To the Graduate Council:

I am submitting herewith a thesis written by Kenneth Monar entitled "Comparative Analyses of Carotenoid Pigments in Euglena gracilis and Astasia pertyi (Euglenophyta) by Reversed Phase-High Performance Liquid Chromatography." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Botany.

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The Graduate School

4

COMPARATIVE ANALYSES OF CAROTENOID PIGMENTS IN *EUGLENA GRACILIS* AND *ASTASIA PERTYI* (EUGLENOPHYTA) BY REVERSED PHASE-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Kenneth Monar

March 1984

ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. P. L. Walne, and my committee, Dr. J. Becker, Dr. L. Jones, and Dr. W. Fletcher, for their help and guidance. I would also like to thank Dr. G. Sayler for the generous use of an HPLC instrument, and his student H-L. Kong for insightful instruction as to its use. Special thanks go to Dr. W. Fletcher for my training in Raman Spectroscopy, and to Dr. R. Pagni and Dr. S. Georghiou for the information and advice they afforded me, and to Dr. P. L. Walne without whose research funding this work would have not been possible. I would also like to thank my fellow graduate students: J. Dunlap, for his patient instruction in electron-microscopy, Dr. P. Kivic for his many informative discussions, and J. Solomon and others for their friendship. I would also like to thank the Botany Department for awarding me a graduate assistantship/teaching assistantship for 3 years and especially Dr. Holton for his guidance.

ABSTRACT

Examination of the carotenoid composition of light-grown and etiolated (dark-grown) Euglena gracilis var. bacillaris by reversed phase-high performance liquid chromatography has shown that these organisms have a similar complement of pigments, although there are quantitative differences. Etiolated cells have a much reduced total pigment concentration; however, with respect to individual carotenoids, some are drastically reduced, others are reduced to a lesser degree, and still others exhibit a slight increase when compared to light-grown cells. The previously reported absence of chlorophyll in etiolated cells is questionable, as this work shows that ca. 4% is being maintained in this cell line. Preliminary results from Astasia pertyi, examined here for the first time, indicate a carotenoid complement quite different from that of Euglena gracilis. The comparative results from Astasia pertyi and Euglena gracilis light-grown and etiolated cells may have implications both for the biosynthetic patterns of carotenoids and phylogenetic considerations in the euglenoid flagellates; the techniques developed here should provide a strong basis on which to develop taxonomies based on carotenoid composition.

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Quantities

ml - milliliter
µl - microliter
g - gram
mg - milligram
µg - microgram
ng - nanogram
pg - picogram

cm - centimeters
mm - millimeters
µm - micrometer
nm - nanometers

Relative Quantities

A	absorbance
°C	degrees Celsius
pH	negative log of the hydrogen ion concentration
psi	pounds per square inch
rpm	rotations per minute
%	parts per 100 parts
wt./vol.	weight per unit volume
W	watts

Chemicals

$CaCO_3$	calcium carbonate
HCl	hydrochloric acid
$LiAlH_4$	lithium aluminum hydride
$NaBH_4$	sodium borohydride

Other Symbols

ATT	attenuation
DF	dilution factor
HPLC	high-performance liquid chromatography
NARP	non-aqueous reversed phase
ODS	octadecasilane
RP	reversed phase
TLC	thin-layer chromatography
var.	variety

CHAPTER I

INTRODUCTION

The presence of photosynthetic, heterotrophic, and phagotrophic organisms with the Euglenophyta render them an interesting group with respect to their carotenoid pigmentation, since one of the principal functions of these compounds involves photosynthesis (Goodwin, 1980).

Euglena can serve as a model system for the study of carotenoid composition since it is capable of losing most of its pigmentation (chlorophylls and carotenoids) and regenerating pigments again, depending upon culture conditions (for review see Schiff, 1978).

As in all photosynthetic organisms, the major site of carotenoids in a Euglena cell is the chloroplast (Goodwin, 1980), but an extrachloroplastidic pigmented structure, known as the stigma or eyespot, is also present in both Euglena and Astasia pertyi (Walne and Arnott, 1967; Kivic and Vesk, 1974a,b; Colombetti et al., 1982) and is known to contain carotenoid pigments (Batra and Tollin, 1964; Bartlett et al., 1972; Heelis et al., 1979; Osafune and Schiff, 1981; Pagni et al., 1981). The stigma appears to play a role in the ability of these organisms to detect light (for review see Colombetti et al., 1982).

The major objectives of this study were to reexamine the carotenoid composition of light-grown Euglena gracilis by the use of modern, sensitive techniques not previously employed, and

to examine how the absence of chloroplasts from Astasia pertyi or their regression to proplastids in etiolated Euglena gracilis affects the carotenoid composition. The carotenoid composition of Astasia pertyi has not been studied previously; it lacks chloroplasts but possesses a pigmented stigma and was chosen as a potential source of stigmatal carotenoids synthesized independently of the chloroplast. Elucidation of the carotenoids present in Astasia and Euglena may provide some insights about the sites of carotenoid synthesis or their coding in the cell, and may also be useful in considerations of euglenoid taxonomy and phylogeny.

Since carotenoids are extremely labile structures (Liaaen-Jensen, 1971b) and many of the previously used chromatographic techniques are now known to cause artifacts (Braumann and Grimme, 1981), recently developed techniques in reversed phase high-performance liquid chromatography (RP-HPLC) were employed here to obtain the most accurate results possible.

CHAPTER II

MATERIALS AND METHODS

Culture Methods

Cultures of Euglena gracilis var. bacillaris (#884) were obtained from the University of Texas Culture Collection of Algae and were grown in Euglena Broth (Difco) or Hutner's Euglena Medium C prepared without a carbon source except for the vitamins, thiamine and B_{12} (Appendix A.1, A.2).

Astasia pertyi (#1204/3) was obtained from the Culture Centre of Algae and Protozoa, Cambridge, England, and was grown in a modified "Cambridge" Medium (Appendix A.3) or Cambridge Medium (Appendix A.4), both supplemented with vitamin B_{12}.

All cultures were grown in 2500 ml "Wide-Form" culture flasks containing 1000 ml of liquid media. Euglena gracilis was grown at 25°C under 2×10^{-3} W/cm^2 continuous illumination. Astasia pertyi and etiolated Euglena gracilis var. bacillaris were grown at 25°C in complete darkness.

Cell number per unit volume was determined by use of a haemocytometer and hand-held counter followed by serial dilutions of known concentrations. The light scattering of these solutions was determined spectrophotometrically (Zeiden and Schiff, 1967) with a Bausch & Lomb Spectronic 20, with detector set at 675 nm. Standard curves were then developed.

Harvesting of Cells

Cells were harvested from actively growing cultures and were separated from the liquid medium by centrifugation in a Sharples Super-Speed Centrifuge, washed once with glass-distilled water, and re-centrifuged at 2500 rpm in a GSA rotor in a Sorvall RC-2B centrifuge. The pellet of cells was resuspended in a minimal volume of glass-distilled water, frozen in a flask by cooling with dry ice in methanol, and subsequently freeze-dried. During freeze-drying, the flasks were covered with aluminum foil to prevent photo-degradation of the pigments. Freeze-dried cells were stored at -10°C in the dark, over desiccant ($CaCO_3$), and under a nitrogen atmosphere pending extraction.

Pigment Extraction

Approximately 50 ml of HPLC-grade acetone was added to a flask containing the freeze-dried cells and set aside for 10 min under a nitrogen atmosphere. The ensuing suspension was apportioned among several polycarbonate centrifuge tubes, and most of the cells were pelleted by spinning in a tabletop centrifuge at maximum speed for 1 min. The pelleted material was re-extracted four or five times with acetone, and extracted once or twice with an aliquot of HPLC-grade methanol until the pellet appeared colorless.

The pooled extracts were kept on ice under a nitrogen atmosphere and were filtered with a 10-μm Millipore filter (LCWP-050) to remove cellular debris. The extract was subsequently taken to dryness in the dark using a Brinkman Roto-Evaporator and a Welch

(Model 1399) vacuum pump. Since the evacuated gases were combustible, the pump was placed in a well-ventilated fume hood to reduce the possibility of an explosion. The dried extract was resuspended in a small volume of freshly distilled diethyl ether and filtered a second time using a 0.22-µm Millipore filter (GVWP-025). The ether extract was apportioned among several test tubes for subsequent analyses.

Total Pigment Concentration

An estimate of the total carotenoid and chlorophyll content in concentrated bulk extracts was made by diluting 10-100 µl of pigment extract with 80% acetone and measuring the absorbance of the solution at 480 nm, 645 nm, 652 nm, and 663 nm in a Gilford Spectrophotometer (Kirk and Allen, 1965). The total carotenoid content in that extract was calculated using the following equation (Davies, 1976):

$$A^*_{480} = A_{480} + 0.114A_{663} - 0.638A_{645}$$

where:

A^*_{480} = the corrected absorbance at 480 nm

A_{480} = the measured absorbance at 480 nm

$0.114A_{663} - 0.638A_{645}$ = the correction factor due to the absorbance of chlorophyll

The corrected absorbance was then substituted into the following equation:

$$\text{Carotenoid (mg/ml)} = \frac{A^*_{480}}{250} \times DF$$

where:

DF = Dilution factor

$250 = (0.1) \times E^{1\%}_{1cm}$; the average extinction value of a 1% solution of the pigment in a 1-cm light path optical cell, corrected to mg/ml (Davies, 1976)

The total pigment content was obtained by multiplication of the pigment concentration by the total extract colume.

Similarly, the total chlorophyll content in 80% acetone was determined using the following equation (Bogorad, 1962; Lewin, 1962).

$$\text{mg/ml (chlorophyll)} = \frac{A_{652}}{36} \times DF$$

Separation of Carotenoid Standards

A few milligrams of each of the carotenoid standards, echinenone, β-carotene, and zeaxanthin (Hoffman-La Roche, Basel, Switzerland), were suspended in a known value of chloroform. The individual carotenoid concentrations were determined using the following equation:

$$X = \frac{[E][Y]}{[0.1][E^{1\%}_{1\ cm}]}$$

where:

X = mg of carotenoid standard

E = measured extinction at the wavelength of maximum absorption (Davies, 1976)

Y = quantity (ml) of chloroform used to dissolve carotenoids

$[0.1][E^{1\%}_{1\ cm}]$ = the extinction value of a 1% solution of the carotenoid in a 1-cm light path optical cell at the wavelength maximum in chloroform corrected to mg (Davies, 1976)

A mixture of the individual standards was made from which a dilution series was developed and used for HPLC analysis.

Analytical Chemical Reactions

Reduction of carbonyl functions: $NaBH_4$. An aliquot of the ether extract was taken to dryness as in the extraction procedure above, resuspended in a mixture of ethanol and benzene (1:1 vol./vol.) and subsequently treated with excess $NaBH_4$ for 1 hr under a nitrogen atmosphere (Hertzberg et al., 1969; Matus et al., 1981). Glass-distilled water was added to the ethanol-benzene mixture causing the water-insoluble benzene together with the pigments to separate from the aqueous phase. The benzene was similarly taken to dryness and resuspended in a minimal volume of chloroform.

Reduction of carbonyl functions: $LiAlH_4$. An aliquot of the ether extract was cooled to -20°C in a dry-ice/methanol bath. Lithium aluminum hydride ($LiAlH_4$) was added to the cooled extract and the mixture brought to room temperature (Liaaen-Jensen, 1971a; Nitsche, 1973). The reaction was permitted to proceed for 2 min at room temperature; then the mixture was re-cooled to -20°C, and the reaction was terminated by the dropwise addition of methanol to the mixture. Glass-distilled water was added to the mixture, and the water-insoluble ether-pigment layer separated from the aqueous phase. The ether layer was taken to dryness and resuspended in a minimal volume of chloroform.

Acid re-arrangement. Enough HCl was added to an aliquot of the ether extract to make a concentration of 0.01% HCl (vol./vol.) (Matus et al., 1981). The acidified extract was kept under a nitrogen atmosphere for 30 min; then the acid was washed free from the mixture by the addition of glass-distilled water, and the upper ether-pigment layer was removed, taken to dryness, and resuspended in a minimal volume of chloroform.

Reversed Phase High Performance Liquid Chromatography (RP-HPLC)

All RP-HPLC analyses were performed on a Perkin-Elmer Series 1 Liquid Chromatograph equipped with a Perkin-Elmer LC-75 single beam, variable wavelength spectrophotometer and a Perkin-Elmer LC Autocontrol. All data were recorded and processed with a Hewlett-Packard 3390A Integrator.

Aliquots of concentrated pigments were separated using a Dupont Zorbax ODS column (250 X 4.6 mm). Separation was achieved without gradient elution (non-aqueous, reversed phase chromatography-NARP) using the following mobile phase: 90% methanol; 5% tetrahydrofuran; 5% hexane. The flow rate was 1-2 ml/min. Pigments were injected onto the column with a Rheodyne (Model 7125) valve.

RP-HPLC of carotenoid standards and whole-cell extracts from Euglena gracilis and Astasia pertyi were performed using the following chromatographic conditions: stationary phase--Dupont Zorbax ODS, 5 μm, column--stainless steel, 250 x 4.6 mm; mobile phase--methanol (90%), tetrahydroforan (5%), hexane (5%), gradient--none; flow rate--1 ml/min; temperature--ambient;

chart speed--0.2 cm/min; detector wavelength--445 nm; spectra--the tick-marks indicate 50-nm spacing.

Analysis of Standards and Whole-Cell Extracts

The wide variation of pigment concentrations used in this study necessitated the adjustment of the spectrometer attenuation to keep the signal output within a range acceptable by the integrator. As such, all peak areas have been normalized to an absorbance (A_{1cm}) of one.

Peak areas are a function of the spectrometer characteristics and the specific integrator used; therefore, the standard curve described in this work is valid only for the equipment described above.

The column and conditions described above can be used analytically, or preparatively with only minor losses in resolution. Peak area versus quantity of pigment injected is linear over a defined range, but the deviation from linearity does not change proportionally with increasing quantities of pigment injected (data not shown). Thus, estimates of pigment concentration can be made beyond the analytical range (limit not determined).

The attenuation was altered during the HPLC chromatographic experiments due to the greater height-to-width ratios of early eluting peaks, combined with the fact that these early peaks were present in much greater quantities than later-eluting peaks in E. gracilis.

As a result of the low concentration of some carotenoids, as much as 408 μg of pigment (largely chlorophyll, 374 μg) were added in some instances. A methodology (not used here) to circumvent this difficulty is presented elsewhere (see Appendix C).

