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THE OCCURRENCE OF UBIQUINONE IN TWO SPECIES OF *PSEUDOARACHNIOTUS*

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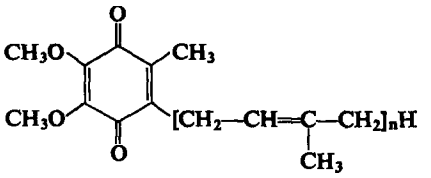
Abstract—*Pseudoarachniotus hyalinosporus* strains A and B and *P. roseus* were cultured on a standard basal medium containing $NaNO_3$ either as the sole nitrogen source, or in combination with one of the following, ammonium tartrate, peptone and beef extract. All three strains were shown to contain the terpenoids squalene, ubiquinone, an ester of ergosterol and free ergosterol. In each case the ubiquinone was identified as the −35 homologue (UQ_7). Although the media had little effect on the growth (dry weight) of the various fungi, the highest yields (mg/g dry weight) of ubiquinone and ergosterol were generally obtained on the media having the more complex nitrogen sources.

INTRODUCTION

UBIQUINONES[1] (Coenzyme Q) (I) are 2-methyl-3-alkyl-5,6-dimethoxybenzoquinones found widely distributed in nature, generally in association with the intracellular organelles concerned with terminal respiration. The alkyl group takes the form of an all-*trans*-polyisoprenoid chain containing from 6 to 10 isoprene units.



I (n = 6 to 10)

Generally it would appear that the higher orders of the plant and animal kingdoms contain either ubiquinone-50 or -45 (UQ_{10} or UQ_9). Ubiquinone-50 is the only isoprenologue occurring in many mammalian, avian, higher plant and some fish and insect tissues.[2,3,4] Exceptions do occur, an important example being the rat which contains UQ_9 as the major homologue together with lesser amounts of UQ_{10}, UQ_8 and UQ_7.[5] UQ_9 has also been reported in fish, insects, vegetable oils and algae.[2,6] Lester and Crane[2] have found all the homologues

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[1] For a general review see:
(*a*) R. A. MORTON, *Vitamins and Hormones* **19**, 1 (1961).
(*b*) O. SCHINDLER, *Fortschritte der Chemie organischer Naturstoffe* **20**, 73 (Springer-Verlag) (1962).
[2] R. L. LESTER and F. L. CRANE, *J. Biol. Chem.* **234**, 2169 (1959).
[3] J. F. PENNOCK, R. A. MORTON and D. E. M. LAWSON, *Biochem. J.* **73**, 4P (1955).
[4] D. L. LAIDMAN and R. A. MORTON, *Biochem. J.* **84**, 386 (1962).
[5] D. E. M. LAWSON, D. R. THRELFALL, J. GLOVER and R. A. MORTON, *Biochem. J.* **79**, 201 (1961).
[6] A. C. PAGE, JR., P. H. GALE, F. KONIUSZY and K. FOLKERS, *Arch. Biochem. Biophys.* **85**, 874 (1959).

from UQ_6 to UQ_{10} in micro-organisms. From a survey of the literature[1b,2,7,8,9] it is apparent that fungi (Eumycetes) also can contain any of these ubiquinone homologues. Thus UQ_6 has been found in the Ascomycete, *Ashbya gossypii*; UQ_7 in the Ascomycetes, *Endomyces lindneri* and *Endomycopsis fibuliger* and the Deuteromycete, *Mycoderma monosa*; UQ_8 in the Deuteromycete *M. monosa*; UQ_9 in the Basidomycetes, *Agaricus campestris*, *Ustilago zeae* and *Polyporus schweinitzii* the Ascomycetes, *Penicillium brevi-compactum* and *P. chrysogenum* and the Phycomycete, *Mucor corymbifer*; UQ_{10} was found in the Basidomycetes, *Ustilago zeae* and *Polyporus schweinitzii* and the Ascomycetes, *Neurospora crassa* and *Aspergillus fumigatus*. Gale and co-workers[10,11] have reported the isolation from the Ascomycetes, *Giberella fujikuroi* and *Penicillium stipitatum* of an analogue of ubiquinone-50 (UQ_{10}) having a saturated terminal isoprene unit.

