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FREE AND BOUND LIPIDS OF *NEUROSPORA CRASSA*

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Key Word Index—*Neurospora crassa*; fungi; free and bound lipids; sterols; fatty acids; carotenoids; 3,4-dehydrolycopene.

Abstract—Extraction of light and dark grown cells of *Neurospora crassa* with chloroform–methanol gave a free lipid extract in which the relative amounts and compositions of sterols, fatty acid and carotenoid fractions were determined. Further extraction of the cells with methanolic potassium hydroxide solution liberated a bound lipid fraction from the cells. The levels of the bound lipid fraction were much lower than those of the free lipids but analysis showed that the composition was similar to that of the free lipids.

INTRODUCTION

Recent reports [1–8] have shown that a variety of microorganisms contain both a $CHCl_3$–MeOH extractable lipid pool (free) as well as a second lipid fraction (bound) which is released only after treatment of the cells with methanolic KOH solution. It has also been noted that the relative amounts of the two lipid pools are dependent on environmental factors such as O_2 levels [1], light [3–7], temperature [8] and also the age of the cells [8]. *Neurospora crassa* is a fungus in which lipid levels are markedly affected by growth in the presence or absence of light. It was therefore of interest to establish the presence of the free and bound lipid pools and to determine the effect of light on their relative concentrations.

RESULTS

Sequential extraction of the freeze-dried dark and light grown *N. crassa* cells with $CHCl_3$–MeOH followed by extraction with boiling methanolic KOH solution gave the two lipid extracts. The dark-grown cells were found to contain appreciable quantities of bound sterols and fatty acids but only traces were isolated from the light grown cells (Table 1).

The integrity of the fatty acid and sterol fractions from the two cell lipid pools was confirmed by examination of the individual components of these fractions (Tables 2 and 3). Both the light-grown and dark-grown sterol fractions contained ergosterol and episterol as the only components. The relative levels of the two sterols in the free and bound fractions from the light-grown cells were similar but the bound fraction from the dark-grown cells had significantly lower levels of ergosterol than the free lipid fraction. The fatty acid composition of the light-grown and dark-grown cells were significantly different, with the fatty acids from the latter having a lower degree of unsaturation. Although the degree of unsaturation of the solvent and KOH extractable fatty acids were similar for both the light and dark-grown cells there were significant differences in the levels of the individual fatty acids. The base-extractable fractions contained a higher percentage of the 18:2 acid with the levels of 18:1 being lower than those in the lipid-extractable fraction.

Table 1. Percentage composition of fatty acid, sterol and carotenoid fractions in the lipid pools of *N. crassa*

Fraction (Growth conditions)†	$CHCl_3$–MeOH extract	KOH extract
Sterols (D)	0.2	0.02
Sterols (L)	0.4	tr
Fatty acids (D)	2.2	0.4
Fatty acids (L)	3.3	0.2
Carotenoids (D)	0.003	tr
Carotenoids (L)	0.04	0.0002

* Based on wt of freeze-dried cells. † D = dark-grown cells; L = light-grown cells. A second $CHCl_3$–MeOH extract yielded only trace amounts of additional lipids (<2% by wt of the first extract).

The carotenoid fractions from the light and dark grown cells were isolated using a modified extraction procedure (see Experimental) because of the instability of these compounds. The procedure yielded both solvent and KOH extractable fractions. Phytoene was the major solvent-extractable carotenoid from both the light and

Table 2. Percentage composition of the sterol fractions from *N. crassa*

Sterol	Light-grown cells		Dark-grown cells	
	$CHCl_3$–MeOH extractable	KOH extractable	$CHCl_3$–MeOH extractable	KOH extractable
Ergosterol	76	75	74	65
Episterol	24	25	26	35

Table 3. Percentage composition of the fatty acid fractions from *N. crassa*

Growth conditions	Cell fraction	Fatty acid composition 14:0	16:0	16:1	18:0	18:1	18:2	18:3	Degree of unsaturation
Light-grown	$CHCL_3$–MeOH-extractable	1.1	21.5	2.6	5.0	17.1	49.1	3.5	1.32
	KOH-extractable	0.6	27.2	2.8	1.7	9.6	54.9	3.2	1.32
Dark-grown	$CHCl_3$–MeOH-extractable	0.5	31.0	4.5	5.8	20.7	33.3	4.2	1.04
	KOH-extractable	0.9	27.9	1.8	9.3	16.7	39.1	4.2	1.09

* GLC analysis was repeated (3×) and the above data were an average of these runs. The data obtained were all within ±5% of the percentage composition value for each fatty acid.

dark-grown cells although the amounts of this component were ten times higher in the light-grown cells (Table 1). These cells also gave a KOH-extractable carotenoid fraction which consisted primarily of 3,4-dehydrolycopene; the dark grown cells did not contain any carotenoids in the KOH-extractable fraction.

