Biochem. J. (1968) **109**, 877
Printed in Great Britain

The Characterization and Distribution of Hexahydropolyprenyl Esters in Cultures of *Aspergillus fumigatus* Fresenius

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(*Received* 2 *May* 1968)

The total mycelial lipid of *Aspergillus fumigatus* was analysed and over half of its hexahydropolyprenol was shown to be esterified with fatty acids. Comparison of the fatty acid content of the prenyl esters with the sterol ester and the total lipid indicated a marked predominance of saturated fatty acids in the polyprenyl esters. The predominant acids esterified to the prenols were palmitic acid, linoleic acid, oleic acid, lignoceric acid, stearic acid and palmitoleic acid. Most of the unesterified polyprenol was found in the mitochondrial fraction, but the esterified prenol was equally distributed throughout the cell fractions. This distribution was unlike that found for ergosteryl ester in the same tissue.

Isoprenoid alcohols (prenols) esterified with fatty acids have frequently been observed in many essential oils. Such prenols have less than five isoprene residues and may be designated 'short-chain prenols'. Recently, fatty acid esters of long-chain prenols (containing more than four isoprene residues) have also been observed, and in one case the polyprenyl ester was purified and characterized; thus fatty acid esters of all-*trans*-prenol-9 (solanesol) in tobacco were shown by Rowland & Latimer (1959) to consist mainly of palmitate and linolenate with smaller amounts of linoleate, myristate and oleate. However, no comparison with the sterol ester constituent of the tobacco was made. Esters of predominantly-*cis*-polyprenols were also observed in the wood of *Betula verrucosa* (Lindgren, 1965) and in pig liver (Butterworth, 1964), though their complete purification and characterization have not been reported.

A study of the intracellular distribution of the polyprenyl ester in pig liver indicated that the ester and the free prenol were concentrated in different cell fractions (Butterworth, 1964). Polyprenols were recently implicated in mucopolysaccharide biosynthesis (Higashi, Strominger & Sweeley, 1967; Wright, Dankert & Robbins, 1967), and thus the specific localization of polyprenyl ester and free polyprenol within the cell may be of some significance in this context also.

The mycelium of the pathogenic mould *Aspergillus fumigatus* Fresenius contains a complex of polyprenols consisting of hexahydroprenol-18, -19, -20, -21, -22, -23 and -24. Each alcohol has a saturated α- (hydroxy-terminal) isoprene residue, a saturated ω-terminal isoprene residue and a saturated ψ-isoprene residue (adjacent to the ω-residue). Two of the unsaturated residues in each alcohol are *trans* and the remainder are *cis* (Stone, Butterworth & Hemming, 1967). Preliminary experiments on the composition of the total mycelial lipid of this mould indicated that a large proportion of the polyprenol complex was naturally esterified. The isolation, characterization and intracellular distribution of this prenyl ester are the subject of the present paper.

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MATERIALS AND METHODS

Materials. Benzene, light petroleum (b.p. 40–60°) and diethyl ether were dried over sodium wire and distilled. Diethyl ether was distilled over reduced iron immediately before use to remove peroxide contaminants. Chloroform was a general analytical reagent containing ethanol (1%, v/v) as stabilizer. Neutral Woelm alumina was weakened to Brockmann grade III by the addition of water before use.

Micro-organism and culture methods. Aspergillus fumigatus Fresenius (L.S.H.T.M. A.46; C.M.I. 89353) was grown on Raulin–Thom medium in Roux bottles and harvested as described by Stone *et al.* (1967). The incubation temperature was 30° and growth was terminated after 9 days, when it was maximal (Stone & Hemming, 1967).

Extraction of lipid from whole tissue. The mycelia from 30 cultures were dried between filter papers and weighed before the extraction of total lipid by the Folch procedure (Folch, Ascoli, Lees, Meath & LeBaron, 1951). The mycelia (700g.) were sliced into small pieces and homogenized in 6l. of chloroform–methanol (2:1, v/v) with an Ultra-Turrax homogenizer. Filtration through fluted filter paper removed insoluble material, which was discarded. An

equal volume of water was added to the filtrate and the mixture was vigorously shaken. After overnight separation into two phases the brownish lower layer was removed and evaporated under vacuum to yield 8·3g. of a dark-brown oil.

