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The Neutral Carotenoids of Wild-Type and Mutant Strains of *Neurospora crassa*

A. Hughes Goldie[1] and Ronald E. Subden[1]

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The neutral carotenoids of wild-type Neurospora crassa *and of carotenoid mutants at four discrete genetic loci were isolated using gradient elution chromatography on deactivated alumina columns. Carotenoids were identified by absorption spectrophotometry and thin layer cochromatography with carotenoid standards. Phytoene, phytofluene, ζ-carotene, β-carotene, neurosporene, torulene, lycopene, and 3,4-dehydrolycopene were isolated from wild type. Phytoene, phytofluene, ζ-carotene, β-carotene, neurosporene, γ-carotene, lycopene, and one unknown carotenoid, tentatively identified as 15,15′-*cis-*β-carotene, were isolated from a yellow mutant,* ylo-1. ylo-1 *also contained residual carotenoids having similar absorption spectra to, but very different chromatographic behavior from, phytofluene, ζ-carotene, β-carotene, and lycopene. Albino and colored* al-1 *mutants contained large amounts of phytoene and only traces of other neutral carotenoids. Albino* al-2 *and* al-3 *mutants contained only traces of neutral carotenoids.*

INTRODUCTION

To elucidate the carotenoid biosynthetic pathway in *Neurospora crassa*, a number of carotenoidless mutants were obtained and their carotenoid intermediate pools examined.

Biochemical analysis of *N. crassa* carotenoid mutants has been in progress since 1949 (Haxo, 1949), but reports disagree with respect to the positioning of mutant lesions in the carotenoid biosynthetic pathway, the actual carotenoids present in wild-type and mutant strains, and the quantitative

[1] Department of Botany and Genetics, University of Guelph, Guelph, Ontario, Canada.

aspects of carotenogenesis (Haxo, 1949, 1952; Zalokar, 1954; Huang, 1964; Jensen, 1965; Harding, 1968; Subden, 1969).

These inconsistencies may have arisen through: use of older cultures (Subden, 1969), which tend to accumulate end products through the residual activities of some mutant enzymes; use of morphological mutants such as *peach* microconidial (Harding, 1968), which indirectly result in different carotenoid ratios; and use of strains lacking any genetic or complementation analysis of the carotenoidless marker to preclude the possibility of secondary effects of chromosomal aberrations. One of the original albino strains (*al-1* 4637T) (McClintock, 1955) was involved in a translocation.

The recent isolation of new carotenoidless mutants from *N. crassa* and the development of improved chromatographic and extraction procedures (Davies, 1965, 1970; Lakshmanan and Lieberman, 1964) have justified a reexamination of the nature of biosynthetic lesions in *Neurospora* carotenogenesis. This work makes use of a representative range of genetically characterized *N. crassa* mutants, and a new albino mutant, *al-3*, which has been located in linkage group V, is also studied (Wang *et al.*, 1971, with permission of the authors).

METHODS

Strains Used

Wild-type *N. crassa* and carotenoid mutants of *N. crassa* at four discrete genetic loci were used. Wild types 74A-OR23-1A and 74-OR8-1a, *ylo-1* mutant Y30539y, *al-2* mutants 15300 and Y256M220, and *al-1* 34508 (formerly *aur*) were obtained from the Fungal Genetics Stock Center, Dartmouth College, New Hampshire. *al-1* strains RES-6 and RES-25 (formerly *ylo-b*) and *al-2* RES-8 were isolated in this laboratory from a colonial strain obtained from A. L. Schroeder, Washington State University, Pullman, Washington. *al-3* was obtained from Dr. R. L. Phillips, University of Minnesota, St. Paul, Minnesota. All mutants were backcrossed to wild type to obtain a homogeneous genetic background and mating type A.

Culture Conditions

Mycelium was grown at 25 C in 12-liter batches in 5-gal carboys for 6 days with heavy aeration and ambient illumination. The culture medium was that used by Westergaard and Mitchell (1947) with glucose as a carbon source.