This communication is concerned with the occurrence of, and effect of nitrogen source on the levels of, ubiquinone and related terpenoids in two species of *Pseudoarachniotus*, an ascomycete from the sub-class Euascomycetes; order Aspergillales; family Gymnoascaceae. Some members of this family constitute the perfect stages of the dermatophytes, fungi which cause skin diseases of man and animals. *Pseudoarachniotus hyalinosporus* (one of the test organisms) was first described by Kuehn *et al.*[12] These workers reported that of 54 strains of this organism, 24 were isolated from rodent lungs, 9 from dung, 19 from soil and one from a *Tinea pedis* (ringworm) condition. This species was found in two collections from India and appears to be widely distributed in California. *P. roseus* (the second test organism) was first isolated from an ulcerated gizzard of a chicken in Arizona and described as a new species by Kuehn.[13] Neither of these species has been reported before in Egypt.

RESULTS AND DISCUSSION

Pseudoarachniotus hyalinosporus strains A and B, and *P. roseus* were cultured, for 80, 24 and 40 days, respectively, on the basal medium (*medium 1*) or on the basal medium supplemented with ammonium tartrate (*medium 2*), peptone (*medium 3*) or beef extract (*medium 4*). At the end of the growth period the mycelial mats were washed and the total lipid extracted and chromatographed on columns of Brockman Grade III acid-washed alumina (see Methods). The u.v. absorption spectra of the various fractions eluted from the column were determined and the levels of various u.v. absorbing components calculated. Table 1 summarizes the pertinent details of a typical extraction and chromatography.

Fraction 1 was in all cases transparent to u.v. light and exhibited only weak end-absorption. From i.r. studies it was shown to be predominantly hydrocarbon in nature. Fraction 1 from all extracts was examined for the presence of squalene; aliquots were spotted on thin layers of Kieselgel G along with authentic samples of squalene, the plates were then developed with light petroleum (60–80). On staining with iodine vapour brown spots of identical R_f to the authentic squalene marker, were observed in all cases.

Fractions 2 and 5 (2% and 20% ether in light petroleum) from all the fungi had the same selective u.v. absorption curves, characteristic of $\Delta^{5,7}$-sterols. Since ergosterol is the typical

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[8] N. M. PACKTER and J. GLOVER, *Biochim. Biophys. Acta* **58**, 531 (1962).
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TABLE 1. CHROMATOGRAPHY OF THE TOTAL LIPID FROM *P. hyalinosporus* STRAIN B GROWN ON THE BASAL MEDIUM*

Fraction No.	Ether in petrol (%)	Wt. of fraction (mg)	λ_{max} (mμ) and $E^{1\%}_{1cm}$ of fractions†
1	0	3·2	weak end-absorption
2	2	1·6	263 (50), 272 (65), 282 (65), 295 (39)
3	5	31·2	275 (1·8)
4	10	4·2	weak absorption 260, 270, 282
5	20	13·2	262 (193), 271·5 (265), 281·8 (276), 293·4 (167)

107 mg lipid, isolated from 5 mats, chromatographed on 10 g Brockmann Grade III acid-washed alumina. 100 ml of each fraction were collected.

* See text.

† Spectra in cyclohexane, except for the 5% fraction which was determined in ethanol. $E^{1\%}_{1cm}$ in brackets after absorption peak.

mould sterol the two fractions were tentatively identified as an esterified (fraction 2) and the free form (fraction 5) of this sterol. A comparison of the amounts of ergosterol estimated spectrophotometrically with the weights of the fractions showed that the ester sterol contributed little of the weight of fraction 2, whereas the free sterol accounted for all the weight in fraction 5. That fraction 2 contained a sterol ester was confirmed by saponifying the fraction and rechromatographing the unsaponifiable fraction on a column of grade III alumina; the sterol was now eluted by 20% ether in light petroleum demonstrating that alkaline hydrolysis has split off an esterifying group. All the sterol fractions (free and sterol split from the esterifying moiety) when treated with Liebermann-Burchard reagent (see Methods) gave an immediate pink flush which turned via purple and blue to a green colour, showing the presence of a fast reacting sterol such as ergosterol. The extinction of the coloured solution was read at λ_{max} 675 mμ, 90 sec after mixing the reagent and test solutions, and from a comparison with a standard curve it was calculated that in all cases ergosterol would account for > 97% of the sterol present in the fractions. The free sterol fractions were crystallized several times from acetone–petroleum ether mixtures and gave crystals m.p. 162–164° (uncorrected) (ergosterol, 165°) either alone or on mixing with authentic ergosterol. The i.r. spectra determined as KBr-discs were found to be superimposable on a spectrum of similarly prepared authentic ergosterol. The free sterol fraction and the sterol originally esterified were finally examined by gas–liquid chromatography. In all cases the samples showed a major peak having the same retention times as authentic ergosterol (A. Dennis, personal communication).

