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Norman I. Bishop

The photosynthetic activities, the pigmentation and the chloroplast membrane polypeptides of several mutants of the green alga, Scenedesmus obliquus, with complete or limited deficiencies in the light-harvesting complex of photosystem II are characterized and compared to those of the normal phenotype.. The principal phenotype examined in this study, K0-9, behaves similarly to the WT Scenedesmus in that it grows autotrophically, mixotrophically and heterotrophically; cells grown under these various conditions retain near normal photosynthetic capacity without alteration of either photosystem I or photosystem II activities. Also, the associated chloroplast membrane polypeptides, as well as the polypeptides of the apoproteins of the LHC-II, appear identical to those of the WT. However, absorption spectra analyses and gel electrophoresis studies revealed that the mutant lacks the major light-harvesting complex, LHCP-II (as shown in "green" gels) and the corresponding chlorophyll a and chlorophyll b of the light-harvesting complex.

Examination of total pigment extracts of WT and K0-9 by HPLC revealed several obvious differences. The most apparent ones noted were the absence of chlorophyll b and the associated decreased level of chlorophyll a, a decreased level of neoxanthin and an increased concentration of violaxanthin in extracts of K0-9. The level of these two xanthophylls appear to show an inverse relationship with neoxanthin decreasing by approximately 70% and violaxanthin increasing by a comparable amount. This noted relationship suggests that violaxanthin, a presumed precursor to neoxanthin, undergoes only limited conversion in K0-9. Previous mutant studies on chlorophyll b-deficient phenotypes of algae and higher plants also noted decreased levels of neoxanthin and in vitro reconstitution studies with the apoproteins of LHC-II showed that rebinding of chlorophyll b required various xanthophylls, specifically neoxanthin, also to be present.

The other light-harvesting complex mutants of Scenedesmus examined in this study, including LF-1-LHC, LF-15 hf-17-LHC, CP-13-LHC, PS-28-LHC and LF-23-LHC, exhibit similar general deficiencies of chlorophyll b, lack the green bands of the LHCP-II but retain the apoproteins of this complex and show the same relationship between neoxanthin and violaxanthin as noted for K0-9. Even though these strains were derived from mutant phenotypes already suffering major deletions in other parts of the photosynthetic apparatus, their inabilities to synthesize or to accumulate chlorophyll b show the same common factor as noted for K0-9, i.e., the apparent accumulation of violaxanthin and decrease of neoxanthin. This suggests that the mutual site of mutation in these various phenotypes may reside at the point of conversion of violaxanthin to neoxanthin. This interpretation supports the concept that the precursor(s)

to chlorophyll b must be initially bound near to or at the site within the chloroplast membrane where it will eventually function in the LHCP.

Examination by detergent polyacrylamide gel electrophoresis (PAGE) of the light-harvesting complexes of isolated chloroplast fragments of the several phenotypes employed in this investigation confirmed that the inability to synthesize chlorophyll b was paralleled by the loss of the highly pigmented LHCP-II. However, in no case was it observed that the synthesis of the apoproteins of LHC-II, or their binding to the chloroplast membrane, was affected by the mutation causing chlorophyll b deficiency. Also no changes in electrophoretic mobility, i.e., apparent molecular weight, of either the 26 or 28 kDa polypeptides were noted in electrophoretic studies on control or heat denatured chloroplast fragments of the various phenotypes. Similarly PAGE of chromatographically purified samples of the LHC apoproteins of the WT and several mutants showed no alteration of electrophoretic mobility. These observations suggest, but do not prove, that the LHC-II apoproteins of the various phenotypes studied are not modified by the mutation causing loss of chlorophyll b synthesis.

The Carotenoids and Membrane Polypeptides of the Light Harvesting Complex of Mutants of *Scenedesmus obliquus* Deficient in Chlorophyll *b*

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EMS	Ethyl methane sulfonate
kDa	Kilodaltons
LDS	Lithium dodecyl sulfate
LHC	Light harvesting complex
LHCP I	Light harvesting protein complex of photosystem I
LHCP II	Light harvesting protein complex of photosystem II
M	Molar
m A	MilliAmpere
n m	Nanometer
PAGE	Polyacrylamide gel electrophoresis
PCV	Packed cell volume
PSI	Photosystem I
PSII	Photosystem II
WT	the wild type, or unmutated strain

The Carotenoids and Membrane Polypeptides of the Light-Harvesting Complex of Mutants of Scenedesmus obliquus Deficient in Chlorophyll b

INTRODUCTION

The general mechanism of photosynthesis in higher plants is a light-induced oxidation-reduction reaction in which water is oxidized to provide the electrons and protons which are eventually utilized to reduce carbon dioxide to a carbohydrate form. Two photosystems are required to carry out the primary oxidation reaction (photosystem II) and the generation of reduced NADP (photosystem I). The combined action of these two photosystems within the chloroplast membrane structure also results in the generation of ATP (photophosphorylation). The functioning of the individual photosystems is dependent upon a discrete arrangement of pigmented lipoproteins within the chloroplast membrane such that an efficient utilization of available light energy is possible.

To insure an efficient utilization of the diffuse sunlight available in Nature, plants in general have developed a unique system of two reaction center complexes (for each of the photosystems) and associated light collecting (or antennae) systems. The fundamental role of the chlorophylls and accessory pigments associated within each of these systems is to capture and convert the light energy of the available quanta, within the wavelength region of the electromagnetic spectrum between 350 and 750 nm, to a stable and useful chemical form which can be utilized directly in the appropriate reaction center complex. The typical absorption spectrum of a solution of chlorophyll a demonstrates two major absorption bands in the blue (420 nm) and red (680 nm) regions. In a simplified interpretation

these absorption bands represent the two principle excited states of the chlorophyll a molecule, i.e., the 2nd and 1st excited states respectively. All evidence indicate that it is the 1st excited singlet state, which arises directly by absorption of "red" quanta or by decay from the 2nd excited singlet state, that functions in the photosynthetic process. This stabilized energy form can be used directly if energy stabilization occurred through chlorophyll a of a reaction center or migrates randomly to overlapping pigment molecules with sequentially lower energy levels until it either reaches a reaction center, decays as fluorescence or dissipates as heat. In algae and higher plants the two reaction centers and their immediate antennae systems are characterized by the in vivo absorption maxima of the specific chlorophyll a molecule, i.e., 680 nm for photosystem II (PS-II) and 700 nm for photosystem I (PS-I).

The rate limiting step in the primary reactions of photosynthesis is determined by the turn-over rate of the reaction centers. One of the factors involved in this is the immediate availability of excitation energy for the reaction center chlorophyll a. This is the primary function of the accessory pigment systems associated with each of the photosystems. In green algae, such as the organism used in this study (Scenedesmus obliquus), as in most algae and higher plants, a light harvesting-chlorophyll-protein complex (LHCP) comprised of chlorophyll a, chlorophyll b and specific carotenoids is associated with each of the photosystems. The ability of plants to form the LHCP is determined by genetic factors governing the synthesis of the pigments, both chlorophylls and carotenoids, and the associated proteins. This involves, consequently, the cooperative action of both the chloroplast and nuclear genomes.

The studies for this thesis were initiated to determine (1) if the loss of the ability of certain mutant phenotypes of the green alga Scenedesmus obliquus to synthesize chlorophyll b was correlated with changes in the types or amounts of specific carotenoids normally associated with the LHCPs and/or (2) whether specific alterations of the apoproteins associated with the LHCP resulted in such mutant phenotypes. It is hoped that the additional characterizations of these mutants in this thesis will help provide for some further understanding of how the association of the various components of the chloroplasts leads to the development of a functional LHCP.

Nature of the light-harvesting chlorophyll a/b complex

Specific evidence for the existence of chlorophyll-protein complexes was obtained when detergent solubilized thylakoids were subjected to electrophoresis (SDS-PAGE) without prior thermal denaturation (Ogawa, et al., 1966; Thornber, et al., 1967). Originally only three major green bands were seen on unstained gels; two of these represented chlorophyll-protein complexes (CP-I and CP-II) while the other was a "free" pigmented zone containing chlorophylls and carotenoids. The slower moving band, CP-I, contained only chlorophyll a and migrated at an apparent molecular weight of 110 kDa in the gel system employed. CP-II contained equal amounts of chlorophylls a and b and migrated with an apparent molecular weight of 35 kDa. Studies with a mutant strain of Scenedesmus lacking photosystem I activity led to the conclusions that CP-I represented at least the reaction center of PS-I, P-700, and its immediate light-harvesting chlorophyll a-system (Thornber, et al., 1967). By analogy, CP-II was

thought to represent the total chlorophyll-protein complex of PS-II. Later research demonstrated that high-speed centrifugation of spinach thylakoids partially solubilized with either Triton X-100 or digitonin resulted in a clear separation of a PS-II complex containing only chlorophyll a from an inactive complex containing equal amounts of chlorophylls a and b (Vernon, et al., 1972; Wessels, et al., 1973). In 1974 Thornber and Highkin showed that thylakoids obtained from the chlorophyll b-less mutant of barley (chlorina f2) contained CP-I but lacked CP-II even though it was known to possess both PS-I and PS-II activities. This observation suggested that CP-II and PS-II were separable entities with CP-II serving as the major light harvesting component of the total PS-II complex. As a result of these findings, CP-II was renamed, accordingly, as the light-harvesting chlorophyll a/b protein (LHCP or LHC a/b). The dominant form, LHCP-II, is associated with PS-II and constitutes approximately half of the total chlorophyll and one-third of the protein in thylakoids of green plants. LHCP-II is the most abundant of the LHCPs of the chloroplast; recent work (Peter and Thornber, 1988) has resolved it further into four separate pigment protein complexes termed LHCP-IIa, LHCP-IIb, LHCP-IIc and LHCP-IId. LHCP-IIb constitutes 85-90% of the total associated chlorophyll. Only two principle apoproteins, with apparent molecular weights of 27 and 25 kDa, appear to be associated with each of the LHCP-II types (Green, 1988).

Recent research employing reduced concentrations of the surfactant, Triton X-100, has revealed that a light-harvesting complex, containing both chlorophyll a and b, is also associated with photosystem I (Mullet, et al., 1980). This procedure has also demonstrated that like the corresponding LHCP of PS-II, the LHCP-I is made up of three different pigment-binding

proteins. The apoprotein of these show apparent sizes of 24 (LHC-Ia), 21 (LHC-Ib) and 17 (LHC-Ic) kilodaltons and have been purified to homogeniety (Vainstein, et al., 1989). None of these cross-reacted with antibodies raised against the major light-harvesting complex, LHC-IIb, or against the PS-I or PS-II reaction center core complexes.

The predominance of specific antennae system in green plants is determined by inherent genetic systems of a given species and by the light intensity in a plant's local environment (Apel and Kloppstech, 1978; 1980). In addition to the chlorophyll b, the carotenoids of this complex also function as efficient absorbers of light energy thus expanding the useful range of light absorption by this complex. Certain of the carotenoids may also be involved in the binding (synthesis?) of chlorophyll b to the appropriate polypeptide subunit of the complex (Schmidt, et al., 1986). This potential relationship will represent a major theme of this thesis and will be discussed in more detail in later sections.

Synthesis and localization of the light-harvesting chlorophyll protein complex

It is well known that the chloroplast is a true plastid with its own distinctive genome capable of both the transcription and translation of the information stored in chloroplast DNA. However, the total amount of DNA within a chloroplast is insufficient to code for the large number of proteins required for total photosynthesis. Only those polypeptides that are specifically involved in the early photochemistry of PS-I and PS-II, i.e., the apoproteins of the immediate antennae of the PS-I and PS-II reaction center cores, the D1- and D2-polypeptides of the PS-II reaction center core,

the apoprotein of cytochrome b-559 and a few other smaller proteins, are coded for by chloroplast DNA. The apoproteins of the LHCs are encoded by a small gene family of the nuclear genome (Dunsmuir, et al., 1983; 1985), synthesized on cytoplasmic ribosomes, released as soluble precursor forms and transported to the chloroplast in an inactive zymogen form (Ellis, 1981; Grossman, et al., 1982). Through a "clipping" mechanism not yet fully understood, these pre-proteins are incorporated into the thylakoids (Cuming and Bennett, 1981; Schmidt, et al., 1981; Bennett, et al., 1984); this system must involve a unique process for crossing the multiple membrane system of the chloroplast. Current evidence suggests that the amino acid leader sequence of the LHC pre-protein undergoes sequential clipping as the LHC-apoproteins are recognized and passed on into the chloroplast where they eventually become associated with the thylakoid membrane system. At specific stages in this process, the pre-proteins are cleaved to their mature molecular forms and become ligated with chlorophyll a, chlorophyll b and various specific carotenoids. It is at this stage that further development of a mature chloroplast is dependent upon the co-ordinated regulation of gene expression in the chloroplast and the nucleus since the synthesis of the pigments appears to be regulated through the chloroplast.

The mature LHC-II polypeptides are extremely hydrophobic and according to the deduced amino acid sequences contain three (Kalrin-Neumann, et al., 1985) or four (Anderson and Goodchild, 1987) membrane spanning regions. The model with three transmembrane domains was proposed from hydropathy plot analysis (Kalrin-Neumann, et al., 1985) while the model with four transmembrane domains developed from agglutination studies with an antiserum generated against a synthetic

octapeptide corresponding to the carboxyl-terminus of LHC-II (Anderson and Goodchild, 1987). The three-domain membrane spanning model places the amino- and carboxyl-termini at the outer (stromal) and inner (luminal) surfaces of the chloroplast while the four-domain model predicts them to be located only at the outer membrane surface. Verification of either model is still incomplete but for this thesis the four-domain model offers a greater number of conserved, potential-chlorophyll binding sites which would be protected from protease action.

Biosynthesis of the chlorophylls

Although the study reported in this dissertation is not directly concerned with the mechanism for the biosynthesis of the chlorophyll a per se, it is of importance to recognize how the synthesis of one of the major components of the LHCP, chlorophyll b, can be prevented in the types of mutations used for the outlined experiments. The biosynthesis of chlorophyll a, as presented in detail in numerous reviews (e.g., Bogorad, 1976; Schneider, 1980; Britton, 1983; Rudiger and Benz, 1984; Griffith and Oliver, 1984) proceeds through the reaction sequences normally involved in the formation of a pyrrole and an isoprene unit. These are (1) the synthesis of 5-aminolevulinic acid (ALA), (2) the conversion of ALA to a tetrapyrrole (protoporyphyrin IX), (3) its subsequent conversion to protochlorophyllide a in a series of dark reactions in most etiolated plant tissue and (4) the light-dependent conversion of protochlorophyllide a to chlorophyllide a, its conversion to an intermediate form of chlorophyll a by a transesterification with geranylgeranyl pyrophosphate (GGPP), a precursor of the

hydrophobic, C-20 alcohol, phytol, and the stepwise reduction of the digeranyl moiety through the chlorophyll synthase.

Upon illumination of etiolated plant systems, either of higher plant material or specific mutants of unicellular algae, protochlorophyllide a (Pchlide) is reduced to chlorophyllide a (Chlide) through the NADPH-Pchlide oxidoreductase (Griffiths, 1978; Oliver and Griffith, 1982). The initial burst of chlorophyll a synthesis is followed by a lag of variable length, which depends upon various experimental parameters, and then both chlorophyll a and chlorophyll b accumulate at proportional rates (Oelze-Karow and Mohr, 1978; Oelze-Karow, et al., 1978). In this sequence, chlorophyll b is not yet synthesized until chlorophyll a attains a "threshold" level. Whereas chlorophyll a synthesis requires light, chlorophyll b formations appears to depend upon the previous production of chlorophyll a rather than directly on light.

Although details of the mechanism for chlorophyll a biosynthesis have existed for some time the mechanism for chlorophyll b formation remains obscure. As is suggested above, chlorophyll b arises either from chlorophyll a or from a common precursor such as Pchlide a or Chlide a. However, the precise mechanism of the conversion of Chl a to Chl b, a reaction which seemingly involves the oxidation of the C-7 methyl group of ring II to an aldehyde, remains unknown at this time. Recent findings by (Senger, et al., 1989) on the greening mutant of Scenedesmus, C-2A', suggest that a protochlorophyllide b can be detected in dark grown cells which have been provided with excess ALA. Whether this observation is significant for the overall biosynthetic pathway for chlorophyll b biosynthesis remains to be evaluated (Figure 1).

Carotenoid function and biosynthesis

Carotenoid function in photosynthesis:

Typical carotenoids contained in chloroplast membranes, either as a part of a reaction center or of an antennae system, fulfill the role of accessory light absorbing pigments through radiationless transfer of energy from the excited singlet state of the carotenoid to a chlorophyll molecule. This mechanism is summarized in Eqs. 1 and 2 below.

(a) Light harvesting function: Singlet-singlet energy transfer

$$\text{Eq. 1} \quad \text{Car} \xrightarrow{h\nu} {}^1\text{Car}^*$$

$$\text{Eq. 2} \quad {}^1\text{Car}^* + \text{Chl} \longrightarrow \text{Car} + {}^1\text{Chl}^*$$

A second and major role of these pigments is to serve as agents which prevent excessive damage of chloroplast components as caused by the triplet state of chlorophyll or the resulting excited singlet state of oxygen, $^1O_2$. As indicated earlier in this section, the excited singlet state of chlorophyll, ^{1}Chl*, is produced by the absorption of a light quantum; its excess energy is utilized either in productive photochemistry or decays as fluorescence as summarized in Eqs. 3 and 4 below.

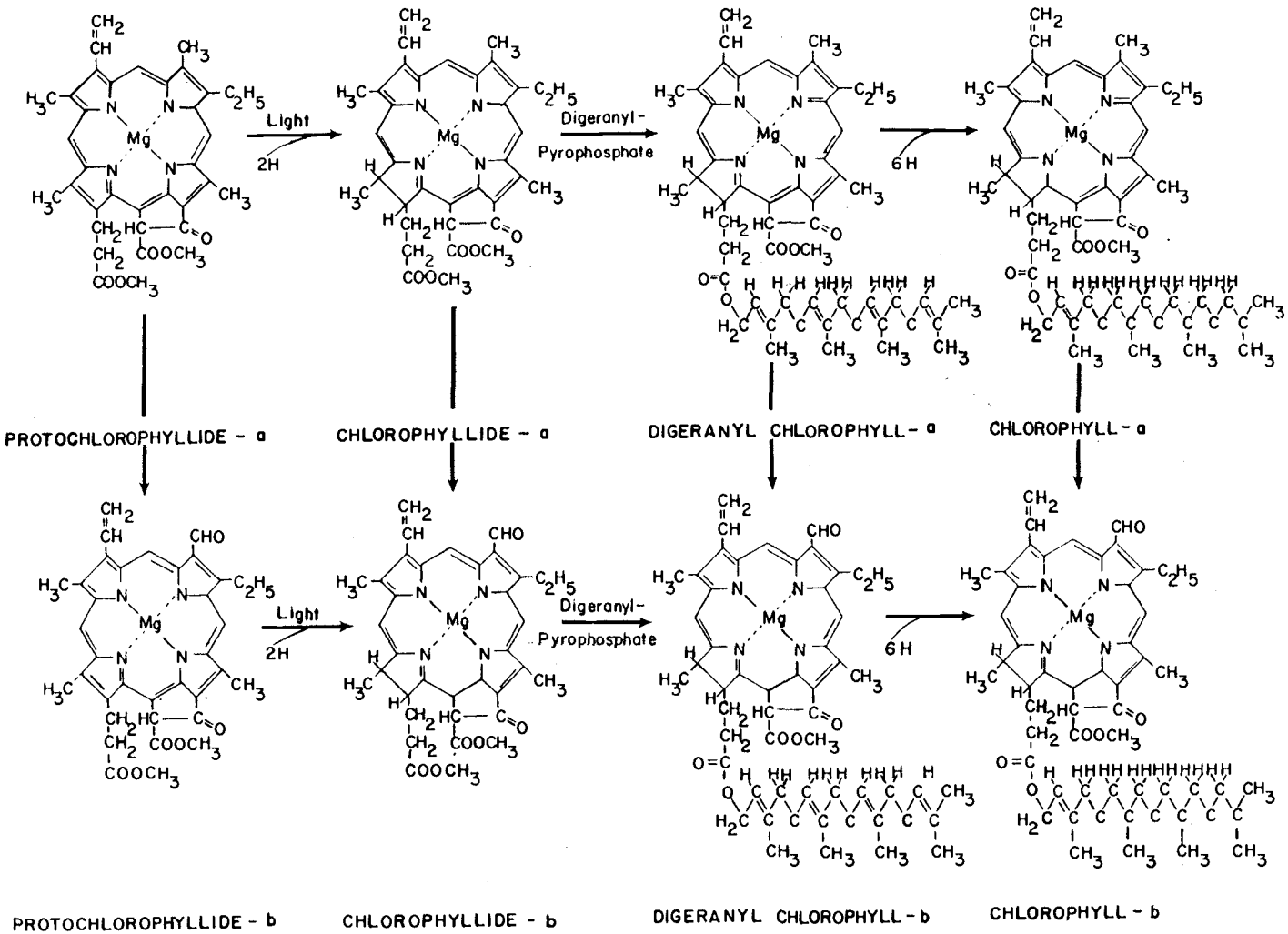

Figure 1. Alternate pathways for the biosynthesis of chlorophyll b beginning with protochlorophyllide a as the initial precursor.

