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Isolation and Identification of Carotenoids Produced by a Green Alga (*Dictyococcus cinnabarinus*) in Submerged Culture

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1. Six carotenoid pigments were produced by and isolated from the green alga *Dictyococcus cinnabarinus* grown in submerged culture in the presence of glucose. 2. The first, second and fourth pigments were identified respectively as β-carotene, echinenone and canthaxanthin; the physicochemical properties of the other three are described and their similarity to other oxo-carotenoids is shown. 3. Culture techniques, isolation and identification procedures are described.

In previous work (Dentice di Accadia, Gribanovski-Sassu, Romagnoli & Tuttobello, 1965) it was shown that the green alga *Dictyococcus cinnabarinus* grown in the presence of glucose in submerged culture changed its colour from green to red owing to accumulation of carotenoids.

The first investigations showed that at the end of the growth period chlorophyll was absent although six other different pigments were isolated, one of which appeared to be echinenone.

In the present paper the isolation and identification of the different pigments are described.

MATERIALS AND METHODS

Strain. The strain used was *Dictyococcus cinnabarinus* 280 (Kol–F. Chodat) Vischer, obtained from the algal collection of the Botanical Institute of the University of Geneva, Switzerland.

Culture medium. For all stages of the culture, Detmer medium was used. Its composition in g./l. is as follows: $Ca(NO_3)_2,4H_2O$, 1·00; $MgSO_4,7H_2O$, 0·25; KCl, 0·25; KH_2PO_4, 0·25; 1% $FeCl_3$ solution, 1 drop/l.; final pH 5·7–6·0.

Culture technique. Erlenmeyer flasks (500 ml.) containing 100 ml. of Detmer medium diluted 1:3 with a 0·1% solution of yeast autolysate were inoculated from an agar slant of a green culture of the alga. The flasks were put on a rotatory shaker running at 220 rev./min. They were maintained at 25° and illuminated by 20 fluorescent tubes of 14 w 'warm white' type installed at about 40 cm. from the shelf of the shaker, supplying a constant illumination of 3500–4200 lux.

After about 2 weeks the shake cultures (green) were transferred to fresh medium, with the addition of glucose (2%, w/v), so that the initial population was 200000 cells/ml. The second stage lasted 2 weeks.

The cultures were green initially but changed gradually during the growth to an intense orange–red colour.

Extraction of the pigments. About 12 g. of fresh cells (corresponding to about 3 g. dry wt.) was harvested from five flasks and collected in an International model HR-1 refrigerated centrifuge at 4° for 15 min. at 5000 rev./min. (1800*g*). The residue was washed twice with 100 ml. portions of saturated aq. $CaCO_3$ solution and centrifuged again for 10 min. at 5000 rev./min. It was then ground for 30 sec. in a refrigerated mortar with 8 g. of Celite and 100 mg. of $CaCO_3$, and 50 ml. of acetone was added gradually. The acetone suspension was then decanted and the residue was treated with 50 ml. of aq. 85% (v/v) acetone, filtered on a Buchner filter, extracted again with 50 ml. of pure acetone and washed three or four times with pure acetone to give a final volume of 250 ml.

The combined acetone extracts were diluted with 2 vol. of water and extracted with 200 ml. of light petroleum (b.p. 40–70°). After extraction 20 g. of anhydrous Na_2SO_4 was added to the light-petroleum solution, and after 30 min. the Na_2SO_4 was removed and washed again with light petroleum, which was then added to the solution containing pigments to give a final volume of 250 ml.

Pigment separation. The pigments were separated by chromatography on a column, after previous thin-layer chromatography of a sample to determine the nature of the pigments present. For the thin-layer chromatography a glass plate covered with a 25 μ layer of silica gel (nach Stahl; E. Merck, A.-G., Darmstadt, W. Germany) in water (1:2, w/v) was used; the plate was dried in an oven at 110° for 30 min.; when chlorophylls were present 6% (v/v) propanol in hexane as eluent was employed, whereas for the carotenoids alone the eluent was 12% (v/v) acetone in hexane.

For column chromatography of extracts from red cultures the procedure was the following. Crude extracts concentrated to small volume in a stream of N_2 were passed through a column (10 cm. long × 2 cm. diam.) of Florisil (100–200 mesh; Fisher Scientific Co., Fair Lawn, N.J., U.S.A.). Three fractions were separated. Fraction I was rapidly eluted with 10% (v/v) acetone in hexane, and fractions II and III were eluted respectively with 20% (v/v) and 40% (v/v) acetone in hexane.