Reagents

Petroleum ether (boiling point 30–60 C), acetone, diethyl ether, and benzene

were all A.C.S. approved solvents, obtained from Fisher Scientific Co. Ltd., Toronto, Ontario. Digitonin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

Extraction of Carotenoids

The extraction procedure is that developed by Goodwin and Davies (Davies, 1965), but extensively modified for extraction of large volumes of fungal mycelium. The mycelium was harvested in cheesecloth and placed in a shallow tray with just enough medium (10–12 ml/g fresh weight) to prevent conidiation and illuminated with 155 ft-candles (cool-white fluorescent light as measured by a Weston model 756 intensity meter) for 3 hr to maximize carotenoid production. It was then harvested again in cheesecloth, squeezed dry, and disrupted in 1 liter of acetone in a ball mill for 2 hr. Dry weight determinations were done on the acetone slurry, which was filtered through a Whatman 44 filter paper. The filtrate was extracted three times with 10% benzene (v/v) in petroleum ether. The pooled epiphase (benzene and petroleum ether) was washed three times with water and dried under N_2 in a flash drier.

The extract was then saponified overnight in 500 ml of saponification mixture (10 ml of 30% KOH, w/v, per 90 ml of 95% ethanol). The saponification mixture was extracted three times with 10% benzene in petroleum ether. The pooled epiphase (benzene and petroleum ether) was washed five times with water and dried under N_2 in a flash drier.

Steroids were removed by crystalization overnight in a small volume of petroleum ether (1 ml petroleum ether/g dry weight of mycelium) at -17 C. This was followed by digitonin precipitation of 3-β-hydroxysterols. The extract was dried and dissolved in 200 ml of 50% acetone (v/v) in 95% ethanol, and 40 ml of 0.5% (w/v) solution of digitonin in 50% ethanol (v/v) was added. After 1 hr on a shaker, the 3-β-hydroxysterol digitonides were removed by centrifugation for 10 min at $8000 \times g$. The digitonin treatment was repeated as necessary by addition of 100 ml acetone, 100 ml 95% ethanol, and 40 ml digitonin solution, until no further precipitate was obtained. The extract was then transferred to 10% benzene (v/v) in petroleum ether, washed three times with water, dehydrated with anhydrous Na_2SO_4, and dried under N_2.

Chromatography

Carotenoid extracts were chromatographed on columns (30 by 2 cm) of deactivated neutral alumina (80–200 mesh, 6.0% water) (Davies, 1965; Kushwaha *et al.*, 1970). The column was eluted with 300 ml petroleum ether, followed by a concave elution gradient of diethyl ether in petroleum ether

Table I. Chromatography of Wild-Type *N. crassa* Carotenoids[a]

Carotenoid	Absorbance maxima	Percentage diethyl ether required for elution
Phytoene	275, 285, 298	0
Phytofluene	330, 347, 366	0
ζ-Carotene	376, 397, 421	0.6–3.4
β-Carotene	427, 450, 478	3.4–3.5
Neurosporene	414, 438, 467	3.5–4.3
Torulene	460, 483, 515	4.1–4.8
Lycopene	442, 469, 502	4.7–5.5
3,4-Dehydrolycopene	462, 491, 524	5.6–6.9
Residual carotenoids	—	32–37

[a] The (30 by 2 cm) alumina column (6.0% water) was developed with 300 ml petroleum ether, followed by a concave elution gradient of diethyl ether in petroleum ether up to 50% diethyl ether (v/v); 260 5-ml fractions were collected. The gradient mixer apparatus was similar to that used by Parr (1953) and described by Bock and Ling (1954) with cross-sectional areas $A_1 = 29.61$ cm^2 and $A_2 = 9.19$ cm^2, total volume $V = 10^3$ ml, and reservoir concentrations $C_1 = 0\%$ and $C_2 = 67\%$ diethyl ether (v/v) in petroleum ether. Diethyl ether concentration, C, was calculated using the formula $C = C_2 - (C_2 - C_1)\ (1 - v/V)^{A2/A1}$, where v is the elution volume. A short communication describing the use of gradient elution chromatography for separating carotenoids is being prepared for publication.