The peak area is a function of concentration of the pigment, and the concentration is a function of the volume of culture used and the cell density. Since the latter aspect is difficult to control, all comparative quantities are made on a per-cell basis, using the following equation:

$$\left[\frac{\text{peak area x attenuation factor}}{\text{total cell number} \div \text{total carotenoid (mg)}}\right] \times \left[\begin{array}{c}\text{quantity}\\ \text{carotenoid}\\ \text{injected}\\ \text{(mg)}\end{array}\right] = \frac{\text{area}}{\text{cell}}$$

Although other authors have usually presented these data on a dry-weight basis, the cell weight was found to vary among light-grown and dark-grown cells.

Since the chromatographic performance is dependent on the solvent system used, minor variations in solvent composition can affect retention times. The results for etiolated Euglena appeared qualitatively similar to light-grown cells, but retention times were extended. Linear regression showed the retention times to be equivalent (slope = 0.967; y-intercept = -0.013).

Growth of Astasia pertyi was slow and sporadic, and the low pigment content per cell required larger-scale culture methods than could be employed in this study in order to obtain definitive results.

CHAPTER III

RESULTS

Effects of Culture Media on Growth

Growth curves of Euglena gracilis var. bacillaris and Astasia pertyi in the various liquid media used throughout this study are shown in Figure 1.

Growth of Euglena gracilis cultured in the autotrophic Hutner's "C" medium was sparse (Fig. 1, Curve "A"); maximum density was less than 2×10^5 cells/ml. Mixotrophic growth of Euglena gracilis on Euglena Broth medium under continuous illumination resulted in a cell density of $2.0\text{-}2.5 \times 10^6$ cells/ml within 130 hrs. (Fig. 1, Curve "B"). In both media, Euglena gracilis exhibited a doubling time of ca. 24 hrs. in actively growing cultures.

Curve "C" (Fig. 1) shows the heterotrophic growth pattern of Euglena gracilis grown in Euglena Broth in complete darkness. The maximum density for cells grown heterotrophically was ca. 1.5×10^6 cells/ml. The doubling time under these conditions was 47 hrs. in actively growing cultures.

The growth of Astasia pertyi is shown in Curves "D" and "E" (Fig. 1). Successful growth in Modified Cambridge medium required an inoculation density of 0.5×10^5 cells/ml. A long lag period indicated that the cells had some difficulty in acclimating to this culture at low cell densities. The maximum cell density of A. pertyi achieved in this medium was 0.35×10^6 cells/ml.

Figure 1. Comparative growth of *Euglena gracilis* var. *bacillaris* and *Astasia pertyi* in liquid media.

A. *Euglena gracilis* var. *bacillaris* grown under continuous illumination in Hutner's Medium C.

B. *Euglena gracilis* var. *bacillaris* grown under continuous illumination in Euglena Broth.

C. *Euglena gracilis* var. *bacillaris* grown in darkness in Euglena Broth.

D. *Astasia pertyi* grown in darkness in Modified Cambridge Medium.

E. *Astasia pertyi* grown in darkness in Cambridge Medium.

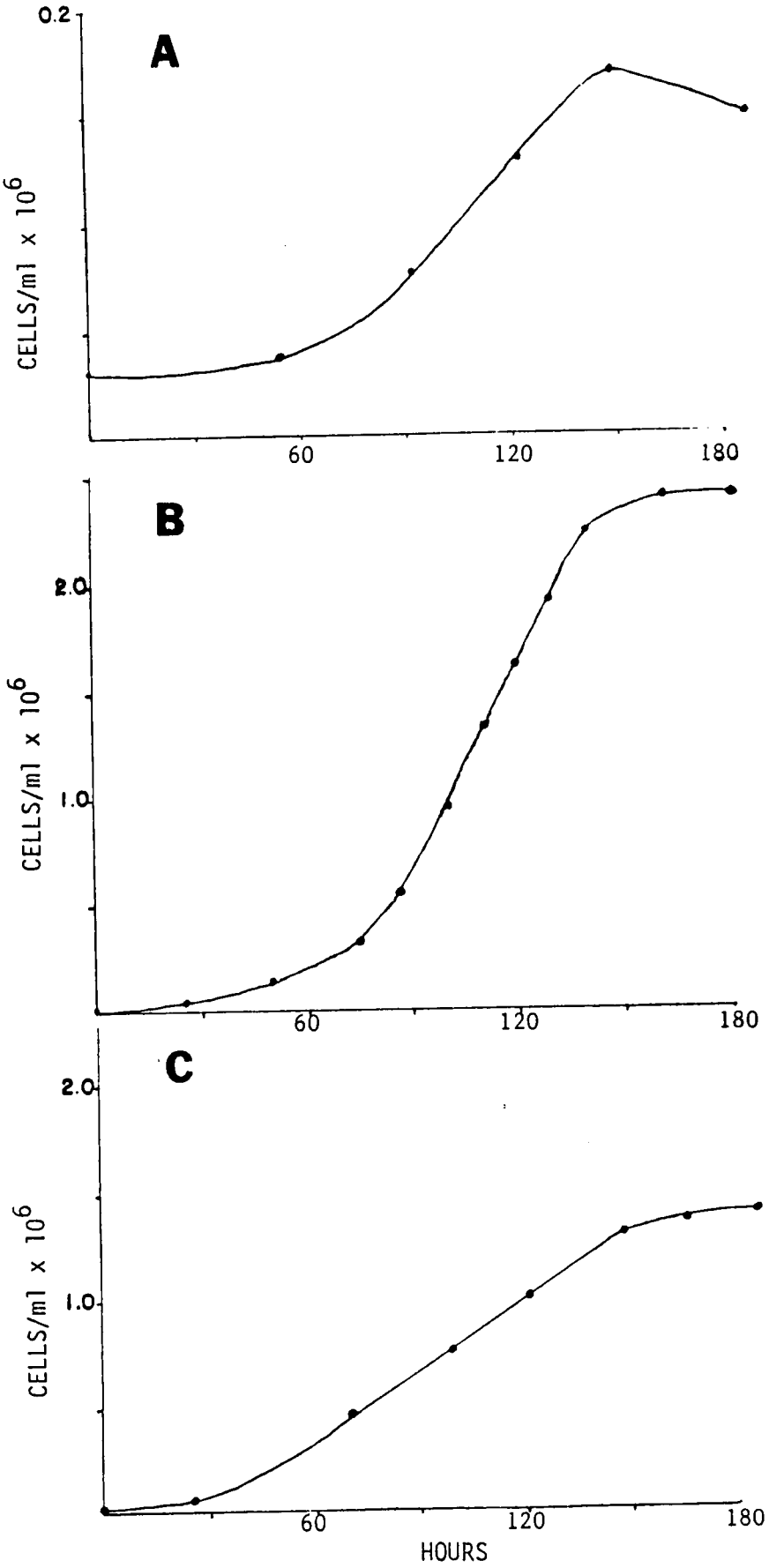
A
0.2
CELLS/ml x 10^6
60
120
180
B
2.0
1.0
CELLS/ml x 10^6
60
120
180
C
2.0
1.0
CELLS/ml x 10^6
60
120
180
HOURS

Figure 1

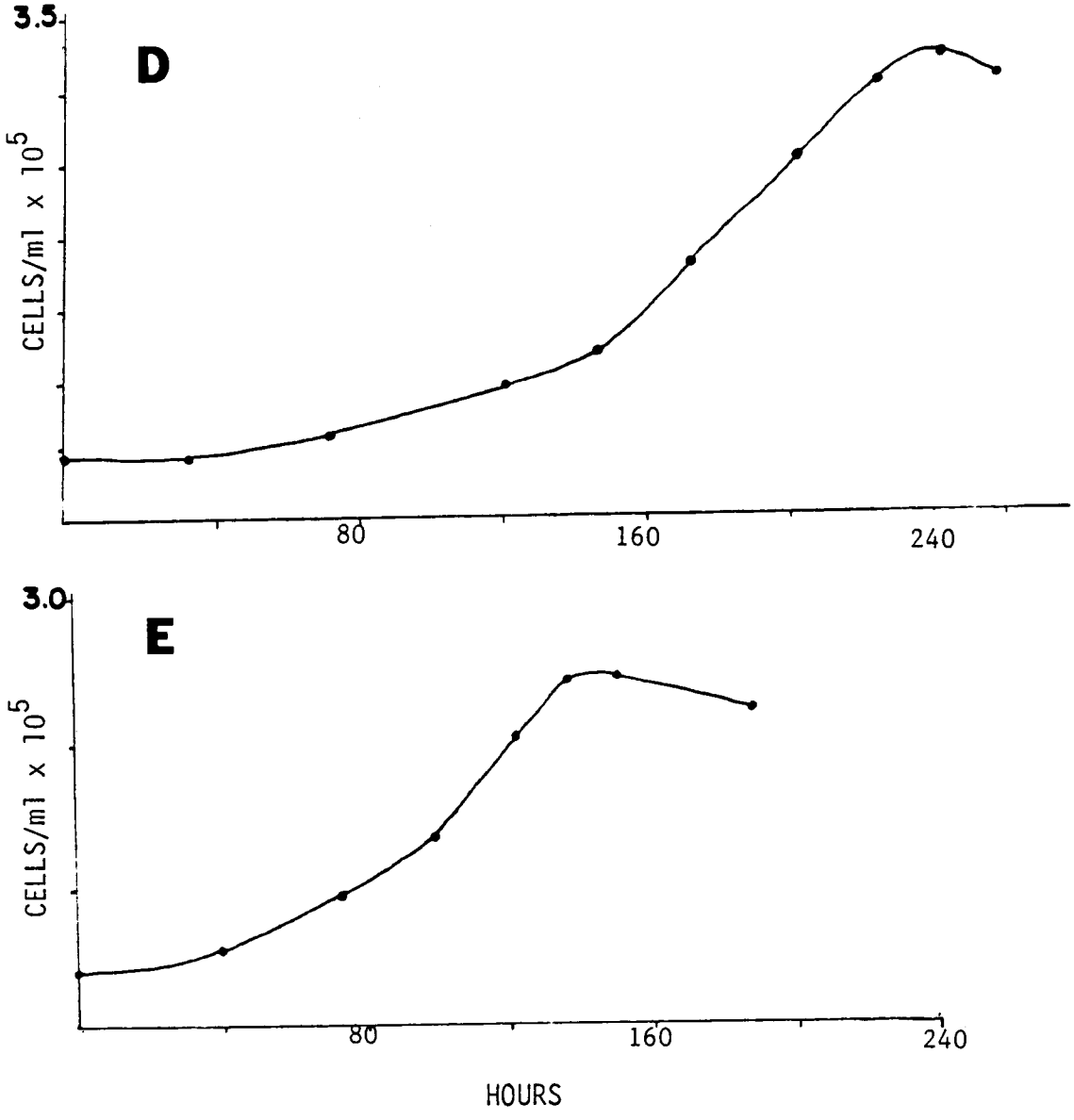

Figure 1 (continued).

When grown in the unmodified Cambridge Medium, A. pertyi achieved maximal growth in less time, but the cultures were also less dense than in the Modified Cambridge Medium.

RP-HPLC of Carotenoid Standards

Column performance with carotenoid standards. The utility of the solvent system developed in this work for separating a variety of carotenoids (in less than 37 minutes) is shown in Figure 2, together with an absorption spectrum for each standard. The three carotenoid standards rendered five separate peaks, labeled peak St A to St F in the order of their elution from the column. Zeaxanthin yielded peaks St A and St B, echinenone yielded peaks St C and St D, and β-carotene yielded peak St F. Peak St E (inset) shows a spectrum of α-carotene obtained "on-line" in other experiments but not shown in Figure 2 (Appendix B).

Table 1 summarizes the data obtained from the carotenoid standards; numerous successive experiments indicated that the results were reproducible. The capacity factor (k') was included, as it is independent of the flow rate. These peaks were also well resolved using a flow rate of 2 ml/min (data not shown). The information included in Table 1 illustrates the difficulty of matching carotenoid spectral properties in 90% methanol, 5% hexane, and 5% tetrahydrofuran, with those reported in the literature.

Figure 2. HPLC chromatograms of carotenoid standards.

Order of Elution	
1	St A - Unknown xanthophyll
2	St B - Zeaxanthin
3	St C - Unknown ketocarotenoid
4	St D - Echinenone
5	St E - α-carotene (inset)
6	St F - β-carotene

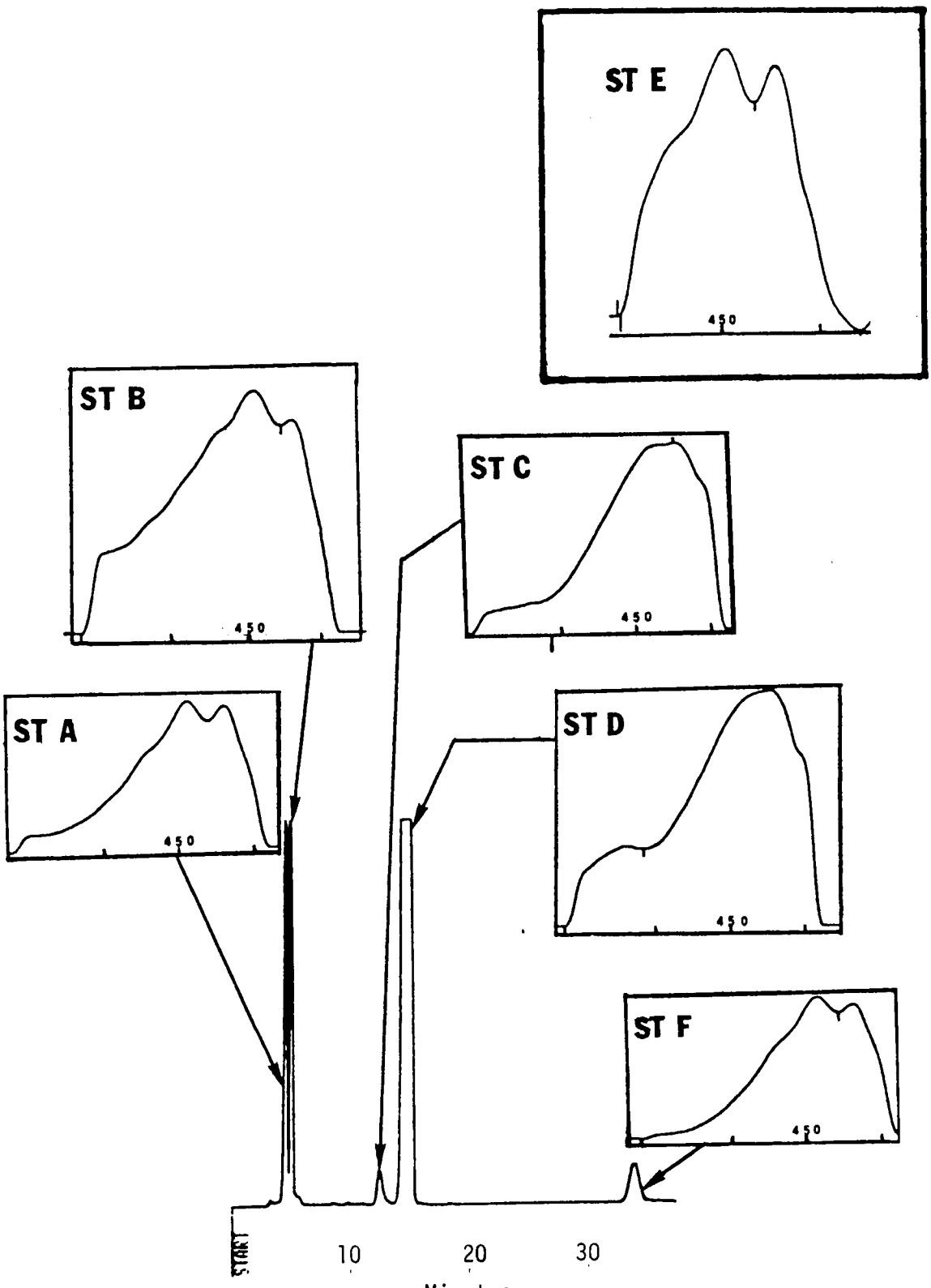
ST E
450
ST B
450
ST C
450
ST A
450
ST D
450
ST F
450
START
10
20
30
Minutes

Figure 2

Table 1. Chromatographic and spectral data for carotenoid standards.

