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THE CAROTENOIDS OF BLUE-GREEN ALGAE—I.

THE CAROTENOIDS OF *OSCILLATORIA RUBESCENS* AND AN *ATHROSPIRA* SP.

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Abstract—The carotenoid composition of *Oscillatoria rubescens* has been re-examined in a quantitative manner. β-Carotene, zeaxanthin, echinenone (myxoxanthin), myxoxanthophyll and oscillaxanthin were found together with small amounts of cryptoxanthin and a hydroxylated ketocarotenoid whose probable structure is 4-keto-3′-hydroxy-β-carotene (I). The carotenoids of an *Athrospira* sp., whose systematic position is close to that of *O. rubescens*, were qualitatively and quantitatively very similar to those of *O. rubescens*, and the *Athrospira* sp. can be used as a more convenient source for myxoxanthophyll and oscillaxanthin—the structures of which are not yet established.

INTRODUCTION

THE carotenoids of various blue-green algae were extensively studied in the early days;[1,2] further representatives of this algal class have been examined more recently.[3–5] A summary of the present position has been made by Goodwin[3,6] who states that the blue-green algae are the only photosynthetic organisms in which β-carotene often constitutes about one-half of the total carotenoid content, additional characteristic carotenoids being echinenone (myxoxanthin, 4-keto-β-carotene)[7,8] and myxoxanthophyll.

Several problems have remained unsolved, such as the suggested identity of aphanin and echinenone,[9] of myxoxanthophyll and aphanizophyll[3,10] and the chemical structures of some of the carotenoids encountered—flavacin, aphanicin, myxoxanthophyll, aphanizophyll and oscillaxanthin. To justify generalizations, the carotenoid composition of further members of the Cyanophyceae ought to be examined in a quantitative manner.

The aim of the present project was to clarify some of these points, and this paper represents the first of our contributions.

RESULTS AND DISCUSSION

The carotenoids of *Oscillatoria rubescens* have previously been studied qualitatively by Heilbron and Lythgoe[11] and Karrer and Rutschmann.[12] Their findings are summarized in Table 1, together with the quantitative data from the present investigation.

[1] T. W. GOODWIN, *The Comparative Biochemistry of the Carotenoids*. Chapman & Hall, London (1952).
[2] P. KARRER and E. JUCKER, *Carotinoide*. Verlag Birkhäuser, Basel (1948).
[3] T. W. GOODWIN, *J. Gen. Microbiol.* **17**, 467 (1957).
[4] J. TISCHER, *Z. physiol. Chem.* **311**, 140 (1958).
[5] H. H. STRAIN, Annual Priestley Lecture 1958, *Chem. Abstr.* 13890f (1958).
[6] T. W. GOODWIN, in *Encyclopedia of Plant Physiology* Vol. 5, 394 (1960).
[7] T. W. GOODWIN and M. M. TAHA, *Biochem. J.* **48**, 513 (1951).
[8] T. W. GOODWIN, *Biochem. J.* **63**, 481 (1956).
[9] T. W. GOODWIN and M. M. TAHA, *Biochem. J.* **47**, 244 (1950).
[10] I. M. HEILBRON, *J. Chem. Soc.* 79 (1942).
[11] I. M. HEILBRON and B. LYTHGOE, *J. Chem. Soc.* 1376 (1936).
[12] P. KARRER and J. RUTSCHMANN, *Helv. Chim. Acta* **27**, 1691 (1944).

The previous identification of β-carotene, zeaxanthin (3,3'-dihydroxy-β-carotene) and echinenone (4-keto-β-carotene) from the epiphasic fraction of *O. rubescens* was confirmed by the visible light absorption spectra and chromatographic behaviour of these carotenoids compared directly with authentic samples. In addition a small amount of cryptoxanthin (3-hydroxy-β-carotene) was found. Lutein (3,3'-dihydroxy-α-carotene) was absent; the structure of a minor keto-carotenoid will be discussed under *Athrospira* sp. below. The hypophasic fraction comprised myxoxanthophyll and oscillaxanthin.

The carotenoids of *Athrospira* have not been studied before. The systematic position of this genus relative to *Oscillatoria* and *Aphanizomenon* can be summarized as follows:[13] all are in the order Nostocales of the Cyanophyta, *Athrospira* and *Oscillatoria* being in the family Oscillatoriaceae, and *Aphanizomenon* in the family Nostacaceae.

The carotenoid composition of the *Athrospira* sp. examined was qualitatively and quantitatively very similar to that of the closely related *O. rubescens* (Table 1).

