Polyprenols of *Aspergillus niger*

THEIR CHARACTERIZATION, BIOSYNTHESIS AND SUBCELLULAR DISTRIBUTION

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A polyprenol complex of *Aspergillus niger* was shown, by using spectrometric methods, to consist of a family of *exo*-methylene-hexahydroprenols that contain between 18 and 24 isoprene residues per molecule. Each prenol contains two *trans* residues, three saturated residues (α , ω and ψ) and an *exo*-methylene substituent on the carbon atom β to the isopropyl group in each ω -residue. The ubiquinone complex consisted of 90% ubiquinone-9, 9% ubiquinone-8 and 1% ubiquinone-10. The amount of polyprenol complex present reached a maximum of 1.7 mg/culture bottle after 9–10 days of growth, coincident with the maximum weight of mycelium. The amount of ergosterol (10 mg/culture bottle) and ubiquinone (1 mg/culture bottle) reached a peak at 8 days. By the 13th day of growth the yield of ergosterol had fallen by 20% and that of ubiquinone by 85%. A study of the incorporation of [2-¹⁴C]mevalonate over different time-intervals confirmed that there was a slow turnover of prenol, a more rapid turnover of ergosterol and a very rapid turnover of ubiquinone. At any one time each member of the prenol complex had essentially the same specific radioactivity as other members of the complex. A similar conclusion was made about the ubiquinone mixture. Just over half of the polyprenol present was esterified to fatty acids. Subcellular fractionation studies indicated that the unesterified prenol is associated primarily with a mitochondrial fraction, whereas the ester is more widely distributed.

The results of a detailed study of the nature and biosynthesis of the polyisoprenoid compounds of *Aspergillus fumigatus* have been reported (Stone *et al.*, 1967; Stone & Hemming, 1967). In particular, the presence of a family of hexahydropolyprenols containing between 18 and 24 isoprene residues in each molecule was described and evidence for the fairly rapid incorporation of mevalonic acid into these compounds was presented. Our interests have now turned to the function of these compounds in the *Aspergilli*. However, in attempting to avoid the pathogenic hazards of *Aspergillus fumigatus* we have investigated first the possibility of using *Aspergillus niger* in these studies. In the present paper, the results of studies on the rate of biosynthesis of the polyprenols in relation to the biosynthesis of ubiquinones and sterols and to the growth of the fungus are reported. Evidence is presented showing that the polyprenols are closely related to those of *A. fumigatus* but modified in the ω -isoprene residue. Results of studies on the subcellular distribution of the polyprenols and their esters are also reported.

Experimental

Materials

3RS-[2-¹⁴C]Mevalonic acid lactone was obtained from The Radiochemical Centre, Amersham, Bucks.,

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U.K. Diethyl ether was dried over lead-sodium alloy and was then distilled from reduced iron. Light petroleum was dried over lead-sodium alloy and was distilled (b.p. 40–60°C). Acetone was dried over Hi-Drite (Hi-Drite Ltd., London W.1, U.K.) and was distilled (b.p. 56.5°C). Chloroform was a general analytical reagent containing ethanol (1%, v/v) as stabilizer. Activated acid-washed alumina was purchased from M. Woelm, Eschwege, Germany. Kieselgel G (E. Merck A.G., Darmstadt, Germany) and Whatman cellulose CC41 (W. and R. Balston Ltd., Maidstone, Kent, U.K.) were used for t.l.c.

All other reagents, unless otherwise specified, were of A.R. purity.

Methods

Micro-organisms and culture methods. *Aspergillus niger* van Tiegh (C.M.I. 59354) was maintained on slopes of potato dextrose agar (Oxoid) which were prepared according to the manufacturer's instructions. The slopes were incubated at 30°C for 7–8 days after inoculation with a loop of spore suspension. After growth the slopes were stored at 0–4°C for up to 10 weeks. Each slope was shaken with 10 ml of sterile water. The resulting spore suspension was used to inoculate up to five Roux bottles, each containing 200 ml of the medium described by Raman *et al.* (1965) that had been sterilized in an autoclave for 15 min at 120°C. After growth in static culture at 28°C the medium was decanted and the thallus was washed

with water. The washed thallus was dried between filter papers and the weight of the thallus was recorded as the 'damp-dry' weight. The pH of the growth medium at harvesting was measured by using a Pye Unicam model 292 pH-meter.

