The Fatty Acid Composition of Sporangiospores and Vegetative Mycelium of Temperature-adapted Fungi in the Order Mucorales

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SUMMARY

The lipid content and fatty acid composition of sporangiospores and vegetative mycelium of mesophilic, thermotolerant and thermophilic fungi in the Mucorales were examined. In each fungus the spores contained less lipid than the vegetative mycelium. The mesophiles accumulated less lipid in spores and mycelium than did thermotolerants and thermophiles. No unusual fatty acids were detected by gas-liquid chromatography in the lipids of spores or mycelium. The fatty acid compositions of spores and vegetative mycelium were qualitatively very similar, but spore lipids were always more highly saturated than mycelial lipids. Lowering growth temperature from 48 to 25° increased the synthesis of unsaturated fatty acids in the spores and the mycelium of the thermotolerant and thermophilic fungi examined.

INTRODUCTION

There have been several investigations of the fatty acid composition of the lipids of fungal spores, in a number of which a significant proportion of the spore lipid has been shown to comprise 'unusual' fatty acids. In the order Uredinales (rusts) *cis*-9, 10-epoxyoctadecanoic acid formed up to 40% of the spore oil (Tulloch, Craig & Ledingham, 1959; Tulloch & Ledingham, 1962). In the order Erysiphales (powdery mildews) the spore lipid of *Sphaerotheca humuli* was shown to contain 42% behenic acid, which is rarely found in fungal lipids and then only in trace quantities, while in *Erysiphe graminis* the spore oil contained 45% of an unidentified fatty acid, thought to have a branched chain, or cyclic system (Tulloch & Ledingham, 1960). Sclerotia of *Claviceps purpurea* contained 34% ricinoleic acid (12-hydroxystearic acid) but this acid could not be demonstrated in other members of the Hypocreales (Shaw, 1965).

Linoleic acid has been found as a major component of the lipids of spores of widely different groups of fungi. It comprised as much as 60% of the total fatty acids of sclerotia of *Sclerotium rolfsii* (Howell & Fergus, 1964) and 65% of the spore fatty acids of *Penicillium atrovenetum* (Van Etten & Gottlieb, 1965). In the fruiting bodies of some Basidiomycetes it accounted for over 70% of the total fatty acids (Hughes, 1962; Bentley, Lavate & Sweeley, 1964; Shaw, 1966*a*), while spore lipid of the smut *Tilletia oetens* contained 63% of it (Tulloch & Ledingham, 1960).

The class Phycomycetes is conspicuously absent from the groups so far examined. The fatty acid composition of the spores of phycomycete fungi was therefore examined

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firstly to determine whether it was similar to that of related mycelium, and secondly to determine whether spore lipids were influenced by the incubation temperature in the same way as were mycelial lipids. There is abundant experimental evidence that micro-organisms, in common with other poikilotherms, synthesize increased proportions of unsaturated fatty acids, at the expense of saturated fatty acids, as the incubation temperature is lowered (see Kates, 1964, 1966; and Farrell & Rose, 1967*a, b*; for reviews). Curiously, Long & Williams (1960) found that the spore lipids of the thermophile *Bacillus stearothermophilus* became more unsaturated as the temperature was raised.

A number of mesophilic, thermotolerant and thermophilic fungi in the order Mucorales were therefore grown at different temperatures, and the lipid content and fatty acid composition of sporangiospores and mycelium determined. The definition of Cooney & Emerson (1964) that a thermophilic fungus has a maximum temperature for growth at or above 50° and a minimum temperature for growth at or above 20°, was used to distinguish thermophilic from thermotolerant fungi, the latter having a minimum growth temperature below 20°.

METHODS

Organisms. The mesophilic fungi used in this investigation were obtained from the Commonwealth Mycological Institute, Kew, England. They were: *Mucor mucedo* Auct. (IMI 103731), *M. racemosus* Fresen. (IMI 103730), *M. ramanniaaus* Moller. (IMI 35044*a*) and *M. hiemalis* (+) Wehm. (IMI 21216). The thermotolerant and thermophilic fungi were obtained from Dr H. C. Evans, Biology Department, University of Keele. They were: *Rhizopus* sp. (thermotolerant), *Mucor miehei* and *M. pusillus* (thermophilic).

