AN ABSTRACT OF THE THESIS OF

Geoffrey W. Harvey (Name) for the Doctor of Philosophy (Degree)

in Botany and Plant Pathology (Major Department) presented on May 23, 1974 (Date)

Title: Characteristics of Two Noncarotenogenic Light Sensitive Mutants of Scenedesmus obliquus Strain D_3

Redacted for privacy

Abstract approved: Norman I. Bishop

Two light sensitive mutants of the green alga Scenedesmus obliquus strain D_3 have been characterized. The photodynamic nature of their sensitivity has been established, the subchloroplast site of photodamage has been investigated, and their noncarotenogenic character has been verified. The two mutants possess several similarities, although mutant L. S.-41 demonstrates a more extreme damage in high intensity light than does mutant L. S.-4.

Mutant L. S.-41 suffers the photodynamic loss of photosynthesis with an accompanying bleaching of chlorophyll. The damage to the photosynthetic electron transport system is general, comprising an initial loss of photosystem I followed by a later impairment of photosystem II. Associated with photosystem II impairment is the disappearance of recognizable α-tocopherol and plastoquinone A, which suggests the photoprotective role of these chloroplast components.

Fluorescence measurements indicate that generalized irreversible disruption of the photosynthetic thylakoids is the end result of prolonged exposure to intense light. A normal complement of carotenoids was demonstrated in mutant L. S.-41 both initially and following high intensity irradiation, confirming the mutant's noncarotenogenic nature.

Mutant L. S.-4 suffers a similar photodynamic impairment of photosynthesis, but this mutant gains chlorophyll content during irradiation. The damage within the photosynthetic mechanism is very specific. Results indicate an initial impairment of cyclic photophosphorylation, followed by an interruption of acyclic electron transfer immediately on the reducing side of cytochrome f. Subsequently, damage occurs to the cytochrome f, plastocyanin, and P700 components of electron transfer. Unlike mutant L. S.-41, mutant L. S.-4 is able to recover from photoimpairment to a certain degree. Photosynthetic capacity can be fully restored under dark aerobic conditions, but those characteristics of the electron transport system dependent upon tight coupling of the two photosystems do not fully recover. Recovery may be blocked by anaerobic conditions or inhibitors of protein synthesis which interfere with the normal function of 70 S ribosome. Carotenoids, quinones, and α-tocopherol all appear in normal levels in the mutant.

Characteristics of Two Noncarotenogenic Light Sensitive Mutants of *Scenedesmus obliquus* Strain D_3

by

Geoffrey W. Harvey

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1975

APPROVED:

Redacted for privacy

Professor of Plant Physiology
in charge of major

Redacted for privacy

Head of Department of Botany and Plant Pathology

Redacted for privacy

Dean of Graduate School

Date thesis is presented May 23, 1974

Typed by Suelynn Williams for Geoffrey W. Harvey

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Norman I. Bishop, whose guidance, assistance and constructive criticism has been invaluable during this research and the thesis preparation. I also wish to thank Dr. R. R. Becker, Dr. T. C. Moore, Dr. K. E. Van Holde, Dr. I. Isenberg and Dr. W. K. Ferrell for serving on my graduate committee.

Very special appreciation is extended to my wife, Kathie, whose support and sacrifice has made this work possible. She has also contributed considerably to the secretarial preparation of the manuscript. The aid of Janet L. Williams in the proofreading of the manuscript is gratefully acknowledged.

Financial support for the investigation was provided by a traineeship from the National Science Foundation (GZ-2465). Support in part was also provided by the National Science Foundation (GB-33925X) and the U. S. Atomic Energy Commission (AT(45-1)-2227).

TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

LIST OF FIGURES

LIST OF FIGURES CONTINUED

LIST OF TABLES

ABBREVIATIONS

ATP	adenosine triphosphate
CCCP	m chlorocyanocarbonylphenyl hydrazone
DPIP	2, 6-dichlorophenol-indophenol
DCMU	3-(3, 4-dichlorophenyl)-1, 1-dimethyl urea
nm	10^{-9} meters
μm	10^{-6} moles
μl	10^{-6} liters
PCV	packed cell volume

CHARACTERISTICS OF TWO NONCAROTENOGENIC LIGHT SENSITIVE MUTANTS OF SCENEDESMUS OBLIQUUS STRAIN D_3

I. INTRODUCTION

Photosynthetic phenomena have been found distributed universally among a large number of prokaryotic and eukaryotic algae, bryophytes, lower tracheophytes and spermatophytes. The general functional photosynthetic mechanism remains quite similar throughout this wide range of plant forms, although bacterial photosynthesis appears to possess only the photosystem I fragment of the normal photosynthetic electron transport system. The light requiring reactions function by means of two photosystems which are linked in series (Figure 1). These photosystems act in concert to strip electrons from water, producing molecular oxygen, and conduct these electrons to generate reduced pyridine nucleotide and adenosine triphosphate (Kok, 1965). The dark or non-light requiring photosynthetic reactions, which utilizes the light reaction products to fix carbon dioxide, function according to the scheme discovered by Calvin and Benson (Bassham, 1965). Recently, modifications of the scheme, comprised of added cycles for carbon dioxide concentration (C_4 fixation pathways and Crassulacean acid metabolism) have been found in plants adapted to arid environments (Hatch and Slack, 1970). Similar adaptations in the apportionment of the light reactions are now becoming evident

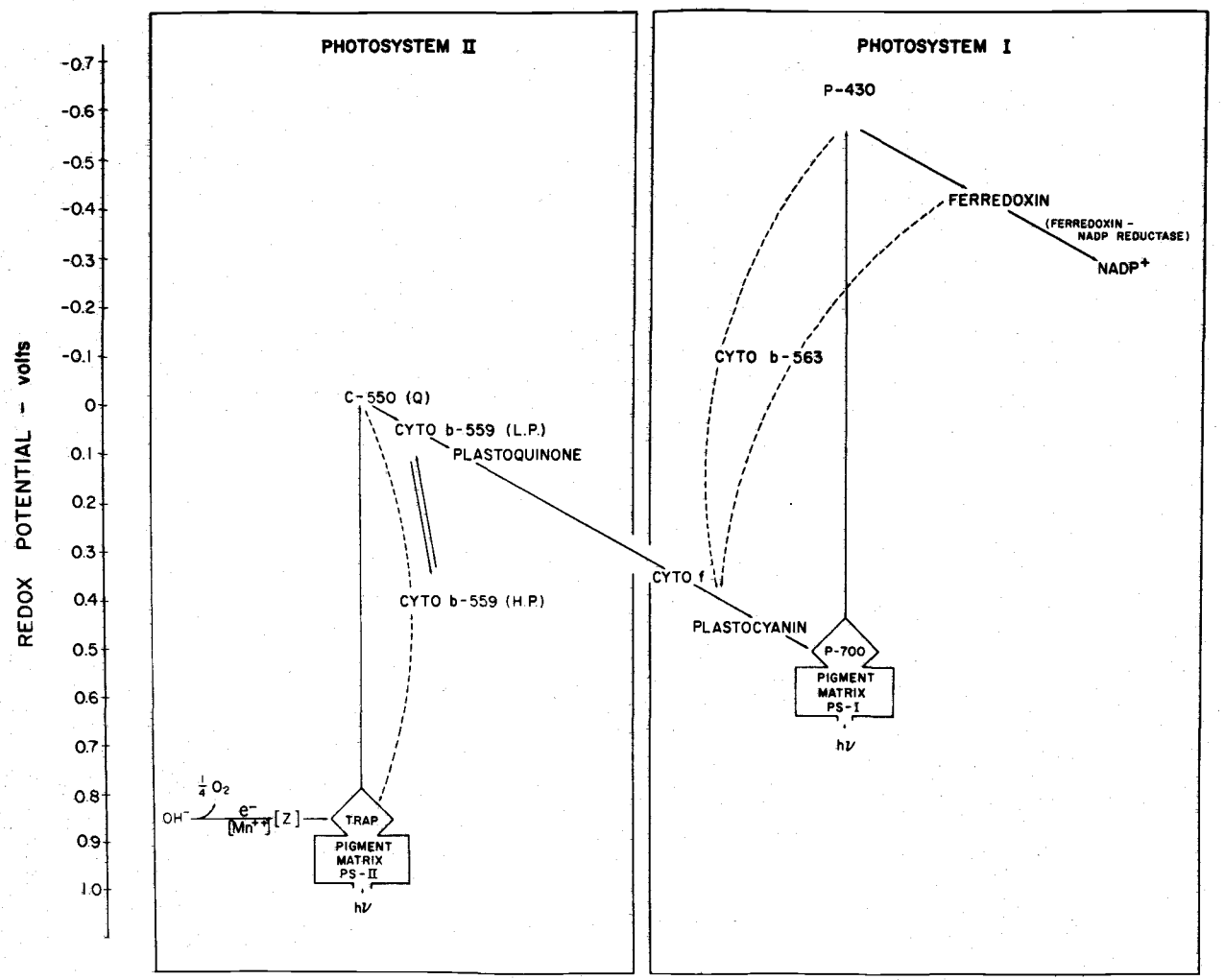


Figure 1. The photosynthetic electron transfer scheme, depicting the action of both photosystems.

(Boardman, 1971; Laetsch, 1971). Yet the information accrued to date indicates that the basic photosynthetic machinery has changed little in its design during the evolutionary process. The stability of the chronologically primitive process of photosynthesis, makes it reasonable to assume that experimental work concerning the mechanism in green algae is generally applicable to the photosynthetic mechanism of other plants.

The primary bioenergetic importance of photosynthesis to plants is reflected at the morphological, anatomical and ultrastructural levels. The evolutionary tendencies toward thin flattened modified stem systems as photosynthetic organs and chloroplast orientation mechanisms are two of the many adaptations allowing for the maximum exposure of surface area to incident irradiation. However, such exposure to potentially damaging radiation is not without difficulties. Chlorophyll, the universal photosynthetic pigment, is <u>in vitro</u> a photosensitizing dye, capable of spawning singlet oxygen (O_2^1), the major agent of photooxidations (Spikes, 1968). Incident radiation in the visible and infrared wavelengths causes thermal increases, which hasten the loss of great amounts of water through transpiration. Radiation is capable of initiating free radical chain reactions, which disrupt subcellular structure and cause damaging alteration of important molecular components (Scott, 1965; Demopoulos, 1973).

In normal plant cells and chloroplasts such disruptive effects

are not evident. The chloroplast is subjected to widely varying regimes of light intensity (ranging from 10^3 to 10^5 ergs/sec-cm^2) often with limiting carbon dioxide, the terminal electron sink, but photodamage is rarely observed. Thus mechanisms must exist within the photosynthetic machinery to forestall the harmful effects of radiant energy. The recent understanding of cells as an aggregation of biochemical systems structurally and functionally related, suggests that a number of photoprotective systems may exist which synergistically reinforce the effectiveness of each other.

Early Studies of Light Sensitivity

The phenomenon of light sensitivity, a light intensity dependent damage to cells and their components, has been induced in the laboratory by several investigators. Ursprung (1917), one of many early investigators, who used magnifying lenses to concentrate light on plant surfaces, found a net loss in starch reserves, normally a photosynthetic product, under high light intensity conditions. He used the term solarization, as derived from photography, to describe the phenomenon. Even earlier similar techniques were used to destroy a large portion of the chlorophyll present in irradiated cells (Ewart, 1898; Pringsheim, 1879-1881). Later experiments performed separately by Noack (1925), Arnold (1931) and Emerson (1933) using different approaches, confirmed the observation that intense light

inhibits the photosynthetic mechanism and detrimentally affects cellular components.

More detailed studies involving very accurate respiratory techniques with the alga Chlorella were published at nearly the same time by Myers and Burr (1940) and Franck and French (1941). Both investigations indicated that the inhibition of photosynthesis induced by intense light was intensity dependent and required oxygen. These requirements are now used to define a photodynamic process. Myers and Burr (1940) made the important observation that in the dark or at very low light intensities, the photosynthetic capacity of solarized cells was recovered. Franck and French (1941) obtained photodynamic damage by removal of carbon dioxide under intense light conditions. Their results can be explained in the light of our present knowledge as the removal of the terminal electron sink. This would cause the photosynthetic electron transfer components to remain in the reduced condition, allowing little probability that incoming quanta could do normal photochemical work. Thus the quanta are left to act in alternate degradative processes.

At a later date Kok (1956) studied the inhibitory effect of intense light by repeating the work of Myers and Burr (1940). He confirmed an intensity dependent inhibition of photosynthesis and its dark recovery. The work was extended by measuring the quantum yield of photosynthesis during the inhibition process. These data

indicated a sharp rise in the number of quanta required to drive photosynthesis during inhibition. Interpretation of the data in view of the two photosystem theory of photosynthetic electron transport leads to the conclusion that photosystem II is impaired. In a subsequent paper this conclusion is reached (Kok, Gassner and Rurainski, 1965), but the claim that the inactivation was a nonphotodynamic process was not adequately demonstrated.

