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KETOCAROTENOID BIOSYNTHESIS BY *HAEMATOCOCCUS LACUSTRIS*

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Key Word Index—*Haematococcus lacustris*; Chlorophyceae; biosynthesis; carotenoids; ketocarotenoids.

Abstract—*Haematococcus lacustris* incubated on a nutrient-depleted medium utilised acetate-[2-^{14}C] from the medium and carbon fixed photosynthetically for the biosynthesis of ketocarotenoids. Conversion of β-carotene to astaxanthin occurs via the intermediates echinenone and canthaxanthin.

INTRODUCTION

Ketocarotenoids are synthesised as secondary carotenoids by some species of green algae when growth is arrested by unfavourable environmental conditions other than carbon deficiency [1]. Goodwin and Jamikorn [2] studied the formation of the secondary pigment astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) in *Haematococcus lacustris*. They concluded that synthesis did not occur at the expense of the primary carotenoid pigments associated with photosynthesis, and that a colourless precursor already present in the green cells, or carbon fixed photosynthetically was therefore used. Photosynthesis was considered essential to provide either. fixed carbon or energy. Droop [1,3,4] working on the same species, demonstrated that the secondary pigment could be formed at a comparatively low rate in the absence of light if acetate were provided as a carbon source. Encystment and associated ketocarotenoid formation in the dark required greater quantities of acetate than for an equivalent yield of vegetative cells [3]. This demonstrated a continued demand for carbon following the cessation of cell multiplication.

Species of green algae have been cultured in nutrient-depleted media for periods of up to 50 days. After 20 days the concentrations of chlorophylls and primary carotenoids were substantially reduced while ketocarotenoids concentrations rose [5,6]. Based on this and further observations, Czygan [7] postulated the formation of ketocarotenoids from degradation products of chlorophyll. Similar changes were observed in a preliminary study of ketocarotenoid biosynthesis by *H. lacustris* (B. H. Davies and V. Hunn, personal communication). The kinetics of primary carotenoid decline and secondary carotenoid increase were consistent with conversion of β-carotene (β,β-carotene) and the primary xanthophylls (based on β-carotene) into the ketocarotenoids. Changes in the concentrations of echinenone (β-β-caroten-4-one) and canthaxanthin (β,β-carotene-4,4′-dione) were consistent with their possible role as intermediates on the pathway to astaxanthin.

Two basic proposals as to the source of ketocarotenoids emerge. The first is that they arise by *de novo* synthesis or from a colourless precursor, and the second is that they are formed from primary pigments present within the green cell. The present study was designed to give further insight into which was the dominant process. *De novo* synthesis would have a substantial demand for carbon, whereas synthesis from primary pigments would not.

RESULTS AND DISCUSSION

Algae were cultured in the presence of sodium acetate-[2-^{14}C]. Acetate was used in preference to bicarbonate-[^{14}C] since its assimilation would be less influenced by changes in growth medium pH, or by changes in photosynthetic rate associated with encystment [8]. *Haematococcus* species have been shown to assimilate organic nutrients; acetate may be assimilated at all stages of development [1–4,9–11]. Green cells were grown on a nutrient-rich medium, then transferred to a nutrient-depleted medium to induce rapid reddening. The study consisted of three separate experiments. In the first, acetate-[2-^{14}C] was provided throughout to determine the pattern of incorporation into ketocarotenoids at all phases of development. In the second, acetate-[2-^{14}C] was provided for initial growth but not during reddening, and in the third, during reddening only.

Acetate-[2-^{14}C] was utilised for the biosynthesis of ketocarotenoids even at an advanced stage of culture development, but despite a continuous supply in Expt. 1, dilution of specific radioactivity occurred (Fig. 1). Dilution was accentuated in the cultures of Expt. 2 by incorporation of unlabelled acetate. Demand for carbon on encystment is greater than for vegetative growth [3]. The low concentration of acetate added on resuspension may therefore have been rapidly exhausted. However, dilution of specific radioactivity demonstrated the presence of an alternative unlabelled, or a less highly labelled carbon source.

