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ON FUNGAL CAROTENOIDS AND THE NATURAL DISTRIBUTION OF SPIRILLOXANTHIN

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Abstract—The carotenoid composition of the fungi *Lycogala epidendron* (L.) Fr., *Aleuria aurantia* (Fr.) Fuckel, *Neurospora crassa* Shear and Dodge and *N. sitophila* Shear and Dodge has been re-examined in a quantitative manner. A carotenoid component assumed by others to be spirilloxanthin has been shown to be 3,4-dehydrolycopene. Methoxylated carotenoids were absent, and the natural distribution of spirilloxanthin appears to be restricted to certain photosynthetic purple bacteria. The presence of spirilloxanthin in the non-photosynthetic bacterium *Corynebacterium poinsettae* Starr and Pirone was not confirmed. The carotenoid composition of *Scutellinia scutellata* (L. ex St. Amans) Lambotte has also been investigated. The fungus examined produced a high proportion of monocyclic carotenes and minor amounts of xanthophylls. Axanthophyll present in *Aleuria* (*Peziza*) *aurantia*, recently claimed to be identical with rubixanthin was found by direct comparison to be different from rubixanthin. This xanthophyll, here designated aleuriaxanthin, is possibly (IV) and was present as an ester.

INTRODUCTION

THE methoxylated carotenoid spirilloxanthin (I) is a characteristic carotenoid of several photosynthetic purple bacteria in the families Athiorhodaceae[1] and Thiorhodaceae.[2] It is also present in *Rhodomicrobium vannielii* Duchow and Douglas.[3]

Spirilloxanthin (rhodoviolascin) has also been claimed to be present in some fungi, namely *Lycogala epidendron*,[4] *Aleuria aurantia*,[4] *Neurospora crassa*[5] and *N. sitophila*.[6] In addition, its presence in the non-photosynthetic bacterium *Corynebacterium poinsettae* has been reported.[7]

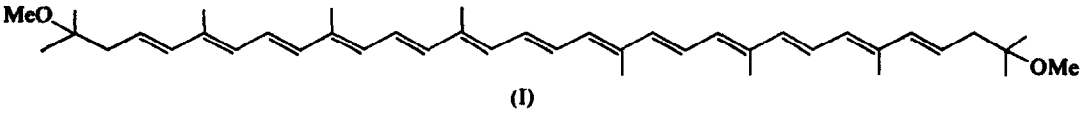
Two alternative pathways for the biosynthesis of spirilloxanthin have been established.[8–10] When considering the accompanying carotenoids in the non-photosynthetic organisms mentioned above, it is striking that the other methoxylated carotenoids of the so-called normal or alternative spirilloxanthin series[1] are virtually absent. This throws some doubt on the correct identification of spirilloxanthin.

In the present paper a re-investigation of the carotenoid distribution in these organisms has been undertaken using modern methods in order to settle the natural distribution of spirilloxanthin. For a survey of the early literature on fungal carotenoids the reader is referred to the pertinent review by Haxo.[5]

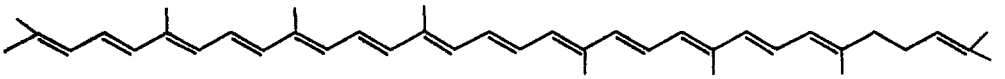
[1] S. LIAAEN JENSEN, in *Bacterial Photosynthesis*, (Edited by H. GEST, A. SAN PIETRO and L. P. VERNON,) p. 19. Antioch Press, Yellow Springs, Ohio (1963).
[2] K. SCHMIDT, S. LIAAEN JENSEN and H. G. SCHLEGEL, *Arch. Mikrobiol.* **46**, 138 (1963).
[3] L. RYVARDEN and S. LIAAEN JENSEN, *Acta Chem. Scand.* **18**, 643 (1964).
[4] E. LEDERER, *Bull. Soc. Chim. Biol.* **20**, 611 (1938).
[5] F. HAXO, *Arch. Biochem.* **20**, 400 (1949).
[6] F. HAXO, *Fortschr. Chem. Org. Naturstoffe.* **12**, 169 (1955).
[7] M. P. STARR and S. SAPERSTEIN, *Arch. Biochem.* **43**, 157 (1953).
[8] S. LIAAEN JENSEN, G. COHEN-BAZIRE, T. O. M. NAKAYAMA and R. Y. STANIER, *Biochim. Biophys. Acta* **29**, 477 (1958).
[9] S. LIAAEN JENSEN, G. COHEN-BAZIRE and R. Y. STANIER, *Nature* **192**, 1168 (1961).
[10] K. E. EIMHJELLEN and S. LIAAEN JENSEN, *Biochim. Biophys. Acta* **82**, 21 (1964).