Under conditions where the rate of utilization of $^1Chl^*$ is decreased, the triplet state (Eq. 5) of chlorophyll arises through the phenomenon of intersystem crossing (ISC).

(b) Protection function: The triplet state

1. Photodamage:

$$\text{Eq. 3} \qquad {}^1Chl^* \xrightarrow{Kp} \text{Photochemistry}$$

$$\text{Eq. 4} \qquad {}^1Chl^* \xrightarrow{Kf} Chl + h\nu \text{ (fluorescence)}$$

$$\text{Eq. 5} \qquad {}^1Chl^* \xrightarrow{Kisc} {}^3Chl^*$$

Photodamage may result through this more stable, excited state of chlorophyll, but more likely results through the production of the excited state of oxygen (Eq. 6); this entity is a potent oxidant and reacts rapidly, for example, with a number of unsaturated fatty acids of chloroplast lipids (Eq. 7).

$$\text{Eq. 6} \qquad {}^3Chl^* + {}^3O_2 \longrightarrow Chl + {}^1O_2^*$$

$$\text{Eq. 7} \qquad {}^1O_2^* \longrightarrow \text{harmful oxidations}$$

The carotenoids protect against such a scenario by quenching either the triplet state of chlorophyll or the singlet state of oxygen wherein the less reactive triplet state of the carotenoid is produced (Eqs. 8 and 9). This state

rapidly returns to the normal "ground" state of the carotenoid molecule by a radiationless conversion process (Eq. 10).

2. Protection:

Eq. 8 $^3Chl^* + Car \text{-------} ^3Car^* + Chl$

Eq. 9 $^1O_2^* + Car \text{--------} ^3O_2 + {}^3Car^*$

Eq. 10 $^3Car^* \text{------} Car$ (radiationless conversion)

3. Structural function:

An additional important function of the carotenoids may result from their involvement in the development of chloroplast membrane structure. It is well known that the carotenes and carotenoids are unequally distributed between the reaction centers (β-carotene) and the light-harvesting complexes (lutein); the extent of this specific distribution patterns is just beginning to be recognized as it becomes possible to purify the individual reaction centers and LHCP without loss of pigments (Vainstein, et al., 1989). Considerable evidence exists which demonstrates that the loss of ability in a number of unicellular algae to synthesize the normal carotenoids of the chloroplasts, as caused either by mutation or by treatment with select chemical inhibitors, results in the loss of normal chloroplast structure, loss of photosystem II reaction center components and inhibition of the development of the various light-harvesting components even when the organism(s) are cultured in the absence of light.

The observation (Plumley and Schmidt, 1987) that the in vitro rebinding of chlorophylls a and b to the apoproteins of LHC-II requires the presence of specific carotenoids, i.e., neoxanthin or violaxanthin, also indicates an alternate role for these pigments in the photosynthetic apparatus. It is difficult, however, not to become involved in a cause-and-effect relationship in considering a possible alternate role for these ubiquitous compounds in the photosynthetic apparatus.

Carotenoid Biosynthesis:

Because of Man's long fascination with the carotenoids, details of their biosynthesis have been known for considerable time and much of this information is covered in numerous texts, reviews and copendia (Isler, O., 1971; Britton and Goodwin, 1981; Britton, 1983; Britton 1986). The mechanism for the biosynthesis of early precursors to the C40-polyenes is identical with that of phytol up to the formation of geranyl-geranyl pyrophosphate (GGPP). The condensation of two molecules of GGPP leads to the formation of the initial C-40 carotene, phytoene. Although a moderately soluble enzyme, phytoene synthetase, catalyzes the incorporation of GGPP into phytoene (Britton, 1986) other steps involved in its desaturation and cyclization are not well known. The various carotenes (α- or β- carotene) of mature plant tissue arise from a series of dehydrogenation and cyclizations of phytoene (see Figure 2). Current research in this area (Sandmann and Kowalezyk, 1989) has demonstrated a phytoene desaturase in detergent extracts of the cyanobacterium,

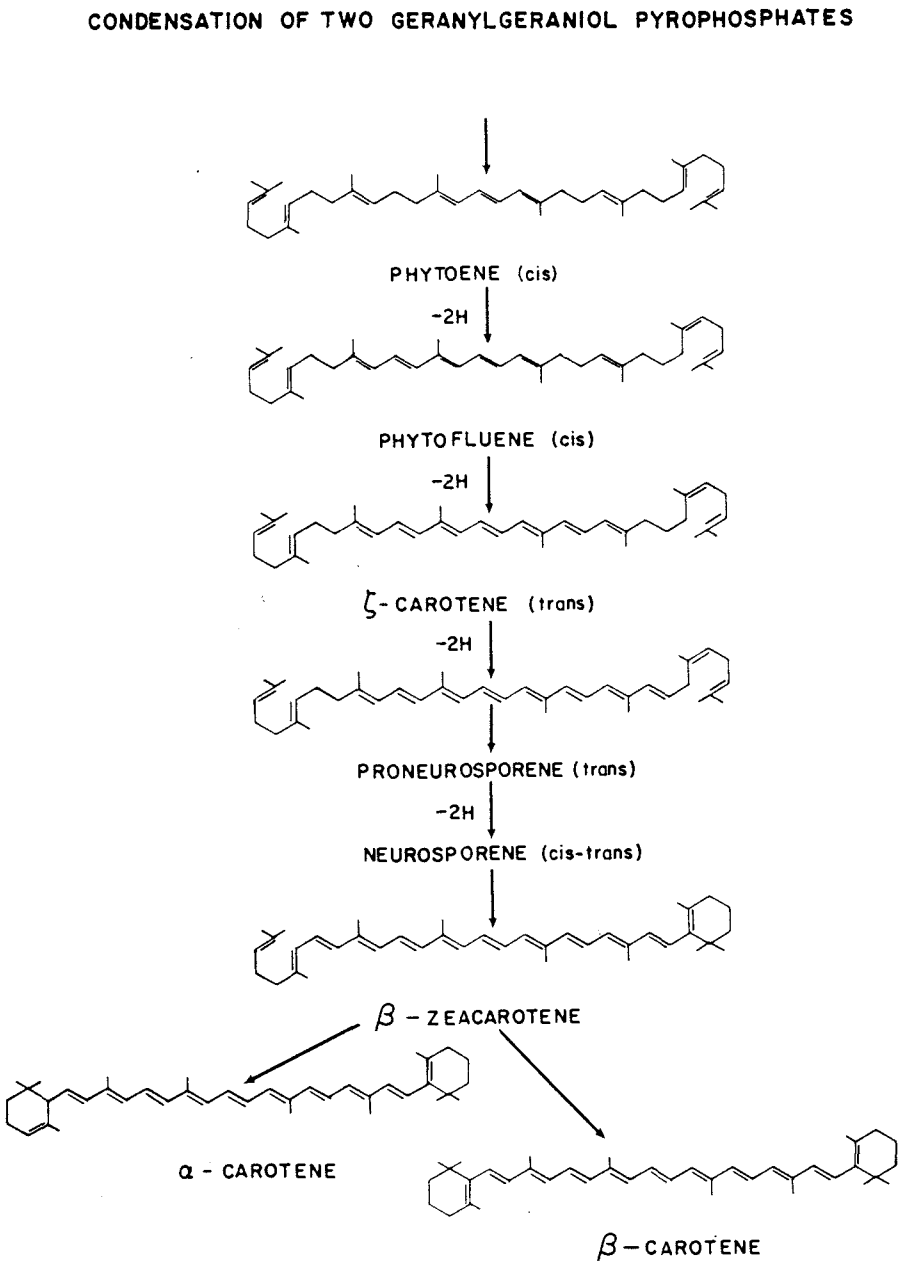

Figure 2. Biosynthetic pathway for the conversion of the C-40 polyene, phytoene, to α- and β-carotene.

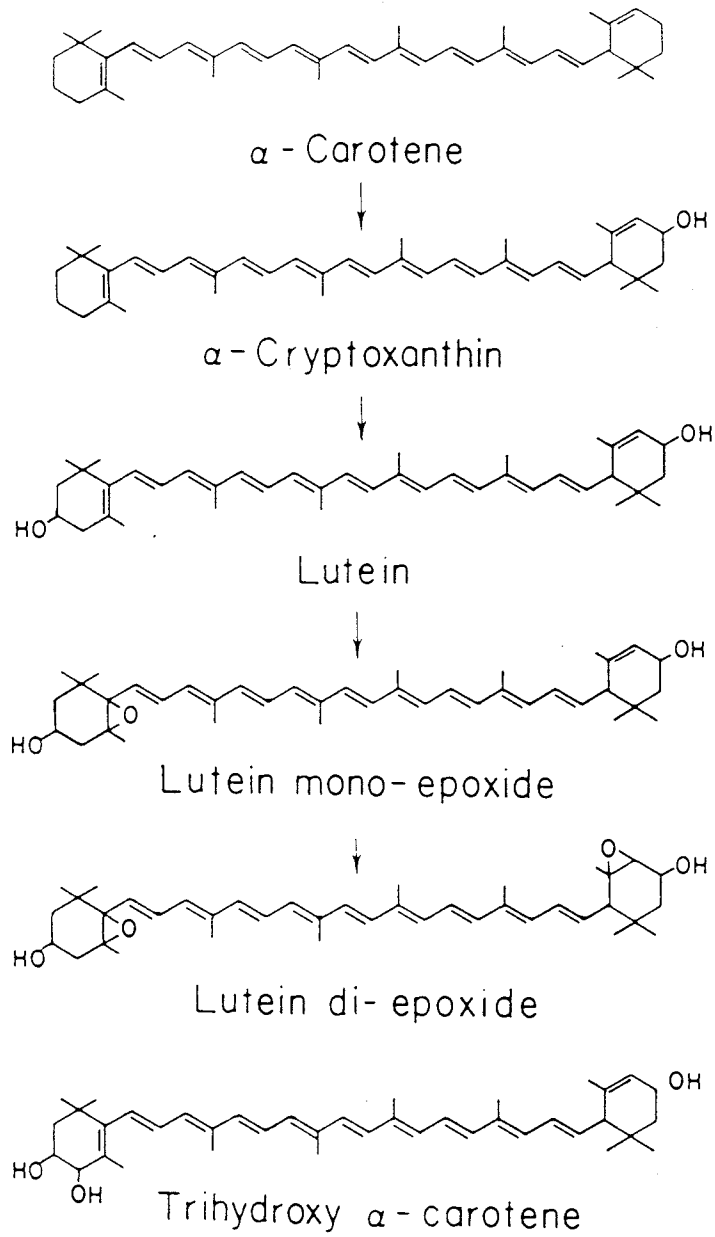

Figure 3. Suggested pathway for the conversion of α-carotene to the related, and more common xanthophylls, e.g., lutein, loraxanthin (trihydroxy-α-carotene). Molecular oxygen is the preferred substrate for each of the indicated steps.

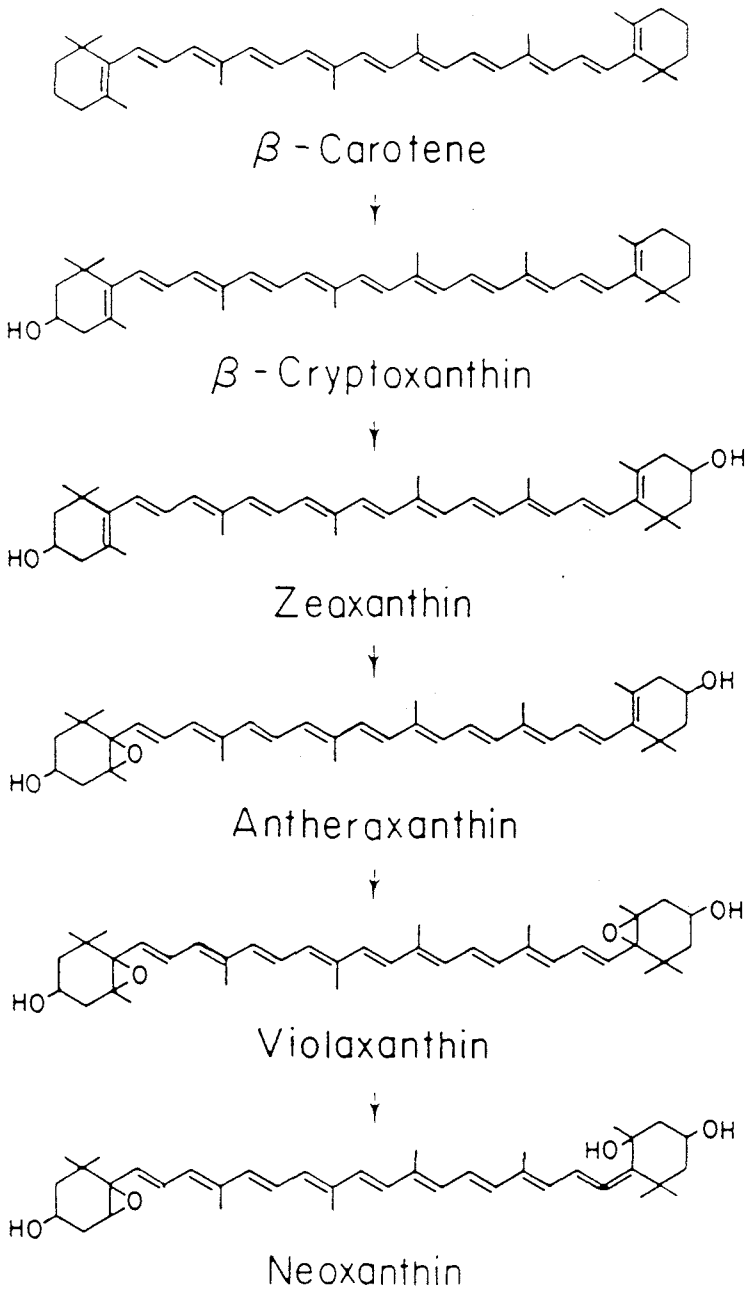

Figure 4. Suggested pathway for the conversion of β-carotene to the related, and more common xanthophylls, e.g., zeaxanthin, violaxanthin, neoxanthin, etc. As is indicated in the legend for Fig. 3, molecular oxygen is the preferred substrate for the introduction of oxygen in either the hydroxyl or epoxide groups of the various intermediates.

Anacystis, which preferentially converts phytoene to α-carotene and to a lesser extent into β-carotene. The formation of the xanthophyll, i.e., the addition of hydroxyl or epoxide groups to either α- or β-carotene, occurs through the addition of molecular oxygen, and not water, through the function of a mixed function oxidase (Britton, 1983). Studies utilizing either $^{18}O_2$ or $H_2{}^{18}O$ as substrates for production of the xanthophylls have consistently shown that both the hydroxy and epoxy groups arise from $^{18}O_2$. In Figures 3 and 4 the general pathways for the syntheses of the most abundant types of xanthophylls found in Scenedesmus are presented. Specific details, however, for each conversion step shown remain poorly defined. From the information currently available it appears that the sequence of reactions necessary for the formation of the carotenes and xanthophylls is brought about by a multi-enzyme complex which requires a high degree of organization and is probably part of a cytoplasmic membrane system (Sandmann and Kowalezyk, 1989). The inherent desaturase enzymes may also require some kind of simple electron transport system, such as in cytochrome P-450, as also would the mixed function oxidases involved in xanthophyll biosynthesis. Although model reactions have been proposed for the formation of the various xanthophylls, such as neoxanthin (Isler, 1971), specific biochemical pathways have not been identified to date.

Alternate light-harvesting chlorophyll complexes in chlorophyll b-less plants.

Similar antennae systems are also found in other algae which characteristically do not synthesize chlorophyll b. As much as 50% of the

light-harvesting capacity of cyanobacterial and red algal cells is found in a family of colored proteins called phycobiliproteins. These proteins are assembled in vivo into macromolecular aggregates, phycobilisomes, which are attached to the outer surface of the photosynthetic lamellae. These pigmented proteins are highly efficient (approaching 100%) in transferring absorbed light energy to the reaction center chlorophylls of PS-II. The properties of the phycobilisomes and phycobiliproteins have been extensively reviewed in recent years (Glazer, 1982; Glazer, 1983).

In other algal systems lacking chlorophyll b certain carotenoid-protein complexes serve as light-harvesting systems. In the Dinoflagellates a water soluble chlorophyll a-peridinin protein complex has been isolated and characterized; this system shows 100% efficient energy transfer from peridinin to chlorophyll a. In the diatoms and brown algae chlorophyll a-fucoxanthin protein complexes represent the light-harvesting complex associated with PS-II (Barrett and Anderson, 1980). It is of interest that the structures of peridinin and fucoxanthin are similar to that of neoxanthin (Isler, 1971).

MATERIALS AND METHODS

Autotrophic cultures, when used, were grown in nitrate medium at 25°C in glass bubble tubes (Bishop and Senger, 1971). Light was provided by a bank of warm-white and grow-lux fluorescent lamps which gave a light intensity of 1 mW/cm^2 at the surface of the bubble tubes. A mixture of 3% CO_2 in air was bubbled through the tubes.

Heterotrophic cultures were grown on the nitrate medium (Kessler, Arthur, and Brugger, 1957) enriched with (NGY) 0.5% glucose and 0.25% yeast extract (Bacto yeast extract, Difco Labs., Detroit, MI). Cultures of individual phenotypes were grown at 30°C in 250 ml of medium in 500 ml screw-cap Erlenmeyer flasks on a gyrotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.). This size of culture provided sufficient cells for analysis of the various photosynthetic parameters examined with the various strains studied. For larger scale cultures 1.25 L of growth medium in Fernbach flasks was inoculated directly from the smaller cultures. 2.5 to 3 day old cultures were normally used for experimentations since the photochemical characteristics of the WT maximizes at this age (Berzborn and Bishop, 1973).

Mixotrophic cultures were obtained by growing cells under the same conditions used for autotrophic growth except the enriched medium described above was employed. Generally, only samples two days old were analyzed.

Packed cell volume (PCV) was used to quantitate the amount of cell material used for each experiment. This was determined by centrifuging an aliquot of a sample in a modified, calibrated, cytocrit centrifuge tube

using a Sorvall table top centrifuge (Model GLC-1) for five minutes at 300 x g.

Mutant induction, isolation and selection

Mutants deficient in either photosynthetic capacity and/or the light-harvesting pigment complex (LHCP) used in this study were induced by treatment of the green alga, Scenedesmus obliquus, strain D_3, (to be referred to as wild-type [WT]) with ethyl methane sulfonate (EMS) according to the methods described by Bishop (1971, 1982). The initial selection of the mutant strains deficient in the LHCP was made by careful visual examination of matured algal clones (14 days old) which had been grown heterotrophically on agar plates at 30°C. Strains lacking or deficient in chlorophyll b could be detected by their lighter green, often bluish-green, color. Clones showing this characteristic were transferred to individual culture tubes and subsequently analyzed spectrophotometrically for any alterations in the LHCP.