Fraction 4 (10% ether in light petroleum) usually showed no selective absorption in the u.v. region, but occasionally a trace of ergosterol was present in this fraction. In these cases allowance for this absorption was made when calculating the levels of ergosterol in the fungi.

Fraction 3 (5% ether in light petroleum), the ubiquinone-containing fraction, had a single selective absorption band at 275 mμ in ethanol, indicative of this quinone. The level of ubiquinone in the fraction was estimated at this stage by treatment of a suitable aliquot with sodium borohydride (see methods). Since the fraction in several cases, notably *P. hyalinosporus* strain B, contained a large quantity of extraneous material which would interfere with the identification of the ubiquinone isoprenologue by reversed phase chromatography, the

samples were first purified by semiquantitative chromatography on thin layers of Kieselgel G. The fraction was taken up in the minimum volume of cyclohexane and applied as a stripe to the thin layer, marker spots were placed at each end. After development of the plate with a benzene–chloroform mixture [1 1, v v], the UQ-band (R_f 0·32) was located by spraying the marker spots with leucomethylene blue,[14] and scraped off and the quinone eluted with ether. The recovered material, of negligible weight, had an u.v. spectrum qualitatively identical to authentic UQ. The nature of the isoprenologues in the UQ-fractions recovered from thin layers was determined by reversed phase partition chromatography on thin layers of Kieselgel G impregnated with paraffin.[15] The chromatograms were developed in 95 5 (v/v) acetone water. Table 2 gives the R_f values for UQ_{10}–UQ_6. In all cases the ubiquinones isolated behaved as UQ-, the -35 homologue.

TABLE 2 R_f VALUES FOR UBIQUINONES CHROMATOGRAPHED ON REVERSED PHASE THIN LAYERS

Homologue	R_f
UQ_{10}	0·46
UQ_9	0·55
UQ_8	0·63
UQ_7	0·70
UQ_6	0·79

Kieselgel G plates impregnated by dipping in a 5% w/v solution of liquid paraffin (medicinal) in 60–80 light petroleum

The plates were developed using 95% acetone water as solvent.

The stoicheiometric results for the levels of ubiquinone, esterified sterols and the free sterol, for the different fungi on the various media are given in Table 3. From a consideration of the dry weights (lipid-extracted cells) it can be seen that variation of the nitrogen source makes little difference to the density of growth of the fungi, except in the case of *P. roseus* which failed to grow on *medium 1* because of its inability to utilize nitrate-nitrogen. The lipid values are of interest in that *P. hyalinosporus* strain A shows little significant variation between the four media, except perhaps that growth is slightly better on *media 2, 3* and *4*, this may reflect a preference for nitrogen in a more complex form than nitrate. However, *P. hyalinosporus* strain B and *P. roseus* show marked variation in the lipid yields from the various media, the levels fluctuating markedly for each organism and showing a contradictory behaviour between the two species. This may be due to the variation in N-source or possibly the pH of the different media; the former explanation is the more probable since only on *medium 2* was any marked variation in the final pH observed (*med. 1, 3* and *4* final pH 7·6–8·4; *med. 2* final pH 3·6–4·7).

The u.v. absorbing terpenoids show several patterns which are probably a reflection of the media on which the fungi were cultured. For the two strains of *P. hyalinosporus* the highest concentrations of UQ_7 are obtained on *medium 3* (peptone as the supplementary source); the enhancement is more marked with strain B. *P. roseus* showed similar variations in the UQ-

[14] T. W. GOODWIN, *Lab. Pract.* April (1964).
[15] D. R. THRELFALL and T. W. GOODWIN, *Biochim. Biophys. Acta*, **78**. 532 (1963).