After washing the fractions with water to eliminate the acetone, they were dried over Na_2SO_4, concentrated again in a stream of N_2 and again chromatographed.

Fraction I was passed through a column (30 cm. long × 1·5 cm. diam.) of alumina (Brockmann grade 1). From this fraction four pigments were separated. The first pigment (P_1), sharply divided from the others, was eluted with hexane alone, but the second (P_2), third (P_3) and fourth (P_4) required gradient elution with hexane containing from 1% to 12% (v/v) of acetone. Pigments P_2, P_3 and P_4 were rechromatographed on a column (20 cm. long × 1 cm. diam.) of alumina and eluted with hexane containing respectively 3%, 8% and 10% (v/v) of acetone.

Fraction II, containing only one pigment (P_5), was also rechromatographed on a column (20 cm. long × 1 cm. diam.) of Florisil (100–200 mesh) and eluted with 20% (v/v) acetone in hexane.

On fraction III, containing only one pigment (P_6), identification tests were carried out without any further chromatography.

For extracts from green cultures, grown with no addition of sugars, the following procedure was used. The crude extract, concentrated to small volume in a stream of N_2, was chromatographed on a column (40 cm. long × 2 cm. diam.) of cellulose (Whatman standard grade). Four fractions were separated.

The first fraction, eluted with pure hexane, was again concentrated to a small volume in a stream of N_2 and eluted from a column (15 cm. long × 1 cm. diam.) of alumina (Brockmann grade 1) with hexane. It contains only one pigment.

The second fraction, eluted with 10% (v/v) acetone in hexane, was rechromatographed on a column (30 cm. long × 1·5 cm. diam.) of Florisil (100–200 mesh); two pigments were separated and eluted with hexane containing respectively 5% and 10% (v/v) of acetone; a third, faintly pink, band was also noted.

The third and fourth fractions were eluted respectively with 15% (v/v) and 20% (v/v) acetone in hexane, rechromatographed on a cellulose column and eluted again with hexane containing respectively 12% and 15% (v/v) of acetone.

Saponification. All the carotenoids after separation were saponified with 6% (w/v) KOH in methanol in the dark for 5 min. at 40° (Krinsky & Levine, 1964). The mixture was then rapidly cooled and an equal amount of water was added. It was then extracted with peroxide-free diethyl ether three or four times until no more colour could be observed in the ethereal solution. After removal of alkali by washing with water, the solution was dried with Na_2SO_4.

Lipid determination. Lipids were determined by gas–liquid chromatography. After saponification carried out directly on the culture by refluxing with 2 N-KOH in aq. 50% (v/v) ethanol for 3 hr., extraction with peroxide-free diethyl ether was carried out. Almost all the lipids passed into the solution. Fatty acids still present in the aqueous phase were neutralized with aq. $NaHCO_3$ solution. The two phases were separated and the aqueous phase, after acidification with HCl, was again extracted in diethyl ether. The solvent was eliminated by vacuum distillation and the residue was methylated by refluxing with methanol containing 3% (v/v) of conc. H_2SO_4 for 2–3 hr. The volume was reduced to about one-quarter by distillation and the solution was diluted with water and again extracted with ether. The solvent was again eliminated by vacuum distillation. The residue of methyl esters of fatty acids was then ready for gas–liquid chromatography, which was carried out in a Fractovap model C apparatus (Carlo Erba, Scientific Apparatus Division, Milan, Italy). The separation was carried out on a stainless-steel capillary column (50 m. long × 0·25 cm. diam.). Apiezon L was used as a stationary phase; the temperature of the oven was 175° and the temperature of the vaporizer 300°; a flame ionization detector with helium as transport gas was used. The column was calibrated with a known mixture of methylated fatty acids.

Identification of pigments. The pigments were identified by the following determinations: (*a*) absorption spectra, both in the visible and ultraviolet regions, obtained with a Cary spectrophotometer, in various solvents, namely light petroleum (b.p. 40–70°), ethanol, chloroform, carbon disulphide, hexane, pyridine, benzene, aq. 80% (v/v) acetone, diethyl ether; (*b*) partition coefficient between hexane and aqueous methanol (Petracek & Zechmeister, 1956*a*), with measurement of phase concentrations by extinction determinations; (*c*) reduction with $LiAlH_4$ in diethyl ether at room temperature, with subsequent destruction of the excess of reagent by ethanol after 15 min. (Goodwin, 1956); (*d*) comparison of physical and chemical properties of the extracted pigments with pure samples.

