Biochem. J. (1971) **123**, 331–339
Printed in Great Britain

Isoprenoid Phenol and Quinone Precursors of Ubiquinones and Dihydroubiquinones [Ubiquinones (H_2)] in Fungi

By AH LAW, D. R. THRELFALL AND G. R. WHISTANCE
Department of Biochemistry and Agricultural Biochemistry, University College of Wales, Penglais, Aberystwyth, Cards. SY23 3DD, U.K.

(*Received 2 November* 1970)

1. Ten moulds and two yeasts were analysed for the presence of 2-polyprenylphenols, 2-polyprenyl(H_2)phenols, 6-methoxy-2-polyprenylphenols, 6-methoxy-2-polyprenyl(H_2)phenols, 6-methoxy-2-polyprenyl-1,4-benzoquinones, 6-methoxy-2-polyprenyl(H_2)-1,4-benzoquinones, 5-demethoxyubiquinones, 5-demethoxyubiquinones(H_2), ubiquinones and ubiquinones(H_2). 2. The organisms were found to be of three types: (*a*) those that contained only ubiquinones (*Aspergillus fumigatus* and *Penicillium brevi-compactum*) or ubiquinones(H_2) (*Alternaria solani*, *Claviceps purpurae* and *Penicillium stipitatum*); (*b*) those that contained 5-demethoxyubiquinones and ubiquinones (*Agaricus campestris*, *Aspergillus niger*, *Phycomyces blakesleeanus*, *Rhodotorula glutinis* and *Saccharomyces cerevisiae*) or 5-demethoxyubiquinones(H_2) and ubiquinones(H_2) (*Aspergillus quadrilineatus* and *Neurospora crassa*); (*c*) one that contained 2-decaprenyl(H_2)phenol,6-methoxy-2-decaprenyl(H_2)phenol,6-methoxy-2-decaprenyl(X-H_2)-1,4-benzoquinone, 5-demethoxyubiquinone-10(X-H_2) and ubiquinones(H_2) (*Aspergillus flavus*). 3. Studies were made on the biosynthesis of ubiquinones and ubiquinones(H_2) by *Asp. flavus*, *Phyc. blakesleeanus* and *S. cerevisiae*. These provided evidence that in *Phyc. blakesleeanus* 5-demethoxyubiquinone-9 is a precursor of ubiquinone-9 and that in *S. cerevisiae* 5-demethoxyubiquinone-6 is a precursor of ubiquinone-6. In addition they yielded results that may be interpreted as providing evidence that in *Asp. flavus* 6-methoxy-2-decaprenyl(X-H_2)-1,4-benzoquinone and 5-demethoxyubiquinone-10(X-H_2) are precursors of ubiquinone-10(X-H_2).

Daves, Friis, Olsen & Folkers (1966) and Friis, Nilsson, Daves & Folkers (1967) proposed pathways for the biosynthesis of ubiquinones from *p*-hydroxybenzoic acid in the photosynthetic bacterium *Rhodospirillum rubrum* (Scheme 1). Their proposals resulted from the isolation from *Rsp. rubrum* of a group of isoprenoid phenols and quinones with structural affinities to ubiquinones (Olsen *et al.* 1966; Friis, Daves & Folkers, 1966; Whistance, Threlfall & Goodwin, 1966; Friis *et al.* 1967) and the demonstration that in *Rsp. rubrum* two of the compounds, 2-decaprenylphenol and 6-methoxy-2-decaprenylphenol, are precursors of ubiquinone-10 (Parson & Rudney, 1965; Olsen *et al.* 1966). More recently, Whistance, Brown & Threlfall (1970) have isolated a number of similar phenols and quinones from the non-photosynthetic bacteria *Pseudomonas ovalis* Chester and *Proteus mirabilis*. Moreover, they have shown that, in addition to 2-polyprenylphenols and 6-methoxy-2-polyprenylphenols, 6-methoxy-2-polyprenyl-1,4-benzoquinones and 5-demethoxyubiquinones (6-methoxy-3-methyl-2-polyprenyl-1,4-benzoquinones) are precursors of ubiquinones in these organisms.

In the present paper we report the results of our investigations to determine whether polyprenylphenols and quinones of the type described above are intermediates in the biosynthesis of ubiquinones and ubiquinones(H_2) by fungi. A brief account of some aspects of this work has appeared previously (Ah Law, Threlfall & Whistance, 1970).

EXPERIMENTAL

Radiochemicals

L-[U-^{14}C]Tyrosine hydrochloride (12.6mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. *p*-Hydroxy[U-^{14}C]benzoic acid (9.8mCi/mmol) was prepared by alkaline fusion of L-[U-^{14}C]-tyrosine (Parson & Rudney, 1964; Whistance, Threlfall & Goodwin, 1967).