Cell fractionation. The subcellular components of the mycelium of *A. niger* were fractionated by a method similar to that used by Boulter & Hurst (1960) for the fractionation of fragments of *Neurospora crassa* and *Polystictus versicolor*. The 8-day-old thallus was washed with three changes of ice-cold water. The thallus was then cut into small pieces with scissors and mixed with silver sand (acid-washed, then washed with 0.1 M-EDTA followed by ice-cold water). To the mixture was added a solution of sucrose (0.5 M) in 0.03 M-KH₂PO₄ (adjusted to pH 7.4 with 2 M-KOH) containing 0.01 M-EDTA, and the whole was ground in a mortar. The slurry was filtered through two layers of fine muslin and the precipitate was re-ground. After the process had been repeated, the combined filtrates were centrifuged at 500g for 10 min. The supernatant was centrifuged again in the same way. The pooled precipitates were resuspended in the sucrose-buffer mixture and re-centrifuged at 500g for 10 min. This final 500g pellet was retained and the supernatant was discarded.

The original 500g supernatant was centrifuged at 15000g for 20 min at 2°C and the supernatant so produced was then re-centrifuged at 15000g for 20 min. The two combined 15000g pellets were washed by resuspending them in buffer and centrifuging them at 15000g for 20 min. The 15000g pellet was retained and the 15000g supernatant was then centrifuged at 75000g for 100 min. The supernatant was centrifuged a second time at 75000g for 100 min and the combined precipitates were washed by resuspending them in sucrose-buffer and centrifuging them again at 75000g for 100 min. The washings were discarded but the 75000g pellet and the original 75000g supernatant were retained.

Incorporation of mevalonate. In experiment A mevalonate was added to sets of three Roux bottles containing cultures of *A. niger* at 0, 3, 6 or 9 days after inoculation with spores. The cultures were harvested 72 h after mevalonate had been added. In experiment B the mevalonate was added to each Roux bottle at the same time as spore inoculation. Sets of three Roux bottles were then harvested at intervals of 48 h.

In both experiments, the solution of 3*RS*-[2-¹⁴C]-mevalonate (1 µCi, 0.08 µmol/bottle) in 0.01 M-KH₂HPO₄ (adjusted to pH 7.4 with 0.5 M-KOH; 1 ml/bottle) that had been sterilized by passage through a Millipore filter (Millipore U.K. Ltd., London N.W.10, U.K.) was injected into each Roux bottle through the cotton-wool plug.

Isolation of polyisoprenoid compounds. (a) From unsaponifiable lipid. The unsaponifiable lipid of the

harvested cultures was recovered by the usual methods (Stone & Hemming, 1967). Ergosterol, ubiquinone and prenol (as prenol acetate) were isolated from this lipid as described by Stone & Hemming (1967). The ergosterol was purified by t.l.c. on Kieselgel G (0.5 mm thick layers) with chloroform as the developing solvent. The chromatograms were sprayed with fluorescein (0.01 %, w/v, in ethanol) and the ergosterol was located as a dark band under u.v. light. The sterol was extracted from the adsorbent with diethyl ether. A portion of the extract was assayed for ergosterol spectrophotometrically (Stone & Hemming, 1967).

The ubiquinone was purified by t.l.c. and assayed spectrophotometrically (Stone & Hemming, 1967).

The prenol acetate fraction was subjected to preparative t.l.c. on Kieselgel G (0.5 mm thick layers) with benzene-light petroleum (b.p. 40–60°C) (7:13, v/v) as solvent. The prenol acetate was located after spraying the chromatogram with fluorescein and was observed to have the same *R_F* value (0.41) as marker spots of the acetylated hexahydropolyprenols of *A. fumigatus* (Stone *et al.*, 1967). The prenol acetate was extracted from the Kieselgel G with diethyl ether and was then saponified (Stone *et al.*, 1967). The liberated prenol was weighed on a Mettler balance (model H 20).

Preparative reversed-phase partition chromatography of samples of ubiquinone and prenols was done on kieselguhr-G (0.25 mm thick layers) impregnated with paraffin (5 %, v/v, paraffin in petrol). The developing solvent for the ubiquinone sample was dry acetone-water (47:3, v/v) saturated with paraffin, and that for the prenol samples was dry acetone saturated with paraffin. Ubiquinones were located as dark bands under u.v. light after spraying with fluorescein. Bands of ubiquinone corresponding to markers of ubiquinone-8, -9 and -10 were observed. Prenols were made visible as light bands under u.v. light by staining with fluorescein (Dunphy *et al.*, 1965). Seven bands of prenols were usually present. The position of radioactive prenols was confirmed by radioautography before they were extracted for measurement of radioactivity.

Ubiquinones and prenols were extracted from the appropriate areas of preparative chromatograms by using acetone-diethyl ether (1:1, v/v).

Analytical reversed-phase partition t.l.c. and adsorption t.l.c. were done as described above except that the polyisoprenoids were located by using anisaldehyde as a staining reagent (McSweeney, 1965).