Peak No.	Pigment	Retention Time (Min)	Capacity Factor k'*	Maxima Found (nm)	Maxima Reported **	III/II Found ***	III/II Reported
ST A	Unknown	4.33 ± .07	0.7	429, 458, 483	---	77	--
ST B	Zeaxanthin	4.73 ± .05	0.9	429, 456, 482	434, 456, 485	18.6	26
ST C	Unknown	23.27 ± .02	3.8	466, 481	---	--	--
ST D	Echinenone	14.30 ± .14	4.7	469, 482	480	--	--
ST E	α-carotene	---	---	424, 454, 480	433, 457, 484	70	61
ST F	β-carotene	33.16 ± .14	12.0	431, 458, 482	428, 451, 480	55	26

*k': Capacity factor; $k' = (V_R - V_0)/V_R$, where V_R and V_0 are the eluted volumes of a retained and unretained solute in a given system, respectively (Appendix D).

**In $CHCl_3$ (Davies, 1976).

***III/II indicates the relation between the long wavelength maximum (III) and the absorbance at wavelength maximum II (underlined). Reported values in $CHCl_3$ (Hager and Stransky, 1970).

Chemical treatment of standards. The application of the analytical chemical treatments described in Materials and Methods to the above standards is shown in Figure 3. No epoxides or di-epoxide carotenoids were used as standards, and as expected, treatment with HCl did not alter the retention times of any of the peaks when compared with the untreated sample. The reducing treatments ($NaBH_4$ and $LiAlH_4$) altered both the presumed keto-carotenoid (Fig. 3, St C) and the known ketocarotenoid, echinenone (Fig. 3, St D) as anticipated, resulting in an increase in polarity of this standard due to the introduction of a hydroxyl group and a concomitant decrease in retention time. Figure 3 also shows the superiority of the two-minute $LiAlH_4$ reduction technique over the standard one-hour $NaBH_4$ treatment in reducing echinenone. The data on chemical treatment also indicate a solubility difference between St A and St B, as shown by the relative decrease in St A after chemical treatment and re-extraction into ether with glass-distilled water. The chromatographic behavior of St A, St B, and St F are not modified by these procedures.

The spectral properties of the $LiAlH_4$-modified peaks, C' and D', are presented in Figure 4; although the unmodified spectral properties of St C and St D are similar (Fig. 2, cf. St C, St D; Table 1), the spectra of the modified compounds are quite different (Fig. 4, cf. C', D'). The data shown in Figure 4 are summarized in Table 2.

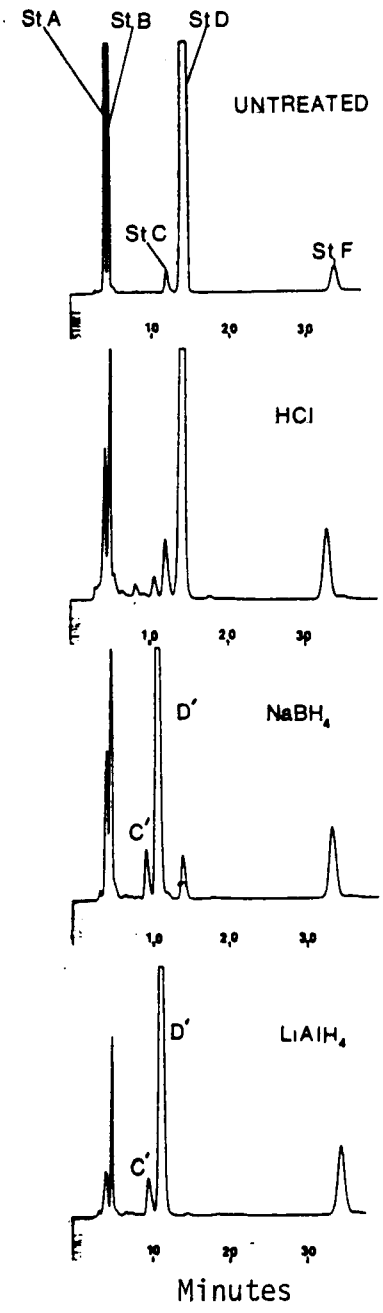

Figure 3. The effect of chemical modification on the retention times of carotenoid standards.

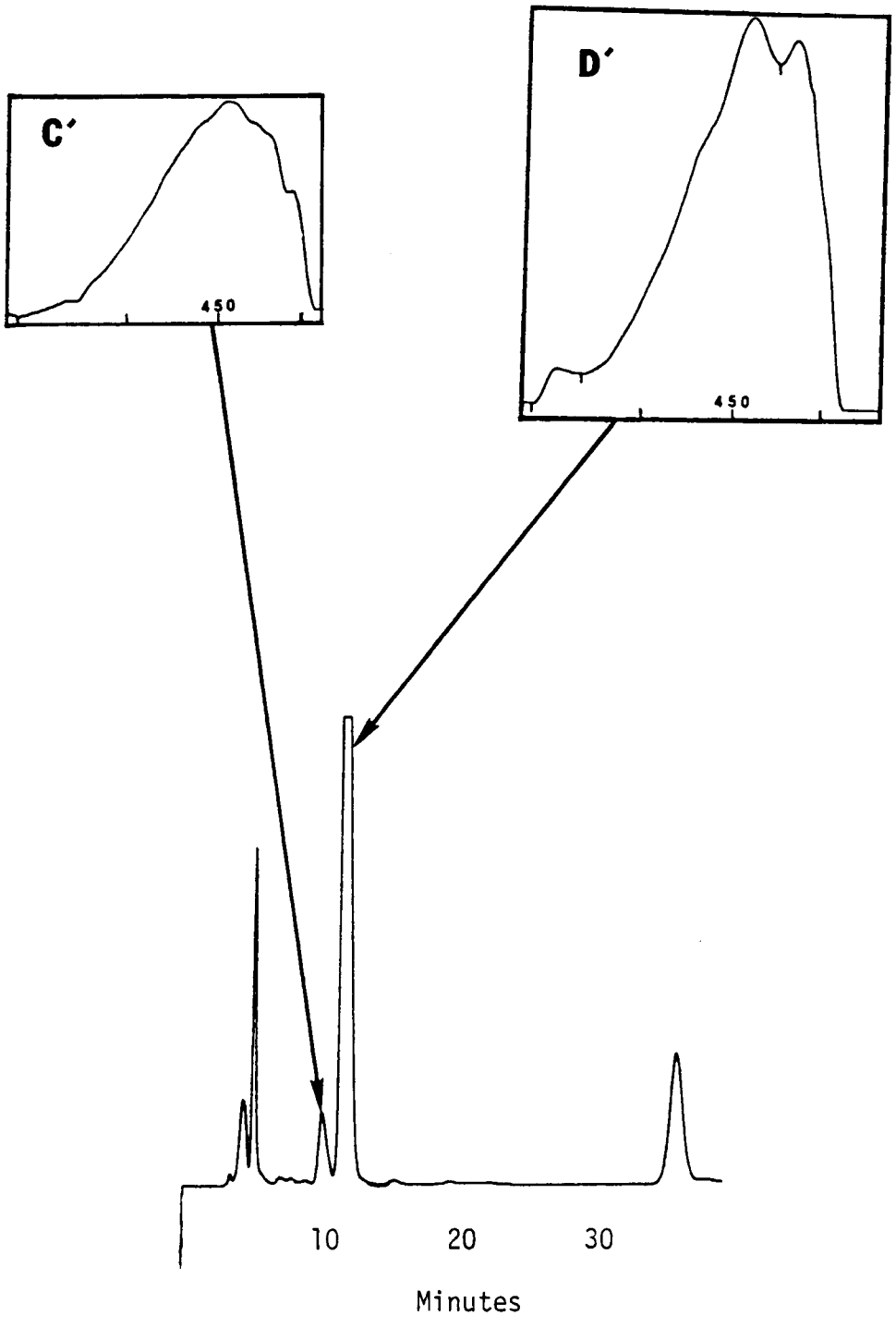

Figure 4. HPLC chromatogram of $LiAlH_4$-modified peaks and their corresponding absorption spectra.

Table 2. Chromatographic and spectral properties of $LiAlH_4$-modified standards.

Peak	Pigment	Retention Time	Capacity Factor	Maxima Found	III/II Found*
C'	Unknown	9.38	2.7	464, 476, 484	--
D'	4-hydroxy, β-carotene	10.85	3.3	430, <u>460</u>, 486	53

*III/II indicates the relation between the long wavelength maximum (III) and the absorbance at wavelength maximum II (underlined).

Dose-response curve. The plotted peak area as a function of the quantity of standard injected is shown in Figure 5. The minimum detectable quantity of each standard compound was 10 pmoles; quantitication of pigment as a function of peak area was possible in the range of 10-500 pmoles (6-350 ng). Beyond this range, the peaks were still well-resolved, and pigment quantities of at least 36 nmole (19 μg) caused only a minor loss in resolution.

RP-HPLC of Pigments from Light-Grown Euglena gracilis var. bacillaris

Untreated extracts. Figure 6 shows the chromatographic analysis of an acetone extract from Euglena gracilis var. bacillaris grown under continuous illumination. Twenty-five peaks, and spectra corresponding to those peaks, are shown in Figure 6. Six components represent over 94% of the recorded extinction (peak area at 445 nm) for extracts from photosynthetically active E. gracilis. [Figure 6: peak 1 (6.2%); peak 2 (72.5%); peak 4 (5%); peak 5 (4.2%); peak 18 (3%); and peak 21 (6%); of these peaks, the first four eluted in less than 11 minutes.]

In Figure 6, peaks 4 and 5 represent chlorophylls b and a, respectively, as shown by their spectra (Fig. 6, cf. peaks 4, 5). The retention time of peak 21 matched that of the β-carotene standard; peaks 1, 2, 3, 22, 23, and 24 exhibited carotenoid spectra. All other peaks for which spectra were obtained were either chlorophyll-like (Fig. 6, cf. peaks 6, 8, 9, 10) or of questionable origin (Fig. 6, cf. peaks 20, 25).

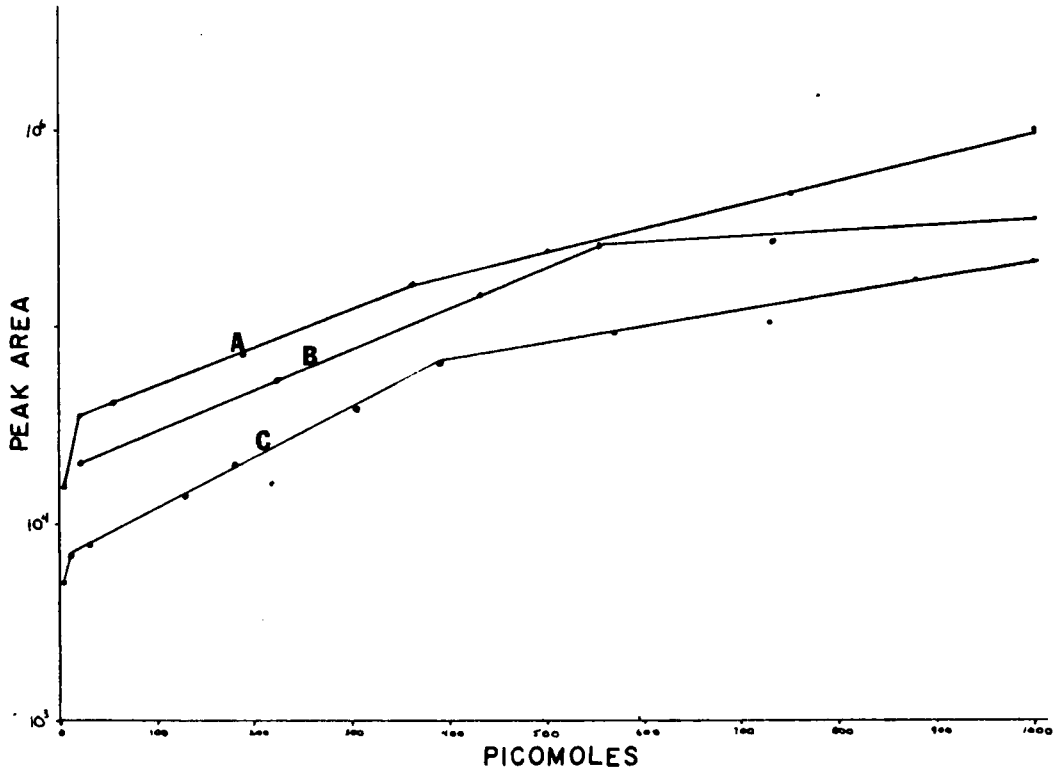

Figure 5. Calibration curve of three carotenoid standards, zeaxanthin (A), echinenone (B), and β-carotene (C). Area is given in arbitrary units as produced by the integrator; area was normalized to $1A_{1cm}$. Detector wavelength was 445 nm.