Mutational techniques were first employed in investigations of photosensitivity by Griffiths et al. (1955). A mutant of the photosynthetic bacterium Rhodopseudomonas spheroides, which was found to be devoid of the cyclic carotenoids (carotenogenic), was developed. The mutant exhibits strong light sensitivity, which is of a photodynamic nature. Wild-type cells with the normal carotenoid complement are non-photodynamically sensitive (Griffiths and Stainier, 1956; Sistrom, Griffiths and Stainier, 1956). The use of the agent diphenylamine, which blocks carotenoid synthesis, demonstrated similar results (Anderson and Fuller, 1958). This evidence, combined with earlier evidence regarding carotene's in vitro role in the inhibition of chlorophyll photooxidation (Aronoff and Mackinney, 1943), spawned the theory that carotenoids are photoprotective agents. The theory has been termed the Stainier hypothesis (Krinsky, 1971).

The mutational work with photosynthetic bacteria stimulated interest in similar work with more advanced photosynthetic organisms.

Claes (1954) developed a number of carotenogenic mutants of the green alga, Chlorella. These mutants were photodynamically sensitive, contained much lower chlorophyll levels than the wild-type cells and did not form cyclic carotenoids (Kandler and Schötz, 1956). Subsequent work with carotenogenic mutants of Chlamydomonas (Sagar and Zalokar, 1958) and Scenedesmus (Williams, 1971) has shown that these mutants produce few thylakoids, which are not stacked and bear little chlorophyll. More significantly, Kandler and Sironval (1958) isolated a Chlorella strain, which was photosensitive, but contained nearly normal levels of chlorophyll and carotenoids. When subjected to high intensity light the cells suffered irreversible bleaching of chlorophyll and carotenoids, while the normal strains were unaffected. Further discussion of experiments conducted with this light sensitive strain will be deferred until later.

Experiments with carotenogenic mutants in higher plants have been conducted, even though problems with maintaining the mutant in higher plants, which will not grow heterotrophically, limit such experiments. Several mutants of sunflower (Helianthus annuus) have been shown to be carotenoidless. These mutants do make small amounts of chlorophyll, but it is quickly bleached (Wallace and Schwarting, 1957). Similar mutants in corn (Zea mays) were developed (Smith and Koski, 1951), which contain the carotene precursor phytoene as the major C-40 polyene (Anderson and Robertson, 1959).

Again chlorophyll is bleached as rapidly as it is synthesized in intense light, but in weak light chlorophyll is formed (Anderson and Robertson, 1959; Smith, Durham and Wurster, 1959). More extensive reviews of carotenogenic mutants of both photosynthetic and non-photosynthetic organisms have been prepared by Krinsky (1966; 1968).

Mechanism of Photodynamic Oxidations

The mechanism of photodynamic oxidations has been studied (Spikes and Straight, 1967; Gollnick, 1968; Scott, 1965). The most widely accepted scheme for photodynamic oxidations is diagrammed below (Schenk, 1954).

$$S + h\nu \longrightarrow S^*$$

$$S^* \longrightarrow S^3$$

$$S^3 + O_2^3 \longrightarrow S + O_2^1$$

$$O_2^1 + A \longrightarrow AO_2$$

A sensitizing dye (S) absorbs a quantum and an electron is raised to a higher energy state (S^*). Eventually, the excited state decays either to the ground state or, of greater interest, to the triplet state (S^3). The triplet state of the sensitizer is then able to transfer its energy to triplet (ground state) oxygen (O_2^3) and decay to the ground state (S). In the process triplet oxygen is excited to the singlet state (O_2^1), which is able to react with several substrates (A) in a rather nonspecific

manner. It is not certain that the O_2^1 is a free species, and for this reason the alternate theory of a sensitizer-oxygen complex involved in the oxidation has persisted (Gollnick, 1968).

Recent experiments conducted by Kornhauser et al. (1973), bear on the scheme presented above. Photodynamic oxidation of guanosine was compared with O_2^1 oxidation. It was found that the photodynamic oxidation consisted of two distinct modes of oxidation, type I and type II, which give rise to different products. The type II products were similar to oxidation products caused by singlet oxygen produced by radiofrequency discharge. However, the nature and mechanism of type I oxidations remain obscure.

Early observations of the photodynamic destruction of chlorophyll in vitro and carotene's protective effect were made by Aronoff and Mackinney (1943). Evidence for singlet oxygen generation by photosensitations of chlorophyll in vitro has also been presented (Foote and Wexler, 1964). Further, Foote (1968) and Foote and Denny (1968) provide evidence that carotene quenches singlet oxygen as shown in the scheme below. Chlorophyll acts as a photosensitizing dye, as in the

$$\text{Chl} + h\nu \longrightarrow \text{Chl}^1$$

$$\text{Chl}^1 \longrightarrow \text{Chl}^3$$

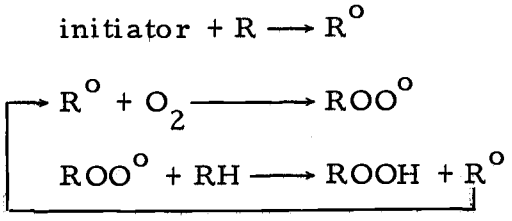
$$\text{Chl}^3 + O_2^3 \longrightarrow \text{Chl} + O_2^1$$

$$O_2^1 + \text{car} \longrightarrow O_2^3 + \text{car}^3$$

$$\text{car}^3 \longrightarrow \text{car} + \text{heat}$$

scheme presented earlier, forming singlet oxygen. The singlet oxygen reacts with ground state carotene forming ground state oxygen and triplet carotene. Triplet carotene then decays to the ground state by radiationless heat loss (Mathis and Kleo, 1973). The scheme appears to be a plausible mechanism for photoprotection in photosynthetic thylakoids, which are rich in carotene and carotenoids; these structures are also rich in quinones which quench chlorophyll triplets (Fujimori and Livingston, 1957). However, the possibility that carotenes and carotenoids as well as quinones function by quenching chlorophyll triplets rather than singlet oxygen has not been excluded.

Another effect of intense light is the initiation of free radicals. Free radical chain reactions are started by initiating phenomena and proceed through several cycles as diagrammed below (Scott, 1965).



The initiator acts by stripping an electron from or adding an electron to a compound, transforming it into a free radical. Oxygen can add to the radical to produce an oxygenated free radical, which is quenched by formation of the initial free radical species. Thus a chain reaction is realized. Visible and ultraviolet light, as well as peroxides and transitional metal ions have been demonstrated to be

initiators. Quinones and the antioxidants (especially α-tocopherol) have been suggested as agents, retarding free radical oxidations. Such agents act either by counteracting the initiator or by interfering with the free radical cycle. Plant phenolics, carotene and carotenoid triplet quenching and quinone triplet quenching may act by initiator suppression. Ascorbic acid and α-tocopherol, as well as other agents, most likely act by electron or hydrogen ion donations which break the free radical chain. Unchecked, such reactions would cause detrimental alterations of cellular components.

Subcellular Sites of Photodamage

The possible subcellular sites of photodamage have been reported in only a few cases. Such subcellular localization provides a degree of insight into the mechanism of photodamage and protective mechanisms. The most common form of damage is to the pigment matrix in the form of pigment bleaching. Bleaching has been reported, as stated earlier, by several investigators in both mutants and wild-type cells (Griffiths and Stainier, 1956; Kandler and Sironval, 1958; Smith and Koski, 1951; Wallace and Schwarting, 1954; Abeliovich and Shilo, 1972). Photobleaching has similarly been reported in isolated chloroplasts deprived of their normal electron source (Yamashita, Tsuji and Tomita, 1971).

Impairment of electron transport processes have been reported.

Kandler and Sironval (1959) provided evidence indicating an impairment of respiration in their light sensitive Chlorella strain. Their findings implicated an uncoupling of oxidative phosphorylation as an initial effect of intense light. After respiratory impairment photobleaching began. Prebble and Huda (1973) have demonstrated damage at several points in the respiratory electron transport chain of a carotenogenic mutant of the bacterium, Sarcina lutea.

Impairment of photophosphorylation has been shown in isolated chloroplasts. Forti and Jagendorf (1960) demonstrated an anaerobic light dependent impairment. This damage appeared to be localized in the cyclic photophosphorylation site, since agents which short circuit cyclic electron flow (PMS) restore a part of the phosphorylation. Kendeleva et al. (1972) have recently reported similar damage to cyclic photophosphorylation in pea chloroplasts when laser illumination is used. Although the role of oxygen was not clearly defined, it was shown that cyclic photophosphorylation could be impaired to levels greater than 70% with less than 30% impairment of pyridine nucleotide photoreduction. An anaerobic photoinactivation of photosystem II has been claimed in isolated chloroplasts (Kok, Gassner and Rurainski, 1965). The evidence supports an inactivation of photosystem II, but it is not clearly demonstrated that the process is anaerobic.

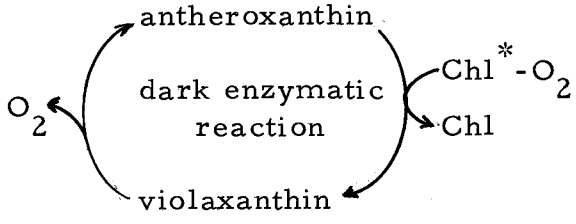
Theories of Photoprotection

The capability of organisms to survive and grow in intense light, when many of their components are photosensitizing dyes, has been explained by a number of theories. The mutant work and experiments with the carotenoid synthesis inhibitor, diphenylamine, support the hypothesis that carotenoids are photoprotective agents (Krinsky, 1966; 1968; 1971). However, the mode of carotenoid protection is not settled.

Singlet oxygen or triplet chlorophyll quenching by carotene and carotenoid, which has been demonstrated in vitro (Foote and Denney, 1968), has been thought to occur in vivo. Supportive evidence for this hypothesis has been supplied by Witt and Wolff (Witt, 1971; Witt and Wolff, 1972). These investigators have shown that the 520 nm absorbency change has an action spectrum very similar to the absorption spectrum of a carotene or carotenoid. They argue that the 520 change is an indicator of the metastable triplet carotene. Although these in vivo studies agree with Foote's in vitro work, many investigators believe that the 520 nm absorbency change reflects a positional perturbation of a carotenoid within the membrane, which is related to the proper membrane conformation required for photophosphorylation (Fleischman and Clayton, 1968; Baltscheffsky, 1969). Recently Krinsky has moved closer to the singlet oxygen quenching view by

presenting evidence dealing with the in vitro photolysis of synthetic liposomes, which is best explained by carotenoid quenching of singlet oxygen or a dye-oxygen complex triplet quenching (Anderson and Krinsky, 1973).

In earlier papers, Krinsky (1966) has been the proponent of carotenoid protective action as demonstrated by the scheme below. The carotenoid antheroxanthin is epoxidated by a chlorophyll*-O_2 complex producing violaxanthin and ground state chlorophyll. In a



subsequent dark enzymatic reaction violaxanthin is de-epoxidated with the liberation of oxygen. The scheme is based in part on observations of Dworkin (1959), who noted that wild-type Rhodopseudomonas spheroides irradiated aerobically at low temperatures suffer photodynamic killing. From this work, an oxycarotenoid reductase enzyme was suggested which could operate only at moderate temperatures. However, alternate explanations of the photokilling are possible. Among these is a diminution of the Calvin cycle reactions causing in effect the same phenomenon observed by Franck and French (1941). Patterns of carotenoid flux in the green alga Scenedesmus do not

support the scheme (Williams, 1971; Williams, Frick and Bishop, 1974). Rather than the expected increase in violaxanthin in irradiated cells, a decrease is observed with a concurrent increase in the dihydroxylated forms, lutein and zeaxanthin.

Many investigators have developed evidence consistent with the theory that carotenoids act in a structural capacity to insure the proper membrane conformation. The electron microscopic examination of carotenogenic mutants indicate that these mutants possess aberrant thylakoids as compared to wild-type cells. This result has been obtained in the green algae Chlamydomonas (Sagar and Zalokar, 1958) and Scenedesmus (Williams, 1971). Such mutants also contain considerably less chlorophyll, much of which is not bound to the membrane, and are highly photosensitive. Using fluorescence techniques, Brill (1963) determined that carotenoid loss affects the structure of the lamellae and their ability to undergo conformational changes. Recent evidence, suggesting that conformational changes in the thylakoids during illumination exclude oxygen from the pigment matrix, denotes the importance of carotenoids as structural entities capable of cis-trans isomerizations (Papageorgiou, Isoakidous and Argoudelis, 1972). The requirement of specific carotenoids to insure proper structure has been demonstrated with mutants containing partially cyclized polyenes. These mutants exhibit photosensitized damage to photosystem II and possess aberrant thylakoid structure (Faludi-Daniel

and Nagy, 1972). Additionally, the structure of the membrane seems alterable by the plant to respond to changing environmental conditions. In Vaccinium myrtillus the ratios of chlorophyll:carotenoid have been shown to vary markedly at different times of the year (Schultz, 1973). Thus evidence is present to argue that the carotenoids major role is to provide a proper membrane assembly, which in turn allows for the development of a proper structural surface for certain essential free radical reactions to occur. This situation would be readily alterable according to changing needs.