TABLE 1. CAROTENOIDS ISOLATED FROM *O. rubescens* AND *Athrospira* sp.

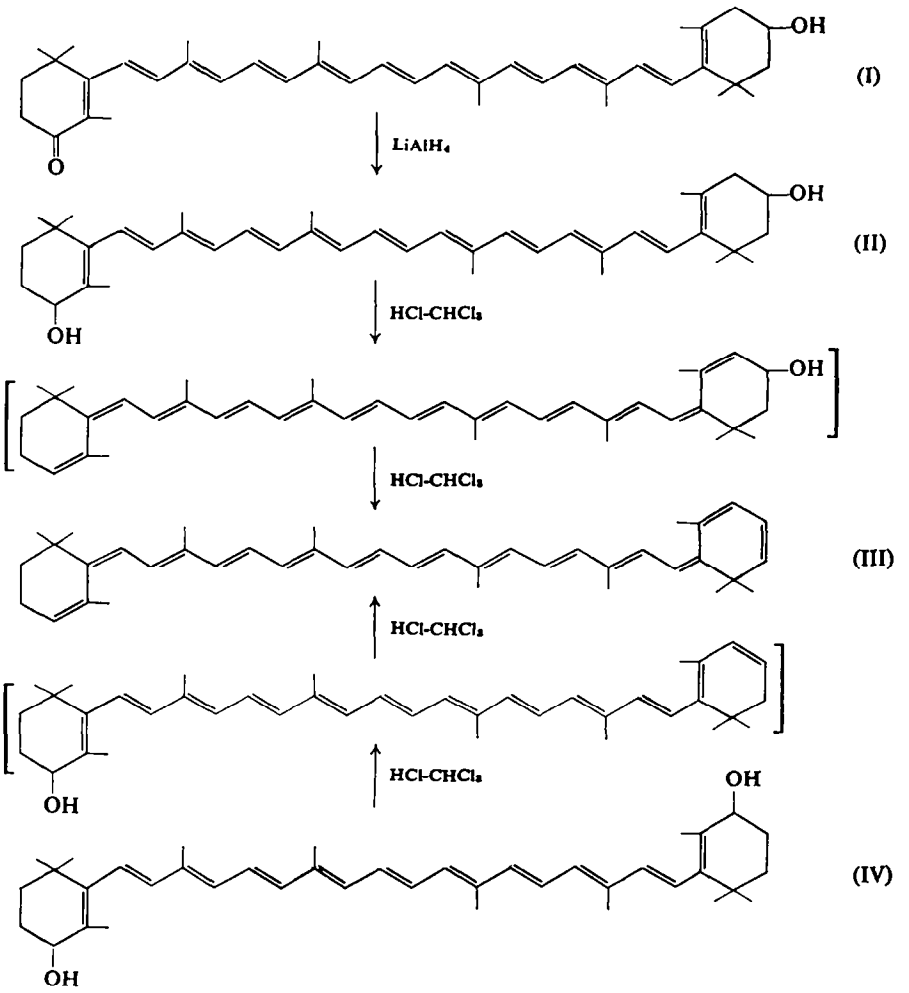
Carotenoid	*Oscillatoria rubescens*			*Athrospira* sp.
	Heilbron and Lythgoe[7]	Karrer and Rutschmann[8]	Present investigation	Carotenoid in % of total
β-Carotene	+	+	29	27
Cryptoxanthin	−	−	4	2
Zeaxanthin	−	+	8	15
Lutein	+	−	—	—
Echinenone ≡ myxoxanthin	+	+	19	3
4-Keto-3'-hydroxy-β-carotene	−	−	1	2
Myxoxanthophyll	+	+	30	46
Oscillaxanthin	−	+	10	5

β-Carotene and zeaxanthin were isolated in the crystalline state and a number of criteria for the identification of these carotenoids were obtained. The cryptoxanthin present had an absorption spectrum in visible light identical with that of β-carotene; the partition ratio and chromatographic behaviour were as for a mono-hydroxy-β-carotene, and no allylic dehydration product was obtained with acid chloroform.[14] It might be mentioned that Tischer[4] fairly recently isolated cryptoxanthin from *O. amoena*. Echinenone was identified by direct spectral and chromatographic comparison with an authentic specimen, and gave on hydride reduction a mono-ol identical with isocryptoxanthin (4-hydroxy-β-carotene).