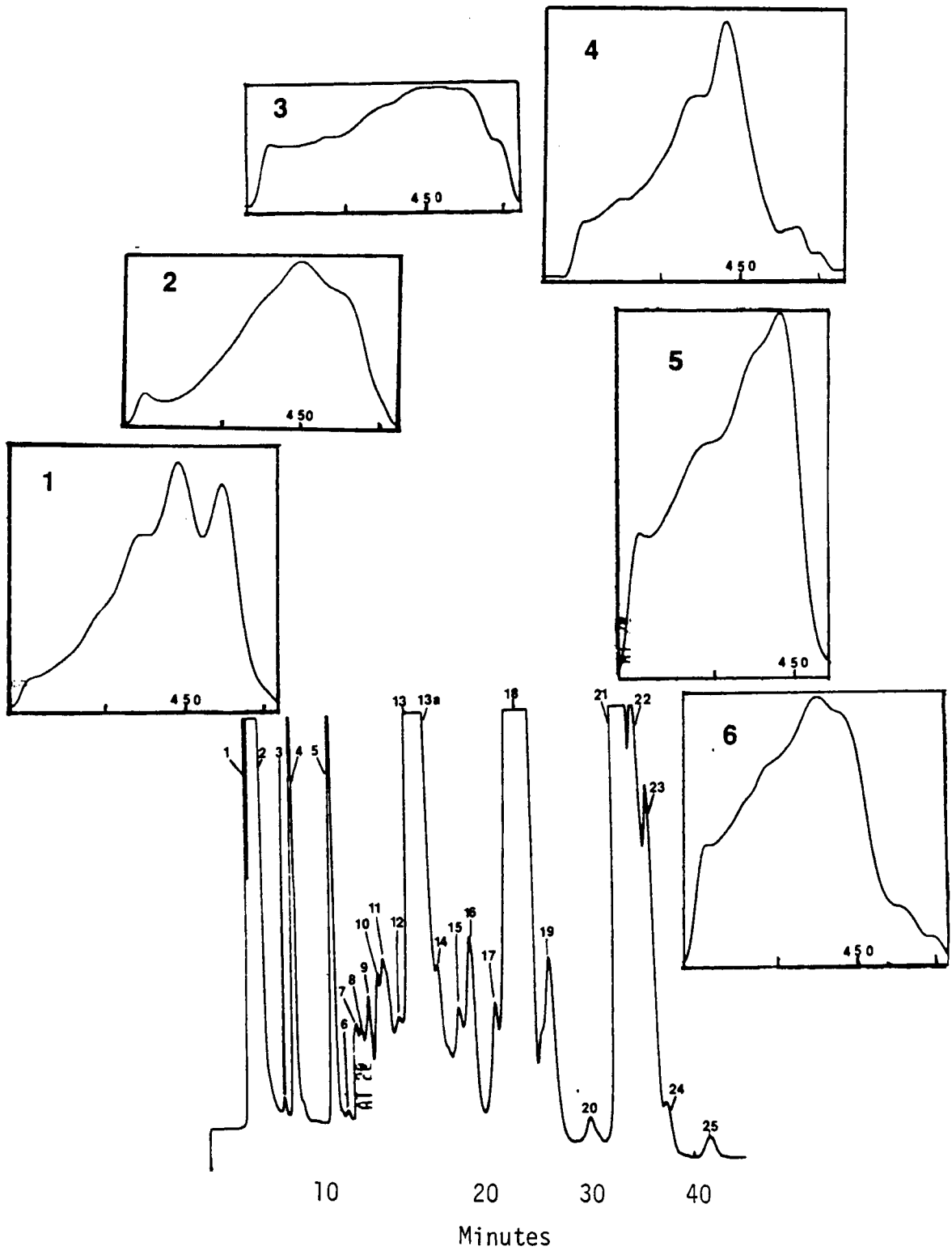

Figure 6. HPLC chromatogram of untreated, whole-cell extracts from Euglena gracilis var. bacillaris grown under continuous illumination. Symbol "2@" marks a sixteen-fold increase in detector sensitivity.

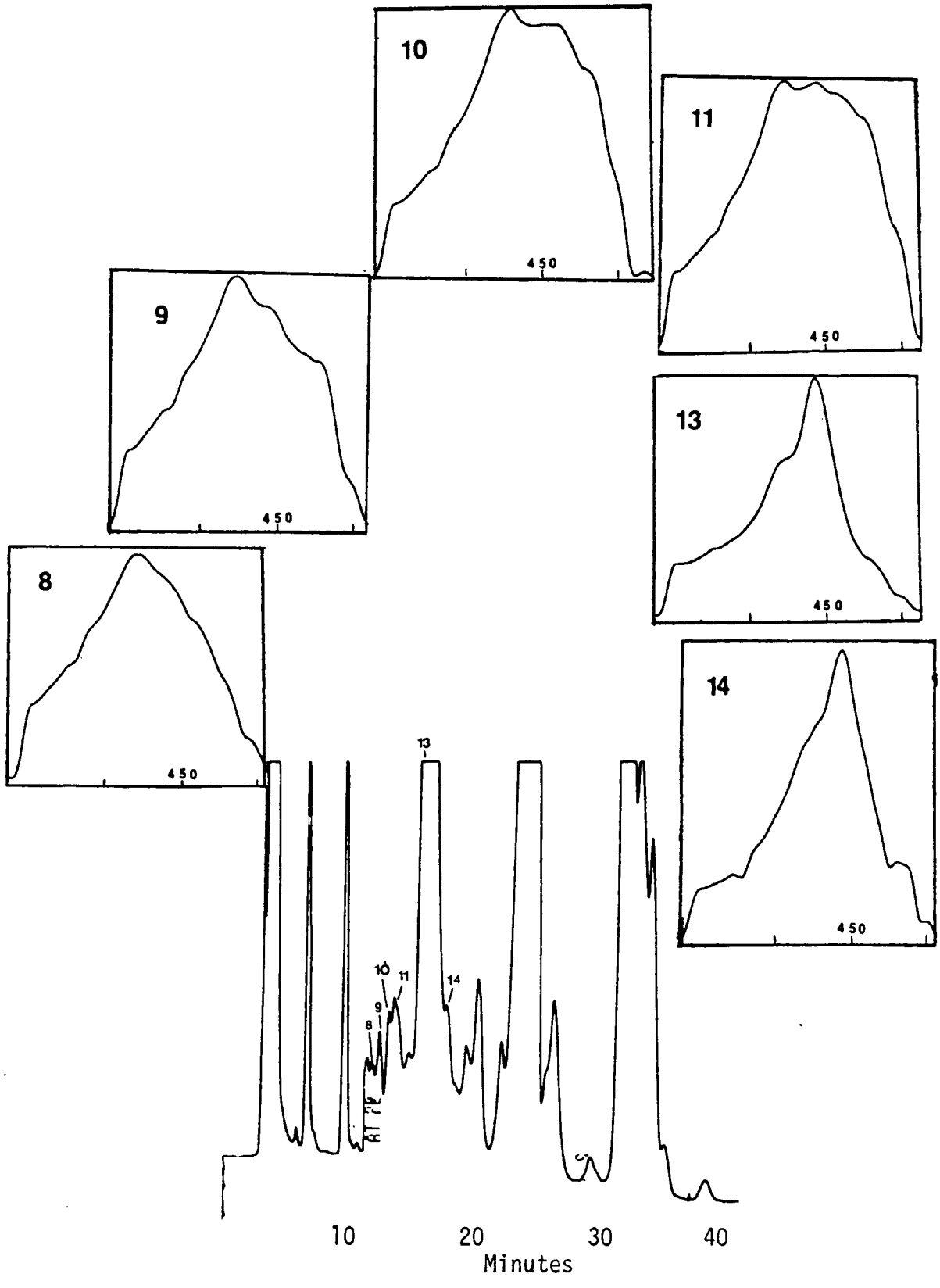

Figure 6 (continued).

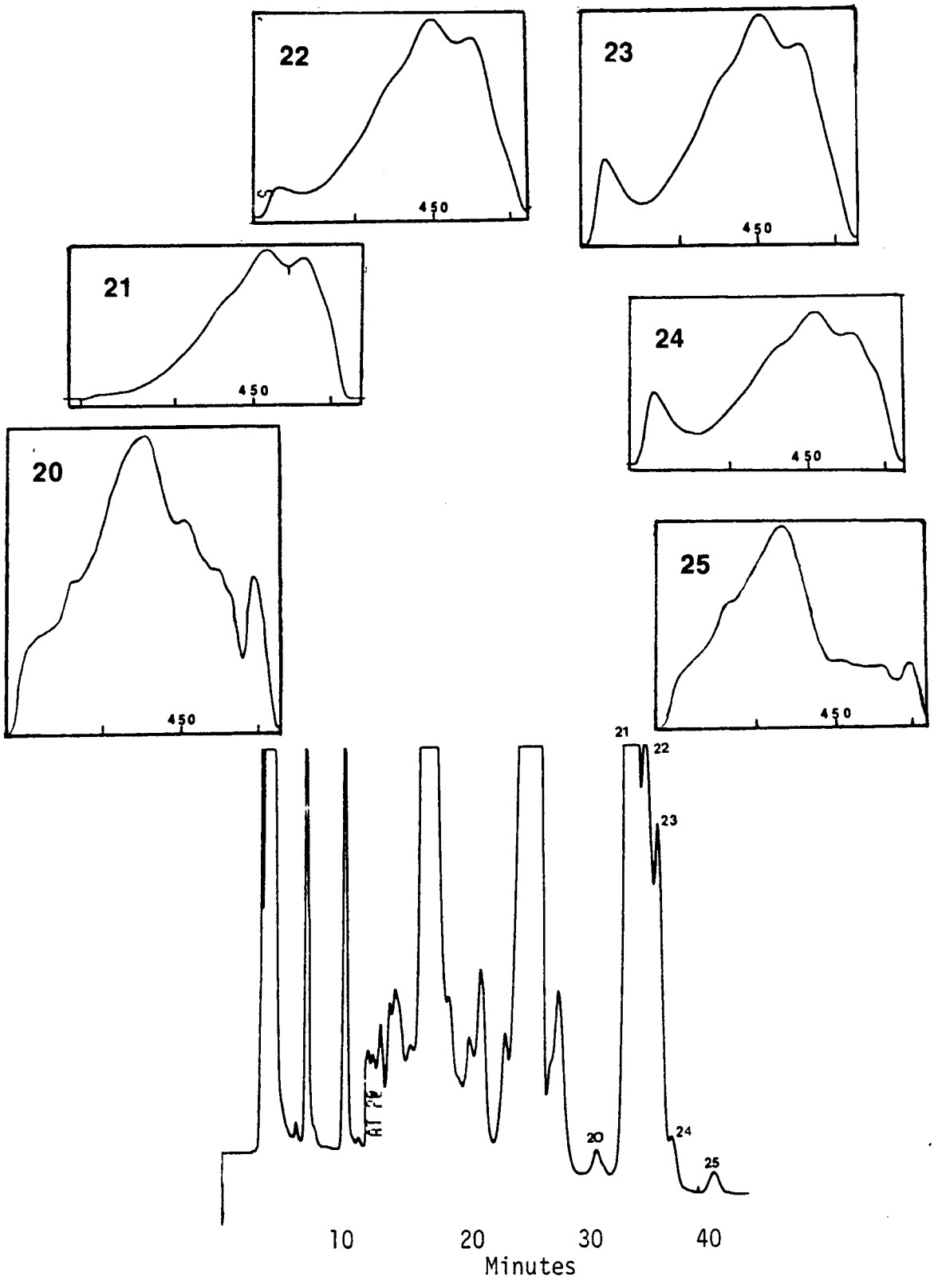

Figure 6 (continued).

Figure 7 shows comparative chromatographic data on the light-grown Euglena scanned at two different wavelengths (445 nm and 480 nm) as a test for the occurrent of di-epoxides. In Figure 7, peaks 13 and 13A show a marked decrease in extinction, but the results are ambiguous. Chlorophyll a shows very little extinction at 480 nm.

Figure 8 was included to show the effect of storage on the extract during the course of the analyses. A decrease in the height of peak 11 and the appearance of a new peak (Fig. 8, peak 2a) were observed. The latter appeared intermittently and was not considered artifactual on the basis of other results. Peak 2a was assigned to zeaxanthin on the basis of similarities to retention times, absorption maxima, and the III/II ratio of the zeaxanthin standard.

Chemical treatment of extracts. Figure 9 summarizes the results obtained by treating whole-cell extracts from Euglena gracilis var. bacillaris with $LiAlH_4$. The reduction of peak 4 (cf. Fig. 9B, Fig. 7) was observed in other chromatograms so treated (data not shown). The $LiAlH_4$ treatment appeared to remove the background pigments that interfered with the acquisition of spectra from peaks 3, 7, 8, and 16 (Fig. 7); these spectra are shown in Figure 9.3, 9.7, 9.8, and 9.16. New peaks resulting from this treatment are indicated in Figure 9 (arrows), and these changes (i.e., the appearance of the new peaks) make the positions of peaks 10 and 11 unclear (Fig. 7, cf. peaks 10, 11).

The results of treating the whole-cell extracts with HCl caused a decrease in the quantities of chlorophylls a and b.