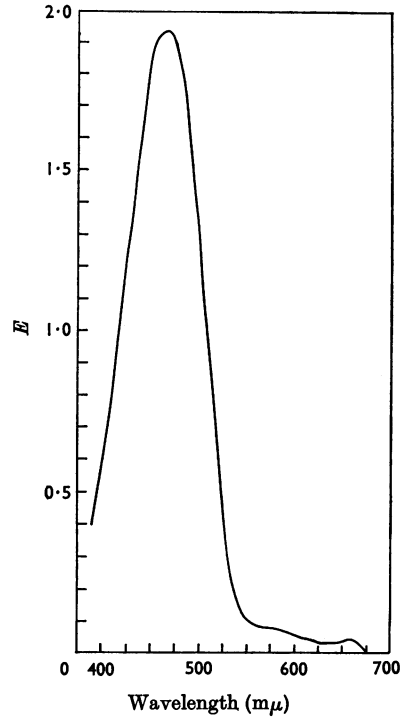

Fig. 1. Absorption spectrum of total pigments extracted from *Dictyococcus cinnabarinus* grown in submerged culture in the presence of 2% glucose.

Table 1. *Partition coefficients of the carotenoids of* Dictyococcus cinnabarinus *grown in submerged culture in the presence of* 2% *glucose*

Pigment	Ratio of pigment in epiphase (hexane) to pigment in hypophase (aq. methanol): Hexane–95% methanol	Hexane–85% methanol
P_1	100:0	—
P_2	92:8	—
P_3	44:56	—
P_4	52:48	—
P_5	—	0:100
P_6	—	40:60

RESULTS

For red cultures, the crude extract before pigment separation showed an absorption spectrum with $\lambda_{max.}$ at 470–475 mμ (Fig. 1). The results obtained in the isolation and identification of the pigments are shown in Tables 1 and 2.

Lipid determination by gas–liquid chromatography showed that all the fatty acids present belong to the normal series from C_{14} to C_{18}, saturated and unsaturated with 1 and 2 double bonds/mol., with a net predominance of oleic acid (nearly 60%) (Table 3).

For green cultures, grown in the absence of

Table 2. *Spectral characteristics of the carotenoids of* Dictyococcus cinnabarinus *grown in submerged culture in the presence of* 2% *glucose*

Compound	$\lambda_{max.}$ (mμ): Light petroleum (b.p. 40–70°)	Ethanol	Chloroform	Carbon disulphide	Hexane	Pyridine	Benzene
Pigment P_1	448, 475	—	—	485, 515	(425), 450, 478	—	—
Pigment P_2	456	—	—	488–490	458	—	—
Reduction product from pigment P_2	(425), 449, 475	—	—	—	—	—	—
Pigment P_3	462	473	481	497	462–464	483	481
Reduction product from pigment P_3	(425), 447, 475	—	—	—	—	—	—
Pigment P_4	465–469	475	—	501	465–470	—	480
Reduction product from pigment P_4	—	—	—	—	(426), 450, 475	—	—
Pigment P_5	(390), 413, 437, 466	(390), 414, 437, 467	(420), 445, 475	—	—	—	—
Furanoid product obtained from pigment P_5	—	(378), 400, 424, 450	—	—	—	—	—
Pigment P_6	472–475	—	—	505	475	490	—

Table 3. *Fatty acids of the lipids of* Dictyococcus cinnabarinus *grown in submerged culture in the presence of* 2% *glucose*

Fatty acids	Fatty acid composition (% of total): Saturated	Unsaturated: With 1 double bond/mol.	Unsaturated: With 2 double bonds/mol.
C_{14}	Trace	Trace	Trace
C_{15}	Trace	Trace	Trace
C_{16}	23·3	3·2	3·8
C_{17}	Trace	Trace	Trace
C_{18}	2·2	58·4	8·7

glucose and employed as seed culture, as mentioned above, the absorption spectrum of the total pigments showed peaks at 430, 460 and 660mμ (Fig. 2). The first fraction contained a single pigment, which was identified as β-carotene as described below for the red cultures. The two pigments contained in the second fraction were identified as xanthophylls; their absorption spectra in light petroleum showed peaks respectively at 421, 444 and 473mμ and at 420, 441 and 471mμ.

