AN ABSTRACT OF THE THESIS OF

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Title: A STUDY ON THE CAROTENOIDS OF WILD-TYPE AND MUTANT STRAINS OF THE ALGA, SCENEDESMUS OBLIQUUS

Abstract approved: Redacted for Privacy
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The primary objective of this thesis is to study, characterize and compare four types of Scenedesmus obliquus pigment mutants with the wild-type strain. The specific nature of the carotenoids synthesized in both heterotrophically and myxotrophically-grown cells, Hill reaction activity, photoreduction activity, and total photosynthetic activity of these cells are examined.

Mutant C-X-6E, lacking carotenoids (dark-grown cells), bleaches when placed in the light; is unable to do photosynthesis; shows no Hill reaction; but does exhibit photoreduction activity. Another mutant, C-X-18, which has only high levels of acyclic carotenoids and low amounts of chlorophyll, also bleaches in the light and gives results similar to those seen in the photosynthetic studies with C-X-6E. C-X-6D, when grown in the dark, forms appreciable amounts of acyclic carotenoids and trace amounts of the cyclic and

oxygenated carotenoids; when placed in the light it synthesizes normal carotenoids but retains the high levels of the acyclic carotenoids. This mutant shows very low levels of photosystem II activity, low levels of photosystem I activity, and very low levels of overall photosynthesis. The final mutant studied, C-X-72, which has normal carotenoids and low levels of chlorophyll, is able to green in the light; exhibits low levels of photosystem I and II activity; and has a total photosynthetic activity lower than that of the wild-type.

The results of these studies seem to indicate the close association between the carotenoids and the photosystem II and the necessity for a normal carotenoid biosynthetic pathway for the prevention of chlorophyll photooxidation and for maximum photosynthetic activity.

A Study on the Carotenoids of Wild-Type and Mutant Strains of the Alga, *Scenedesmus obliquus*

by

Janet Lynn Williams

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A STUDY ON THE CAROTENOIDS OF WILD-TYPE AND MUTANT STRAINS OF THE GREEN ALGA, SCENEDESMUS OBLIQUUS

I. INTRODUCTION

General Definition and Description

Carotenoids -

> any of several highly unsaturated pigments (as carotenes and xanthophylls) most of which are yellow, orange, or red . . . being characterized chemically by a long aliphatic polyene chain composed of isoprene units (82, p. 341).

The carotenoids are one of the most widespread groups of naturally-occurring pigments found in living organisms. These yellow, orange, and occasionally red pigments are ubiquitous to all natural photosynthetic organisms. They occur independently of the chlorophyll in higher plants but are often masked by it and become evident only when the chlorophyll is absent. For example, the brilliant colors of many leaves in the fall are due mainly to the carotenoids which become more apparent when the chlorophyll bleaches. In addition to higher plants, the bryophytes, pteridophytes and lichens all have similar carotenoids. Goodwin (32) has proposed a pattern of evolution of the algae based on the distribution of the carotenoids among the various classes. The photosynthetic bacteria in general have a different group of carotenoids.

These pigments are also found in non-photosynthetic tissues of

the plant kingdom. Many of the yellow, orange and red shades of flowers are due to the presence of carotenoids in the chromoplasts. Color changes associated with the ripening of bananas, apples, pears, tomatoes, and peppers are due either to the disappearance of the chlorophylls thus exposing the carotenoids, or chlorophyll disappearance in combination with the formation of additional carotenoid pigments. A few roots such as carrots, sweet potatoes and turnips synthesize significant amounts of carotenoids. Recent work (32) has shown that certain genera of the fungi, originally thought not to synthesize these pigments, have been found to be carotenogenic.

Although carotenoids are found in animals, all evidence indicates that they are not synthesized by animal tissues. When carotenes are taken into the body via foods, they can be converted into vitamin A and retinenes which are required by man and other animals for normal growth, development and vision. The yellowness of cream from cows is due to the carotenes obtained from forage plants. The yellow color of the egg yolk is due to the presence of xanthophylls. Brightly colored birds such as canaries and flamingos obtain their yellow or reddish colors from the carotenoids of plants they eat (60). This brief introduction to the distribution of this wide variety of pigments gives one an idea of the extensiveness of their occurrence and their importance to every day living.

Carotenoid Chemistry

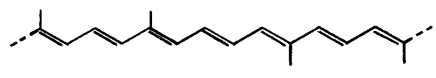
Although the pigments now known as carotenoids have been reported in the literature for a century or more, it has just been in the last 40 years or so that attempts have been made to understand their chemistry. In the 1930's the structures of the more common carotenoids were determined by Karrer, Kuhn, Zechmeister, Heilbron and their respective co-workers (83). In 1948, Karrer and Schwyzer (52) synthesized a trace of β-carotene from vitamin A. But the first synthesis of carotenoids in isolatable amounts was done using β-carotene and lycopene as substrates in 1950 by several laboratories independently - Karrer and Eugster (50), Inhoffen et al. (43, 44, 45, 46), and Milas et al. (59). Following this achievement, the range of synthetic methods in the field was extended by the laboratories of Karrer, Inhoffen, Weedon, Isler and Pommer (47). Today a large number of carotenoids have been synthesized, some even on an industrial scale. However, in the last few years most of the information learned about the carotenoids has been derived from studies on the biosynthetic pathway.

The carotenoids are fat-soluble pigments which can be extracted by the usual organic solvents and subsequently purified by various chromatographic techniques. In practice they can be characterized by visible, infra-red and nuclear magnetic resonance spectroscopy. The

carotenoids which serve as accessory pigments for photosynthesis absorb light strongly in the blue (424-491 nm) and in the green (491-550 nm); they usually have a triple-banded spectra in the region of 400-550 nm (60). The absorption properties of a polyene vary with the number of conjugated double bonds, the solvent used, the side groups present on the molecule, the amount of cyclization, and the cis-trans configuration of the molecule (83).

Because of the instability of many carotenoids and their tendency to undergo stereomutation, the techniques used should be carried out in an inert atmosphere whenever possible; solvents should be purified (the epoxide forms of the carotenoids are especially sensitive to acids); and carotenoid solutions should not be exposed to extremes of light or heat (21, 83).

The majority of the carotenoids have a C_{40} carbon skeleton (tetra-terpene) composed of eight isoprene units $\begin{matrix} CH_2 \\ CH_3 \end{matrix}\!\!>\!C - CH_2 - CH_3$). Common to all carotenoids is a C_{18} central unit, consisting of seven conjugated double bonds and four side-chain methyl groups:

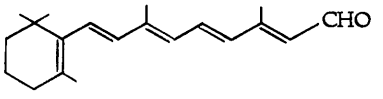


In many carotenoids the isoprene unit on one or both ends of the molecule can be in the form of a six-membered ring. These two end groups can be the same as in lycopene, β-carotene and zeaxanthin; or

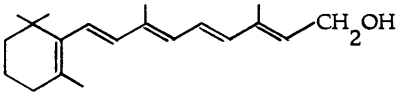
different as in α-carotene, lutein, and echininone (Tables 1 and 2).

The carotenoids can be subdivided into two groups: the hydrocarbon types or carotenes, and the oxygenated forms such as the xanthophylls. The xanthophylls have a much greater structural diversity than the carotenes in that the added oxygen can be present in a hydroxyl, keto, or methoxyl group. The most common of the carotenes seen in green plants and algae are α-carotene and β-carotene in that order. The most prevalent xanthophylls seen are lutein, violaxanthin and zeaxanthin with cryptoxanthin, neoxanthin and antheraxanthin less frequently encountered (32).

Carotenoids with fewer than 40 carbon atoms exist but this present study was not concerned with these compounds. As noted earlier, there is evidence for the conversion, in mammals, of β-carotene and some other carotenoids into the C_{20} compounds, retinene and vitamin A (51).



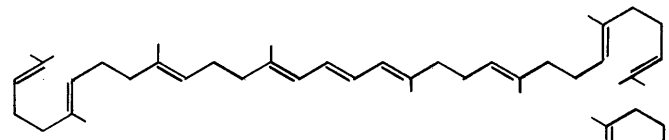
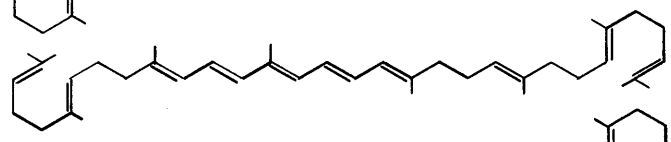
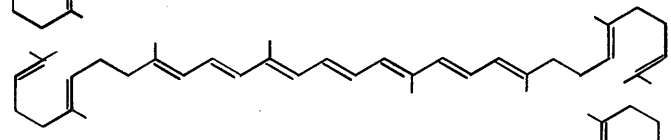
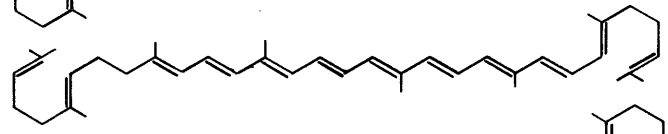
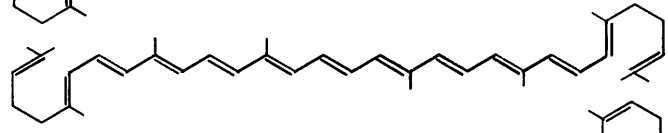
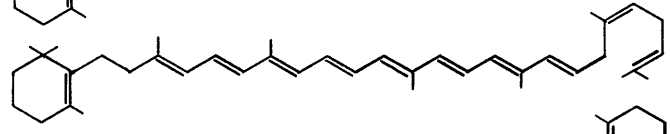
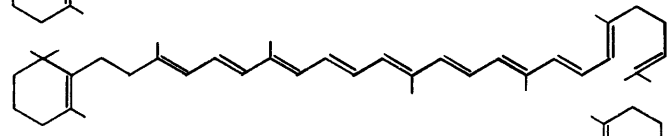
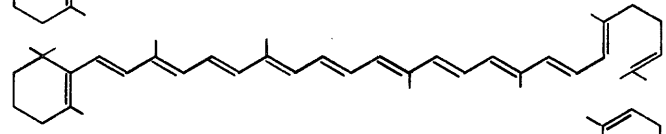
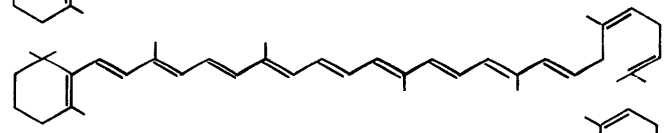
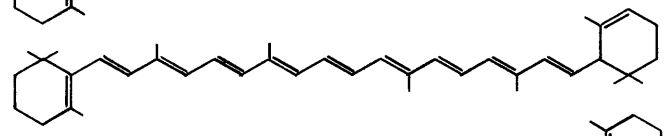
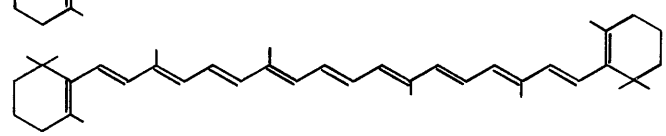
Retinene



Vitamin A

It was through the pioneering work of Gillam and later the extensive studies of Zechmeister and his colleagues (89) that the importance of geometrical isomerization in carotenoid chemistry was revealed. It was shown that under the proper conditions (elevated

Table 1. The structures of some naturally-occurring carotenes.