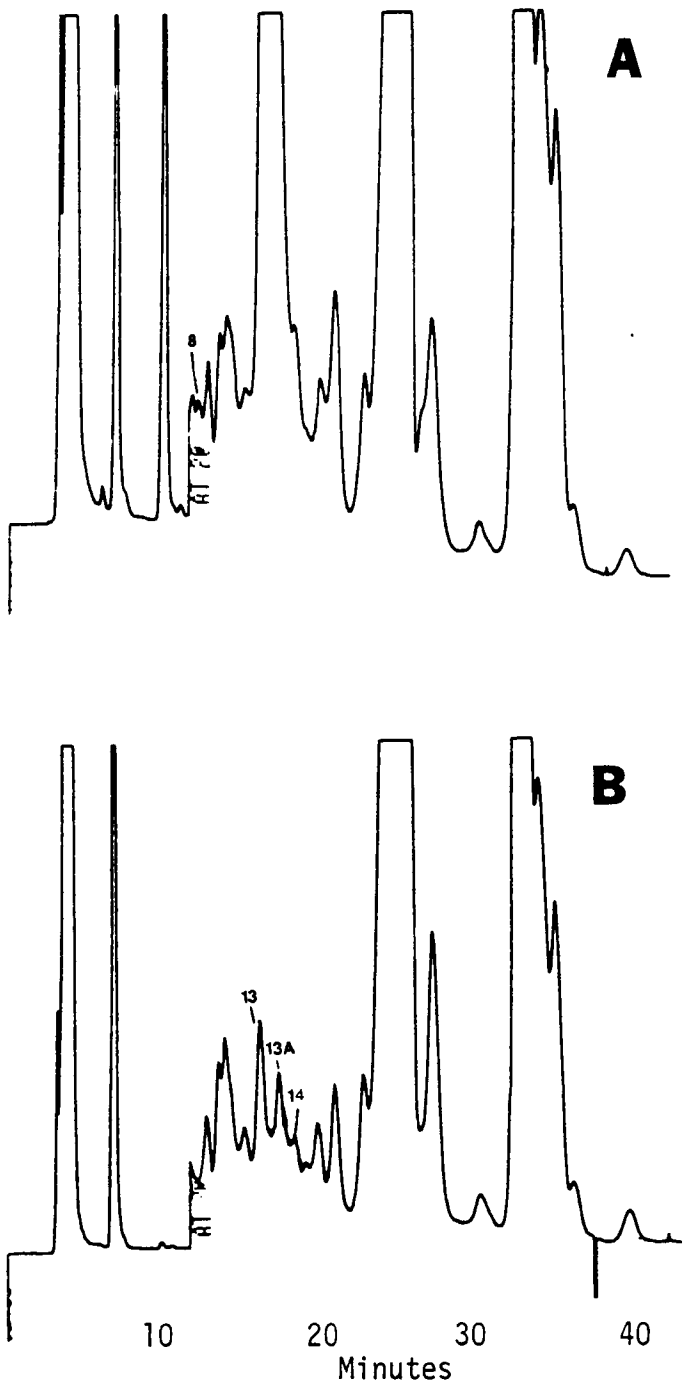

Figure 7. HPLC chromatograms of untreated, whole-cell extracts from Euglena gracilis compared at two detector wavelength settings. A indicates a 445 nm setting, B indicates a 480 nm setting. Symbol "At 2@" marks a sixteen-fold increase in detector sensitivity. Concentrations equivalent.

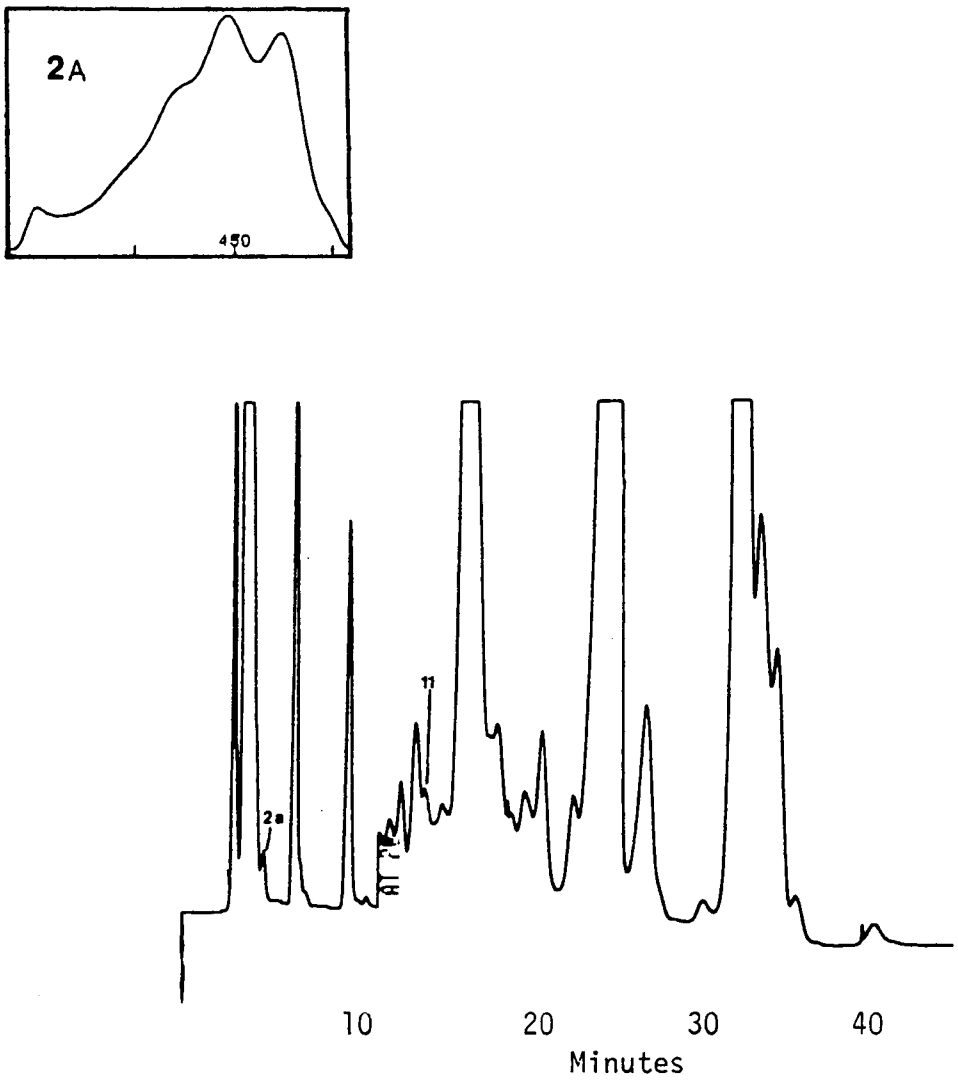

Figure 8. HPLC chromatograms of whole-cell extracts from Euglena gracilis var. bacillaris, grown under continuous illumination, detector setting at 480 nm. Effect of storage (50 hr.). Symbol "At 2@" marks a sixteen-fold increase in detector sensitivity.

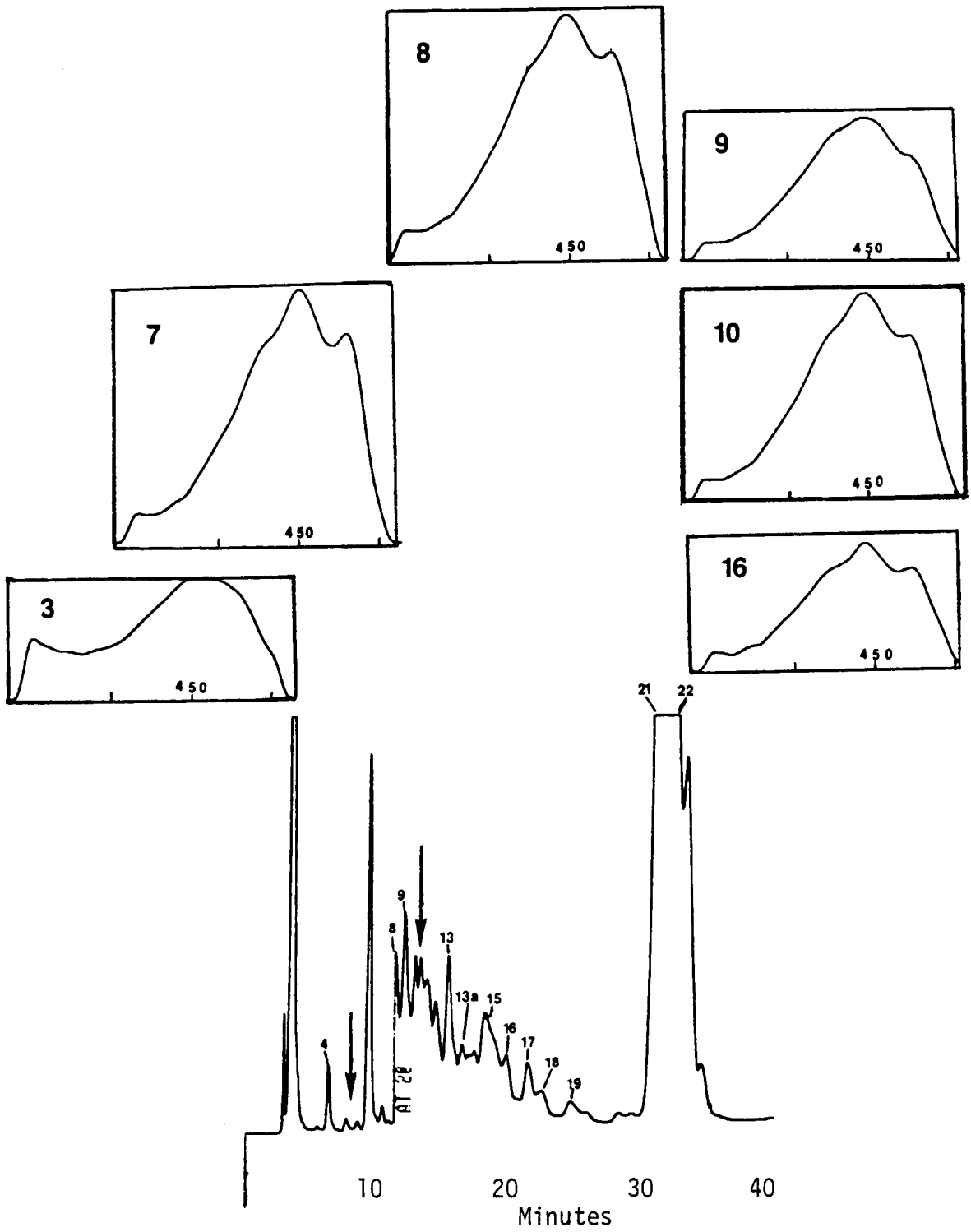

Figure 9. HPLC chromatograms of whole-cell extracts from Euglena gracilis var. bacillaris, grown under continuous illumination, treated with $LiAlH_4$. Symbol "At 2@" marks a sixteen-fold increase in detector sensitivity. Arrow denotes new peaks.

Chlorophyll a also disappeared in one experiment (Figure 10); although the disappearance could not be correlated with any visible changes in the spectra, the ratio of peak 1 to peak 2 changed by 1% (Fig. 7, cf. peaks 1 and 2; see Discussion). Figure 10 also shows a new peak.

RP-HPLC of Pigments from Dark-Grown (Etiolated) Euglena gracilis var. bacillaris

Table 3 presents comparative data on pigments from both light-grown and etiolated cells of Euglena gracilis. The etiolated cells contained ca. 4% of the pigment found in wild-type cells. The carotenoid/chlorophyll ratio was slightly higher (ca. 12%) in the etiolated cells.

Figure 11 shows a HPLC chromatogram obtained from the etiolated cells. The post-chromatographic data indicated that peak 1 (cf. Fig. 7.1) was not present; however, two different retention times (one smaller, one larger) were listed in its place (see Discussion).

Figure 11 is also different from Figure 6 in the region of peaks 12, 13, and 13a. Lack of an interfering pigment (probably chlorophyll) permitted a spectrum to be obtained for peak 13a. In contrast, Figure 11 is similar to Figure 7.B, the untreated extract of light-grown cells scanned at 480 nm. The pigments in 7.B, peaks 13 and 13a, may represent the extinction of carotenoids present in this region after the interfering peak (low extinction at 480 nm) is removed.

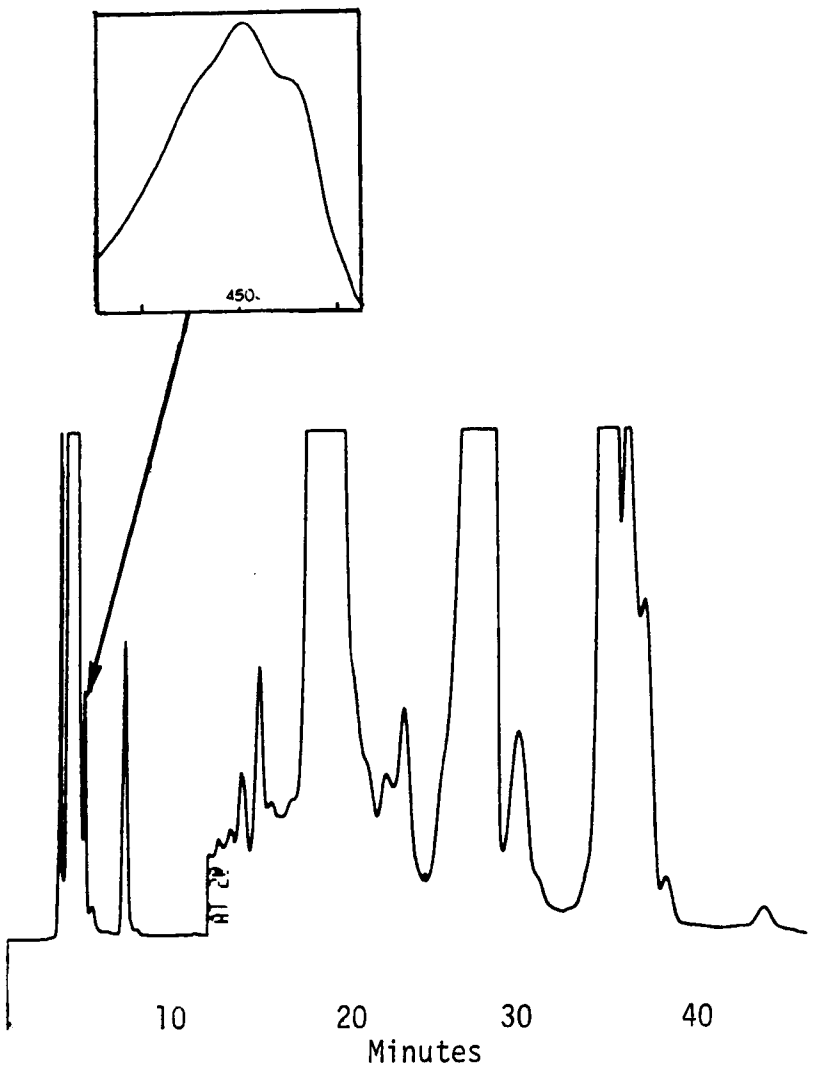

Figure 10. HPLC chromatogram of whole-cell extracts from Euglena gracilis var. bacillaris, grown under continuous illumination, treated with HCl. Symbol "At 2@" marks a sixteen-fold increase in detector sensitivity. Spectra denotes new peak.