The enzyme superoxide dismutase may also act as a photoprotective agent. This protein was first recognized in animal cells, in which the protein was known as erythrocuprin or hemocuprin, but no function was known. The enzyme catalyzes the reaction shown below (McCord and Fridovich, 1969). It has been recognized in

$$O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

animals and plants (Asada, Urano and Takahaski, 1973) as a copper containing enzyme and in bacteria as a manganous enzyme (Keele, McCord and Fridovich, 1970). In plants the subcellular location has been demonstrated to be in the chloroplast (Asada et al., 1973). One of its roles may be to dismutate superoxide radicals which are formed in the photosynthetic water-splitting process or by electron donation to oxygen. In this regard it might act in the univalent oxygen

reduction known as the Mehler reaction (Mehler, 1951a; 1951b; Asada et al., 1973). The potential for biological protection against superoxide radicals by this enzyme has been convincingly demonstrated (Lavelle, Michelson, and Dimitrijevic, 1973), but it is also possible that the high levels of ascorbate in chloroplasts scavenge O_2^- in the aqueous regions (Allen and Hall, 1973).

Liver microsomal research has uncovered the deleterious effect of free radicals upon membrane configuration. The best known of these is the microsomal lipid peroxidation, initially discovered by Hochstein and Ernster (1963). Lipid peroxidation occurs by a free radical chain reaction similar to that shown earlier. The prime substrate for lipid peroxidations are mono, di and tri-unsaturated fatty acids. These fatty acid species are abundant among thylakoid lipids, particularly in the two glycolipids, monogalactosyldiglyceride and digalactosyldiglyceride. The major product of lipid peroxidation is the free radical malonyldialdehyde, a compound capable of cross linking reactions similar to those of glutaraldehyde, the cross linker frequently used for fixation in electron microscopy. Fatty acid chain schism is another product of lipid peroxidation and would cause major alternations in membrane conformation (Tappel, 1973).

Lipid peroxidation has been demonstrated to occur in isolated chloroplasts (Heath and Packer, 1965). It was shown that light and exogenous doses of the unsaturated fatty acid, linoleic acid,

stimulated the peroxidation, which yielded the major product, malonyldialdehyde (Heath and Packer, 1968a, b). The stimulation by light is explained either by its role as a chain reaction initiator or by the photosynthetic production of peroxides. Transitional metal divalent cations, excepting Mn^{+2}, also stimulated malonyldialdehyde production. Damage to the chloroplasts was found localized in photosystem II.

Two means of suppressing free radical reactions have been suggested (Scott, 1965). The first of these is to suppress the initiator before the chain reaction begins. Demopoulos (1973) has suggested that two of the most common features of cellular activities are designed for this purpose. One control over free radical reactions is the retention of these reactions, which are necessary to the cells economy, on the hydrophilic surface of a highly structured membrane. Thus the free radicals of electron transport phenomena are not free to initiate deleterious free radical reactions. In a similar way enzyme-substrate complexes may play the important role of keeping free radical intermediates of cellular reactions in a non-reactive state. As discussed earlier carotene and quinones may act to quench triplet initiators, while plant phenolics may act as a screening agent against ultraviolet radiation (Andersen and Kasperbauer, 1973).

Another mechanism is through agents which break the free radical chain reaction. Such agents act by donating electron or

hydrogen ions to free radicals in order to break the chain. α-Tocopherol, which is known to be an antioxidant and to suppress lipid peroxidation (Zalkin and Tappel, 1960), is thought to function in this manner (Scott, 1965). Ascorbic acid might play a similar role in an aqueous medium. Quinone compounds might also scavenge free radicals (Demopoulos, 1973).

Evidence supporting the role of antioxidants has been rare in photosensitive systems. Dworkin (1960), suggested a "photochemical" buffer to explain resistance to photokilling in a carotenogenic mutant. Although α-tocopherol has not been found in bacteria, some similar antioxidant could be involved. The protective effect of antioxidants was noted by Brill (1963) in his system. Heath and Packer (1968b) and Anderson and Krinsky (1973) also observed the benefits of α-tocopherol in photodynamic in vitro systems. Recently, Bishop (1974) has isolated a mutant of Scenedesmus, which does not form α-tocopherol. The mutant is very photolabile, suffering the photodynamic loss of photosystem II. Thus it is reasonable to assert that α-tocopherol is a photoprotective agent in photosynthetic organisms and may function as shown below (Figure 2).

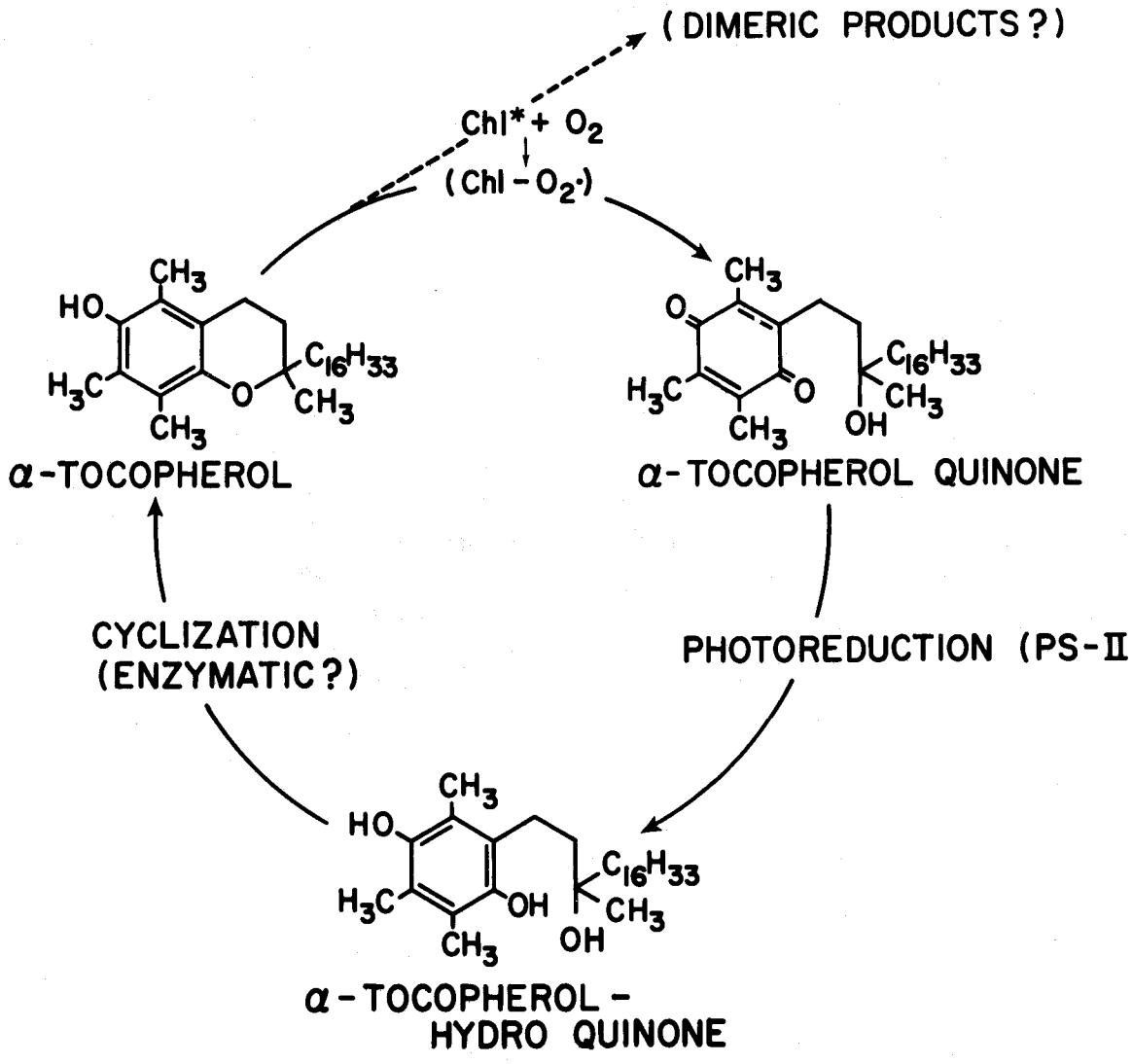


Figure 2. Hypothetical scheme depicting the function of α-tocopherol in photosystem II.

II. STATEMENT OF PURPOSE

A survey of the literature concerning light-sensitive mutants indicates that all such mutants are of a carotenogenic nature. Close examination of the methodology used to isolate these mutants reveals the common feature of their selection on the basis of abnormal coloration. This uniform selection method explains not only the homogenous nature of these mutants developed from widely varying plant groups but also the failure of previous researchers, using the mutation approach, to suspect alternate mechanisms of photoprotection in photosynthetic organisms. In addition the apparent relation between the carotenogenic syndrome and light sensitivity seems to have stifled other selective approaches.

In the work reported here, a different selective approach was employed. A light sensitive mutant was defined as any mutant which would not grow autotrophically or myxotrophically, but would grow and exhibit normal photosynthesis under heterotrophic conditions. Using this approach, our purpose has been to find new types of light sensitive mutants, investigate the possibility of alternate photoprotective mechanisms within the photosynthetic mechanism, and understand the sites of photodamage within the photosynthetic electron transfer system of the mutants.

III. METHODS AND MATERIALS

Culture of Algal Strains

Scenedesmus obliquus strain D_3 was cultured heterotrophically on nitrate medium (Kessler, Arthur and Brugger, 1957) enriched with 0.5% glucose and 0.25% yeast extract. Cells were grown in 250 ml of medium in a 500 ml screw top erlenmeyer flask agitated on a shaker at 25°C (Pratt and Bishop, 1968). Autotrophic cultures were grown on the nitrate medium in bubble tubes (Senger, 1970), before fluorescent light banks composed of soft white and Gro-Lux elements. Light intensity was approximately 1×10^4 ergs/sec-cm^2; air - 4% CO_2 was bubbled through the culture. Myxotrophic cultures were grown under conditions identical to autotrophic growth, but the glucose and yeast extract enriched nitrate medium was used.

Two day cultures were harvested for all experiments, since these cultures were approaching the end of logarithmic growth and showed optimal photosynthetic capacity (Berzborn and Bishop, 1973).

Mutant Isolation

General photosynthesis mutants were isolated after x-ray or chemical treatment using the $^{14}CO_2$ uptake technique outlined by Bishop (1971), or the fluorescence technique described by Bennoun and Levine (1967). The light sensitive mutants employed in this study

were selected upon the basis of their failure to grow under both autotrophic and myxotrophic culture conditions, while exhibiting growth and photosynthesis when cultured heterotrophically.

High Intensity Irradiation Conditions

High intensity irradiations were performed with a light source equipped with a 1000 W Sylvania tungsten-halogen lamp (DNX). The light beam was focused and collimated to produce a nearly uniform field of light. Light intensities were regulated with copper wire screens; light intensity was measured with a YSI model 65 radiometer. Cells were irradiated in a Kolle culture vessel, of 3 cm depth, held in a constant temperature bath at 25°C; 2 μl packed cell volume/ml were resuspended in 0.05 M K_2H-KH_2PO_4 buffer (pH = 6.5) and normally bubbled with air - 4% CO_2 (exceptions will be noted in appropriate places). These conditions permitted no increase in cell numbers of the suspended cultures.

Rates of photosynthesis and respiration were determined with a Clark Ag-AgCl electrode in association with a Gilson model KM oxygraph. The oxygraph was equipped with a light source providing an intensity to the cells of 2.5×10^5 ergs/sec-cm^2, composed of wavelengths greater than 580 nm.

Chlorophyll Determinations

Hot methanol extraction of whole cells was used to determine chlorophyll. Chlorophyll concentration was determined spectrophotometrically and calculated using the equation developed by Holden (1965), which has been adapted to provide data in micromoles.

$$\mu M\ Chl = 25.5\ (A_{650}) + 4.0\ (A_{665})\ X\ D.F.\ X\ V\ ml\ X\ 0.001104$$

The equation is based upon the extinction coefficients of chlorophyll a and b in methanol as determined by MacKinney (1941).

Photoreduction and p-Benzoquinone Hill Reaction

Photoreduction was performed using a Gilson Differential Respirometer (Bishop, 1972). Cells (100 µl packed cell volume) were collected by centrifugation and resuspended in 2.5 ml of 0.05 M KH_2-K_2HPO_4 buffer (pH = 6.5); 0.5 ml of $5 X 10^{-6}$ M DCMU was placed in the side arm well. The samples were gassed with hydrogen - 4% CO_2 and allowed to adapt their hydrogenase for 8 hours. The DCMU was then added from the side arm. The samples were pre-illuminated with the measured vessel exposed to $2 X 10^4$ ergs/sec-cm^2 for 5 minutes to exclude lag effects often observed. Rates of photoreduction were then measured as microliters of hydrogen and carbon dioxide consumed at a light intensity of $9 X 10^4$ ergs/sec-cm^2.

p-Benzoquinone Hill reaction was measured with the Gilson

model KM oxygraph using 20 µl packed cell volume of cells resuspended in 2 ml of 0.05 M KH_2-K_2HPO_4 buffer (pH = 6.5) made to 10 mM $MgCl_2$, 20 mM KCl and 23 mM p-benzoquinone (Bishop, 1972).

