The Appearance of Neoxanthin during the Regreening of Dark-grown Euglena [1, 2]

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Euglena gracilis has been shown to contain large amounts of the epoxide carotenoids, antheraxanthin and neoxanthin, when cultured heterotrophically in the light (13). This green flagellate can be changed by a variety of agents and conditions into yellow or colorless forms which can no longer carry out photosynthetic reactions. This change is apparently due to the inability of these modified strains either to synthesize chlorophyll or replicate chloroplasts. There have been several reports of altered patterns in the carotenoid pigments in these yellow and colorless forms.

Pringsheim and Pringsheim (16) demonstrated that high temperature treatment (34–35°) would convert *E. gracilis* to an irreversibly bleached form, and Goodwin and Gross (8) have studied the carotenoid pigments of such a heat-bleached substrain. They found that, as with the parent strain, xanthophylls were present to a much larger extent than the hydrocarbon polyenes. The differences noted were that the heat-bleached strain contained phytofluene and a ζ-carotene-like pigment in the hydrocarbon fraction, unlike the green cells. In addition, the xanthophyll fraction was altered; neoxanthin was absent, and they reported only echinenone, zeaxanthin, and lutein in this fraction. In view of the fact that Krinsky and Goldsmith (13) identified the major xanthophyll of *E. gracilis* as antheraxanthin, and that this had been mistakenly identified as lutein in an earlier publication of Goodwin and Jamikorn (9), we have assumed that there is some antheraxanthin in heat-bleached substrains of *E. gracilis,* but this remains to be proven. It would therefore seem as if heat-bleached *E. gracilis* lacks at least one of its 2 epoxide carotenoids, neoxanthin, and may or may not retain some antheraxanthin.

The drug-bleached substrains have been investigated more thoroughly. Certain antibiotics, such as streptomycin (17) and erythromycin (5) and various antihistamines (10) are capable of bleaching *E. gracilis.* Seven bleached substrains were studied by Goodwin and Gross (8) and they concluded that the synthesis of the typical chloroplast carotenoids, β-carotene, lutein (antheraxanthin?), and neoxanthin was upset. Indeed, they were unable to detect neoxanthin in any of these strains, and only traces of lutein (antheraxanthin?).

Darkness will also induce chlorotic, or etiolated forms of *E. gracilis,* but unlike heat or drugs, this procedure is reversible. Goodwin and Jamikorn (9) concluded that the pigments in dark-grown *E. gracilis* were the same as those found in the light-grown cells and in the same relative amounts, although at a lower concentration. This was modified by a later study, in which Goodwin (7) reported that he found only lutein (antheraxanthin?) with β-carotene, neoxanthin and violaxanthin being absent or present in only minute traces.

All of these studies would indicate that there is an alteration in the carotenoid pigments in *E. gracilis* when they are made chlorotic. With the recent demonstration of the kinetics of regreening of dark-grown *E. gracilis* and the measurement of the rate of appearance of photosynthetic capacity during this process (21) we have investigated what changes, if any, occur in the carotenoid pigments during this process. A preliminary report of this work has appeared (14).

Materials and Methods

Isolation and Separation of Pigments. E. gracilis var. bacillarus Pringsheim (15) which had been maintained in the dark for over 2 years, was exposed to 100 to 150 ft-c of white light in a resting medium as described earlier (21). After varying periods of time, the cells were harvested and frozen. All pigment analyses were started 2 to 48 hours after harvesting.

All of the following manipulations were carried out under dim light. Between 1.5 and 3.5 g (wet wt) of cells were suspended in 6% KOH in methanol (ca. 5 ml/g wet weight) and saponified for 5 minutes at 40°. The suspension was chilled in an ice bath and centrifuged for 5 minutes at 5000 × *g*. The residue was reextracted 4 times wtih absolute methanol. The pooled supernatant fractions were diluted to a 50% methanol concentration by the addition of an equal volume of a 5% NaCl solution, and ex-

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tracted several times with diethyl ether (peroxide-free), until the ether extract was colorless. No acidic pigments were observed. The ether extract was washed with water until the washings were neutral, and checked for completion of saponification by momentarily viewing it with an ultraviolet lamp (3650 A). If any reddish fluorescence was observed, indicating the presence of unsaponified chlorophyll, the solution was resaponified and the above extraction procedure repeated.