Biosynthesis of ketocarotenoids from the primary pigments of green cells. Superficially the changes in concentration of primary β-carotene and related xanthophylls

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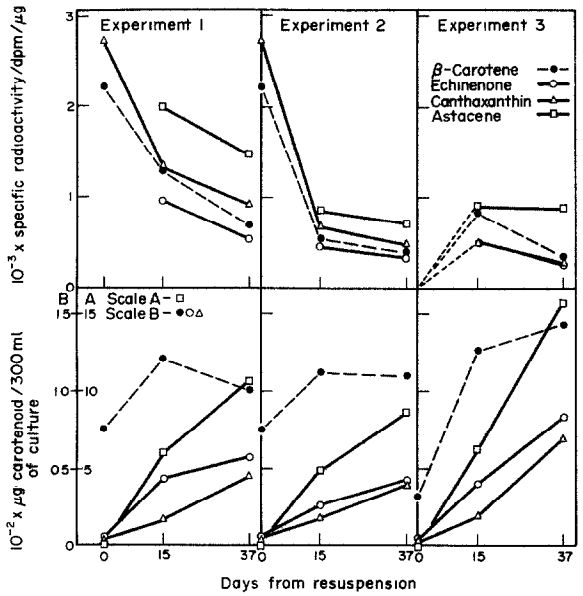

Fig. 1. Quantity and specific radioactivity of β-carotene and major ketocarotenoids following incubation of *H. lacustris* with acetate-[2-^{14}C]. Acetate-[2-^{14}C] present throughout Expt 1; prior to resuspension, Expt 2, and after resuspension, Expt 3. Green cultures of Expts 1 and 2 were grown under identical conditions and therefore bulked (600 ml) for analysis.

appeared insufficient to account for the quantities of ketocarotenoids produced, but no measure of turnover of the xanthophylls was made. With sampling restricted to 3 points, the peak of production may not have been observed (Fig. 1 and Table 1). These pigments would undoubtedly share, however, the same early biosynthetic steps as the ketocarotenoids, resulting in comparable specific radioactivity. In addition, the period of maximum rate of dilution of β-carotene and canthaxanthin, 0–15 days from resuspension, was associated with the maximum rate of biosynthesis of all pigments. A high rate of turnover of primary carotenoids would be required to supply the needs of ketocarotenoid formation. Since these pigments are integral components of the chloroplast structure, such turnover is unlikely.

Chlorophylls were not analysed, but analysis of *H. lacustris* cultured under the same conditions (P. Donkin and B. H. Davies, unpublished work), and of other green algal species cultured in nutrient-depleted media [5,6] have shown that peaks of primary xanthophyll concentration are mirrored by peaks of chlorophyll concentration. The changes in chlorophyll concentration were quantitatively great enough to provide carbon for ketocarotenoid formation [5,6, P. Donkin and B. H. Davies, unpublished work]. Parshikov and Drokova [12] observed that acetate-[2-^{14}C] was more readily incorporated into the carotenoids of the green alga *Dunaliella salina* than into chlorophylls. Thus biosynthesis of ketocarotenoids from chlorophyll degradation products would result in a reduction in their specific radioactivity. As with primary xanthophylls, however, a high rate of chlorophyll turnover would be required for conversion to ketocarotenoids during the early stages of culture reddening. This is considered unlikely to occur.

Colourless reserve materials such as lipids or carbohydrates are potential precursors but these also increase on encystment, necessitating rapid turnover 6,8]. Any endogenous carbon source utilised must possess a specific radioactivity sufficiently low to account for the value for echinenone after 37 days from resuspension. The existence of pools of low specific radioactivity carbon was improbable as acetate-[2-^{14}C] may be readily incorporated into all major metabolic pathways.

De novo *biosynthesis of ketocarotenoids.* Over the first 15 days from resuspension, the high rate of ketocarotenoid metabolism was paralleled by that of primary xanthophylls and β-carotene (Fig. 1 and Table 1). This suggested an increase in the quantity of photosynthetic tissue in response to nutrient-depleted conditions, in order to supply the carbon demanded by encystment [1]. The data of other authors could be interpreted in this way [5,6]. Sprey [8] observed a continuation of CO_2 assimilation in cultures of *H. lacustris* which appeared red, although incorporation declined with maturity. The high rate of dilution of specific radioactivity in β-carotene and canthaxanthin over the first 15 days from resuspension may therefore have resulted from a combination of an increasing rate of photosynthesis and, initially, the small pool size of these pigments. In more mature cultures the rate of dilution declined with declining photosynthesis rate and increasing pigment pool size.

The sequence of intermediates on the pathway to astaxanthin: ketocarotenoids. The relative intensity and parallelism of changes in ketocarotenoid specific radioactivity in Expts 1 and 2 (Fig. 1) were consistent with a pathway from echinenone through canthaxanthin to astaxathin. In conditions of dilution of an already labelled series of intermediates (Expts 1 and 2), those intermediates early in the pathway will be diluted first. Where there is a direct conversion from one intermediate to another with only a single pool of each, established dilution curves will be almost parallel. Further evidence for the interconversion between the ketocarotenoids analysed was the sequential nature of changes in total radioactivity (Table 2).

