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QUINONES AND RESPIRATORY ACTIVITY IN *SPOENDONEMA EPIZOUM*

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Abstract—Conidia, gemmae, submerged mycelium and surface cultures of different ages of *Sporendonema epizoum* were analyzed for isoprenoid quinones. Respiratory activities of the fungus, metabolizing endogenous substrates and glucose, were determined. *Q*-9 was the only quinone detected; its concentration, lowest in conidia, increases during germination and then decreases in submerged culture. *Q*-9 concentrations in surface cultures are much higher than those in submerged cultures and are not related to culture age. Changes in *Q*-9 concentration and respiratory activity are roughly parallel; a peak is reached during gemma formation.

INTRODUCTION

THE DISTRIBUTION of ubiquinone (*Q*) and other quinones involved in electron transport has not been so thoroughly investigated in fungi as it has been among bacteria.

It was initially thought that the pattern of *Q* distribution among fungi was fairly simple,[1] but recently it has been shown to be more complex. Most fungi contain several *Q* homologues, one of them constituting up to 80–90% of the total;[2,3] some others contain, additionally or exclusively, one or more types of dihydroquinone.[3–5] The pattern can be even more complex. In *Aspergillus flavus*, for example, *Q*–10, dihydrohomologues of *Q*–8, *Q*–9, *Q*–10, 5 demethoxy-*Q*-10 and another benzoquinone closely related to *Q*–10 (6-methoxy-2-decaprenyl 1,4 benzoquinone) have been reported.[3]

The functional role of *Q* in fungal respiratory chain was demonstrated by Anderson.[6]

The quinone content of the *Sporendonema epizoum* group,[7] a highly aerobic organism involved in the spoilage of salted food, has not yet been investigated. Neither the changes in the ubiquinone content of fungi with age, culture condition, or phase of growth, nor their relationship to respiratory activity have received much attention. In the investigations described in this paper we have been concerned with identifying the type of quinone present in *S. epizoum*, the variations of its level with the phase of growth and its relationship with respiratory activity.

[1] CRANE, F. L. (1965) in *Biochemistry of Quinones* (MORTON, R. A., ed.), p. 183, Academic Press, London.
[2] DAVES, G. D., MURACA, R. F., WHITTICK, J. S., FRIIS, P. and FOLKERS, K. (1967) *Biochemistry Easton.* **6**, 2861.
[3] LAW, A. H., THRELFALL, D. R. and WHISTANCE, G. R. (1971) *Biochem. J.* **123**, 331.
[4] LAVATE, W. V. and BENTLEY, R. (1964) *Archs Biochem. Biophys.* **108**, 287.
[5] LAVATE, W. V., DYER, J. R., SPRINGER, G. M. and BENTLEY, R. J. (1962) *J. Biol. Chem.* **237**, PC 2715.
[6] ANDERSON, J. A. (1964) *Biochim. Biophys. Acta* **89**, 450.
[7] CIFERRI, R. and REDAELLI, P. (1934) *J. Trop. Med. Hyg.* **37**, 167.

RESULTS

Extractions

Separate aliquots of a wet mass of conidia were submitted to four extraction procedures; lipid yields were recorded and *Q* determined; results are summarized in Table 1. Similar experiments performed with gemmae and mycelium confirmed that the most convenient method of extraction was to combine methanolic extractions as in method one and acidified acetone–ethanol as in method three, since refluxing with methanol extracts as much total lipid and *Q* as the combined method of chloroform–methanol (2:1, v/v) extraction and methanol refluxing. Furthermore, the time-consuming freeze drying step is avoided. Extraction by methanol of naphthoquinones and *Q* seems equally efficient.[8]

TABLE 1. EXTRACTION OF LIPIDS FROM CONIDIA OF *Sporendonema epizoum*

Method*	Step	Total lipid % of dry wt	*Q* μg/g of dry wt	Method	Step	Total lipid % of dry wt	*Q* μg/g of dry wt
1		2·70	10 20	4	a	0·90	5·60
2		2·10	1·10		b	0·20	
3	a	1·52	10·50		c	0·42	7·40
	b	1 40			Total	1 62	13·00
	c	0·74	6·70				
	Total	3 66	17·20				

* Details of extraction methods are presented in Experimental.