Common name	Structure
phytoene*	
phytofluene*	
ζ-carotene	
neurosporene	
lycopene	
α-zeacarotene	
β-zeacarotene	
γ-carotene	
δ-carotene	
α-carotene	
β-carotene**	

*cis configuration in the 15, 15' bond

**Example of numbering system of β-carotene

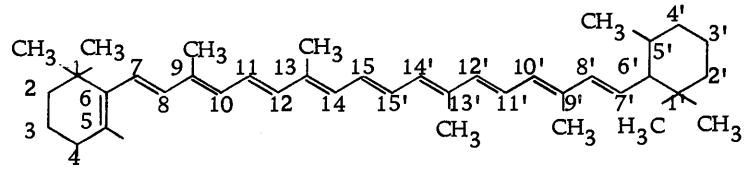
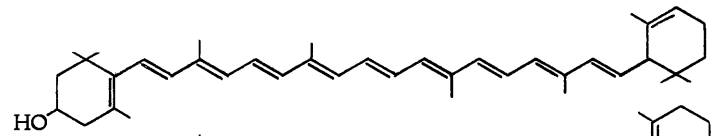
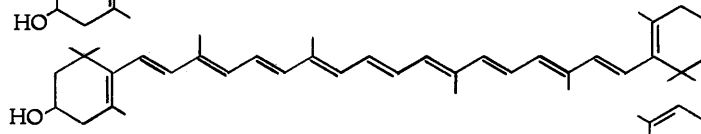
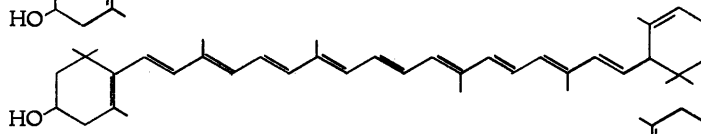
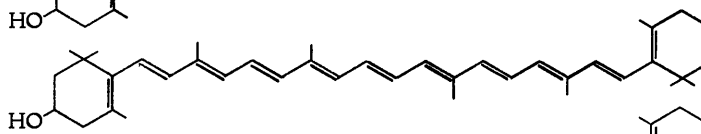
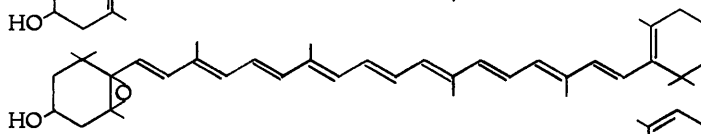
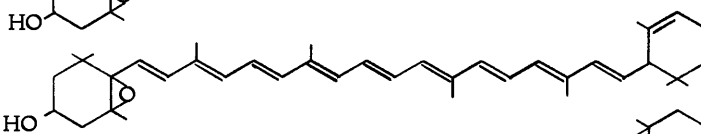
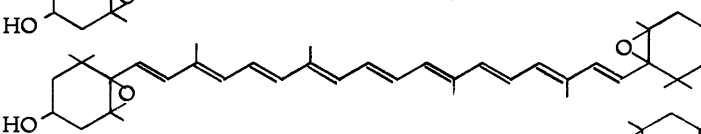
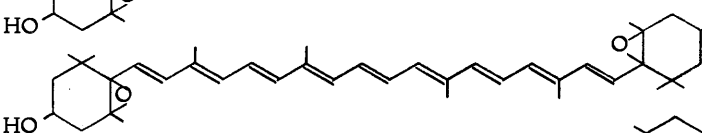
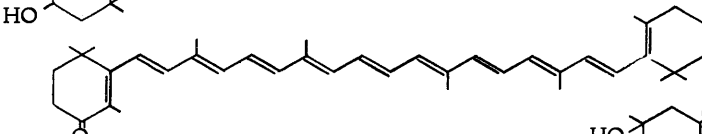
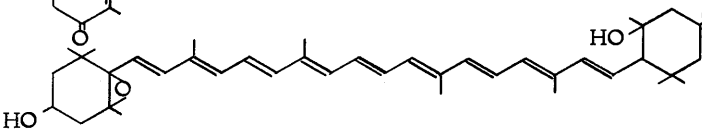


Table 2. The structures of some naturally-occurring xanthophylls.

Common name	Structure
α-cryptoxanthin	
β-cryptoxanthin	
lutein	
zeaxanthin	
antheraxanthin	
lutein mono-epoxide	
violaxanthin	
lutein di-epoxide	
echininone	
neoxanthin	

temperature and irradiation at wave lengths corresponding to the main absorption band) all carotenoids can be converted to mixtures of stereoisomers. It is theoretically possible, for example, for lycopene with a symmetrical carbon skeleton and 11 conjugated double bounds, to exist in 1056 different forms. Of these only 40 have been encountered experimentally. But due to the methyl side-groups which hinder isomerization, the number of sterically unhindered isomers of lycopene is 72 (83).

Normally the most stable isomer of a carotenoid is the all trans form; the 15, 15' cis bonds are particularly labile (83). Phytoene must be an exception because it is believed to be all trans except for a central 15, 15' cis bond (63). A number of cis and poly-cis isomers of carotenoids have been isolated from naturally-occurring sources, although a few of these may be the result of improper isolation, which caused stereomutation of the more common trans forms (83).

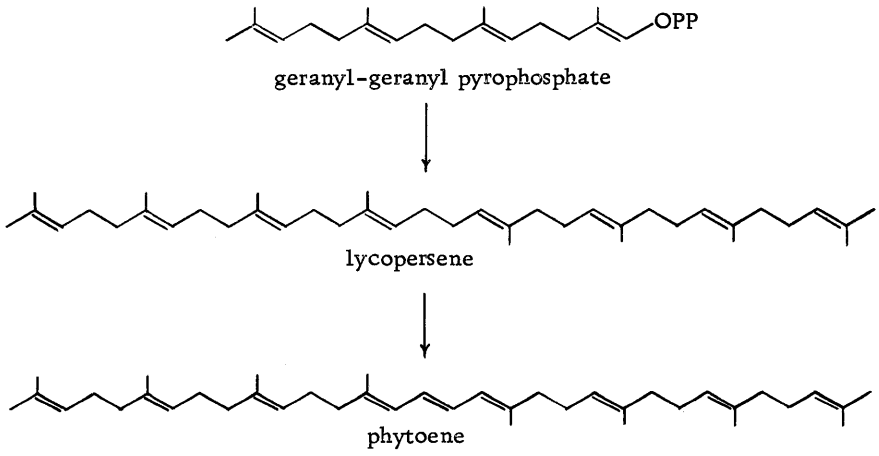
Biosynthesis of Cyclic Carotenes

There have been two major developments in carotenoid biochemistry in recent years. One is the work of Porter and his colleagues in isolating cell-free systems which will carry out the steps of carotene biosynthesis, and the second is the utilization of the Cornforth-Popjak technique using stereospecifically ^{3}H-labeled mevalonate to clarify the stereochemistry of the biosynthetic pathway of the

carotenoids by Goodwin and his co-workers.

The discovery of mevalonic acid and its role in the biosynthesis of cholesterol in 1956 (78) was a milepost in the biochemistry of isoprenoid compounds. Subsequently, biosynthetic studies during the late 1950's and early 1960's were concentrated on searching for a five-carbon basic unit which could lead to the synthesis of terpenoids. The discovery of isopentenyl pyrophosphate fulfilled this requirement for a "biological isoprene unit" (31). The main building block for isopentenyl pyrophosphate was found to be acetyl-CoA, and the steps involved in the synthesis of phytoene from acetyl-CoA are summarized in Figure 1. See a review by Porter and Anderson in 1967 for a detailed discussion of this pathway (63).

There has been some controversy as to the mechanism of the formation of phytoene from geranyl-geranyl pyrophosphate. Some workers reported lycopersene as an intermediate and proposed the head-to-tail condensation of two geranyl-geranyl pyrophosphates to give lycopersene which in turn could be converted to phytoene (37, 38):



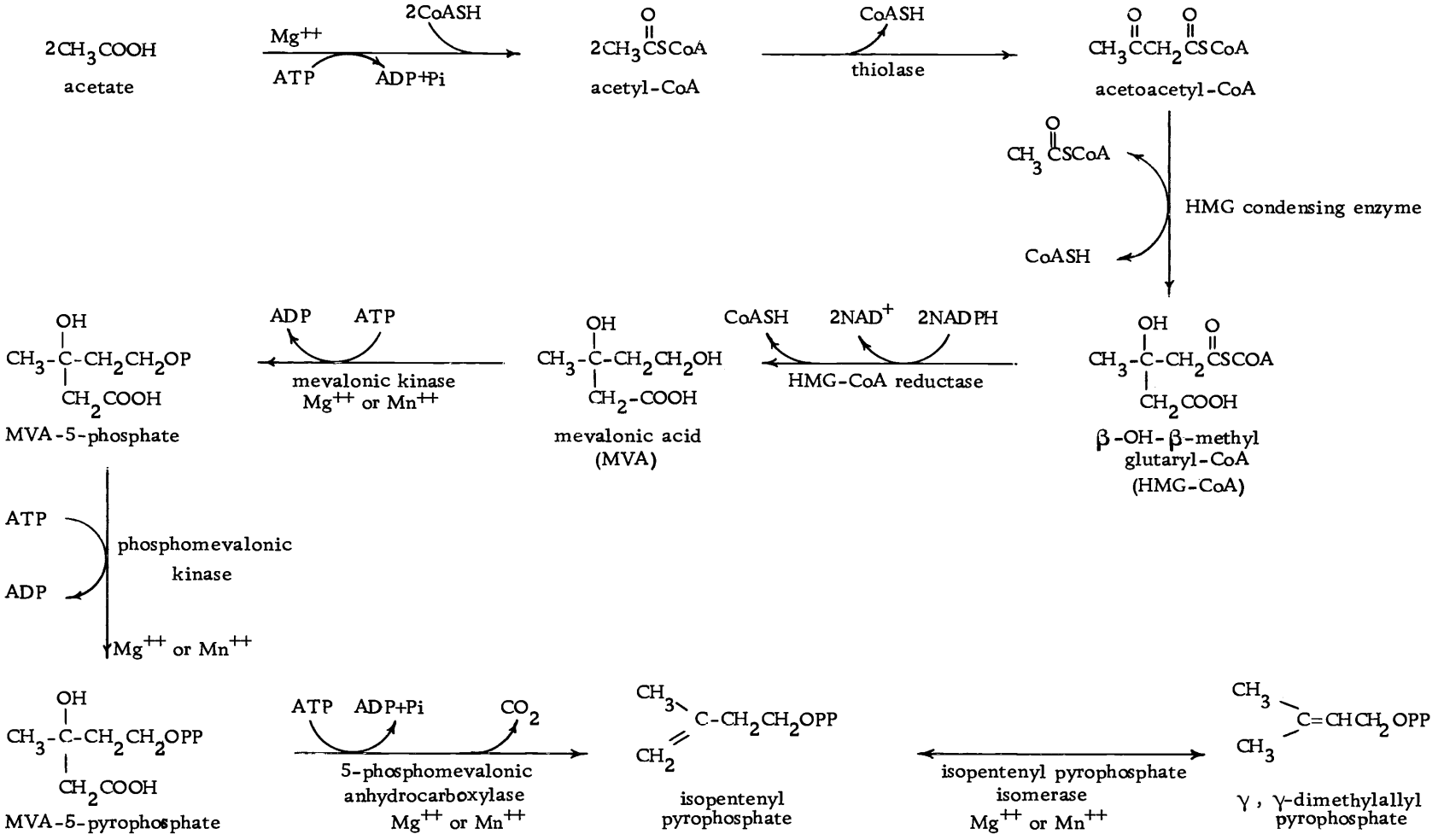


Figure 1. Biosynthesis of phytoene from acetate.

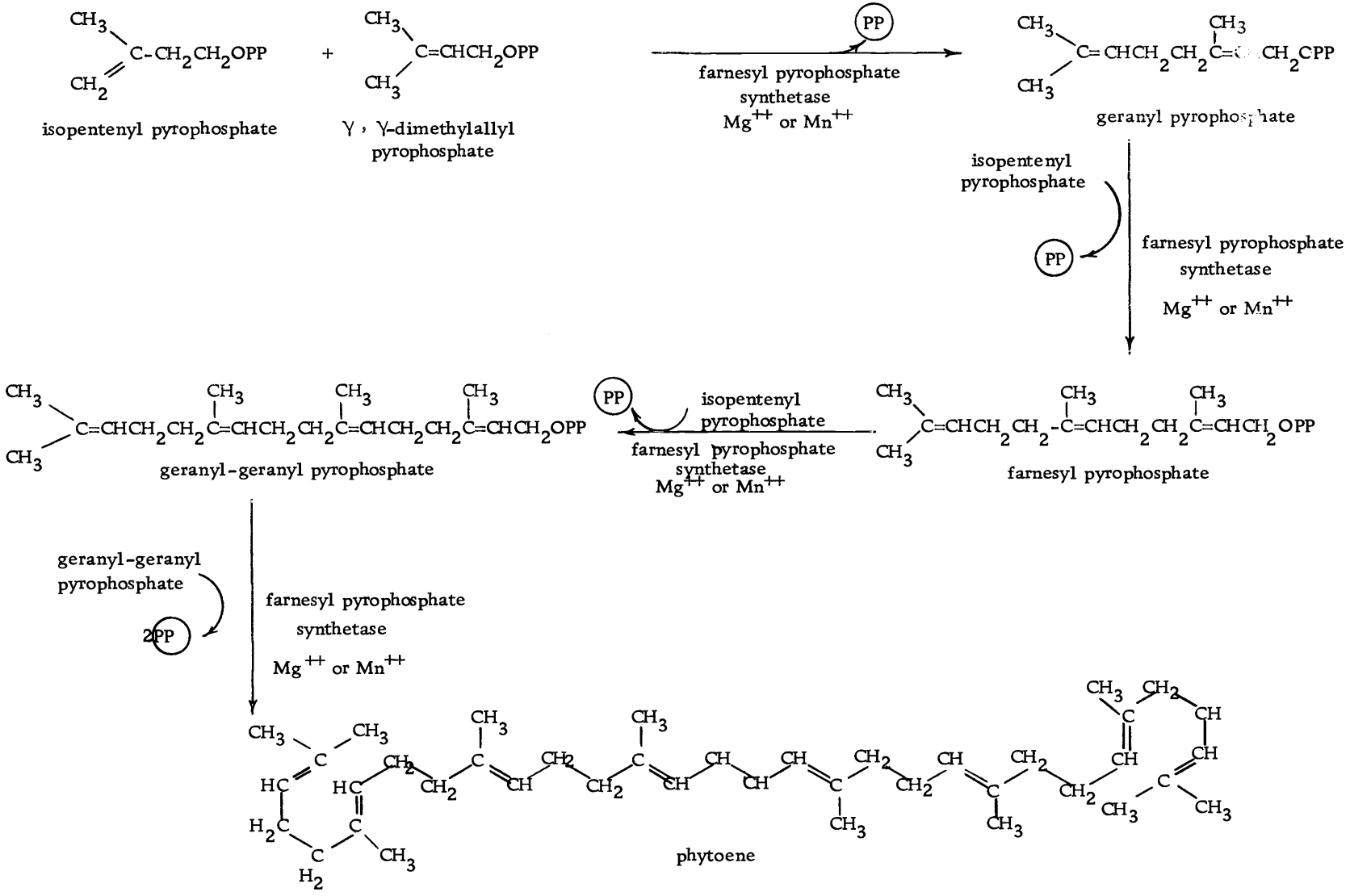


Figure 1. (continued)

But if lycopersene was formed prior to phytoene, then this conversion would require an oxidation step. Rilling (66) has reported that the synthesis of phytoene occurs in the absence of an electron acceptor. Other investigators have been unable to detect lycopersene in many plants that synthesize carotenoids and it is questionable whether it exists in any plant material (63). With the condensation of two geranyl-geranyl pyrophosphates tail to tail, a change in the oxidation state of the system would not be needed. Studies on the geometrical configuration of phytoene show that the central double bond is cis and that all other double bonds have the trans configuration (22, 48). This suggests geranyl-geranyl pyrophosphate must be all trans, which is indeed the case (24, 35).