The maximum separation between the dilution curves of the ketocarotenoids occurred in Expt 1 (Fig. 1). Provision of acetate-[2-^{14}C] throughout culture development resulted in highly labelled pools requiring substantial biosynthesis of unlabelled pigment for dilution. Astaxan-

Table 1. Quantities of chloroplastidic xanthophylls at 0, 15 and 37 days from resuspension of *H. lacustris* on a nutrient-depleted medium (Expt. 3)

Days from resuspension	Carotenoid*			
	Lutein	Antheraxanthin	Violaxanthin	Neoxanthin
0	93	6	27	13
15	282	13	78	55
37	137	5	34	21

* μg in 300 ml of culture.

Table 2. Total radioactivity in β-carotene and major ketocarotenoids.

Days from resuspension	Radioactivity in each carotenoid (10^{-3} × total ^{14}C activity in dpm*)				% ^{14}C recovery§
	β-Carotene	Echinenone	Canthaxanthin	Astaxanthin (Astacene)	
Expt. 1†					
0	166.1	−‡	9.8	−‡	0.09
15	153.8	41.5	21.6	1,178.8	0.35
37	68.4	29.1	40.7	1,561.4	0.43
Expt. 2‡					
0	166.1	−‡	9.8	−‡	0.09
15	58.1	11.7	11.1	402.2	0.24
37	42.6	13.8	19.0	602.9	0.34
Expt. 3					
0	—	—	—	—	—
15	103.0	19.9	9.3	552.6	0.34
37	47.4	20.0	20.9	1,394.4	0.74

* Calculated from the specific activity and the quantity of pigment measured before radiochemical purification. † Green cultures of experiments 1 and 2 were grown under identical conditions and therefore bulked (600 ml) for analysis; results are expressed per 300 ml of culture. ‡ Inadequate recovery from chromatography. § Recovery in carotenoids is based on the total quantity of ^{14}C added to the cultures, i.e. 0.18 mCi at 15 and 37 days, Expt. 1; 0.09 mCi for all others.

thin with a large pool size and late in the pathway was slow to dilute. In Expt 2 a greater proportion of ketocarotenoid was formed from unlabelled acetate resulting in small readily-diluted labelled pigment pools. However, observation of the total post-resuspension counts in astaxanthin (Table 2) shows that considerable radioactivity occurred in this pigment although only 3 μg were present in three bulked green cultures. Insufficient astaxanthin was available to determine the specific radioactivity in green cultures but during a preliminary study a value within 10% of that of canthaxanthin was observed. Allowing for substantial error, a predicted value would not be more than twice that for canthaxanthin of which the quantities present were similar. Thus the astaxanthin present at resuspension could provide only a small proportion of the total radioactivity observed in this pigment in brown and red cells, the remainder coming from a series of precursors present in the green cell, of which the pigments measured would be later components.

The labelling pattern observed in Expt 3 may have resulted from two opposing processes. Immediately following resuspension when the acetate-[2-^{14}C] concentration would be greatest and photosynthetic rate less than maximal, the dominant process would be incorporation of labelled acetate. With the increase in photosynthetic pigments and reduction in acetate concentration, dilution of specific radioactivity in ketocarotenoids by carbon derived from CO_2 would accelerate. Radioactivity incorporated into a metabolic sequence where pools of unlabelled intermediates already exist results in greatest radioactivity occurring in the precursors at the beginning of the sequence. As already discussed, a dilution pathway would give the opposite result. At a certain stage of culture development a crossover of the labelling sequence would occur. Measurement of specific radioactivity at only 2 points precluded observation of this phenomenon, but the results shown for Expt 3 represent an intermediate stage.

The specific radioactivity of astaxanthin did not reach as high a value as in Expt 1 where acetate-[2-^{14}C] was provided throughout. This supports the conclusion of Expt 2, that significant incorporation of label into precursors occurred in the green cells. However, quantitative comparisons are complicated by the greater quantity of pigment biosynthesis observed in Expt 3. This increased both the demand for carbon and presumably the supply of fixed labelled CO_2. The total radioactivity in astaxanthin after 37 days from resuspension approached that after 37 days in Expt 1. Thus the greatest proportion of astaxanthin was synthesised after resuspension and from exogenous sources of carbon. Since the primary pigments also reached a peak following resuspension, synthesis of astaxanthin from these cannot be excluded. However, the more plausible mechanism is a direct incorporation of acetate or fixed CO_2 into ketocarotenoids by normal terpenoid biosynthetic routes.