Fluorescence Measurements

Fluorescence measurements were made with the instrumentation and methods developed by Senger and Bishop (1972). Two samples of cells (15 µl packed cell volume) were resuspended in 3.0 and 2.5 ml respectively, of 0.05 M KH_2-K_2HPO_4 buffer (pH = 6.5); one was made to 4 µM DCMU by the addition of 0.5 ml of 5 X 10^{-5} M DCMU. Base level and DCMU induced fluorescence were measured using a 436 nm actinic light. Variable yield fluorescence was measured by noting the effects of 650 nm and 712 nm wavelengths of light each at an intensity of 2 X 10^2 ergs/sec-cm^2, upon the base level fluorescence.

Anaerobic Glucose Photoassimilation

Anaerobic glucose photoassimilation was measured using a modification of the scheme reported by Pratt and Bishop (1968). Erlenmeyer flasks (50 ml) were supported in the control temperature bath of a Gilson differential respirometer, held at 25°C. Samples of algae, containing 100 µl packed cell volume of cells, were resuspended in 3 ml of 0.05 M KH_2-K_2HPO_4 (pH = 6.5) made to 31 mM glucose. The vessels were continuously gassed with argon, while they were

irradiated for 2 hours at 2 X 10^4 ergs/sec-cm^2. Glucose concentration was measured with the Nelson method on an aliquot of the supernatant buffer following centrifugation of the cell suspension. The amount of glucose consumed during the irradiation was calculated by comparison of the amounts of glucose remaining in the darkened control vessel and the illuminated one.

Hydrogen Photoproduction

The method of Stuart was utilized for hydrogen photoproduction measurements (1971). The Gilson differential respirometer was used in conjunction with Warburg flasks, equipped with center wells and double side arms. Cells (100 µl packed cell volume/sample) were resuspended in 2.5 ml of 0.05 M KH_2-K_2HPO_4 buffer (pH = 6.5; 0.5 ml of 3 X 10^{-4} M CCCP was placed in the side arm and 0.2 ml of 10% KOH was placed with a filter paper wick in the center well. The vessels were gassed with hydrogen and allowed to adapt their hydrogenase during an 8 hour period. Before measuring hydrogen photoproduction, the CCCP was added from the side arm, and the vessels were gassed with argon. Preillumination was then carried out for 10 minutes at an intensity of 2 X 10^4 ergs/sec-cm^2 to avoid lag effects often observed. Light dependent hydrogen evolution was measured directly in microliters at an intensity of 9 X 10^4 ergs/sec-cm^2.

520 nm Absorbency Change

An Aminco-Chance (DW-2) spectrophotometer was used to measure the 520 nm absorbence change. Cells (15 µl packed cell volume/sample) were resuspended in 3 ml of KH_2-K_2HPO_4 buffer (pH = 6.5) and placed in the sample cuvette. A measuring wavelength of 520 nm was used with the isosbestic reference wavelength set at 540 nm. A Balzer K-4 broad band interference filter and a Corning green 4-77 filter were used to exclude wavelengths of light generated by the actinic beams of light. A high pressure Hg arc lamp (150 W) served as the actinic light source; its output was filtered through a Corning infrared filter (No. 1-69) and a Schott filter transmitting light at 663 nm wavelength with a half bandwidth of 11 nm. The light beam was collimated and focused upon the reaction cuvette with appropriate lenses; light intensity at the surface of the cuvette was 2.5×10^2 ergs/sec-cm^2.

Chloroplast Isolation and Reactions

Chloroplasts were islated from Scenedesmus using techniques developed by Berzborn and Bishop (1973).

Methyl viologen reduction systems, using both water and DPIP-ascorbate as electron sources, were developed after systems originally described by Kok, Rurainski, and Owens (1965) and Izawa et al. (1967).

For methyl viologen photoreduction with water as the electron source, the standard reaction buffer (20 mM tricine-KOH pH 7.5, 30 mM KCl, 0.4 M sucrose, and 1% (W/V) bovine serum albumin) was made 0.1 mM methyl viologen, and 0.3 mM NaN_3 with chloroplasts (90 μg chlorophyll/sample) added to a final volume of 2 ml. A second, control reaction contained 2 μM DCMU as well. Reactions were also run which included saturating levels (0.03 μM) of purified Scenedesmus plastocyanin.

Methyl viologen photoreduction, using DPIP-ascorbate as an electron source, was carried out by making 2 ml of the standard buffer (see above), to 0.1 mM methyl viologen, 0.3 mM NaN_3, 0.2 μM DPIP, 20 μM Na ascorbate, 2 μM DCMU, and 0.03 μM plastocyanin. Chloroplasts were added to provide the reaction mixture with 90 μg chlorophyll. Both photoreductions were measured as oxygen uptake with the Clark (Ag-AgCl) electrode in conjunction with a Gilson model KM oxygraph, when irradiated with red light (> 580 nm) of an intensity of 2.5×10^5 ergs/sec-cm^2. The results obtained with Scenedesmus chloroplast preparation, using dye-ascorbate as the electron source, are consistent with those reported by Izawa (1968). Since oxygen uptake was DCMU insensitive, and light dependent, it was assumed to be a photosystem I driven photoreduction of methyl viologen, which was followed by electron donation to oxygen for ascorbate oxidation (Allen and Hall, 1973). The methyl viologen photoreduction, with H_2O

as the electron donor, was assumed to be an acyclic electron flow requiring both photosystems.

The absorbency change in Scenedesmus chloroplasts, attributable to cytochrome 552 (cyto. f), was monitored with the Aminco-Chance (DW-2) spectrophotometer at 552 nm. Chloroplasts (45 μg chlorophyll/ml) were placed in 3 ml of the standard buffer containing exogenous plastocyanin (0.03 μM). The measuring beam was set at 552 nm and the reference isosbestic point at 575 nm. The phototube was protected against extraneous irradiation with a Balzer K-4 broad band interference filter and a Corning blue-green 4-71 filter. Two high pressure Hg arc lamps (150 W) served as the actinic light sources. One output was filtered through a Corning infrared filter (No. 1-69) and a Schott filter transmitting light at 663 nm wavelength with a half bandwidth of 11 nm. The second output was filtered through a similar arrangement, but a Schott filter, transmitting light at 705 nm with a half bandwidth of 12 nm, replaced the 663 nm transmittance filter. Both light beams were collimated and focused upon the reaction cuvette with appropriate lenses; light intensities at the surface of the cuvette were 5×10^2 ergs/sec-cm^2 and 1×10^2 ergs/sec-cm^2 respectively. The actinic sources were controlled with shutter systems and a timer to provide alternating 15 second illumination.

Photosynthesis Recovery Inhibition Experiments

Recovery of photosynthetic capacity in the presence of protein synthesis inhibitors was performed with erlenmeyer flasks (125 ml) suspended in the regulated temperature bath of a Gilson respirometer, held at 25°C. The photoinactivated cells (2 µl packed cell volume/ml) were suspended in 0.05 M KH_2-K_2HPO_4 buffer (pH = 6.5) and air - 4% CO_2 was bubbled continuously through the darkened vessels. Recovery of photosynthetic capacity was monitored each 30 minutes.

To determine if recovery of photosynthesis was dependent upon synthesis of new protein, varying levels of antibiotic inhibitors cycloheximide (0 - 7.0 µg/ml) and chloroamphenicol (0 - 60 µg/ml) were included in separate flasks (Bishop, unpublished results).

Plastoquinone, α-tocopherol and Carotenoid Analyses

Plastoquinone, α-tocopherol and carotenoid separation and identification were performed with methods similar to those described by Bishop and Wong (1971). Separation of plastoquinone A and α-tocopherol was achieved using a single plate chromatography of an aliquot of hot methanol whole cell extract. This procedure, employing silica gel G as absorbant and benzene-heptane (85-15 V/V) as solvent, replaced the column and TLC procedure previously employed (Bishop and Wong, 1971). The area on the plate containing plastoquinone was

removed, the plastoquinone eluted from the absorbent with chloroform, and then evaporated to dryness. The sample was redissolved in 3 ml absolute ethanol and the absorbencies of the oxidized and reduced form were determined at 255 nm; sodium borohydride was used to reduce plastoquinone. The change in absorbency was used in the equation below,

$$\mu M\ PQ = \Delta A_{(255)}(0.2005)\frac{V\ (total)}{V\ (streaked)}$$

to calculate total μ moles in a 3 ml sample. The area corresponding to α-tocopherol (R_f = 0.4) was removed and rechromatographed with the same TLC system. After elution of the purified sample, its concentration was determined spectrophotometrically at λ = 292 nm using an $E^{1\%}_{1\ cm}$ = 75 in absolute alcohol as determined in this laboratory with authentic α-tocopherol. The concentration was calculated from the equation below,

$$\mu M\ \alpha\text{-tocopherol} = A_{292}(0.9411)\frac{V\ (total)}{V\ (streaked)}$$

assuming a sample volume of 3 ml.

Carotenoids were separated and quantified in accordance with the methods developed by Williams (1971) and Williams, Frick and Bishop (1974). An averaged $E^{1\%}_{1\ cm}$ of 2500, in absolute alsohol, was used to determine the concentrations of all the carotenoids investigated.

IV. RESULTS AND DISCUSSION

Mutant L.S.-41

General Characteristics and the Photodynamic Nature of the Light Sensitivity

The photosynthetic behavior of wild-type Scenedesmus and that of the mutant L.S.-41 in high intensity irradiation are quite different (Figure 3). When heterotrophically grown cells are exposed to high intensity white light (1.5×10^6 ergs/sec-cm^2) under autotrophic conditions, wild-type cells exhibit a normal and anticipated increase in photosynthetic capacity, while the cells of L.S.-41 reveal a rapid decline in photosynthetic activity. The responses of photosynthesis, respiration and chlorophyll concentrations to irradiation time were examined in greater detail for L.S.-41 (Figure 4). The decline in respiratory activity occurs at approximately the same rate as that of photosynthesis. The loss of chlorophyll in the mutant is a slow process, unlike the rapid chlorophyll bleaching exhibited by many carotenogenic mutants of Scenedesmus (Williams, 1971) and by Kandler's (1958) strain of Chlorella. Chlorophyll loss is, however, a significantly different feature of the mutant, since wild-type cells gain in chlorophyll content during irradiation.

Two lines of evidence suggest that the damage to photosynthesis in mutant L.S.-41 is of a photodynamic nature. The first of these is