Table 3. Comparative bulk properties of light- and dark-grown *Euglena gracilis* var. *bacillaris* and *Astasia pertyi*.

	Light-Grown EGB	Dark-Grown EGB	Dark-Grown AP
Dry Wt. Cell	667 pg	485 pg	576 pg
Total Carotenoid/Cell	0.97 pg	0.045 pg	0.005 pg
Total Chlorophyll/Cell	10.70 pg	0.38 pg	---
Carotenoid/Chlorophyll (%)	9.1	11.9	---

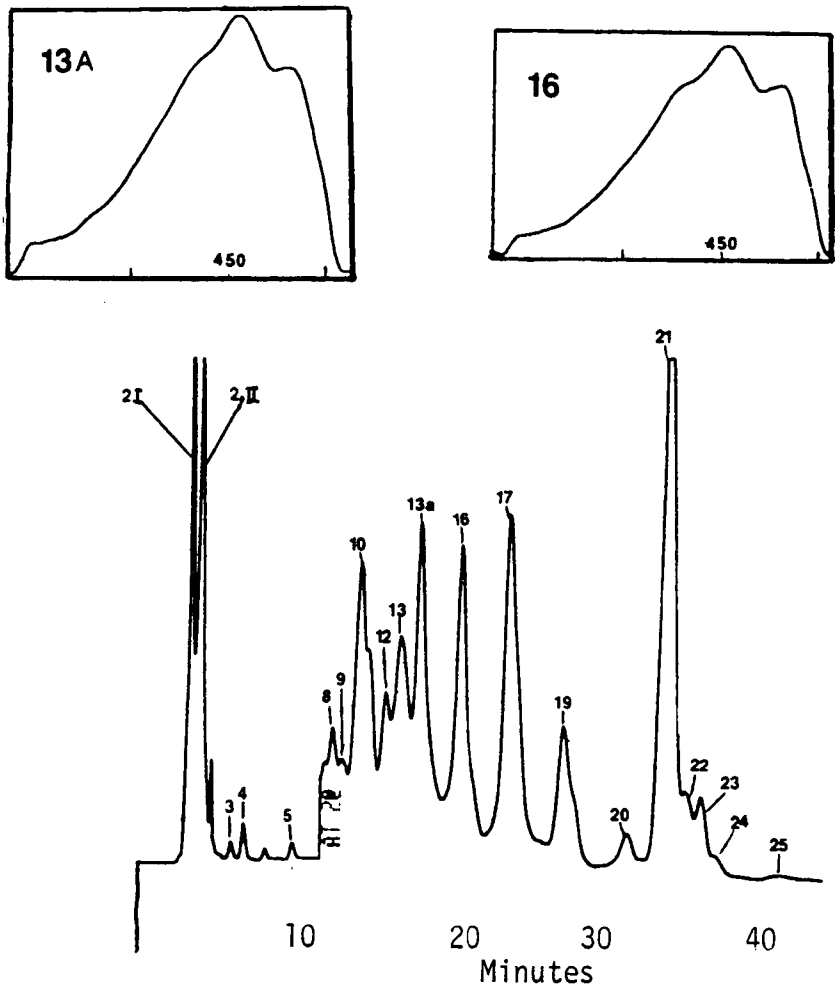

Figure 11. HPLC chromatogram of untreated, whole-cell extracts from dark-grown (etiolated) Euglena gracilis var. bacillaris. Symbol "At 2@" marks a sixteen-fold increase in detector sensitivity.

Table 4 summarizes the chromatographic and spectral data obtained from both the etiolated and light-grown Euglena cells.

RP-HPLC of Pigments from Dark-Grown Astasia pertyi

Data on the pigment properties of Astasia pertyi are compared with those from light-grown and etiolated Euglena gracilis in Table 3.

Figure 12 and Table 5 present preliminary results obtained from A. pertyi. Peaks R and T have the same height-to-width ratios and separation times as α- and β-carotene (data not shown), but were too dilute to permit spectra to be obtained. Peak B exhibited the broad absorption band of a ketocarotenoid, but had a lower retention time than did peak 1, Figure 6 (cf. Fig. 2, St D and Discussion). None of the peaks could be correlated by retention time or spectral properties with those peaks for which spectra were obtained. No data regarding chemical modification of whole-cell extracts were collected.

Table 4. Comparative HPLC chromatographic and spectral properties of dark- and light-grown Euglena gracilis var. bacillaris.

Light-Grown				Dark-Grown				
Peak No.	Retention Time	Maxima Found	III/II	Peak No.	Corrected* Retention Time	Maxima Found	III/II	Relative % (Lt./Drk.)**
1	3.10	392, 426, 446, 475	70.6	1	3.10	--	--	1
				2D I	3.30	{DI:DII::6:1}	--	--
2	3.7	--	--	2D II	3.89		--	--
2A	4.72	426, 455, 480	64.4	2A	--	--	--	14
3	5.91	352, 458	N.A.	3	5.90	--	--	76
4	6.78	376,425,445	N.A.	4	6.68	--	--	4
5	9.93	400, 430, 446	N.A.	5	9.81	--	--	3
7	11.82	430, 456, 483	14.5	7	11.78	--	--	51
8	12.25	430, 450, 483	15.7	8	12.24	--	--	100
9	12.84	456, 482	N.A.	9	12.85	--	--	33
10	13.64	427, 450, 480	5.7	10	13.91	--	--	200
11	14.08	--	--	11	14.43	--	--	38
12	15.35	--	--	12	15.55	--	--	98
13	15.92	--	--	13	16.50	--	--	--
13A	17.00	--	--	13A	17.60	430, 458, 483	16.3	--
16	21.15	431, 454, 483	17	16	21.70	431, 454, 483	16	100
21	33.4	431, 459, 484	54	21	33.30	--	--	11
22	34.57	424, 452, 478	25	22	34.41	--	--	10
23	35.59	425, 453, 478	15.6	23	35.48	--	--	122
24	36.71	429, 457, 482	19	24	--	--	--	--

*Linear regression was used to correct retention times. Scope = .967; Y - intercept = -0.013.

**Based on peak area/cell at λ 445.

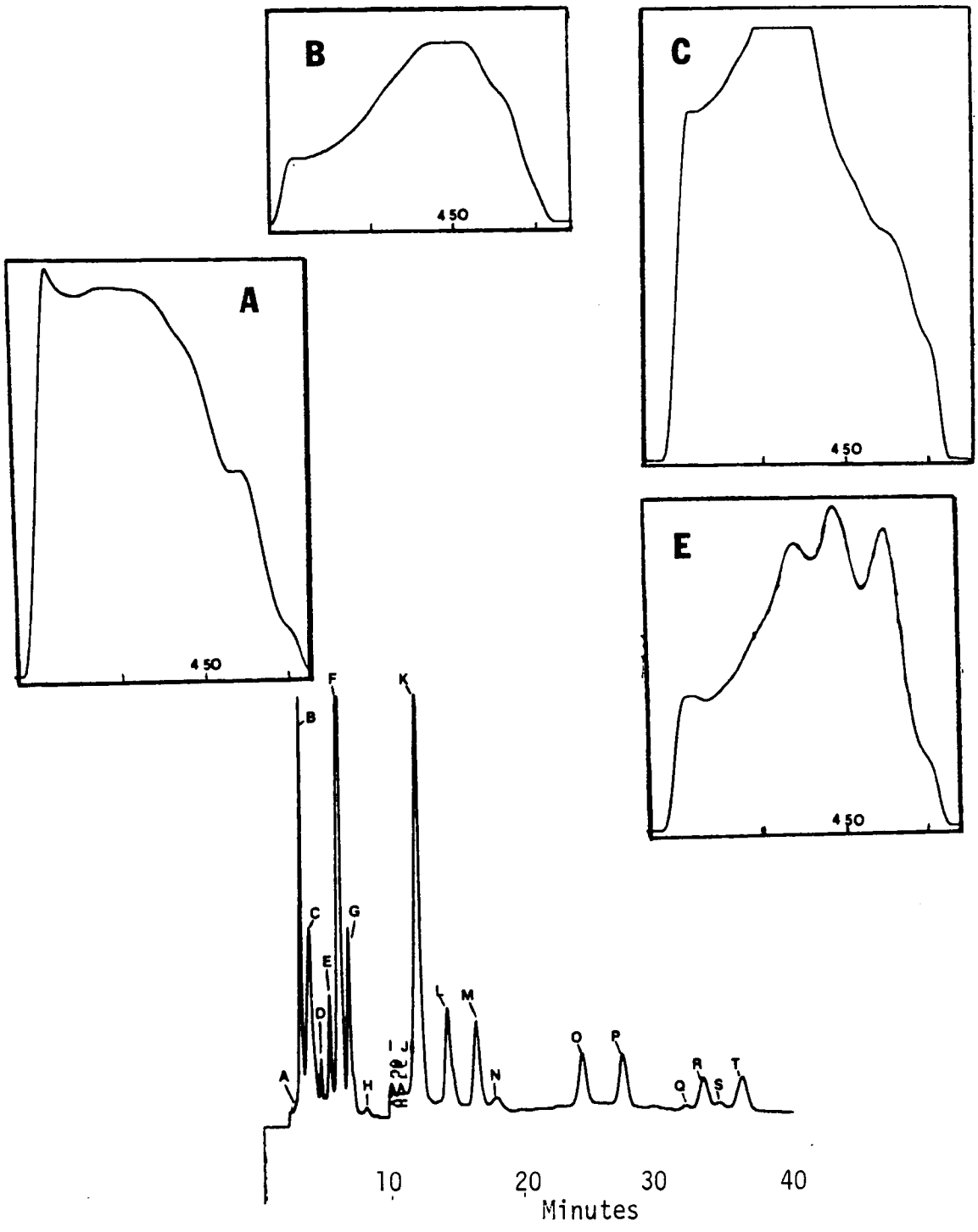

Figure 12. HPLC chromatogram of untreated, whole-cell extracts from Astasia pertyi grown in darkness. Symbol "At 2@" marks a four-fold increase in detector sensitivity.

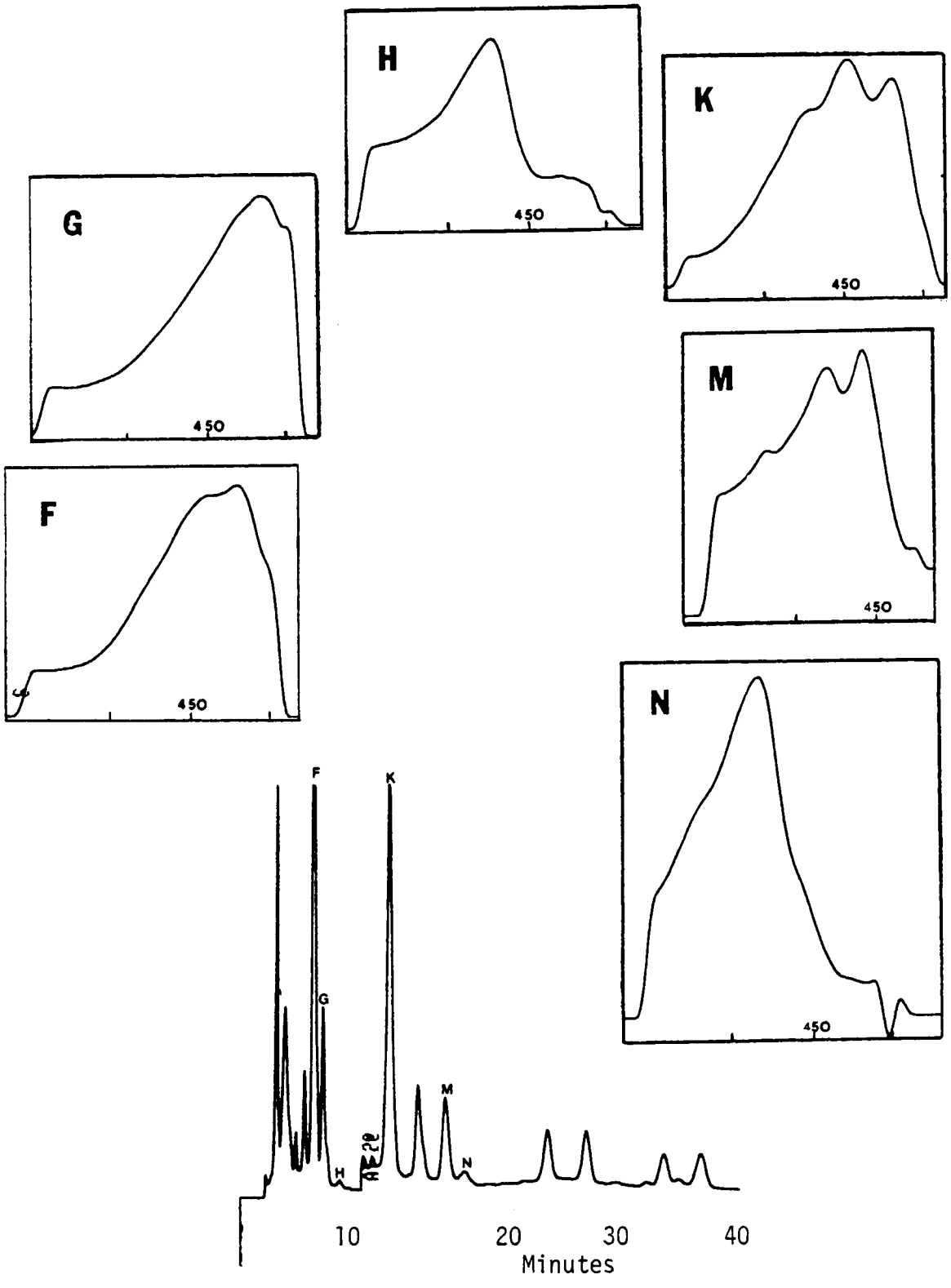

Figure 12 (continued).

Table 5. HPLC chromatographic and spectral properties of *Astasia pertyi*.