RESULTS AND DISCUSSION

The quantitative carotenoid composition of *Lycogala epidendron* and *Aleuria aurantia* is given in Table 1. Neither contained spirilloxanthin (I), but both contained 3,4-dehydrolycopene (II) as a minor carotenoid.



(I)



(II)

TABLE 1. THE CAROTENOID COMPOSITION OF VARIOUS FUNGI*

	Per cent of total carotenoid					
	Myxomycetes	Ascomycetes				
	Lycogalaceae	Pezizales			Spaeriales	
Carotenoid	*Lycogala epidendron*	*Aleuria aurantia* Lot 1	*Aleuria aurantia* Lot 2	*Scutellinia scutellata*	*Neurospora crassa*	*Neurospora sitophila*
Phytofluene					10	6
ζ-Carotene					28	23
Neurosporene					19	13
β-Carotene	39	34	26		14	21
γ-Carotene	27	39	50	91	4	10
Lycopene	18	0·2	1			5
Torulene	13			6	11	3
3,4-Dehydrolycopene	3	1			2	3
Aleuriaxanthin ester		25	22			
Unidentified xanthophylls		1		3	2	7
Acid carotenoids					10	9
Carotenoids in per cent of acetone extracted residue	0·07	0·14	0·10	0·18		
Total carotenoid in sample in mg	2·12	1·65	1·50	0·33	1·03	3·30

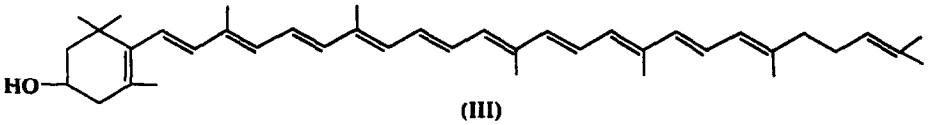
* Classification based on Ursing,[11] Alexopoulos[12] and Ainsworth.[13]

Both organisms synthesized monocyclic carotenoids in relatively high proportion.

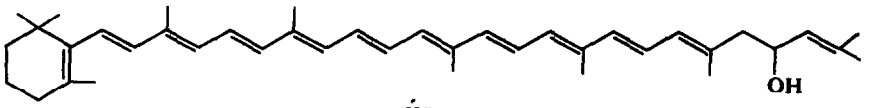
The carotenoids of *Aleuria aurantia* (*Peziza aurantia*) were recently investigated by Valadon,[14] who claimed the presence of rubixanthin (III) in this fungus. This finding could not be confirmed. However, a natural carotenoid ester of a rubixanthin-like carotenoid, here called aleuriaxanthin, was present. Aleuriaxanthin exhibited an absorption spectrum in visible light and adsorptive and partition properties indicative of its being a monohydroxy-γ-carotene. Acetylation evidence is presented for the secondary (or primary) character of

[11] B. URSING, *Svenska Växter. Kryptogamer*, Nordisk Rotogravyr, Stockholm (1949).
[12] C. J. ALEXOPOULOS, *Introductory Mycology*, J Wiley, New York (1960).
[13] G. C. AINSWORTH, *Ainsworth & Bisby's Dictionary of the Fungi* (5th Ed.) Butler & Tanner, London (1961).
[14] L. R. G. VALADON, *Biochem. J.* **92**, 19P (1964).

the non-allylic (to the polyene chain) hydroxyl group. Since aleuriaxanthin was found to be different from rubixanthin (III) and 1',2'-dihydro-1'-hydroxy-γ-carotene,[15] the hydroxy group is probably located in 2 or 3'-position. Aleuriaxanthin possibly has the structure (IV). Judging from the R_f-values of aleuriaxanthin acetate and the natural ester, the esterifying acid in the natural ester is a long-chain fatty acid. The relationship between aleuriaxanthin and gazaniaxanthin[16] is unknown.



(III)



(IV)

The carotenoid composition of *Scutellinia scutellata* was also examined. γ-Carotene was the major carotene, and torulene (3',4'-dehydro-γ-carotene) was also present.

The carotenoids of *Neurospora crassa* have been studied by many workers; first by Haxo[5] in a qualitative manner. The quantitative carotenoid composition of the cells examined here is listed in Table 1. 3,4-Dehydrolycopene (II), which has previously been mistaken for spirilloxanthin (I),[5] was present. The observation by Grob[17] that this carotenoid is rapidly formed in *N. crassa* from more saturated carotenoid precursors, is intelligible from its being a 3,4-dehydrolycopene (II) rather than the methoxylated spirilloxanthin (I). The main carotenoid acid synthesized by *N. crassa* has been designated neurosporaxanthin by Zalokar.[18] The structural elucidation of neurosporaxanthin has now been carried out and will be reported elsewhere.[19]

Haxo[6] has stated that the carotenoid composition of *N. sitophila* was very similar to that of *N. crassa*. This is in agreement with the present findings (see Table 1).