Direct measurement of the difference absorption spectrum between the mutant phenotype and the WT with the Aminco DW-2 dual beam spectrophotometer provided the most convenient, quantitative evaluation of the difference in pigment composition between the WT cells and each of the phenotypes evaluated.

Total pigment extraction

For the quantitative determination of the individual chlorophylls and carotenoids contained in the separate phenotypes studied, a volume of cell suspension sufficient to provide 1 ml packed cell volume was

centrifuged for five minutes at 4,100 x g in a Sorvall table top centrifuge. This volume was determined from a PCV determination on an aliquot of the culture sample as outlined above. 1 ml PCV of pelleted cells was resuspended in a slurry of absolute ethanol and 1 mm. diameter glass beads and placed in the medium size (110 ml.) stainless steel cup of the Vibrogen Zellmuhle (Tubingen, W. Germany). The cell and its content was then shaken at full velocity for eight to nine minutes; the temperature was maintained at 4°C throughout the breakage and extraction procedure. Glass beads were removed from the slurry by filtration through nylon screen. The beads were rinsed with additional cold ethanol and the filtrates combined. Cell debris was removed from the filtrate by centrifugation for 5 minutes at 4,100 x g in a Sorvall GS-2 centrifuge head and the supernatant saved. The pellet of broken cells was extracted twice with 96% ethanol to remove any residual pigments. An aliquot of the combined extracts was analyzed for total chlorophyll (Arnon, 1949). The remaining pigments were transferred to ethyl ether by vigorously shaking one volume of the extract with one volume of ether and two volumes of 2 M NaCl (plus a trace of 80% acetone) in a separatory flask (Braumann, et al., 1981). The ether layer was removed and washed with double distilled water to remove traces of acetone and ethanol. This total procedure was performed in near darkness and at 4°C, keeping samples covered, to avoid oxidation and isomerization of the carotenoids.

Identification of pigments

Chlorophylls and carotenoids of the wild type strain were initially identified by comparison of their absorption spectra and their Rf values

obtained from thin layer chromatography to those of published standards. 0.5 ml of the total pigment extracts, as prepared above, was streaked onto a TLC plate coated with silica gel G and then developed with a solvent system of petroleum ether-isopropanol-H_2O (100:12.5:0.5). The migration distance of each band was determined, the area corresponding to that band scraped from the TLC plate and the pigment eluted from the adsorbent with freshly redistilled chloroform (containing 1% ethanol). The sample was then dried under vacuum and either redissolved in diethyl ether for later HPLC analysis or in ethanol or heptane for spectrophotometric analysis. The absorption spectra of the individual pigment fractions were determined with an Aminco DW-2 spectrophotometer over the range of 350 to 500 nm. Similar determinations were also made with fractions obtained by direct HPLC purification of the total pigment extract. The Rf values obtained by TLC, the HPLC retention times and the absorption wavelength maxima were compared to those of known standard carotenes and xanthophylls for specific identification of each isolated pigment.

For quantitative evaluation of the amounts of each of the identified pigments present in extracts of the various phenotypes evaluated in this study, high performance liquid chromatography elution profiles of known amounts of pure pigment were run and the area under the peak determined. Extinction coefficients used for these calculations were determined by Braumann and Grimme (1981).

High performance liquid chromatography: Analysis of Carotenoids

With a Beckman HPLC system, consisting of two 110 B Solvent Delivery Modules, a Model 420 Controller, a Model 340 Organizer and a Model 163 Variable Wavelength Detector, pigment analyses were conducted on samples according to the procedure developed by Braumann and Grimme (1981). 20-25 ul of whole cell extract in ethyl ether (see above) were injected onto a system consisting of a 250 mm x 46 mm LiChrosorb C-18 reverse phase column (10 micron) and a Vydac GCH-4 guard column. Runs were made with a flow rate of 1.5 ml/min., a full-scale sensitivity of 0.2 absorbancy units and a chart speed of 2.0 mm/min. Absorbance was measured at 440 nm with the variable wavelength detector of the HPLC apparatus. The solvent gradient consisted initially of 85% solvent B (acetonitrile-methanol, 75:25) and 15% solvent A (double distilled water) which was brought to 100% solvent B in 15 minutes where it remained for an additional 55 minutes in most elution programs. All solvents were of HPLC grade and prefiltered with a 45 micron filter (Nylon 66, Rainin).

Individual bands were collected, concentrated either under vacuum or under a stream of nitrogen gas and stored at -45°C for later spectrophotometric analysis.

Preparation of Scenedesmus chloroplast fragments

Algal cells collected from two Fernbach flasks each containing 1.25 L. of NGY medium were washed once in distilled water and centrifuged

for 5 minutes at 5,000 rpm in the GSA rotor. The algal pellet was resuspended in Berzborn #2 buffer (per liter: 139.6 g sucrose, 2.24 g KCl, 3.58g tricine, pH 7.5) and divided into three aliquots. Breakage of the algal cells and preparation of chloroplast fragments were performed with the previously published procedures (Berzborn and Bishop, 1973).including a high speed centrifugation step (77,000 x g, 30 rotor). For pigment extraction, chloroplast fragments were resuspended in a medium consisting of 20 mM HEPES, 5 mM MgCl2, 15 mM NaCl, pH 7.5 and brought to a volume of 30 ml for a sucrose step gradient (1.8M-,1.5M-, 1.0M-, 0.75M sucrose) centrifugation. The resuspended pellet was divided equally among 6 tubes which were then centrifuged for 60 min. at 83,000 x g in the Beckman SW-27 swinging-bucket rotor. The main, deep green band was collected, diluted with BZ-#1 buffer (sucrose omitted) and centrifuged at 77,000 x g (30 rotor); the pelleted chloroplast fragments were then frozen for pigment analysis as described previously. To remove traces of soluble proteins the pelleted chloroplast material obtained from the high speed centrifugation step was resuspended in a high salt buffer (1.0M NaCl, 5 mM $MgCl_2$, 50 mM tricine [pH=8.0]) and stirred in the cold for 30 minutes. This sample was then diluted 1:1 by the addition of distilled water and the chloroplast fragments repelleted by centrifugation.

Extraction of proteins and pigments from chloroplast particles with chloroform-methanol

The procedure developed for the solubilization of the intrinsic polypeptides of bacterial chromatophore and the membrane-limited components of the coupling factor (CFo) (Tandy, et al., 1982, 1983; Boyan

and Clement-Cormier, 1984), with modifications, was used for the initial stages of separation of the organic solvent-soluble components of the algal chloroplast. The salt-washed pellet of chloroplast fragments was thoroughly resuspended in approximately 50 ml of a solution composed of 50mM sodium carbonate and 50mM dithiothreitol. Total chlorophyll concentration was determined (Arnon, 1949) and a sufficient volume of a 1:1 mixture of chloroform methanol was added to the chloroplast suspension to give a solvent/chlorophyll ratio of 20/1 (v/v). Normally this required a solvent volume of approximately 250-300 ml. This ratio of solvent to chloroplast suspension volume resulted in a maximum phase separation upon centrifugation of the total extract at 5,000 rpm. The yellowish-green upper phase was collected by suction and the lower deep green phase was separated from the interphase precipitate by decanting. Both phases were saved for further purification steps.

The addition of an equal volume of distilled water to the upper phase caused an immediate milky precipitation which was then collected by centrifugation for 10 minutes at 12,000 x g. The reddish-brown supernatant was generally discarded and the pale-green pellet resuspended in approximately 50 ml chloroform-methanol (1:1). Following centrifugation (4,100 x g) the pellet was normally discarded and the supernatant saved for chromatography.

Separation of proteins from pigments by column chromatography on Sephadex LH-20 and LH-60

The chloroform-methanol supernatant obtained in the last step was applied to a Sephadex LH-20 column (2.5 x 40 cm) and the major portion of

the applied protein was separated from contaminating pigments by elution with a 1:1 mixture of chloroform-methanol. 3 ml fractions were collected and their absorption at 280 nm determined; normally the contents of tubes 21-35 were pooled and the protein collected by precipitation with diethyl ether (5/1) and centrifugation. The pellet obtained was dissolved either in the standard digestion medium for detergent gel electrophoresis or in 0.1% octyl glucoside-Tris buffer (pH 7.5). The above samples were also applied to Sephadex LH-60 in an attempt to achieve greater separation of pigments from proteins. The elution was also carried out with a 1:1 mixture of chloroform-methanol.

High performance liquid chromatography: Purification of the Apoproteins of the Light-Harvesting Complex

For further purification of the two primary apoproteins of the LHC-II reversed phase HPLC was attempted. For this purpose a 5 micron, C-18 reverse phase column (Vydac 218TQ54) with an attached Vydac guard column provided the stationary phase The mobile phase consisted of a step gradient of 0%, 40%, 55% and 100% methanol in acetonitrile (containing 0.1% Trifluoroacetic acid) over 60 minutes plus a final step of methanol-chloroform (1:1). Samples (250 µl) of the chloroform-methanol extract purified by LH-20 and LH-60 chromatography, containing known concentrations of protein, were injected into the HPLC system described above (see HPLC carotenoid analysis for additional details of the system). Elution profiles were run at a flow rate of 1.5 ml/min. at 500-2000 psi (depending upon solvent mixture composition). Absorbance at 280 nm

was monitored with the Beckman Model 163 variable wavelength detector at a full-scale sensitivity of 0.2 absorbance units.

All solvents employed were of HPLC grade and filtered prior to use with a 0.45 micron filter (Nylon 66, Rainin). Protein samples were concentrated, prior to HPLC, under vacuum in an ice bath. Precipitation during concentration was prevented by the careful addition of aliquots of chloroform-methanol (1:10). All protein samples were filtered through Teflon filters (0.45 micron) prior to HPLC analysis.

Polyacrylamide gel electrophoresis

The polypeptide composition of the chloroform-methanol soluble fractions derived from the algal chloroplast fragments was examined using lithium dodecyl-sulfate (LDS) polyacrylamide gel electrophoresis (PAGE). The procedure employed is based on the original method described by Lammeli (1970) for a sodium dodecyl sulfate gel system. Modifications of this original method as made by Delepelaire and Chua (1979) were incorporated into the procedure used in this study except for the following alterations. Electrophoresis was routinely performed with a vertical dual slab gel apparatus (Bio-Rad) employing the short gels of 10 x 14 cms with a thickness of 1.5 mm. The stacking gel contained 5.5% acrylamide and the running gel was normally a 9% to 16.5% acrylamide gradient gel. In some cases the running gel included 4 or 6M urea.

A final concentration of not more than 100 ug protein was applied to each gel slot. Protein concentration was determined with the Lowry method (Lowry, et al, 1951) as modified by Peterson (1977, 1983). Electrophoretic separation was carried out with a Bio-Rad power supply

(Model 1420 B) at a constant current setting of 12.5 mA/plate; temperature was maintained at 10°C. Electrophoresis was terminated when the migration of the marker dye, thymol blue, neared the bottom of the gel. Subsequently the gel slabs were removed and stained overnight in a solution of 0.25% Coomassie briliant blue R-250 (Bio-Rad) in 10% acetic acid and 50% methanol. Slabs were destained by several sequential washings in a 7% acetic acid-50% methanol solution. Photographic records of the gel patterns were made for evaluation and molecular weight calibration.

The molecular weights of the proteins separated by LDS-PAGE were estimated by comparing migration distances of the various polypeptides to those of protein standards of known molecular weights (Weber and Osborn, 1969). Protein standards obtained from Sigma Chemical Co. were dissolved in the same solubilizing solution used for the hydrophobic proteins of the chloroplast membrane preparations. The eight protein standards used were: bovine serum albumin, 66,000 Da; ovalbumin, 45,000 Da; Glyceraldehyde-3-PO_4 dehydrogenase, 36,000 Da; carbonic anhydrase, 29,000 Da; trypsinogen, 24,000 Da; soybean trypsin inhibitor, 20,100 Da; β-Lactoglobulin, 18,400 Da and lysozyme, 14,300 Da. A standard calibration curve was constructed through a computer program for each gel.

RESULTS AND DISCUSSION

Characterization of the mutant phenotypes of Scenedesmus obliquus employed in this dissertation

The unicellular green alga, Scenedesmus obliquus, is an extremely useful organism for biochemical-genetic analysis of mechanisms of photosynthesis since it possesses all of the typical components of the two photosystems found in higher plants but can be grown in large number under a variety of experimental conditions in the laboratory. When provided with an energy source (glucose) and sufficient reduced nitrogen (urea or amino acids) the organism will grow in total darkness and produce a fully competent photosynthetic apparatus including all the chlorophyll-protein complexes. Consequently, it is possible to develop a variety of mutations in which select portions of the photosynthetic machinery are altered. For this study a number of mutant phenotypes were developed from the normal wild-type and specific mutants deficient in various partial reactions of photosynthesis which preferentially lack the chlorophyll a and chlorophyll b or only chlorophyll b of the light-harvesting pigment-protein complex, LHC. The general features of each of the phenotypes employed are described below.

The principal phenotype examined in this study, K0-9, behaves like the WT Scenedesmus in that it grows autotrophically, mixotrophically and heterotrophically, has near normal photosynthetic capacity, retains both photosystem I and photosystem II activities (Table I) and the associated membrane polypeptides as well as the polypeptides of the apoproteins of the

LHC (Figure 17). This strain was derived directly (Bishop and Oquist, 1979) from the WT Scenedesmus (see Methods for mutagenic procedures) and lacks both the chlorophyll a and chlorophyll b of the light harvesting complex (LHC). The absence of chlorophyll b is apparent from the general bluish-green color of the culture and was experimentally verified by column, thin layer and high performance liquid chromatographic procedures. The absorption spectrum in the range 400-700 nm shows a major shift in the absorption peak at 485 nm to 491, coincidental with the disappearance of the Soret band of chlorophyll b, the disappearance of the chlorophyll b at 651 nm and the smoothing of the shoulder at 672 nm (Figure 5). By running equal density samples (i.e., equal PCVs) of the WT versus K0-9 in the Aminco DW-2 recording spectrophotometer the absorbance difference spectrum is obtained which emphasizes these noted differences, as is shown in Figure 5. This spectrum also reveals the loss of an approximately equal amount of chlorophyll a through the appearance of peaks at 433, 669 and 678 nm in the difference spectrum. Other mutant phenotypes used in this study which have pigment characteristics comparable to K0-9 include PS-28-LHC and LF-1-RVT-LHC. The absorption spectra and absorbancy difference spectra for PS-28-LHC (and the original strain from which it was derived, PS-28) are shown in Figure 8. Although this strain is unique in that it develops the digeranyl derivative of chlorophyll a because of its inability to synthesize phytol, the spectral qualities of both PS-28 and the LHC-deficient strain appear nearly identical to that of the WT and K0-9.

Four additional strains (PS-11-LHC, LF-15-hf-17-LHC, LF-1-LHC and LF-23-LHC) were studied which showed loss principally of chlorophyll b and with only minor changes in chlorophyll a concentration (Figures 6 and

7). One difference noted in the absorbancy difference specrtra of these strains (because of the nearly identical spectra obtained from all of these strains, only data obtained from LF-1-LHC and LF-23-LHC are presented) suggests the appearance of a new type of chlorophyll because of an apparent increase in absorption at 686 nm (Figure 6); this result most likely is an experimental artefact since no other chlorophyll type was noted in the HPLC elution profiles. These mutant phenotypes were derived from existing strains of Scenedesmus which had previously been shown to lack photosynthetic capacity because of loss of one or more of the major components of the photosystems. As is indicated in Table I, strain PS-11-LHC was derived from PS-11 which is a pleiotropic mutant lacking all components of photosystem II; strain CP-I-13-LHC was obtained from a photosystem I deficient strain lacking the principal pigment-protein of this system, CP-I; LF-15-17-LHC was produced from a strain lacking the coupling factor, CF-1, and CP-I; LF-1-LHC was derived from a strain possessing the unprocessed form of one of the polypeptides of the PS-II reaction center core heterodimer, the D1-polypeptide and LF-23-LHC originated from a strain having reduced levels of this polypeptide complex. Despite the wide differences in the overall photosynthetic apparatus of these strains, the change in their spectral qualities resulting from their inability to synthesize chlorophyll b are very similar. A more detailed analysis of their total chloroplast composition follows.

Pigment analyses of whole cells of WT and various mutant strains

The major underlying similarities in the different phenotypes of Scenedesmus studied are (1) the general deficiency of chlorophyll b and the

absence of the light-harvesting complex, LHC-II, in "green" gels from LDS-PAGE. Despite the apparent absence of this complex from chloroplast membranes, the apoproteins associated with it appear to be synthesized at normal levels in all of the strains. As noted earlier, chlorophyll b-deficient strains of higher plants and of the green alga, Chlamydomonas reinhardtii, also show decreased levels of certain carotenoids, notably neoxanthin. Although kinetic evidence suggest that chlorophyll b is derived from chlorophyll a, the actual mechanism by which chlorophyll b is formed remains obscure. In vitro studies by Plumley and Schmidt (1984) suggest that the xanthophylls are essential for rebinding of chlorophyll b to the LHC apoproteins. This suggests that the binding or stabilization of this chlorophyll to a matrix protein may be part of the biosynthetic sequence.

To examine this hypothesis the total pigment complementation of each of the Scenedesmus strains used in this study were evaluated by thin layer chromatography and high performance liquid chromatography. Because of the extensive isomerization of certain of the xanthophylls during TLC, notably the epoxides, violaxanthin and neoxanthin, only data obtained with the HPLC procedure will be presented here. Initial purification of most of the carotenoids was performed, however, with TLC procedures.

When freshly prepared extracts of young cells of the WT were examined immediately after preparation the HPLC elution profile shown in Figure 9 was regularly obtained. From existing published data and by HPLC analysis of standards prepared in this laboratory the identity of each of the principal individual peaks was determined. The identification (by peak number, general name and chemical name) and general light absorption characteristics are summarized in Table II. Many of the minor peaks apparent in Figure 9 were either not consistently seen or remained at

insignificant low levels and no attempt was made to identify or quantify them in this study except for antheraxanthin which elutes between violaxanthin and lutein. With the HPLC elution program and column employed in this study (see Methods and Materials) lutein and zeaxanthin only resolved when the concentration of lutein was decreased; consequently values expressed for lutein in Table III represent a summed value for the two xanthophylls. Other peaks corresponding to chlorophylls a' and b' were noted when aged extracts were used for the HPLC. These represent the allomerized form of the respective chlorophylls and should be considered as degradatory products. The digeranyl derivatives of chlorophyll a and b, which have dramatically different retention times from the normal chlorophylls, were noted only in extracts of strains PS-28 and PS-28-LHC (Figures 15 and 16). Peaks 7 and 8 were identified as α- and β-carotene, respectively. Because of the long retention times quantification of these two pigments, when performed, was made directly form TLC preparations.

In Figures 9-16 the HPLC elution profiles of the pigment extracts from young cells of WT and K0-9 grown mixotrophically, autotrophically and heterotrophically are shown. The most obvious differences noted when the patterns for K0-9 are compared to those of the WT are the absence of chlorophyll b, a decreased level of neoxanthin and an increased concentration of violaxanthin in extracts of K0-9. Quantitation of the content of each of the identified components in cells of the WT and K0-9 was performed by graphic computation of the area under each peak and comparison against comparable values from authentic standards (see Methods and Materials). These values, as moles per 100 moles of chlorophyll a, are presented in Table III. The differences noted above are

more sharply defined in this tabluation. Comparison of the values of K0-9 to those of the WT for either autotrophic or mixotrophic cultures shows that the concentration of neoxanthin is decreased by approximately 70% while that of violaxanthin is increased by a comparable amount. No other consistent variations are apparent for loroxanthin (trihydroxy α-carotene), antheraxanthin and lutein. The observed accumulation of violaxanthin, an apparent intermediate in the biosynthetic pathway for neoxanthin (Figure 4), suggests that the inability of the various phenotypes to accumulate/synthesize chlorophyll b may be a result of a mutation at this point.