Peak. No.	Retention Time	Maxima Found	III/II	Relative Amount Per Cell
A	--	--	--	--
B	2.7	458, 484	N.A.	4
C	3.4	--	--	54
D	4.3	--	--	<1
E	4.9	424, 448, 476	71.2	2
F	5.6	462, 482	N.A.	20
G	6.4	485	N.A.	<5
H	7.8	423	N.A.	<1
I	9.7	--	--	<1
J	10.4	--	--	1
K	11.6	428, 452, 481	57	4
L	14.0	--	--	1
M	16.2	388, 422, 444	N.A.	1
N	17.8	420	N.A.	<1
O	24.5	--	--	<1
P	27.6	--	--	<1
Q	32.4	--	--	<1
R	33.8	--	--	<1
S	35.0	--	--	<1
T	36.8	--	--	<1

CHAPTER IV

DISCUSSION

Significance of Chromatographic Techniques Used in This Study

A new chromatographic system for the analysis of complex mixtures of carotenoids was developed in this study, representing the second report of the use of NARP-HPLC; this is the first application of NARP-HPLC to whole-cell extracts from a photosynthetic organism and the first comparative report on the pigment composition of related organisms.

Since the technology of plant pigment separation using RP-HPLC is still in the phase of rapid development (see Nelis and De Leenheer, 1983), much of the methodology employed in this study was developed during the course of the investigation.

Preliminary studies indicated the necessity of using a very hydrophobic stationary phase (22% carbon loading) to effect separation of complex mixtures of carotenoids. Similar extracts have been examined previously on highly retentive columns (Braumann and Grimme, 1981; Ben-Amotz et al., 1982), but the investigators used water gradients and a 10-μm diameter support material. The use of 5-μm support material in this work doubles the number of theoretical plates (vs. 10 μm) (Krstulović and Brown, 1982), yet the size and hydrophobicity of the material decrease the column's capacity to accept water gradients due to excessive back-pressures (Krstulović and Brown, 1982).

Previous studies have relied on the use of co-elution of standards as the sole criterion for the identification of unknowns (Ben-Amotz et al., 1982; Nelis and De Leenheer, 1983). This is a probable although not absolute criterion for identification. Braumann and Grimme (1981) collected eluted fractions and quantified their pigment data using absorption spectroscopy. The present work employed carotenoid standards and explored the feasibility of "on-line" absorption spectroscopy, as well as the correlation of adsorption characteristics to carotenoid structure. These adsorption characteristics are reciprocal to the results obtained with reversed-phase systems (Krstulović and Brown, 1982) and are supported by the data presented here and elsewhere (Braumann and Grimme, 1981; Nelis and De Leenheer, 1983).

The Carotenoids of Light-Grown Euglena gracilis var. bacillaris

The data presented for light-grown Euglena gracilis confirm the presence of carotene and zeaxanthin in these cells, as reported previously (Goodwin and Jamikorn, 1954; Krinsky and Goldsmith, 1960), and suggest also the presence of at least five other pigments: neoxanthin, diadinoxanthin, diatoxanthin, chlorophyll a, and chlorophyll b, as reported by Aitzetmüller et al., 1968; Hager and Stransky, 1970; Johannes et al., 1971; and Bjørnland, 1982.

Peak 1, the least retained of the pigments, had the highest III/II ratio and the shortest wavelength maximum of the major peaks; it also had a relative concentration of ca. 6% (at 445 nm). These results suggest a greater number of oxygen substitutions

and a shorter chromophore than the later-eluting zeaxanthin (Moss and Weedon, 1976). In comparison, the related genus, Eutreptiella, has neoxanthin as its most retained pigment; it, too, was present in a relative concentration of ca. 6%, and had a similar III/II ratio and absorption maxima as peak 1. Thus, peak 1 of Euglena in this work is probably neoxanthin.

Peak 2 contained ca. 70% of the total peak area. Its absorption spectrum and III/II ratio differ from both diadinoxanthin and diatoxanthin, both of which have been reported in Euglena (Aitzetmüller et al., 1968; Hager and Stransky, 1970) and in Eutreptilla (Bjørnland, 1982); these pigments would be expected to elute between zeaxanthin and neoxanthin on the basis of adsorption affinities (Moss and Weedon, 1976; Davies, 1976). Peak 2 is further discussed in relation to etiolated Euglena gracilis later in the Discussion.

Peak 3 exhibits the broad absorption band indicative of a ketocarotenoid, but it elutes much earlier than the echinenone standard; its wavelength maximum is shifted substantially from echinenone; these results suggest a di- or trisubstituted keto-carotenoid with a shortened chromophore. Other ketocarotenoids reported for Euglena (Goodwin and Gross, 1959) are ruled out on the basis of their chromophore length (see Hager and Stransky, 1970, for absorption maxima, and Davies, 1976 for absorption maximum vs. chromophore length). Neither could peak 3 be assigned to heteroxanthin, a ketocarotenoid reported in Euglena (Nitsche,

1973) since its structure would indicate a retention time less than the zeaxanthin standard (peak 2A).

Bjørnland (1982) showed the presence of an unknown keto-carotenoid ester in Eutreptiella, and Heelis et al. (1979) reported a similar compound in the stigma of E. gracilis St "Z". The effect of esterification on retention times of carotenoids has not been examined to date; therefore, no correlation with peak 3 can be made at this time.

Since neoxanthin would be expected to undergo epoxide-furanoid rearrangement upon treatment with HCl, the new peak following such treatment was not unexpected, but it cannot be correlated with neoxanthin (see discussion regarding diadinoxanthin in etiolated Euglena).

The Carotenoids of Etiolated Euglena gracilis var. bacillaris

The results obtained from untreated extracts of etiolated cells illustrate three important points regarding the major pigments, including a clarification of the nature of peak 2 in chromatograms from light-grown cells.

First, the HPLC chromatographic data confirm earlier reports of reduced neoxanthin content in etiolated cells as compared to light-grown Euglena gracilis (Krinsky et al., 1964; Vaisberg and Schiff, 1976). Vaisberg and Schiff (1976) reported that this reduction is correlated with the de-differentiation of the plastids in etiolated cells.

Second, the data suggest that peak 2 of the light-grown cells is, in reality, composed of two peaks, which are designated diadinoxanthin (Fig. 11, peak 2.I; 70% of total peak area at 445 nm) and diatoxanthin (Fig. 11, peak 2.II; 12% of total peak area) known to be present in etiolated cells (Gross et al., 1975) and eluting prior to zeaxanthin (Fig. 11, peak 2A; Davies, 1976).

Bjørnland reported the percentages of diadinoxanthin to diatoxanthin to be 18% and 30%, respectively, for light-grown Eutreptiella, and this reversal in relative quantities in etiolated Euglena probably reflects the light-catalyzed, enzyme-mediated de-epoxidation of diadinoxanthin to diatoxanthin known as the xanthophyll cycle (Goodwin, 1980).

Third, the chromatographic results obtained for etiolated cells confirm the existence of only quantitative differences in the pigments of etiolated and light-grown cells (Goodwin and Jamikorn, 1954; Gross et al., 1975) and disagree with Vaisberg and Schiff (1976) regarding the absence of chlorophyll in etiolated cells; our results suggest that ca. 4% of the total chlorophyll in light-grown cells is maintained in the etiolated line. However, this may reflect a brief exposure to light or a light-leak in the growth chamber in which these cells were grown.

Comparative Data: Etiolated vs. Light-Grown Euglena

The total amounts of carotenoid per gram dry weight in both etiolated and light-grown Euglena are less than reported values (Goodwin and Jamikorn, 1954) and may reflect methodological

differences in the determination of dry weight. On a per cell basis, the etiolated cells have 4.6% of the carotenoid present in light-grown cells, a result in agreement with Kirk and Tilney-Bassett (1967). However, for comparative purposes, the data indicate that it is not the total carotenoid content per cell which should be compared, but rather the relative concentrations of the individual pigments.

The data presented in Tables III and IV (pp. 34 and 37) comparing etiolated and light-grown Euglena pigments suggest that although total carotenoid per cell is greatly reduced, several of the minor peaks from etiolated cells either changed only slightly or were present in greater amounts than in light-grown cells (peaks 3, 8, 10, 12, 16, and 23). Since the reactions controlling carotenoid production are enzyme-mediated, the results may suggest that light, or light-mediated reactions, are suppressing/inducing carotenoid synthesis. Alternatively, the data may reflect branch points in biochemical pathways.

The Carotenoids of Dark-Grown Astasia pertyi: A Preliminary Report

Astasia pertyi belongs to a group of euglenoids classified in the subgenus or genus Khawkinea (see e.g., Jahn and McKibben, 1937; Pringsheim, 1956; and Leedale, 1967, for further discussion of this confusing taxonomic controversy), a group representing those non-photosynthetic Euglena-like protists which possess a pigmented stigma.

Astasia ocellata (another Khawkinea type) has been reported to contain seven carotenoids (Thomas et al., 1967): α-carotene, β-carotene, echinenone, 4-keto-α-carotene, canthaxanthin, and two additional unidentified ketocarotenoids.

The present work represents the first enumeration of the carotenoid composition of A. pertyi. Nineteen peaks were resolved, and of the ten for which spectra were obtained, six were definitely carotenoid. Of these six absorption spectra, three had substantial fine-structure, results in conflict with those of Thomas et al., (1967) who found only ketocarotenoids (which lack fine-structure).

In addition, it is noteworthy that no spectra or retention times from the A. pertyi data could be correlated with those from either etiolated or light-grown E. gracilis (see following discussions).

Carotenoid Biochemical Systematics and Euglenoid Phylogeny

"Despite the vast inventory of carotenoids identified in protists, only a handful have proven useful in constructing phylogenies and taxonomies" (Ragan and Chapman, 1982). The techniques developed here and elsewhere (Braumann and Grimme, 1981; Nelis and De Leenheer, 1983) should provide a stronger basis on which to develop taxonomies based on carotenoid composition: first, by clarifying the distribution of carotenoids which appear sporadically and/or in low concentrations (e.g., α-carotene) (Goodwin, 1980) through the increased sensitivity of procedures used in this study; second, by permitting the separation of very similar carotenoids

in a reproducible manner (e.g., α- and β-analogs); and third, by identifying those carotenoids which may appear or disappear as a consequence of physiological factors such as nutrition (Goodwin, 1980).

As mentioned earlier, the euglenoids pose some particularly interesting and difficult evolutionary problems in that photosynthetic, heterotrophic, and phagotrophic organisms all occur with the same group. Leedale (1978) proposed the following evolutionary scheme for euglenoids: *Eutreptiella* $\xrightarrow{1}$ *Euglena* $\xrightarrow{2}$ *Khawkinea* $\xrightarrow{3}$ *Astasia* with the events as follows: 1--loss of a flagellum; 2--secondary loss of the chloroplast; 3--loss of the stigma. This scheme does not address the absence, in *E. gracilis*, of the enzyme responsible for synthesis of α-carotene (results presented here and Vaisberg and Schiff, 1976) compared to the presence of α-carotene in both *Eutreptiella* (Bjørnland, 1982) and *Astasia ocellata* (Thomas et al., 1967). It may be that the enzyme (α-cyclase) responsible for ε-ring synthesis is repressed in light-grown *Euglena*.

Furthermore, the carotenoid composition of *Astasia pertyi*, based on preliminary studies, differs substantially from *Astasia ocellata* (Thomas et al., 1967) and may suggest that these two organisms are more removed from *Euglena* than proposed by Leedale (1967). The complete absence of carotenoids from *Astasia longa* (non-*Khawkinea*-type) (Gross et al., 1955) further complicates Leedale's proposal (Leedale, 1978).

An alternative evolutionary hypothesis, which would better fit the carotenoid profiles, is the multiple evolution of the monoflagellated green and colorless euglenoids from a common phagotrophic, biflagellated ancestor. This scheme would also eliminate the need for postulating the "secondary loss" of an organelle (e.g., plastid: Euglena→Khawkinea) or the complete loss of carotenoid-synthesizing machinery (Khawkinea→Astasia). It must be reiterated, however, that these suggestions are based on preliminary results, and Astasia ocellata and A. longa, together with A. pertyi, must be further characterized using the modern procedures developed in this work and elsewhere (Nelis and De Leenheer, 1983) before the above suggestions can be seriously considered.

The Site of Carotenoid Synthesis

The presence of carotenoids in A. ocellata (Thomas et al., 1967), A. pertyi (this work), and numerous other euglenoids (e.g., bleached Euglena gracilis) that lack plastids but retain stigmata (Kivic and Vesk, 1974a,b) would argue against any absolute requirement for the presence of plastids in carotenoid synthesis. Clearly, however, plastid presence can affect the types and amounts of carotenoids present (this work; Goodwin and Jamikorn, 1954; Gross et al., 1975; Vaisberg and Schiff, 1976; Steinitz et al., 1981).

Margulis (1981) postulated that, in Euglena, neoxanthin and β-carotene are under control of plastid genes, while zeaxanthin and antheraxanthin are under control of nuclear genes. The results obtained thus far for euglenoids do not support this view since

β-carotene is present in the aplastidic Astasias, and although neoxanthin appears to fluctuate with plastid de-differentiation and re-differentiation, zeaxanthin does so to a lesser degree (see Results). Similarly, the synthesis of C-4 substituted ketocarotenoids is a non-plastid event (Goodwin, 1980), but the site of synthesis is not known.

The control of carotenoid synthesis during re-greening has been examined through the use of differential inhibitors of cytoplasmic and orgenellar ribosomes (Evans, 1971; Kivic and Vesk, 1972; Neumann and Parthier, 1973; and Kronestadt and Walles, 1975). A proposal for further research may involve the use of these techniques, as well as inhibitors of respiration and photosynthesis and labeled substrates for carotenoid synthesis, to determine the site(s) of synthesis of specific carotenoids.

CHAPTER V

SUMMARY

The results presented have confirmed the presence of neoxanthin and zeaxanthin in both light-grown and etiolated Euglena gracilis and support the earlier suggestions that diadinoxanthin and diatoxanthin are also major pigments. Additionally, the results confirm that etiolated Euglena cells have a carotenoid content which differs only quantitatively from the light-grown Euglena. The contention that no chlorophyll is present in Euglena cells cultured for long periods in the dark is questionable, since this work has shown that ca. 4% of the pigment is maintained in the etiolated cell line compared to light-grown cells.

Although the above results suggest that alterations in carotenoid synthesis do exist and appear to be related to the presence of actively photosynthetic plastids, it was not possible to determine specifically which carotenoids are affected.

The preliminary results obtained from Astasia pertyi are too different from Euglena and other related organisms to suggest thata definite carotenoid complement is associated with the stigma. Greater quantities of test material for this organism are needed for reliable pigment determinations. In both Euglena and Astasia, comparison of experimental material with a larger number of standards is essential for precise pigment identification in future studies.