Finally the carotenoid composition of *Corynebacterium poinsettae* was re-investigated. The presence of spirilloxanthin (I), lycoxanthin (3-hydroxy-lycopene) and cryptoxanthin (3-hydroxy-β-carotene) had previously been suggested by Starr and Saperstein.[7] This could not be confirmed. The spirilloxanthin-like component in the cells used in the present investigation was different from both spirilloxanthin (I) and monodemethylated spirilloxanthin. The carotenoids of this bacterium will be a subject for further investigation.

In summary, it may be concluded that spirilloxanthin (I) and methoxylated carotenoids in general, seem to be restricted to certain photosynthetic purple bacteria, a fact that strengthens the disputable taxonomic value of carotenoids.

MATERIALS AND METHODS

Except for acetone and light petroleum (boiling range 40–70°) all solvents and reagents used were of analytical grade.

Saponifications of crude extracts were carried out at room temperature for 1–2 hr in

[15] R. Bonnett, A. A. Spark and B. C. L. Weedon, *Acta Chem. Scand.* **18**, 1739 (1964).
[16] L. Zechmeister and W. A. Schroeder, *J. Am. Chem. Soc.* **65**, 1535 (1943).
[17] E. C. Grob, *Chimia Switz.* **12**, 86 (1958).
[18] M. Zalokar, *Arch. Biochem. Biophys.* **70**, 568 (1957).
[19] A. J. Aasen and S. Liaaen Jensen, *Acta Chem. Scand.* (To be published).

ether–methanol with a final alkali concentration of 5% KOH. The unsaponifiable matter was transferred to ether–light petroleum in a separating funnel on dilution with 5% aqueous NaCl-solution in the usual manner. The epiphase was washed with aqueous salt solution and dried over anhydrous Na_2SO_4. The procedure used for column chromatography has been described elsewhere,[8] the adsorbent was Woelm neutral alumina, activity grade 2.[20]

Paper chromatography was carried out on Schleicher & Schüll No. 287 paper (with kieselguhr filler) and on Schleicher & Schüll No. 288 paper (aluminium oxide filler) according to the method described previously.[21] For co-chromatography tests, the 3-divided paper technique was employed.[22]

Absorption spectra in visible light were recorded on a Beckman DB Instrument. Extinction coefficients used in the quantitative determinations are listed in Table 2. Partition tests were performed according to the procedure of Petracek and Zechmeister.[23] All operations

TABLE 2. PROPERTIES OF SOME FUNGAL CAROTENOIDS

Carotenoid	$\lambda_{max.}$ in light petroleum (mμ)	$E^{1\%}_{1cm}$ at main $\lambda_{max.}$ used	Eluant required from deactivated alumina activity grade 2 (%)*	R_f Schleicher & Schüll No. 288 0%†	1%	10%	R_f Schleicher & Schüll No. 287 0%	2%	5%
Phytofluene	332 347 368	1500	5	0·90					
ζ-Carotene	376 398 418	2000	10	0·58					
β-Carotene	(425)450 478	2500	10–15	0·46	0·98		0·95		
γ-Carotene	434 460 491	2600	15–25		0·70		0·63		
Aleuriaxanthin ester	434 460 491	2500	25		0·33				
Neurosporene	414 439 466	2900	25	0·20			0·66		
Torulene	460 484 518	3200	35		0·40		0·25		
Lycopene	445 472 504	3400	35–50				0·40		
3,4-Dehydrolycopene	463 493 527	3000	75						0·52
Aleuriaxanthin	434 460 491	2600	8*			0·63		0·65	

* Ether in light petroleum.
† Acetone in light petroleum.

were carried out as far as possible in subdued light and inert atmosphere. Concentrations were made under vacuum at 40°; extracts were stored at −20°.

Lycogala epidendron

Biological material. Lycogala epidendron (L.) Fr. (young aethalia) was collected at Viksåsen, near Trondheim, in September 1964.

Pigment extraction. The squeezed fungus was kept under acetone for 4 days at −20°.