Biological methods

Source, growth and maintenance of organisms. Altenaria solani Herb. I.M.I. 46816, *Aspergillus flavus* Herb. I.M.I. 16145, *Aspergillus niger* Herb. I.M.I. 17454, *Aspergillus quadrilineatus* Herb, I.M.I. 89349, *Claviceps purpurae* Herb. I.M.I. 44613, *Neurospora crassa* Herb. I.M.I. 53240, *Penicillium brevi-compactum* Herb. I.M.I. 40225, *Peni-*

Pathway A

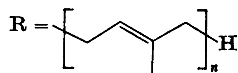
p-Hydroxybenzoic acid → 4-Carboxy-2-polyprenylphenol → 2-Polyprenylphenol → [6-Hydroxy-2-polyprenylphenol] → 6-Methoxy-2-polyprenylphenol → 6-Methoxy-2-polyprenyl-1,4-benzoquinone

Pathway B

2-Polyprenylphenol → 3-Methyl-2-polyprenyl-1,4-benzoquinone → 5-Demethoxyubiquinone

6-Methoxy-2-polyprenyl-1,4-benzoquinone → 5-Demethoxyubiquinone → 5-Hydroxy-6-methoxy-3-methyl-2-polyprenyl-1,4-benzoquinone → Ubiquinone

Scheme 1. Proposed pathways for the biosynthesis of ubiquinones from *p*-hydroxybenzoic acid in Gram-negative bacteria (after Daves *et al.* 1966; Friis *et al.* 1967), where



cillium stipitatum Herb. I.M.I. 89713 and *Phycomyces blakesleeanus* Herb. I.M.I. 22191 were obtained from the Commonwealth Mycological Institute, Kew, Surrey, U.K. *Aspergillus fumigatus* (from mouldy hay) was a gift from Mr A. Bye of this Department. *Rhodotorula glutinis* I.F.O. 559 was obtained from the Institute of Fermentation, Osaka, Japan. *Saccharomyces cerevisiae* (baker's yeast) was purchased as a compressed block from The Distillers Co. Ltd., Liverpool, U.K. *Agaricus campestris* (edible mushroom) was purchased locally.

Alt. solani, *Asp. flavus*, *Asp. fumigatus*, *Asp. niger*, *Asp. quadrilineatus*, *N. crassa* and *Pen. stipitatum* were grown for 3, 4 or 5 days at 28°C in shake (300rev./min; Gallenkamp orbital shaker) culture on the medium of Lavate & Bentley (1964). *C. purpurae* and *Phyc. blakesleeanus* were grown for 5 days at 28°C in shake (300rev./min) culture on the media of Castagnoli & Mantle (1966) and Goodwin & Lijinsky (1952) respectively. *Rt. glutinis* was grown for 5 days at 28°C in shake (300rev./min) culture on malt extract broth (Oxoid). In all cases the media were dispensed as 1-litre volumes in 2-litre conical flasks. *Pen. brevi-compactum* was grown for 14 days at 28°C in static culture on the medium of Lavate & Bentley (1964). The medium was dispensed as 150ml volumes in Roux bottles.

All organisms were maintained by periodic subculture on Czapek-Dox agar (Oxoid) slopes.

Harvesting of organisms. Moulds were harvested by straining through cheese-cloth. *Rt. glutinis* was harvested by centrifugation in a Sharples Super centrifuge.

Analytical methods

Extraction and preliminary fractionation of lipids. In most cases the organisms were saponified in the presence of pyrogallol, as described by Lavate, Dyer, Springer &

Bentley (1965) and Spiller, Threlfall & Whistance (1968). The unsaponifiable lipids were recovered from the saponification mixtures by the procedure of Spiller *et al.* (1968). In the experiment with *p*-hydroxy[U-^{14}C]benzoic acid designed to determine whether 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols and 6-methoxy-2-polyprenyl-1,4-benzoquinones occur in *S. cerevisiae*, the yeast cells (2g/ml) were suspended in 0.05M-potassium phosphate buffer, pH 5.7, and disintegrated by exposure for 1h at 12°C to the output of an MSE 500W ultrasonic disintegrator at an average frequency of 20000Hz, 1.6C of output energy being used/20ml of cell suspension. The resulting broken-cell suspension was refluxed for 20min with 30vol. of ethanol, after which the debris was removed by centrifugation and the lipids were extracted into diethyl ether (Spiller *et al.* 1968). In similar experiments with *Asp. flavus* and *Phyc. blakesleeanus* the fungal cells were freeze-dried and their lipids were extracted by refluxing for 6h with 20vol. of light petroleum (b.p. 40–60°C).

After precipitation of most of the sterol in cold (−20°C) light petroleum (b.p. 40–60°C) (Spiller *et al.* 1968), the lipids were chromatographed on columns of acid-washed alumina (Brockmann grade III; M. Woelm, Eschage, Germany) developed by stepwise elution with 0.25%-, 1%-, 3%-, 5%-, 8%-, 12%- and 20%-(v/v) E/P* (Threlfall & Goodwin, 1967). In this chromatographic system 6-methoxy-2-polyprenylphenols and 6-methoxy-2-polyprenyl(H_2)phenols are eluted by 1% E/P, 2-polyprenylphenols and 2-polyprenyl(H_2)phenols by 3% E/P, ubiquinones, ubiquinones(H_2) and small amounts of 5-demethoxyubiquinones and 5-demethyoxyubiquinones(H_2) by 5% E/P and 6-methoxy-2-polyprenyl-1,4-benzoquinones, 6-methoxy-2-polyprenyl(H_2)-1,4-benzoquinones and most of the 5-demethoxyubiquinones and 5-demethoxyubiquinones(H_2) by 8% E/P.

Detection and purification of quinones. All quinones were detected and purified by a combination of adsorptive and reversed-phase chromatography on thin layers of Silica gel G (E. Merck A-G., Darmstadt, Germany) (Table 1 and Whistance, Brown & Threlfall, 1969*a*, 1970).

Characterization of quinones. (1) 5-Demethoxyubiquinones. These were characterized by column chromatography and t.l.c. (adsorptive, reversed-phase and Ag^+ ion) (Table 1), by u.v. spectroscopy, by mass spectrometry and by the u.v. spectra of their chromenols (Daves, Wilczynski, Friis & Folkers, 1968; Whistance *et al.* 1969*a*, 1970).