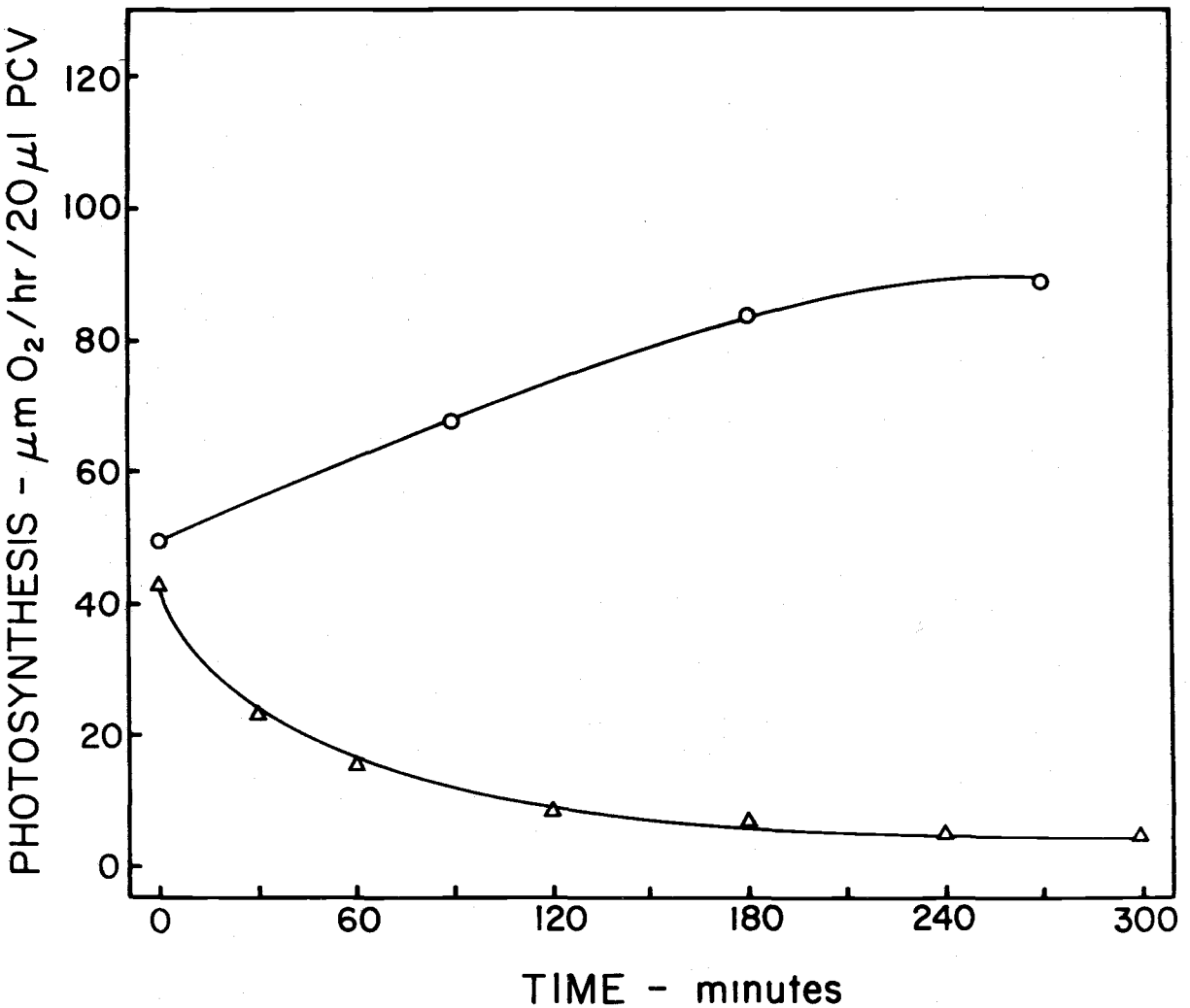


Figure 3. Photosynthetic capacity of wild type (o-o) and mutant L.S.-41 (Δ-Δ) during a time course in high intensity (1.5×10^6 ergs/sec-cm^2.

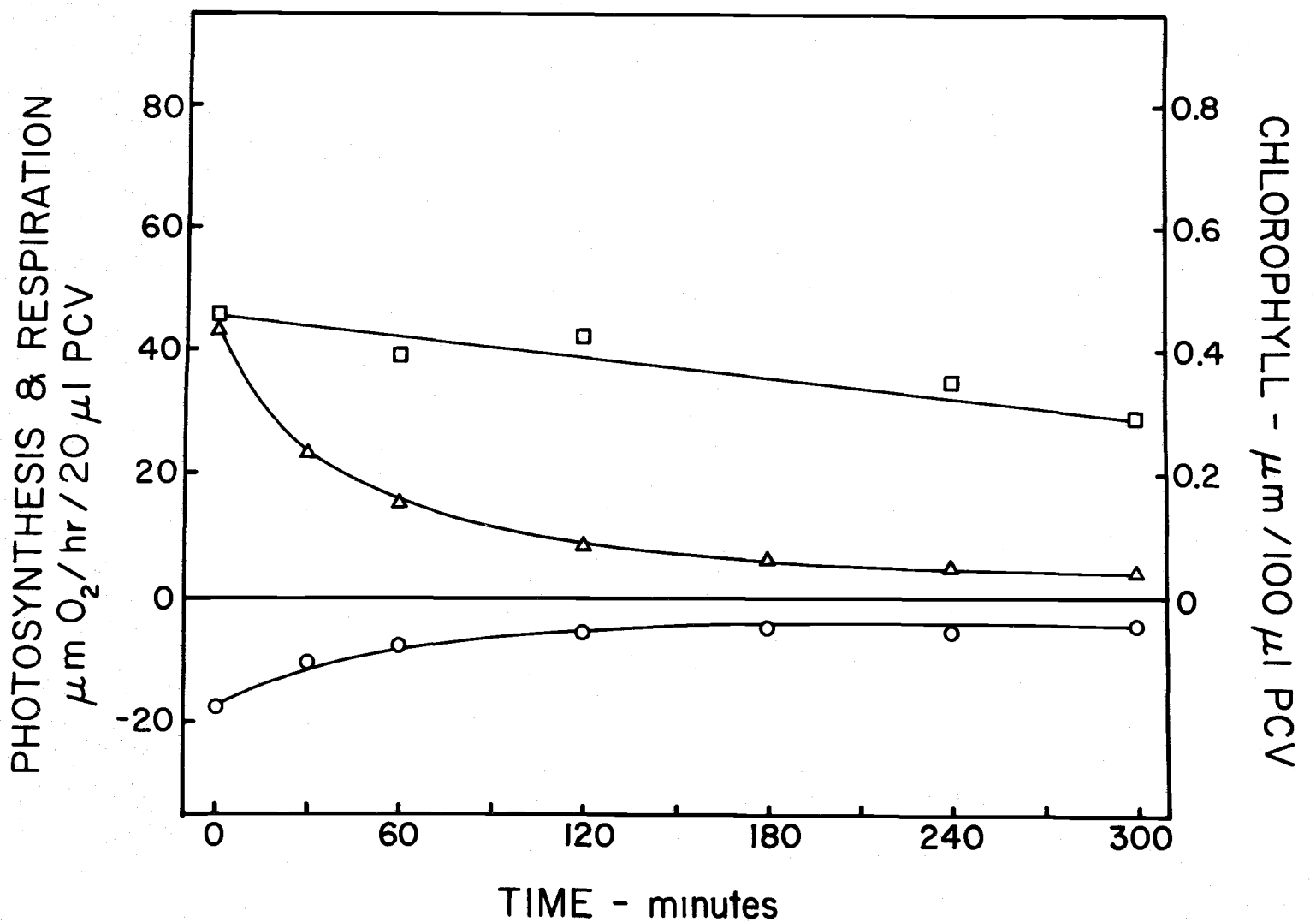


Figure 4. Photosynthesis (Δ-Δ), respiration (o-o), and chlorophyll (□-□) levels for the mutant L.S.-41 while subjected to white light with an energy of 1.5×10^6 ergs/sec-cm^2.

the photosynthetic behavior of the cells when exposed to different light intensities in air - 4% CO_2 (Figure 5). The data demonstrate that the degree of inhibition is a function of light intensity. The second line of evidence involves the role of oxygen in the observed inhibition. When cells are illuminated at one light intensity (1.5×10^6 ergs/sec-cm^2) in the presence or absence of oxygen, no inhibition is noted in the latter case (Figure 6). If air - 4% CO_2 is bubbled through the culture vessel during irradiation, a typical loss of photosynthetic capacity is observed. However when the cells are held in a gas phase of N_2 - 4% CO_2 an initial activation of photosynthesis occurs prior to a much slower inactivation. Since little or no loss of photosynthesis is observed when a gas phase of pure nitrogen is used, a requirement for oxygen in the inactivation process is apparent. The delayed photodamage observed in the N_2 - 4% CO_2 gas phase most likely is a result of the in situ photosynthetic oxygen generation. The observations summarized in Figures 5 and 6 reveal the requirements of high light intensity and oxygen for photoinactivation; hence, the inactivation process by definition is photodynamic.

General Sites of Photodamage

In attempts to localize the site of photodamage in mutant L.S.-41 and later in the mutant L.S.-4, various in vivo studies of reactions and absorbency changes known to be representative of specific parts of

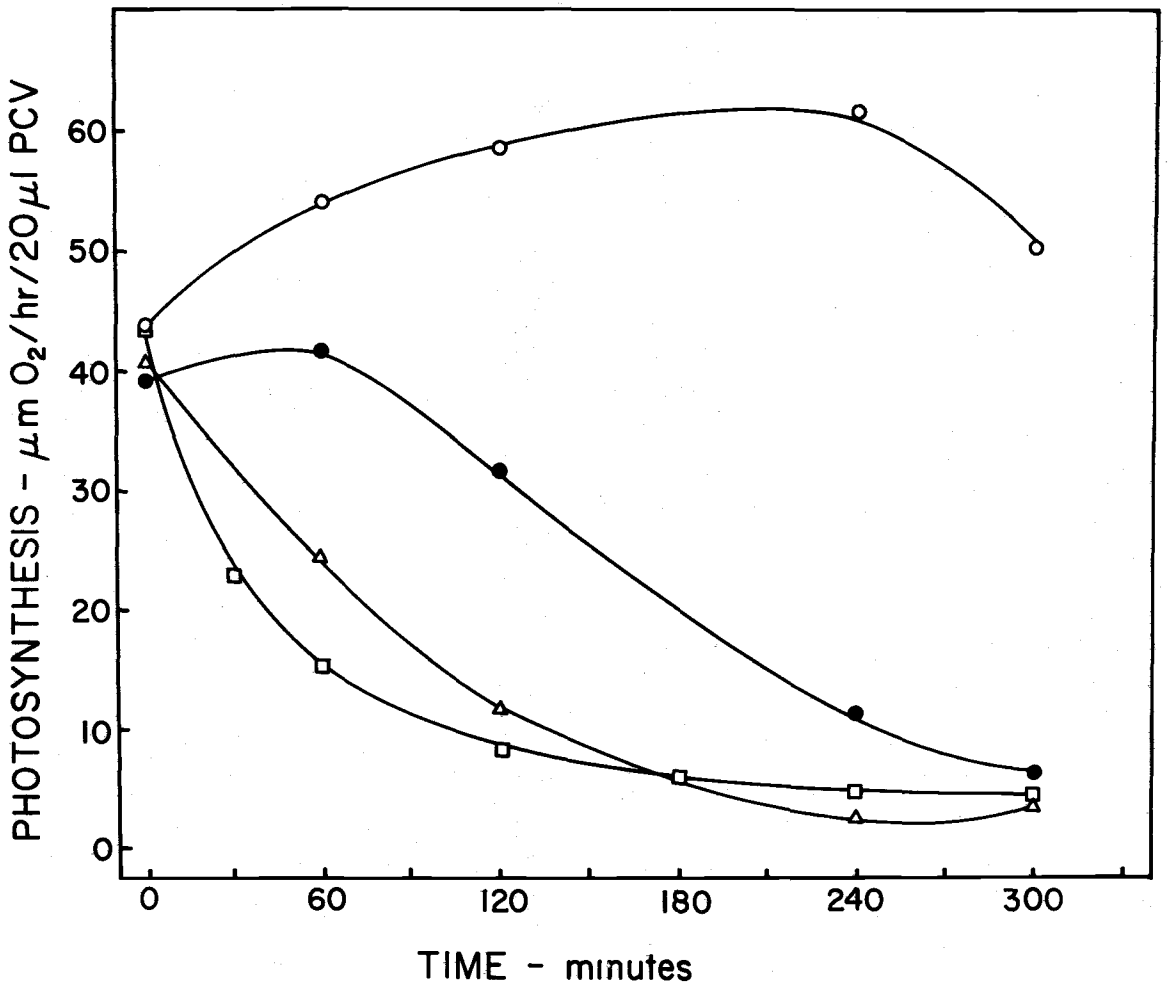


Figure 5. The effect of light intensity (1.5×10^4 ergs/sec-cm^2, o-o, 1.5×10^5 ergs/sec-cm^2, ●-●, 7.5×10^5 ergs/sec-cm^2, Δ-Δ, and 1.5×10^6 ergs/sec-cm^2, □-□) upon the photoinactivation of photosynthesis in mutant L.S.-41.