When the saponification and extraction were complete, the pigments were taken to dryness, following the addition of absolute ethanol to facilitate the removal of water, in vacuo at 40°. The extract was resuspended in 2 % ethanol in petroleum ether for column chromatography. The initial separation was carried out on columns of aluminum oxide, as described earlier (13). Three fractions (I, II, III) could be readily eluted from such columns with 2 %, 4 %, and 50 % ethanol in petroleum ether respectively. A fourth fraction (IV) tightly bound to the adsorbant, was eluted with a combination of water, ethanol, and traces of glacial acetic acid. The first 3 fractions were fractionated further as follows:

Fraction I. Adsorbed from petroleum ether on a Sea Sorb-celite (1: 1) column and developed with 0.75 % ethanol in petroleum ether. An orange band (I-A) was eluted with this solvent, and 2 pink bands and a yellow band were eluted from the extruded column as a single fraction (I-B) with absolute ethanol.

Fraction II. This fraction, in 10 % acetone in petroleum ether, was adsorbed on a column of Microcel-C, and when developed with this solvent, 3 bands appeared. These were, from the bottom up, orange (II-A), dark yellow (II-B) and yellow (II-C) respectively. The column was extruded, and the 3 zones were eluted with absolute ethanol.

Fraction III. This fraction, suspended in 4 % ethanol in petroleum ether, was adsorbed on a column of Sea Sorb-celite (1: 1) and development with 5 % ethanol in petroleum ether led to the separation of the pigments into a lower, greenish-yellow zone (III-A) and an upper yellow zone (III-B). The fractions were eluted from the extruded column with absolute ethanol.

Absorption spectra of the individual pigments were determined in petroleum ether (fraction I) or absolute ethanol (fractions II, III, IV), and identifications were made as described previously (13). The procedure of Krinsky (12) was used to obtain M_{50} values from the quantitative partition coefficients as a final means of identification. M_{50} values represent the percentage methanol required to give a partition coefficient of 50: 50 in the solvent system petroleum ether-aqueous methanol. This M_{50} value is directly proportional to the number and types of polar substituents in carotenoid molecules, and is an extremely sensitive means of differentiating closely related carotenoid pigments (12).

Reagents. The solvents used were acetone, ethanol, and petroleum ether (30°–60°), reagent grade, without further purification. Adsorbants included aluminum oxide (Merck, suitable for chromatographic adsorption), magnesium oxide (Sea Sorb 43, Fisher), celite (Johns-Manville Co.) and Microcel-C (Johns-Manville Co.).

Results

The carotenoid pigments isolated from *E. gracilis* var. bacillaris, both dark-grown and those exposed to 100 to 150 ft-c for 6 days in resting medium, are shown in table I.

Fraction I-A, β-carotene, was identified by its spectral and chromatographic properties.

Fraction I-B, which was not separated into its component carotenoids due to its small size, was assumed to contain cryptoxanthin and the keto pigments echinenone and euglenenone from the color of the bands, relative position on the column, and the fact that these 3 pigments had all been reported previously in *E. gracilis* (13).