Cell fractionation and lipid extraction. The cell fractionation was based on the method of Boulter & Hurst (1960), who obtained enzymically active mitochondrial preparations from *Neurospora* and *Polystictus* sp. The mycelia from two cultures of *Aspergillus* were harvested and allowed to stand for 1hr. with three 500ml. changes of ice-cold distilled water. Washed tissue (6–10g.) was ground in a mortar for 15min. with twice its volume of 80–120-mesh quartz sand and 100ml. of 0·5M-sucrose–0·03M-sodium phosphate buffer, pH7·3, containing neutralized 0·01M-EDTA. During the grinding ice was added to keep the temperature below 1° and Na_2HPO_4 soln. was added to keep the pH just above neutral. The slurry was filtered twice through muslin and centrifuged at 500*g* for 10min. in a Sorvall centrifuge. The resulting light-brown pellet, containing cell debris and nuclei, was washed with buffer and centrifuged at 500*g* for 10min.; the washings were discarded. Similarly the supernatant was centrifuged again at 500*g*, to remove all traces of cell debris. The supernatant fluid was spun at 15000*g* for 15min. in the Sorvall centrifuge to sediment the mitochondria. These were resuspended in buffer with a small glass homogenizer and centrifuged at 15000*g* for a further 15min.; the washings were discarded. The supernatant solution was again spun at 15000*g* for 15min. to remove last traces of mitochondria, and was then spun at 105000*g* in a Spinco centrifuge for 4hr., to yield a grey-brown pellet of 'microsomes' and supernatant. The pellet was resuspended in buffer and centrifuged again at 105000*g*. The supernatant was centrifuged again at 105000*g* to remove traces of microsomes. All operations were performed at approx. 1° and all vessels and materials were precooled to this temperature before use. Each cell fraction was suspended in 10ml. of water, 40ml. of ethanol–ether (1:1, v/v) was added and the mixture was refluxed on a boiling-water bath. After 30min. it was cooled and extracted three times with light petroleum. The organic phase was washed with water, dried over Na_2SO_4 and evaporated to dryness under N_2.

Lipid fractionation. Each lipid sample was dissolved in light petroleum and fractionated by column chromatography with alumina as the adsorbent and E/P solutions* as the eluents. Substances eluted with 1% (v/v) E/P, 2% (v/v) E/P, 12% (v/v) E/P and ether respectively were collected. The 1%-E/P and 2%-E/P fractions were shown, by i.r. spectroscopy, to contain all the ester material by their characteristic carbonyl absorption at 1730cm.$^{-1}$. The 12%-E/P fraction contained all the ubiquinone and free prenol, and the ether fraction consisted mainly of free ergosterol. This free ergosterol was estimated by a modification of the Morton & Stubbs procedure (Mercer, 1960).

Lipid fractions from whole tissue were further fractionated and analysed to determine the structures of the ester constituents (Scheme 1). Lipid chromatographic fractions from the cell fractions were each saponified, by refluxing in 60% KOH, to liberate the free alcohols and to remove the large amount of triglyceride. Before saponification, 1vol. of methanolic 5% (w/v) pyrogallol was added to prevent breakdown of the ubiquinone.