A second keto-carotenoid in the epiphasic fraction had an absorption spectrum in visible light identical with that of echinenone. R_f-value and partition ratio were indicative of the presence of one free hydroxyl group. This compound could not be chromatographically separated from 4-keto-4'-hydroxy-β-carotene, prepared by *p*-chloranil oxidation of isozeaxanthin,[15] but differed from the latter in resistance toward allylic oxidation with *p*-chloranil. Hydride reduction gave a diol similar to zeaxanthin. However, treatment of this diol with acid chloroform[14] gave a red elimination product with the same R_f-value and absorption spectrum in visible light as *retro*-bis-dehydro-β-carotene (III), obtained by acid

[13] R. A. LEWIN, *Physiology and Biochemistry of Algae*. Academic Press, New York (1962).
[14] P. KARRER and E. LEUMANN, *Helv. Chim. Acta* **34**, 445 (1951).
[15] S. LIAAEN JENSEN, *Acta Chem. Scand.* **19**, 1166 (1965).

chloroform treatment of synthetic isozeaxanthin (IV) according to the report of Bodea and Nicoara.[16] In agreement with our structural interpretation of the new keto-carotenoid being 4-keto-3′-hydroxy-β-carotene (I) and its reduction product the corresponding diol (II), Bodea and Tamas[17] obtained the same elimination product (III) on treatment of their partly synthetic diol (II) with acid chloroform. The presence of cryptoxanthin and echinenone together in both these organisms lends further support to the identification of the minor keto-carotenoid from *Athrospira* sp. and *O. rubescens* as I. This is apparently the first report of the natural occurrence of I.



The major component of the hypophasic carotenoid fraction was identified as myxoxanthophyll on the basis of absorption spectra in visible light and R_f-values as compared with those of authentic myxoxanthophyll isolated from *O. rubescens*. In a similar manner, comparison of the final acetates and the allylic dehydration products derived from myxoxanthophyll isolated from the two sources supported the identity. Finally oscillaxanthin and its

[16] C. Bodea and E. Nicoara, *Rev. Chim. Acad. Rep. Populaire Romaine* **7**, 79 (1962).
[17] C. Bodea and V. Tamas, *Ann. Chem.* **67**, 57 (1964).

acetate from *Athrospira* sp. exhibited the same absorption spectra and R_f-values as free and acetylated oscillaxanthin from *O. rubescens*. *Athrospira* sp. is therefore a convenient source of myxoxanthophyll and oscillaxanthin. The chemical structures of these two carotenoids are being studied. The result will be published elsewhere.[18]

EXPERIMENTAL

Materials and Methods

Reagents and solvents used, except for acetone and light petrol (b.p. 40–60°), were of analytical grade.

Pigment extraction was performed at room temperature with successive portions of acetone–methanol (7:3). The extract was concentrated and the pigments were transferred to ether on admixture with aq. NaCl in a separating funnel. The concentrated ether extract was saponified for 2 hr at room temperature with KOH–methanol (final alkali concentration 5%), and the unsaponifiable matter transferred to ether in a separating funnel in the usual manner. The total carotenoid content was spectrophotometrically estimated using $E^{1\%}_{1\,cm}=2500$ at the main λ_{max} in ether. The ether extract was concentrated to dryness, and the pigments were submitted to partition between light petrol and 85% aq. methanol.

The epiphasic carotenoids were chromatographed on columns of Woelm neutral alumina, activity grade 2,[19] and the hypophasic carotenoids were separated on columns of Linters cellulose powder. Circular paper chromatography was carried out on Schleicher & Schüll No. 287 (kieselguhr paper)[20] or Schleicher & Schüll No. 288 (aluminium oxide paper).[21] For co-chromatography tests the 3-divided paper technique was used.[22]

Partition ratios were determined according to the method of Petracek and Zechmeister.[23] Acetylations,[24] hydride reduction[25] and acid-chloroform treatment[25] were carried out as described elsewhere. Melting points were measured in evacuated capillary tubes and are given uncorrected.

Visible light absorption spectra were recorded on a Beckman DB recording spectrophotometer and i.r. spectra on a Perkin Elmer Model 21 instrument in KBr-discs as described elsewhere.[26]

Oscillatoria rubescens

Biological material. A unialgal culture of *O. rubescens*, prepared by the Norwegian Institute for Water Research, Oslo, was used.

Carotenoid content. A total of 3·8 mg carotenoids, or 0·28 per cent of the extracted algal residue, was isolated. The carotenoid composition is given in Table 1, and the identification of the individual components is described below in order of increasing adsorption.