(Table I); 260 fractions (5 ml) were collected. Individual carotenoids were rechromatographed on smaller columns (20 by 2 cm or 20 by 1 cm) of deactivated alumina (80–200 mesh, 2.5% water), developed with petroleum ether or gradients of diethyl ether in petroleum ether.

Spectrophotometry

Absorption spectra were recorded from 600 to 220 nm with a Beckman Acta III recording spectrophotometer, calibrated with a holmium filter.

Carotenoid fractions having the same absorption spectra were pooled and estimated quantitatively using extinction coefficients reported by Davies (1970). Some carotenoid fractions having absorption peaks for two different carotenoids were estimated quantitatively using Beer's law equations for two-component mixtures. This procedure was more successful than rechromatography for small amounts of two-component mixtures and produced higher recovery of carotenoids. The necessary extinction coefficients (Table II) were obtained from spectra of rechromatographed carotenoids or carotenoid standards (kindly supplied by Hoffman-LaRoche & Co., Ltd., Basel).

Table II. Absorbancies Used for Estimation of Carotenoid Concentrations[a]

Carotenoid	Wavelength	Extinction coefficient ($E^{1\%}$ cm)	Absorptivity ($1\ g^{-1} \cdot cm^{-1}$)
ζ-Carotene	397	2270	227.0
	452		3.96
β-Carotene	397		120.8
	452	2505	250.5
	438		225.9
Neurosporene	452		210.9
	438	2990	299.0
	483		86.05
Torulene	438		158.4
	483	3240	324.0

[a] Small amounts of two-component mixtures were resolved by solving two equations in two unknowns. Standard carotenoid mixtures were used to demonstrate the validity of Beer's law for mixtures of two carotenoids. Absorptivity values for this purpose were derived from absorbtion spectra of pure carotenoids.

Thin-Layer Chromatography

Extracts were cochromatographed with carotenoid standards on thin layers (0.25 mm) of Silica Gel G (Merk), developed with petroleum ether or with 1% or 10% benzene (v/v) in petroleum ether.

RESULTS

Chromatography

Crystallization of steroids, present in quantities of up to 1% of dry weight (Ottke, 1949; Tsuda and Tatum, 1959), followed by digitonin precipitation of 3-β-hydroxysterols was required to prevent chromatography columns from jamming. Concave gradient elution, which has been used successfully in steroid chromatography (Lakshmanan and Lieberman, 1964), produced the best separations of carotenoids. Stepwise elution (Davies, 1970; Kushwaha *et al.*, 1970) and linear gradient elution (Davies, 1965) of deactivated alumina columns produced very poor separations of *N. crassa* carotenoids, probably due to the presence of large amounts of steroids in all strains examined.

Wild Type

Phytoene, phytofluene, ζ-carotene, β-carotene, neurosporene, torulene,

Table III. Wild-Type Carotenoids[a]

Carotenoid	Weight of carotenoid (μg)	Concentration of carotenoid (μg/g dry weight)	Percent of total neutral carotenoids
Phytoene	2890	88.0	85.8
	3980	86.6	87.8
Phytofluene	180	5.48	5.34
	124	2.71	2.74
ζ-Carotene	18.6	0.567	0.553
	55.1	1.20	1.22
Neurosporene	179	5.45	5.31
	126	2.74	2.78
Lycopene	11.9	0.361	0.352
	23.8	0.518	0.525
3,4-Dehydrolycopene	4.13	0.126	0.122
	tr[b]		
β-Carotene	48.7	1.49	1.44
	224	4.89	4.96
Torulene	37.5	1.14	1.11
	tr[b]		
Total	3370	103	
	4530	98.7	

[a] Two determinations.
[b] Refers to traces of carotenoid identified by thin layer chromatography.

lycopene, and 3,4-dehydrolycopene were eluted from the (30 by 2 cm) alumina column (6.0% water, Tables I and III). This is consistent with previous studies of wild-type *N. crassa* carotenoids (Haxo, 1949, 1952; Zalokar, 1954; Huang, 1964; Jensen, 1965; Harding, 1968; Subden, 1969). γ-Carotene and β-zeacarotene, reported in some previous investigations of wild-type *N. crassa* (Harding, 1968; Subden, 1969), were not found in quantities sufficient for spectrophotometric identification, less than 0.1 μg/g dry weight.