(b) From whole lipid. The whole lipid of the thallus or of cell fractions was extracted by homogenizing the material with chloroform-methanol (2:1, v/v) or with ethanol-ether (1:1, v/v). Water was added to the extract and the organic solvent phase was washed several times with water. The solvent was removed by evaporation and the lipid was then

chromatographed on a column of alumina by the method of Stone *et al.* (1967) by successive elution with 1:49 and 3:22 (v/v) mixtures of diethyl ether and light petroleum (b.p. 40–60°C), then finally diethyl ether. Polyprenyl esters and ergosteryl esters were eluted in the first fraction, unesterified polyprenol and ubiquinone in the second fraction and unesterified ergosterol in the third fraction. Each fraction was saponified (Stone *et al.*, 1967) both to remove saponifiable material and also to liberate polyprenol and ergosterol from their esters. Each fraction was then assayed for polyprenol, ubiquinone and ergosterol.

In one experiment with whole thallus, the first fraction from alumina chromatography was not saponified but was subjected to preparative chromatography on a thin layer of Kieselgel G with benzene–light petroleum (b.p. 40–60°C) (7:13, v/v) as solvent. Seven bands of material were apparent when the chromatogram was viewed under u.v. light after spraying with fluorescein. One of these (band 4) had the same R_F (0.58) as a marker sample of ergosteryl stearate. The R_F of band 3 was 0.67. Markers of polyprenol and ergosterol remained very close to the origin. Each band of the chromatogram was removed, then extracted with diethyl ether before the extract was saponified. A sample of the ether-soluble portion of each unsaponifiable lipid was subjected to chromatography on Kieselgel G with chloroform as solvent. The saponified extracts of bands 3 and 4 contained material corresponding in R_F value to marker spots of polyprenol (0.71) and ergosterol (0.22) respectively. The extracts of the other bands did not correspond chromatographically to any of the available authentic compounds and they were not studied further. The extracts containing polyprenol and ergosterol were purified finally by preparative t.l.c. by using the same system.

Determination of radioactivity. Fractions were assayed for ^{14}C with the Beckman LS-100 liquid-scintillation counter. Each sample was dissolved in

toluene (10 ml) containing 2,5-diphenyloxazole (50 mg) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene (3 mg) and was assayed for ^{14}C in a glass vial. The radioactivity measurement was corrected for quenching and was accurate to within 1%.

Radioautography. Radioautograms of thin-layer chromatograms were obtained by using 'Kodirex' X-ray film (Kodak, London W.C.2, U.K.) which was developed in 'Ilford Contrast FF' developer and fixed in Ilfofix (both from Ilford Ltd., Knutsford, Cheshire, U.K.).

Spectroscopy. U.v.-absorption spectra were determined by using a Unicam SP.800 instrument. I.r. absorption spectra were obtained on Perkin Elmer Infracord spectrometers, models 137 and 237, with solvent-free films of the samples between rock-salt discs.

N.m.r. studies were conducted with either a Perkin Elmer R12 60 MHz spectrometer or (by courtesy of Dr. R. J. Abraham and Mrs. A. Spencer of the Department of Organic Chemistry, University of Liverpool) a Varian HA100 100 MHz instrument. Spectra obtained with the Perkin Elmer instrument were accumulated on the Perkin Elmer R12 n.m.r. Digital Signal Averager. Solvents used for n.m.r. were CCl_4 and benzene (both Uvasol grade; Merck) containing tetramethylsilane at a concentration of 1.2% (v/v).

Mass spectra were determined by Mrs. A. Ball on an AEI MS12 instrument and also on a MS 902 spectrometer at the Physico-Chemical Measurements Unit, Harwell, Didcot, Berks., U.K.

Results and Discussion

Growth of mycelium

Under the growth conditions used, the fungus reached its maximum damp-dry weight of 65–70 g at 9–10 days (Table 1). This peak coincided with the

Table 1. *Variation in pH of the medium and yield of thallus, polyprenol, ubiquinone and ergosterol with the age of the culture*

For experimental details see the text.

Age of culture (days)	pH of medium	Weight of thallus (g)	Weight of polyisoprenoids (mg)		
			Polyprenol	Ubiquinone	Ergosterol
0	2.9	0	0	0	0
2	2.0	1.4	0.06	0.10	0.24
4	2.6	4.4	0.25	0.28	2.40
6	2.5	5.2	0.98	0.68	7.50
8	2.6	5.4	1.58	1.02	10.40
11	3.2	6.5	1.66	0.58	8.00
13	4.0	5.9	1.68	0.16	7.90

appearance of black conidial spores at the edge of the thallus, which had covered the whole thallus by the 13th day. By this stage the damp-dry weight had fallen to 55g and the pH of the medium had risen to 4; both these phenomena probably resulted from autolysis.