TABLE 3. THE LEVELS OF UBIQUINONE AND ERGOSTEROL IN THREE STRAINS OF *Pseudoarachniotus* GROWN ON DIFFERENT NITROGEN SOURCES

Fungus	Medium* No.	Age of culture (days)	Dry wt.† (g)	Lipid wt. (mg)	Ubiquinone-35		Ergosterol			
							Ester		Free	
					(μmole)	(μmole/g d.w.)	(mg)	(mg/g d.w.)	(mg)	(mg/g d.w.)
P. hyalinosporus strain A	1	80	0·599	39·2	0·048‡	0·08	0·136	0·227	9·3	15·6
	2	—	0·755	43·5	0·267	0·344	0·208	0·269	9·9	12·8
	3	—	0·653	43·4	0·251	0·385	0·618	0·947	11·9	18·4
	4	—	0·655	43·2	0·142	0·217	0·360	0·550	8·2	12·6
P. hyalinosporus strain B	1	25	1·53	107	0·288	0·188	0·285	0·186	10·8	7·1
	2	—	1·34	60·6	0·302	0·225	0·238	0·176	12·1	9·0
	3	—	1·43	135·3	0·698	0·448	0·284	0·199	15·4	10·8
	4	—	1·34	48·7	0·302	0·226	0·239	0·178	11·9	8·9
P. roseus	1	40	failed to grow							
	3	—	1·09	44·0	0·416	0·381	0·332	0·303	10·3	9·4
	4	—	0·91	106	0·539	0·593	0·242	0·266	15·1	16·6

Values represent amounts from five flasks.

* N-source in med. 1, 0·2% $NaNO_3$; in med. 2, 0·2% $NaNO_3$+0·2% ammonium tartate; in med. 3, 0·2% $NaNO_3$+0·2% peptone (Evans); and in med. 4, 0·2% $NaNO_3$+0·2% beef extract (Oxoid).
† Dry wt. of lipid extracted cells.
‡ Unexpectedly low.

level for cultures grown on different media; however, in this case the highest increase (~ 2-fold) was obtained on *medium 4* (beef extract). In order to exclude the possibility of the organisms deriving their UQ from an exogenous source, the peptone and beef extract used in the experiments were exhaustively extracted with ethanol/ether mixtures and the extract examined for ubiquinone; in no case was this quinone detected.

This variation in ubiquinone levels was also clearly reflected in the values recorded for ergosterol. The highest levels of free and esterified sterol being obtained for those cultures having the highest UQ-levels, an exception being the esterified sterol levels of *P. roseus* which showed the reverse behaviour. A study of the ester sterol expressed as a percentage of a total-sterol gave some interesting results (Table 4). The values show that there is a clear trend in *P. hyalinosporus* strain A for the level of ester relative to free-sterol to rise with increasing

TABLE 4. ERGOSTEROL ESTER EXPRESSED AS A PERCENTAGE OF THE TOTAL ERGOSTEROL

	P. hyalinosporus		
	A	B	*P. roseus*
Med.			
1	1·4	2·6	—
2	2·1	1·9	—
3	4·9	1·8	3·1
4	4·2	2·0	1·4

medium complexity, the highest value occurring in the *medium 3* which supports maximum ubiquinone synthesis. In direct contrast to this, strain B shows little variation in relative amounts on all media used. *P. roseus* shows the same trends for *media 3* and *4* with regard to the sterols as does strain A, but differs markedly in that this relationship is not paralleled by the ubiquinone values.

The finding of ubiquinone-35 as the isoprenologue occurring in these organisms was not unexpected, since it has been demonstrated that the Ascomycetes may contain any of the homologues from UQ_6 to UQ_{10} except UQ_8. From the limited studies that have been carried out it is impossible to say if the Eumycetes exhibit any relationship between the chain-length of the ubiquinone homologue occurring and the degree of organization of the fungi. This aspect would seem to be worth further investigation from a systematic viewpoint.

EXPERIMENTAL

Organisms

Two strains A and B of *Pseudoarachniotus hyalinosporus* Kuehn *et al.** and one strain of *P. roseus* Kuehn were used in this investigation. The three strains were isolated in Alexandria from the floor of a chicken house. For their isolation a penicillin–streptomycin mixture was used to check bacterial growth and actidione† to suppress growth of other fungi. The fungi were maintained on dextrose–peptone–beef agar slopes at room temperature. Strain A has white, erect hyphae which on ageing turn greenish-blue. Strain B did not have conspicuous erect hyphae, but its more or less prostrate mycelium is dark yellow with exudates of greenish yellow droplets.