β-Carotene required 2% ether–light petrol for elution from deactivated alumina; λ_{max} (427), 450 and 478 nm in light petrol; $R_f=0{\cdot}24$ on aluminium oxide paper (light petrol).

[18] S. HERTZBERG and S. LIAAEN JENSEN, *Acta Chem. Scand.* To be published.
[19] H. BROCKMANN and H. SCHODDER, *Ber. Deut. Chem. Ges.* **74**, 73 (1941).
[20] A. JENSEN and S. L. JENSEN, *Acta Chem. Scand.* **13**, 1863 (1959).
[21] A. JENSEN, *Acta Chem. Scand.* **14**, 2051 (1960).
[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).
[24] S. LIAAEN JENSEN, *Kgl. Norske Videnskab. Selskabs Skrifter* No. 8 (1962).
[25] S. LIAAEN JENSEN, *Acta Chem. Scand.* **17**, 489 (1963).
[26] S. LIAAEN JENSEN, *Acta Chem. Scand.* **17**, 303 (1963).

Co-chromatography tests in the latter system did not permit separation from synthetic *trans* β-carotene.

Myxoxanthin≡echinenone required ~7% acetone–light petrol for elution from deactivated alumina; λ_{max} 455 nm (broad peak) in light petrol; R_f=0·79 on kieselguhr paper (2% acetone–light petrol). This compound could not be distinguished spectrophotometrically or chromatographically (co-chromatogram) from *trans* synthetic echinenone.

Cryptoxanthin required 20–30% acetone–light petrol for elution from deactivated alumina. The absorption spectrum in visible light was identical with that of β-carotene, and the R_f-value (0·68) on kieselguhr paper (5% acetone–light petrol) was similar to that of isocryptoxanthin.

4-Keto-3'-hydroxy-β-carotene (I) required 30% acetone–light petrol for elution from deactivated alumina. The absorption spectrum in visible light was identical with that of echinenone, and a co-chromatography test on kieselguhr paper (R_f=0·77; 10% acetone–light petrol) supported its identity with I isolated from *Athrospira* sp. (see below).

Myxoxanthophyll required 25–30% acetone–light petrol for elution from the cellulose column; R_f=0·35 on kieselguhr paper (30% acetone–benzene). The *trans* isomer had λ_{max} at 450, 476 and 506 nm in acetone.

The final acetate obtained on acetylation had R_f=0·47 (*trans*) and R_f=0·58 (neo A) on kieselguhr paper (10% acetone–light petrol).

The main non-polar dehydration product obtained on treatment with acid chloroform had R_f=0·35 on aluminium oxide paper (2% acetone–light petrol) and λ_{max} at (465), 489 and 520 nm in acetone.

Oscillaxanthin. Presumably because of crystallization on top of the column, oscillaxanthin required pyridine for complete elution from the cellulose column. This pigment exhibited λ_{max} at 466, 490 and 522 nm in acetone (*cis*-isomerized).

On acetylation an acetate with R_f=0·59 on kieselguhr paper (20% acetone–light petrol) was obtained.

Athrospira *sp.*

Biological material. The *Athrospira* sp. used occurs in almost unialgal culture in Lake Aranguadi, near Debre Zeit, Ethiopia, and the biological material was supplied by Dr. R. M. Baxter, University College, Haile Sellassie I University, Addis Ababa.

Carotenoid content. A total of 125 mg carotenoid or 0·16% of the extracted algal residue, was obtained. The carotenoid composition is given in Table 1, and the identification of the individual components is described below in order of increasing adsorption.

β-Carotene required 5% ether–light petrol for elution from deactivated alumina. The *trans* (R_f=0·23) and neo A (R_f=0·44) isomers could not be separated from the corresponding stereoisomers of synthetic β-carotene on aluminium oxide paper (light petrol). Crystalline *trans* β-carotene was obtained from ether–methanol; m.p. 176–179°, λ_{max} 453 and 480 nm in acetone. The i.r. spectrum was identical with that of synthetic β-carotene (cf. Ref. 27).

Echinenone required ~8% acetone–light petrol for elution from deactivated alumina; λ_{max} 455 nm in light petrol; R_f=0·79 on kieselguhr paper (2% acetone–light petrol). It could not be separated from synthetic echinenone in the latter system.