Biosynthesis of polyisoprenoid compounds

The content of polyprenol in the thallus increased with time, reaching a maximum after approx. 10 days (Tables 1 and 2). The results of experiments A and B showed that the rate of prenol biosynthesis reached a maximum between days 3 and 6 of growth and then decreased slowly. The increase in incorporation of ^{14}C in experiment B followed the increase in weight of polyprenol closely up to day 8. The results indicate that only after day 7 or 8 did the specific radioactivity of the initial pool of mevalonate become markedly lowered by endogenous mevalonate. The constancy in yield of prenol per culture, coupled with a fall in its ^{14}C content after day 9 or 10, suggests that at this stage the rate of biosynthesis of prenol is balanced by the rate of its catabolism. Reversed-phase partition chromatography indicated the presence of a family of prenols, each member of the family differing only in the number of isoprene residues. The specific radioactivity of the whole family changed slightly as the culture aged.

The rate of biosynthesis of ubiquinone continued to rise until day 7 to 8 (Tables 1 and 2). There was evidence of a rapid decomposition of ubiquinone after day 8, and the fact that the total incorporation of ^{14}C by day 8 (experiment B) was little more than that incorporated between days 6 and 9 (experiment

A) suggests that the turnover of ubiquinone is rapid. It is probably much greater than that of ergosterol or prenol. Reversed-phase partition chromatography of the ubiquinone fraction showed this to be a mixture of ubiquinone-8 (9%), ubiquinone-9 (90%) and ubiquinone-10 (1%). The relative proportions of these three compounds remained constant throughout the growth period.

The changes in rate of biosynthesis of ergosterol are also summarized in Table 2. As with the prenols, the rate of incorporation of [^{14}C]mevalonate reached a maximum between days 3 and 6, but the rate decreased more slowly than with the prenol. This is also reflected in the peak of total incorporation of ^{14}C occurring 1–2 days later than with the prenols. The results differ from those with *A. fumigatus* (Stone & Hemming, 1967) as there is no evidence that the increase in rate of biosynthesis is biphasic. However, the fact that the total weight of ergosterol reaches a peak 2 days before the peak of total incorporation suggests that there are two sites of synthesis that make use of two physically separate pools of endogenous mevalonate, one of which equilibrates with exogenous [^{14}C]mevalonate more readily than the other. Probably the turnover of first one pool of ergosterol and then the other can be more rapid than that of the prenols, although probably less than the turnover of ubiquinone.

Because the specific radioactivities of the prenols, ergosterol and of the side chains of the ubiquinones were approximately the same (7000 d.p.m./mg) at day 6 (experiment B) it is clear that, even if there are different pools of mevalonate, they equilibrate with exogenous mevalonate equally well up to day 6. The differences in specific radioactivities that are apparent

Table 2. *Change in recovery of ^{14}C in polyisoprenoid compounds with the period of growth after administration of [$2\text{-}^{14}\text{C}$]mevalonate at day 0 (experiment A) and after administration of [$2\text{-}^{14}\text{C}$]mevalonate 3 days before harvesting (experiment B)*

For experimental details see the text.

Age of culture (days)	$10^{-3} \times \text{Recovery of } ^{14}\text{C} \text{ (c.p.m.)}$					
	Polyprenol complex		Ubiquinone		Ergosterol	
	Expt. A	Expt. B	Expt. A	Expt. B	Expt. A	Expt. B
2	0.2		0.2		0.2	
3		2.2		1.0		22.0
4	1.5		2.0		12.0	
6	6.9	6.9	3.4	2.8	54.8	42.2
8	10.4		6.5		83.0	
9		5.0		4.8		41.5
11	10.2		4.0		97.5	
12		4.5		2.5		18.0
13	9.2		3.4		66.4	

at day 8 and on subsequent days may be a result of the marked physiological and morphological changes associated with sporulation.

Characterization of the polyprenol complex

From the biosynthetic studies the maximum yield of polyisoprenoid compounds was expected to be between days 9 and 10. The fungus was therefore grown in 40 Roux bottles and was harvested on day 9. The polyprenol complex (64mg) was isolated from the unsaponifiable lipid of the thallus as described in the Experimental section. Some studies were done with the complex but most experiments were performed by using individual members of the complex after they had been separated from each other by preparative reversed-phase partition chromatography.