Porter and Lincoln in 1950 proposed a scheme for the stepwise conversion of phytoene to lycopene, which in turn was thought to be the precursor of β-carotene (64) (Figure 2). This proposal was made some time before the exact structures of the intermediates were known, and was based on pigment distribution in various tomato mutants. Claes observed similar patterns in a series of experiments on Chlorella mutants (11, 12, 13, 14). In the Chlorella mutants, which exhibited characteristic blocks in the biosynthetic pathway of the carotenoids, there was a noticeable build-up of carotenoids of a higher saturation level (phytoene, phytofluene and ζ-carotene) not seen in the normal wild-type (11). Other studies with the inhibitor of

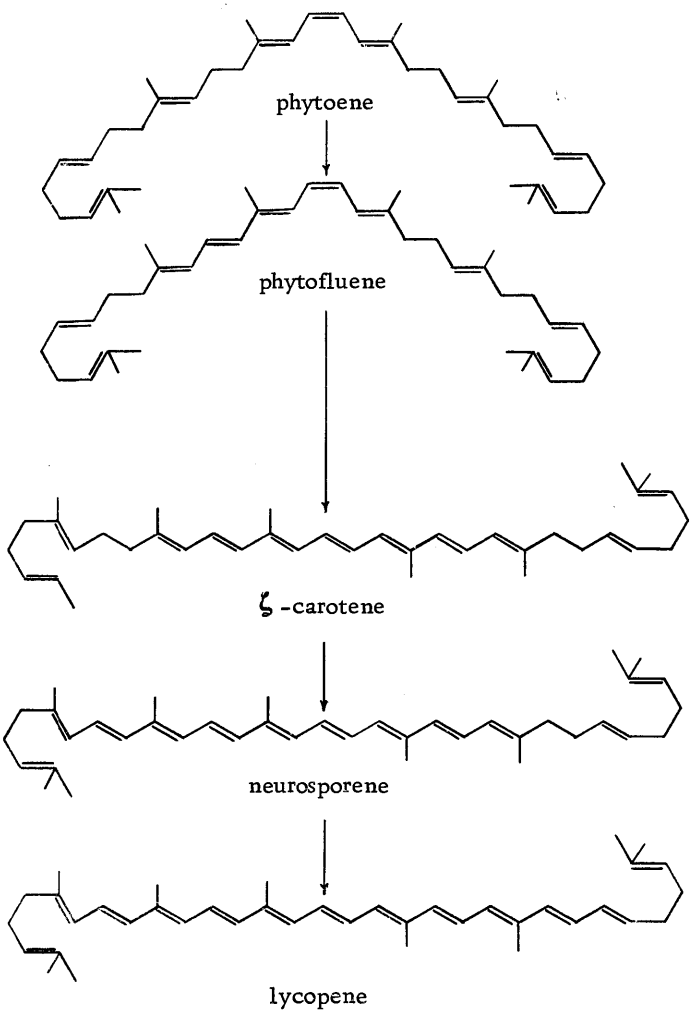
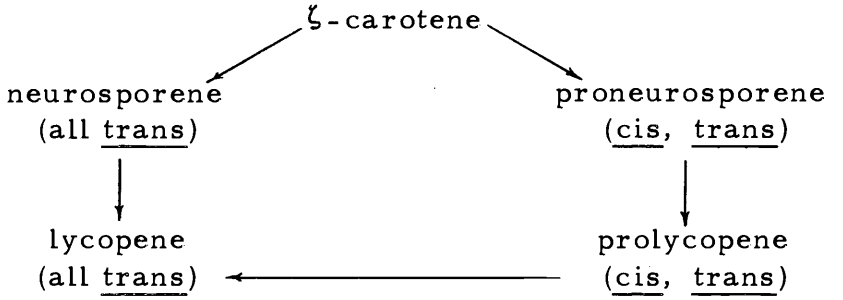


Figure 2. The conversion of phytoene into lycopene.

carotene formation, diphenylamine, demonstrated the same accumulation of phytoene, phytofluene, etc. When the diphenylamine was removed by washing, the synthesis of carotenes resumed (33). Beeler and Porter have demonstrated the conversion of phytoene to phytofluene with tomato plastids via dehydrogenation (2). This conversion does not seem to alter the configuration of the molecule (48). It has been reported that in Staphylococcus aureus, phytoene is converted to ζ-carotene (76, 77). This step would require both a dehydrogenation and a change in confirmation since cis ζ-carotene has not been observed in biological materials (63). It is possible that a single enzyme could do this (63). ζ-carotene is presumably the precursor for neurosporene, and proneurosporene in tissues which form it. If so, there would probably be two enzymes involved. Then lycopene could be made from neurosporene directly or from proneurosporene through prolycopene in the following manner (63):



Claes (12) demonstrated that proneurosporene and prolycopene are formed in a Chlorella mutant grown in the dark, but on exposure to blue light the cis-carotenes were converted to trans-neurosporene

and trans-lycopene. Incorporation of radioactivity of terpenol-pyrophosphates into lycopene and neurosporene has been seen (3). Just recently the requirement for nicotinamide dinucleotide phosphate (NADP) in the conversion of phytoene to phytofluene and from phytofluene through the intermediates to lycopene has been demonstrated with a spinach enzyme system (75). This further indicates that there are at least two dehydrogenation steps in the synthesis of lycopene from phytoene.

Considerable evidence is available to support the hypothesis that neurosporene gives rise to the cyclic carotenes and their derivatives. However, it is also claimed that lycopene is cyclized to form the carotenes. Porter and Lincoln (56) first proposed lycopene as a precursor from studies on the inheritance of carotenes in tomatoes, in which the usual high level of lycopene was replaced by β-carotene. Claes' experiments with Chlorella (11, 12, 13, 14) supported this finding. Decker and Uehleke provided more direct evidence when they isolated chloroplasts which were able to convert radioactive lycopene into β-carotene (23). Although this work reportedly has been confirmed by Godnev and Rotfarb (30), other laboratories have not been able to substantiate these findings (63). It is known that in a number of systems lycopene is not cyclized to β-carotene (31).

Porter and Anderson revised their scheme for the biosynthesis of the carotenes by suggesting that the cyclic carotenes were formed

from neurosporene as well as lycopene (62). Goodwin has proposed a pathway for the formation of both α-carotene and β-carotene from neurosporene with α-cryptoxanthin and β-cryptoxanthin as intermediates (31) (Figure 3). In studies testing the effects of inhibitors on carotene synthesis, α- and β-zeacarotenes have been isolated from maize (65), and β-zeacarotene from both Phycomyces blakesleeanus (84) and yeast (70). It is likely that the pigment "X" of Goodwin and Osman (34) and the unidentified pigment found by Claes (14) in a Chlorella mutant are both β-zeacarotene. Currently the exact pathway for the formation of the cyclic carotenes is not known. It is evident that they are formed from the unsaturated hydrocarbons and at least some are formed from neurosporene. It is possible that different pathways are utilized in different organisms or even in the same organism.

Xanthophyll Biosynthesis and Interrelationship

In studies on a mutant strain of Chlorella, which is dependent upon light and molecular oxygen to synthesize xanthophylls, Claes (15) found that the biosynthesis of xanthophylls in the dark was always accompanied by a corresponding decrease in α- and β-carotene accumulated in a prior light period. Strain (73, 74), in analyses of fall leaves, found a decrease in the carotene level followed by a net increase in the xanthophylls. These and other studies would indicate

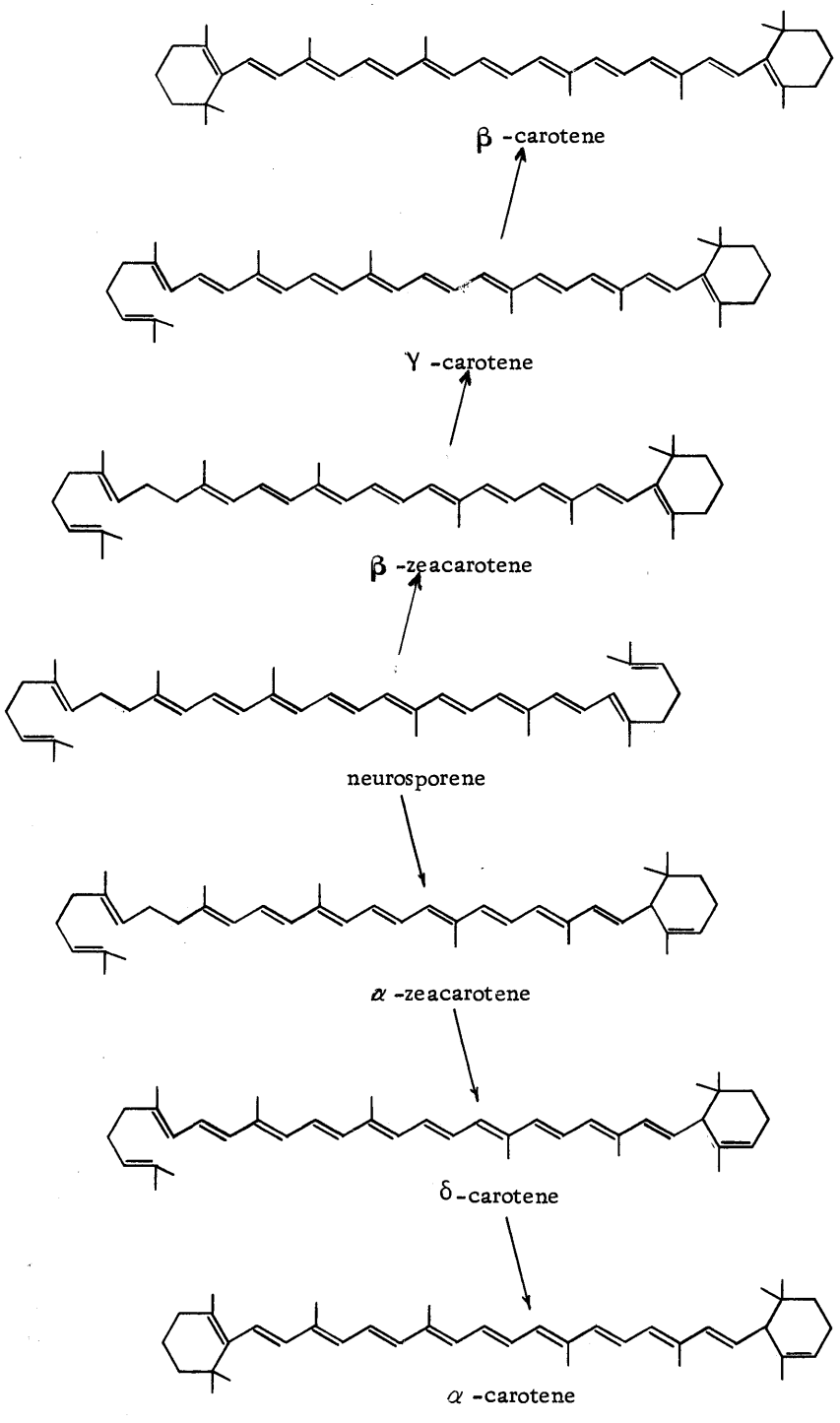


Figure 3. Proposed scheme for the biosynthesis of α and β carotene from neurosporene.

that both α-carotene and β-carotene are the immediate precursors of the xanthophylls in normal cells and that there exists such a stoichiometric relationship in photosynthetic organisms such that the carotenes can be converted on a mole per mole basis to their respective oxygenated forms.