Fraction II-A was identified as zeaxanthin by its

Table I

Carotenoid Pigments in Euglena Gracilis var. Bacillaris

Fraction	Pigment	Dark-grown		Light-grown*	
		μg/g wet wt.	%	μg/g wet wt.	%
I-A	β-carotene	2.1	2.0	192.5	13.8
I-B	echinenone, euglenanone, cryptoxanthin	3.1	3.0	26.0	1.9
II-A	zeaxanthin	22.1	21.2	72.3	5.2
II-B	*trans*-antheraxanthin	67.4	64.4	998.9	71.8
II-C	*cis*-antheraxanthin				
III-A	neoxanthin	0.0	0.0	66.9	4.8
III-B	trollein	3.8	3.6	18.4	1.3
IV	hydroxyechinenone	5.9	5.6	15.6	1.1
	Totals	104.5		1390.6	

* Six days in resting medium (0.054 M mannitol, 0.01 M $MgCl_2$ and 0.01 M KH_2PO_4) at 100 to 150 ft-c.

characteristic M_{50} value (12) and by cochromatography with an authentic sample of zeaxanthin isolated from yellow corn meal. This pigment had not been previously reported in either bacillaris or Z strain grown in the light (13) or in the dark (7). However, the introduction of Microcel-C as an adsorbant (22) has enabled us to effect a separation of II-A and II-B. On magnesium oxide columns, zeaxanthin cannot be readily separated from antheraxanthin.

Fractions II-B and II-C were *trans-* and *cis-*antheraxanthin, respectively.

Fraction III-A, present only in the illuminated cultures, was identified as neoxanthin, as described earlier (6).

Fraction III-B, present at a level of 1 to 2 % of the total pigments in light-grown cells, has now been observed in both bacillaris and Z strain, and appears to be very similar to trollein, a trihydroxy carotenoid originally isolated from oranges (3).

Fraction IV was identified as hydroxyechinenone (13).

The effect of light on the development of carotenoids during the regreening process in this alga is shown in figure 1.

Antheraxanthin is synthesized at a linear rate for about 100 hours, at which time its synthesis ceases. β-carotene however continued to increase at a linear rate out to at least 141 hours, the longest illumination time studied in this experiment. In figure 1-B, it can be seen that trollein behaves similarly to β-carotene, in that its synthesis continued linearly over the entire experimental period.

Neoxanthin, which is not present in the dark-grown cells is first identified at 4 hours, at a level of 0.4 μg/g wet weight, or less than 0.3 % of the total pigment found at that time. Numerous extractions of dark-grown cells have always confirmed the original observations that neoxanthin is absent from such cultures (7). Its synthesis stops sharply at the same time that the synthesis of antheraxanthin stops.

Zeaxanthin levels, although fluctuating widely, can be interpreted as increasing linearly with time during the entire course of illumination.

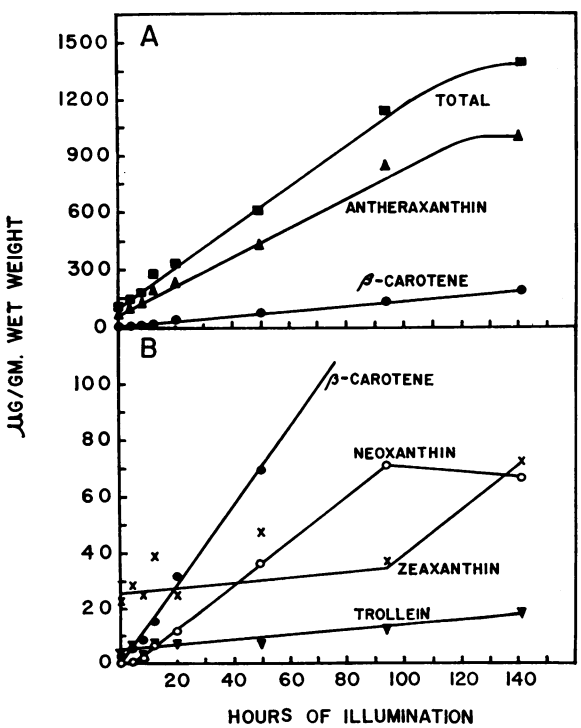

FIG. 1. The rate of appearance of carotenoid pigments with illumination at 100 to 150 ft-c in *E. gracilis* var. bacillaris. A. The total carotenoid pigments (■), antheraxanthin (▲) and β-carotene (●) are plotted using the upper ordinate. B. β-carotene (●), neoxanthin (O), zeaxanthin (×), and trollein (▼) are plotted using the lower ordinate.