(2) Ubiquinones. These were characterized by column chromatography and t.l.c. (adsorptive, reversed-phase and Ag^+ ion) (Table 1), by u.v. spectroscopy, by m.p. and by mass spectrometry (Isler *et al.* 1961; Daves *et al.* 1966; Whistance, Dillon & Threlfall, 1969*b*).

(3) 6-Methoxy-2-decaprenyl(X-H_2)-1,4-benzoquinone. This was characterized by column chromatography and t.l.c. (adsorptive, reversed-phase and Ag^+ ion) (Table 1) by u.v. spectroscopy, by mass spectrometry and by the fact that in *Asp. flavus* it is a precursor of ubiquinone-10(X-H_2) (Ah Law *et al.* 1970).

(4) 5-Demethoxyubiquinones(H_2). These were characterized by column chromatography and t.l.c. (adsorptive, reversed-phase and Ag^+ ion) (Table 1), by u.v. spectro-

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

Table 1. *T.l.c. systems used for the detection, characterization and purification of isoprenoid quinones*

The quinones were purified by using the systems *A*, *B* and *C* in the sequence *A*, *C*, *B*.

Compounds	R_F values on silica-gel G layers: System *A* [benzene–chloroform (1:1, v/v)]	R_F values on silica-gel G layers: System *B* (benzene)	Homologue: *N*	Homologue: *n*	R_F values: $AgNO_3$-impregnated silica-gel G layers developed with acetone–butan-2-one (1:4, v/v) Ubiquinone-*N*	R_F values: System *C* [paraffin-impregnated silica-gel G layers developed with aq. 90% (v/v) acetone] 6-Methoxy-2-*n*-1,4-benzoquinone	System *C*: 5-Demethoxy-ubiquinone-*N*	System *C*: Ubiquinone-*N*
6-Methoxy-2-polyprenyl-1,4-benzo-			6		—	—	0.60	0.53
quinones	0.40	0.10	7		—	—	0.54	0.45
			7(H_2)		—	—	—	0.37
6-Methoxy-2-polyprenyl(H_2)-1,4-			8	Octaprenyl	0.52	0.50	0.47	0.37
benzoquinones	0.40	0.10	8(H_2)		0.55	—	—	0.29
			9	Nonaprenyl	0.50	0.43	0.38	0.29
5-Demethoxyubiquinones	0.45	0.15	9(H_2)		0.53	—	0.29	0.20
5-Demethoxyubiquinones(H_2)	0.45	0.15	10		0.48	—	0.29	0.20
Ubiquinones	0.53	0.23	10(X-H_2)	Decaprenyl-(X-H_2)	0.51	0.29	0.22	0.11
Ubiquinones(H_2)	0.53	0.23	11		0.46	—	—	0.11

scopy and in the cases of 5-demethoxyubiquinones-9(H_2) and -10(H_2) by mass spectrometry and by the u.v. spectra of their chromenols (Ah Law *et al.* 1970). The positions of the saturated isoprene units are discussed in the Results section.

(5) Ubiquinones(H_2). These were characterized by column chromatography and t.l.c. (adsorptive, reversed-phase and Ag^+ ion) (Table 1), by u.v. spectroscopy, by m.p. and by mass spectrometry (Gale, Arison, Trenner, Page & Folkers, 1963; Lavate & Bentley, 1964; Lavate *et al.* 1965). The position of the saturated isoprene unit in the side chains of the ubiquinones-10(H_2) from *Asp. flavus*, *Asp. quadrilineatus* and *N. crassa* was determined by n.m.r. spectrometry. In each case the n.m.r. spectrum was identical with those reported for ubiquinone-10-(X-H_2) from *Giberella fujikuroi* and *Pen. stipitatum* (Gale *et al.* 1963; Lavate *et al.* 1965).

Detection of phenols. The appropriate column fractions were chromatographed as bands against authentic samples of the nona [isolated from *Ps. ovalis* Chester (Whistance *et al.* 1970)] and deca [isolated from *Rsp. rubrum* (Whistance *et al.* 1966)] homologues of 2-polyprenylphenol (R_F 0.52) and 6-methoxy-2-polyprenylphenol (R_F 0.65) on thin layers of Rhodamine 6G-impregnated silica gel G developed with benzene. In this system 2-polyprenyl(H_2)-phenols and 6-methoxy-2-polyprenyl(H_2)phenols migrate with the same R_F values as 2-polyprenylphenols and 6-methoxy-2-polyprenylphenols respectively. The areas of the developed plates between the markers [located as pink spots under u.v. light (Whistance *et al.* 1969*b*)] were removed and the compounds present were eluted with diethyl ether. These were then chromatographed as spots or short bands with marker phenols on paraffin-impregnated silica gel G layers developed with aq. 95% (v/v) acetone. [In this system 2-polyprenyl(H_2)-phenols and 6-methoxy-2-polyprenyl(H_2)phenols migrate more slowly than the corresponding unsaturated forms, e.g. 2-decaprenyl(H_2)phenol and 6-methoxy-2-decaprenyl-(H_2)phenol migrate with the same R_F values as 2-undecaprenylphenol (R_F 0.39) and 6-methoxy-2-undecaprenylphenol (R_F 0.25) respectively (cf. 2-decaprenylphenol, R_F 0.47, and 6-methoxy-2-decaprenylphenol, R_F 0.32)]. After removal of the solvent the plates were sprayed with Gibbs reagent and heated at 90°C (Whistance *et al.* 1969*b*). Under these conditions 2-polyprenylphenols and 2-polyprenyl(H_2)phenols appear turquoise blue and 6-methoxy-2-polyprenylphenols and 6-methoxy-2-polyprenyl(H_2)-phenols appear blue–violet. Gibbs reagent is extremely sensitive to isoprenoid phenols (Whistance *et al.* 1969*b*), and through its use the presence of as little as $0.5\mu g$ of material can be detected.