To test the extent of the possible destruction of ubiquinone in the acidified acetone–ethanol extraction step, a *Q*-containing sample of *S. epizoum* lipids in acetone–ethanol (1:1) containing 1% HCl was boiled for 1 hr. After solvent removal, the remaining *Q* was estimated; 99% *Q* was recovered.

Quinone Contents

Three experiments were performed with conidia after microscopic examination to establish their purity. The lipids extracted in each of the two steps of the method finally adopted were independently chromatographed on alumina and the eluates spectrophotometrically analyzed in cyclohexane. Naphthoquinones were not detected. The 4 and 6% Et_2O–petrol fractions showed the presence of *Q* which was quantitatively determined.

Two experiments were carried out with gemmae. Microscopic examination showed a germination rate of about 20% and no more than 5% had grown into hyphae. No naphthoquinones were detected and *Q* was eluted in the 4 and 6% Et_2O–petrol fractions.

Three experiments were initially performed with surface mycelium. Harvesting took place after 12 days of incubation in the first and after 10 days in the second and third experiments. *Q* was determined in fractions showing absorption peaks in cyclohexane at 272 nm. To test the influence of age in *Q* content another two experiments were later performed with mycelium 4- and 6-days-old.

Two experiments were carried out with 36 and 51 g of wet submerged mycelium (collected after 60 hr of incubation) under the same experimental conditions.

[8] BISHOP, D H L., PANDYA, K. P. and KING, H. K. (1962) *Biochem. J* **83**, 606.

The results of the experiments are summarized in Table 2, which shows total lipid and *Q* extracted.

TABLE 2. *Q* CONTENTS AND RESPIRATORY ACTIVITIES OF *Sporendonema epizoum* IN DIFFERENT GROWTH PHASES

	Conidia			Gemmae		Submerged mycelium		Surface mycelium of following age (days): 4	6	10	10	12
Dry weight (g)	1·50	4 20	18 00	1·26	9·25	10 25	7 20	2·60	0 52	11 27	3 93	8 31
Lipid (mg)	49 00	160.00	760 00	60 40	453 00	1 152 00	693 00	156 00	36·00	725 00	280·00	540 00
*Q*45 (μg)	21·10	62·30	276·00	251·20	2 620 00	305·00	89 80	690·00	99 20	2 219·00	711·00	1 557·00
(μmol/g dry wt)	0·018	0·019	0·019	0·255	0·362	0·038	0·016	0·340	0·244	0·252	0·232	0·240
Lipid (% dry wt)	3 26	3 80	4·22	4 80	4 90	11·24	9·62	6·00	6·92	6 43	7·12	6 50
Quinones (% lipid)	0·843	0·839	0·836	0·426	0·386	0·326	0·313	0·442	0·275	0·366	0·254	0·292
Endog respiration*	7 2	7·2	7·2	10 0	11 0	8 9	8 9	22 0	27 0	15 0	N.D.	N D.
Respiration with glucose*	10 0	10 0	10 0	31·0	34 0	13 68	13 15	38 00	31·00	25 40	N.D.	N.D.

* Oxygen uptake, μl O_2/hr/mg prot.

Q *Characterization*

An aliquot of every *Q*-containing fraction was chromatographed (reversed-phase TLC) together with *Q*-5, *Q*-8, *Q*-9 and *Q*-10 standards. Initially, the crude fractions were used and test samples exhibited consistently lower R_f values than standards, a phenomenon that has already been reported.[9]

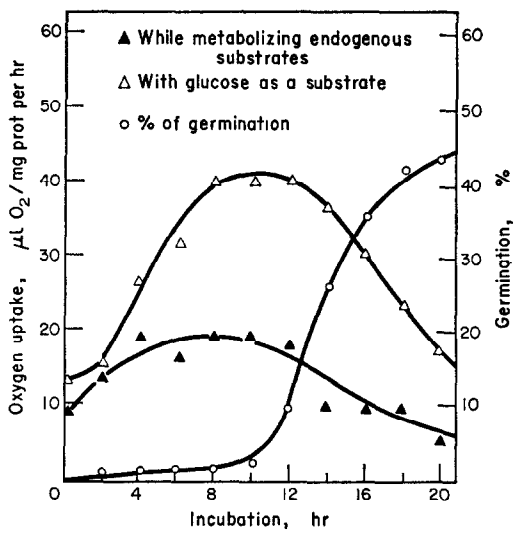

FIG. 1. OXYGEN UPTAKE DURING THE GERMINATION OF *Sporendonema epizoum* CONIDIA.