When chromatographed on thin layers of Kieselgel G with chloroform as solvent each individual polyprenol had the same R_f value (0.71). Reversed-phase partition chromatography showed that the complex contained at least seven components, A to G, evenly spaced over the R_f range 0.14–0.42. On the same chromatogram, hexahydropolyprenols-22, -21 and -20 of *A. fumigatus* (Stone *et al.*, 1967) were evenly spaced over the R_f range 0.27–0.38. The pattern was consistent with the existence of a complex containing a series of seven prenols of increasing chain length and of a size similar to the hexahydropolyprenols of *A. fumigatus*. The quantities of polyprenols recovered and the intensity of stain on the analytical reversed-phase partition chromatograms indicated that the complex had the approximate composition 1% A, 6% B, 15% C, 37% D, 29% E, 12% F and 1% G.

The green-grey colour stain obtained on t.l.c. with the anisaldehyde reagent (McSweeney, 1965) is typical of polyisoprenoid compounds. The fact that the compounds could be acetylated and the acetates saponified to regenerate these compounds supports

the idea that they are a family of polyisoprenoid alcohols.

Infrared spectroscopy

I.r. spectra of the individual polyprenols confirmed that they differed from each other only in chain length. The spectrum of the polyprenol complex showed that it contained only polyprenols and that no impurities were apparent. Fig. 1 is the i.r. spectrum of the polyprenol D of *A. niger*. Comparison of this with the i.r. spectrum of the hexahydropolyprenol-21 of *A. fumigatus* shows that the former spectrum contains all of the peaks present in the latter and (in most cases) with the same intensity relative to the other peaks in the spectrum.

The C–O stretching (O–H) deformation absorption band occurs at 1060cm^{-1} showing the compound to be a primary alcohol with the β -carbon atom fully saturated. The polyisoprenoid nature of the structure is confirmed by the bands at 835cm^{-1} (C–H deformation of a trisubstituted olefin), at 1665cm^{-1} (C=C stretching of a non-conjugated system) and at 3028cm^{-1} (C–H stretching of a =CH group), together with the relative intensities of the bands at 1450cm^{-1} (C–H deformation of $-\text{CH}_2-$ and $-\text{CH}_3$) and at 1365cm^{-1} (C–H deformation of $-\text{CH}_3$) (Bellamy, 1958). A predominantly *cis* configuration of the polyprenol is suggested by the small peaks at 1090cm^{-1} , at 1130cm^{-1} with a shoulder at 1150cm^{-1} and at 1310cm^{-1} with a shoulder at 1330cm^{-1} together with the absence of a shoulder at 795cm^{-1} on the intense peak at 835cm^{-1} (Burgos *et al.*, 1963).

However, in addition to these absorption bands characteristic of poly-*cis*-prenol, the spectrum exhibits absorption bands consistent with the presence of the grouping $\begin{smallmatrix} \text{R}^1 \\ \text{R}^2 \end{smallmatrix} \text{C}=\text{CH}_2$ in the structure (Bellamy, 1958). Out-of-plane deformation of the =CH₂ group

Fig. 1. Infrared-absorption spectrum of a thin film of polyprenol D

A_1 in successive isoprene residues, this produced four series of peaks throughout the spectrum.

It was clear that the major peaks in the mass spectrum could be explained by the fragmentation of the structure shown in Fig. 2. This was thus consistent with the evidence from i.r. and n.m.r. spectroscopy (see below).

Nuclear-magnetic-resonance spectrometry

The n.m.r. spectra of the polyprenol complex and of the individual polyprenols were qualitatively the same, showing the expected quantitative differences caused by differences in chain length. The 60 MHz spectrum obtained of a solution of the complex dissolved in carbon tetrachloride (containing tetramethylsilane) is shown in Fig. 3. The results from the spectrum are summarized in Table 3. The spectrum shows striking similarities with that of the hexahydropolyprenol complex of *A. fumigatus* (Stone *et al.*, 1967). In particular the spectra of both compounds exhibit olefinic proton resonance (4.95τ), a triplet for the methylene group adjacent to the hydroxyl ($6.35, 6.45, 6.56\tau$), strong peaks for $-\text{CH}_2-$ groups adjacent to a C atom carrying a double bond ($7.98, 8.02\tau$) and strong peaks for methyl groups on a C atom carrying a double bond ($8.35, 8.44\tau$). There is also significant resonance in both spectra corresponding to $-\text{CH}_2-$ and $-\text{CH}_3$ groups in a saturated environment ($8.6-9.3\tau$).

A close inspection of the $8.2-8.6\tau$ region of a 100 MHz spectrum of a solution of the *A. niger* prenol in benzene revealed that the peaks on either side of the internal *trans* peak at 8.38τ were absent,

Fig. 3. Nuclear-magnetic-resonance spectrum obtained at 60 MHz of a solution of the polyprenol complex in carbon tetrachloride (1.5%, w/v)

A total of 149 spectra were accumulated by using a digital signal-averager accessory.

confirming that the ω -terminal residue lacked the isopropylidene group present in fully unsaturated prenols and contained instead an isopropyl group, as in the hexahydropolyprenols of *A. fumigatus* (Feeney & Hemming, 1967).