Two processes, hydroxylation and epoxidation, are involved in the formation of the xanthophylls from either α- or β-carotene. The hypothesis that the biochemical hydroxylation of the methyl group involves the direct participation of molecular oxygen and that subsequent oxidation involves the oxygen from water was suggested by Gillette in 1959 (29). Yamamoto et al. in 1962 (86) demonstrated in studies using $^{18}O_2$ and $H_2{}^{18}O$, that the hydroxyl groups of lutein, violaxanthin and neoxanthin of Chlorella did indeed arise from molecular oxygen. There was some evidence that the epoxy group of neoxanthin arose from water but this notion has since been withdrawn (85). Yamamoto and his associates were not able to determine whether $^{18}O_2$ or $H_2{}^{18}O$ gave rise to the epoxy oxygens in violaxanthin (88). But in 1965, Yamamoto and Chichester (85) concluded from studies on lima bean that there was incorporation of $^{18}O_2$ into the epoxy group(s) of antheraxanthin and thus violaxanthin. It would seem that in the conversion of the carotenes to their oxygenated forms, oxygen from air, and not from water or photosynthetically derived oxygen, is utilized. On the basis of these studies, the scheme in Figure 4 was

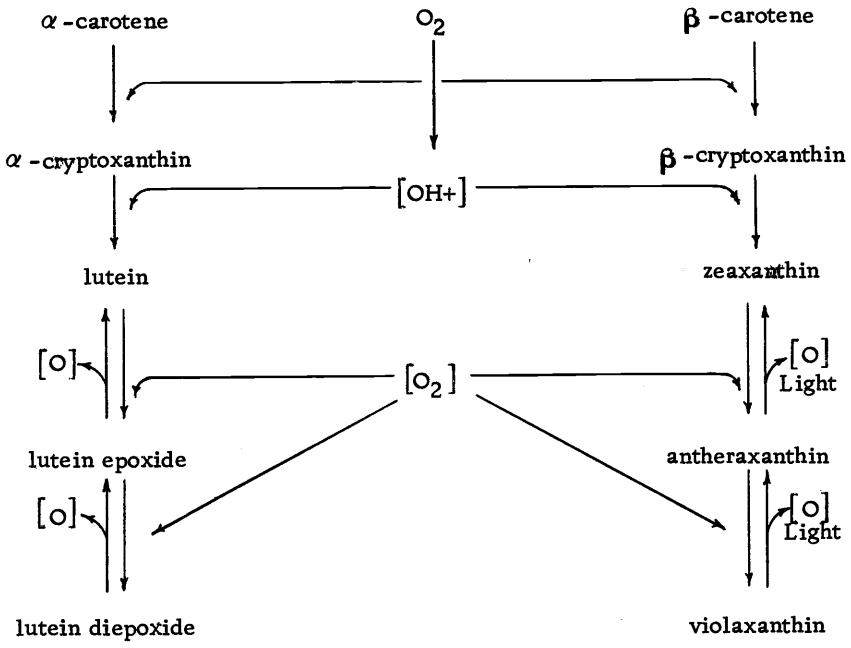


Figure 4. Interrelationship of the carotenes and their oxygenated forms.

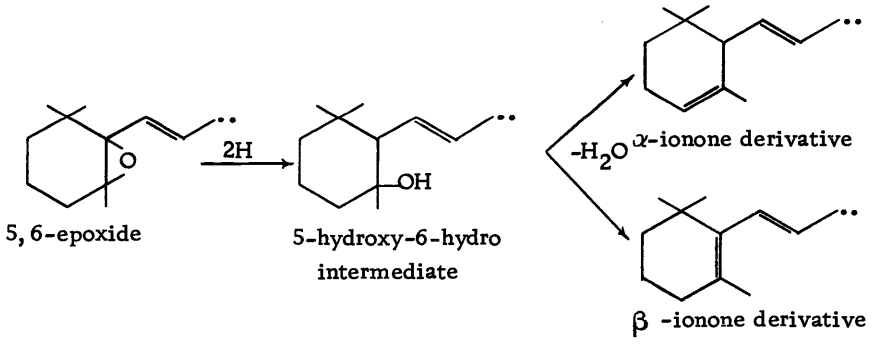
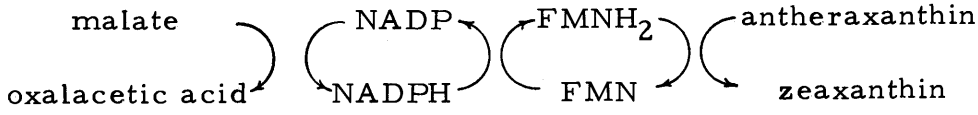


Figure 5. Interconversion of α- and β-ionone derivatives.

proposed (25). There is direct evidence for the β-carotene portion of the pathway, but little is known about the further conversion of α-carotene. α-Cryptoxanthin, an obvious precursor to lutein (the major xanthophyll of leaves), occurs in plants, but in what manner lutein is made and whether it is epoxidated is not known.

Early reports of Sapozhinikow et al. (68), suggesting that violaxanthin could be converted to lutein under anerobic conditions in the dark, were disputed by Yamamoto et al. (87); these latter workers supported instead the interconversion of violaxanthin to zeaxanthin with antheraxanthin as an intermediate. A possible alternate route to the α and β ionone-related compounds has been suggested to explain this discrepancy (54) (Figure 5). Schimmer and Krinsky (68) and Curl (20) have suggested a structure for neoxanthin which would fit the structure of the 5-hydroxy-6-hydro intermediate (Table 2). Studies by Bamji and Krinsky (1) recently have shown that the dark conversion of antheraxanthin to zeaxanthin is enzymatic in nature and requires a source of reducing potential as seen below:



They concluded that the source of NADPH necessary for de-epoxidation is light dependent. Krinsky (35) has shown more recently that light inhibits the above reaction but that the addition of DCMU overcomes this inhibition. This would suggest that photosynthetically

evolved oxygen inhibits this reaction.

Function of the Carotenoids in Photosynthesis

Since all normal photosynthetic tissue contains carotenoids in close association with chlorophyll, they might be expected to participate in some way in the mechanism of photosynthesis. The classic experiments of Engelmann in 1880 (28) indicated that oxygen-sensitive bacteria accumulated in regions illuminated by blue light as well as red light. Since a major part of the total absorption in the 400-500 nm region is light absorbed by the carotenoids, it was concluded that they must serve as accessory pigments for photosynthesis. In purple photosynthetic bacteria the energy absorbed by the carotenoids can be transferred to bacteriochlorophyll, and eventually to the photosynthetic reaction center, with an efficiency ranging from 30-70% (17). With the diatom Nitzschia closterium this efficiency can vary from 38-93% (26), the light abosrbed by the carotenoids in the blue region being as great as the chlorophyll absorption in the red (9). The quantum yield of the light absorbed in the 400-500 nm region (27) is about equal to that of the chlorophyll region in the green algae, but much lower for the red and blue-green algae. This difference may be due to variations of the chloroplast structure in the different algae.

Carotenoids appear necessary for the normal function of the chloroplast. At first it was postulated that the carotenes were

someway involved directly in initiating photosynthesis. Lynch and French (58) found that in lyophilized chloroplasts, which performed the Hill reaction, there was a loss of 80% of the normal activity if the carotenoids were removed by petroleum ether extraction. However, the activity could be restored if either the extract or partially purified β-carotene was added back. But oddly enough the crude extract was more efficient than the crystalline β-carotene. Bishop (4) was able to duplicate their work but he also noted this discrepancy between the effects caused by the crude extract and crystalline β-carotene. He concluded that the carotenes themselves must not, or at least not entirely, be responsible for the restoration of the Hill reaction. In experiments with vitamin K (6), which was known to be present in chloroplasts, he found a greater stimulation than with β-carotene. But upon analysis of the crude extract no vitamin K was detected; this latter observation has been confirmed by other workers. Further examination of the crude extract showed that plastoquinone was the actual compound responsible for the restoration of the Hill reaction rather than β-carotene and most likely vitamin K (4).

In 1958, Sager and Zalokar (67) described a pale-green mutant strain in the alga Chlamydomonas reinhardi, which contained low amounts of chlorophyll and traces of carotenoids. This mutant has a greatly reduced but otherwise apparently normal lamellar structure. On a chlorophyll basis its photosynthetic rate was greater than the

wild-type but the mutant overall had a poor growth rate (67). This led Sager to hypothesize that the carotenoids are not essential for photosynthesis except possibly in catalytic amounts (67). A blue-green mutant of the anaerobic photosynthetic bacterium Rhodopseudomonas spheriodes has also been described which contains bacteriochlorophyll and no colored carotenoids, although the mutant does have phytoene equivalent on a weight basis to the colored carotenoids of the wild-type. This mutant is still capable of photosynthesis (36).

Another possible function attributed to the carotenoids is that they can protect the cell from the photodynamic action of chlorophyll. This was first demonstrated by Griffiths et al. (36) with the mutant photosynthetic bacterium described above. Since neither light nor oxygen alone killed the mutant lacking colored carotenoids, but the combination of the two was lethal, they concluded the death must result from photodynamic action and that the primary function of the carotenoid pigments in phototrophs is to act as chemical buffers against photooxidation (71). Other studies have shown this same phenomenon. The blue-green mutant of Chlamydomonas reinhardi, which contains only traces of α- and β-carotene and has a low chlorophyll content, will die when exposed to light and air (67). Similar observations have been made by Claes (11) with mutants of Chlorella vulgaris. Since the possibility existed that the mutants previously described had undergone multiple gene, rather than single gene mutations, it was possible no

simple causal relationship existed. Therefore experiments were run (18) in which normal bacterial cells were grown in the presence of diphenylamine, DPA (a suppressor of carotenoid synthesis). It was found that the treated cells behaved like the mutants by being photosensitive to aerobic conditions, but if the DPA was washed out and the cells placed under the same conditions, they eventually returned to normal.

In higher plants the absence of colored carotenoids leads to photooxidative destruction of the chlorophyll. In two β-caroteneless mutants of Helianthus annuus (sunflower) chlorophyll destruction occurs in white light. The resulting leaf color is dependent upon the other pigments present (39, 81). In the albina mutant (80), which lacks both β-carotene and xanthophyll, the bleached seedlings were white like the dark-grown seedlings. Normal protochlorophyll and prolamellar bodies were synthesized in the plastids of the dark-grown leaves of the mutant. The chloroplasts of the mutant were comparable to the immature chloroplasts of greening wild-type leaves, except they contained a group of large globuli. Under weak light some chlorophyll and a small amount of grana formation occurred. Under normal light the plastids lost their regular form and assumed an amoeboid shape, the discs swelled and disintegrated, and the globuli disappeared. The second mutant (80), a xanthophyll mutant, is missing only β-carotene and upon bleaching has a yellow color. In the dark, the proplastids

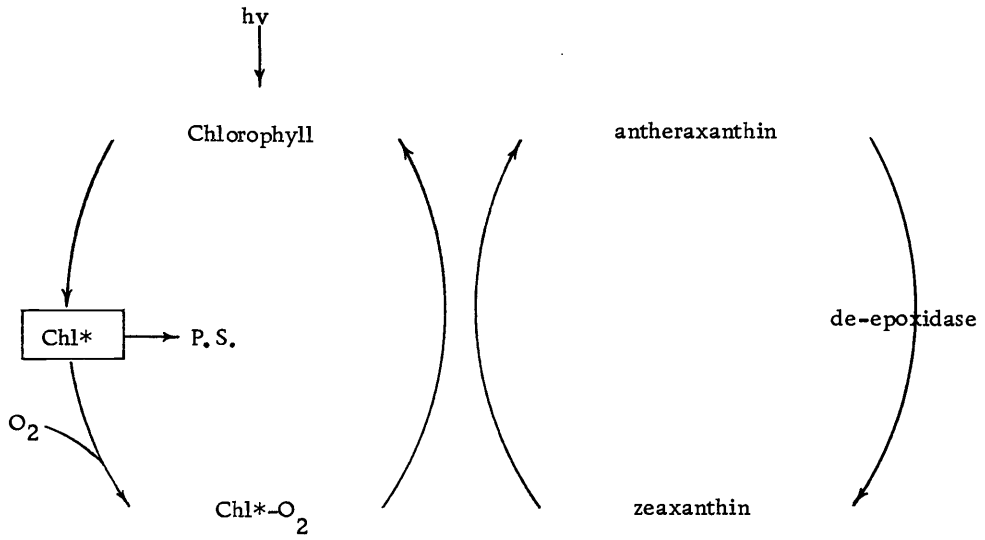
have crystalline prolamellar bodies and appear identical to the dark-grown wild-type seedlings. The chloroplasts of the xantha mutant contain vesicles and discs and frequently a few grana of large size; these chloroplasts are more resistant to bleaching. Some traces of chlorophyll still exist in the colorless leaves. After bleaching, the plastids degenerate and become amoeboid in shape, but the grana persist for some time.