Subsequently the *Q*-containing fractions were further purified on kieselgel thin-layers (see Experimental). After this purification all samples ran consistently with *Q*-9 in reversed-phase TLC and its $\Delta E^{1\%}_{1cm\ 275nm}$ oxidized reduced (158) was used for quantitative estimation of *Q*.

[9] JONES, C. W. and REDFEARN, E. R. (1966) *Biochim. Biophys. Acta* **113**, 467.

Respiratory Activities

Before proceeding to lipid extraction, aliquots of each batch were removed for measurements of oxygen uptake with endogenous substrates and after addition of glucose. Results are presented in Table 2.

Evolution of Respiratory Activities During Submerged Growth

In view of the differences in Q content and respiratory activity of spores, gemmae and submerged mycelium it was thought convenient to follow the evolution of respiratory activities during submerged growth. Samples from duplicate 100-ml cultures were periodically removed and bulked; cells were separated, washed and aliquots used for protein determination, counting of germinated cells and oxygen uptake measurement while metabolizing endogenous substrates and glucose. Results of one experiment are shown in Fig. 1. Values were similar in several experiments, although the peak of the curve was sometimes 1 or 2 hr earlier or later, closely following shifts of percentage germination. Quinone content could not be similarly followed since insufficient material was available for reliable analysis.

DISCUSSION

The usual method of Q assay in biological material, involving saponification and extraction of the unsaponifiable fraction, cannot be used for *S. epizoum* conidia since Q is destroyed during the prolonged periods of saponification required.

Lipid extraction of the different growth phases, especially conidia, of *S. epizoum* requires careful attention. Chloroform–methanol mixtures (2:1, v/v) of Folch[10] do not extract more than about 50% of the total lipids of conidia while refluxing with methanol extracts about 75%. The efficiency of the chloroform–methanol extraction in other growth phases is a little higher (75 in the surface mycelium, 80 in gemmae and 60% in submerged mycelium) but is always significantly lower than that obtained by refluxing with methanol. It is also interesting to note that the percentage of ubiquinone in the bound lipids is higher than in the easily extractible lipids.

The concentration of Q in *S. epizoum* conidia is about six times lower than that reported for the oidia of *Pleurotus ostraetus* by Yusef *et al.*[11] and cannot be properly compared to those reported for other fungi,[4,12–14] since all of them refer to mycelium.

The observed Q content of germinated conidia (gemmae) is from 10 to 20 times higher than that of the resting conidia and it seems reasonable to conclude that the actual figures for pure gemmae should be still higher since the experimental material contained some ungerminated conidia and some with hyphae 10–20 μm long. Several synchronization procedures were tried without success.

Total lipids represent about 4% of the dry weight in conidia and nearly 5% in gemmae. Q Contents represent an average of 0·04% of lipids in conidia and 0·40% in gemmae. Therefore the large increase in Q content when conidia germinate does not reflect changes in the general pattern of lipid evolution during germination but is, rather, an independent phenomenon.

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[11] YUSEF, H. M., THRELFALL, D. R. and GOODWIN, T. W. (1965) *Phytochemistry* **4,** 559.
[12] LESTER, R. L. and CRANE, F. L. (1959) *J. Biol. Chem.* **234,** 2169.
[13] PAGE, A. C, GALE, F., WALLICK, J., WALTON, R. D. and MCDANIEL, L E. (1960) *Archs. Biochem. Biophys.* **89,** 318.
[14] TAKEDA, T., TSUCHIMOTO, N. and MIWA, S. (1968) *Appl. Microbiol* **16,** 1806.

Q Level drops after germination in submerged culture to values close to those found in conidia. For reasons already stated, the analytical data probably underestimate the extent of this decrease. Expressed in terms of percentage lipid, the drop in Q level is even more marked, since lipid content rises during submerged growth.