Cultural Conditions

The fungi were grown in liquid media of the following basal composition per litre: dextrose, 10 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0·5 g; $NaNO_3$, 2 g and 0·5 ml of a micro-element solution (equivalent in ppm to 0·005B, 0·02 Cu, 0·10 Fe, 0·01 Mn, Mo and 0·09 Zn, prepared from H_3BO_3, 28·5 mg; $CuSO_4.5H_2O$, 78·5 mg; $Fe(NH_4)_2(SO_4)_2.6H_2O$, 702 mg; $MnSO_4.4H_2O$, 40·5 mg; $ZnSO_4.7H_2O$, 39·5 mg, molybdic acid, 18 mg; distilled water to make 500 ml). To this basal solution was added either 0·2% ammonium tartrate, peptone (Evans) or beef extract (Oxoid). *P. roseus* failed to grow on the basal medium owing to its inability to utilize nitrate-nitrogen.

Inoculation of 100 ml liquid media dispensed in 250 ml. Erlenmeyer flasks was carried out with a spore and hyphal suspension prepared from an agar culture. The organisms were allowed to grow in five-replicate static cultures at 30° until thick mycelial mats were obtained. Strains A and B were grown for 50 and 25 days respectively, while *P. roseus* was grown for 40 days.

Extraction of the Mat

The mycelial mats were harvested by filtration at the pump and washed thoroughly with distilled water to remove adhering media. The tissue was then chopped finely with a pair of scissors and extracted successively with hot acetone, ethanol and finally diethyl ether. The lipid extracted cells were oven dried (50°) and weighed. The extracts were bulked in a separat-

* Kindly identified by the Commonwealth Mycological Institute, Kew.

† Appreciation is expressed to the Upjohn Co., Kalamazoo, Michigan, for the gift of the Actidione used.

ing funnel and the acetone and alcohol removed by washing with water. The ethereal lipid extract was dried overnight over anhydrous sodium sulphate (Analar), filtered and taken to dryness under N_2. All manipulations were carried out in subdued light.

Chromatography of the Lipid

The lipid (50–100 mg) was suspended in a small volume of petroleum ether (40–60°) and chromatographed on 5 or 10 g of deactivated alumina (Brockmann grade III, Woelm (aniontropic)). The column was developed by stepwise elution, 50 or 100 ml., fractions of ether and light petroleum being collected (0, 2, 5, 10 and 20% ether in light petroleum). The ubiquinone was eluted with 5% ether in light petroleum. Ergosterol ester and ergosterol were eluted by 2 and 20% ether in light petroleum, respectively.

Saponification

The 2% ether in light petroleum fractions containing ergosterol ester, were saponified by refluxing for 30 min with 2 ml 60% aqueous KOH and 3 ml ethanol. The solution was cooled, diluted with 2 vol of water and extracted three times with ether. The bulked ethereal extracts were washed with water until the washings were no longer alkaline to phenolphthalein. The extract was dried over anhydrous Na_2SO_4, filtered and taken to dryness under N_2.

Thin-layer Chromatography

Thin-layer chromatography was carried out on layers of "Kieselgel G" (E. Merck, Darmstadt, Germany). Reversed phase chromatography was carried out on thin layers impregnated with paraffin (5% in 40–60° petrol).[15] The plates were developed in a saturation chamber.[16] The solvents used to develop the plates are detailed in the text. Ubiquinones were visualized by spraying with leucomethylene blue, the quinone spots becoming blue immediately.[14] Squalene was visualized by staining with iodine vapour.[17]

Estimation of Ubiquinone and Ergosterol

The amounts of ubiquinone in the chromatographic fractions were determined spectrophotometrically (in ethanol) using the procedure described by Pumphrey and Redfearn.[18] Ergosterol levels (free and esterified) were determined spectrophotometrically in cyclohexane at 282 mμ after correction for irrelevant absorption;[19] $E^{1\%}_{1\,cm}$ at 282 mμ for pure ergosterol was taken as 310. The extinctions (E) of the extracts were read at 276 mμ (E_1), 282 mμ (E_2) and 288 mμ (E_3). The equation obtained was: E_{corr}, 282 mμ (λ_{max}) = 1·54 ($2E_2 - E_1 - E_3$).

All spectra were recorded with the Perkin-Elmer Uvicord, 137 u.v. spectrophotometer. The Liebermann-Burchard reaction was carried out under the conditions described by Davies.[20]

Infra-red spectra were determined as KBr-discs[21] using the Perkin-Elmer Model 137E Infracord.

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[20] W. E. Davies, Ph.D. Dissertation, U.C.W. (Aberystwyth).
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Solvents

Petroleum ether (40–60) and ethyl ether were dried over sodium wire and redistilled; the ethyl ether was also distilled over reduced iron immediately prior to use. Ethanol and cyclohexane were of spectroscopic grade.

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