There is one important qualitative difference between the spectra of the prenols of *A. niger* and *A. fumigatus*. This is the presence in the former of a small sharp peak at 5.29τ corresponding to the protons of a terminal methylene group. The area of the peak corresponded to only one of these groups.

There is also a close quantitative correlation between most of the spectrum of the prenol complex of *A. niger* and that of the hexahydropolyprenols of *A. fumigatus*. It is clear that in both cases, for every 20 isoprene residues present, 17 are unsaturated and internal and these are in the ratio of 2 *trans* to 15 *cis*. (The ratio of *cis/trans* residues was determined from the $8.2-8.5\tau$ region of a spectrum of a benzene solution at 60 MHz and was confirmed by results from studies at 100 MHz (Feeney & Hemming, 1967). However, important quantitative differences are apparent in the region $8.6-9.3\tau$ and these are coupled with a relative increase in the number of protons resonating close to 8.0τ . These differences can be explained by the substitution of a terminal methylene group for two protons of the saturated ω -residue. Not only does this eliminate the resonance of two protons in the $8.6-8.8\tau$ region but it also causes the shift of the resonance of the four protons on the two C atoms adjacent to the substituent from this region to the 8.0τ region. This substitution therefore decreases the number of protons resonating in the $8.6-8.8\tau$ region from 17 to 11. The relative intensity of the methyl resonance in the $9.0-9.2\tau$ region remains the same as in the spectrum of the *A. fumigatus* prenols.

Not only does the n.m.r. spectrum support the structure proposed in Fig. 2, but it also shows that the terminal methylene group is most likely to be in the position shown. Its presence in any of the unsaturated residues would have resulted in obvious differences in the spectrum owing to the production of either a conjugated system of double bonds or of an arrangement containing one (or two) $-\text{CH}_2-$ groups adjacent to two C atoms carrying double bonds. The quantitative differences discussed in the previous paragraph also demand that the terminal methylene group is present between two $-\text{CH}_2-$ groups of a saturated residue. These considerations, together with the evidence that resonance of the protons of the terminal methylene group at 5.29τ appears as a singlet, provide strong evidence that the group

occurs as part of a saturated residue.

Table 3. Assignment of the peaks in the nuclear-magnetic-resonance spectrum of the *A. niger* polyprenol complex

For experimental details see the text. The measurements at 60 MHz were done in carbon tetrachloride with the polyprenol complex; those at 100 MHz were done in benzene with polyprenol-20. For *A. niger*, the theoretical relative peak area is the actual number of protons expected assuming the structure of polyprenol-20 to be that shown in Fig. 2 with 2 *trans* and 15 *cis* isoprene residues and assuming the complex to contain 12% prenol-19, 29% prenol-20, 37% prenol-21, 15% prenol-22 and 6% prenol-23. For *A. fumigatus*, the theoretical relative peak area is the actual number of protons expected from the hexahydropolyprenol complex of *A. fumigatus* (Stone *et al.*, 1967). The protons that resonate are underlined.