The values obtained for dark grown cultures (heterotrophic) are less dramatic. However, the general pattern of a decreased level of neoxanthin in the chlorophyll-b-deficient mutant persists. Apparently the dark grown cultures, although producing a near normal pigment profile, synthesize less of the xanthophylls, particularly lutein. An observation related to this is that the level of α- and β-carotene in dark grown cells is about equal whereas light grown cells possess primarily β-carotene with only trace amounts of α-carotene (personal communication, N. I. Bishop). This suggests obviously, that the formation of lutein from α-carotene is strongly influenced by light.

Table 1

Comparison of various whole cell and isolated chloroplast particle photochemical reactions of the WT and selected phenotypes deficient in the Light Harvesting Complex.

Strain	PS	PR	H_2O-DCPIP	DCP-DCPIP	DCPIP-MV	H_2O-DAD
Wild Type	1084[a]	348[b,a]	220[c]	180	310[d]	270
K0-9	980	360	240	228	340	260
LF-1-LHC	90	340	0	278	380	0
LF-15hf-17-LHC	tr	0	258	178	0	260
CP-I-13-LHC	tr	0	220	178	0	254
PS-28-LHC	80	170	0	94	280	0
LF-23-LHC	80	342	0	tr	320	0

[a] Rates of photosynthesis (PS) and photoreduction (PR) given as μ mol O_2/mg Chl/h and μ mol CO_2/mg Chl/h as measured at saturating red light and at 25°C.
[b] Photoreduction measured in an atmosphere of 4% CO_2 + 96% H_2 after a 4 h adaptation period.
[c] Rates of cell free photochemical reaction given as μ mol DCPIP reduced/mg Chl/h at saturating light intensities with wavelength > 620 nm.
[d] PS-I catalyzed reduction of methyl viologen measured polarographically as μ mol O_2 uptake/mg Chl/h.

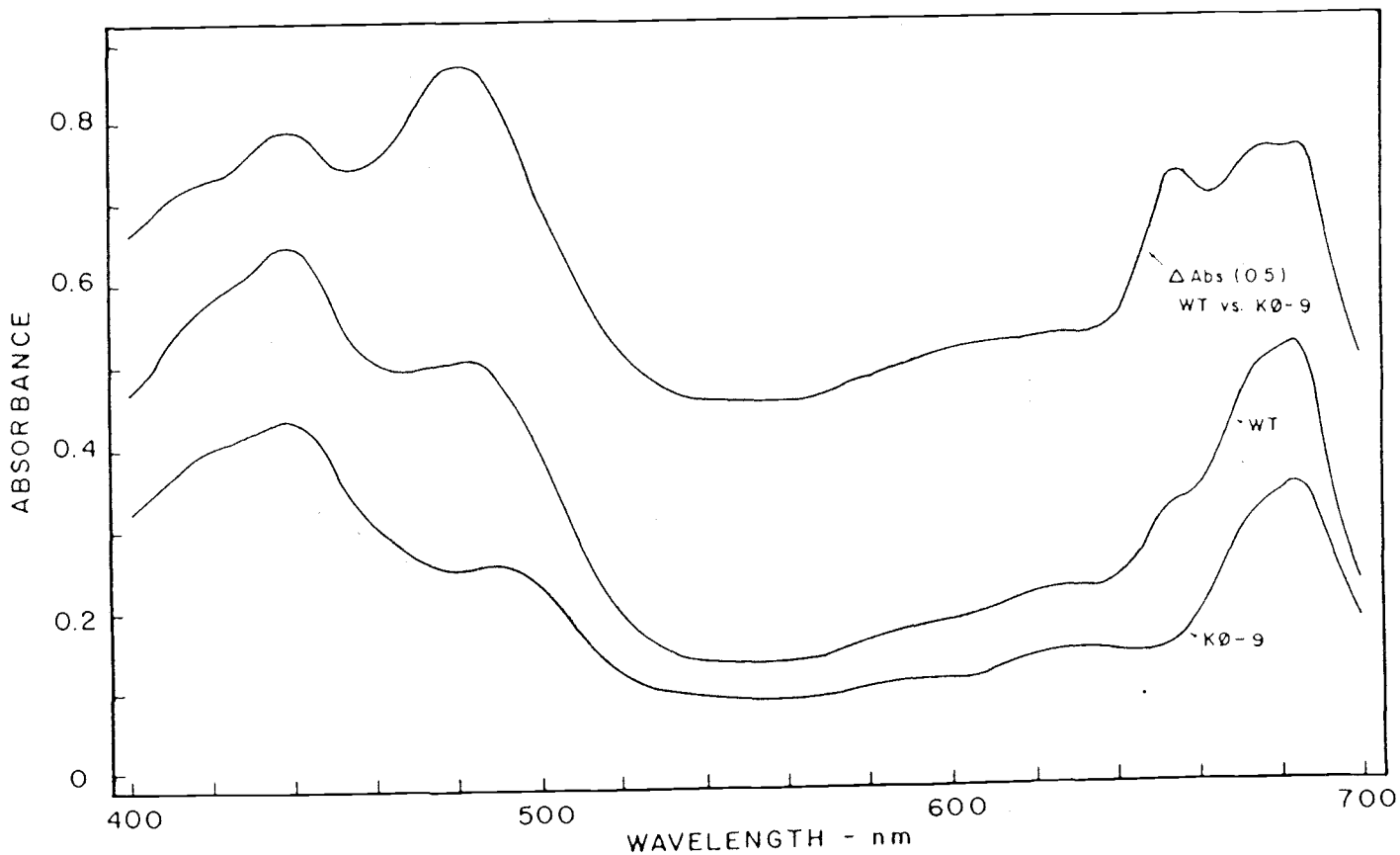

Figure 5. Absorption spectra of whole cells of WT and mutant K0-9 of Scenedesmus obliquus and the absorption difference spectrum of these two strains. Spectral analysis made with the DW-2 spectrophotometer. Packed cell volume = 1 μl/ml.

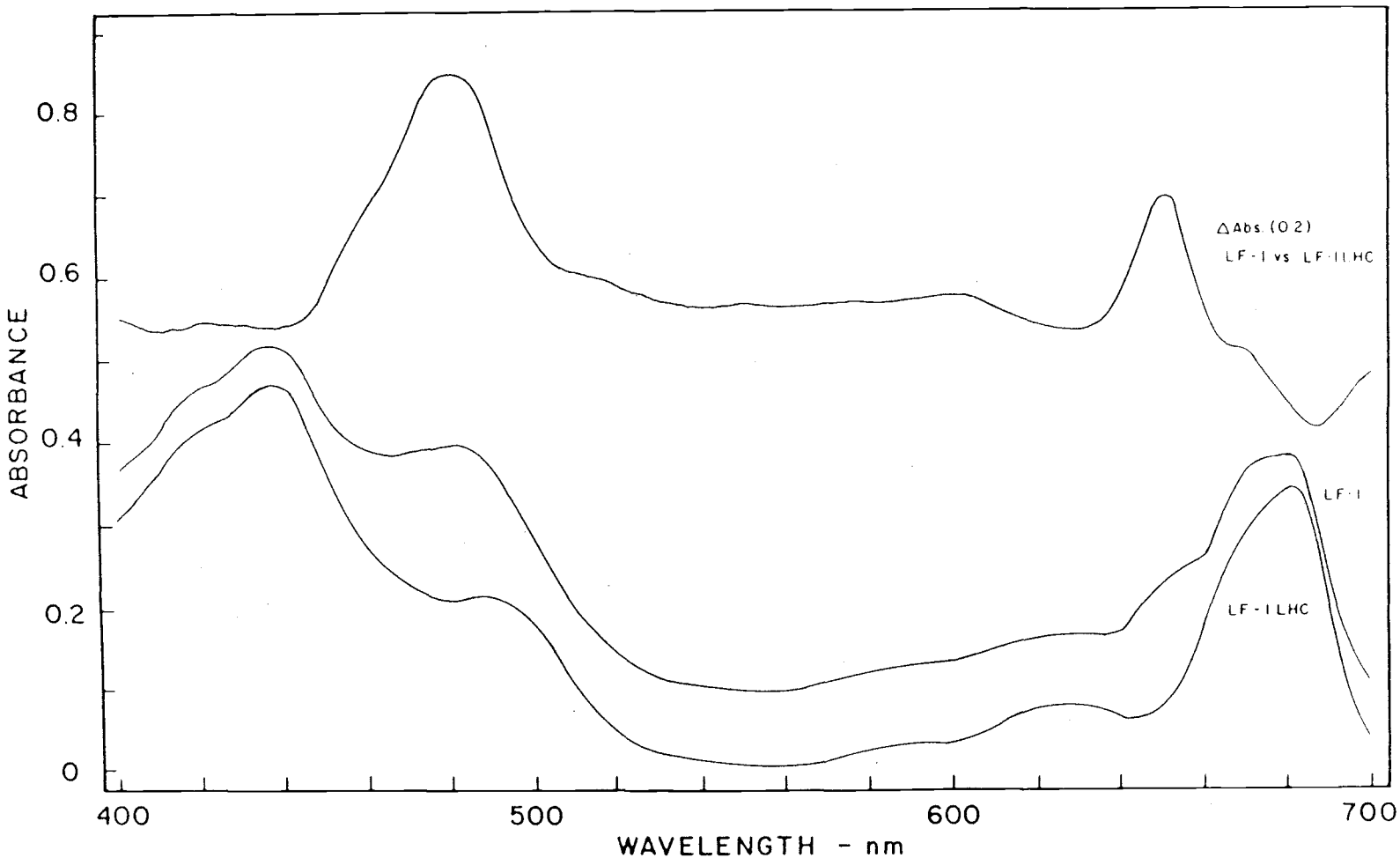

Figure 6. Absorption spectra of whole cells of LF-1 (a mutant strain lacking the water-side of PS-II) and LF-1-LHC (a secondary mutant of LF-1 unable to synthesize chlorophyll b) and the absorption difference spectrum derived from these two strains. PCV = 1 µl/ml. Cells obtained by heterotrophic culture.

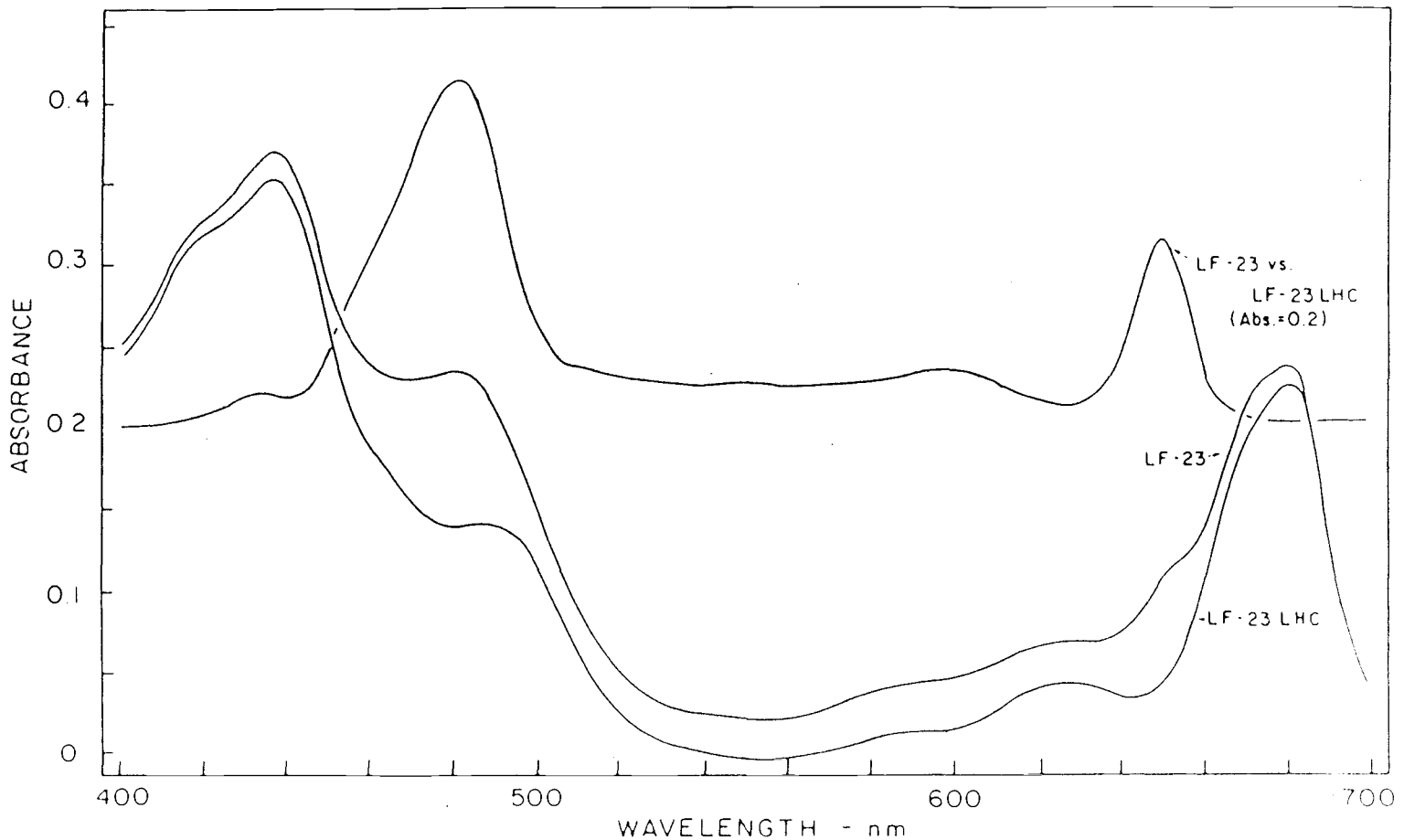

Figure 7. Absorption spectra of whole cells of LF-23 (a mutant strain lacking the reaction center polypeptide, D1) and LF-23-LHC (a secondary mutant of LF-23 unable to synthesize chlorophyll b) and the absorption difference spectrum derived from these two strains. PCV = 1 µl/ml. Cells obtained by heterotrophic culture.

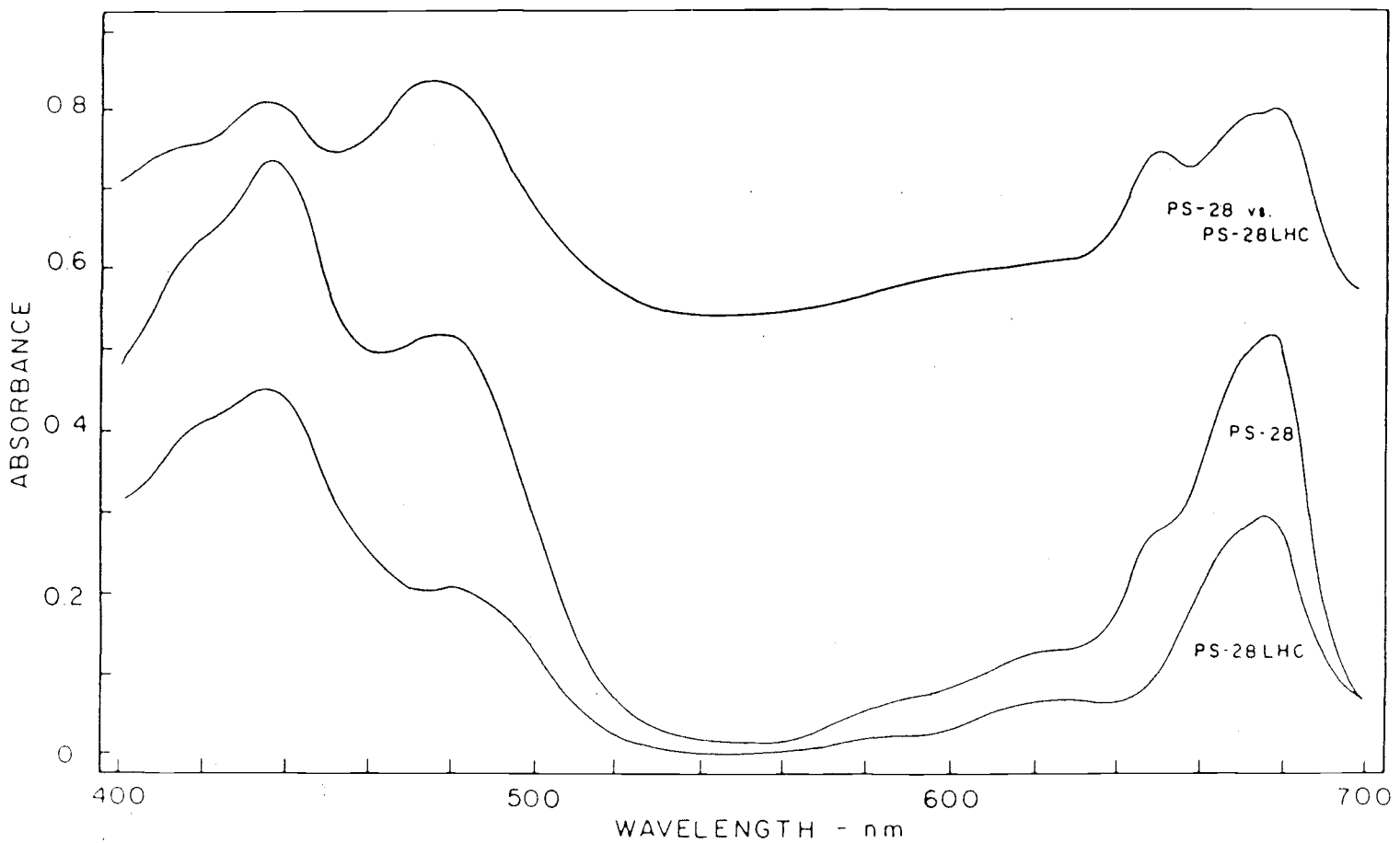

Figure 8. Absorption spectra of whole cells of mutant PS-28 (a mutant strain unable to synthesize phytol and, consequently, normal chlorophyll b) and the absorption difference spectrum of these strains. Cells grown heterotrophically. PCV = 1 µl/ml.

Table 2

Pigment composition of whole cells of the wild-type and the mutant K0-9 grown autotrophically,heterotrophically and mixotrophically.

	moles/100 moles Chlorophyll					
	autotrophic		heterotrophic		mixotrophic	
	WT(6)	K0-9(6)	WT(3)	KO-9(3)	WT(4)	K0-9(4)
Compound						
neoxanthin	5.58±.27	1.69±.07	4.97±.23	2.10±.15	6.02±.36	1.83±.20
loroxanthin	1.50±.13	2.50±.12	3.00±.17	1.50±.14	3.74±.20	3.45±.15
violaxanthin	0.54±.08	5.70±.32	0.95±.08	0.84±.08	1.67±.13	5.70±.24
anthera						
xanthin	0.22	0.29	ND	ND	0.22	0.32
lutein	10.7±.47	9.71±.38	3.17±.19	3.26±.20	9.80±.42	8.62±.37
chlorophyll b	61.5±3.2	0	64.9±2.9	0	48.7±4.6	0
chlorophyll a	100	100	100	100	100	100

The numbers in parenthesis represent the total number of pigment analysis run on each of the culture types. NC = not calculated.

Table 3

Comparison of the pigment composition of heterotrophically grown whole cells of wild-type and various mutant phenotypes deficient in photosynthetic activity and chlorophyll b

	moles/100 moles chlorophyll a						
	WT	PS-28 LHC	LF-1 LHC	LF-15hf-17-LHC	CP-I_a-13 LHC	LF-23 LHC	PS-11 LHC
Compound							
neoxanthin	4.97	0.78	2.10	2.50	2.40	1.90	2.10
loroxanthin	3.00	3.86	5.20	6.20	5.50	4.80	5.70
violaxanthin	0.95	1.20	5.50	4.10	4.30	5.20	4.80
lutein	3.17	1.45	6.10	5.50	5.90	7.00	5.80
chlorophyll a	100	100*	100	100	100	100	100

*This calculation is based on the concentration of the digeranyl-digeraniol derivative of chlorophyll a which is produced in mutant PS-28-LHC.