Finally, through application of the suggested refinements in technique, the methods presented here should contribute significantly to detailing the distribution of a wide variety of "minor" carotenoids present in organisms, and may also be instrumental in the reconsideration of these molecules as more reliable biochemical taxonomic markers. Furthermore, this technique may find application in many areas where carotenoid analysis is important.

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APPENDIXES

APPENDIX A

MEDIA

1. Euglena Broth

To rehydrate suspend 8.7 g of powder in 1000 ml glass-distilled water and heat to boiling. Sterilize in autoclave for 15 min. at 15 psi (121°C).

L-Glutamic Acid	5.0 g
DL-Malic Acid	2.0 g
Boric Acid	1.14 g
Thiamine Hydrochloride	1.0 mg
Vitamin B_{12}	0.2 µg
Ammonium Phosphate	0.2 g
Monopotassium Phosphate	0.4 g
Calcium Carbonate	0.5 g
Magnesium Sulfate	0.5 g
Ferric Chloride	24.0 mg
Zinc Sulfate	26.4 mg
Manganese Sulfate	12.4 mg
Ferrous Ammonium Sulfate	14.0 mg
Cobalt Sulfate	2.4 mg
Cupric Sulfate	0.4 mg
Ammonium Molybdate	0.36 mg
Sodium Vanadate	0.37 mg

Reference: Greenblatt and Schiff (1959).

2. Hutner's Euglena Medium C Modified by Deleting Carbon Sources

Dissolve 0.78 g Hutner's Dry Mix in glass-distilled water and bring to a final volume of 1000 ml. Add 50 pg of Vitamin B_{12}. Autoclave at 15 psi (121°C) for 20 min.

Hutner's Dry Mix

Monopotassium Phosphate	3.0 g
Magnesium Sulfate	4.0 g
Thiamine Hydrochloride	0.6 g
*Metals 45 Mix	0.22 g

*Metals 45 Mix

Ferrous Ammonium Sulfate	14.0 g
Zinc Sulfate	4.4 g
Manganese Sulfate	1.55 g
Cupric Sulfate	0.31 g
Cobalt Sulfate	0.48 g
Boric Acid	0.57 g
Ammonium Molybdate	0.64 g
Sodium Vanadate	0.093 g

Reference: Hutner and Bach (1956)

3. Modified Cambridge Medium

Dissolve the following components in 1000 ml of glass-distilled water, adjust to pH 5.1 using 1N HCL. Autoclave at 15 psi (121°C) for 25 min.

Sodium Acetate	2 g
Bacto Tryptone	30 g
Difco Yeast Extract	2 g
Potassium Nitrite	0.5 g
Monopotassium Phosphate	1.5 g
Magnesium Sulfate	0.1 g
Calcium Chloride	0.01 g
Ferric Chloride	trace
Vitamin B_{12}	0.05 µg

Reference: Von Dach (1940)

4. Cambridge Medium

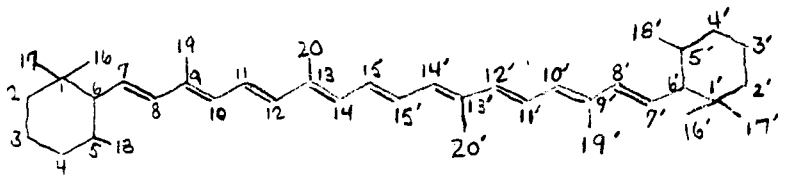
Dissolve the following components in 1000 ml of glass-distilled water. Adjust pH to 5.1 with 1N HCl. Autoclave at 15 psi (121°C) for 25 min.

Sodium Acetate	1 g
Beef Extract (Difco)	1 g
Bacto Tryptone (Difco)	2 g
Yeast Extract (Difco)	2 g
Calcium Chloride	0.01 g
Vitamin B_{12}	0.05 µg

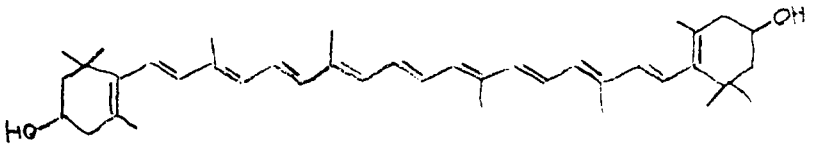
Reference: Starr (1974)

APPENDIX B

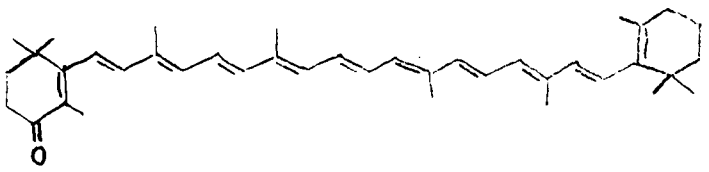
STRUCTURE OF CAROTENOID PIGMENTS MENTIONED IN TEXT



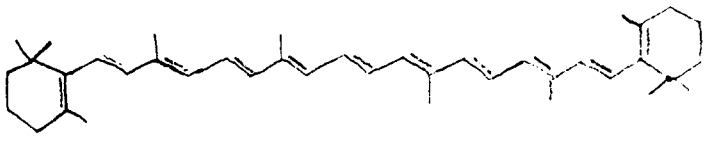
The Hydrocarbon Skeleton



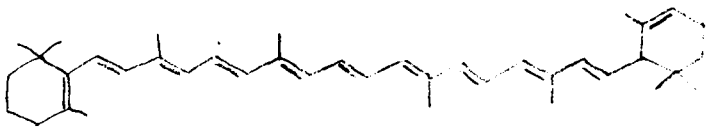
1. Zeaxanthin: β,β-carotene-3,3'-diol



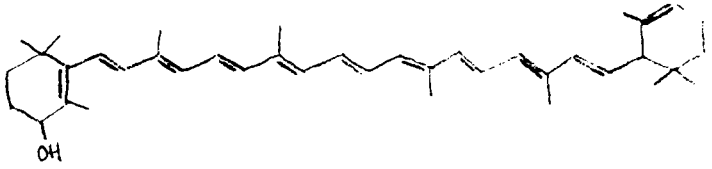
2. Echinenone: β,β-carotene-4-one



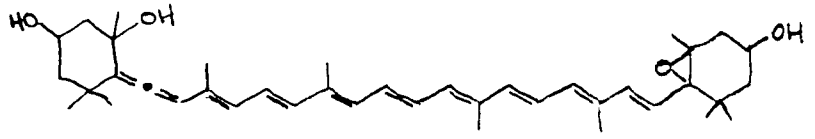
3. β-Carotene: β,β-carotene



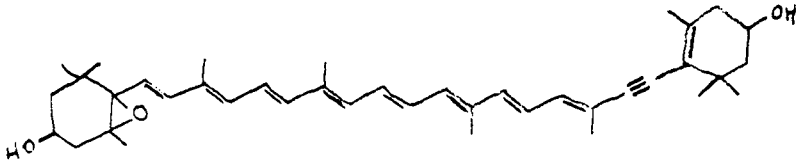
4. α-Carotene: β,ε-carotene



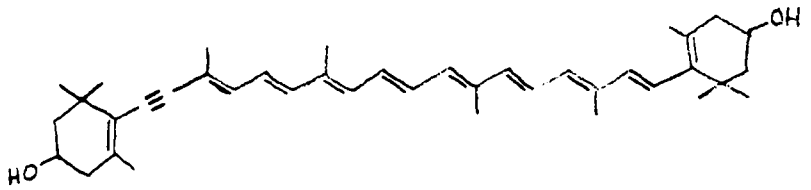
5. 4-Hydroxy-α-carotene



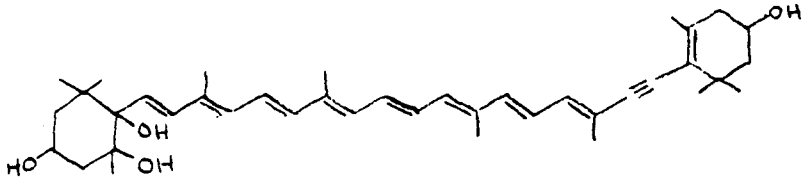
6. Neoxanthin: 5',6'-epoxy-6,7-didehydro-5,6,5',6' tetrahydro-β,β-carotene-3,5,3'-triol



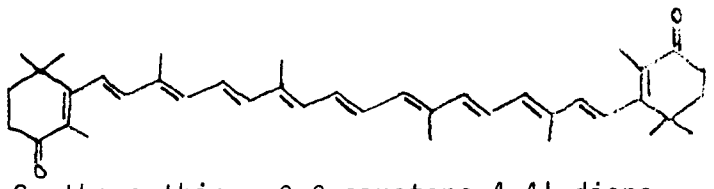
Diadinoxanthin: 5,6-epoxy-7',8'-didehydro-β,β-carotene-3,3'-diol



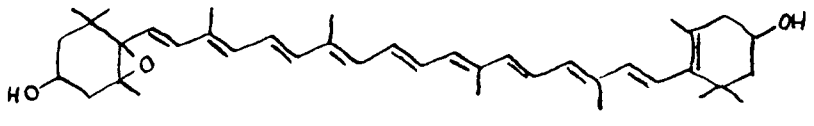
7. Diatoxanthin: 7,8-didehydro-β,β-carotene-3,3'-diol



9. Heteroxanthin: 7',8'-didehydro-5,6-didehydro-β,β-carotene-3,5,6,5'-tetrol



10. Canthaxanthin: β,β-carotene-4,4'-dione



11. Antheraxanthin: 3,3'-dihydroxy-5,6-epoxy-β-carotene

APPENDIX C

ADDITIONAL CHROMATOGRAPHIC PROCEDURES

Final Sample Storage

Pigments should be suspended in hexane instead of chloroform since the latter may contain trace impurities of HCl (Davies, 1976).

Removal of Chlorophylls and the Concentration of Minor Peaks

Saponification of the whole-cell extracts would effectively remove the chlorophylls, as well as any steroids present in the extracts (Davies, 1976). This technique, however, does not address the problems of the wide variation in concentrations of the minor carotenoids and the major carotenoids active in the photosynthetic apparatus.

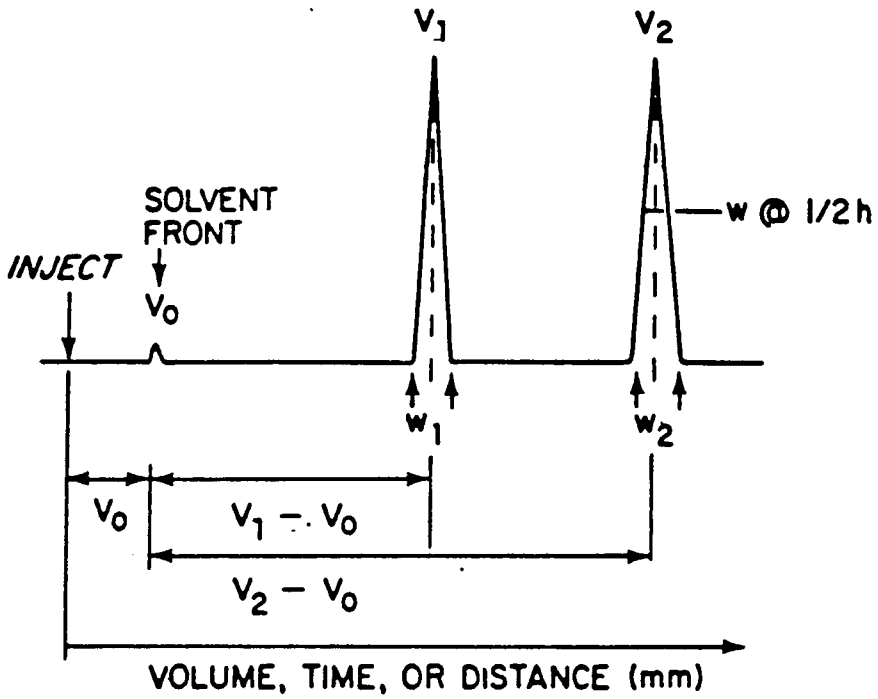
The partition of carotenoids of different solubilities between two immiscible solvents prior to chromatography can be used to remove chlorophylls (Hager and Stransky, 1970), but this technique does not effect 100% separation of the different fractions.

These problems can be circumvented by using the "preparative" capabilities of the column and solvent system described in this work, as well as the high flow rates permitted with NARP-HPLC. This would allow the separation of three fractions (early xanthophyll, chlorophyll, later xanthophylls and carotenes) in less than 17 minutes. The minor fraction would

be concentrated over numerous runs and re-chromatographed with a flow rate of 1.5 ml/min (time:27 min). Column equilibration with 100% methanol for 5 minutes would be preceded by chromatography of the early xanthophyll fraction with this solvent system.

APPENDIX D

SOME HPLC CHROMATOGRAPHIC PARAMETERS PERTAINING TO THIS STUDY



THEORETICAL PLATES(N) = $16\left(\frac{V}{W}\right)^2$ or $5.5\left(\frac{V}{w\ @\ \frac{1}{2}h}\right)^2$

HEIGHT EQUIVALENT TO THEORETICAL PLATE(HETP) = $\frac{\text{COL. LENGTH(mm)}}{N}$

RESOLUTION (R_s) = $\frac{V_2 - V_1}{\frac{1}{2}(w_2 + w_1)}$

CAPACITY FACTOR (k') = $\frac{V_1 - V_o}{V_o}$ and $\frac{V_2 - V_o}{V_o}$

SEPARATION FACTOR(α) = $\frac{k'(V_2)}{k'(V_1)}$

Reference: Perkin-Elmer (1981).

VITA

Kenneth Monar was born on March 18, 1956 to Paul V. and Jeanette A. Monar. He attended Blessed Sacrament Grammar School in Margate, New Jersey and graduated Holy Spirit High School in 1974. The following September he entered Boston University and in 1978 was awarded a Bachelor of Arts degree with a major in Biology and a minor in Chemistry. The summer of 1978 was spent in the Experimental Marine Botany Course at the Marine Biological Laboratory, Woods Hole, Massachusetts. In September, 1979, he entered Graduate School in the Department of Botany of The University of Tennessee, Knoxville, where he has held a Graduate Assistantship and Graduate Teaching Assistantship. He completed his studies in March, 1984 and was awarded a Master's degree in Botany.