Walles concluded that β-carotene must be necessary for normal lamellar systems as well as for the protection of the chlorophyll and the grana structure against photodestruction (80). The majority of the carotenoid mutants of higher plants can synthesize chlorophyll but generally it is susceptible to photooxidation. The extent of this destruction varies but is greatest where plastid differentiation comes to an end at an early proplastid stage. In order to resist bleaching, the chlorophyll must presumably be bound to a thylakoid protein and be protected by the carotenoids in a lamellar system (80). Smith et al. (72) found it was not the total amount but the type of carotenoid present which was important against photooxidation, since some mutants with as much carotenoid pigment as the wild-type strain were still bleached when exposed to light.

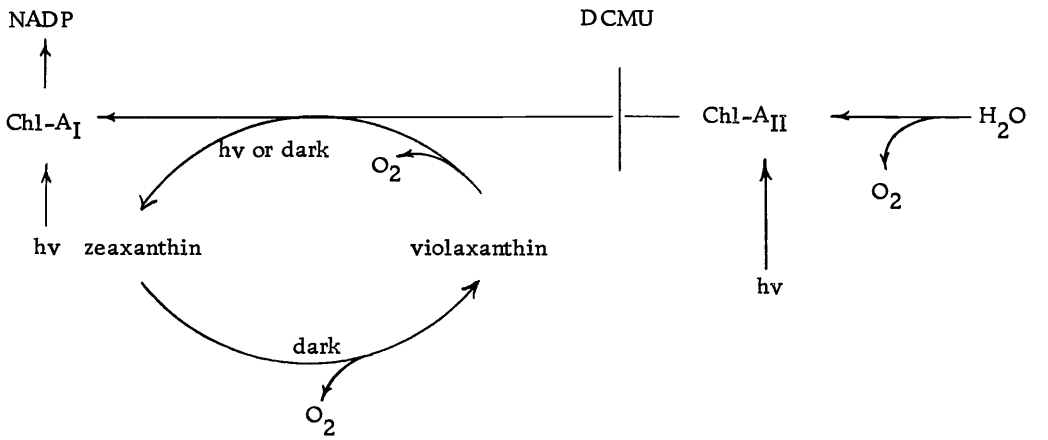
Not all the carotenoids are effective in preventing the photo-destruction of chlorophyll. The extent of the protection appears to be dependent upon the length of the chain and the degree of unsaturation.

Studies on a sequence of carotenoid mutants of Rhodopseudomonas spheroides (19) indicated the following: mutants with only phytoene and phytofluene were photosensitive; mutants which synthesize a carotene with seven conjugated double bonds (ζ-carotene) showed variable photosensitivity but mutants which synthesize carotenes with nine or more conjugated double bonds had full protection. Claes (16) has reported the same pattern of activity with Chlorella mutants. These results were interpreted to mean that the carotenoids with nine or more conjugated double bonds quench the triplet state of the chlorophyll and thus give a protective effect. Livingston and Pugh (57) also reported that in flash photolysis experiments, the carotenoids quench the triplet state of chlorophyll a and b.

The exact manner by which the carotenes or carotenoids prevent photooxidation in the cell is not known. Krinsky (55) feels that the carotenoid pair, antheraxanthin-zeaxanthin, could fulfill the requirement of being "chemical buffers" to protect cells from lethal photosensitized oxidations. The epoxide cycle in this case would serve as a protective agent in the following way:



Hager (40) has proposed an hypothesis which is slightly modified in that the light-induced transformation of violaxanthin to zeaxanthin is not identical with the process which causes the release of oxygen in photosynthesis, but instead is coupled with that electron transport system which proceeds between reduced plastoquinone and oxidized P-700. Energy-rich compounds formed in the electron flow would be responsible for the cleavage of oxygen from violaxanthin to form zeaxanthin.



Evidence seems to support the speculation that the carotenoids in most photosynthetic tissues are involved in the photosynthetic mechanism in some way. From the proposed models of Krinsky and Hager above, the conversion of the epoxys to the xanthophylls appears as a net electron-accepting reaction, whereas the reverse system acts as an electron-donating reaction. While these pigments do not seem to play a direct role in the transport of oxygen, they may serve as an electron buffering system, being able to either donate or accept electrons from the photosynthetic system upon demand.

Within recent years, several workers (10, 61, 79) have attempted to physically separate the chloroplasts into particles representing the two photosynthetic systems. Treatment of chloroplasts with such detergents as Triton X-100, digitonin, or sodium dodecyl sulfate (79) resulted in the separation of a light particle associated with photosystem I and of a heavier particle showing photosystem II activity. Upon examination of the two particles, β-carotene was found to be in higher concentrations in the photosystem I particle and the oxygenated carotenoids such as lutein, neoxanthin, and violaxanthin were found associated more closely with photosystem II particles. This observation suggests that the xanthophylls are related functionally more closely with the oxygen-evolving system. This distribution of the carotenes and xanthophylls may be accounted for by the fact that photosystem I supposedly generates only a mild oxidizing potential

while photosystem II, because of its fundamental role in the photolysis of water, produces a strong oxidizing potential.

II. STATEMENT OF PURPOSE

Intensive studies have been made on different mutants of bacteria, algae, and higher plants in order to understand better the biochemical role of the carotenoids in the chloroplasts of green plants. The Introduction of this thesis reviewed several examples of work already accomplished in this area. It is known that in the absence of the carotenoids, the chlorophylls bleach in light and photosynthesis ceases. If a series of carotenoid-pigment mutants could be segregated into several groups of different levels of development along the carotenoid biosynthetic pathway, hopefully one might learn at what step of this pathway the actual interplay between the carotenoids and the chlorophylls takes place. Since a number of pigment mutants of the alga Scenedesmus obliquus was available in Professor N. I. Bishop's collection, at Oregon State University, there existed an excellent opportunity to segregate and examine a diverse number of pigment mutants. It was possible to divide these strains into four separate groups: those with no carotenoids; those with only acyclic carotenoids; those with α- and β-carotene but no oxygenated carotenoids; and those with normal carotenoids but unable to green in the dark.

For this study, two mutants of each group were chosen and grown both heterotrophically and myxotrophically. These were analyzed for the type of carotenoids present and compared to the

wild-type. (No attempt was made at this time to analyze the pigments quantitatively.) One mutant of each phenotype was examined for its ability to do total photosynthesis and again compared to the wild-type. Attempts were made to associate the type of mutant with either photosystem I or II by measuring photoreduction and the Hill reaction. Hopefully, information obtained from this study will result in a more detailed understanding of the genetic lesions in each of the mutants examined, and lead to a clearer picture of the role of the various carotenoids in photosynthesis.

III. MATERIALS AND METHODS

Culturing of the Algae

Selected pigment mutants of Scenedesmus obliquus, strain D_3 (ScD_3), were used for these studies. The eight mutants analyzed (C-X-2A', C-X-6D, C-X-6E, C-X-9, C-X-15, C-X-18, C-X-28, C-X-72; abbreviated 2A , 6D, 6E, 9, 15, 18, 28, 72) were X-ray induced mutants isolated by Professor N. I. Bishop according to methods already described (7). The cultures were grown in a nitrate medium (53) to which was added 0.5% glucose and 0.25% yeast extract. Heterotrophic cultures were maintained at 28°C in Erlenmeyer flasks on a rotary shaker. Myxotrophic cultures were maintained at 25°C in glass bubble-tubes through which a mixture of 4% CO_2 in air was bubbled. Light was provided by a combination of warm white and Gro-Lux fluorescent lamps; light intensity at the surface of the tubes was 10,000 lux. Cells were usually two or three days old when analyzed.

Analysis for Carotenes and Carotenoids

One ml packed-cell volume (PCV) of cells was obtained from young cultures of the wild-type (WT) and each of the above mutants. Identical analyses of each strain, grown both myxotrophically and heterotrophically, were made in the following manner. The cells were extracted with hot methanol, centrifuged, the supernatant saved, and

the pellet extracted twice more to give a total volume of 100 ml. The chlorophyll concentration was determined according to the method of Holden (42). The total sample was then saponified with 20 ml of 5N NaOH, and the mixture subsequently partitioned three times with 100 ml of petroleum ether (b. r. = 37-45°C) and 15 ml of 5% (w/v) NaCl. The petroleum ether extracts were combined, washed with 50 ml of H_2O, dried of excess water with Na_2SO_4, and evaporated to dryness. Finally, the sample was taken up in 2 ml chloroform; 0.5 ml was streaked onto the desired thin-layer chromatography plate; the plates were pre-incubated in a saturated atmosphere of the developing solvents, and then developed in the appropriate solvent system.

A preliminary examination of extracts of ScD_3 WT and the pigment mutants was made using silica gel G plates and a solvent system consisting of petroleum ether : isopropanol : water (200:20:1). Since the separation of the pigments was not as complete as desired, a survey was made to obtain a better system of analysis. (See Table 3 for remarks on the various adsorbent and solvent systems tried.) All solvents and chemicals used in the purification of the carotenes and carotenoids were of chemically pure grade and precautions were taken during the entire analysis to avoid both light and excess heat. When the samples had to be maintained for a short period of time they were kept at -40°C. The thin-layer plates were kept under anaerobic conditions immediately after being removed from the solvent system.

Table 3. Various adsorbents and solvent systems employed for carotene and carotenoid separation.

	Adsorbent	Solvent System	Reference	Remarks
1.	Silica gel G	Petroleum ether:isopropanol: water (200:20:1)	Bishop (8)	Three major separations into carotenes, xanthophylls and epoxides. Zeta-carotene isomerizes very easily in this solvent.
2.	Silica gel G	Methylene chloride:ethyl acetate (80:20)	Davies (21)	Does not resolve the carotenes and the carotenoids do not move appreciably from the origin.
3.	Silica gel G impregnated with paraffin (5% in petroleum ether)	Methanol:acetone (5:2)	Bishop (5)	Similar to Silica gel G (see #1) except zeta carotene does not isomerize so easily. A time consuming and messy process.
4.	$CaCO_3$:MgO:$Ca(OH)_2$ (59:12:10)	Ligroin (b. r. = 100-140°C)*: acetone:chloroform (5:5:4)	Hager (41)	Very good separation of the carotenoids. Carotenes move with the solvent front.
5.	"	Ligroin (b. r. = 100-140°C): acetone: benzene (4:1:1)	Hager (41)	Separates only carotenes but not enough to allow separation of individual carotenes.
6.	"	Isopropanol:isooctane (1:100)	Bishop (5)	Very good for carotenes. Clear and wide separation. Carotenoids remain at the origin.
7.	"	Isopropanol:isooctane (10:100)	Bishop (5)	Very good separation of the carotenoids. Carotenes move with the solvent front.
8.	"	Isopropanol:isooctane(10:100) then isopropanol:isooctane (1:100)	Bishop (5)	Fairly good separation but cannot oveload the plate and thus there is not enough material to analyze.

*Abbreviated, CMC

This eliminated most isomerization, especially that of ζ-carotene and violaxanthin.

The carotenes and carotenoids are generally strongly colored bands on the thin-layer plates and thus easily detected visually. Phytofluene and phytoene are colorless but fluoresce blue under ultraviolet (UV) light, and ζ-carotene, though a pale yellow color, fluoresces blue-green. The desired zones were scraped off the plates, eluted with acetone and evaporated to dryness. The individual pigments were tentatively identified from their absorption spectra, measured in various solvents such as petroleum ether, chloroform, ethanol, and benzene. The absorption spectra were determined with a Beckman DK-2A recording spectrophotometer. The maximal absorption varies with the number of conjugated double bonds -- shifting from the UV, to longer wavelengths as this number increases (50). The introduction of a hydroxy group (lutein, zeaxanthin) or an epoxy group (violaxanthin, neoxanthin, antheraxanthin) into the molecule has little effect on the absorption maxima (50). A method of detecting differences in the carotenoids, which may vary in absorption only by a few nm, was shown by Karrer in 1945 (49). He found that in the presence of traces of HCl, an ethanolic solution of a compound having a 5, 6-epoxide isomerizes to the furanoid epoxide (5, 8-epoxide) within three minutes. If the carotenoid is a mono-5, 6-epoxide, a shift of 18-25 nm is seen and if the carotenoid is diepoxy, the shift is

about 40 nm (50). This reaction also proceeds in chloroform when traces of HCl are present. It is for this reason that only freshly redistilled chloroform should be used in analysis.