In studies with *p*-hydroxy[U-^{14}C]benzoic acid designed to determine whether 2-polyprenylphenols, 2-polyprenyl-(H_2)phenols, 6-methoxy-2-polyprenylphenols and 6-methoxy-2-polyprenyl(H_2)phenols occur in *Asp. flavus*, *S. cerevisiae* and *Phyc. blakesleeanus* $100\mu g$ of a mixture of 2-undecyl- through to 2-hexa-prenylphenol (isolated from *Rsp. rubrum* and *Ps. ovalis* Chester) were added to the cells before extraction. After extraction the phenols were purified and detected as described above, and the layers were examined for ^{14}C radioactivity by radioautography.

Syntheses of 7-demethoxyubichromenols and 7-demethoxyubichromenols(H_2) from 5-demethoxyubiquinones and 5-demethoxyubiquinones(H_2). These were carried out by refluxing the quinones in pyridine (Whistance *et al.* 1970).

Quantitative determination of quinones. All quinones were determined spectrophotometrically by measuring the fall in extinction at their wavelength of maximum u.v. absorption when an ethanolic solution of the quinone was treated with $NaBH_4$ under the conditions described by Threlfall & Goodwin (1967). In all cases the $\epsilon_{ox.} - \epsilon_{red.}$ value for ubiquinones, 12 250 (Lawson, Threlfall, Glover & Morton, 1961), was used for the calculations.

U.v. spectroscopy. U.v. spectra were determined in a Unicam SP. 800 spectrophotometer.

Mass spectrometry. Mass spectra were determined in an MS-9 (A.E.I. Ltd.) mass spectrometer.

N.m.r. spectrometry. N.m.r. spectra were determined at 60 Hz in a Varian spectrometer. CCl_4 was used as the solvent and tetramethylsilane as the internal standard.

Radioassay. The methods employed have been reported by Threlfall, Whistance & Goodwin (1968) and Whistance *et al.* (1967). All counts were corrected for background and instrument efficiency.

Solvents. Light petroleum (b.p. 40–60°C), benzene (A.R.) and diethyl ether were dried over sodium wire and redistilled; ether was redistilled over reduced iron immediately before use. Spectroscopic solvents were of the appropriate grade; all other solvents were of A.R. or equivalent grade.

RESULTS

Analyses of moulds and yeasts for 2-polyprenylphenols, 2-polyprenyl(H_2)-phenols, 6-methoxy-2-polyprenylphenols, 6-methoxy-2-polyprenyl(H_2)-phenols, 6-methoxy-2-polyprenyl-1,4-benzoquinones, 6-methoxy-2-polyprenyl(H_2)-1,4-benzoquinones, 5-demethoxyubiquinones and 5-demethoxyubiquinones-(H_2). The organisms examined were the ubiquinone-containing moulds *Ag. campestris*, *Asp. fumigatus*, *Asp. niger*, *Pen. brevi-compactum* and *Phyc. blakesleeanus* (Packter & Glover, 1960; Lavate & Bentley, 1964; Spiller *et al.* 1968), the ubiquinone-10(H_2)-containing moulds *Alt. solani*, *Asp. quadrilineatus*, *C. purpurae*, *N. crassa* and *Pen. stipitatum* (Lavate & Bentley, 1964; Lavate *et al.* 1965) and the ubiquinone-containing yeasts *S. cerevisiae* and *Rt. glutinis* (Lester & Crane, 1959). Reference to Tables 2, 3 and 4 shows that as far as their quinone complements are concerned the organisms are of three distinct types: (*a*) those that contain only ubiquinones or ubiquinones(H_2) (Table 2), (*b*) those that contain 5-demethoxyubiquinones and ubiquinones or 5-demethoxyubiquinones(H_2) and ubiquinones(H_2) (Table 3) and (*c*) one that contains 6-methoxy-2-decaprenyl(H_2)-1,4-benzoquinone, 5-demethoxyubiquinone-10(H_2) and ubiquinones(H_2) (Table 4). None of the organisms contained detectable amounts of 2-polyprenylphenols, 2-polyprenyl(H_2)phenols, 6-methoxy-2-polyprenylphenols or 6-methoxy-2-polyprenyl(H_2)-phenols (however, see below). In *S. cerevisiae* a *p*-benzoquinone was detected in the

Table 2. *Analyses of organisms which contain only ubiquinones or ubiquinone*(H_2)

Organism	Age (days)	Wet wt. (g)	Ubiquinone	
			Homologue	(mg)
Alt. solani	5	700	10(H_2)	10.94
			9(H_2)	0.19
			8(H_2) and 7(H_2)	Present
			Ubichromenol-9(H_2) present also	
Asp. fumigatis	3	600	10	11
			9	0.13
			8	0.02
			7	Present
Cl. purpurae	5	184	10(H_2)	11.75
			9(H_2)	Present
Pen. brevi-compactum	14	211	9	13.22
			8	0.67
			7	0.06
			Ubichromenol-8 present also	
Pen. stipitatum	4	500	10(X-H_2)*	2.64

* Characterized by Lavate *et al.* (1965).