DISCUSSION

Examination of light and dark grown *N. crassa* cells at log phase growth indicated the presence of both free and bound sterols, fatty acids and carotenoids. Recent results [9] have shown that the relative proportions of the free and bound sterols in *N. crassa* were dependent on the age of the cells with the older cells containing relatively more free sterol than early log-phase cells or the conidia. The data reported herein confirm this result since the cells contain relatively high amounts of the free sterol fraction. Elliott and coworkers [9] also report that ergosterol is the main component of the free and bound fraction and this was confirmed in the present study. Moreover we have also identified the second major sterol component as episterol. Since isolation of the sterols was obtained via hydrolysis no attempt was made to investigate separately the ester and free sterol fractions as previously reported [9]. Preliminary TLC examination of the $CHCl_3$–MeOH extracts of our strain of *N. crassa* in late log-phase growth indicated only small amounts of sterol esters. The sterol results thus confirm the presence of a tightly bound cellular sterol fraction which has also been observed in yeast [12–14], fungi [11], algae [3,5], higher plants [7] and lichens [15–17]. Fatty acids and carotenoids were isolated in the free and bound fractions. Although the relative levels of the KOH extractable fractions were low in all cases the integrity of the extracts was confirmed by showing that the composition of the individual components of this fraction were significantly different in composition from those of the comparable $CHCl_3$–MeOH-extractable fraction. The dark-grown cells contained relatively higher levels of base-extractable sterols and fatty acids than were observed in the light-grown cells whereas only the former cells contained a bound carotenoid fraction. Inconsistencies in the occurrence of 3,4-dehydrolycopene in previous reports [18–20] can be attributed to the extraction and culture conditions.

EXPERIMENTAL

Isolation and analysis of the free and bound fatty acid and sterol fractions. Cultivation and growth of *N. crassa* was carried out as previously described [9]. The freeze-dried cells were macerated in a blender with $CHCl_3$–MeOH (2:1) and the mixture refluxed for 2 hr. The mixture was filtered, the filtrate concentrated to dryness and the resulting extract hydrolyzed with methanolic KOH soln (10%) for 2 hr. The soln was diluted with H_2O and the lipids removed by Et_2O extraction. The sterol fraction was isolated from this fraction by preparative TLC using $CHCl_3$ as running solvent. The sterol band was removed from the plate, eluted with Et_2O, taken to dryness and weighed to give the free sterol fraction. The relative lability of the sterol components in the extraction procedure was not evaluated although it is anticipated that the workup procedures would result in some decomposition of the labile ergosterol [9]. It was assumed that the sterols are decomposed to the same extent in all fractions [9] and thus the data which are given are uncorrected. The solvent extracted cells were refluxed with methanolic KOH soln (10%) for 2 hr, filtered and the soln diluted with H_2O and extracted with Et_2O. The bound sterols were isolated from this extract as described above.

Analysis and separation of the sterol fraction. Sterol fractions were converted into their corresponding acetate derivatives by treatment with Ac_2O–C_5H_5N (1:2) and analysed by GLC using a 2 m × 6 mm glass column packed with 3% OV17 at 250° using He as carrier gas. The steryl acetate fractions gave only 2 major peaks with RR_S (cholestane) of 4.30 and 4.50.

Table 4. Percentage composition of the carotenoid fractions from *N. crassa*

	Light-grown $CHCl_3$–MeOH Extractable	Light-grown KOH Extractable	Dark-grown $CHCl_3$–MeOH Extractable	Dark-grown KOH Extractable
Neutral carotenoids				
Phytoene	89	—	100	—
*Carotenoid intermediate	10.8	—	tr	—
3,4-Dehydrolycopene	0.12	80	—	tr
Unidentified	—	20	—	—
Acid carotenoids				
Neurosporaxanthin	100	—	tr	—

* This fraction includes the carotenoid intermediates, phytofluene, ζ-carotene, neurosporene, lycopene, β-carotene, torulene.

The R_f values were identical to those of ergosteryl and episteryl acetates respectively. The two components were isolated by $AgNO_3$–Si gel TLC to give: ergosteryl acetate, *m/e* 438 (M^+), 378 (M^+–MeCOOH), 363 (M^+–MeCOOH–Me) 253 (M^+–side chain–MeCOOH); PMR(220 M Hz), (δ, ppm) 5.65 (*m*, C-22/23), 5.35 (*m*, C-6/7), 4.68 (*m* 3αH), 1.02 (*d*, $J = 6H_6$, C-21 Me) 0.96 (*s*, C-19 Me), 0.91 (*d*, $J = 7$ Hz, C-28 Me), 0.83 (*d* $J = 7$ Hz, C-27 Me), 0.81 (*d* $J = 7$ Hz, C-26 Me), and 0.63 ppm (*s*, C-18 Me); episteryl acetate, *m/e* 440 (M^+), 423 (M^+–Me), 255 (M^+–side chain–MeCOOH); PMR (220 M Hz) (δ, ppm) 5.15 (*m*, C-7), 4.70 (*d*, $J = 11$ Hz, C-24–CH_2), 4.60 (*m*, 3αH), 1.03 (*d*, $J = 7$ Hz, C-27,26,21 Me), 0.81 (*s*, C-19 Me) and 0.54 ppm (*s* C-18 Me).

Analysis of fatty acids. The crude fatty acid fractions were transmethylated by refluxing for 90 min in C_6H_6–MeOH–H_2SO_4 (20:10:1). The resultant fatty acid Me esters were purified by TLC (petrol–Et_2O; 97:3) and weighed. The fractions were analyzed on a stainless steel column (2 m × 3 mm) packed with 8% HIEFF-IBP (Applied Science) at 140° using He as a carrier gas.

Isolation and identification of the carotenoid fractions. The freeze-dried cells were extracted with $CHCl_3$–MeOH (1:1) and Me_2CO and the combined extracts concentrated. The extract was saponified with 20% methanolic KOH soln for 10 hr at 20°. The saponification mixture was saturated with petrol and the organic phase washed repeatedly with H_2O to give the neutral carotenoid fraction. The basic soln was acidified and re-extracted with petrol to give the acidic carotenoid fraction containing neurosporaxanthin as the sole carotenoid component. Identification of the individual carotenoids and their quantitative analysis were carried out as described in refs [10,11].

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