hatched areas indicate the positions of material stainable with the anisaldehyde reagent. A, Marker geranylgeraniol; B, marker farnesol; O, origin; SF, solvent front.

Fraction (*c*) contained no ^{14}C corresponding in R_F (0.2–0.35) in system B to marker sterols. The radioactivity recovered in this fraction was probably due to trailing of [^{14}C]farnesol and [^{14}C]geranylgeraniol, most of which was eluted in fraction (*b*).

Large-scale extraction of polyprenols and ubiquinone

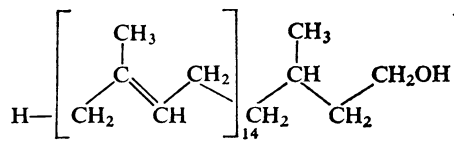
The unsaponifiable lipid from 590g damp weight of *P. cactorum* was extracted in the usual way. Separation on an alumina column yielded 87mg of fraction (*a*), 35mg of fraction (*b*) and 312mg of fraction (*c*) (see the Materials and Methods section). Investigation of fraction (*b*) by analytical t.l.c. indicated the presence of approx. 10–12mg of ubiquinone and 5–8mg of polyprenol as determined by the size of the spot compared with those given by marker solutions of ubiquinone and polyprenol of known concentration.

Preparative adsorption t.l.c. of fraction (*b*) yielded a ubiquinone fraction and a polyprenol fraction. The former was separated into fractions corresponding to ubiquinone-9 (2.5mg) and ubiquinone-10 (0.5mg) by preparative reversed-phase partition t.l.c. The polyprenol fraction was similarly separated into prenols corresponding to dolichol-14 (0.5mg), -15 (1.5mg) and -16 (0.5mg).

Analytical reversed-phase partition t.l.c. of the ubiquinone fractions indicated that they were pure. Mass spectrometry confirmed their identification. Ubiquinone-9 and ubiquinone-8 showed molecular ions for the quinol forms at *m/e* 796 and 728 as well as for the quinone forms at *m/e* 794 and 726 respectively. Both compounds also gave spectra with prominent peaks at *m/e* 235 and 197 and a series of weaker peaks between *m/e* 700 and 300 separated by 68 *m/e* units, caused by successive loss of isoprene residues. These patterns are characteristic of mass spectra of ubiquinones (Bowie *et al.*, 1966; Muraca *et al.*, 1967).

The main polyprenol appeared pure when judged by analytical reversed-phase partition t.l.c., although the two minor polyprenols contained traces of the main component. The slow-moving component (R_F 0.38) was chromatographically identical with the main component (dolichol-16) of the family of dolichols isolated from yeast (Dunphy *et al.*, 1967; Feeney & Hemming, 1967). The main component of the *P. cactorum* mixture corresponded to yeast dolichol-15 and the fast-moving component corresponded to yeast dolichol-14. In addition there was evidence of a prenol corresponding to yeast dolichol-13.

Mass spectrometry of the main *P. cactorum* polyprenol confirmed its identification as dolichol-15 (I). A molecular ion at *m/e* 1040 was prominent. The fact that this was much more intense than $(M-18)^+$, corresponding to loss of water from the molecular



(I)

ion, supported the idea that the α-isoprene residue is saturated. A similar observation was made in the spectra of hexahydropolyprenols (Stone *et al.*, 1967) and of rat liver dolichols (Gough & Hemming, 1970*b*), whereas in the spectra of fully unsaturated prenols, for example castaprenols, $(M-18)^+$ is more intense than M^+ (Wellburn *et al.*, 1967). The rest of the spectrum revealed three series of peaks: (i) where the loss of the ω-isoprene residue to give a peak at *m/e* 971 $(M-69)^+$ was followed by the successive loss of isoprene units (*m/e* 68) from the ω-end of the chain to give peaks at 903, 835, 767 etc.; (ii) where the loss of water and of the ω-isoprene residue to give a peak at *m/e* 953 was followed by the successive loss of isoprene residues; (iii) where the loss of two H atoms, water and of the ω-isoprene residue to give a peak at *m/e* 951 was followed by the successive loss of isoprene residues. Of the three series (i) was the most abundant at *m/e* greater than 1000, but (ii) became more abundant at *m/e* below 850, and at *m/e* below 400 series (iii) was dominant.

Mass spectrometry of the two minor polyprenol fractions revealed the presence of dolichol-16 in one and of dolichol-14 in the other. These spectra showed features similar to those of the spectrum of dolichol-15.