τ value	Assignment	Relative areas of peaks		
		Experimental	Theoretical	
			<i>A. niger</i>	<i>A. fumigatus</i>
Information obtained at 60 MHz				
4.95	$>\text{C}=\underline{\text{CH}}-\text{CH}_2-$	17	17.8	17.8
5.29	$>\text{C}=\underline{\text{CH}}_2$	2	2	—
6.35 } 6.45 } 6.56 }	$-\text{CH}_2\underline{\text{CH}}_2-\text{OH}$	2	2	2
7.98 } 8.02 }	$\begin{array}{c} \text{CH}_3 \\ \\ -\text{CH}_2-\text{C}=\text{CH}-\text{CH}_2- \end{array} \text{ and } \begin{array}{c} -\text{CH}_2-\text{C}-\text{CH}_2- \\ \\ \text{CH}_2 \end{array}$	74	74.9	71.3
8.35 } 8.44 }	$\begin{array}{c} \underline{\text{CH}}_3 \\ \\ -\text{C}=\text{CH}- \end{array}$	57	53.1	53.4
8.60 } 8.80 }	$\begin{array}{c} \text{CH}_3 \qquad \qquad \text{CH}_3 \\ \qquad \qquad \\ -\text{CH}_2-\text{CH}_2-\text{CH}-\text{CH}_2-\text{CH}_2-, \text{CH}_3-\text{CH}-\text{CH}_2- \end{array} \text{ and } \left. \begin{array}{c} \text{CH}_3 \\ \\ -\text{CH}_2-\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}_2- \\ \\ \text{CH}_3 \end{array} \right\}$	23	24	30
9.00 } 9.30 }	$\begin{array}{c} \text{CH}_3 \\ \\ (\text{H})-\text{CH}_2-\text{CH}-\text{CH}_2 \end{array} \text{ and } \underline{\text{OH}}$			
Additional information obtained at 100 MHz				
7.80 } 7.83 } 7.96 }	$\begin{array}{c} \text{CH}_3 \\ \\ -\text{CH}_2-\text{C}=\text{CH}-\text{CH}_2- \end{array} \text{ and } \begin{array}{c} -\text{CH}_2-\text{C}-\text{CH}_2- \\ \\ \text{CH}_2 \end{array} \left. \vphantom{\begin{array}{c} \text{CH}_3 \\ \\ -\text{CH}_2-\text{C}=\text{CH}-\text{CH}_2- \end{array}} \right\}$	72	72	68
8.25 } 8.38 }	$\begin{array}{c} \text{cis} \\ \text{trans} \end{array} \left\{ \begin{array}{c} \underline{\text{CH}}_3 \\ \\ -\text{C}=\text{CH}- \end{array} \right.$	46 6	45 6	45 6
8.59 } 8.66 } 8.76 }	$\begin{array}{c} \text{CH}_3 \qquad \qquad \text{CH}_3 \\ \qquad \qquad \\ -\text{CH}_2-\text{CH}_2-\text{CH}-\text{CH}_2-\text{CH}_2-, \text{CH}_3-\text{CH}-\text{CH}_2- \end{array} \text{ and } \left. \begin{array}{c} \text{CH}_3 \\ \\ -\text{CH}_2-\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}_2- \\ \\ \text{CH}_3 \end{array} \right\}$	10	11	17
9.01 } 9.05 } 9.09 } 9.19 }	$\begin{array}{c} \text{CH}_3 \\ \\ (\text{H})-\text{CH}_2-\text{CH}-\text{CH}_2 \end{array} \text{ and } \underline{\text{OH}}$	12	13	13

There is strong i.r., mass- and n.m.r.-spectrometric evidence of a saturated α and ω residue. The last two techniques indicate the presence of a third saturated residue. Mass spectrometry favours the placement of this third residue in the ψ position and analogy with the hexahydropolyprenols of *A. fumigatus*, the structures of which were also confirmed by degradative studies, supports this.

Clearly the group

cannot be accommodated in the α -saturated residue. It could be accommodated in the ψ residue, but this would result in one of the $-\text{CH}_2-$ groups being between two C atoms carrying double bonds, with a consequent shift downfield of its proton resonance. Since there is no evidence for this, the terminal methylene group is most likely to be in the ω -residue and in the position shown.

The evidence for the precise position of the terminal methylene group is obviously heavily dependent on the n.m.r. spectrum and especially on measurements of the areas of the peaks in the 7.8–8.1 τ and 8.5–9.2 τ regions of the spectrum. The measurements were checked several times in several different spectra and although in the spectrum shown in Fig. 3 (and Table 3) the area of the 8.6–8.8 τ region was larger than expected, the areas of the relevant peaks in other spectra (especially those at 100 MHz) were very close to the expected values. It is probably reasonable to argue that although the area measurements fit the structure proposed in Fig. 2 more closely than any other structure, the margin of error possible in such measurements make it difficult to eliminate entirely the possibility that the methylene group is on the carbon atom α , γ or ϵ to the isopropyl group in the ω -residue. However, it is easier to understand the insertion of a methylene group on the α or ϵ carbon atom in view of what is known about alkyl substitution of sterols.

There is thus strong evidence in favour of the idea that the polyprenol complex consists of a family of prenols ranging from *exo*-methylene-hexahydropolyprenol-18 to *exo*-methylene-hexahydropolyprenol-24, the *exo*-methylene group occurring on the carbon atom that is β to the terminal isopropyl group in each prenol (Fig. 2). The percentage composition of the mixture of prenols-18 to -24 approximates respectively to 1% -18, 12% -19, 29% -20, 37% -21, 15% -22, 6% -23 and 1% -24.

The family of prenols is clearly very similar to that found in *A. fumigatus*, differing only in the presence of the *exo*-methylene group. Although the position of this methylene group is surprising from the biosynthetic point of view, the spectrometric evidence is quite strong. However, the spectrometric studies give

Table 4. Yields per culture (60 g damp weight of mycelium) of the esterified and non-esterified forms of polyprenol and ergosterol

For experimental details see the text.