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

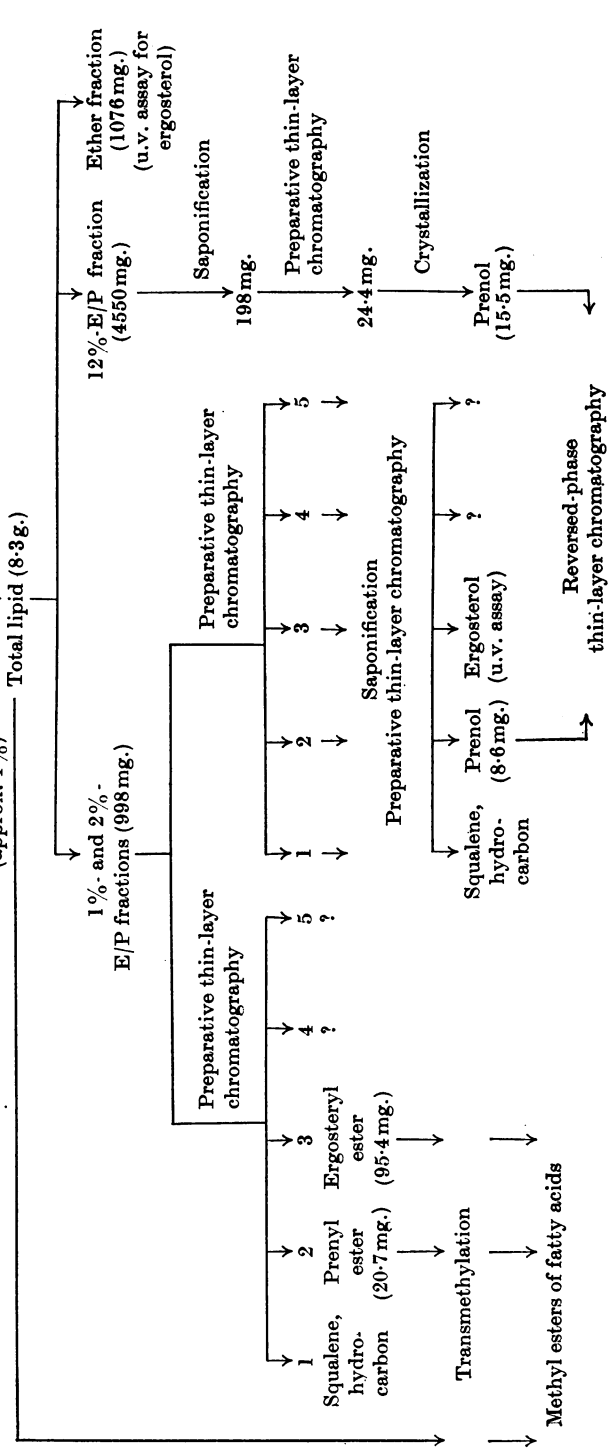
Free polyprenol in the resulting unsaponifiable lipids was measured by a direct comparison of spot size (Truter, 1963) with a range of spots of standard amounts of polyprenol after thin-layer chromatography (Kieselgel G; 275μ thickness; developing solvent, chloroform) and staining with phosphomolybdic acid. Ubiquinone was determined by measuring the decrease in E_{275} on reduction with a few grains of $NaBH_4$ in ethanolic solution (Crane, 1959).

Analysis of the ester constituents. The combined 1%-E/P and 2%-E/P fractions were chromatographed preparatively as lines on thick layers (500μ) of silica gel G with light petroleum–benzene (13:7, v/v) as the developing solvent. On staining the plates with fluorescein (Dunphy, Whittle & Pennock, 1965), bands of lipid were evident with R_F values 0·87, 0·67, 0·54, 0·31 and 0·20. These were labelled bands 1, 2, 3, 4 and 5 respectively. Each band of material was eluted with ether and a portion of each (one-third) was saponified by refluxing a solution of it in benzene (3mg./ml.) with an equal volume of 15% (w/v) KOH in ethanol–water (17:3, v/v) for 20min. After extraction of the lipid from this saponification mixture, portions were analysed by thin-layer chromatography alongside authentic marker compounds. Band 1 contained lipid that co-chromatographed with squalene and also material (probably saturated hydrocarbon) that travelled close to the solvent front. Lipid from band 2 co-chromatographed with *Aspergillus* hexahydropolyprenol [isolated by saponification of the 12%-E/P chromatography fraction and purified by preparative thin-layer chromatography, with 1% (v/v) methanol in benzene as the developing solvent, followed by recrystallization from ethanol containing a little ether]. That from band 3, which strongly absorbed u.v. light, co-chromatographed with ergosterol. The lipid fractions from bands 2 and 3 each chromatographed as a single component, and i.r. spectra of these fractions confirmed the assignments. Lipid fractions from bands 4 and 5 did not co-chromatograph with any of the reference compounds.