The final confirmation of the characterization of these polyprenols as dolichols depends on their having the correct stereochemistry. Unfortunately, insufficient material was isolated to give a satisfactory n.m.r. spectrum. Therefore evidence of the stereochemistry had to be obtained from biosynthetic studies. These are discussed fully below.

Fraction (*c*) from the original chromatography of the total unsaponifiable lipid showed the presence of traces of material with the same chromatographic mobility as cholesterol on adsorption t.l.c. and with the same staining characteristics as cholesterol (an intense blue after heating for approx. 10min) with the anisaldehyde reagent. Further alumina-column chromatography and preparative adsorption t.l.c. yielded 2mg of a white solid. This material was identical with cholesterol on both adsorption t.l.c. and reversed-phase partition t.l.c. (acetone–water, 4:1, v/v). The presence of the sterol was almost certainly due to cholesterol from the original slopes of *P. cactorum* being transferred to the liquid cultures and being recovered unchanged.

Incorporation of 3R-[2-^{14}C,(4R)-4-$^{3}H_1$]mevalonate and of 3R-[2-^{14}C,(4S)-4-$^{3}H_1$]mevalonate into the polyisoprenoids

Having established the molecular size of the polyprenols it was decided to establish the stereochemistry of the isoprene residues involved. Insufficient material was recovered to obtain a satisfactory n.m.r. spectrum. The only other method that gives reliable quantitative information on the stereochemistry of prenols is to follow the incorporation of 3*R*-[2-^{14}C,(4*R*)-4-^{3}H]- and 3*R*-[2-^{14}C,(4*S*)-4-^{3}H]-mevalonate. The original studies on squalene biosynthesis (Cornforth *et al.*, 1966) and on rubber biosynthesis (Archer *et al.*, 1966) established that in the biogenesis of *trans*-isoprenoid residues from the above species of mevalonate 4*R*-^{3}H is retained and 4*S*-^{3}H is lost, whereas in the biogenesis of *cis*-isoprenoid residues 4*S*-^{3}H is retained and 4*R*-^{3}H is lost. It has been confirmed with betulaprenols (Gough & Hemming, 1970*a*) and undecaprenol of *Lactobacillus plantarum* (Gough *et al.*, 1970) that, assuming the ω-residue to incorporate 4*R*-^{3}H and not 4*S*-^{3}H (i.e. to be biogenetically *trans*), the number of biogenetically *trans* residues agrees precisely with the number of physically *trans* residues as determined by n.m.r. spectrometry. The same agreement was found between the number of biogenetically *cis* and physically *cis* residues. The n.m.r. spectra of all polyprenols of mixed stereochemistry containing an unsaturated α-residue also show that this α-residue is always physically *cis*. With rat liver dolichols, which contain a saturated α-residue, the biogenetic results were as expected if one assumed that the α-residue was biogenetically *cis* and was subsequently hydrogenated (Gough & Hemming, 1970*b*). That saturated α-residues are biogenetically *cis* and ω-residues are biogenetically *trans* was confirmed by degradation of the polyprenols of *Aspergillus fumigatus* after incorporation of the two forms of doubly labelled mevalonates (Stone & Hemming, 1967). There is therefore strong evidence for believing that studies of the incorporation of 3*R*-[2-^{14}C,(4*R*)-4-^{3}H]- and of 3*R*[2-^{14}C,(4*S*)-4-^{3}H]-mevalonate can give reliable information about the stereochemistry of polyprenols.

The two forms of mevalonate were incorporated into separate cultures of *P. cactorum* and the unsaponifiable lipid of the mycelium was recovered as described above. Fraction (*b*) was obtained from this lipid by chromatography on alumina in the usual way. The individual polyisoprenoid compounds listed in Table 1 were recovered from this fraction by preparative adsorption t.l.c. followed by preparative reversed-phase partition t.l.c. Since the quantities of each compound isolated from the fungus were too small to be detected by the methods described in the Materials and Methods section authentic compounds were used either as markers or carriers to locate their presence on chromatograms. Approx. 1 mg of yeast dolichol-complex was added to a portion of the dolichol fraction as carrier during reversed-phase partition t.l.c. Each compound was freed of paraffin and fluorescein as described above before radioactivity counting.

The results of this experiment are shown in Table 1.

Table 1. *Proportions of ^{3}H and ^{14}C in samples of polyisoprenoid compounds isolated from the mycelium of P. cactorum grown in the presence of doubly labelled mevalonates*

For experimental details see the text. The corrected ratio is the ratio that would have been obtained if the original mevalonate had had a $^{3}H/^{14}C$ ratio of 1:1 and allowing for the number of 2-C atoms of the mevalonate expected to be incorporated into each molecule of the compound concerned. The actual $^{3}H/^{14}C$ ratios of the 4*R* and 4*S* forms of mevalonate when counted on the same day as the samples below were 9.37:1 and 7.20:1 respectively.