As shown in Fig. 1, during the first stages of germination an increase in respiratory activity, both with endogenous material and added glucose as substrates, takes place. Oxygen consumption while metabolizing added glucose/mg of protein increases by a factor of 3 after about 10–12 hr of incubation, when the first gemmae are formed. This increase is not sustained and respiratory activity soon drops to reach, in mycelium, levels close to those of resting conidia.

The pattern of Q distribution and respiratory activities are roughly parallel. These results are consistent with a possible involvement of Q in the respiratory chain of *S. epizoum*. The changes in Q content could be partially responsible for the variations in respiratory activity during growth; it seems likely that, to meet the comparatively high rate of respiration needed for germination, this organism would preferentially synthesize ubiquinone and perhaps other electron transport carriers; later on this necessity might disappear and the rate of synthesis of these products decrease, while the global synthesis of organic material would proceed.

The comparatively low Q concentration in submerged culture, related to gemmae, is strikingly different from the recorded behaviour of other microorganisms such as *Achromobacter*[15] and *Tetrahymena pyriformis*[14] where an increase in Q content with age has been observed.

The remarkable difference between the Q content of submerged and surface mycelium (which is similar to that of gemmae), either in terms of dry weight or percentage of total lipids, agrees with the observed differences in respiratory activity, but is not, at the moment, easy to explain. Sugimura and Rudney,[16] Bishop *et al.*[8] and Lester and Crane[12] have shown great differences in Q content in organisms aerobically and anaerobically grown, but differences in oxygen availability do not seem to be the cause of the differences in Q content of submerged and surface culture of *S. epizoum*, since submerged cultures were efficiently aereated; perhaps they reflect considerable differences in their methabolic pathways.

EXPERIMENTAL

Materials. Sporendonema epizoum was obtained from the Centraalbureau voor Schimmelcultures (Baarn, Holland). It was maintained by periodic subculture every 3–4 weeks on malt agar slopes containing 7·5% of NaCl (w/v). Conidia were obtained by the method of Sala and Burgos.[17] Gemmae were obtained by growing the organism in a modified Vaisey[9] medium consisting of casein hydrolysate (10 g), dextrose (10 g), $MgSO_4$ (200 mg), $FeSO_4$ (20 mg), K_2HPO_4 (1 g), NaCl (75 g) and deionized water up to 1000 ml. This medium was distributed in 100-ml amounts into 500 ml conical flasks and sterilized during 15 min at 121°. Each conical flask was inoculated with 2 ml of a suspension containing $1·8 \times 10^9$ conidia/ml which proved to give the fastest and most uniform germination, and incubated in a reciprocal shaker at 25° and 230 strokes/min. Gemmae were harvested after 16 hr of incubation, by centrifugation at 2400 *g* for 4 min. The pellet was washed 2× by resuspension in water followed by centrifugation. For production of submerged mycelium, 9 l. of modified Vaisey[18] liquid medium were sterilized in a 12 l. glass cylindrical container: after sterilization and medium was inoculated with 180 ml of a suspension containing $1·8 \times 10^9$ conidia/ml and incubated at 25° in a thermostated H_2O bath for 60 hr: vigorous aereation of culture was effected by forcing filtered air into the medium through two sintered glass plates (No. 1). Foaming was prevented with a few drops of

[15] Crane, F. L. (1962) *Biochemistry* **1**, 510.
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[17] Sala, Fco. J. and Burgos, J. (1972) *Appl. Microbiol.* **24**, 504.
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liquid silicone. Harvesting of mycelium was done by decantation and centrifugation at 4500 rpm in a Martin Christ Universal centrifuge for 5 min

In surface culture, batches of modified liquid Vaisey[18] medium were inoculated and incubated for 14 hr (as described for gemmae production) and were kept still at 25° for 3, 6, 10 and 12 day periods Mycelium was harvested by filtration through coarse filter paper, washed on a funnel with tap water and blotted between two sheets of filter paper.

Extraction of lipids. It was necessary to find an extraction procedure able to give maximum ubiquinone yield, while avoiding destruction of naphthoquinones and changes in the structure of *Q* A variety of procedures were compared for conidia; all of them were performed under subdued light.