	Non-esterified		Esterified	
	(μg)	(%)	(μg)	(%)
Polyprenol	744	45	928	55
Ergosterol	5384	74	1856	26

no indication of the precise position in the molecule of the *trans*-isoprene residues. The analogy with the hexahydropolyprenols of *A. fumigatus* and consideration of biosynthetic studies on these compounds (Stone & Hemming, 1967) and of betulaprenols (Gough & Hemming, 1970) favour the presence of the two *trans* residues adjacent to the ψ saturated residue.

Subcellular distribution of the free and esterified prenols

It was decided to determine the proportion of the prenol complex present as an ester of long-chain fatty acids and the proportion present in an unesterified form. The distribution of the two forms throughout fractions of the cell was also studied.

Analysis of the whole lipid from 25 cultures showed that just over half of the prenol present was esterified to long-chain fatty acids (Table 2). This ester was eluted from a column of alumina by a mixture (1:49, v/v) of diethyl ether and light petroleum (b.p. 40–60°C), and on thin layers of Kieselgel G had R_F 0.67 with benzene–light petroleum (7:13, v/v) as solvent and R_F 0.85 with chloroform as solvent, and yielded prenol on alkaline hydrolysis. The unesterified prenol was eluted from the same column of alumina by a 2:23 (v/v) mixture of diethyl ether and light petroleum and had R_F values of 0.04 and 0.71 respectively in the same t.l.c. systems. Reversed-phase partition chromatography on a thin layer of paraffin-impregnated kieselguhr with dry acetone as solvent gave a chromatographic pattern for the prenol complex derived from the ester which was identical with that for the prenol complex that was isolated in an unesterified form.

It is notable that a much smaller proportion (approximately one-quarter) of the ergosterol was present as ester (see Table 4). The values for prenol and sterol are very similar to the patterns in *A. fumigatus* (Stone & Hemming, 1968) and for the corresponding compounds in pig liver (Butterworth & Hemming, 1968).

The results of the analyses of the subcellular fractions are given in Table 5. Most of the unesterified prenol was recovered in the 15000g pellet whereas the esterified prenol was distributed throughout the

Table 5. *Yields of isoprenoid compounds per culture (60g damp weight of mycelium) after subcellular fractionation*

For experimental details see the text.

Fraction	Polyprenol				Ergosterol				Ubiquinone	
	Free		Ester		Free		Ester			
	(μg)	(%)	(μg)	(%)	(μg)	(%)	(μg)	(%)	(μg)	(%)
	500g pellet	130	19	210	34	580	10	1110	49	130
15000g pellet	450	66	210	34	3350	55	430	19	460	59
75000g pellet	40	6	170	28	1530	25	400	18	110	14
75000g supernatant	60	9	20	3	640	10	320	14	90	11

particulate fractions much more evenly. It is well established that most of the ubiquinone of living cells is present in the mitochondria (Crane, 1965) and for this reason ubiquinone can be taken as a marker for mitochondria or mitochondrial fragments. Table 5 shows that the 15000g pellet, as expected, contained most of the mitochondria of the tissue. The good correlation between the distribution of ubiquinone and prenol is consistent with the idea that the prenol is also associated principally with the mitochondria. However, particulate material other than that of mitochondrial origin was almost certainly present in this fraction, which could have been the source of the prenol. Certainly the free prenol is associated with a particulate cell fraction that sediments at 15000g, but not specifically with nuclear or microsomal fractions, which one would expect to sediment at 500g and 75000g respectively. The small quantity of unesterified prenol found in these fractions may well be contamination with particles or fragments of particles that would normally be found in the 15000g sediment. In contrast, the esterified prenol occurs in all three sediments.

The distribution of esterified and unesterified prenol and of ubiquinone-9 throughout the cell fractions observed is very similar to that of the equivalent components in cell fractions of *A. fumigatus* (Stone & Hemming, 1968) and in pig liver (Butterworth & Hemming, 1968). Prenol phosphates have been implicated in bacterial wall biosynthesis and in liver glycoprotein biosynthesis. It is not yet clear if the distribution of prenol is a reliable guide to the distribution of prenol phosphates. If prenol phosphates are involved in fungal wall biosynthesis one might expect them to be concentrated in fragments of the Golgi bodies, i.e. in the 75000g sediment. The distribution of unesterified prenols obviously lends no support to the possibility of their phosphates having such a role. The significance of the striking association of unesterified prenol with the 15000g sediment

and the difference between this and the more general distribution of the esterified prenol throughout the particulate fractions is not yet clear.

The distribution of unesterified ergosterol is also similar to that found in *A. fumigatus* and pig liver but the concentration of the esterified ergosterol in the 500g pellet is different. The reason for this difference is not understood but it may have physiological significance, for sterols are known to play an important role in the growth and development of fungi.

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