Composition of media, inoculation and incubation

The culture medium had the following composition (per litre deionized water): KH_2PO_4 1 g, $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ 0.5 g, glucose 20 g, ammonium sulphate 1.2 g, sodium succinate 5 g, yeast extract 5 g. Its pH was adjusted to 6.5 prior to autoclaving. Twenty ml. medium was dispensed into 100 ml. Erlenmeyer flasks, and in 50 ml. medium containing 2% agar into 8 oz. flat medicine bottles. Inocula were grown on agar slopes for 14 days, mesophiles at 25° and thermotolerants and thermophiles at 48°.

Two types of cultures were grown, 'mycelial' and 'spore'. 'Mycelial' cultures were grown by inoculating each 100 ml. Erlenmeyer flask with 1 ml. spore suspension and incubating in still culture for 14 days. Preliminary experiments had shown that under these conditions sporulation was particularly sparse, spores forming less than 0.1% of the dry weight of the culture. 'Spore' cultures were grown by inoculating each flat medicine bottle with a small inoculum transferred by sterile wire.

All cultures were incubated at the specified temperatures for 14 days and then harvested. Mycelial mats were removed from six replicate Erlenmeyer flasks, washed with deionized water, filtered on a sintered glass disc, dried overnight at 80° and used as a combined sample. Spores were removed from the surface of the agar slopes by lightly scraping them with a spatula into deionized water. Spore suspensions from a number of replicate flat medicine bottles (usually 25) were pooled, filtered through three layers of cheesecloth and centrifuged. The spore pellet was dried overnight at 80°.

Extraction and saponification of the fungal lipid

Dried spores and mycelia were each quickly powdered in a small grinder and the lipid extracted for 8 hr in a Soxhlet apparatus with benzene and ethanol (594 + 297 v/v) (Shaw, 1965). The amount of lipid in each sample was calculated by subtracting the weights of material before and after extraction. The lipid was saponified by refluxing with 0.6 M-ethanolic KOH for 1 hr.

Fatty acid analysis

The mixture of fatty acids was methylated with BF_3 /methanol reagent and analysed by gas-liquid chromatography. A $9'' \times \frac{1}{4}''$ glass column of diethyleneglycolsuccinate (5%) on chromosorb G (100 to 120 mesh) was used in a Pye Model 64 gas chromatograph. The operating temperature was 175° and the nitrogen carrier gas flow 40 ml./min. The fatty acid methyl esters were tentatively identified by comparison of retention times with those of authentic standards. Standard fatty acid methyl esters were obtained from Fluka A.G., Bucks, Switzerland and included methyl esters of C 12 to C 20 saturated acids, as well as the methyl esters of palmitoleic, oleic, linoleic and α -linolenic acid. Fatty acid esters were identified completely by combined mass spectrometry and gas-liquid chromatography. The percentages of fatty acid methyl esters were calculated from the peak areas.

RESULTS

Lipid content of spores and mycelium

In all the species examined the lipid content of the spores from 'spore' cultures was lower than that of mycelium from 'mycelial' cultures. Also the lipid contents of spores and mycelium of mesophilic fungi were lower than those of thermotolerant and thermophilic fungi. These results are summarized in Tables 1 and 2.

Table 1. *Lipid content of spores and mycelium of mesophilic fungi grown at 25°*

Spores and mycelium were harvested from agar-slope cultures and stationary liquid cultures, respectively, after 14 days growth. They were dried and weighed, and the lipid extracted in a Soxhlet apparatus. The amount of lipid present was calculated by weighing each sample before and after extraction.

Organism	Fungal phase examined (spores or mycelium)	Lipid content (% of dry weight)
<i>M. mucedo</i>	Spores	3.7
	Mycelium	12.0
<i>M. ramannianus</i>	Spores	7.6
	Mycelium	15.2
<i>M. racemosus</i>	Spores	4.1
	Mycelium	9.8
<i>M. hiemalis</i>	Spores	8.4
	Mycelium	18.1

Fatty acid composition of spores and mycelium

No unusual fatty acids were found in the lipids of spores of any of the species examined. Seven fatty acids occurred in measurable amounts: myristic, palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acids; α -linolenic acid was absent. The

triply unsaturated C 18 acid was identified as γ -linolenic acid. This agrees with previous reports that only the γ -isomer is present in phycomycete fungi (Bernhard & Albrecht, 1948; Shaw, 1965, 1966*a, b*; White & Powell, 1966; Tyrrell, 1967 and Sumner, Morgan & Evans, 1969). These results are summarized in Tables 3 and 4.