When the saponification mixture was partitioned with 10% benzene in petroleum ether, the hypophase (KOH ethanol) was highly colored, indicating the presence of acidic carotenoids.

A very pale orange band remained at the origin of the (30 by 2 cm) alumina column after elution of 3,4-dehydrolycopene. This residual carotenoid (Table I) could be eluted with 32–37% ether (v/v) in petroleum ether but was not present in quantities sufficient for identification and was not included in calculating total neutral carotenoids (Table III).

ylo-1 Mutant

Phytoene, phytofluene, ζ-carotene, β-carotene, neurosporene, γ-carotene, and lycopene were eluted from the (30 by 2 cm) alumina column (Table IV), using the same elution gradient as for wild type.

When the saponification mixture was extracted with 10% benzene in petroleum ether, the hypophase (KOH ethanol) was almost colorless.

β-Carotene spectra were obtained from two distinct chromatographic components in the *ylo-1* mutant. One component chromatographed between ζ-carotene and neurosporene (as in wild type) and had an absorption spectrum very similar to that of all *trans*-β-carotene (Weedon, 1971). The other component chromatographed between γ-carotene and lycopene (Table I) and had an absorption spectrum similar to those of 15,15′-*cis*-β-carotene (*cis* peak at 343 nm) and other β-carotene isomers having one double bond in the *cis* configuration (Weedon, 1971).

Larger amounts of residual carotenoids were present in *ylo-1* than in wild types. This residual carotenoid was eluted by 32–37% ether in petroleum ether and consisted of at least three chromatographic components: a component which fluoresced green when irradiated with long-wave ultraviolet light, one yellow component, and one orange component. These mixtures of carotenoids had absorption peaks resembling those of mixtures of phytofluene, ζ-carotene,

Table IV. Carotenoids of *ylo-1* Mutant[a]

Carotenoid	Weight of carotenoid (μg)	Concentration of carotenoid (μg/g dry weight)	Percent of total neutral carotenoids
Phytoene	2300	86.0	92.9
	2450	77.0	85.4
Phytofluene	50.0	1.87	2.02
	111	3.50	3.88
ζ-Carotene	2.15	0.0805	0.0870
	15.4	0.484	0.537
Neurosporene	19.0	0.713	0.771
	73.4	2.31	2.56
Lycopene	36.1	1.35	1.46
	141	4.45	4.93
γ-Carotene	6.12	0.229	0.248
	19.5	0.613	0.680
β-Carotene	61.4	2.30	2.49
	57.1	1.79	1.99
Total	2470	92.6	
	2870	90.1	

[a] Two determinations.

Table V. Carotenoids of *al-1* Mutants

Strain	Carotenoid	Weight of carotenoid (μg)	Concentration of carotenoid (μg/g dry weight)	Percent of total neutral carotenoids
RES-25A	Phytoene	6050	130	99.2
		3860	87.7	100
	Phytofluene	40.2	0.863	0.658
		tr[a]		
	ζ-Carotene	9.48	0.204	0.155
		tr[a]		
34508A	Phytoene	2800	64.3	100
		3120	62.5	100
ALS-14A	Phytoene	6400	115	100
		4560	144	100
RES-6A	Phytoene	8832	341	100

[a] Refers to traces of carotenoid identified by thin layer chromatography. All *al*-1 mutants had traces of phytofluene, ζ-carotene, and other carotenoids.