Table 4

Characterization and identification of pigments noted by high performance liquid chromatography

Peak #	Common Name	Chemical Name	Adsorption Maxima (nm)
1	Neoxanthin	5,5-Epoxy-6',7'-didehyro 5,6,5',6'-tetrahydro-β,β-carotene-3,3',5'-triol	465.5; 436.5; 413
2	Loroxanthin	19-Hydroxy lutein (Trihydroxy-α-carotene)	472.5; 445
3	Violaxanthin	5,6,5',6'-diepoxy 3,3'-dihydroxy-β-carotene	470; 439; 416
4	Antheraxanthin	3,3'-dihydroxy-5,6-epoxy-β-carotene	474; 448.5; 419
5	Lutein	3,3'-dehydroxy-α-carotene	473; 444.5; 419
6	Chlorophyll b		645; 445
	Chlorophyll b'		650; 462.5
	Chlorophyll b digeranyl		645; 445
7	Chlorophyll a		661; 429
	Chlorophyll a'		663; 431
	Chlorophyll a digeranyl		661; 429
8	α-carotene	β,ε-carotene	472; 444; 420
9	β-carotene	β,β-carotene	477; 450

Pigments eluted from the HPLC column were collected, pooled over several runs, concentrated and redissolved in various solvents for spectral characterization. Ethanol was used for the xanthophylls neoxanthin, loroxanthin, antheraxanthin, violaxanthin and lutein, acetone for the chlorophylls and hexane for the carotenes.

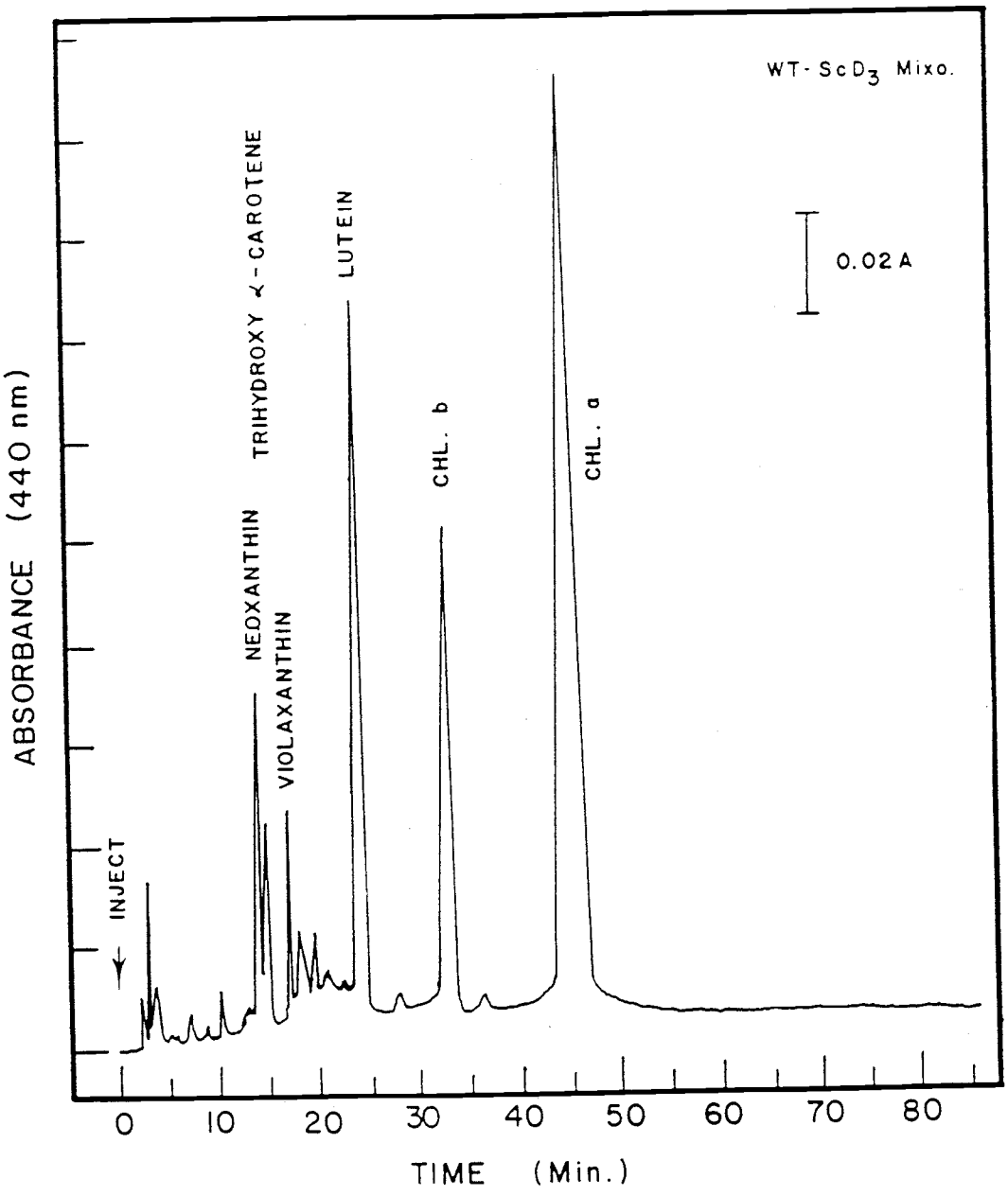

Figure 9. Reversed phase high performance liquid chromatography elution profile of total pigment extract of mixotrophically grown WT cells of Scenedesmus. For this assay the total pigments of 1 ml of cells were extracted according to the procedure outlined (see Methods) and 20 µl of the sample injected onto a LiChrosorb C-18 reverse phase column and eluted according to the described program.

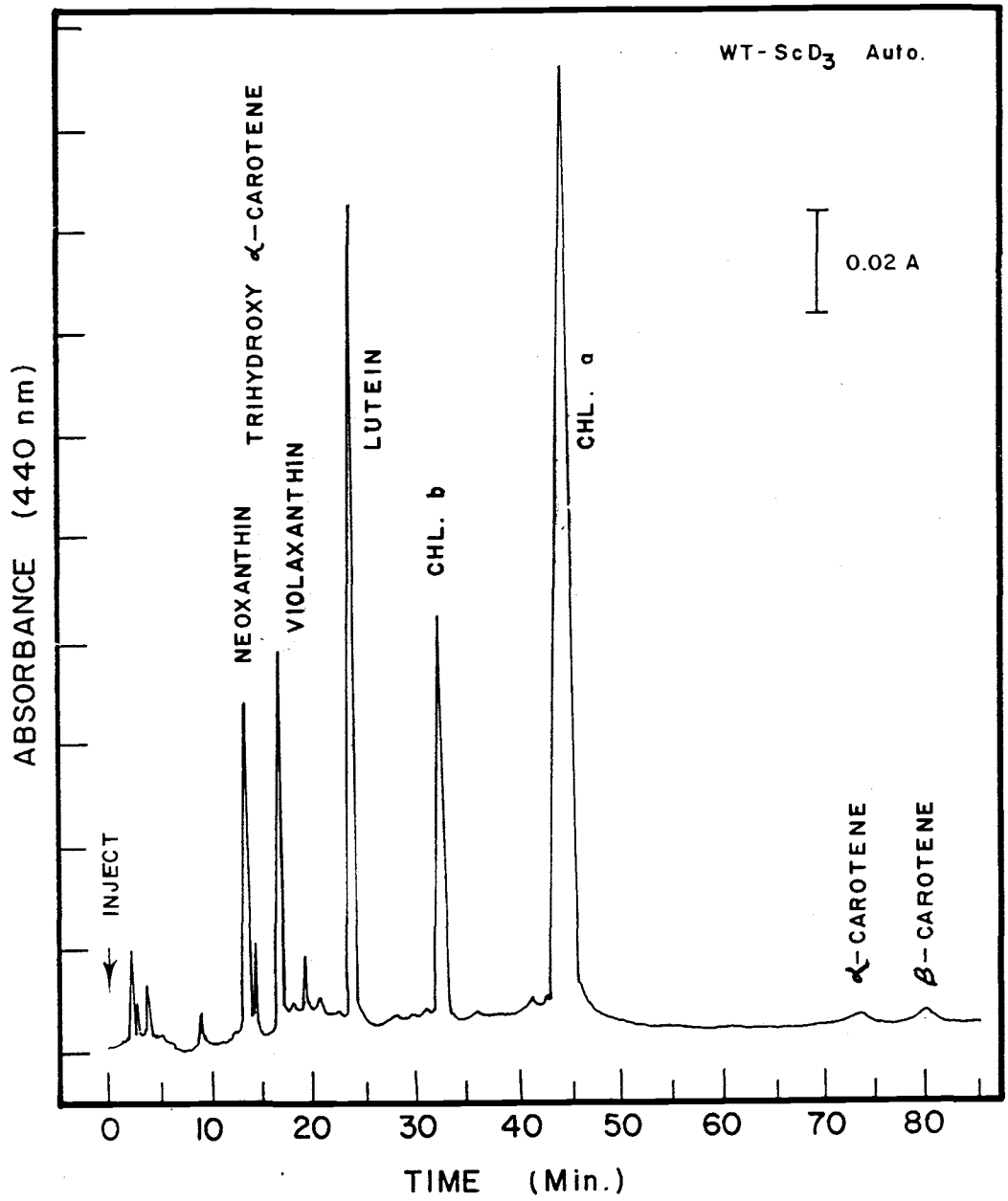

Figure 10. Reversed phase high performance liquid chromatography elution profile of total pigment extract of autotrophically grown WT cells of Scenedesmus. Conditions as indicated in legend of Fig. 9 and in Methods.

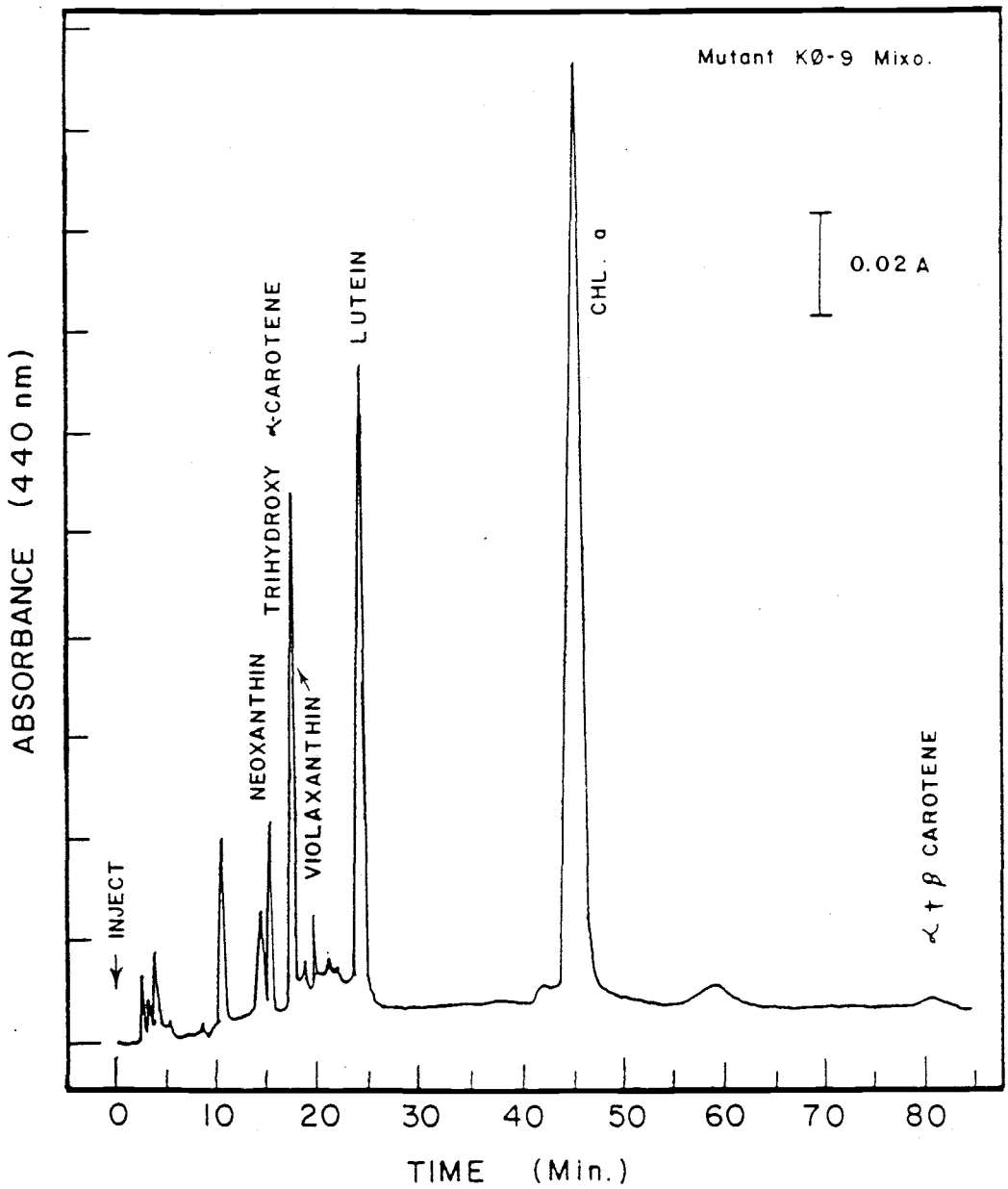

Figure 11. Reversed phase high performance liquid chromatography elution profile of total pigment extract of mixotrophically grown cells of mutant K0-9 of Scenedesmus. Conditions as indicated in legend of Fig. 9 and in Methods.

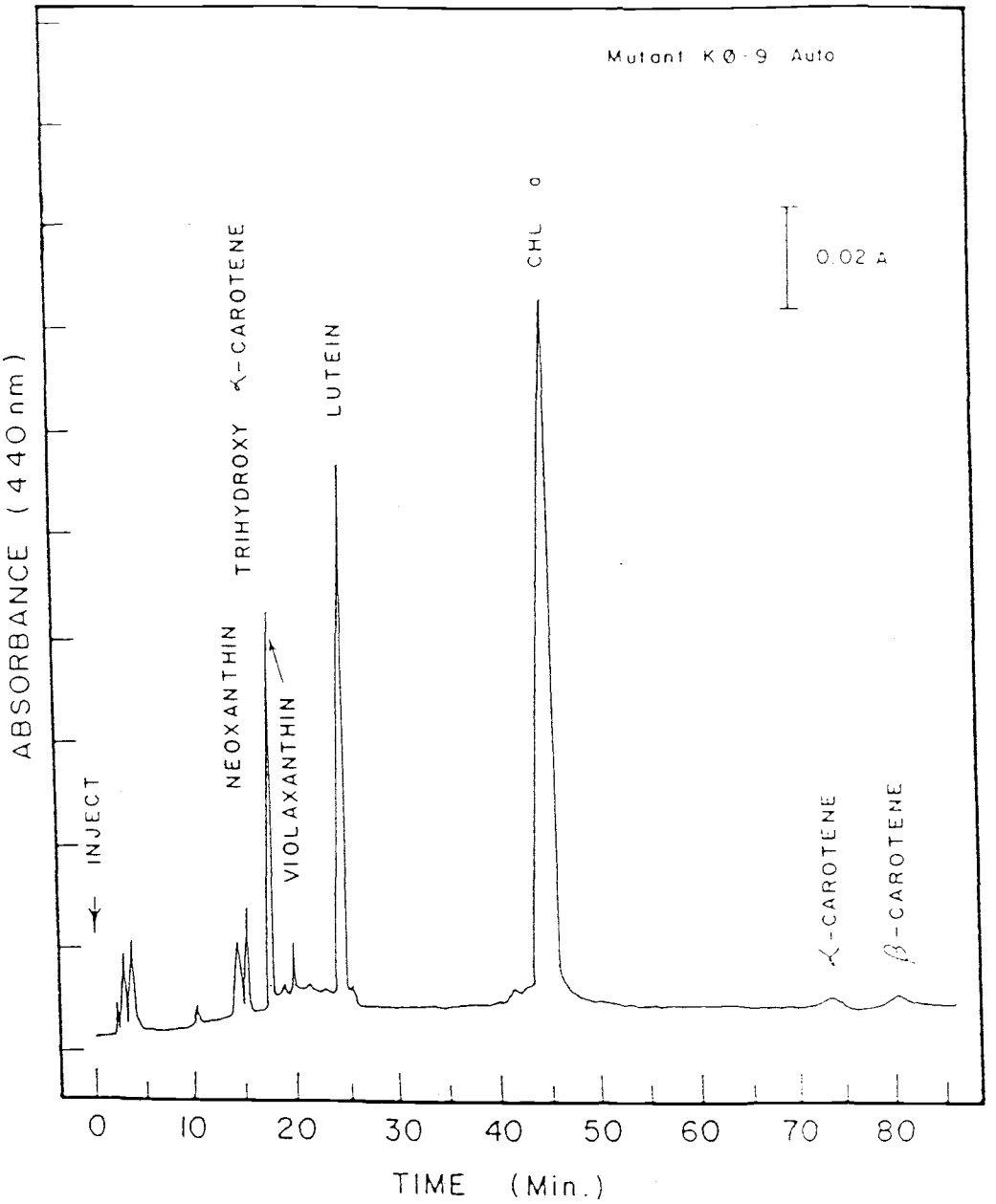

Figure 12. Reversed phase high performance liquid chromatography elution profile of total pigment extract of autotrophically grown cells of mutant K0-9 of Scenedesmus. Conditions as indicated in legend of Fig. 9 and in Methods.

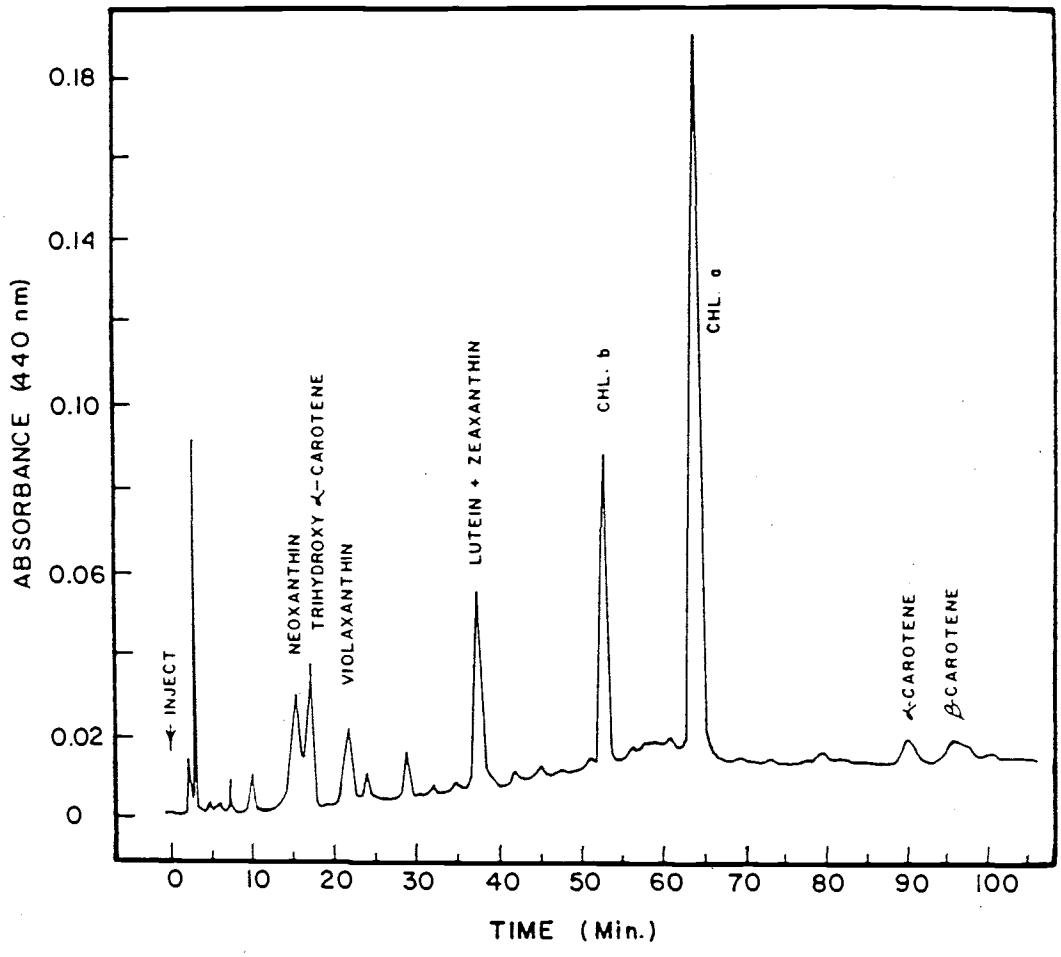

Figure 13. Reversed phase high performance liquid chromatography elution profile of total pigment extract of heterotrophically grown WT cells of Scenedesmus. Conditions as indicated in legend of Fig. 9 and in Methods.