Table 3. *Analyses of organisms that contain 5-demethoxyubiquinones and ubiquinones or 5-demethoxyubiquinones*(H_2) *and ubiquinones*(H_2)

Organism	Age (days)	Wet wt. (g)	Ubiquinone		5-Demethoxyubiquinone	
			Homologue	(mg)	Homologue	(mg)
Ag. campestris	Purchased locally	1000	9	3.69	9	0.01
			8	Present		
Asp. niger	3	360	9	4.53	9	0.06
			8	0.03		
			7	Present		
Asp. quadrilineatus	5	660	10(X-H_2)	8.23	10(H_2)	0.16
			9(H_2)	0.4		
			10	Present		
N. crassa	4	350	10(X-H_2)	5	10(H_2)	0.08
			9(H_2)	0.06		
R. glutinis	5	191	9	3.6	Present	
			8	0.21		
S. cerevisiae	Purchased from The Distillers Co. Ltd.	960	6	66	6*	1.5
			5	Present		
			4	Present		

* Previously reported by Imamoto & Senoh (1968).

8% E/P fraction from column chromatography that was less polar than ubiquinone-6 and 5-demethoxyubiquinone-6 on adsorptive and reversed-phase t.l.c. and that absorbed u.v. light ($\lambda_{max.}$ 246nm and shoulder at 255nm in cyclohexane; $\lambda_{max.}$ 248nm in ethanol, changing to 293nm on treatment with sodium borohydride). Only small amounts of the compound were present (it could not always be detected) and so it was not possible to determine its structure.

The position of the saturated isoprene unit in the side chains of 6-methoxy-2-decaprenyl(H_2)-1,4-benzoquinone, 5-demethoxyubiquinone-9(H_2) and 5-demethoxyubiquinone-10(H_2) proved to be difficult to locate, because the small amounts of material available precluded the use of n.m.r. spectrometry. In the cases of 6-methoxy-2-decaprenyl(H_2)-1,4-benzoquinone and 5-demethoxyubiquinone-10(H_2) from *Asp. flavus* it was shown that both are precursors of ubiquinone-10(X-H_2) (Table 6), which

Table 4. *Analysis of isoprenoid quinone content of 3-day-old* Asp. flavus (1.4 kg *wet wt.*)

Compound	Amount (mg)
Ubiquinone-10(X-H_2)	26
Ubiquinone-9(H_2)	0.5
Ubiquinone-8(H_2)	Present
Ubiquinone-10	Present
Demethoxyubiquinone-10(X-H_2)*	0.48
6-Methoxy-2-decaprenyl(X-H_2)-1,4-benzoquinone*	0.14

* The saturation was assigned to the isoprene unit furthest from the nucleus because these compounds are precursors of ubiquinone-10(X-H_2).

established that it is their tenth unit that is saturated. The position of the saturated units in 5-demethoxyubiquinone-9(H_2) and -10(H_2) from *N. crassa* and 5-demethoxyubiquinone-10(H_2) from *Asp. quadrilineatus* were not determined. However, the fact that both organisms contain ubiquinone-10(X-H_2) suggests that, as in *Asp. flavus*, it is their terminal unit that is saturated.

Analyses of Asp. flavus, Phyc. blakesleeanus *and* S. cerevisiae *for 2-polyprenylphenols, 2-polyprenyl-(H_2)phenols, 6-methoxy-2-polyprenylphenols, 6-methoxy-2-polyprenyl(H_2)phenols and 6-methoxy-2-polyprenyl-1,4-benzoquinones biosynthesized from* p-*hydroxy*[U-^{14}C]*benzoic acid.* In the analyses of *Asp. flavus* (Table 4), *Phyc. blakesleeanus* (Table 3) and *S. cerevisiae* (Table 3) described above no evidence was found of the presence of 2-polyprenylphenols, 2-polyprenyl(H_2)phenols, 6-methoxy-2-polyprenylphenols, 6-methoxy-2-polyprenyl(H_2)-phenols or 6-methoxy-2-polyprenyl-1,4-benzoquinones, although all three organisms contained a homologue of either 5-demethoxyubiquinone or 5-demethoxyubiquinone(H_2) [*Asp. flavus* also contained 6-methoxy-2-decaprenyl(X-H_2)-1,4-benzoquinone]. To investigate further the apparent absence from these organisms of 2-polyprenylphenols, 2-polyprenyl(H_2)phenols, 6-methoxy-2-polyprenylphenols, 6-methoxy-2-polyprenyl(H_2)-phenols and 6-methoxy-2-polyprenyl-1,4-benzoquinones labelling experiments with *p*-hydroxy-[U-^{14}C]benzoic acid were carried out. This it was considered would be more effective than the usual methods, i.e. staining with Gibbs reagent and detection under u.v. light, in detecting submicrogram amounts of the phenols and quinones. In these experiments milder procedures than usual were used for extracting the lipids (see the Experimental section); this was to minimize any risk of degradation occurring.

(*a*) Experiment with *Asp. flavus*. Three-day-old cells (350g fresh wt.; 30g dry wt.) were suspended in 2 litres of 0.1M-potassium phosphate buffer, pH 6.0, containing 6μCi of *p*-hydroxy[U-^{14}C]-benzoic acid, and incubated for 5h at 28°C in a Gallenkamp orbital shaker (300rev./min). At the end of the incubation the cells were freeze-dried and extracted with light petroleum (b.p. 40–60°C) in the presence of carrier 2-polyprenylphenols and 6-methoxy-2-polyprenylphenols.