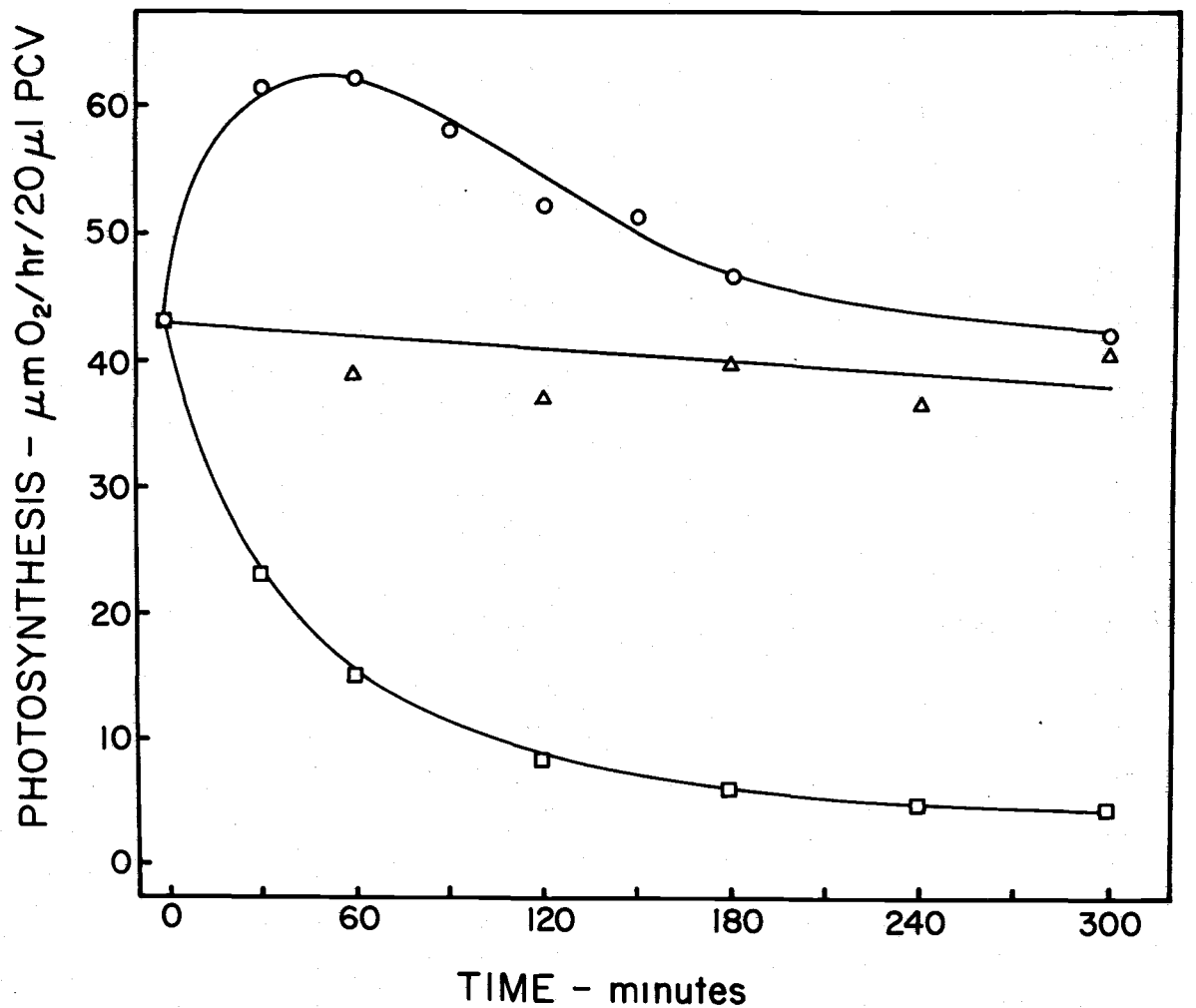


Figure 6. The photoinactivation of photosynthesis in L.S.-41 in air - 4% CO_2, (□-□), N_2 - 4% CO_2 (o-o), and N_2 (Δ-Δ) during high intensity (1.5×10^6 ergs/sec-cm^2) white light irradiation.

the photosynthetic mechanism were made. One of the oldest methods, light saturation curves, was used to differentiate between impairment in the electron transfer reactions and the carbon dioxide reduction reactions of photosynthesis. However this approach was not exercised with mutant L.S.-41, due to the obvious generality of the photodamage. The reaction termed photoreduction was utilized to monitor the condition of photosystem I, while the p-benzoquinone Hill reaction was similarly used in the case of photosystem II. Fluorescence studies were made during the inactivation process to support evidence gained from photoreduction and p-benzoquinone Hill reaction studies and to monitor the condition of the pigment matrix. With mutant L.S.-4 more specific in vivo reactions and absorbency changes were necessary.

In Scenedesmus, as well as several other genera of green algae, the process of anaerobic photoreduction (Gaffron, 1940) has been demonstrated to be a photosystem I mediated reaction (Bishop, 1958); photosystem II does not participate in the reaction and, in fact, its operation can be deleterious to photoreduction. The process of photoreduction consists of the removal of electrons from the donor, molecular hydrogen, by the action of the enzyme, hydrogenase, coupled to the reaction center of photosystem I. The electrons are utilized to reduce pyridine nucleotide and to generate ATP via cyclic photophosphorylation. Carbon dioxide is reduced through the normal mechanism, thus acting as the terminal electron sink. When photoreduction was

measured following varying exposure times to high intensity irradiation in the mutant, its activity diminished with kinetics similar to those for complete photosynthesis (Figure 7). The similarity in inactivation kinetics indicates that photodamage to photosystem I is the principal site involved in the photodynamic impairment of photosynthesis in mutant L.S.-41.

The in vivo capacity for photosystem II activity was assessed by measuring the p-benzoquinone Hill reaction. Gimmler and Avron (1971) have demonstrated that p-benzoquinone has two effects on green algal chloroplasts. They noted that the compound functions to both crosslink the thylakoid membranes and to act as an electron acceptor (Hill oxidant) for electrons obtained from the photolysis of water by photosystem II. Measurement of the rates of the p-benzoquinone Hill reaction during irradiation of mutant L.S.-41 indicates a slower photoinactivation of photosystem II, compared to photosystem I (Figure 7). During the first 90 minutes of irradiation, Hill reaction activity remains high while photoreduction activity falls to 10 percent of the original value. From this study it can be concluded that photosystem I in the mutant is impaired well in advance of photosystem II.

By evaluation of the fluorescent characteristics of the normal and the mutant strains during irradiation, evidence can be obtained to support the hypothesis that photosystem I is the principal site of light inactivation in L.S.-41. Many investigators have demonstrated

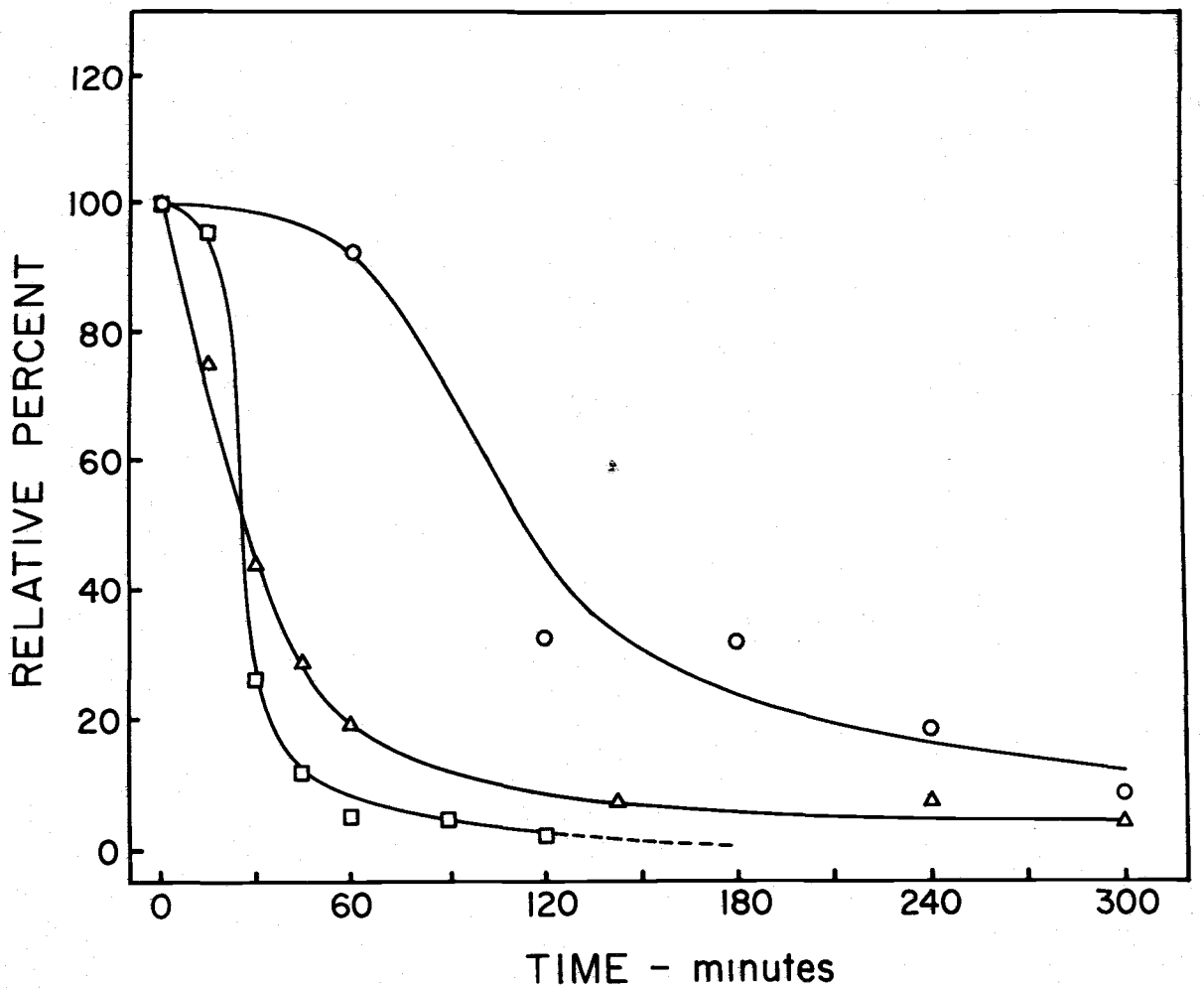


Figure 7. Photosynthesis, (Δ-Δ), photoreduction, (□-□), and Quinone Hill reaction (o-o) expressed in percent of peak activity when mutant L.S.-41 is placed in a high intensity (1.5×10^6 ergs/sec-cm^2) light field.

in vivo fluorescence of photosynthetic pigments when excitating light is provided. The fluorescence yield maintains a base level, which is dependent upon the state of the quencher, Q. The location of the quencher as the immediate electron acceptor of the photosystem II pigment system allows the phenomenon of variable yield fluorescence. If Q has been reduced by electrons from photosystem II, the fluorescence yield increases. Light of 650 nm reduces Q. If Q has been oxidized by electron donation to photosystem I, the fluorescence yield is lowered. Light of 712 nm causes a photosystem I mediated oxidation of Q (Butler, 1966). Variable yield fluorescence, the effect of 650 nm and 712 nm actinic light sources on base level fluorescence produced by excitation of 436 nm light, was monitored to determine the status of the two photosystems. Photosystem I wavelengths (712 nm) lost their effect much sooner than photosystem II wavelengths (650 nm) as demonstrated in Figure 8. This result supports the interpretation that photosystem I is initially impaired, followed by damage to photosystem II. In addition the total fluorescence produced by 436 nm light increases to a maximal value during the irradiation procedure. The attainment of this optimal value coincides with the maximal inhibition of photosynthesis produced in the irradiation period. It is also apparent from the data (Figure 8) that this maximum level of fluorescence coincides with the fluorescence level produced by the addition of DCMU. The fact that fluorescence levels continued to increase

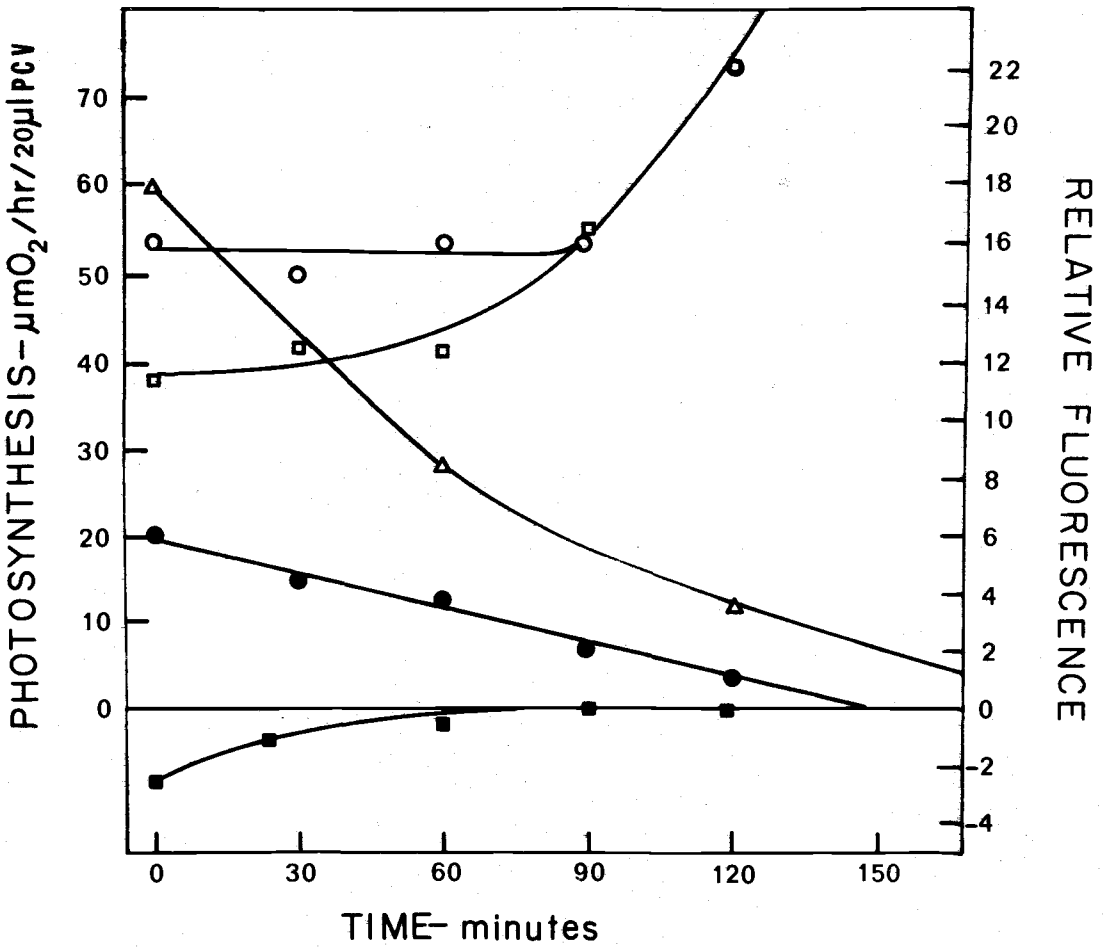


Figure 8. The base fluorescence yield (□-□), DCMU induced fluorescence yield (o-o), variable yield fluorescence (650 induced change (●-●) and 712 induced change (■-■)), and photosynthesis (Δ-Δ) of mutant L.S.-41 when subjected to illumination. Measurements were made on 10 μl PCV/3 ml of 0.05 M KH_2-K_2HPO_4 buffer; pH = 6.5.

rather than reaching a maximal level, might result from secondary effects of the photoinactivation on the chloroplast structure such that more chlorophyll molecules would be freed from association with the quencher to fluoresce at maximum fluorescence yield.