Saponification, chromatographic separation and identification of the individual carotenoids. The acetone extract was concentrated to dryness and saponified. Paper chromatographic examination prior to and after saponification revealed no change in polarity of the carotenoid

[20] H. BROCKMANN and H. SCHODDER, *Ber. Deut. Chem. Ges.* **74**, 73 (1941).
[21] A. JENSEN and S. LIAAEN JENSEN, *Acta Chem. Scand.* **13**, 1863 (1959).
[22] A. JENSEN, O. AASMUNDRUD and K. E. EIMHJELLEN, *Biochim. Biophys. Acta* **88**, 466 (1964).
[23] F. J. PETRACEK and L. ZECHMEISTER, *Anal. Chem.* **28**, 1484 (1956).

components; for R_f-values see Table 2. The carotenoid mixture was resolved on a column of deactivated alumina. Eluants required are given in Table 2. The individual components were identified from their absorption spectra in visible light and co-chromatography tests on circular paper with authentic carotenoids, cf. Table 2. The result is presented in Table 1. *Trans*-3,4-dehydrolycopene could not be separated from a synthetic sample and was less strongly adsorbed than spirilloxanthin on kieselguhr paper.

Aleuria aurantia

Biological material. Aleuria aurantia (F.) Fuckel Lot 1 was collected in Kjerringdal, Øvre Gjervan near Jonsvannet in October 1964; Lot 2 near Wilhelmsmyr, Heimdal 3 weeks later.

Pigment extraction, saponification, chromatographic separation and identification of the individual carotenoids. These operations were carried out in an analogous manner to that described for *Lycogala epiendron* above. The results are given in Tables 1 and 2.

Aleuriaxanthin ester exhibited an absorption spectrum in visible light similar to that of γ-carotene. However, as seen from Table 2, this compound is more strongly adsorbed than *trans*-γ-carotene on aluminium oxide paper. As revealed by co-chromatography tests, aleuriaxanthin ester is also more strongly adsorbed than chlorobactene (aromatic γ-carotene)[24] and less strongly adsorbed than rubixanthin isolated from *Rosa rubigenosa*.[24] This compound was entirely epiphasic when partitioned between light petroleum and 95% methanol. Aleuriaxanthin ester was resistant towards saponification under the conditions specified above for 1 hr, but the chromatographically purified ester was quantitatively converted to aleuriaxanthin upon saponification (5% KOH-methanol) for 21 hr.

Aleuriaxanthin exhibited the same absorption spectrum as the ester. Its more polar character is inferred from the data given in Table 2 and the observed partition ratio of 12:88 in light petroleum:95% methanol. Aleuriaxanthin ($R_f = 0{\cdot}63$) was easily separated from rubixanthin ($R_f = 0{\cdot}42$) on circular aluminium-oxide paper (10% acetone–light petroleum), and was further slightly more strongly adsorbed than 1′,2′-dihydro-1′-hydroxy-γ-carotene ($R_f = 0{\cdot}70$)[15] and less strongly adsorbed than hydroxychlorobactene ($R_f = 0{\cdot}56$)[24] as revealed by co-chromatography tests in the same system.

Aleuriaxanthin was readily acetylated in dry pyridine with acetic anhydride in the usual way. On co-chromatography on aluminium-oxide paper (1% acetone–light petroleum) the acetate ($R_f = 0{\cdot}24$) was more strongly adsorbed than natural aleuriaxanthin ester ($R_f = 0{\cdot}33$). Saponification of aleuriaxanthin acetate in 5% KOH-methanol for 19 hr gave a quantitative recovery of aleuriaxanthin.

The possible presence of allylic hydroxyl groups in aleuriaxanthin was tested according to the acid chloroform procedure of Karrer and Leumann (cf.[25]). No products with longer chromophores were produced. Nor were products with longer chromophores produced on *p*-chloranil oxidation of aleuriaxanthin using the method of Warren and Weedon.[26]

Scutellinia scutellata

Biological material. Scutellinia scutellata (L. ex St. Amans) Lambotte=*Lachnea scutellata* was collected near Wilhelmsmyr, Heimdal, in early October 1964.

Pigment extraction, saponification, chromatographic separation and identification of the

[24] S. Liaaen Jensen, E. Hegge and L. M. Jackman, *Acta Chem. Scand.* **18**, 1703 (1964).
[25] S. Liaaen Jensen, *Acta Chem. Scand.* **17**, 489 (1963).
[26] C. K. Warren and B. C. L. Weedon, *J. Chem. Soc.* 3972 (1958).

carotenoid components was performed as for *Lycogala epidendron*. The results are presented in Tables 1 and 2. The yellow, unidentified component had $\lambda_{max.}$ at 441 and 470 mμ in light petroleum with adsorptive properties indicative of a mono-hydroxy carotenoid.