The sequence of intermediates on the pathway to astaxanthin:β-carotene. The first pigment precursor of ketocarotenoids in *H. lacustris* may be β-carotene. However, the specific radioactivity of this pigment was always greater than that of its presumed metabolite echinenone. With a single β-carotene pool being converted directly to echinenone, this could not occur. Further evidence that the results could not be interpreted in this way was the lack of parallelism between the specific radioactivity curves of β-carotene and canthaxanthin in Expt 1 (Fig. 1).

β-Carotene functions as part of the photosynthetic pigment complex, as a precursor of the xanthophylls (with β,β ring structure) associated with photosynthesis, and of ketocarotenoids. The rise in β-carotene concentration between 0 and 15 days from resuspension paralleled by rises in concentration of primary xanthophylls may have resulted from an increase in the quantity of photosynthetic tissue. A proportion of the carotene synthesised would be turned over to form the xanthophylls, but a substantial proportion would remain fixed in the chloroplast membrane structure and inert to further metabolic change. However, a labile pool of β-carotene must also have been present and rapidly and continously turned over to form ketocarotenoids. Turnover of β-carotene was confirmed by the continued decline in specific radioactivity associated with a decline in concentration between 15 and 37 days from resuspension. Since the

levels of primary xanthophylls declined during this period, conversion of β-carotene to ketocarotenoids was the likely pathway of removal.

In Expt 2 the rate of β-carotene specific radioactivity decline between 15 and 37 days from resuspension was much reduced despite continued formation of ketocarotenoids and the incorporation of unlabelled acetate and CO_2 into the cultures. The total radioactivity also declined little, and a similar base level was reached 37 days after resuspension in Expt 3. This radioactivity may have represented β-carotene fixed in residual chloroplast membrane structure, a conclusion supported by the significant concentrations of primary xanthophylls present at 37 days. The rapid decline in total radioactivity from green to brown cultures may have resulted from turnover of β-carotene-[^{14}C] to primary xanthophylls and ketocarotenoids (Table 2).

The β-carotene labelling pattern observed in Expt 3 resulted from the influence of the 2 pools and the complex crossover of specific radioactivity dominance already described.

If the chloroplast develops into a chromoplast [8], β-carotene may become separated into 2 pools only after a proportion is incorporated into the chloroplastidic membranes. Biosynthesis of β-carotene would have a common location. The primary xanthophylls may then be formed within the membrane structure of the chloroplast, and the ketocarotenoids within the interstitial fluid.

The quantitative significance of the proposed pathways. The results have been interpreted as demonstrating that *de novo* synthesis of ketocarotenoids from exogenous carbon sources is the major pathway. However, despite the possible existence of residual chloroplast structure in mature red cultures, chloroplastidic β-carotene, and perhaps the β-carotene derived primary xanthophylls [13] may be utilised for ketocarotenoid biosynthesis at a late stage of culture development. Chlorophyll degradation products could also be utilised but by a less direct metabolic route. The extent and rapidity of ketocarotenoid formation in green algae is controlled extensively by culture conditions [1–11]. The relative contributions of the proposed pathways to ketocarotenoid biosynthesis may be related to the culture medium, light intensity, temperature, and maturity of the cells subjected to nutritional deficiency. However, during preliminary studies of acetate-[2-^{14}C] incorporation, cells were resuspended on a medium containing acetate and levels of trace metals half those present in the growth medium. This resulted in a rapid decline in photosynthetic pigments and a similar increase in ketocarotenoids during a 24-hr period. Despite the rate of change, exogenous sources of carbon appeared to be the major ketocarotenoid precursors.