Discussion

Our observations of a marked increase in the carotenoid pigments of dark-grown *E. gracilis* following exposure to 100 to 150 ft-c of light while suspended in a resting medium are similar to the results reported by Stern et al. (21). On a wet weight basis, the carotenoids show a linear increase for about 100 hours, and appear to level off by 140 hours. This increase in total carotenoids is accounted for primarily by antheraxanthin which represents 70 % of the total carotenoids in both dark-grown and light-grown cells of *E. gracilis* var. bacillaris and 80 % of the total carotenoid in light-grown *E. gracilis* strain Z (13).

The hydrocarbon carotenoid, β-carotene, does not follow the pattern of antheraxanthin, but continues to increase linearly for at least 141 hours, the longest time studied in these experiments. This continued increase in β-carotene may be related to the observation that old cultures of *E. gracilis* contain flecks of hematochrome throughout the cytoplasm (11), in comparison to the single eyespot found in growing cells.

The 4 hour lag period in the appearance of neoxanthin during the regreening of dark-grown *E. gracilis* seems to be of particular importance in view of the results reported recently on the development of photosynthetic competence under identical experimental conditions. Stern et al. (21) found a 6 to 8 hour lag in the appearance of CO_2 fixation and O_2 evolution and in the linear rate of synthesis of chlorophyll and carotenoid following exposure of dark-grown *E. gracilis* to light. This apparent discrepancy in the initial appearance of carotenoid pigments may be due to the fact that we are reporting our results on the basis of wet weight, whereas Stern et al. reported their results on a per cell basis (21). This onset of photosynthetic competence is reflected in the morphological development of the plastid, for Ben-Shaul et al. (1) have shown that unfused discs begin invaginating from the inner proplastid membrane at 2 hours, and the first multidisced lamellae appear at 4 to 6 hours. Finally, it should be pointed out that Schiff and French (19), using an O_2 electrode, have demonstrated that the time at which net photosynthetic

O_2 evolution appears is about 4 hours. Prior to this, there appears to be a photostimulation of respiration.

The appearance of neoxanthin concomitantly with the appearance of photosynthetic competence, and its complete absence in dark-grown cells, raises the question of the possible relationship between neoxanthin and photosynthetic processes. Epoxide carotenoids had been suggested as participants in both photosynthetic O_2 evolution (4, 18) and O_2 transport (2), but little information was available until 2 recent experiments using $H_2{}^{18}O$ appeared (20, 22). Although both experiments rule out the diepoxide violaxanthin as an intermediate O_2 transporter during the Hill reaction in spinach chloroplasts, the evidence with respect to neoxanthin is less certain. Yamamoto et al. (22) analyzed neoxanthin under adverse instrumental conditions, while Shneour and Calvin (20) did not have a sufficient quantity of neoxanthin to analyze.

Of the 2 epoxide carotenoids in *E. gracilis,* only neoxanthin parallels the course of development of photosynthetic competence with respect to both the initial lag period and the leveling off at about 90 hours (21). It still remains to be demonstrated that antheraxanthin is present in other etiolated forms of *E. gracilis,* such as those produced by heat, antibiotics or antihistamine. In the drug-bleached and heat-bleached strains investigated by Goodwin and Gross (8), neoxanthin was lacking. It would therefore appear as if neoxanthin is closely related to the photosynthetic system of *E. gracilis.*

Trollein and zeaxanthin both appear to increase with time, although the data for zeaxanthin show rather wide variations. The minor pigments cryptoxanthin, echinenone and euglenanone were not separated; the total amount also shows an approximate linear increase with time. Similar results are found with hydroxyechinenone.