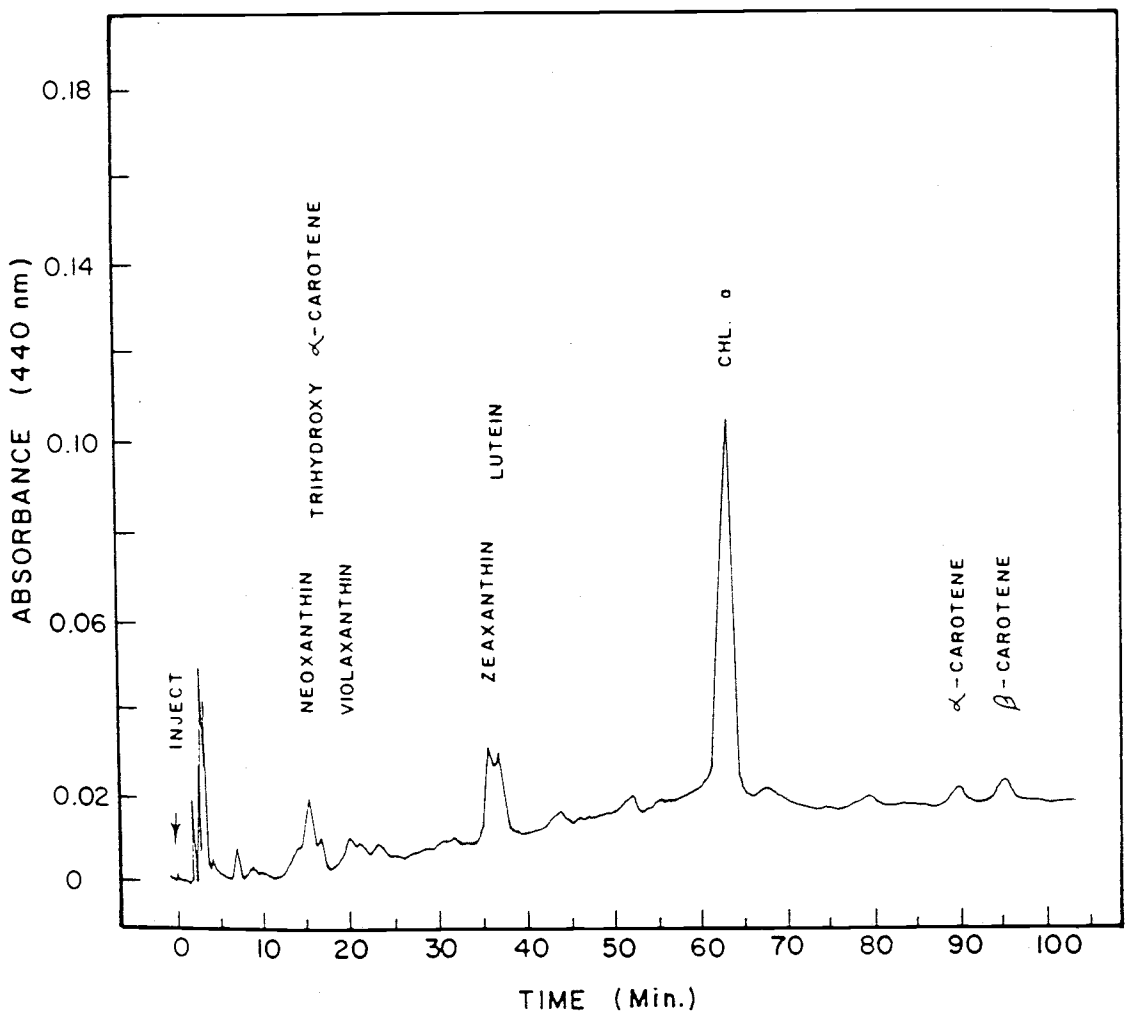

Figure 14. Reversed phase high performance liquid chromatography elution profile of total pigment extract of heterotrophically grown cells of mutant K0-9 of Scenedesmus. Conditions as indicated in legend of Fig. 9 and in Methods.

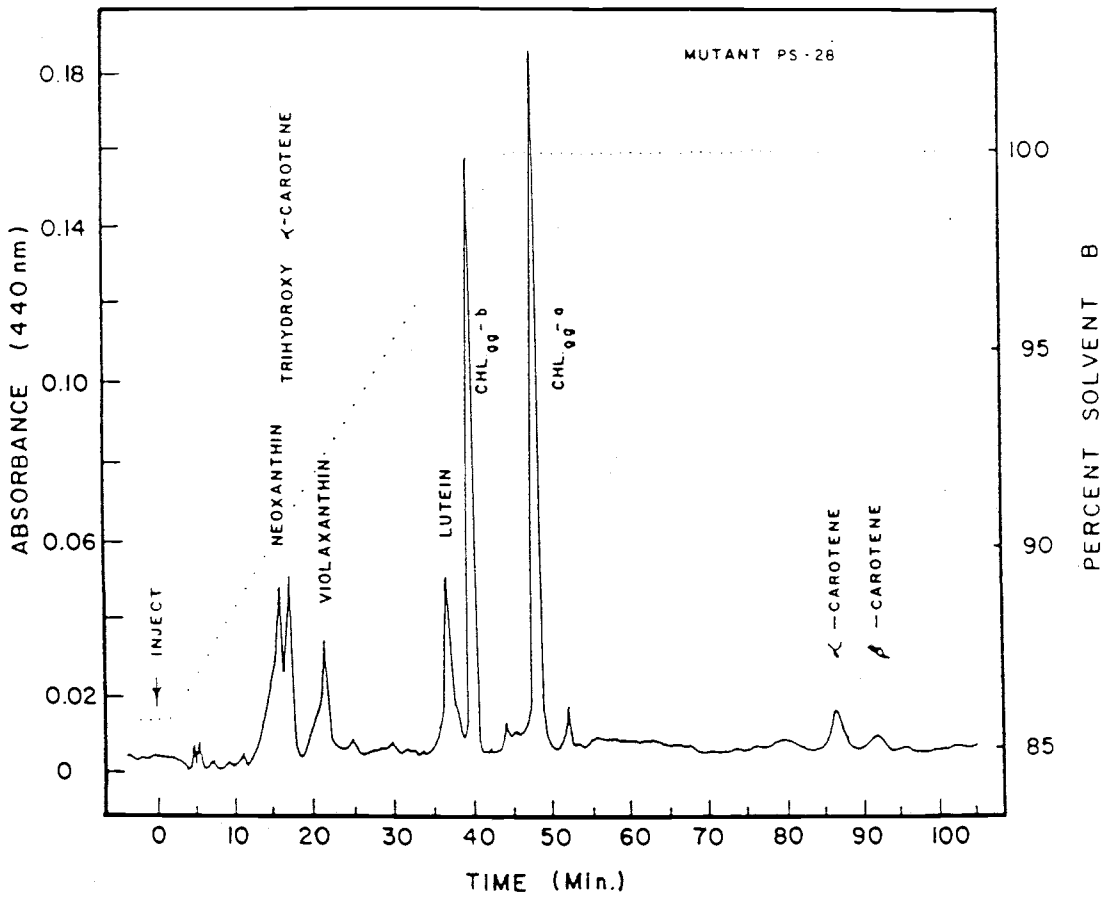

Figure 15. Reversed phase high performance liquid chromatography elution profile of total pigment extract of heterotrophically grown cells of mutant PS-28 of Scenedesmus. Conditions as indicated in legend of Fig. 9 and in Methods. Note the different elution program used for the HPLC run.

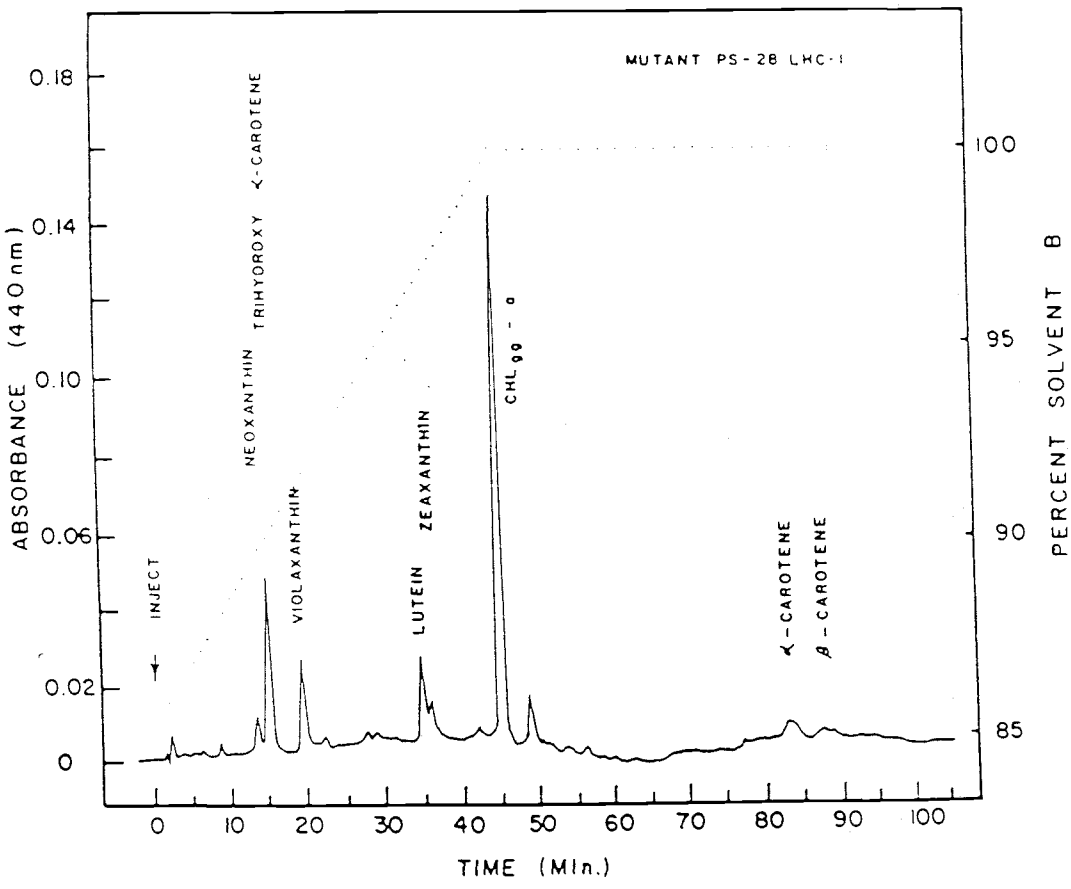

Figure 16. Reversed phase high performance liquid chromatography elution profile of total pigment extract of heterotrophically grown cells of mutant PS-28-LHC of Scenedesmus. Conditions as indicated in legend of Fig. 9 and in Methods. Note the different elution program used as in Fig. 15.

Chloroplast membrane polypeptides of the WT and LHC deficient polypeptides: Polyacrylamide Gel Electrophoresis.

The majority of the proteins associated with the reaction centers and the light-harvesting systems are hydrophobic in nature and are closely associated with the chloroplast membrane. Their separation and identification has been most successfully accomplished by detergent-polyacrylamide gel electrophoresis (PAGE). Those proteins with which this study has been most concerned, i.e., the polypeptides of LHC-II, are also hydrophobic in nature and are normally separated by PAGE as a green complex which under appropriate electrophoretic conditions will separate minimally into two green bands when a non-dissociating gel system is employed. In Figure 17 a typical photographic reproduction of a gel electrophoretic pattern for chloroplast pigment-protein complexes of chloroplasts obtained from the WT and three mutant phenotypes of Scenedesmus is shown. The mutant phenotypes were LF-1, a normal pigmented strain, a secondary mutant of LF-1 lacking the LHC-II, LF-1-LHC-1 and strain K0-9 whose general photosynthetic characteristics were summarized previously (Table I). A general feature of the mutant phenotypes of Scenedesmus deficient in chlorophyll b is the striking absence of the strongly pigmented band of LHC-II. However, in all the chlorophyll b-deficient strains evaluated in this study none of them was found to lose the apoproteins of the complex. The presence of several additional lower molecular weight polypeptides (lanes c and d of Figure 17) for LF-1-LHC and K0-9 compares closely with patterns noted for samples of normal chloroplast membranes which had been heat treated (see Methods)

Figure 17. Polypeptides of thylakoid membranes extracted from dark-grown cells of WT Scenedesmus (lane a), mutant LF-1 (lane b), mutant LF-1-LHC (lane c) and mutant K0-9 (lane d). The samples (250 μg of protein) were separated by LiDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue R250.

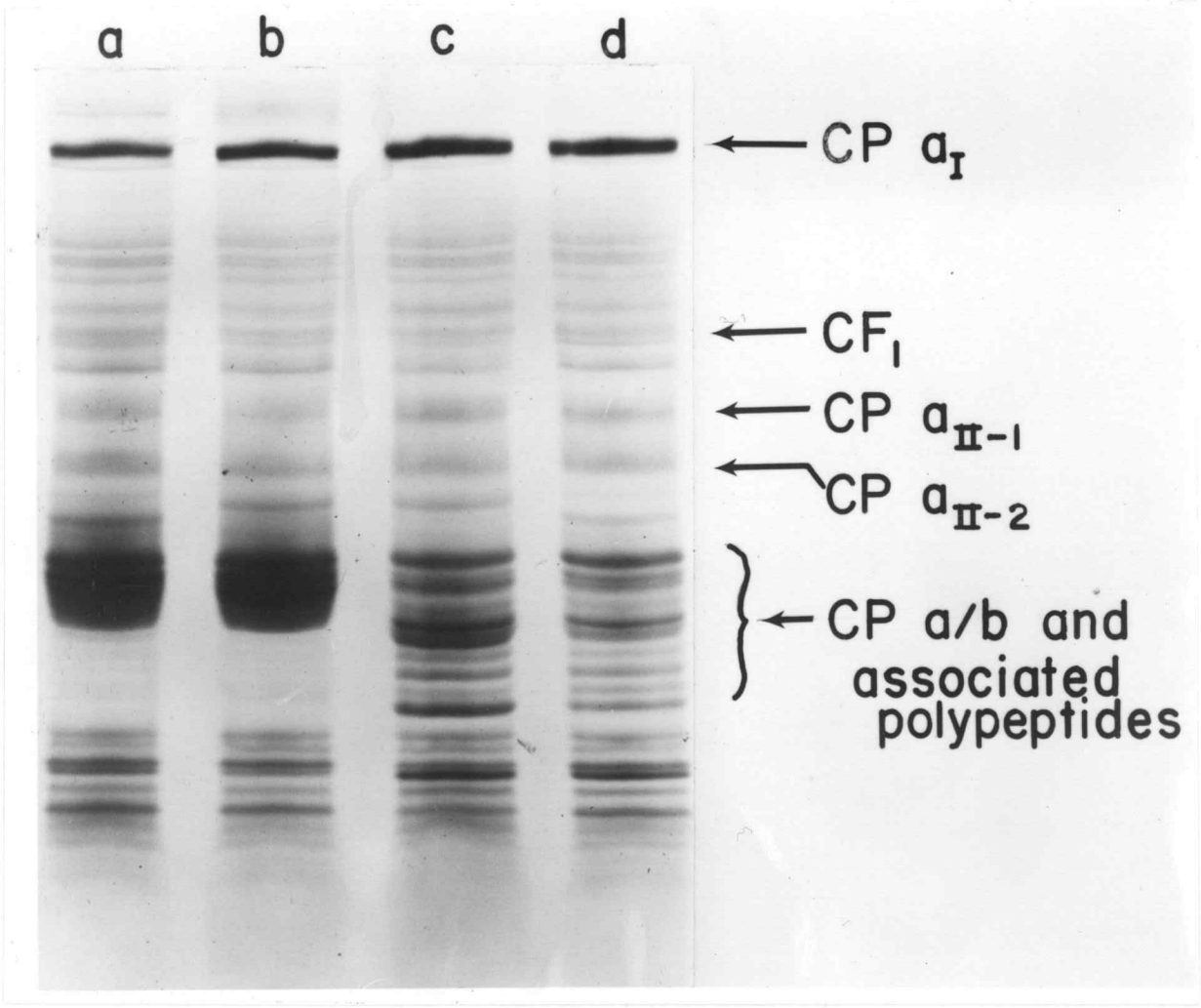
a
b
c
d
CP a_I
CF_1
CP a_{II-1}
CP a_{II-2}
CP a/b and associated polypeptides

Figure 17.

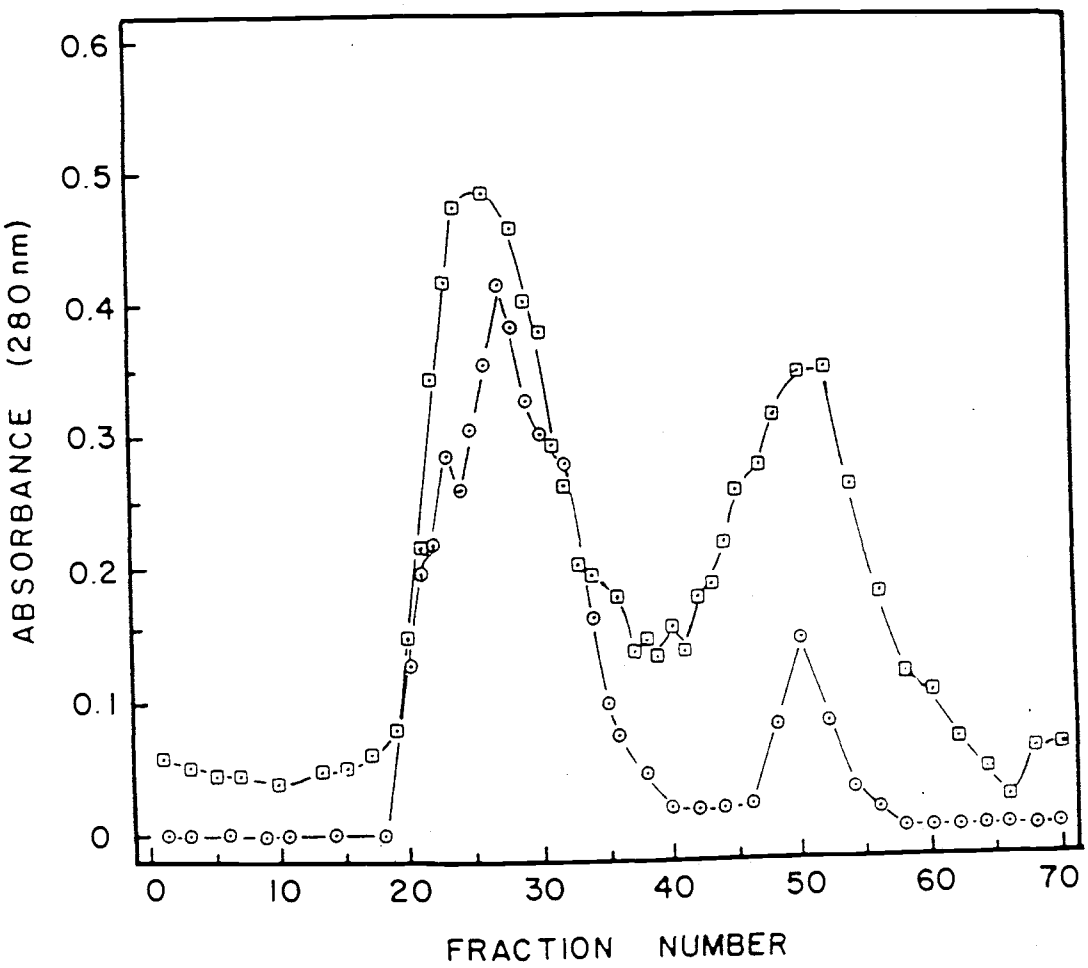

Figure 18. The elution profiles observed when the chloroform/methanol extract of chloroplast fragments (fraction 3) is applied either to a LH-20 (□—□) or a LH-60 column (○—○) and eluted with a 1:1 mixture of C/M. Absorbancy monitored at 280 nm with a Zeiss PMQ-II spectrophotometer. Fractions 20-40 contained primarily carotenoids and traces of chlorophyllin. Tubes 40-60 contained proteins consisting primarily of the 26 and 28 kDa polypeptide of the LHC-II (See Fig. 19).

prior to electrophoresis. This treatment results in the dissociation of the pigment-protein complexes, a more complete saturation of the freedpolypeptides with the anion of the detergent employed (which in turn results in a more precise determination of the electrophoretic mobility of the polypeptides) and a more intensive staining of the denatured protein bands. Evaluation of the area of the gel indicated in Figure 20 as LHC shows that the electrophoretic mobilities of the apoproteins of LHC-II derived from the WT or from the mutants are identical. This suggests that if the mutation resulting in the chlorophyll b-less strains affects either the amino acid composition or sequence, or both, of the apoproteins, which in turn would affect the presumed binding sites of the chlorophylls, such alterations are not detectable by changes in electrophoretic mobilities in the gel system employed.