Examination of the lipid extract by column chromatography showed that most (96%; 1428400 d.p.m.) of the radioactivity in the column chromatographic fractions was in those containing ubiquinones(H_2), 5-demethoxyubiquinones(H_2) and 6-methoxy-2 polyprenyl(H_2)-1,4-benzoquinones (5%- and 8%-E/P fractions). However, radioactivity (1840d.p.m. and 36600d.p.m. respectively) was also associated with the fractions that would contain 6-methoxy-2-polyprenyl(H_2)phenols and 2-polyprenyl(H_2)phenols should they be present (1%- and 3%-E/P fractions). Fractionation of the 1%- and 3%-E/P fractions by adsorptive and reversed-phase t.l.c. established that all the radioactivity in the 1%-E/P fraction and 10% of that in the 3%-E/P fraction migrated with the R_F values expected for 6-methoxy-2-polyprenyl(H_2)-phenols and 2-polyprenyl(H_2)-phenols respectively [the rest of the radioactivity in the 3%-E/P fraction migrated with ubiquinones(H_2)]. In each case more than 90% of the radioactivity migrated as the decaprenyl(H_2) homologue.

(*b*) Experiment with *Phyc. blakesleeanus*. Three-day-old cells (300g fresh wt.; 25g dry wt.) were incubated for 5h with 6μCi of *p*-hydroxy[U-^{14}C]-benzoic acid under the conditions used in the experiment with *Asp. flavus*. At the end of the incubation the cells were freeze-dried and extracted with light petroleum (b.p. 40-60°C) in the presence of carrier 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols and 6-methoxy-2-nonaprenyl-1,4-benzoquinone (50μg, isolated from *Ps. ovalis* Chester).

Fractionation of the lipid extract by column chromatography showed that most (95%; 1640000 d.p.m.) of the radioactivity in the column-chromatographic fractions was in those containing ubiquinones (19.4μmol; 1600000d.p.m.) and 5-demethoxyubiquinones (0.8μmol; 240000d.p.m.) (5%- and 8%-E/P fractions). Radioactivity was also present in the 3%-E/P fraction (50000d.p.m.), but the 1%-E/P fraction was not radioactive, showing that no 6-methoxy-2-polyprenyl[*ring*-^{14}C]-phenols were present. Adsorptive and reversed-phase t.l.c. of the 3%-, 5%- and 8%-E/P fractions established that 2-polyprenyl[*ring*-^{14}C]phenols and 6-methoxy-2-polyprenyl-1,4-[*ring*-^{14}C]benzoquinones were absent, the radioactivity in the fractions being associated entirely with ubiquinones and 5-demethoxyubiquinones.

(*c*) Experiment with *S. cerevisiae*. Cells (30g fresh wt.) were suspended in 50ml of 0.05M-potassium phosphate buffer, pH 5.7, containing 1.2% (w/v) of glucose and 2μCi of *p*-hydroxy-[U-^{14}C]benzoic acid, and incubated for 3h at 28°C in a Gallenkamp orbital shaker (200rev./min). On completion of the incubation the cells were disintegrated by sonication, after which the lipids were extracted in the presence of carrier 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols and 6-methoxy-2-nonaprenyl-1,4-benzoquinone (50μg; isolated from *Ps. ovalis* Chester) from the broken-cell suspension with hot ethanol and fractionated by column chromatography.

As in the case of *Phyc. blakesleeanus*, ubiquinones (3.7μmol; 44720 d.p.m.) and 5-demethoxyubiquinones (0.09 μmol; 16400 d.p.m) were highly labelled, but no ^{14}C-labelled 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols or 6-methoxy-2-polyprenyl-1,4-benzoquinones could be detected.

Conversion of 5-demethoxyubiquinones into ubiquinones by Phyc. blakesleeanus *and* S. cerevisiae *and of 6-methoxy-2-decaprenyl(H_2)-1,4-benzoquinone and 5-demethoxyubiquinone-10(H_2) into ubiquinone-10(X-H_2) by* Asp. flavus. To obtain evidence that 5-demethoxyubiquinones are precursors of ubiquinones in *Phyc. blakesleeanus* and *S. cerevisiae* and that 5-demethoxyubiquinone-10(H_2) and 6-methoxy-2-decaprenyl(H_2)-1,4-benzoquinone are precursors of ubiquinone-10(X-H_2) in *Asp. flavus* three similar pulse labelling experiments with *p*-hydroxy[U-^{14}C]benzoic acid were carried out. In the experiments with *Phyc. blakesleeanus* and *S. cerevisiae* it was found that when cells that had been preincubated with *p*-hydroxy[U-^{14}C]benzoic acid were incubated with unlabelled *p*-hydroxybenzoic acid the total radioactivity contents of the 5-demethoxyubiquinones decreased (Table 5). Moreover, it was found that these decreases could be accounted for almost entirely by the increases in the total radioactivity contents of the ubiquinones (Table 5), findings in keeping with a precursor–product relationship between the two groups of compounds. In the experiment with *Asp. flavus* the results obtained were not so clear cut. Thus, although there were marked decreases in the specific radioactivities and total radioactivity contents of 6-methoxy-2-decaprenyl(H_2)-1,4-benzoquinone and 5-demethoxyubiquinone-10(H_2) when cells which had been preincubated with *p*-hydroxy-[U-^{14}C]benzoic acid were incubated with unlabelled *p*-hydroxybenzoic acid (Table 6), the corresponding rise in the total radioactivity content of ubiquinone-10(X-H_2) was too small to be experimentally significant, i.e., 5% of the total radioactivity (Table 6). However, the fact that the small increase in the total radioactivity content of ubiquinone-10(X-H_2) coincides with decreases in the specific