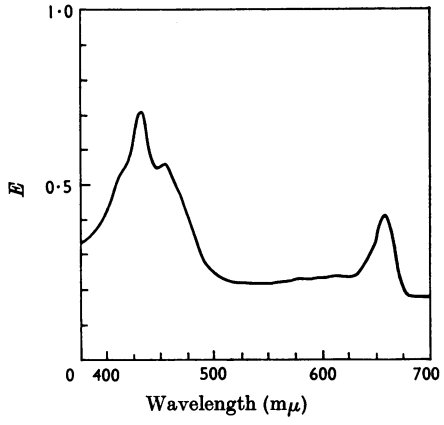

Fig. 2. Absorption spectrum of total pigments extracted from *Dictyococcus cinnabarinus* grown in submerged culture in the absence of glucose.

The third and fourth fractions were identified respectively as chlorophyll a and chlorophyll b, showing $\lambda_{max.}$ in aq. 80% (v/v) acetone and diethyl ether at 430 and 660mμ for chlorophyll a and at 454 and 642mμ for chlorophyll b (Fig. 3).

DISCUSSION

Pigment P_1 was identified as β-carotene from the form and the position of the absorption peaks in hexane, light petroleum and carbon disulphide, and from the partition coefficient. When compared with an authentic sample of β-carotene (Hoffmann–La Roche, Basle, Switzerland) it was found to assume the same position on the chromatography column and to possess the same absorption spectrum in the different solvents mentioned above; in thin-layer co-chromatography a single spot was obtained. Moreover, it crystallized from light petroleum (b.p. 40–70°) after 24hr. at −20° in orange–yellow plates, m.p. 174°.

Pigments P_2, P_3 and P_4 formed over 80% of the total pigments, but the relative amounts changed from culture to culture, though the amount of pigment P_2 was always greater than that of the other two. They all showed similar absorption spectra with a single well-defined peak in the visible region that is typical of carotenoids containing a keto group conjugated with the chromophore system (Karrer & Jucker, 1950).

Pigment P_2 was identified as echinenone (4-oxo-β-carotene) (Lederer, 1935, 1938; Goodwin & Taha,

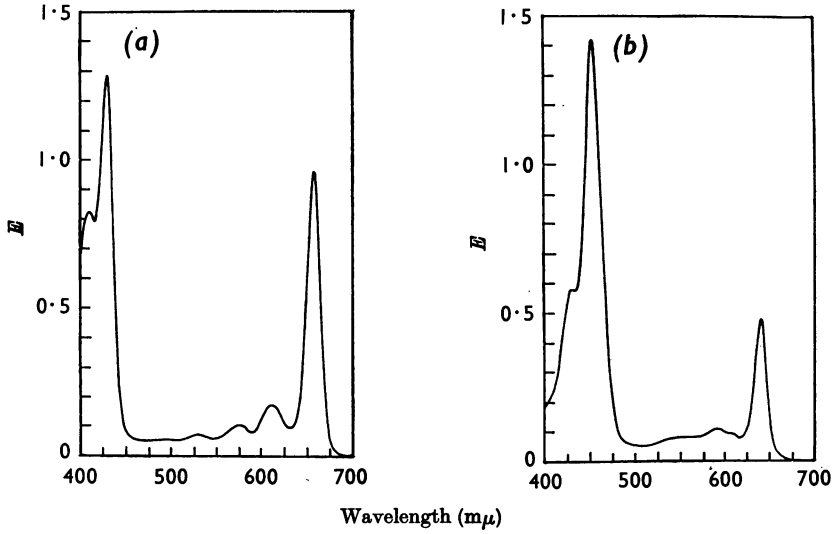

Fig. 3. Absorption spectra in diethyl ether of chlorophyll a (*a*) and chlorophyll b (*b*) extracted from *Dictyococcus cinnabarinus* grown in submerged culture in the absence of glucose.