Partial purification of the LHC polypeptides

To extend the examination of the apoproteins of LHC-II in the normal and chlorophyll-b deficient strains of Scenedesmus it would be essential to have sufficient quantities of them in a moderately purified form which could be used for additional electrophoretic analysis to determine minor changes in molecular weight, for peptide fingerprinting of protease digests or for production of antibodies. As an initial step in such a program it was determined that the apoproteins of LHC-II in each of the phenotypes studies appeared to be identical to those of the WT (see Figure 20) in electrophoretic mobility. Although it is possible to isolate the normal LHC-II of the WT by electroeluting the corresponding green band from sections removed from the polyacrylamide gel prior to staining, this approach for

purifying the apoproteins of the chlorophyll b-deficient strains is not too practical since they are colorless and migrate along with several additional chloroplast membrane polypeptides of similar molecular size.

As an alternate and more direct approach it was attempted to apply the techniques utilized for the purification of intrinsic membrane proteins of bacterial chromatophores and the membrane limited component of the coupling factor (CFo) for partial purification of at least the 26 and 28 kDa polypeptides of the LHC-II. When purified chloroplast membranes were extracted with a 1:1 mixture of chloroform/methanol and the extract fractionated by partitioning and precipitation (see Methods) it was possible to gain a rapid separation of the LHC-apoproteins from the majority of the hydrophobic membrane proteins of the chloroplast. Examination of the various fractions obtained by this procedure by PAGE demonstrated (Figure 19) that the initial precipitate resulting from the addition of an equal volume of distilled water to the original chloroform/methanol extract contained most of the proteins seen in the original chloroplast preparations (Figure 19, lane c). When this precipitate was redissolved in C/M, the LHC-II polypeptides appeared to be preferentially redissolved (Figure 19, lane b) with the remainder retained in the insoluble pellet. These proteins were either recovered by ethyl ether precipitation of the C/M extract or were purified further by column chromatography utilizing Sephadex LH-20.

Further purification of the LHC polypeptides

Application of the chloroform/methanol (1:1) soluble fractions of the water-induced precipitate from the upper phase of the organic solvent extraction of chloroplast membranes of the WT and the various LHC-

deficient phenotypes to a Sephadex LH-20 or LH-60 and elution with chloroform/ methanol (1:1) produced a clear separation of proteins and trace amounts of various pigments. A typical elution profile obtained by this procedure is shown in Figure 18. No clear differences were noted for LH-20 or LH-60 in the separation profiles produced. The initial pigment fraction, accumulating in tubes 20-40, contained no protein as determined by either ethyl ether precipitation attempts or by PAGE analysis of a concentrated sample of this area. Thin layer chromatographic analysis showed the pigments to be primarily carotenoids, with lutein predominating, and chlorophyllin, a water-soluble, dephytolized form of chlorophyll a. Whether the presence of this compound is a result of the procedures employed or whether it demonstrates the normal occurrence of it in algal cultures cannot be determined presently.

The fraction showing appreciable absorption at 280 nm and eluting in fractions 40-60 contained protein precipitable by ethyl ether which upon PAGE analysis was shown to consist primarily of the 26 and 28 kDa polypeptides of the LHC-II and trace amounts of an 8-10 kDa component (Figure 19, lane a and Figure 20, c-g lanes). This last component most likely is the apoprotein of the cytochrome b-559. Comparable elution profiles determined on samples of the chlorophyll b-deficient phenotypes were basically identical (data not presented). LDS-PAGE of the protein fractions of four LHC-deficient phenotypes purified by LH-20 chromatography demonstrated that the 26 and 28 kDa components of the LHC-II appeared unaltered (Figure 20).

It is evident that the combined procedures of organic solvent extraction and partitioning and Sephadex LH-20 chromatography results in

Figure 19. Chloroplast membrane polypeptides of WT Scenedesmus noted in various steps in the chloroform/methanol extraction and purification procedures described in the Method section. Individual columns are (from right to left): molecular weight markers, lane e; C/M solubilized polypeptides from interphase precipitate collected in initial centrifugation of C/M extract, lane d; polypeptides contained in the upper phase, lane c; polypeptides solubilized by C/M extraction of the water precipitate of the upper phase, lane b; and polypeptides obtained through LH-20 chromatography (Fig. 18) of this last fraction, lane a. The samples were separated by LiDS-PAGE as described in Methods and in the legend of Fig 17

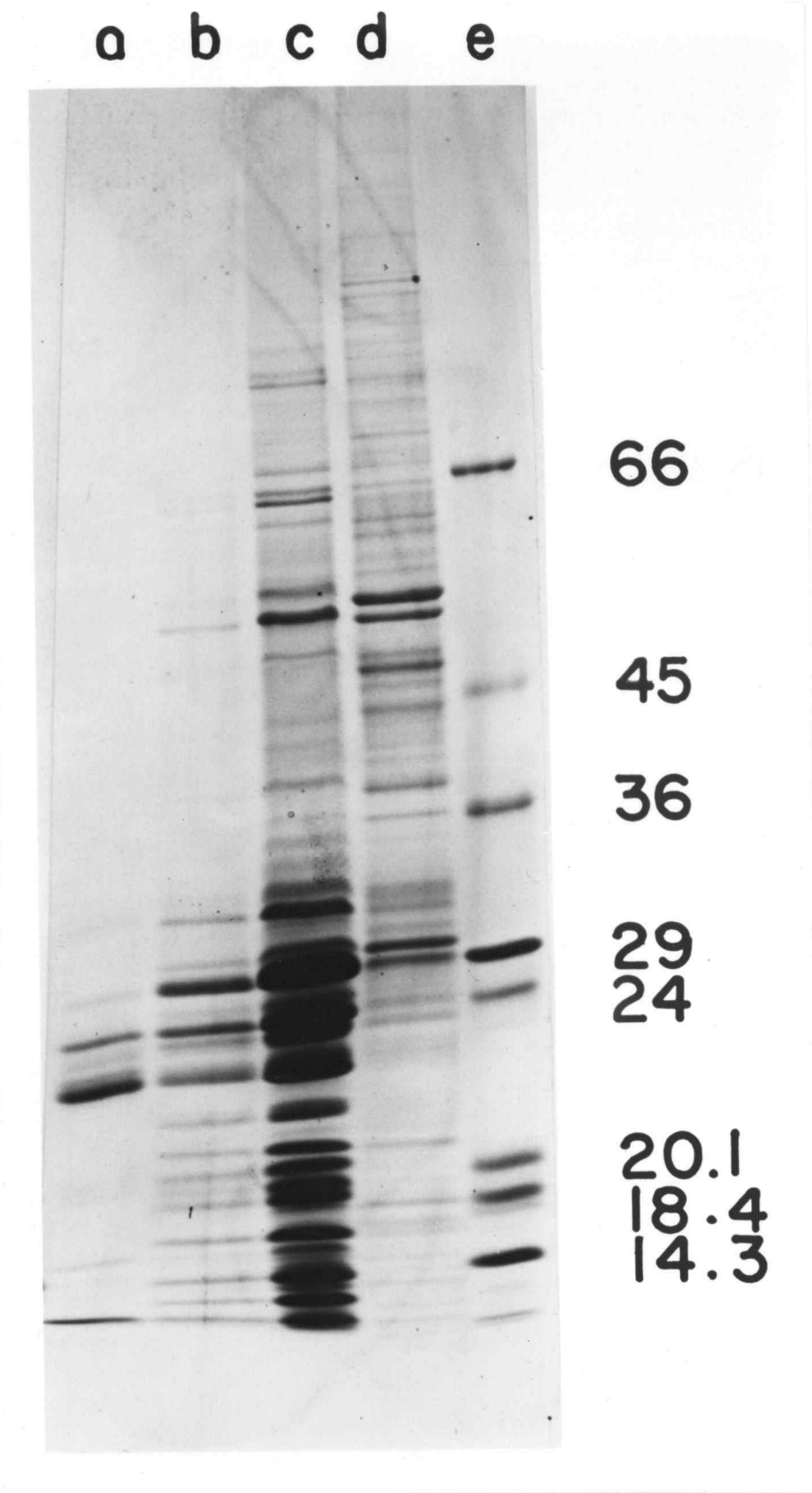

Figure 19.

Figure 20. LiDS-PAGE polypeptide profiles of the LHC-II apoproteins of several chlorophyll-b deficient strains of Scenedesmus purified by organic solvent extraction and Sephadex LH-20 chromatography. Samples are total chloroplast polypeptides of the WT and mutant LF-23 (250 μg protein), lanes a and b; purified samples (15 μg protein) of the LHC-II apoproteins of mutants LF-1-LHC (lane c), K0-9 (lane d), LF-15-hf-17-LHC (lane e), PS-28-LHC (lane f) and of the WT (lane g). Electrophoresis was performed as indicated in Methods.

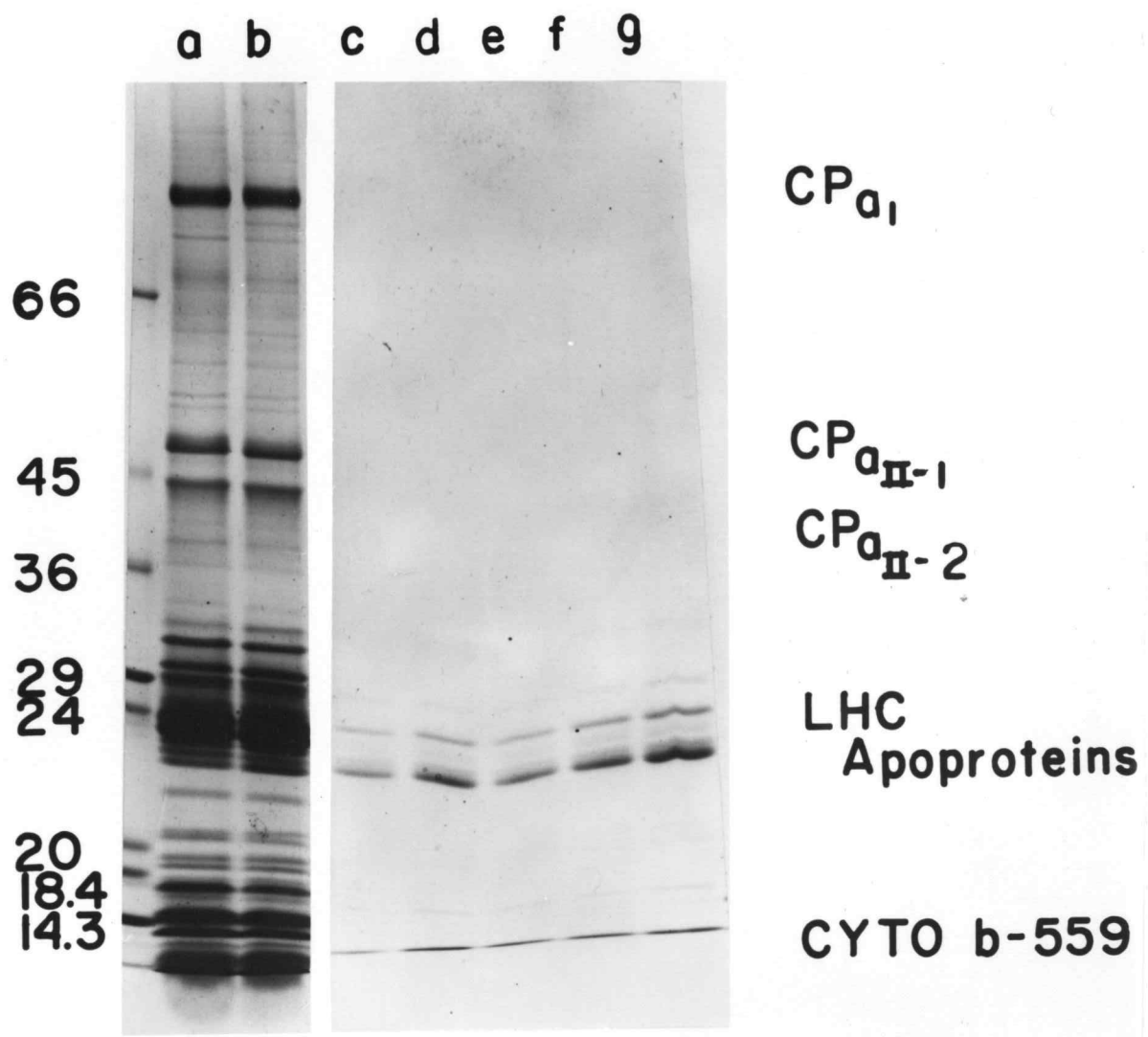
a b c d e f g
66
45
36
29
24
20
18.4
14.3
CP a I
CP a II-1
CP a II-2
LHC
Apoproteins
CYTO b-559

Figure 20.

significant purification of the two major apoproteins of LHC-II. However, because of the known similarities (e.g., amino acid sequence, molecular weights, C/M solubilities) of the two polypeptides the procedures as applied, did not allow for their complete separation.

Attempted purification of LHC polypeptides with HPLC: Reversed Phase Separation

Application of reversed-phase, high-performance liquid chromatography with trifluoracetic acid-acetonitrile gradient systems for separation of soluble peptides (Winkler, et al., 1985) and for purification of the 8 kDa hydrophobic subunit of the energy coupling complex (Tandy, et al., 1983) had been a successful approach to resolving some difficult preparatory procedures. In this study, the attempts made to separate the 26 and 28 kDa polypeptides of the LHC-II with similar techniques were not successful. Contrary to our initial expectations it was noted that when the fraction purified by Sephadex LH-20 chromatography was applied to the HPLC column and solvent systems selected the sample passed through the column just behind the void volume. PAGE examination of this sample showed some removal of impurities, i.e., the sample consisted almost exclusively of the 26 and 28 kDa polypeptides, but no increased separation of the LHC-II polypeptides. The extreme hydrophobic nature of these polypeptides and the fact that the initial purification procedures were highly denaturing are probable major factors in the noted non-retention. Available time and money to continue this portion of the study precluded any further modifications of the procedure.

CONCLUSIONS

The examination of the several LHCP-II-deficient mutants of Scenedesmus obliquus in this study has provided sufficient new information which allows some tentative conclusions about them in four general areas: (1) the modification of the carotenoid pool components, with decreased levels of neoxanthin and increased levels of violaxanthin, accompanies the mutation syndrome, (2) the inability to synthesize chlorophyll b is not always accompanied by a comparable decrease in levels of chlorophyll a of the LHCP, (3) the mutation in Scenedesmus does not involve changes in thylakoid levels of the principal apoproteins of LHCP-II and (4) the 26 and 28 kDa apoproteins of LHC-II are not altered by the mutation sufficiently to cause major changes in electrophoretic mobility.

Modification of the carotenoid pool

Since the earliest study on the chlorophyll b-deficient mutant of barley (Thornber and Highkins, 1974), in which it was reported that the loss of chlorophyll b was accompanied by specific changes in the total carotenoid pool, all subsequent studies on other comparable mutants of vascular plants and green algae (Henry, et al., 1983; Michel, et al., 1983; Plumley, et al., 1985) have reported the same relationship. The data presented in Figures 9-12 and Table III for studies on the pigment composition of normal WT and mutant K0-9 grown both autotrophically and heterotrophically confirm and extend the earlier observations by showing the preferential decrease of neoxanthin and increased levels of violaxanthin in K0-9. This feature was also noted in all other mutant strains of

Scenedesmus examined in this study (Table IV) although the results were somewhat less dramatic when it was necessary to culture the mutants under heterotrophic conditions. A major question arises then to relate this general feature of chlorophyll b-deficient phenotypes to their inability to synthesize (or accumulate) either one or both chlorophyll types of the LHC-II. Since the exact mechanisms for the biosynthesis of chlorophyll b and neoxanthin are currently unknown it is difficult to speculate about any interrelationship between the two pigments which would cause a mutual inhibition. Plumley and Schmidt (1987) suggest that their observed requirement for xanthophylls in reconstitution of the LHCP-II from denatured apoproteins of this complex can be explained by assuming that the xanthophylls, particularly neoxanthin, are intrinsic components of the complex and during reconstitution facilitate refolding of the denatured apoproteins by binding to a specific recognition site within, or on, the folded protein chains. The absence, or modification, of this site, could lead to the decreased level of neoxanthin and, concurrently, prevent the proper folding of the apoprotein and binding of newly synthesized chlorophyll b. This interpretation suggests that apoproteins of the LHCP-II-deficient mutants might lack this binding site and exist, consequently, in the thylakoid membrane in a partially denatured form. Also isolated apoprotein fractions of these strains, in contrast to those of the WT, should not reconstitute to a "normal" LHCP-II. Although reconstitution experiments were not attempted as part of this study, further work along this line would be required to examine this hypothesis. Such in vitro assays with genetically altered LHCP-II apoproteins should facilitate the identification of individual pigment binding sites.

Degree of inhibition of chlorophyll a synthesis in LHCP-II deficient phenotypes

Because it is well established that LHCP-II binds both chlorophylls in approximately equal amounts, it is easily assumed that mutant phenotypes lacking chlorophyll b should also be deficient in the chlorophyll a of the complex. Polyacrylamide gel electrophoresis analysis of membrane fractions of the various mutant phenotypes supports this concept by demonstrating the general absence of the LHCP-II as was shown in Figure 17 for mutants LF-1-LHC and K0-9. By utilizing a less destructive in vivo, analytical system of absorption difference spectra analysis, it was shown (Figures 5-8) that two general types of LHCP-II mutants, one lacking both chlorophylls (as typified by the K0-9 strain) and the other lacking primarily chlorophyll b (strains LF-1 and LF-23) were present in the mutant collection. (Evidence for a chlorophyll b-less LHCP mutant of barley was found also by Duranton and Brown, 1987). Two related interpretations may explain this observation: (1) different binding sites for the two chlorophylls exist in the LHCP-II apoproteins and are preferentially affected in the two mutant forms; (2) the presumed binding sites for the xanthophylls (see above) are less drastically reduced in one type (+-chlorophyll a) than the other. The carotenoid contents of the two mutant types (Table III) do not show significantly different patterns, i.e., both groups show decreased levels of neoxanthin and increased amounts of violaxanthin. However, the limited data available do not allow an extensive interpretation along this line. Whether specific binding sites exist might be resolved through reconstitution studies mentioned previously or through amino acid

sequence determination of representative LHCP-II apoproteins of the two mutant types.