β-carotene, and lycopene but were not included in estimates of total netural carotenoids (Table IV) due to their chromatographic behavior. The residual carotenoids may represent epoxy carotenoids (Britton and Goodwin, 1969) or other oxygenated derivatives (Harding, 1968).

al-1 Mutants

ALS-14A, RES-6A, 34508A, and RES-25A all contained large amounts of phytoene and only traces of other neutral carotenoids (Table V). Thin layer chromatography showed traces of phytofluene (colorless, fluorescent when irradiated with 350 nm ultraviolet light), ζ-carotene (yellow), and from one to three faint orange components which were not identifiable.

al-2 Mutants

15300A, Y256M220A, and RES-8A did not contain enough neutral carotenoids to identify with the spectrophotometer. Thin layer chromatography showed traces of phytofluene, ζ-carotene, and from three to four faint orange components which were not identifiable. In all cases, these traces of carotenoids seemed to be in a higher concentration than in the *al-1* mutants.

al-3 Mutants

The neutral carotenoid content of RP 100 was identical to that of the *al-2* mutants.

Phytoene

In all strains having phytoene, the phytoene eluted from the (30 by 2 cm) alumina column (6% water) was contaminated with substances which fluoresced blue when irradiated with (350 or 254 nm) ultraviolet light. These substances, which absorbed light from 350 to 220 nm, were removed by rechromatography on (20 by 2 cm) alumina columns (2.5% water). The spectra of purified phytoene very closely resembled that of 15,15′-*cis*-phytoene (Maudinas *et al.*, 1972, Kushwaha *et al.*, 1970).

DISCUSSION

In an effort to focus attention on the primary effect of the biosynthetic lesions and avoid the misleading evidence from accumulated intermediates from "leaky" or mutant enzymes with residual activity, only recently induced early stationary phase cultures were used. This technique resulted in essentially the same wild-type distribution of intermediates as previously published (Haxo, 1949, 1952; Zalokar, 1954; Huang, 1964; Jensen, 1965; Harding, 1968; Subden, 1969) but produced significant differences in the mutant strains. The almost colorless hypophase of the *ylo-1* mutant during partition of the saponification mixture with 10% benzene in petroleum ether substantiates previous reports (Haxo, 1952; Harding, 1968; Subden, 1969) that neurosporaxanthin (β-apo-4′-carotenoic acid), present in wild-type *N. crassa*, is absent in the *ylo-1* mutant. Neurosporaxanthin accumulates at the expense of neutral carotenoids when wild-type *N. crassa* is exposed to intense fluorescent light (Harding, 1968). Structural considerations (β-ionone ring and 3′,4′ double bond at cleavage point) suggest torulene as a likely substrate for neurosporaxanthin synthetase, although no evidence was found for torulene or 3,4-dehydrolycopene accumulation. The *ylo-1* lesion probably involves 3′,4′ double bond formation reactions (Harding, 1968) or carboxylation of torulene or γ-carotene (Haxo, 1952; Subden, 1969). The presence of *cis*-β-carotene and the residual carotenoids in the *ylo-1* mutant may be due to isomerization and oxidation of carotenoids accumulating due to the mutant lesion. The residual carotenoids present in *ylo-1* may represent carotenoid epoxides (Britton and Goodwin, 1969).

The *al-1* strains contained large amounts of phytoene (99–100% of the total neutral carotenoids) in concentrations exceeding that of the wild type, indicating a lesion associated with phytoene dehydrogenase activity. The extreme phenotypic variability at this locus (Subden and Threlkeld, 1969, 1970) was due to small quantities of ζ-carotene and other colored carotenoids probably resulting from some residual activity of mutant phytoene dehydrogenases.

Although the evidence is largely negative, the failure of several mutant hunts (Subden, 1969; Schroeder, 1970) to isolate strains with discrete genetic lesions subsequent to phytoene dehydrogenase suggests a model with a multifunctional enzyme for successive dehydrogenations. Both *al-2* and *al-3* affect steps prior to phytoene synthesis but after farnesyl pyrophosphate synthesis, since these strains had large amounts of steroids but no carotenoids. Whether they both affect phytoene synthetase or one affects geranylgeranyl synthetase and the other phytoene synthetase cannot be resolved until the intracellular geranylgeranyl pyrophosphate concentrations are determined.

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