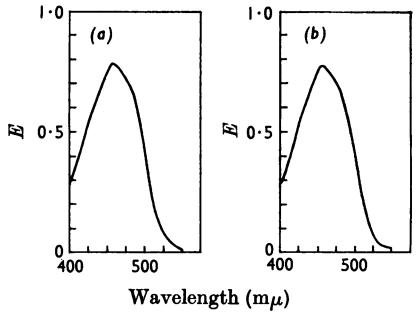

Fig. 4. Absorption spectrum of pigment P_2 extracted from *Dictyococcus cinnabarinus* in submerged culture in the presence of 2% glucose (*a*) compared with the absorption spectrum of a pure sample of echinenone (*b*).

1950). When compared with a pure sample of authentic material it showed the same partition coefficient, the same relative position on chromatographic columns of both alumina and Florisil, a single spot in thin-layer co-chromatography and the same absorption spectra in different solvents (Fig. 4). On reduction with lithium aluminium hydride it gave isocryptoxanthin (4-hydroxy-β-carotene), which after treatment with chloroform saturated with hydrochloric acid absorbed at a higher wavelength owing to an extra conjugated double bond formed by the loss of a water molecule. This reaction indicates the allylic nature of the hydroxyl group with respect to the first ethylenic bond of the conjugated chain (Karrer & Leumann, 1951), and confirms that the keto group of the original carotenoid is at position 4 of the β-ionone ring.

Pigment P_3 had a qualitatively similar absorption spectrum to that of pigment P_2 but it absorbed at a slightly higher wavelength. Its position on the column was very near to that of the next pigment (P_4). On reduction with lithium aluminium hydride it gave a compound with an absorption spectrum similar to the one obtained by the same reduction of pigment P_2; therefore for this pigment also the presence of a keto group in position 4 can be assumed. The slight displacement of the absorption peak of pigment P_3 from that of pigment P_2 suggests the presence of a second carbonyl group in the same ring (in fact if a carbonyl group had been introduced into the β-ionone ring the displacement of the peak would have been nearly double). It did not react with acid chloroform, which indicates the absence of allylic hydroxyl groups at position 4 or 4′ (Karrer & Leumann, 1951). For all these reasons this pigment was tentatively identified as 3,4-dioxo-β-carotene.

Pigment P_4 also had properties very similar to those of echinenone. In fact this pigment also did not react with acid chloroform, which indicates the absence of hydroxyl groups at position 4 or 4′. It had the same spectrum as 4,4′-dioxo-β-carotene (Saperstein & Starr, 1953; Petracek & Zechmeister, 1956*b*). When compared with an authentic sample of canthaxanthin it behaved in the same way on the chromatographic column; thin-layer co-chromatography gave a single spot; the positions of the absorption peaks of unknown and known in light petroleum, ethanol, carbon disulphide, hexane and benzene were also identical. After reduction the form and the position of the peaks indicated the presence of the same chromophoric group as in canthaxanthin; finally, in hexane–95% (v/v) methanol before and after reduction it showed the same partition coefficient as authentic canthaxanthin and its reduction product (22:78).

Pigment P_5 was the only pigment found in the green cultures together with β-carotene. It was strongly absorbed on an alumina column, but was separated on a Florisil column by hexane containing 20% (v/v) of acetone. When traces of hydrochloric acid were added to its solution in ethanol its absorption spectrum moved about 18mμ towards lower wavelengths. This hypsochromic shift is typical of the isomerization of a 5,6-monoepoxide to a 5,8-furanoid oxide, with disappearance of a conjugated double bond in the chromophore chain. Its spectrum was very similar to that of 5,6-epoxy-3,3′-dihydroxy-β-carotene, but its behaviour, which was more hypophasic and showed stronger chromatographic absorption, indicated the presence of a fourth polar group. This pigment could therefore be considered as neoxanthin (Goldsmith & Krinsky, 1960).

Pigment P_6 was present in very small quantity. Its absorption spectrum indicated the presence of a keto group conjugated with the chromophore system. It was distributed 20:80 between the two phases formed by adding equal volumes of light petroleum and 90% (v/v) methanol; when a little water was added it became epiphasic, whereas it remained hypophasic in the presence of alkali, as happens generally for polyketo derivatives in enolic form.

As mentioned above, in the red cultures no chlorophyll could be detected at the end of the growth period. The absence of chlorophyll was confirmed by some observations with the electron microscope where no chloroplasts were revealed.

In conclusion, the production of echinenone as principal pigment and the absence of chlorophyll under the same culture conditions are emphasized.

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