LHCP-II apoprotein synthesis in chlorophyll b-deficient phenotypes

The apoproteins of LHCP-II are encoded through the nuclear genome, synthesized on cytoplasmic ribosomes, released as soluble pre-proteins and processed through a series of clipping enzymes prior to incorporation into the thylakoid membrane. During the final stages of this process the requisite pigments are added and the mature chloroplast is formed. Obviously this involved procedure offers a multitude of potential sites for mutation which would result in a "chlorophyll b-deficient" phenotype. In the original and subsequent studies on chlorophyll b-less strains of higher plants (Thornber and Highkins, 1974; Peter and Thornber, 1988) it was recognized that the LHCP-II apoproteins were not present in isolated thylakoid membranes and the apparent inhibition of their synthesis was thought to be the major cause of the mutation. Subsequent studies (see Green, 1988 for review) showed that the low level or absence of these polypeptides was caused by a rapid turnover of the messenger RNAs for the apoproteins. All of the LHCP-II-deficient strains examined in this study were shown to contain normal levels of the apoproteins (Figures 17 and 18). In studies conducted to date on comparable mutants in other algae, notably Chlamydomonas reinhardtii, similar results have been obtained (Eichenberger, et al., 1986; Michel, et al., 1983). Whether this difference characterizes mutants from higher plants and the algae cannot be decided. However, for the Scenedesmus mutants it is concluded that the

ability of the various phenotypes examined to synthesize the LHCP-II apoproteins is not the determining factor.

Alteration of the 26 and 28 apoproteins of LHCP-II detectable by changes in electrophoretic mobility

As discussed previously, none of the chlorophyll b-deficient strains examined could be characterized by the loss or absence of a LHCP-II-polypeptide. Additionally, PAGE analysis of thylakoids (Figure 17) or of partially purified LHCP polypeptides (Figure 18) derived from these strains never revealed a form of one of the associated polypeptides with an apparent altered molecular mass (i.e., change in electrophoretic mobility). Such an alteration could arise from a genetically-induced change in amino acid composition of one of the polypeptides, thus altering the net electric charge of the polypeptide, or alteration of the post-translational processing of the pre-proteins such that an unprocessed segment, with larger mass, could be incorporated into the chloroplast membrane. Although both mechanisms could result in altered xanthophyll- or chlorophyll-binding sites, with the resulting failure of the chloroplast to accumulate chlorophyll b, no evidence for such changes were noted.

A two dimensional model of the 28 kDa polypeptide of LHCP-II possessing four hydrophobic domains within the thylakoid membrane is presented in Figure 21. This model is similar to that described by Anderson and Goodchild (1987) and is based on the amino acid sequence deduced from the nuclear gene sequence and from agglutination studies with an antiserum generated against a synthetic octapeptide corresponding to the carboxyl-terminus amino acid sequence. In other chlorophyll-binding

proteins, such as the photosynthetic bacteria reaction center complex and the photosystem II reaction center complex, the amino acid, histidine, is involved in the chlorophyll-binding sites. Nearly half of the total chlorophyll of a chloroplast is bound to the LHCP-II and the concentration of histidine in the complex is inadequate to account for this concentration of chlorophyll. As a consequence other amino acids, principally, asparagine and glutamine, have been suggested as possible chlorophyll binding sites. The arrows in the model indicate the various conserved, amino acids that might fulfill this role; if all of them, some twelve, were involved in pigment binding sufficient chlorophyll could be bound to accomodate the above calculation. Where in this model would one expect to locate carotenoid-binding domains whose alteration would influence the binding of chlorophyll a and b? Certainly within the hydrophobic domains but, otherwise, the question begs an answer.

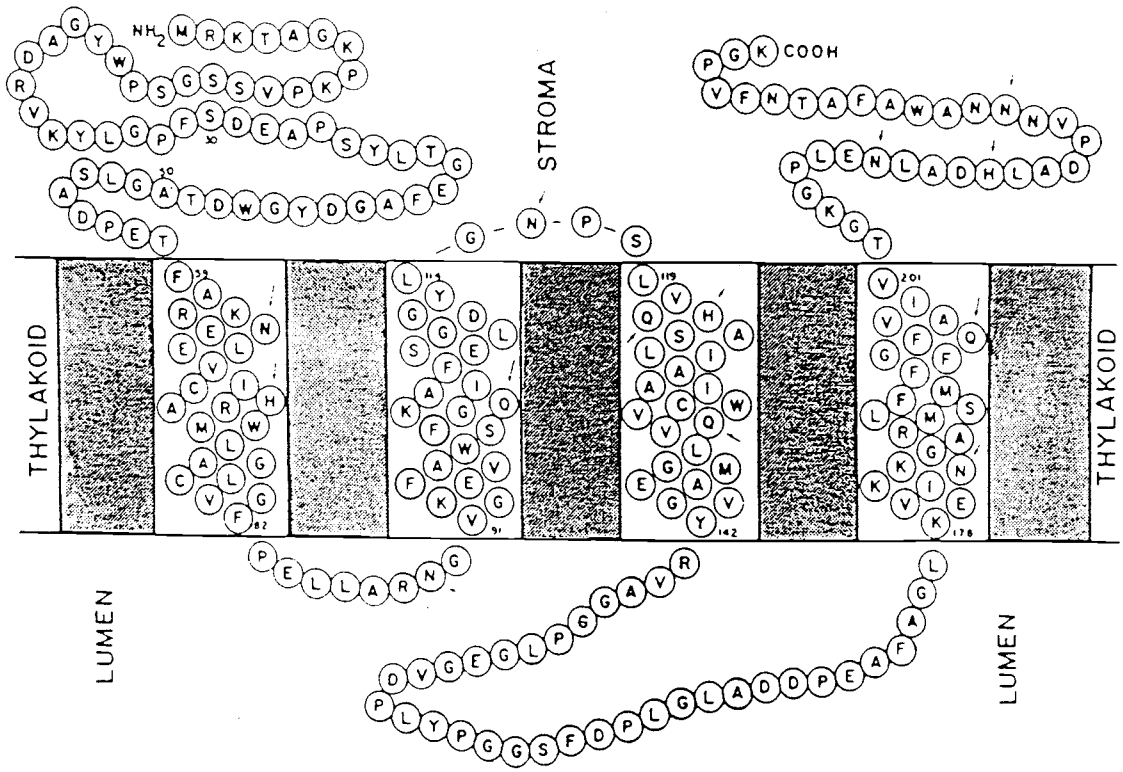

Figure 21. A two dimensional model of the 28 kDa polypeptide of the LHC-II indicating a structure containing four hydrophobic domains being in the stroma phase. Arrows indicate the various amino acids that might be involved in the binding of chlorophyll. The amino acid sequence presented is that deduced from the nuclear gene sequence.

BIBLIOGRAPHY

Anderson, J. M. and D. J. Goodchild. 1987. Transbilayer organization of the main chlorophyll a/b-protein of photosystem II of thylakoid membranes. FEBS Lett. 213:29-33.

Apel, K. and K. Kloppstech. 1978. The Plastid Membranes of Barley (Hordeum vulgare). Eur. J. Biochem. 85:581-588.

Apel, K. and K. Kloppstech. 1980. The effect of light on the biosynthesis of the light-harvesting chlofophyll a/b protein. Planta. 150:426-430.

Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. Plant Physiol. 24:1-15.

Barrett, J. and J. M. Anderson. 1980. The P-700 chlorophyll a-protein complex and two major LHCs of Acrocarpia paniculata and other brown seaweeds. Biochim. Biophys. Acta 590:309-323.

Bennet, J., G. I. Jenkins, A. C. Cuming, R. S. Williams and M. R. Harting. 1984. Photoregulation of thylakoid biogenesis: The case of the light-harvesting chlorophyll a/b complex. In: Chloroplast Biogenesis (Ellis, D., ed.). Cambridge University Press. pp 167-192.

Berzborn, R. J. and N. I. Bishop. 1973. Isolation and properties of chloroplast particles of Scenedesmus obliquus D3 with high photochemical activity. Biochim. Biophys. Acta 292:700-714.

Bishop, N. I. 1971. Preparation and properties of mutants: Scenedesmus: In: Methods in Enzymology (San Pietro, A., ed.) 23A:130-143. Academic Press, New York.

Bishop, N. I. and H. Senger. 1971. Preparation and photosynthetic properties of synchronous cultures of Scenedesmus. In: Methods in Enzymology (San Pietro, A., ed.) 23A:53-66. Academic Press, New York.

Bishop, N. I. and G. Oquist. 1980.. Correlation of the photosystem I and II reaction center chlorophyll-protein complexes, CP-aI and CP-aII, with photosystem activity and low temperature fluorescence emission properties in mutants of Scenedesmus. Physiol. Plant. 49:477-486.

Bishop, Norman I. 1982. Isolation of Mutants of Scenedesmus obliquus defective in Photosynthesis, Methods in Chloroplast

Molecular Biology, Edelman, et al (eds.), Elsevier Biomedicas Press, pp 51-63.

Bogorad, L. 1976. Chlorophyll biosynthesis. In: Chemistry and Biochemistry of Plant Pigments (Goodwin, T. W., ed.) Vol. 1, Academic Press, London. pp 64-148.

Boyan, B. D. and Y. Clement-Cormier. 1984. Organic solvent extraction of membrane proteins. In: Membranes, Detergents and Receptor Solubilization (Venter, J. C., and Harrison, L. C., eds.) Alan R. Liss, Inc. New York. pp 47-64.

Braumann, Thomas, Horst L. Grimme. 1981. Reversed-phase high-performance liquid chromatography of chlorophylls and carotenoids. Biochim. Biophys. Acta 637:8-17.

Britton, G. and T. W. Goodwin. 1981. Carotenoid Chemistry and Biochemistry. Pergamon Press. 399 pages.

Britton, G. 1983. Carotenoids. In: The Biochemistry of Natural Pigments. Cambridge University Press, Chapter II, 23-73.

Britton, George. 1986. Biosynthesis of chloroplast carotenoids. In: Regulation of Chloroplast Differentiation, Vol II, (Akoyunoglou and Senger, ed.), Alan R. Liss, Inc., pp 125-134.

Cuming, A. C. and J. Bennett. 1981. Biosynthesis of the light-harvesting chlorophyll a/b protein: Control of messenger RNA activity by light. Eur. J. Biochem. 118:71-88.

Delepelaire, P. and N.-H. Chua. 1979. Lithium dodecyl sulfate polyacrylamide gel electrophoresis of thylakoid membranes at 4°C: Characterizations of two additional chlorophyll a protein complexes. Proc. Natl. Acad. Sci. USA 76:111-115.

Delepelaire, P. and N.-H. Chua. 1981. Electrophoretic purification of chlorophyll a/b protein complexes from Chlamydomonas reinhardtii and spinach and analysis of their polypeptide composition. J. Biol. Chem. 256:9300-9307.

Dogbo, O., A. Laferriere, A. D'Harlingue, and B. Camara. 1988. Carotenoid biosynthesis: Isolation and characterization of a bifunctional enzyme catalyzing the synthesis of phytoene. Proc. Natl. LAcad.. Sci. USA 85:7054-7058.

Duranton, J. and J. Brown. 1987. Evidence for a light-harvesting chlorophpyll b-less barley mutant. Photosynth. Res. 11:141-151.

Dunsmuir, P., S. M. Smith and J. Bedbrook. 1983. J. Mol. Appl. Genet. 2:285-300.

Dunsmuir, P. 1985. The petunia chlorophyll a/b protein genes: a comparison of Cab genes from different gene families. Nucleic Acid Res. 13:2503-2518.

Eichenberger, W., Boschetti, A. and H.P. Michel. 1986. Lipid and pigment composition of a chlorophyll b-deficient mutant of Chlamydomonas reinhardtii. Physiol. Plant. 66:589-594.

Ellis, R. J. 1981. Chloroplast proteins: synthesis, transport and assembly. Ann. Rev. Plant Physiol. 32:11-37.

Glajch J. L., J. J. Kirkland and J. Kohler. 1987. Effect of column degradation on the reversed-phase high-performance liquid chromatographic separation of peptides and proteins. Journal of Chromatography 384:81-90.

Glazer, A. N. 1982. Phycobilisomes: structure and dynamics. Ann. Rev. Microbiol. 36:173-198.

Glazer, A. N. 1983. Comparative biochemistry of photosynthetic light-harvesting systems. Ann. Rev. Biochem. 52:125-157.

Green B. R. 1988. The chlorophyll-protein complexes of higher plant photosynthetic membranes of just what green band is that: Photosynthesis Research 15:3-32.

Griffith, W. T. 1978. Reconstitution of chlorophyllide formation by isolated etioplast membranes. Biochem. J. 174:681-692.

Griffith, W. T. and R. P. Oliver. 1985. Protochlorophyllide reductase: structure, function and regulation. In: Chloroplast biogenesis (Ellis, R. J., ed.). LCambridge University Press. pp 245-258.

Heide, L. and U. Berger. 1989. Partial purification and properties of geranyl pyrophosphate synthase from Lithospermum erythrorhizon cell cultures. Arch. Biochem. Biophys. 273:331-338.

Henry, L. E. A., J. D. Mikkelsen and B. L. Moller. 1983. Pigment and acyl lipid composition of photosystem I and II vesicles and of photosynthetic mutants in barley. Carlsberg Res. Commun. 48:131-148.

Humbeck, K., S. LRomer and H. Senger. 1988. Changes in carotenoid composition and function of the photosynthetic apparatus during light-dependent chloroplast differentiation

in mutant C-6D of Scenedesmus obliquus. Botanica Acta 101:220-228.

Isler, O. 1971. Carotenoids. Birkhauser Verlag. Basel, Switzerland. 932 pages.

Karlin-Neumann, G. A., B. D. Kohorn J. P. Thornber and E. M. Tobin. 1985. J. Mol. Appl. Genet. 3:45-61.

Kessler, E., W. Arthur and J. E. Brugger. 1957. The influence of manganese and phosphate on delayed light emission, fluorescence, photoreduction and photosynthesis in algae. Arch. Biochem. Biophys. Acta 109:347-356.

Lammeli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-272.

Maloney, M. A., Hoober, J. K. and D. B. Marks. 1989. Kinetics of chlorophyll accumulation and formation of chlorophyll protein complexes during greening of Chlamydomonas reinhardtii y-1 at 38°C. Plant Physiol. 91:1100-1106.

Michel, H., M. Tellenbach and A. Boschetti. 1983. A chlorophyll b-less mutant of Chlamydomonas reinhardtii lacking in the light-harvesting chlorophyll a/b-protein complex but not its apoproteins. Biochim. Biophys. Acta 725:417-424.

Mullet, J. E., J. J. Burke and C. J. Arntzen. 1980. Chlorophyll proteins of photosystem I. Plant Physiol. 65:814-822.

Oelze-Karow, H. and H. Mohr. 1978. Control of chlorophyll b biosynthesis by phytochrome. Photochem. Photobiol. 27:189-193.

Oelze-Karow, H., H. Kasemir and H. Mohr. 1978b. Control of chlorophyll b formation by phytochrome and a threshold level of chlorophyllide a. In: Chloroplast development (Akoyunoglou, G. and J. H. Argyroudi-Akoyunoglou, eds). Elsevier/North-Holland. Amsterdam. 787-792.

Ogawa, T., F. Obata and K. Shibata. 1966. Two pigment proteins in spinach chloroplasts. Biochim. Biophys. Acta 112:223-228.

Peter, G. F. and J. P. Thornber. 1988. In: Photosynthetic light-harvesting systems (H. Scheer and S. Schneider, eds.). W. Gruyter & Co., Berlin. 175-186.

Peterson, G. L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable.. Anal. Biochem. 83:346-356.

Peterson, G. L. 1983. Determination of total protein. In: Methods of enzymology. Academic Press, New York. 91:468-478.

Plumley, F. G., Herrin, D.L., Mishkind, M. L. and G. W. Schmidt. 1985. Light-Harvesting Chlorophyll-protein Complexes In Green Algae. J. Phycol. 21, 14a.

Plumley, F. G., G. W. Schmidt. 1987. Reconstitution of chlorophyll a/b light-harvesting complexes: Xanthophyll-dependent assembly and energy transfer. Proc. Natl. Acad. Sci. USA 84:146-150.

Qoureshi, N. and J. W. Porter. 1981. Conversion of acetyl-coenzyme A to isopentenyl pyrophosphate. In: Biosynthesis of isoprenoid compounds (Portet, J. W. and S. L. Spurgeon, eds.). John Wiley & Sons, New York. 47-94.

Rudiger, W. and J. Benz. 1984. Synthesis of chloroplast pigments. In: Chloroplast biogenesis (Ellis, R. J., ed.). Cambridge University Press. 225-244.

Sandmann, G. and S. Kowalezyk. 1989. In vitro carotenogenesis and characterization of the phytoene desaturase reaction in Anacystis. Biochem. Biophys. Res. Commun. 163:916-921.

Schmidt, G. W., S. G. Bartlett, A. R. Grossman, A. R. Cashmore and N.-H. Chua. 1981. Biosyntheitc pathways of two polypeptide subunits of light-harvesting chlorophyll a/b protein complexes. J. Cell Biol. 91:468-478.

Schneider, H. 1980. Chlorophyll biosynthesis: enzymes and regulation of enzyme activities. In: Pigments in plants (Czygan, F.-C.,ed.). G. Fischer Verlag, Stuttgart and New York. 237-307.

Siefermann-Harms, D. 1985. Carotenoids in photosynthesis. I. Location in photosynthetic membranes and light-harvesting function. Biochim. Biophys. Acta 811:325-355.

Siefermann-Harms, D. 1987. The light-harvesting and protective functions of carotenoids in photosynthetic membranes. Physiol. Plantarum 69:561-568.

Tandy, N. E., R. A. Dilley, M. A. Hermodson, D. Bhatnagar. 1982. Evidence for an interaction between protons released in chloroplast photosystem II water oxidation and the 8000 M_r hydrophobic subunit of the energy-coupling complex. The Journal of Biological Chemistry 257:4301-4307.

Tandy, N. E., R. A. Dilley, F. E. Regnier. 1983. High-performance liquid chromatographic purification of the chloroplast energy coupling complex. Journal of Chromatography 266:599-607.

Thornber, J. P., R. P. F. Gregory, C. A. Smith and J. L. Bailey. 1967. Studies on the nature of the chloroplast lamella. I. Preparation and some properties of two chlorophyll-protein complexes. Biochemistry 6:391-396.

Thornber, J. P. and H. R. Highkin. 1974. Composition of the photosynthetic apparatus of normal barley leaves and a mutant lacking chlorophyll b. lEur. J. Biochem. 41:109-116.

Vainstein, A., C. C. Peterson andJ. P. Thornber. 1989. Light-harvesting pigment-proteins of photosystem I in Maize: Subunit composition and biogenesis. J. Biol. Chem. 264:4058-4063.

Vernon, L. P. and E. R. Shaw. 1969. Photosystem II activities in subchloroplast fragments obtained by detergents. Biochem. Biophys. Res. Commun. 36:878-884.

Weber, K. and M. Osborn. 1969. The reliability of molecular weight determinations of dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.

Wessels, J. S. C., O. Alphen-van Waveren and G. Voorn. 1973. Isolation and properties of particles containing the reaction center complex of photosystem II from spinach chloroplasts. Biochim. Biophys. Acta 292:741-752.

Winkler, G., P. Wolschann, P. Briza and F. K. Christian. 1985. Spectral properties of trifluoroacetic acid-acetonitrile gradient systems for separation of picomole quantities of peptides by reversed-phase high-performance liquid chromatography. Journal of Chromatography 347:83-88.

APPENDIX

Appendix A: A Glossary of Terms

Autotrophic Growth:	Growing of Algae on an Inorganic Culture Medium Aerated with Air Plus 3% CO_2 in the Light.
Heterotrophic Growth:	Growing of Algae in the Dark on a Medium Containing Glucose, Vitamins, Amino Acids and Essential Inorganic Salts.
Mixotrophic Growth:	Combination of the Above.
Photosynthetic Mutant:	A Mutant Deficient in Photosynthetic Activity.
A PS-II Mutant:	A Phenotype Lacking Only PS-II Activity.
A PS-II (Red) Mutant	A Strain Altered on the Reducing Side of PS-II.
A PS-II (Oxid) Mutant:	A Strain Altered on the Oxidizing Side of PS-II.
High Fluorescent Mutant:	A Strain Having High Fluorescence Yield and without a Variable-Yield Component.
Low Fluorescent Mutant:	A PS-Deficient Mutant with a Fluorescence Yield Comparable To or Less Than the Wild-Type's Fluorescence. Probably Lacks the Variable-Yield Component.
Pleiotropic Mutant:	A Phenotype Resulting from Mutation of a Structural Gene Causing Near Complete Loss of a Photosystem.