Neurospora crassa

Biological material. Neurospora crassa Shear and Dodge obtained from Centraalbureau voor Schimmelcultures, Baarn, Netherlands, was cultivated in a light cabinet at room temperature for ~6 days in 5 Fernbacher flasks, each containing ~200 ml of medium. The medium contained 0·1% KNO_3, 0·05% $MgSO_4$. $7H_2O$, 0·01% $CaCl_2$, 0·1% KH_2PO_4, 0·2% NaCl, 5% sucrose, 0·5% yeast extract, 2% yeast autolysate, 0·5% malt extract and trace minerals. The mycelial mat was collected on a cloth filter and was pressed fairly dry.

Pigment extraction. The cells were homogenized with acetone for 3 min in a Waring blendor, whereupon the pigments were immediately released. The cells were left with acetone overnight, and finally the residue was ground with quartz sand in a mortar, followed by a final extraction with acetone.

Saponification. The pigments were transferred to ether–light petroleum in a separating funnel on dilution with 5% aqueous NaCl. Saponification was carried out as outlined above, and the reaction mixture was worked up in the usual manner. The spectrophotometric ratio between the neutral carotenoids of the epiphase and the acidic carotenoids present in the hypophase, prior to acidification of the latter is seen in Table 1.

Chromatographic separation and identification. The neutral carotenoid fraction was chromatographed on columns of deactivated alumina. Phytofluene and ζ-carotene were tentatively identified from their R_f- values and absorption spectra (see Table 2). For the other components the identification included co-chromatography tests with authentic samples. Spirilloxanthin was absent, as judged from the co-chromatography test of the actual pigment with 3,4-dehydrolycopene and spirilloxanthin. The unidentified xanthophyll had an absorption spectrum similar to that of β-carotene and adsorptive properties indicative of a mono-hydroxy compound.

Neurospora sitophila

Biological material. Neurospora sitophila Shear and Dodge was obtained from the same source and cultivated in an analogous manner to that specified for *N. crassa* above.

Pigment extraction, saponification, chromatographic separation and identification was also carried out as for *N. crassa*. The unidentified xanthophyll had $\lambda_{max.}$ at 440, 460 and 490 mμ in light petroleum and required 20% acetone–light petroleum for elution from deactivated alumina.

Corynebacterium poinsettae

Biological material. Corynebacterium poinsettae Starr and Pirone was kindly provided by Prof. R. Y. Stanier, Department of Bacteriology, University of California, Berkeley. The cultivation of Lot 1 was carried out in medium B (10 × 100 ml) according to the procedure of Kunisawa and Stanier.[27] Lot 2 was cultivated in a low-thiamine medium (1 μg thiamine/100 ml culture; 2 × 100 ml) of Starr and Saperstein.[7] The cells were harvested by centrifuging after 3 days of growth.

[27] R. KUNISAWA and R. Y. STANIER, *Arch. Mikrobiol.* 31, 146 (1958).

Pigment extraction and saponification. The centrifuged cells were suspended in water and broken in a cell homogenizer with ballotini beads. The suspension was mixed with an excess of acetone and left for extraction at room temperature for 4 hr. The mixture was diluted with aqueous salt solution and the pigments were transferred to ether in a separating funnel. The ether extract was concentrated, and saponification was performed overnight in a methanol–ether solution containing 5% KOH. The reaction mixture was worked up in the usual manner.

Chromatographic separation. This was performed on a deactivated alumina column. The carotenoid mixture of Lot 1 (0·33 mg/l. culture) consisted of 2% Carotenoid A $\lambda_{max.}$ in light petroleum at 445, 472 and 504 mμ, 5% Carotenoid B $\lambda_{max.}$ 465, 493 and 527 mμ, 46% Carotenoid C $\lambda_{max.}$ 445, 473 and 504 mμ and 47% Carotenoid D $\lambda_{max.}$ (425), 448 and 379 mμ. Carotenoid B required 15% acetone–light petroleum for elution from deactivated alumina and exhibited an absorption spectrum in visible light reminiscent of spirilloxanthin. However, co-chromatography tests on kieselguhr paper (10% acetone–light petroleum) of *trans* Carotenoid B ($R_f = 0{\cdot}50$) with spirilloxanthin ($R_f = 0{\cdot}74$) or mono-demethylated spirilloxanthin ($R_f = 0{\cdot}40$), proved negative. The adsorptive properties and partition ratio (light petroleum:95% methanol 55:45) indicated the presence of a hydroxy group in Carotenoid B. Lot 2 contained 0·22 mg carotenoid/l. culture; Carotenoid B comprised 85 per cent of the carotenoid mixture.

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