(1) *Methanol extraction.* The wet mass of conidia was refluxed with MeOH (5:1, w/v) containing 0·25% pyrogallol. The solid residue was filtered off and refluxed with two further lots of MeOH To the joint methanolic extracts, 3 vols of H_2O were added and the whole was extracted 3× with peroxide-free Et_2O. The Et_2O extract was washed 4× with H_2O, dried over anhyd. Na_2SO_4 and concentrated by distillation; Et_2O was finally removed under N_2

(2) *Saponification.* A portion of conidia slurry was refluxed with 2 vol. MeOH (with 0·25% of pyrogallol) and 1 vol. of KOH (60%, w/v) Since the material was not digested after boiling for 3 hr, the liquid phase was removed, diluted with 3 vol. of H_2O and extracted 3× with Et_2O. The cell residue was boiled again with MeOH and KOH as before; digestion was still unsatisfactory and the liquid phase was decanted and extracted with Et_2O The combined ether extracts were dried over anhyd. Na_2SO_4 and the solvent removed by distillation and finally by evaporation in a stream of N_2.

(3) *Three-step method* (a) Lyophilized samples of conidia were triturated with acid-washed sand and extracted with 10 ml/g of $CHCl_3$–MeOH (2:1, v/v) at room temp. for 2 hr The solid residue was filtered off and reextracted with two further lots of $CHCl_3$–MeOH. The combined extracts were reduced to small vol. in a rotary evaporator and the remaining solvent evaporated in a stream of N_2 Non-lipid material was removed by the method of Folch.[10] (b) The residue was further extracted by refluxing with MeOH as in method 1, but was directly taken to dryness as the $CHCl_3$–MeOH extract. Non-lipid material was also removed by the method of Folch.[10] (c) The cell residue was now refluxed 3× for 1 hr with 10 ml/g of dry wt of acetone–EtOH (1:1, v/v), acidified with HCl (1%, v/v) Solvents and nonlipid material were removed as in step (a) and (b).

(4) *Peck's method*[19] (a) Lyophylized samples of conidia were triturated with acid-washed sand and extracted with EtOH–Et_2O (1:1, v/v, 10 ml/g of dry wt) at room temp., 3× for 1 hr (b) Residues were extracted with $CHCl_3$ (10 ml/g of dry wt) also 3× for 1 hr. Non-lipid material and solvents were removed from the extracts as in the previous method (c) Cell residues from the former extractions were extracted 3× for 1 hr at 50° with 10 ml of EtOH–Et_2O (1:1, v/v) acidified with HCl (1% v/v)/g of dry wt and non-lipid material and solvents were removed as before.

Alumina chromatography was carried out on acid-washed alumina (Brockmann grade III): the columns were loaded with 10 mg of lipid, dissolved in petrol. (b.p. 40–60°)/g of alumina and developed by step-wise elution with 10.1 vol eluant/alumina, of 2%, 4%, 6%, 10% and 15% Et_2O–petrol. After removal of the eluant, each fraction was dissolved in cyclohexane and assayed spectrophotometrically

Q *determinations* Following the above procedure, ubiquinone was eluted with 4–6% Et_2O–petrol Fractions absorbing in the 272 nm region, suspected of containing *Q*, were taken to dryness under N_2 and dissolved in EtOH *Q* was quantitatively determined following the method of Crane[20]

Purification and characterization of Q Purification of *Q* was carried out by TLC on kieselgel after development with $CHCl_3$–C_6H_6 (1:1, v/v) *Q* was recovered by extraction with ether.

Characterization of *Q* was attained by reversed-phase TLC on kieselgel impregnated with paraffin, developed with acetone–H_2O (95.5, v/v); quinones were located by spraying with leuco-methylene blue as described by Goodwin.[21] Standard *Q*-5, *Q*-9, *Q*-10 were run simultaneously with the unknown sample

Other procedures Protein determinations were performed by the method of Johnson, as described by Umbreit *et al*[22] The percentage of conidia germinated was estimated by microscopic counting in a Neubauer chamber. Respiratory activities were determined with whole cells in 0·1 M sodium potassium phosphate buffer (pH 7·4) by conventional manometric techniques. In the case of surface mycelium, a small piece of washed film was used for each analysis

Petrol and Et_2O were dried and redistilled before use Spectrophotometrically pure cyclohexane was prepared by the method of Van Der Ven and Jonge[23] Spectroscopically pure EtOH was obtained by refluxing over NaOH and Zn dust and then distilling.

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