All the results reveal that photodamage to mutant L.S.-41 primarily occurs in photosystem I. However, secondary damage affects photosystem II and apparently thylakoid alteration follows. Thus a general destruction of the photosynthetic electron transport system and eventually the chloroplast membranes is the effect of high intensity irradiation. The finding that respiration is simultaneously impaired exemplified the generality of the damage. Whether respiratory damage was an initial or secondary effect was not investigated.

Carotenoid, Plastoquinone and α-tocopherol Content of Normal and Irradiated Samples of L.S.-41

The possible photoprotective role of carotenoids and α-tocopherol was examined by measurement of the levels of these components in heterotrophically grown and irradiated cells of mutant L.S.-41. These data are compared to similar data obtained with wild-type cells. Plastoquinone was measured since it is a major component of photosystem II. If any of these components functioned in photoprotection, their absence might be the basis of the photosensitivity exhibited by our mutants.

Results of carotenoid analysis of both wild type and L.S.-41 are presented in Table I. The carotenoids were analyzed after 2 days of heterotrophic growth (untreated) and 5 hours of irradiation at 1.5 X 10^6 ergs/sec-cm^2 (treated). In general the data reflect the normal light mediated de-epoxidation of violaxanthin to lutein, and the oxidation of α-carotene to lutein, which we have observed previously in comparison studies of the carotenoids of heterotrophic wild-type cultures versus autotrophic cultures and by difference spectra analyses of methanol extracts obtained from non-irradiated and irradiated algae. However, no significant differences between the carotenoid patterns of wild-type and L.S.-41, either before or after irradiation, were found.

The situation is quite different for both plastoquinone A and α-tocopherol (vitamin E) levels in wild-type and L.S.-41 (Table II). After two days of heterotrophic culture both wild-type and the mutant, have comparable mole ratios of plastoquinone A and α-tocopherol to chlorophyll, even though cells of L.S.-41 exhibit less total chlorophyll in all cases. Yet after 5 hours of irradiation, wild-type cells show increased levels of both plastoquinone A and α-tocopherol, while cells of L.S.-41 contain no measurable amounts of α-tocopherol and greatly reduced quantities of plastoquinone A. The loss of detectable α-tocopherol associated with the loss of photosystem II, supports findings made by Bishop (1974) with mutant PS-28, which is devoid of α-tocopherol and possesses a photosystem II exhibiting a light sensitive

TABLE I

Carotenoid levels of Scenedesmus obliquus wild-type and mutant L.S.-41.

Carotenoid levels of the major components of Scenedesmus wild-type and mutant L.S.-41 expressed as a mole ratio of chlorophyll per carotenoid. Untreated cells were grown heterotrophically for two days while treated cells were grown in a like manner and subsequently, subjected to a five hour irradiation period in a white light field of 1.5×10^6 ergs/sec-cm^2.

Experimental	Chlorophyll	Chlorophyll/ α-carotene	Chlorophyll/ β-carotene	Chlorophyll/ lutein	Chlorophyll/ zeaxanthin	Chlorophyll/ violaxanthin
untreated						
W.T.	2.924	57:1	44:1	18:1	25:1	102:1
L.S.-41	2.373	78:1	64:1	16:1	40:1	79:1
treated						
W.T.	2.974	220:1	58:1	7:1	36:1	311:1
L.S.-41	1.335	258:1	99:1	15:1	23:1	142:1

TABLE II

Plastoquinone A and α-tocopherol levels of Scenedesmus wild-type and L.S.-41.

Plastoquinone A and α-tocopherol levels in Scenedesmus wild-type and mutant L.S.-41. Plastoquinone A and α-tocopherol are presented in both μmoles per 2 ml PCV and as a mole ratio of plastoquinone or α-tocopherol per chlorophyll. Untreated cells were grown heterotrophically for two days, while treated cells were grown in a like manner and subsequently subjected to a five hour irradiation period in a white light field of 1.5×10^6 ergs/sec-cm^2.

	Chlorophyll	Plastoquinone		α-Tocopherol	
	(μmoles/2 ml PCV)	(μmoles/2 ml PCV)	Plastoquinone/ Chlorophyll	(μmoles/2 ml PCV)	α-tocopherol/ Chlorophyll
ScD_3-W.T.					
untreated	15.668	0.1323	0.0084	0.2075	0.0132
treated	17.079	0.1410	0.0083	0.2060	0.0121
ScD_3-L.S.-41					
untreated	8.175	0.0840	0.0103	0.1095	0.0134
treated	6.738	0.0074	0.0011	-	-

nature. The fact that mutant L.S.-4, an alternate type of light sensitive mutant used in this study, retains both α-tocopherol and photosystem II, which will be discussed later, lends additional support to the idea that α-tocopherol functions in the protection of photosystem II by quenching free radicals, or their immediate products. It can be postulated that the loss of plastoquinone in mutant L.S.-41 would also explain the loss of photosystem II activity. However, it is equally feasible that plastoquinone might also serve in a secondary role as a free radical scavenger, especially in higher plant chloroplasts, where it is found in mole ratios as high as one per four chlorophylls.

Mutant L.S.-4

General Characteristics and Photodynamic Nature of the Light Sensitivity

When heterotrophically cultured mutant L.S.-4 is placed in autotrophic conditions, it loses photosynthetic capacity as compared to wild-type Scenedesmus (Figure 9). The kinetics for the photoinactivation of the photosynthetic capacity is composed of two distinct portions. Initially, a phase characterized by increasing photosynthetic capacity is observed. This activation of photosynthesis is comparable to wild-type behavior. A peak is reached within the first 30 to 60 minutes, after which photodestructive processes dominate and photoinhibition ensues. After approximately 5 hours of high intensity

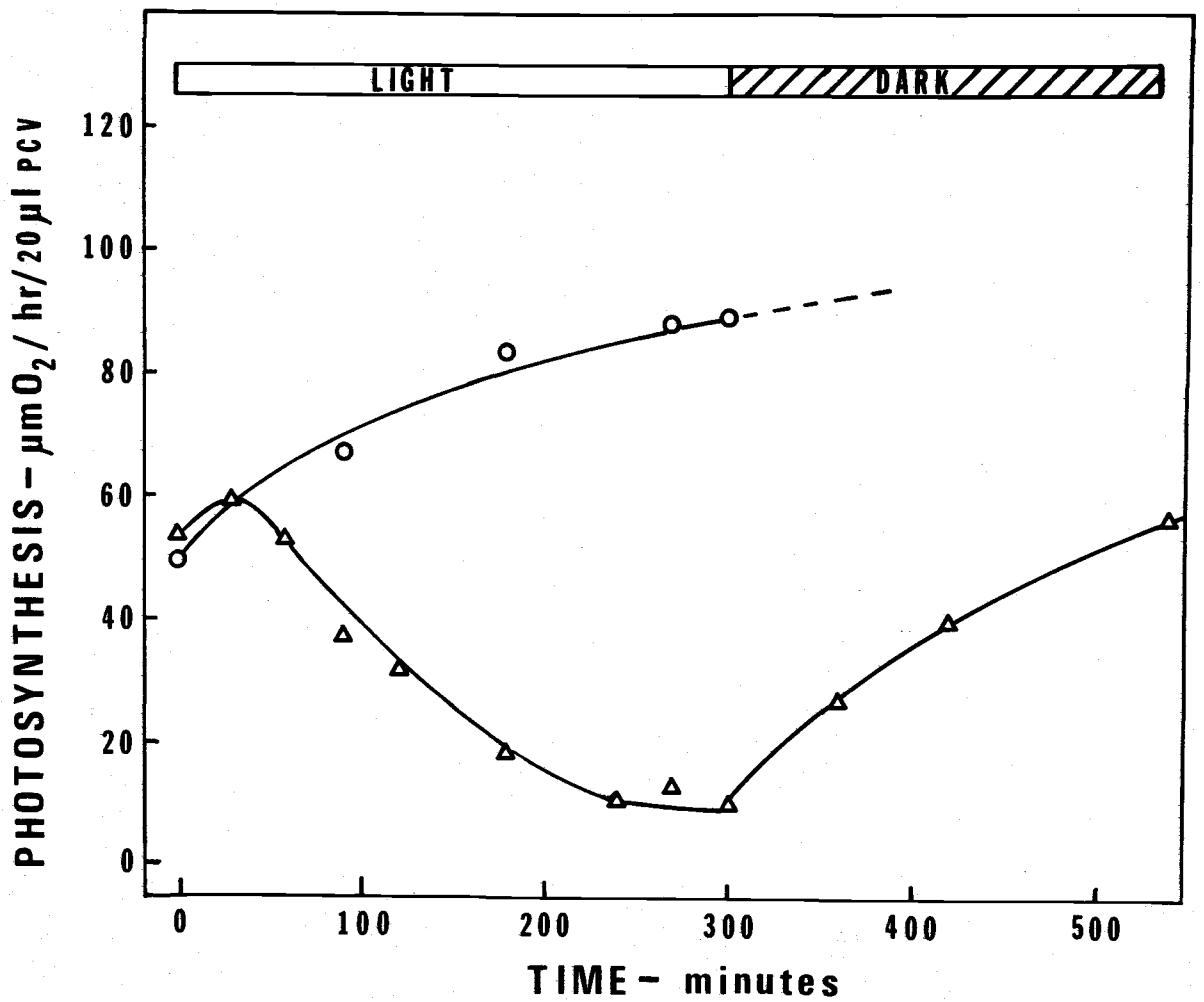


Figure 9. The photosynthetic capacity of wild-type (o-o) and mutant L.S.-4 (Δ-Δ) during a time course of high intensity (1.5×10^6 ergs/sec-cm^2) white light irradiation and in subsequent dark period.

irradiation (1.5×10^6 ergs/sec-cm^2), 90% of the maximal photosynthetic capacity is lost. If these cells are then incubated aerobically in the darkened culture vessel, a fairly rapid recovery of photosynthetic competence occurs. This rather unique restoration results in complete recovery of the mutant's original photosynthetic activity.

The mutant suffers no loss in chlorophyll concentration during the photoinactivation process (Figure 10). Like wild-type cultures, upon exposure to high light intensities L.S.-4 cultures exhibit an increase in chlorophyll content, to a level which obtains saturation only after several hours. Rates of respiration increase immediately upon irradiation of the mutant cells and saturate at a two-fold higher level until photosynthesis is 90% inhibited. Subsequently the respiratory rate drops to its original value. The complete loss of respiratory activity is never evident in mutant L.S.-4 as it is in mutant L.S.-41. This feature, in part, may allow for the recovery of mutant L.S.-4 under dark, aerobic conditions.