Hydride reduction in dry ether gave a product spectrophotometrically (λ_{max} 426, 450 and 476 nm in light petrol), and chromatographically (R_f=0·25 on kieselguhr paper; 2% acetone–light petrol) indistinguishable from isocryptoxanthin (prepared by *N*-bromosuccinimide

[27] O. ISLER, H. LINDLAR, M. MONTAVON, R. RÜEGG and P. ZELLER, *Helv. Chim. Acta* **39**, 249 (1956).

treatment of β-carotene in acetic acid containing chloroform according to the procedure of Karrer and Entschel[28]).

Cryptoxanthin required 25% acetone–light petrol for elution from deactivated alumina; λ_{max} 425, 449 and 475 nm in light petrol; R_f=0·68 on kieselguhr paper (5% acetone–light petrol). The same R_f-value was obtained for partly synthetic isocryptoxanthin. However, cryptoxanthin proved resistant towards allylic dehydration with acid chloroform.

4-Keto-3'-hydroxy-β-carotene (I) required 30% acetone–light petrol for elution from deactivated alumina; λ_{max} 452 nm in light petrol (broad peak) and R_f=0·77 on kieselguhr paper (10% acetone–light petrol). In light petrol–95% methanol the partition ratio was 25:75.

This pigment had the same R_f-value on kieselguhr paper as 4-keto-4'-hydroxy-β-carotene, but was resistant towards allylic oxidation with *p*-chloranil.[15] I (0·57 mg) in 5 ml benzene was treated with *p*-chloranil (1·2 mg) in the presence of iodine. A 100 per cent pigment recovery was obtained but no oxidation product.

Hydride reduction of I (1·5 mg) in dry ether resulted in a 45 per cent pigment recovery. The product (II) had λ_{max} at 425, 450 and 478 nm in ether and R_f=0·59 on kieselguhr paper (10% acetone), as had synthetic zeaxanthin.

Acid chloroform treatment of II (0·7 mg) resulted in a quantitative pigment recovery. The main product (III) had λ_{max} at (467), 488 and 515 nm in acetone and R_f=0·40 on kieselguhr paper (2% acetone–light petrol). The product (III) could not be spectrophotometrically or chromatographically separated from *retro*-bis-dehydro-β-carotene, produced by acid treatment in like manner from synthetic isozeaxanthin (IV) according to the method of Bodea and Nicoara.[16]

Zeaxanthin required from 50% acetone–light petrol to 2% methanol–light petrol for elution from deactivated alumina. Crystalline *trans* zeaxanthin was obtained from acetone–light petrol; m.p. 207–208°. A mixed melting point with synthetic zeaxanthin (m.p. 208°) gave no depression. *Trans* zeaxanthin had λ_{max} at (430), 453 and 479 nm in acetone, and the i.r. spectrum measured in KBr was identical with that of synthetic zeaxanthin (cf. Ref. 29). No separation was obtained on co-chromatography with synthetic zeaxanthin on kieselguhr paper (R_f=0·60; 10% acetone–light petrol). Furthermore, treatment of the natural zeaxanthin (1·0 mg) with acid chloroform gave 92 per cent pigment recovery and no product with prolonged chromophore.

Myxoxanthophyll required 25% acetone–light petrol for elution from the cellulose column. Crystalline myxoxanthophyll, m.p. 172°, was obtained from acetone–light petrol. *Trans* myxoxanthophyll had λ_{max} in acetone at 370, (428), 452, 476 and 508 nm, % D_B/D_{II}=9, % III/II^{24}=60 and R_f=0·38 on kieselguhr paper (30% acetone–benzene). Absorption spectra in visible light and chromatographic properties were found by direct comparison to be identical with those of authentic myxoxanthophyll isolated from *O. rubescens*.

The acetate and allylic dehydration product were prepared in a manner analogous to that described above for myxoxanthophyll from *O. rubescens*. The corresponding products agreed completely in spectroscopic (visible light) and adsorptive properties.

Oscillaxanthin required pyridine for complete elution from the cellulose column; λ_{max} 390, 470, 499 and 532 nm in acetone. The *trans* acetate had λ_{max} in acetone at 390, 470, 499 and 532 nm, % III/II^{24}=60, R_f=0·37 on kieselguhr paper (20% acetone–light petrol), and

[28] P. KARRER and R. ENTSCHEL, *Helv. Chim. Acta* **41**, 402 (1958).
[29] O. ISLER, H. LINDLAR, M. MONTAVON, R. RÜEGG, G. SAUCY and P. ZELLER, *Helv. Chim. Acta* **39**, 2041 (1956).

could not be separated chromatographically from the acetate prepared from authentic oscillaxanthin isolated from *O. rubescens*.

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