EXPERIMENTAL

Algae. Haematococcus lacustris (34/1a) was obtained from the Culture Centre of Algae and Protozoa, Cambridge. The later and therefore not valid synonym *H. pluvialis* Flotow has been used by other authors. Cells were maintained on slopes containing agar (1.0%), peptone (0.1%), KNO_3 (0.02%), K_2HPO_4 (0.002%) and $MgSO_4$ (0.002%) then transferred to liquid cultures in test tubes. A 4 ml inoculation was made into 100 ml starter cultures maintained in 250 ml conical flasks. After 10 days, transfer was made in the same way to 100 ml experimental cultures. The liquid medium was based upon that of Pringsheim [9] and contained KNO_3 (0.01%), $(NH_4)_2HPO_4$ (0.002%), $MgSO_4.7H_2O$ (0.002%), yeast extract (Difco) (0.03%) and NaOAc (0.01%). In addition, 2 ml of a saturated soln of $CaSO_4$ and 0.5 ml of trace element soln [9] was added per 100 ml of medium. Resulting soln pH was 7. All cultures were incubated at 25°, under continuous illumination of 3200 lx from fluorescent strip lighting. They were shaken by hand once in 24 hr. Following an 8-day period of growth, cultures not required for analysis were washed once (Expt 3) or twice (Expt 1 and 2) by harvesting and resuspending in sterile deionised H_2O, then resuspended on a sterilised medium containing KH_2PO_4 (0.01%) and NaOAc (0.01%), pH adjusted to 7. Cultures were harvested by centrifugation at 2500 *g* for 10 min. In the absence of label in green Expt 3 cultures, only one washing was considered necessary. This procedure, however, may have permitted some carry-over of nutrient materials resulting in greater post-resuspension growth than observed in Expts 1 and 2. Three cultures were harvested from each expt at 15 and 37 days from resuspension at which ages they appeared brown and red respectively. This gave a total of 9 cultures analysed for each expt. At resuspension most cells were of the motile vegetative form, the cultures appearing green. At 15 days from resuspension oil droplets were apparent in many cells and motility was reduced. The red (37 day) stage was characterised by the presence of large numbers of thick-walled immotile spores. The culture medium pH at resuspension was between 9.7 and 10. Following resuspension and incubation for 37 days, the medium pH rose to 7.8.

Addition of acetate-[2-^{14}*C*]. NaOAc-[2-^{14}C] (56 mCi/mmol) was added to the growth or resuspension medium to a conc. of 30 μCi/100 ml. It was sterilised as a constituent of the growth medium, but separately from the incubation medium. The wt of NaOAc-[2-^{14}C] represented an addition of only 0.44% to the unlabelled material present in the medium.

Extraction of pigments. The algal pellet was extracted by grinding with sand with a pestle and mortar, first with EtOH, then with Et_2O. Extraction with each solvent was continued until the extracts were only weakly coloured. The Et_2O was removed by red. pres. distillation under N_2 and the remaining EtOH extract saponified at 23° for 14 hr [14]. The product was partitioned between Et_2O and H_2O, the aq. phase extracted 2× with Et_2O, then discarded [14]. A vol. of petrol (bp 40–60°) equal to that of the Et_2O was then added and washing repeated until alkali-free. Addition of petrol aided formation of the solid astacene salt at the solvent interface. The solid was removed and the remainder of the petrol extract dried by freezing to −15° then filtering while cold. Excess solvent was removed as above, and by evaporation under N_2.

Separation of carotenoids. Preliminary separation was on Al_2O_3 (Woelm neutral-Brockmann grade III). β-Carotene, echinenone and canthaxanthin were eluted with 5, 15 and 40% Et_2O in petrol respectively. The chloroplastidic xanthophylls were eluted with 10% EtOH in Et_2O, then further separated on powdered sucrose columns eluted with 15, 20, 35 and 50% Et_2O in petrol to recover lutein (β,ε-carotene-3,3′-diol), antheraxanthin (5,6-epoxy-5,6-dihydro-β,β-carotene-3,3′-diol), violaxanthin (5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol) and neoxanthin (5′,6′-epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β,β-carotene-3,5,3′-triol) respectively. Astacene remained bound to the top of the Al_2O_3 column. The column was extruded, the astacene band excised, and transfered to the top of a short Al_2O_3 column (grade V) to which was added the astacene obtained by partition. The column was washed with EtOH, the pigment eluted with a 10% soln of KOH in 10% aq. EtOH and then partitioned between petrol and H_2O to remove alkali. Astacene salt was removed from the solvent interface, dissolved in a minimum of glacial HOAc and returned to the petrol. Washing was continued until washings were neutral.

Quantitative determination of carotenoids. This was carried out spectrophotometrically [14,15]. Astacene was determined in petrol using an $E^{1\%}_{1cm}$ value determined experimentally by

comparison of the extinction of standard pigment in petrol with that in C_5H_5N [14].

The β-carotene may have contained small quantities of α-carotene (β,ε-carotene). Subsequent purification demonstrated that contamination could not have been greater than 10% at any stage of culture development. Similarly, lutein may have contained zeaxanthin as a minor contaminant.