Analysis of the prenol complex. Equal amounts of polyprenol derived by saponification of prenyl ester and naturally free polyprenol from the 12%-E/P chromatography fraction were subjected to two-dimensional reversed-phase partition thin-layer chromatography on kieselguhr, loaded with a 3·5% (v/v) solution of paraffin in light petroleum as the stationary phase, and dry acetone saturated with paraffin as the mobile phase, in both dimensions. Samples (40μg.) of the polyprenols were applied as spots approx. 1·25in. apart in the bottom left-hand corner of the plate; the plate was then developed in the normal way. When separation in the second dimension was complete the plate was dried and stained with anisaldehyde, and R_F values in the second dimension were measured.

Transmethylation of the ester constituents. Portions (20mg.) of ergosteryl ester, the prenyl derivative and total lipid were transmethylated by the method of Stoffel, Chu & Ahrens (1959) to give methyl esters of fatty acids.

Gas–liquid chromatography. A dual-column F & M model 810 gas chromatograph fitted with flame-ionization detectors was used isothermally. The carrier gas was argon and samples were injected directly on to the column, which was packed in a silane-treated stainless-steel tube having an internal diameter of 0·125in. Analyses were carried out on two different 5ft.-long columns. The support was silane-treated Celite (Gas Chrom CLH, 100–120 mesh) and



Scheme 1. Procedure for the isolation of prenols and fatty acids of the prenyl esters of *Aspergillus*.

the stationary phases were 10% (w/w) polyethylene glycol adipate polyester and 12% (w/w) Apiezon L. The polyethylene glycol adipate column was operated at 197° with a flow rate of 60ml./min.; the Apiezon L column was used at 210° with a flow rate of 70ml./min. Fatty acids were identified by the method of James (1960), with additional results obtained from chromatography after bromination of the mixture of methyl esters (James & Martin, 1956). The percentage of each component of the fatty acid mixtures was calculated by assuming that the weight of ester eluted was proportional to the peak height on the recorder multiplied by the retention time (Carroll, 1961).

Spectroscopy. The i.r. spectra were determined on a Perkin–Elmer Infracord model 237 spectrometer as solvent-free films between rock-salt disks. The u.v. spectra were recorded with a Unicam SP.800 spectrophotometer.

RESULTS

Infrared spectroscopy. Comparison of the i.r. spectrum of the polyprenyl derivative (band 2 of the preparative thin-layer chromatogram of the combined 1%-E/P and 2%-E/P chromatography fractions) with that of the free prenol showed that, though all the characteristic bands of a predominantly *cis*-polyisoprenoid compound were present (Burgos, Hemming, Pennock & Morton, 1963), their intensity was decreased with respect to the other bands. Further, the primary alcohol absorption at 3310cm.$^{-1}$, which was present in the free prenol, was replaced by carbonyl absorptions at 1166cm.$^{-1}$ and 1729cm.$^{-1}$ in the derivative. The position of the C:O stretching frequency (1166cm.$^{-1}$) was consistent with the absence of a double bond $\alpha\beta$ to the carbonyl group (Bellamy, 1958) and also agreed with the structural studies described by Stone *et al.* (1967). The C:O stretching band at 1166cm.$^{-1}$ is characteristic of esters containing a long-chain fatty acid (Bellamy, 1958), and is similar to that shown by synthetic solanesyl palmitate (Rowland & Latimer, 1959). A weak band at 720cm.$^{-1}$ was also present; it arises from a rocking mode of CH_2 groups and is characteristic of a long paraffinic chain, $[CH_2]_n$. The i.r. evidence is thus in accord with the polyprenyl derivative's being a palmitoyl or similar ester derivative.