The fatty acids of spore and mycelial lipids were very similar both qualitatively and quantitatively, but in each species the spore lipids were more saturated than related mycelial lipids. In these respects the lipids of these mucoraceous fungi are similar to those of *Pithomyces chartarum* (dematiaceous imperfect fungus) (Hartman, Hawke, Morice & Shorland, 1960; Hartman, Morice & Shorland, 1962) in which the spore lipids closely resemble the mycelial lipids though they are more saturated.

Table 2. *The effect of incubation temperature on the lipid content of spores and mycelium of thermotolerant and thermophilic fungi*

Spore and mycelial cultures were harvested after 14 days growth at either 25 or 48°. After drying and weighing, the lipid was extracted in a Soxhlet apparatus. The lipid content of each sample was calculated by weighing it before and after extraction.

Organism*	Temperature of incubation (°)	Fungal phase examined (spores or mycelium)	Lipid content (% of dry weight)
<i>Rhizopus</i> sp.	25	Spores	16.1
		Mycelium	25.7
	48	Spores	10.4
		Mycelium	11.6
<i>M. miehei</i>	25	Spores	19.4
		Mycelium	24.3
	48	Spores	11.3
		Mycelium	11.9
<i>M. pusillus</i>	25	Spores	19.3
		Mycelium	26.2
	48	Spores	16.1
		Mycelium	23.1

Table 3. *Fatty acid composition of spores and mycelium of mesophilic fungi grown at 25°*

The lipid of spore and mycelial cultures was saponified, esterified and analysed by gas-liquid chromatography. The percentage of each fatty acid was calculated from the peak area. The degree of unsaturation of the lipid was calculated in terms of the number of double bonds/mole from the formula:

$$\Delta/\text{mole} = 1.0 \times (\% \text{ monoenes})/100 + 2.0 \times (\% \text{ dienes})/100 + 3.0 \times (\% \text{ trienes})/100.$$

Organism	Fungal phase (spores or mycelium)	Fatty acids							Degree of unsaturation
		14:0*	16:0	16:1	18:0	18:1	18:2	18:3	
<i>M.ucedo</i>	Spores	2.5	21.3	3.5	12.6	27.2	21.0	12.0	1.08
	Mycelium	0.8	12.0	3.1	9.3	26.1	25.3	20.2	1.40
<i>M. ramannianus</i>	Spores	4.1	18.7	3.4	5.5	31.2	14.1	20.9	1.25
	Mycelium	1.6	18.5	2.6	4.4	28.0	13.5	30.9	1.50
<i>M. racemosus</i>	Spores	4.9	21.8	3.8	9.1	31.4	14.9	14.1	1.07
	Mycelium	2.7	16.9	2.9	4.6	36.5	16.6	19.4	1.28
<i>M. hiemalis</i>	Spores	2.0	15.0	2.1	17.1	28.0	17.9	18.0	1.20
	Mycelium	2.4	14.8	3.1	9.7	32.6	18.8	19.2	1.29

* The number of carbon atoms: the number of double bonds per molecule.

The spore lipids were influenced by incubation temperature in the same way as mycelial lipids, being more unsaturated when the fungus had been grown at a lower temperature. The sorts and proportions of fatty acids in spore lipids of mesophiles, thermotolerants, and thermophiles were essentially the same as those in the mycelial lipids; the lipids of mesophiles contained greater proportions of the polyunsaturated acids (linoleic and linolenic acid) and lower proportions of oleic acid, compared with lipids of thermotolerants and thermophiles.