	From 3*R*-[2-^{14}C,(4*R*)-4-^{3}H$_1$]mevalonate			From 3*R*-[2-^{14}C,(4*S*)-4-^{3}H$_1$]mevalonate		
	d.p.m.		Corrected ratio	d.p.m.		Corrected ratio
	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
Ubiquinone-9	274900	30150	8.76:9.00	276	4870	0.07:9.00
Ubiquinone-8	65700	7180	7.80:8.00	52	305	0.19:8.00
Geranylgeraniol	371200	42800	3.70:4.00	6930	14050	0.27:4.00
Farnesol	162700	18400	2.83:3.00	318	5590	0.02:3.00
Dolichol-13	17600	7650	3.19:13.00	11700	2050	10.26:13.00
Dolichol-14	34700	16400	3.16:14.00	25100	4350	11.22:14.00
Dolichol-15	38400	19800	3.10:15.00	31100	5350	12.14:15.00
Dolichol-16	5900	3090	3.26:16.00	3580	600	13.16:16.00

The corrected $^3H/^{14}C$ ratios obtained for ubiquinone-9 and -8 are in agreement with the polyisoprenoid side chains of these compounds being all *trans*, and serve as an internal control for the experiment. The identification of farnesol and geranylgeraniol is confirmed by the all-*trans* nature of their biosynthesis. The corrected $^3H/^{14}C$ ratios of four of the dolichols are in agreement with each possessing three biogenetically *trans* isoprene residues, the rest being biogenetically *cis*. This is identical with the situation in rat liver dolichols (Gough & Hemming, 1970*b*). The number of biogenetically *trans* residues also agrees with the number of physically *trans* methyl groups in pig liver dolichols and yeast dolichols (Burgos *et al.*, 1963; Dunphy *et al.*, 1967; Feeney & Hemming, 1967). With dolichol-12 and dolichol-17, insufficient radioactivity was recovered to obtain accurate $^3H/^{14}C$ ratios, but both appeared to be predominantly *cis*.

It is likely that one of the biogenetically *trans* residues is the ω-residue, for in all polyisoprenoid compounds that have been studied in detail the ω-residue has always been biogenetically *trans*. Comparison with other *cis–trans* polyisoprenoid alcohols suggests that all three *trans* residues are at the ω-end of the chain. This is the case with betulaprenols-6–9 of silver-birch wood (Gough & Hemming, 1970*a*) and in *A. fumigatus* the two saturated isoprene residues at the ω-end of the chain of the hexahydropolyprenols have been demonstrated to be biogenetically *trans* (Stone & Hemming, 1967). In addition, the α-residues of each member of each family of *cis–trans* polyisoprenoid alcohols appear always to be physically *cis* and/or biogenetically *cis*. This is so over a large range of chain lengths from compounds containing six isoprene residues, as in betulaprenol-6 (Wellburn & Hemming, 1966), to those containing 24 isoprene residues, as in hexahydropolyprenol-24 of *A. fumigatus* (Stone & Hemming, 1967). This situation is best explained by assuming that the *trans* residues are always at the ω-end of the molecule and that sequential *cis*-addition of isoprene residues leads to the formation of families of *cis–trans* polyisoprenoid alcohols, all members of which have *cis*-α-residues.

Although the incorporation of 3*R*-[2-^{14}C,(4*S*)-4-$^3H_1$]mevalonate was lower than that of 3*R*-[2-^{14}C,(4*R*)-4-$^3H_1$]mevalonate the relative incorporation of each into each member of the dolichol family was almost identical. Of the total ^{14}C incorporated into the dolichols the distribution was approx. 14% into dolichol-13, 30% into dolichol-14, 37% into dolichol-15 and 5% into dolichol-16. The balance was associated with dolichol-17, -12 and shorter members of the family, and radioautography indicated the presence of dolichol-11, -10 and -9 in addition to those already described. A plot of R_m value, i.e. $\log[(1/R_F)-1]$, against assigned chain length produced a straight line, consistent with all of the radioactive materials being members of the same family of polyisoprenoid alcohols.

Incorporation of [2-^{14}*C*]*mevalonate into the total lipid of P. cactorum*

Because there was evidence for the presence of prenol esters in some extracts of *P. cactorum* that had escaped saponification (see above) and because in some tissues, a major part of the polyprenols present are esterified to fatty acids (Wellburn & Hemming, 1966; Stone & Hemming, 1968; Butterworth, 1964) it was decided to study the incorporation of [2-^{14}C]-mevalonate into components of the total lipid of the fungus.