Sodium borohydride, $NaBH_4$, was used as a reducing agent to test for the keto group in hydroxy-echininone, for more positive identification of this compound.

Photosynthetic Studies

Total photosynthesis of two-day-old heterotrophically-grown cells was measured with an oxygen electrode (Ag-AgCl electrode - Gilson Medical Electronics). Twenty µl of cells were suspended in 2 ml of 0.05 M phosphate buffer, pH 6.5. Measurements were made of the rate of oxygen evolved or consumed per minute at 25°C. Hill reaction activity was determined by adding 1 mg p-benzoquinone to the sample and again measuring the rate of oxygen evolved or consumed per minute. Red light of wavelengths greater than 620 nm, at an intensity of 10^5 ergs sec^{-1} cm^{-2}, was used in both experiments.

The rate of photoreduction was determined with a Gilson Respirometer (Gilson Medical Electronics), employing all glass volumometers. One hundred µl of two-day-old heterotrophically-grown cells were suspended in 2.5 ml of 0.05 M phosphate buffer, pH 6.5, and gassed for ten minutes with 96% H_2-4%CO_2 under continuous shaking at 25°C. After a three-hour incubation period, 0.5 ml of 5×10^{-4} M

3(3, 4 dichlorophenyl)-1, 1-dimethyl urea (DCMU) was tipped from the side-arm into the flask and the sample preilluminated for five minutes prior to actual rate determination. Then ten-minute readings were taken, each at different intensities of red light (greater than 620 nm), using a series of neutral density filters (Balzers Lichtenstein), which transmitted light in the range of 5-97%.

IV. RESULTS AND DISCUSSION

Pigment Analysis of the Cultures

Upon visual examination the pigment mutants studied exhibited varying degrees of color. In comparison to the dark green, heterotrophically-grown, wild-type cells: C-X-6E and C-X-9 were very blue-green in color; C-X-18 and C-X-15 were a fluorescent yellow-green; C-X-6D and C-X-28 were a more yellow-olive green; and C-X-72 and C-X-2A' were a bright orange. The visible absorption patterns of a representative mutant of each of the above groups (shown in Figures 6A and 6B) help explain this variation in hue. Mutant 6E completely lacked the carotenoid peak at about 470 nm and had a low absorption by chlorophyll in the region of 420-440 nm. Mutant 18 showed a very low chlorophyll peak at 658 nm, the absence of a chlorophyll peak at 438, the absence of a carotenoid peak at 470 nm, yet it exhibited an unusual series of peaks between 350 and 422 nm. A similar pattern was seen in 6D, except it had a carotenoid peak at 470 nm. Mutant 72 demonstrated a pattern closely resembling the WT except for the low levels of chlorophyll absorption.

Preliminary analysis of the carotenoid pigments of each mutant, using thin-layer chromatographic (TLC) plates of silica gel G and a solvent system of petroleum ether, isopropanol and water, gave support to the results seen in the overall absorption patterns.

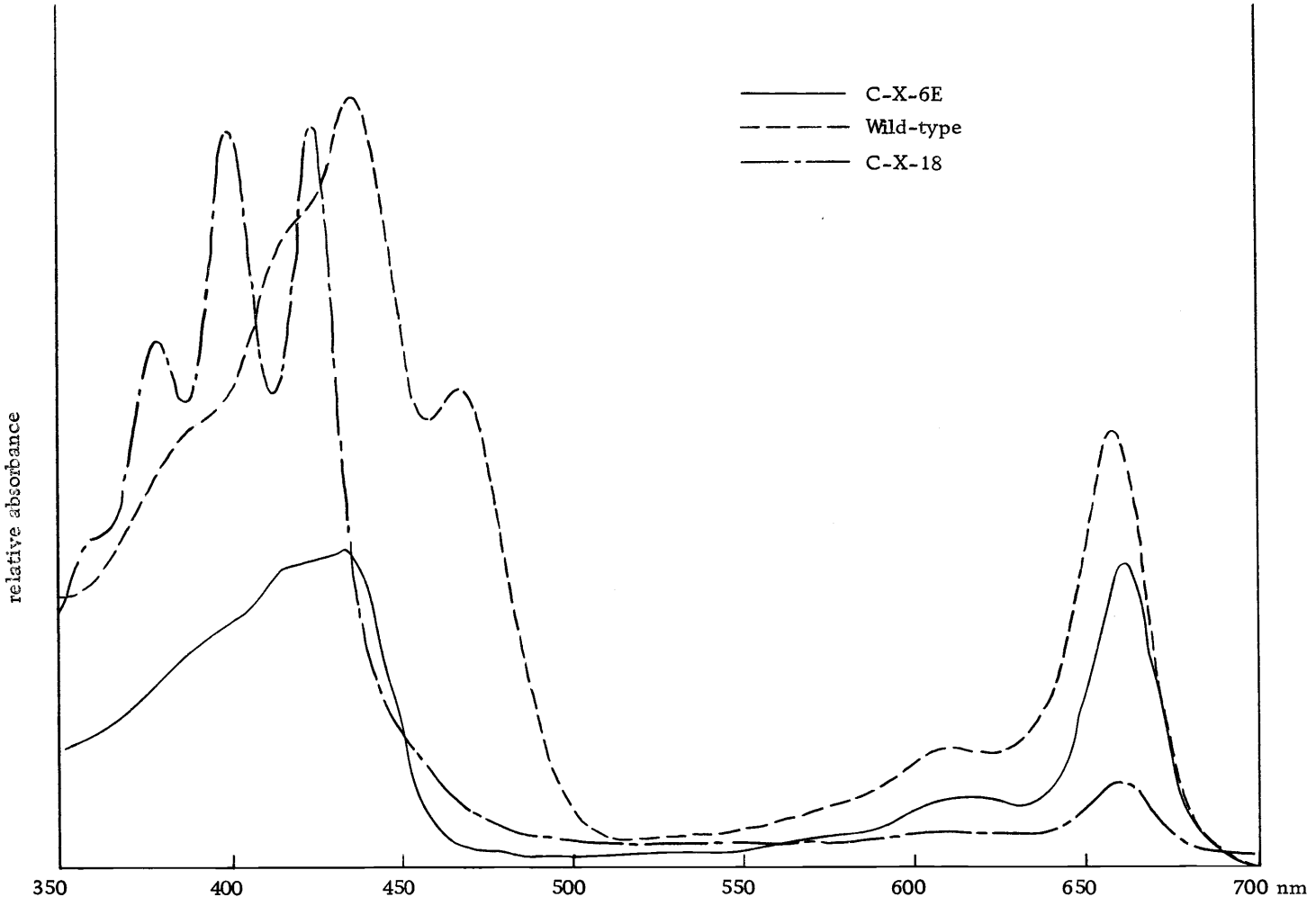


Figure 6A. Absorption pattern of Wild-type, C-X-6E, and C-X-18.

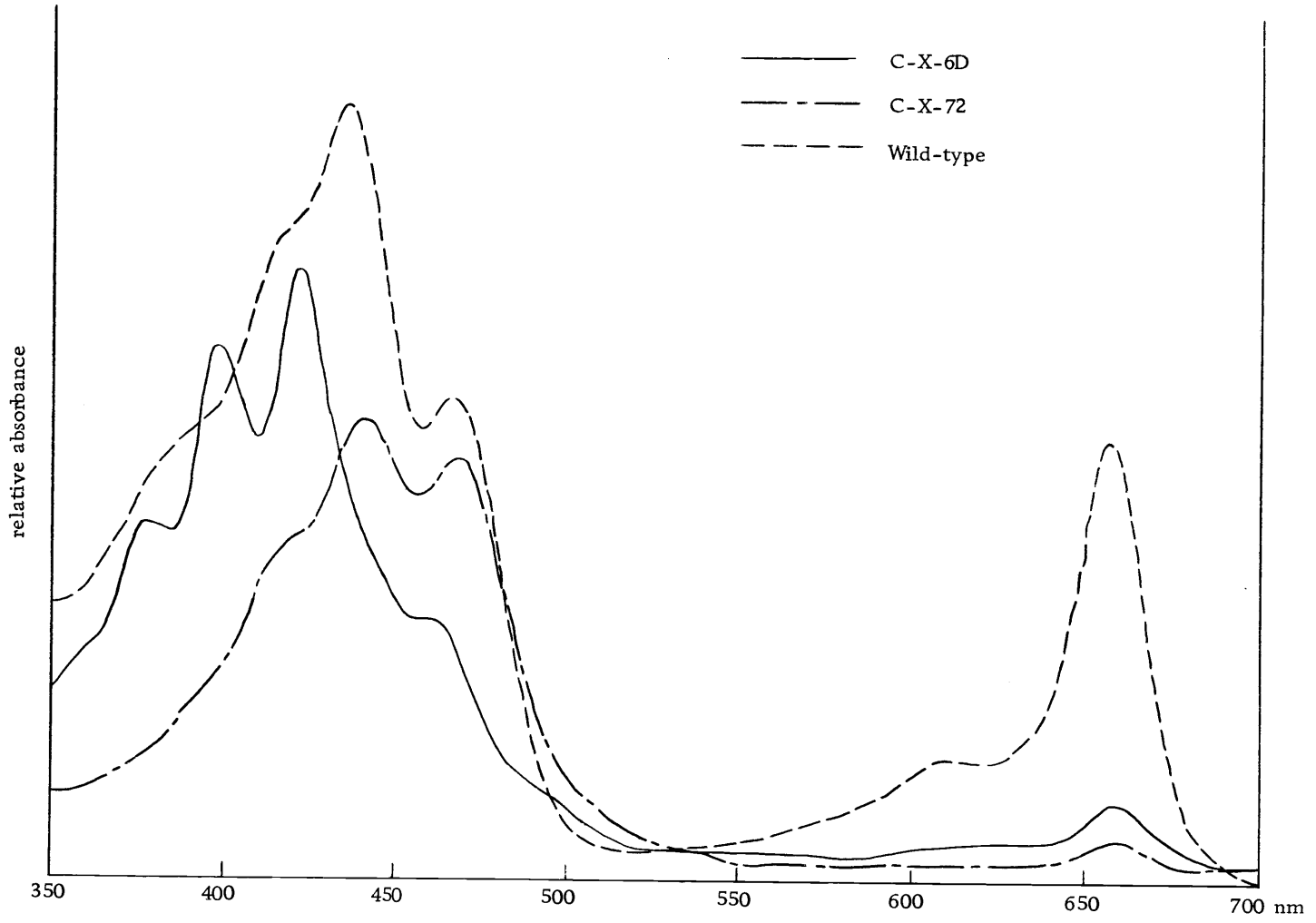


Figure 6b. Absorption pattern of wild-type, C-X-6D, and C-X-72.

The heterotrophically-grown cells of 6E and 9 appeared to be without carotenoids of any kind; cells of 15 and 18 indicated the presence of carotenes but not xanthophylls; 6D and 28 had both carotenes and xanthophylls; 2A' and 72 appeared similar to WT by having a large amount of xanthophylls and only the cyclic carotenes.

Because of the inadequate separation of the various pigments in preliminary analyses, it was decided that a more efficient separating system was needed. Therefore, a series of adsorbents and solvent systems was tested. The results, as reported in Table 2, suggested the use of CMC plates and two separate solvent systems: 1% isopropanol in isooctane for carotene segregation; and petroleum ether: acetone:chloroform (5:5:4) for separation of the xanthophylls. The isolated pigments were then analyzed spectrophotometrically in several solvents and their absorption characteristics compared with the absorption maxima given by Davies (21) (Table 4). The problem arose that many compounds, especially α- and β-carotene, lutein, violaxanthin, zeaxanthin and cryptoxanthin, have very similar absorption spectra. The use of HCl in ethanol as a test for epoxy groups helped to resolve this problem. The relative position of the carotenes and carotenoids on TLC plates can be seen in Figure 7. ζ-Carotene was often seen in three isomeric forms despite precautions taken during extraction and isolation procedures. Lycopene was never identified nor was lutein-mono-epoxide, γ-carotene, or δ-carotene.

Table 4. Absorption maxima (nm) of isolated carotenoids.