Table 5. *Conversion of 5-demethoxyubiquinones into ubiquinones by* Phyc. blakesleeanus *and* S. cerevisiae

In the experiment with *Phyc. blakesleeanus* 4-day-old cells (407g fresh wt.) were suspended in 3 litres of 0.1M-potassium phosphate buffer, pH 6.0, containing 5μCi of *p*-hydroxy[U-^{14}C]benzoic acid, and incubated in a New Brunswick fermenter at 28°C with aeration (10 litres/min) and stirring (300rev./min) for 4h. At the end of the incubation half the cells were taken for analysis. The remaining cells were washed with buffer, resuspended in 10 litres of 0.1M-potassium phosphate buffer, pH 6.0, containing 3mmol of unlabelled *p*-hydroxybenzoic acid and 0.6mol of glucose and incubated in the fermenter for a further 20h before analysis. In the experiment with *S. cerevisiae* two batches of cells (480g fresh wt./batch) were each suspended in 1.5 litres of 0.1M-potassium phosphate buffer, pH 5.7 (in a 5 litre conical flask), containing 2μCi of *p*-hydroxybenzoic acid and aerated (2 litres/min) for 3h at room temperature. At the end of this time the cells from one batch were taken for analysis. The cells from the other batch were washed with buffer, resuspended in 3 litres of 0.1M-potassium phosphate buffer, pH 5.7 (in a 5 litre flask), containing 500μmol of unlabelled *p*-hydroxybenzoic acid and 0.6mmol of glucose and aerated (by bubbling air through at 4 litres/min from a perforated coil laying on the bottom of the flask) for a further 5h at room temperature before analysis.

	Cells incubated with *p*-hydroxy-[U-^{14}C]benzoic acid			Cells incubated with *p*-hydroxy-[U-^{14}C]benzoic acid followed by *p*-hydroxybenzoic acid		
	Amount (mg)	Sp. radio-activity (d.p.m./mg)	Total radio-activity (d.p.m.)	Amount (mg)	Sp. radio-activity (d.p.m./mg)	Total radio-activity (d.p.m.)
Phyc. blakesleeanus						
5-Demethoxyubiquinone-9	0.4	110000	44000	0.36	15000	5400
Ubiquinone-9	11.9	52200	621000	15	43950	659000
S. cerevisiae						
5-Demethoxyubiquinone-6	0.75	260000	195000	0.7	29250	20475
Ubiquinone	31	3906	121200	33.5	9160	307000

Table 6. *Conversion of 6-methoxy-2-decaprenyl(H_2)-1,4-benzoquinone and 5-demethoxyubiquinone-10(H_2) into ubiquinone-10(X-H_2) by* Asp. flavus

Three-day-old cells (250g fresh wt.) were suspended in 2 litres of 0.1M-potassium phosphate buffer, pH 6.0, containing 8 μCi of *p*-hydroxy[U-^{14}C]benzoic acid and incubated for 4h at 28°C in a Gallenkamp orbital shaker (300 rev./min). At the end of the incubation half the cells were taken for analysis. The remaining cells were washed with buffer, resuspended in 4 litres of 0.1M-potassium phosphate buffer, pH 6.0, containing 3 mmol of unlabelled *p*-hydroxybenzoic acid, and incubated for a further 20h at 28°C with vigorous aeration (by bubbling air through a polythene coil at 4 litres/min as in the case of *S. cerevisiae*) before analysis.

	Cells incubated with *p*-hydroxy-[U-^{14}C]benzoic acid			Cells incubated with *p*-hydroxy-[U-^{14}C]benzoic acid followed by *p*-hydroxybenzoic acid		
	Amount (μg)	Sp. radio-activity (d.p.m./mg)	Total radio-activity (d.p.m.)	Amount (μg)	Sp. radio-activity (d.p.m./mg)	Total radio-activity (d.p.m.)
6-Methoxy-2-decaprenyl(H_2)-1,4-benzoquinone	12	450000	5400	13	71400	928
5-Demethoxyubiquinone-10-(H_2)	40	228000	9120	39	90125	3515
Ubiquinone-10(X-H_2)	2038	120000	285500	2400	125000	300000

radioactivities and total radioactivity contents of 6-methoxy-2-decaprenyl(H_2)-1,4-benzoquinone and 5-demethoxyubiquinone-10(H_2) is compatible with the interpretation that in *Asp. flavus* 6-methoxy-2-decaprenyl(H_2)-1,4-benzoquinone and 5-demethoxyubiquinone-10(H_2) are probably precursors of ubiquinone-10(X-H_2).

In the experiment with *Asp. flavus*, although it was not possible to demonstrate a transfer of radioactivity from 2-decaprenyl(H_2)phenol and 6-methoxy-2-decaprenyl(H_2)phenol to ubiquinone-10(X-H_2), the amounts of radioactivity present in these compounds after incubation of the cells with *p*-hydroxy[U-^{14}C]benzoic acid (208 d.p.m. and 410 d.p.m. respectively) fell to almost zero when the cells were incubated for a further period of time with unlabelled *p*-hydroxybenzoic acid.