Analysis of the mycelium after a routine incorporation of [2-^{14}C]mevalonate (5μCi) showed that the yield of ^{14}C in the total lipid was approx. 20% of the available enantiomer of radioactive mevalonate: 16% in the chloroform fraction, 1.5% in the acetone fraction and 2.1% in the 1:1-(v/v)-chloroform–methanol fraction when chromatographed on a column of silicic acid. The chloroform fraction from the silicic acid column was separated into fractions (*a*), (*b*) and (*c*) as described above by alumina-column chromatography. These fractions contained 4.7%, 3.0% and 3.8% of the available ^{14}C respectively. Most of the mass was in fraction (*b*), the weights being 10mg, 360mg and 114mg respectively.

On adsorption t.l.c. of fraction (*a*) with methanol–benzene (1:99, v/v) as solvent two radioactive components were apparent, (*a*1) at R_F 0.7 and (*a*2) at R_F 0.8. A band of dark material, visible under u.v. light, separated components (*a*1) and (*a*2) and this assisted the separation of components (*a*1) and (*a*2) by preparative adsorption t.l.c. in the same system. The recovery of radioactivity from this procedure was 87%, and of that recovered approx. 80% was associated with component (*a*1) and 20% with component (*a*2).

Fractions (*a*1) and (*a*2) were then saponified. Scanning for radioactivity of analytical t.l.c. plates (system B) showed that virtually all of the radioactivity of the saponified fraction (*a*1) had R_F 0.38, corresponding to geranylgeraniol and farnesol markers, whereas most of that from the saponified fraction (*a*2) had R_F 0.59, slightly ahead of the Ficaprenol marker. Analytical reversed-phase partition t.l.c. (system D) showed that approximately half of the radioactivity in saponified fraction (*a*1) corresponded to farnesol (R_F 0.62) and the other half to geranylgeraniol (R_F 0.39). Over 75% of the radioactivity of the saponified fraction (*a*2) corresponded to dolichol-13, -14 and -15 when subjected to analytical reversed-phase partition t.l.c. (system E). It was concluded that the farnesol, geranylgeraniol and dolichols recovered from fraction (*a*) were present in this fraction originally as the fatty acid esters.

Table 2. *Recovery of ^{14}C in the compounds isolated from the whole lipid of P. cactorum grown in the presence of 5 μCi of [2-^{14}C]mevalonate*

For experimental details see the text.

	Radioactivity				
		Esterified		Unesterified	
	Total (d.p.m.)	(d.p.m.)	(% of total)	(d.p.m.)	(% of total)
Farnesol	162000	157000	97	5000	3
Geranylgeraniol	162000	157000	97	5000	3
Dolichol-13, -14 and -15	175000	64000	37	111000	63
Ubiquinone-9 and -10	205000				

Analytical adsorption t.l.c., in methanol–benzene (1:99, v/v) as solvent, separated fraction (*b*) into two radioactive components. One [fraction (*b*1), R_F 0.38] corresponded to ubiquinone marker and the other [fraction (*b*2), R_F 0.25] ran just behind a marker of pig liver dolichols. A preparative version of the same system resulted in the recovery of 63% of the radioactivity in fraction (*b*1) and 34% in fraction (*b*2). Approx. 3% of the radioactivity corresponded to farnesol and geranylgeraniol. Reversed-phase partition t.l.c. (system C) confirmed that the radioactivity in fraction (*b*1) was due to the presence of [^{14}C]ubiquinones-9 and -8 and that in fraction (*b*2) (system E) was due mainly to the presence of [^{14}C]dolichol-13, -14 and -15. This procedure (system D) showed also that the radioactivity in farnesol and geranylgeraniol of fraction (*b*) was approximately equally distributed. T.l.c. analysis of fraction (*c*) both before and after saponification failed to yield any radioactive prenols or sterols. The nature of the radioactive component in fraction (*c*) was not established.

The recovery of radioactivity in the various identified components is summarized in Table 2. Almost all of the farnesol and geranylgeraniol present is esterified. Conversely, only one-third of the dolichols present is esterified. The proportion of radioactivity recovered in ubiquinone was somewhat higher than normal, but as mentioned above this seems to vary with the degree of aeration of the culture. Possibly this culture was aerated slightly more vigorously than the others. Also in this culture rather more ^{14}C was recovered associated with geranylgeraniol than in other experiments. The reason for this was not clear.

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