Pigment	Petroleum ether*			Chloroform			Ethanol			Ethanol:HCl**		
phytoene	298	285	275	-	-	-	-	-	-	-	-	-
phytofluene	368	348	330	-	-	-	-	-	-	-	-	-
ζ-carotene	423	339	377	-	-	-	-	-	-	-	-	-
neurosporene	466	436	413	-	-	-	-	-	-	-	-	-
α-zeacarotene	450	423	400	-	-	-	-	-	-	-	-	-
α-carotene	470	442	420	483	456	(435)	472	444	420	471	445	421
β-carotene	474	448	(426)	487	462	-	472	444	420	471	445	421
lutein	470	442	420	483	453	(431)	472	445	420	472	446	425
zeaxanthin	472	443	420	480	452	(430)	468	444	420	472	445	420
antheraxanthin	473	444	420				465	440	-	450	442	400
violaxanthin	470	442	426	480	450	426	470	441	418	428	402	380
echininone	-	458	-	-	-	-	-	470	-	472	448	420
neoxanthin	462	440	418	473	444	420	465	437	413	425	400	378

*b. r. = 37-45°C

**In the case of echininone, the solvent was Ethanol:$NaBH_4$.

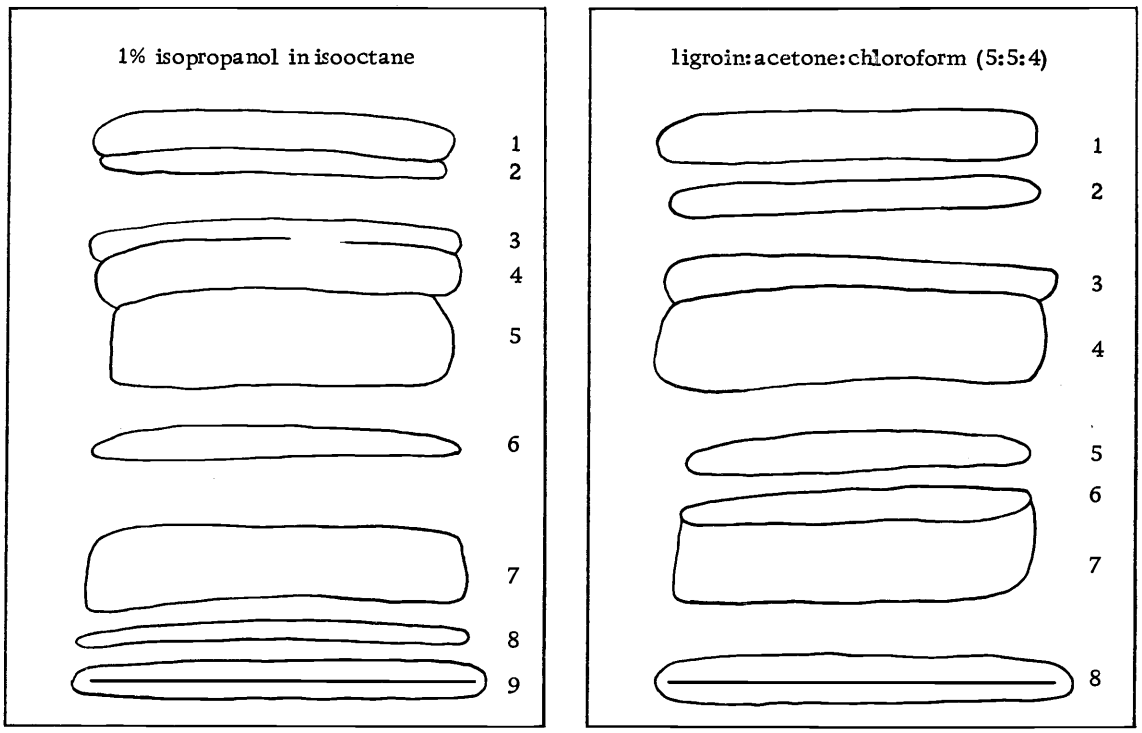


Figure 7. Typical results of pigment separation on CMC plates.

1. Phytofluene - colorless band with high fluorescence.
2. Phytoene and α-carotene - light yellow band with high fluorescence.
3. β-carotene - orange band.
4. α-zeacarotene - dark yellow band.
5. ζ-carotene - fluorescent, yellow band.
6. ζ-carotene - fluorescent, yellow band.
7. ζ-carotene - fluorescent, yellow band.
8. Neurosporene - yellow band.
9. Origin

1. α- and β-carotene, phytoene, phytofluene - orange band.
2. Neurosporene and ζ-carotene - fluorescent, light orange band.
3. Violaxanthin - yellow band, tests for a diepoxy.
4. Lutein - orange band.
5. Neoxanthin - pale orange band, tests for a diepoxy.
6. Antheraxanthin - yellow band, tests for a mono-epoxy.
7. Zeaxanthin - dark yellow band.
8. Origin

The cryptoxanthins were not seen, but easily could have been in the same bands as lutein or zeaxanthin. Since their absorption maxima are very similar to these two compounds, their presence would be difficult to demonstrate. But since the solvent systems employed resolved the isomeric forms of the carotenes (α and β) and the carotenoids (lutein and zeaxanthin), it is unlikely that the cryptoxanthins are present in the mutants. However, this problem was not resolved with satisfaction. A zeacarotene was identified which corresponded to the pattern of the α-form but it is possible that the band seen was the β-form or a mixture of the two. Echininone was only seen in very old cells of WT, 72, 2A' and light-grown cells of 6D.

A summary of the individual pigment analysis of each mutant is presented in Table 5 for heterotrophically-grown cells and Table 6 for myxotrophically-grown cells. A discussion of the results is presented below.

Wild-type - Trace amounts of ζ-carotene and neurosporene were seen in young heterotrophically-grown cells, but these disappeared with the aging of the cells. There was the expected high concentration of β-carotene with a smaller amount of α-carotene. The concentrations of lutein, zeaxanthin and violaxanthin were also high, with less antheraxanthin and only trace amounts of neoxanthin. The latter two compounds increased with the age of the cell and often

Table 5. Carotenes and carotenoids of heterotrophically-grown cells.

Pigment	C-X-6E	C-X-9	C-X-15	C-X-18	C-X-6D	C-X-28	C-X-2A'	C-X 72	WT
Phytoene			+	+	+	+			
phytofluene			+	+	+	+			
ζ-carotene			+	+	+	+	Tr	Tr	Tr
neurosporene					+	+	+	Tr	Tr
α-zeacarotene			Tr	Tr	+	+			
α-carotene					Tr	Tr	+	+	+
β-carotene	Void	Void			+	+	+	+	+
lutein					Tr	Tr	+	+	+
zeaxanthin					Tr	Tr	+	+	+
antheraxanthin							Tr		+
violaxanthin							+	+	+
echininone							Tr	Tr	Tr
neoxanthin							Tr	Tr	Tr

+ = presence of pigment
Tr = trace of pigment

Table 6. Carotenes and carotenoids of myxotrophically-grown cells.

Pigment	C-X-6E	C-X-9	C-X-15	C-X-18	C-X-6D	C-X-28	C-X-2A'	C-X-72	WT
phytoene									
phytofluene					+	+			
ζ -carotene					+	+		Tr	
neurosporene					+	Tr	Tr	Tr	
α -zeacarotene					+	+			
α -carotene	Bleached in the light	Bleached in the light	Bleached in the light	Bleached in the light	+	+	+	+	+
β -carotene					+	+	+	+	+
lutein							+	+	+
zeaxanthin							+	+	+
antheraxanthin					+	+	+	+	+
violaxanthin					+	+	+	+	+
echininone					Tr	Tr	Tr	Tr	Tr
neoxanthin					Tr	Tr	Tr	Tr	Tr

+ = presence of pigment

Tr = trace of pigment

hydroxy-echininone appeared in older cultures. When the WT cells were grown myxotrophically, similar results were seen with the exception of the presence of ζ-carotene and neurosporene. Lycopene was never identified. The chlorophyll content was about 4 mg/1 ml PCV with a chlorophyll a - chlorophyll b ratio varying from 3.0-4.0.

Mutants C-X-6E and C-X-9 - Heterotrophically-grown cells of these mutants completely lacked carotenoids of any kind. The cultures had an average of 0.6 mg chlorophyll/1 ml PCV, which is about 14.6% of the wild-type, and a chlorophyll a - chlorophyll b ratio which was unusually high but variable. These two observations could account for the blue-green color of the cells. When the cultures were transferred to the light and grown myxotrophically, there was immediate bleaching of the chlorophyll and death of the cells. These findings corroborate observations of other workers, who suggested that one role of the carotenoids is their ability to protect the chlorophyll from photooxidation.

Mutants C-X-18 and C-X-15 - In the dark, these two mutant types synthesized only the acyclic carotenoids, such as phytoene, phytofluene, ζ-carotene and α-zeacarotene. The concentration of ζ-carotene was extremely high in proportion to the other pigments, accounting for the fluorescent color of the cultures and the unusual peaks seen in the overall absorption spectrum of mutant 18 in Figure 6A. The α-zeacarotene was seen only in trace amounts, and there

was no evidence of the presence of neurosporene. These results could be explained if the normal biosynthetic pathway of the mutant was blocked such that ζ-carotene could not be converted to neurosporene, causing ζ-carotene to accumulate. If there were enough of a leak (i. e., either decreased synthesis or decreased activity of the appropriate enzyme) in this obstruction, a small amount of neurosporene would be made and quickly converted to α-zeacarotene. The mutant presumably must not be able to synthesize the cyclic carotenes from the small amount of α-zeacarotene present. The chlorophyll concentration was about 14% of the WT. Myxotrophically-grown cells were rapidly photooxidized as would be expected, since ζ-carotene, the major carotene present, has only seven conjugated double bonds and offers little or no protection to the chlorophyll (19).

Mutants C-X-6D and C-X-28 - The acyclic carotenes, phytoene, phytofluene, ζ-carotene and neurosporene, were isolated from the dark-grown cells. α - Zeacarotene, β-carotene, and traces of α-carotene, zeaxanthin and antheraxanthin were also identified. No lutein was seen. The chlorophyll concentration was about 12.8% that of the WT; i. e., about the same as mutants 18 and 15. When these cultures were placed in the light greening took place. The concentrations of the α-carotene and antheraxanthin increased, and the appearance of violaxanthin, neoxanthin and hydroxy-echininone was seen Zeaxanthin did not seem to be present in the light-grown cells but the

concentration of violaxanthin was so high that possibly the zeaxanthin was quickly being converted to violaxanthin, and further to neoxanthin and hydroxy-echininone. Since the early greening cultures of 6D and 28 would still have a low amount of chlorophyll, there would be a great demand for electrons to do maximum photosynthesis. This rapid conversion of zeaxanthin to violaxanthin could supply electrons to the photosynthetic electron transport system according to the method already postulated by Hager (40). Lutein still was not detected, which suggests an alteration of the pathway between α-carotene and lutein. The rate of synthesis of α-carotene during myxotrophic growth appeared to be low. An alternate interpretation might be that the site of mutation was between the formation of α-carotene and α-zeacarotene or neurosporene.

Oddly enough, although these cells would green in the light, they did not lose their high concentration of the acyclic carotenes, as would be expected. Current studies by Bishop (5) on the greening of mutant 6D show electron micrographs of chloroplasts which, after several hours in the light, have a normal stacking of the lamellae but lamellae which are abnormally curled. Possibly this could be due to the inability of the cells to convert all the acyclic carotenes to their cyclic or oxygenated forms.

Mutants C-X-2A' and C-X-72 - The chlorophyll contents of 2A' and 72 are very low (10% or less of the WT), but the carotenoid contents

appear to be identical to the wild-type cells. When placed in the light there is a short adaption period and then rapid greening ensues. There appears to be a block in the conversion of chlorophyll precursors to the chlorophyll a and b, which is overcome only by light. Studies are being made presently on the development of the lamellar structure of these mutants with greening (5).

Photosynthetic Studies

Results of analyses on the total photosynthetic activity of each mutant, as compared to the WT, are summarized in Table 7. Mutant 6E showed no capacity for photosynthesis; quite the contrary, it consumed more oxygen in the light than it did in dark respiration. Mutant 18 consumed less oxygen in the light than 6E, but still had no net photosynthesis. Mutants 6D and 72 evolved oxygen at a rate about 10 and 30% of the WT, respectively. Since the chlorophyll contents of the latter two mutant strains are much lower than the WT, their rates appear to be very efficient.

The results of the photosynthetic measurements require no special interpretation. If the biosynthetic pathway of the carotenoids is not developed to the extent that some cyclic carotenoids can be formed, little or no photosynthetic activity results. This manifestation was also seen when mutant cells were placed in the light. The chlorophyll of mutants 6E and 18 became bleached and subsequent

Table 7. Total photosynthesis.

Culture tested	$\mu M\ O_2$ / 20 µl cells / hour*
Wild-type	+67.71
C-X-6E	-11.70
C-X-18	- 2.31
C-X-6D	+ 5.91
C-X-72	+20.46

*Average of three experiments

Table 8. Hill reaction.

Culture tested	$\mu M\ O_2$ / 20 µl cells / hour*
Wild-type	+20.94
C-X-6E	- 4.80
C-X-18	+ 0.83
C-X-6D	+ 2.87
C-X-72	+ 5.45

*Average of two experiments

death of the cell occurred. C-X-6D does some photosynthesis because it is able to synthesize ζ-carotene and trace amounts of the cyclic carotenes and their oxygenated forms. Mutant 72 exhibited more activity than 6D, and yet it has less chlorophyll than either 6E, 18 or 6D. This seems to emphasize the need for normal carotenoids for normal photosynthesis.