Summary

Dark-grown *Euglena gracilis* var. bacillaris Pringsheim has been shown to contain no chlorophyll and is unable to carry out photosynthetic reactions. In addition, dark-grown cultures contain only 7 % of the carotenoids found in light-grown controls. Neoxanthin, which comprises 5 % of the total carotenoids in the control cultures, is completely lacking in dark-grown cultures.

When dark-grown cultures in a resting medium are exposed to white light carotenoid synthesis begins as soon as the light is turned on, with the exception of neoxanthin which shows a 4 hour lag before its synthesis begins. This 4 hour period corresponds to the lag reported for the first appearance and development of photosynthetic competence. This indicates that neoxanthin synthesis parallels the development of the photosynthetic apparatus in regreening *E. gracilis.*

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Function of Bean Roots and Stems in Sodium Retention [1, 2]

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Sodium is excluded from the tops of many plants (1–4, 7, 8), and hence accumulation of this ion in the leaves of such plants which apparently are rather sensitive to it (2) is avoided. The barrier to sodium transport is effective at moderate external sodium concentrations. At high external concentrations (2, 7, 8), and as a result of prolonged growth in a saline medium (1), sodium contents are found to increase in the stems and sometimes also in the leaves of so-called Na nonaccumulator plants. Gauch and Wadleigh (4) assumed that sodium movement might be restricted by the membranes of certain extrastelar tissue, excluding it from the vascular system. Huffaker and Wallace (8) suggested that the term Na nonaccumulator plant should be considered as a quantitative rather than a qualitative expression. Bernstein et al. (1) found comparable levels of sodium accumulation in the roots, wood, and bark of young apricot trees grown for one year in saline plots; only the leaves and twigs of these plants exhibited restricted sodium accumulation. The authors supposed that sodium had entered the vascular tissue of the trees and that its translocation into the leaves via the transpiration stream had been restricted by retention in the living wood parenchyma cells. During the third year of these experiments sodium levels increased also in the leaves of various stone-fruit trees. Bernstein et al. (1) consider this increase to be due to the development of heart wood during the third year of growth, release of sodium by the senescent parenchyma and its subsequent translocation into the leaves via the transpiration stream.

No direct proof has been brought forward for any of the hypotheses regarding the location of the barrier to sodium transport and the mechanism of its rupture at high external concentrations. The interpretation proposed by Bernstein et al. (1) for sodium leakage to the leaves during the third year of their experiment would not be adequate to explain the gradual advance of sodium in the stem with increasing external concentrations as found in brief tracer experiments (7, 8). The present work is an attempt to supply more data about sodium transport and distribution in bean plants—a Na nonaccumulating species—in order to provide evidence for the elucidation of these phenomena.

Materials and Methods

Brittle wax bean seeds (*Phaseolus vulgaris* L.) were germinated in vermiculite at 25° and grown at this temperature in a light chamber in an aerated 0.1 Hoagland solution—1 (5). Plants with primary leaves fully expanded were then transferred for 24 hours to various experimental solutions. Sodium transport was studied in both intact and derooted plants. For the latter experiments the roots were detached under water before the plants were transferred to the experimental solutions. Except for the composition of the external solution, experiments were carried out under the same conditions as previous cultivation. The plants were hermetically fitted into beakers, dipping only with part of their roots or, in the case of derooted plants, with the basal centimeter of their stems, in the experimental solution. Water loss thus occurred only through the plant and was measured whenever necessary by weight difference, with an accuracy of $\pm$ 0.025 g.

Experimental solutions contained 2×10^{-4} M $CaSO_4$ and NaCl at desired concentrations labeled with 0.2 μc per ml of Na^{22} and/or Cl^{36}. Na^{22} was supplied by the Radiochemical Centre, Amersham, England, as NaCl and was diluted with stable NaCl to the required activity. Chlorine-36 was procured

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