Purification of carotenoids. To obtain radiochemical purity the carotenoids were subjected to the following sequential TLC procedures. β-Carotene: (a) Si gel G, C_6H_6–EtOAc–MeOH (15:4:1; System 1); (b) Si gel G, petrol–Et_2O (19:1), (c) MgO, petrol–C_6H_6 (3:2). Echinenone: (a) System 1; (b) Si gel G, petrol–Et_2O (7:3), (c) MgO,C_6H_6. Canthaxanthin: (a) System 1; (b) cellulose, petrol–Me_2CO (19:1); (c) cellulose, petrol–C_6H_6 (3:2). Astacene was converted to its K salt by the addition of aq. KOH to the petrol soln. The insoluble salt was removed, dissolved in glacial HOAc, washed by partition between petrol and H_2O, dried and purified on 3 consecutive cellulose TLC systems with petrol, petrol–C_6H_6 (1:1) and petrol–Me_2CO (9:1). Pigments were assayed spectrophotometrically and for ^{14}C as described by Davies *et al.* [17]. Carotenoids from red cells, which contained the maximum quantity of pigment and interfering oil, when repeatedly purified by the final TLC system showed no significant change in specific radioactivity.

Identification of carotenoids. The carotenoid constituents of *H. lacustris* have been studied previously [2,6,18–20]. The properties of the pigments analysed in the three expts were consistent with their proposed identities. More extensive investigation was made on samples of unlabelled algae cultured under the same or similar conditions. β-Carotene, echinenone and canthaxanthin co-chromatographed with authentic synthetic carotenoids on the three TLC systems used for the radiochemical purification of each. In addition, β-carotene co-chromatographed (TLC) on Al_2O_3–MgO (3:1) when developed with petrol–EtOAc (24:1); echinenone on MgO–Si gel G (1:1), with C_6H_6–hexane (7:3) and canthaxanthin on Si gel G with Et_2O–hexane (7:3). Astacene co-chromatographed with the authentic synthetic carotenoid on cellulose TLC with petrol–Me_2CO (9:1) and on TLC System 1. The three ketocarotenoids were further characterised by reduction with $NaBH_4$ in EtOH. The hydroxy-carotenoids produced were identified by co-chromatography on TLC with the reduction products of synthetic authentic samples, and by their visible light absorption spectra. Prior to saponification of the extract from unlabelled algae, the chloroplastidic xanthophylls were identified by Si gel G TLC developed with C_6H_6-EtOAc-MeOH (15:4:1) and by the colour produced on exposure of the developed TLC plates to HCl vapour [16]. Following column and TLC purification of saponified extracts the extent of the HCl-induced spectral shift was observed in EtOH [16]. Violaxanthin and neoxanthin co-chromatographed with authentic pigments on cellulose TLC, developed with petrol–Me_2CO (9:1). Lutein co-chromatographed on the same adsorbent developed with petrol–Me_2CI (19:1). Prior to spectrophotometry, lutein was purified by TLC on MgO–Si gel G (1:1), solvent C_6H_6–MeOH (16:1). In red and, to a lesser extent, in brown cultures, violaxanthin became increasingly difficult to purify due to the presence of minor pigment zones. Spectrophotometric evidence suggested the partial conversion of violaxanthin to its 5,8-epoxide (5,6;5′,8′-diepoxy-5,6,5′,8′-tetrahydro-β,β-carotene-3,3′-diol). This may have been an artefact resulting from the presence of greater quantities of acid in the red compared to green cells, the acids being released on extraction. Minor ketocarotenoid zones also increased in red cultures. Because of the small quantities present, these were not analysed; pigments of comparable properties were reported by Czygan [18]. The electronic absorption spectra of all pure pigments were recorded, each in at least two solvents, and compared with those of authentic standards. Absorption maxima were compared with li. values. Further evidence for the identity of echinenone and canthaxanthin was provided by analysis of a green culture from a preliminary acetate-[2-^{14}C] incubation. The carotenoids were separated by sequential columns of powdered sucrose and Al_2O_3 (grade III). Authentic carrier was then added and echinenone purified on a MgO column and canthaxanthin on cellulose TLC. Following chromatography, both pigment fractions were found to contain substantial ^{14}C activity. Labelled constituents from the algae had co-chromatographed with the two authentic pigments.

Authentic carotenoids. β-Carotene, echinenone, canthaxanthin and astacene were gifts from F. Hoffman–La Roche & Co. The chloroplastidic xanthophylls were extracted from fresh lawn cuttings. All pigments were purified prior to use by methods similar to those described above.

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