Table 4. *Effect of temperature on the fatty acid composition of spores and mycelium of thermotolerant and thermophilic fungi*

The lipid extracted from spore and mycelial cultures grown at 25 or 48° was saponified, esterified and analysed by gas-liquid chromatography. The percentage of each fatty acid was calculated from the peak area. The degree of unsaturation of the lipid was calculated in terms of the number of double bonds/mole:

$$\Delta/\text{mole} = 1.0 \times (\% \text{ monoenes})/100 + 2.0 \times (\% \text{ dienes})/100 + 3.0 \times (\% \text{ trienes})/100.$$

Organism	Temperature of incubation (°)	Fungal phase (spores or mycelium)	Fatty acids							Degree of unsaturation
			14:0*	16:0	16:1	18:0	18:1	18:2	18:3	
<i>Rhizopus</i> sp.	25	Spores	6.0	27.3	2.9	12.1	28.8	10.6	12.1	0.89
		Mycelium	1.4	20.6	2.3	5.2	30.0	28.6	11.9	1.22
	48	Spores	5.8	22.6	2.8	13.8	40.1	11.0	4.0	0.77
		Mycelium	Trace	23.3	3.0	18.6	34.9	21.8	5.1	0.94
<i>M. miehei</i>	25	Spores	5.6	24.4	3.1	11.3	32.6	13.1	9.3	0.88
		Mycelium	1.6	22.8	3.0	4.7	47.8	15.8	4.1	0.95
	48	Spores	4.0	26.3	4.8	9.9	44.0	5.9	4.4	0.72
		Mycelium	1.8	27.7	3.0	6.3	48.0	10.3	2.0	0.76
<i>M. pusillus</i>	25	Spores	1.0	25.4	3.0	4.8	42.2	19.0	4.5	0.97
		Mycelium	1.2	23.9	3.0	5.6	39.7	20.2	6.3	1.02
	48	Spores	1.3	28.6	2.9	7.1	39.4	17.2	3.4	0.87
		Mycelium	1.5	26.3	3.1	4.3	42.1	19.2	3.6	0.93

* See footnote to Table 3.

DISCUSSION

The fatty acid compositions of the spores and vegetative mycelium of each organism tested were similar. However, the fact that in each species the spores contained lipid in lower concentration but of a more highly saturated nature than in the mycelium appears to indicate a modification of the fatty acid synthesizing and fatty acid desaturating systems of sporogenous mycelium. Both enzyme systems have cofactor requirements for acetylcoenzyme A (acetyl CoA), acyl carrier protein (ACP) and the reduced forms of nicotinamide adenine dinucleotide (NADH₂) or nicotinamide adenine dinucleotide phosphate (NADPH₂). However, the desaturating enzymes (desaturases) specifically require oxygen, while the synthesizing enzymes (saturases) require carbon dioxide (Mudd & Stumpf, 1961; James, 1963; Stumpf & James, 1963; Nagai & Bloch, 1966).

A correlation between the need for molecular oxygen in the desaturation reaction and the low dissolved oxygen tensions at high temperatures has been used to explain the increased synthesis of highly saturated lipids in response to increases in temperature (Bloomfield & Bloch 1960; Meyer & Bloch, 1963; Harris & James, 1969). In the present context this explanation seems perfectly acceptable when considering the lipid

composition of cultures grown at a high and a low temperature; indeed, preliminary experiments have shown that cultures of *Mucor pusillus* grown at 48° become oxygen deficient, while those at 25° do not. However, it does not explain why spore lipids are more saturated than mycelial lipids, even when 'spore' and 'mycelial' cultures are grown at the same temperature.

A number of studies on the biochemical aspects of fungal morphogenesis have indicated that respiratory changes are associated with spore production; increased spore production in *Mucor hiemalis* and *Phycomyces blakesleeanus* has been correlated with a reduction in the respiration rate of sporogenous mycelium (Hawker & Hepden, 1962), while in mitochondria of sporogenous mycelium of *Neurospora crassa* there is activation of the enzymes of the glyoxylate cycle, and corresponding decrease in activity of enzymes of the Krebs cycle (Turian, 1960; Weiss & Turian, 1966). Also increased activity of proteinase and nuclease enzymes has been demonstrated in spore-producing mycelium of *Penicillium griseofulvum* (Morton, Dickerson & England, 1960). Accumulation of only small concentrations of lipid, but of a highly saturated nature, may therefore be caused by a reduction in the activities of synthetase and desaturase enzymes resulting from a check in the respiration rate of sporogenous mycelium. A depression of respiratory rate would effectively reduce the level of a number of respiratory metabolites, e.g. acetyl CoA, NADH₂, NADPH₂, carbon dioxide, which are also substrates for fatty acid biosynthesis.

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