Gas–liquid chromatography. The results of gas–liquid-chromatographic analysis of the fatty acids

Table 1. *Detailed fatty acid content of the ester fractions from* Aspergillus fumigatus

Designation of fatty acid	Polyethylene glycol adipate (197°) retention time (min.)	Apiezon L (210°) retention time (min.)	Fatty acid content (%, w/w)		
			Prenyl ester	Sterol ester	Lipid
?	1·52	1·52	0·50	—	—
?	2·00	2·20	0·40	—	—
12:0	2·56	3·13	0·90	0·06	—
12:1	2·88	2·60	0·60	0·06	—
13:0*	3·28	4·40	0·60	0·06	0·01
13:1	3·80	3·80	0·70	0·08	—
14:0*	4·28	6·40	2·9	0·19	0·13
14:1	4·82	5·80	1·8	0·14	—
15:0*	5·64	9·45	3·1	0·30	0·79
15:1	6·28	8·48	1·9	0·14	—
16:0*	7·48	13·44	17·2	3·3	15·6
16:1	8·32	12·28	6·4	0·73	0·26
17:0*	10·00	20·1	3·2	0·53	2·5
17:1	11·14	17·36	1·9	0·62	1·0
18:0*	13·46	28·5	8·3	1·6	2·8
18:1	14·86	24·7	12·2	12·5	19·9
18:2	18·16	24·7	15·0	77·2	52·7
18:3	22·4	24·7	—	0·17	0·41
20:0*	24·3	61·3	1·9	0·18	0·25
20:1	26·7	53·7	0·70	0·09	0·25
20:2	32·0	52·5	1·0	0·36	0·75
20:3	35·5	52·5	0·80	0·06	—
20:3	39·0	52·5	0·50	0·02	—
22:0*	43·5	131·8	3·7	0·24	0·37
22:1	47·7	119·3	0·60	0·08	0·21
22:2	59·0	118·6	1·9	0·19	0·44
22:3	72·0	114·5	1·9	0·64	0·44
24:0*	78·5	288·7	9·9	0·53	1·2

* Fatty acids remaining after bromination.

Table 2. *Summary of the fatty acid content of* Aspergillus fumigatus *ester fractions*

	Fatty acid content (%, w/w)					
	Prenyl ester		Sterol ester		Lipid	
Saturated	51·7		7·0		23·6	
Mono-unsaturated	26·8		14·4		21·6	
Di-unsaturated	17·9	47·5	77·8	93·0	53·9	76·4
Tri-unsaturated	2·8		0·8		0·9	

Table 3. *Distribution of ubiquinone*-10 *and free and esterified prenol and ergosterol in subcellular fractions of* Aspergillus fumigatus

	Ubiquinone-10		Unesterified prenol		Prenyl ester		Unesterified ergosterol		Ergosteryl ester	
Cell fraction	(μg.)	(%)	(μg.)	(%)	(μg.)	(%)	(μg.)	(%)	(μg.)	(%)
Nuclei and cell debris	286	13·5	120	9·2	560	34·3	4320	24	720	12
Mitochondria	1468	67·8	1000	77·0	400	24·4	11060	61·3	3240	54
Microsomes	224	10·2	20	1·5	300	18·3	2500	13·8	1800	30
Supernatant	180	8·3	160	12·3	380	23·2	320	0·9	240	4

Table 4. *Distribution of free and esterified polyprenol in the cell fractions of pig liver and* Aspergillus fumigatus *as a percentage of the total in each tissue*

	Pig liver (Butterworth, 1964)		*A. fumigatus*	
Cell fraction	Free	Esterified	Free	Esterified
Nuclei and cell debris	13·2	52·7	9·2	34·3
Mitochondria	76·7	26·6	77·0	24·4
Microsomes	10·1	20·7	1·5	18·3
Supernatant	—	—	12·3	23·2

of the total lipid, ergosteryl ester and polyprenyl ester are correlated in Table 1 and summarized in Table 2.

Identification of the prenol moiety. Saponification of the polyprenyl ester derivative liberated a polyprenol preparation, which was shown by two-dimensional reversed-phase partition thin-layer chromatography to contain seven components, each of which stained with anisaldehyde to give an intensity of stain equal to that of the corresponding component of the naturally occurring free prenol mixture. The seven components of the mixtures from both sources had R_F values 0·49, 0·43, 0·38, 0·33, 0·29, 0·26 and 0·24, and corresponded to hexahydroprenols-18 to -24 in order of decreasing R_F values (see Stone *et al.* 1967). By using two-dimensional chromatography sufficient of the mixture could be chromatographed to show clearly the presence of the quantitatively minor components and still allow separation of the quantitatively major components.