Corroborative evidence for these findings was obtained on analysis of the p-benzoquinone Hill reaction, i. e., a photosystem II activity (Table 8). Mutant 6E showed no oxygen evolution, whereas 18 demonstrated a small amount of activity, slightly less than 5% of the WT; and cultures 6D and 72 showed considerable activity, especially considering their low chlorophyll content. Again the correlation of Hill reaction and carotenoid development can be seen. Mutant 18 did exhibit a small amount of activity, possibly due to the fact that ζ-carotene has been reported (19) to offer some protection against photodestruction of the cell. In both studies the activity of mutant 18 was greater than that of 6E, which is deficient of carotenoids. These findings further suggest a close association between the presence of normal types of carotenoids and photosystem II activity.

Photoreduction experiments revealed almost the opposite results as seen in the Hill reaction studies (Figure 9). On a cell basis the mutants all showed less photosystem I activity than the WT but 6E did exhibit a substantial amount of CO_2 uptake. When these results are

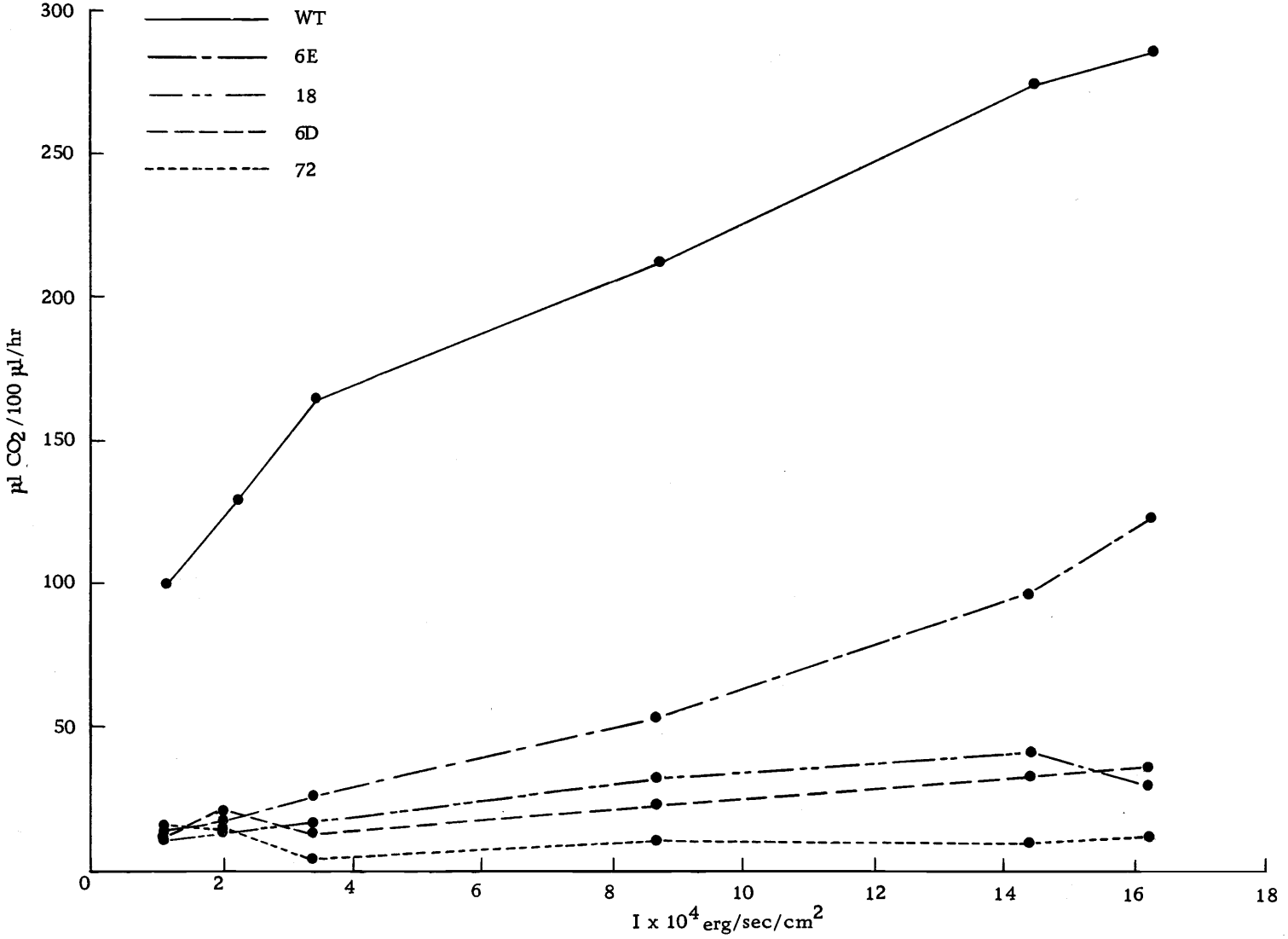


Figure 8. Photoreduction (on a cell basis) versus light intensity.

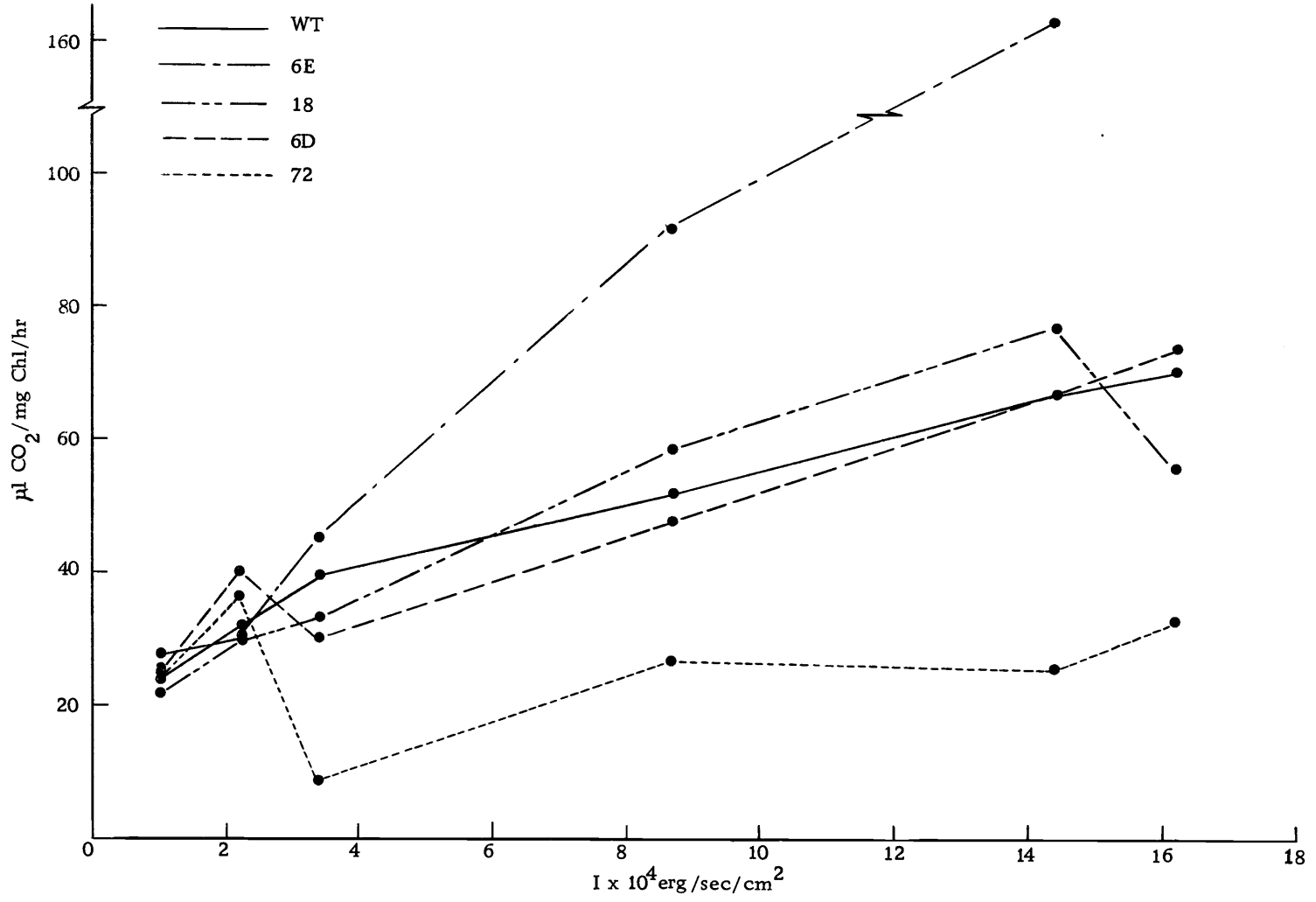


Figure 9. Photoreduction (on a chlorophyll basis) versus light intensity.

calculated on a chlorophyll basis rather than a cell volume basis, as plotted in Figure 10, the mutant 6E actually showed better photoreduction activity than the WT; mutants 18 and 6D had activities equal to the WT. Mutant 72 showed considerably less activity, i.e., about half that of the WT.

The interpretation of these data seems to indicate that the activity of photosystem I is not dependent upon the carotenoids as is photosystem II. This is especially true of mutant 6E, which shows excellent photosystem I efficiency. On an efficiency basis, photoreduction rates of C-X-18 and C-X-6D are about equal to the WT. One explanation for the low efficiency of 72 could be deduced from observations made during the greening of dark-grown cells of this culture, wherein it was noted that the original chlorophyll content dropped appreciably prior to the onset of greening. Why 6E shows such high activity is not known. But the fact that no carotenoids are present in the mutant suggests that the acyclic carotenoids may actually inhibit photosystem I activity as is seen in 6D and 18. Mutant 6E has a very high chlorophyll a - chlorophyll b ratio and thus the chlorophyll present is probably associated with photosystem I. Electron micrographs by Bishop (5) show this mutant to have only a single lamellar system rather than the normal stacking arrangement.

A summary of the general characteristics of mutants studied is presented in Table 9.

Table 9. Summary of the mutant types.

	Pigment composition - Heterotrophic/Myxotrophic				Photosynthetic studies		
	Acyclic carotenoids	Cyclic carotenoids	Oxygenated carotenoids	Chlorophyll	Hill reaction	Photoreduction	Total photosynthesis
Wild type	trace/void	normal/ normal	normal/ normal	normal/ normal	normal	normal	normal
C-X-6E	void/void	void/void	void/void	very low/ void	absent	present	absent
C-X-18	high/void	void/void	void/void	very low/ void	low	present	absent
C-X-6D	high/high	trace/ normal	trace/ abnormal	very low/ normal	low	present	low
C-X-72	trace/ trace	normal/ normal	normal/ normal	very low/ normal	low	low	low

V. CONCLUSION

For the development of a normal, photosynthetically competent chloroplast it is currently held that an involved interplay exists among the events comprising the development of membranes (thylakoids), carotenoids and chlorophyll. Interruption of the biosynthetic pathways leading to production of any of these essential components leads to abnormal development of chloroplasts and decreased photosynthetic activity. For example, if the chlorophyll concentration is low, little or no formation of thylakoids occurs in the cell and the resultant photosynthetic efficiency is poor. The carotenoid biosynthetic pathway itself does not seem to be altered by the chlorophyll content. But when the development of the normal carotenoids is modified, as in mutants 6E and 18, there is a great effect on both the chlorophyll and thylakoid formation. For if no carotenoids are formed, little chlorophyll is seen and that which is present is quickly photooxidized in the presence of light and oxygen. Nor will the thylakoid be in a stacked arrangement as in a normal cell. The same holds true if only the acyclic carotenoids are formed. But once the cyclic and oxygenated carotenoids are synthesized, normal thylakoid stacking takes place and photooxidation of the chlorophyll ceases. Lastly, there is as yet an obscure association between the thylakoid arrangement and chlorophyll production. In mutants with only a single thylakoid arrangement, which is usual in cells without the normal carotenoids,

there is some chlorophyll associated with them. But the chlorophyll is destroyed by light. Yet when the thylakoids are stacked, normal carotenoids are present, and this chlorophyll is protected. In some manner the carotenoids are required for the formation of this double thylakoid and thus serve to protect the chlorophyll from the inherent photodynamic capacity of the cell.

The interpretation of the data presented here is based upon the hypothesis that each of the mutants studied arose from a single gene mutation. Obviously this is the simplest approach, for it is equally feasible that there could be multiple site mutations which result in the phenotypes seen. Thus direct interpretation would be far more complex than that presented here, and would require selective determination of the mode of inheritance of the mutation types. But since the green alga Scenedesmus obliquus is an asexual species, there is no way to test for this situation.

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