DISCUSSION

The results of the studies with *Asp. flavus* (Tables 4 and 6) may be interpreted as providing evidence that in this organism the pathway *p*-hydroxybenzoic acid → 2-decaprenyl(X-H_2)phenol → 6-methoxy-2-decaprenyl(X-H_2)phenol → 6-methoxy-2-decaprenyl(X-H_2)-1,4-benzoquinone → 5-demethoxyubiquinone-10(X-H_2) → ubiquinone-10(X-H_2) is operative for the biosynthesis of ubiquinone-10(X-H_2). This is the first report of information relating to the possible identities of the intermediates in the biosynthesis of ubiquinone-10(X-H_2) from *p*-hydroxybenzoic acid in Nature.

The results of the studies with the other fungi allow no definite conclusions to be drawn about the nature of the pathways by which the organisms synthesize ubiquinones or ubiquinones(H_2). In seven out of the eleven organisms either 5-demethoxyubiquinones or 5-demethoxyubiquinones(H_2) were detected (Tables 2 and 3). Moreover, in two of them, *Phyc. blakesleeanus* and *S. cerevisiae* (the others were not investigated), 5-demethoxyubiquinones were shown to be precursors of ubiquinones (Table 5). The problem is complicated, however, by the apparent absence from all the organisms of polyprenylphenols, 2-polyprenyl(H_2)phenols, 6-methoxy-2-polyprenylphenols, 6-methoxy-2-polyprenyl(H_2)phenols, 6-methoxy-2-polyprenyl-1,4-benzoquinones and 6-methoxy-2-polyprenyl(H_2)-1,4-benzoquinones, and from those organisms in which they could not be detected of 5-demethoxyubiquinones and 5-demethoxyubiquinones(H_2) also. If these compounds are present, but in amounts too small to be detected by our analytical procedures, then it seems likely that the pathways outlined above and in Scheme 1 are operative. If, on the other hand, they are absent, then alternative pathways must exist. Clearly further experimentation is required to clarify this point.

This work was supported by a grant from the Science Research Council. A.L. was in receipt of financial support from the British Council. We thank Dr J. A. Ballantine (University College of Swansea) for the mass-spectral determinations, Dr J. F. Pennock (University of Liverpool) for the n.m.r. spectral determinations and Miss Marian E. Williams for technical assistance.

REFERENCES

Ah Law, Threlfall, D. R. & Whistance, G. R. (1970). *Biochem. J.* **117**, 799.

Castagnoli, N. & Mantle, P. G. (1966). *Nature, Lond.*, **211**, 859.

Daves, G. D., jun., Friis, P., Olsen, R. K. & Folkers, K. (1966). *Vitams Horm.* **24**, 427.

Daves, G. D., jun., Wilczynski, J. J., Friis, P. & Folkers, K. (1968). *J. Am. chem. Soc.* **90**, 5587.

Friis, P., Daves, G. D., jun. & Folkers, K. (1966). *J. Am. chem. Soc.* **88**, 4754.

Friis, P., Nilsson, J. L. G., Daves, G. D., jun. & Folkers, K. (1967). *Biochem. biophys. Res. Commun.* **28**, 324.

Gale, P. H., Arison, B. H., Trenner, N. R., Page, A. C. & Folkers, K. (1963). *Biochemistry, Easton,* **2**, 196.

Goodwin, T. W. & Lijinsky, W. (1952). *Biochem. J.* **50**, 268.

Imamoto, S. & Senoh, S. (1968). *J. chem. Soc. Japan, Pure Chem. Sect.*, **89**, 316.

Isler, O., Rüegg, R., Langemann, A., Schudel, P., Ryser, G. & Wursch, J. (1961). In *Ciba Found. Symp.: Quinones and Electron Transport*, p. 79. Ed. by Wolstenholme, G. E. & O'Connor, C. M. London: J. and A. Churchill Ltd.

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

Lavate, W. V., Dyer, J. R., Springer, C. M. & Bentley, R. (1965). *J. biol. Chem.* **240**, 524.

Lawson, D. E. M., Threlfall, D. R., Glover, J. & Morton, R. A. (1961). *Biochem. J.* **79**, 201.

Lester, R. L. & Crane, F. L. (1959). *J. biol. Chem.* **234**, 2169.

Olsen, R. K., Daves, G. D., jun., Moore, H. W., Folkers, K., Parson, W. W. & Rudney, H. (1966). *J. Am. chem. Soc.* **88**, 5919.

Packter, N. M. & Glover, J. (1960). *Nature, Lond.*, **187**, 413.

Parson, W. W. & Rudney, H. (1964). *Proc. natn. Acad. Sci. U.S.A.* **51**, 444.

Parson, W. W. & Rudney, H. (1965). *Proc. natn. Acad. Sci. U.S.A.* **53**, 599.

Spiller, G. H., Threlfall, D. R. & Whistance, G. R. (1968). *Archs. Biochem. Biophys.* **125**, 786.

Threlfall, D. R. & Goodwin, T. W. (1967). *Biochem. J.* **103**, 573.

Threlfall, D. R., Whistance, G. R. & Goodwin, T. W. (1968). *Biochem. J.* **106**, 107.

Whistance, G. R., Brown, B. S. & Threlfall, D. R. (1969*a*). *Biochim. biophys. Acta*, **176**, 895.

Whistance, G. R., Brown, B. S. & Threlfall, D. R. (1970). *Biochem. J.* **117**, 119.

Whistance, G. R., Dillon, J. F. & Threlfall, D. R. (1969*b*). *Biochem. J.* **111**, 461.

Whistance, G. R., Threlfall, D. R. & Goodwin, T. W. (1966). *Biochem. J.* **101**, 5P.

Whistance, G. R., Threlfall, D. R. & Goodwin, T. W. (1967). *Biochem. J.* **105**, 145.