Cell fractionation. The intracellular distribution of ubiquinone has been studied in various tissues, and in all cases the ubiquinone appeared to be concentrated in the mitochondrial fraction (Crane, 1965). Evidence has been presented for a small percentage of ubiquinone being associated with the debris and submitochondrial fractions (Jayaraman & Ramasarma, 1963), though it is clearly difficult to establish that this is definitely not of mitochondrial origin. The distribution of ubiquinone may therefore be taken as a useful parameter to determine the efficiency of cell fractionation.

In Table 3 it is shown that 67·8% of the ubiquinone in *Aspergillus* is concentrated in the mitochondrial fraction with approx. 10% in each of the debris, 'microsomal' and supernatant fractions. On the basis of ubiquinone content the cell fractionation of *Aspergillus* therefore appears to be reasonably good.

The distribution of prenol and sterol in *Aspergillus* is also shown in Table 3 and it is immediately

apparent that the polyprenyl ester and the free prenol are concentrated in different fractions. The mitochondria contain nearly 80% of the free prenol, whereas the polyprenyl ester appears to be fairly evenly distributed throughout the four fractions. Any contamination by mitochondria, or by mitochondrial fragments, of the non-mitochondrial fractions would therefore be expected to increase the proportion of free prenol in these fractions. As it is inevitable that such contamination occurred to a small extent, it is likely that almost all of the polyprenol in the debris, nuclei, 'microsomes' and supernatant is esterified.

DISCUSSION

Chromatography of the total lipid of *Aspergillus fumigatus* provided a preparation that after saponification was in all respects identical with the mixture of *Aspergillus* $\alpha\psi\omega$-hexahydropolyprenols. The unsaponified preparation had markedly different chromatographic properties from the polyprenol mixture and was much less polar. Its i.r. spectrum suggested that it consisted of polyprenols esterified with fatty acid(s). Transmethylation of the material liberated a number of methyl esters of fatty acids, and thus the evidence is in favour of the preparation being a mixture of *Aspergillus* $\alpha\psi\omega$-hexahydropolyprenyl fatty acid esters.

In the three lipid fractions of *Aspergillus fumigatus* analysed for fatty acid, palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2) formed the bulk of the fatty acid mixtures (Table 1). However, the relative proportions of these varied considerably in the three fractions. If one compares the polyprenyl ester with either the sterol ester or total lipid and then considers the saturated fatty acids and the unsaturated fatty acids, the results (Table 2) are most surprising. Whereas all but 7% of the fatty acids in sterol ester and all but 24% in the total lipid were unsaturated, over 50% of the fatty acid of polyprenyl ester was saturated. This predominance of saturated fatty acids, which was shown throughout the fatty acid constituents of the polyprenyl ester, is in agreement with the composition of solanesyl ester in flue-cured tobacco (Rowland & Latimer, 1959). There is also tentative evidence that dolichyl esters in pig liver may have more saturated fatty acids than the sterol esters in the same tissue (V. Walker & F. W. Hemming, unpublished work). These results suggest that the polyprenyl ester is of some significance to the tissue and is not merely a consequence of random esterification.

The intracellular distribution of free and esterified polyprenol was almost identical with that found in pig liver (Table 4). The distribution of free and esterified ergosterol was, however, rather unusual. In liver preparations, sterol ester normally occurs mainly in the supernatant and is considered to be the form in which the sterol is stored. In *Aspergillus*, however, most of the sterol (free and combined) was found in the mitochondria and very little at all was found in the supernatant fraction. The significance of this is uncertain, but in view of the results obtained for sterol biosynthesis in *Aspergillus* at different stages of growth (Stone & Hemming, 1967) it is likely that this distribution pattern is subject to change.

Polyprenols and polyprenyl esters thus do not appear to be associated in any way with sterol and sterol esters in intracellular distribution and therefore, probably, in function. Also, the evidence of some organization of distribution of polyprenol and polyprenyl esters, when considered with the evidence that polyprenols are selectively esterified with particular fatty acids, suggests that these long-chain isoprenoid alcohols perform a definite function within the tissue.

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