As was done for mutant L.S.-41 the photodynamic nature of the damage to photosynthetic capacity of mutant L.S.-4 is demonstrated by the results of irradiation in varying gas regimes (Figure 11). When air - 4% CO_2 is bubbled through the culture vessel, a typical inactivation curve is obtained. If this gas is replaced with N_2 - 4% CO_2, a much longer activation portion of the curve is demonstrated, followed by a slower loss of photosynthesis. Under a nitrogen gas phase, little

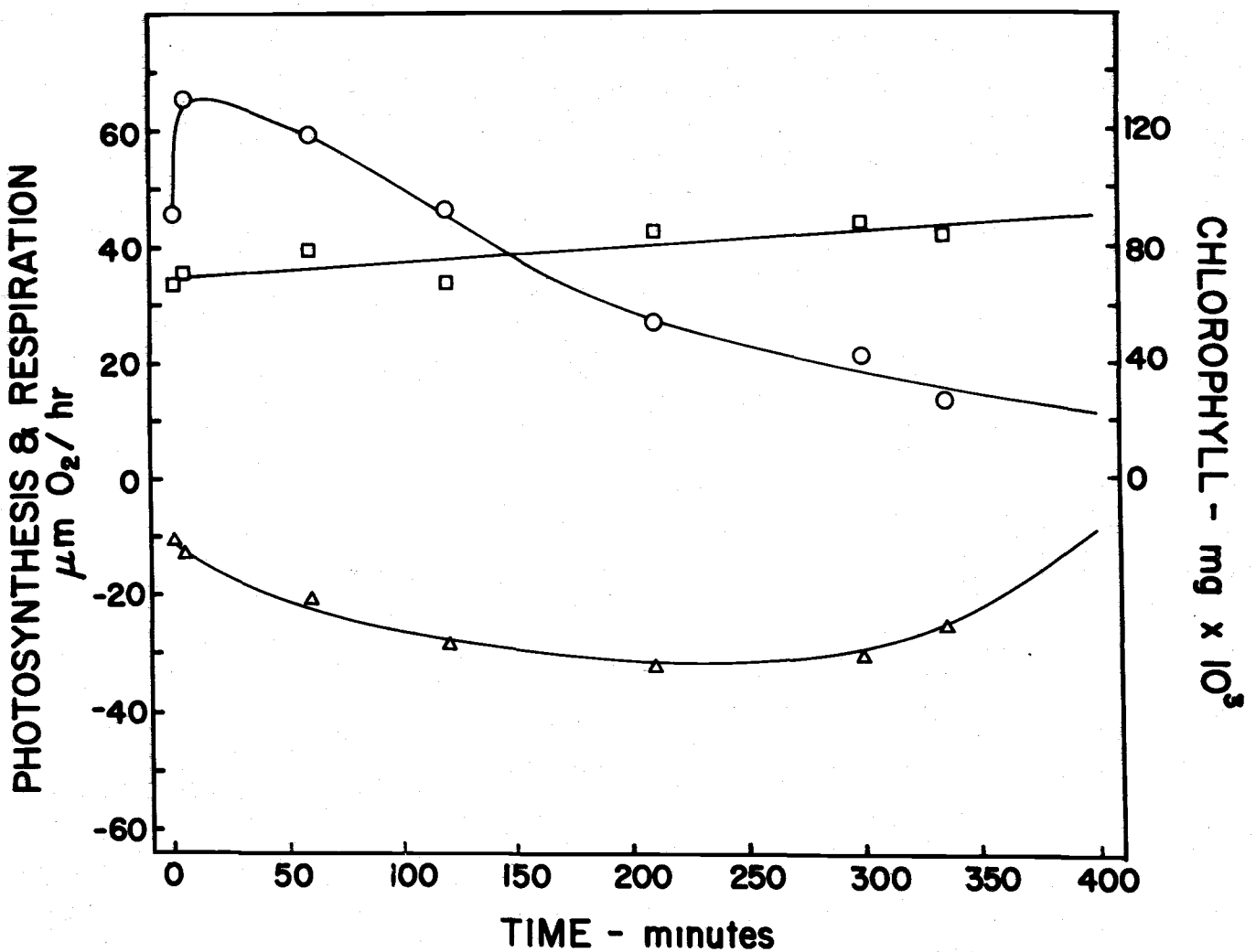


Figure 10. Photosynthesis (o-o), respiration (Δ-Δ), and chlorophyll content (□-□) in mutant L.S.-4 during high intensity (1.5×10^6 ergs/sec-cm^2) irradiation.

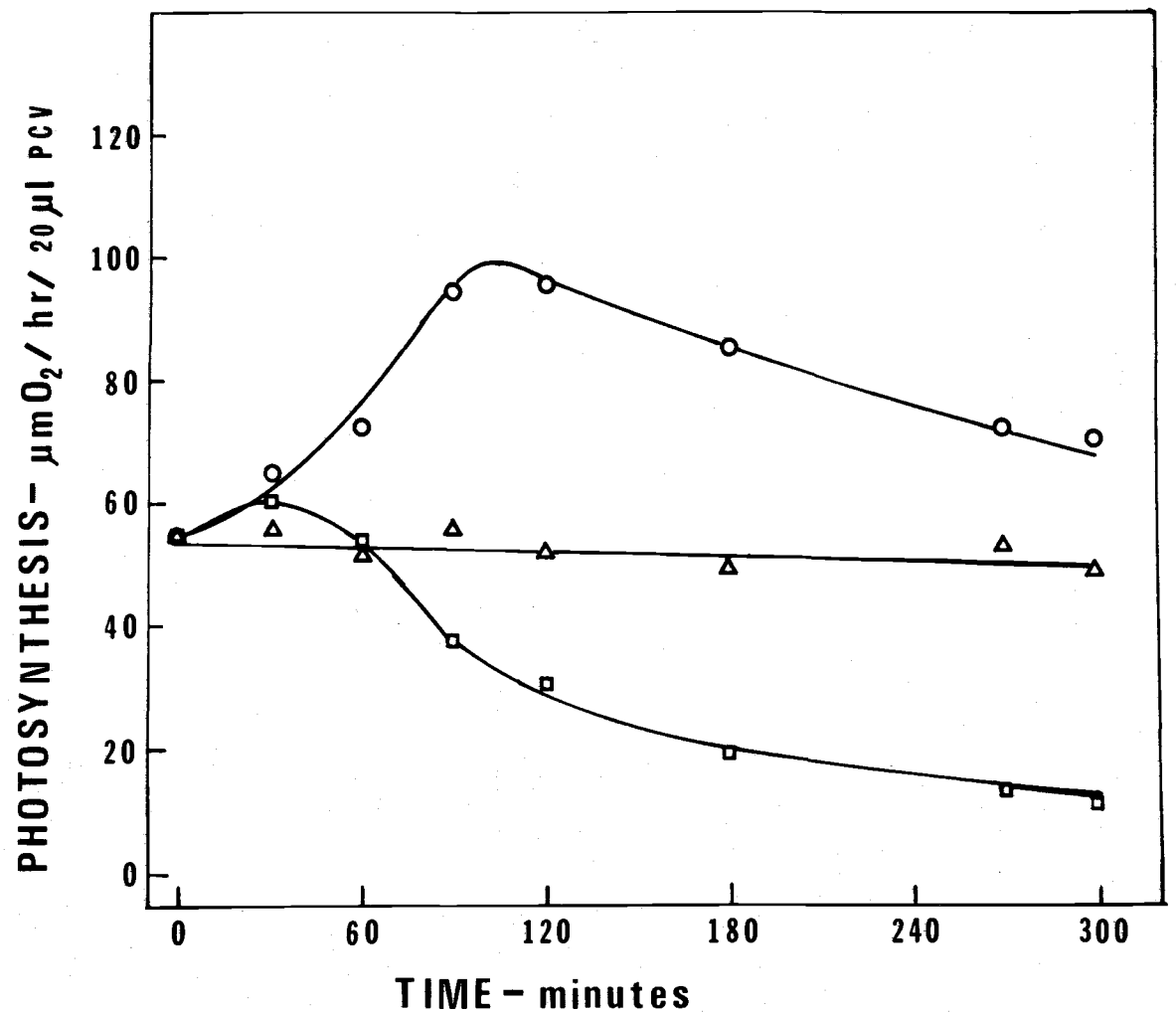


Figure 11. The photoinactivation of L.S.-4 photosynthesis in air - 4% CO_2 (□-□), N_2 - 4% CO_2 (o-o), and N_2 (Δ-Δ) during high intensity (1.5×10^6 ergs/sec-cm^2) irradiation.

change in the photosynthetic capacity is observed. Presumably, photodamage occurs with N_2 - 4% CO_2, because carbon dioxide acting as a terminal electron sink, allows photosynthetic oxygen production. These observations indicate not only the photodynamic nature of the inactivation, but also the oxygen requirement of the initial activation of photosynthesis in both wild-type and the mutant. The light intensity dependence of the photoinactivation was not tested but would presumably be found.

The Subcellular Site of Photodamage

The Photosystem Affected

The photolesion site has been thoroughly investigated from several approaches in mutant L.S.-4. Initial evidence suggesting the photosynthetic electron transport system as the general location was provided by the fundamental approach of light saturation curves of photosynthesis (Figure 12). Characteristics of these curves during the inhibition process allows the differentiation of impairment to the carbon dioxide fixation or photosynthetic electron transfer. Irradiation of wild-type cultures produces no alteration of the light saturation curves, which possess a light limiting portion and a light saturated portion. However, the construction of light saturation curves at points during the irradiation of mutant L.S.-4 reveals changes in the

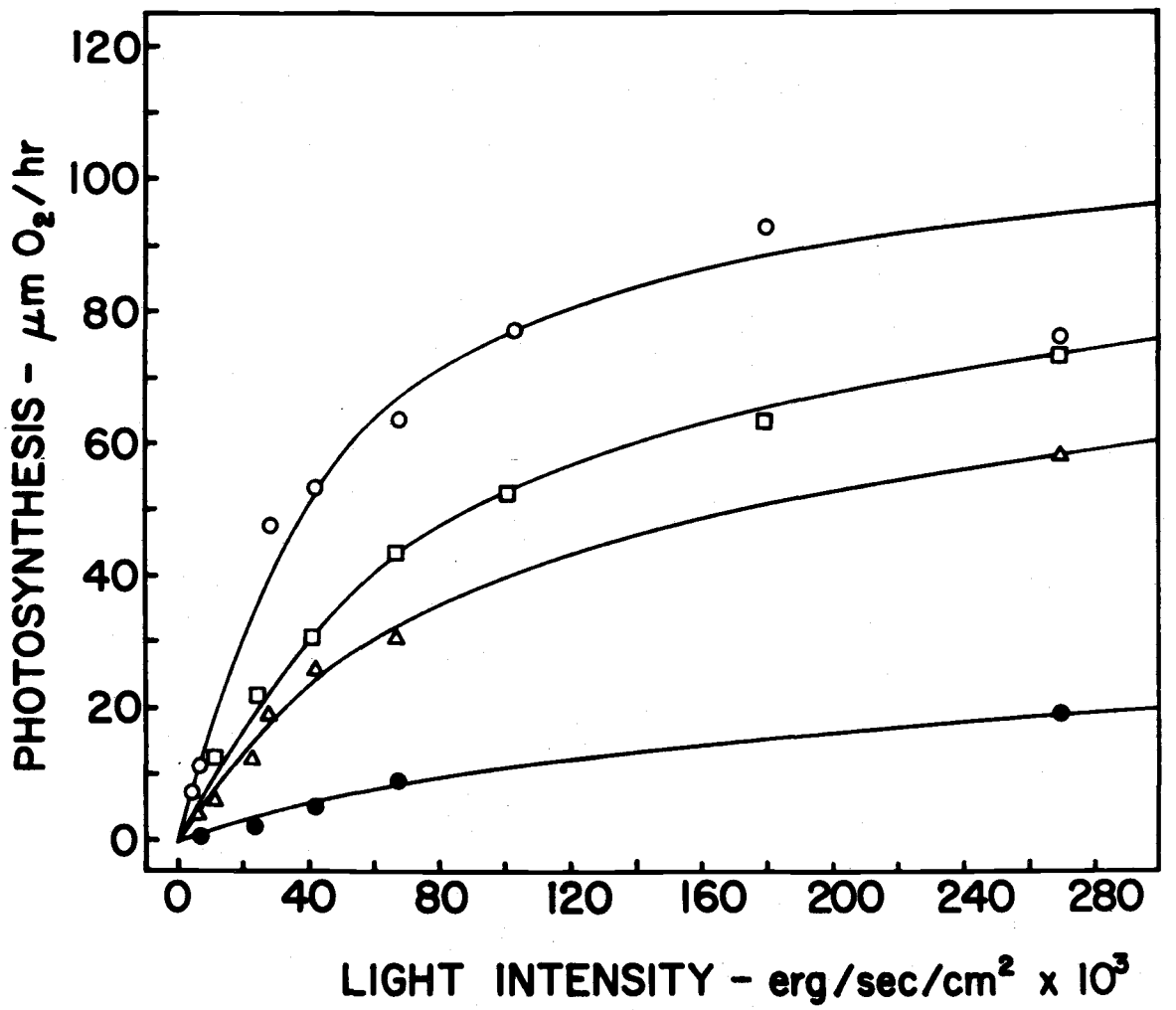


Figure 12. Light intensity versus photosynthetic rate curves constructed with cells of L.S.-4 at zero time (o-o), 120 minutes (Δ-Δ), and 240 minutes (●-●) in high intensity (1.5×10^6 ergs/sec-cm^2) irradiation and after 300 minutes (□-□) of darkness.

light limiting portion. Since the light limiting portion of the curves constructed using mutant L.S.-4 changes in slope with varying exposure to high intensity irradiation, damage to the electron transport system is implicated. Varying levels of the electron transport inhibitor, DCMU, would provide a similar pattern. An inhibitor of carbon dioxide fixation (Calvin-Benson pathway) such as cyanide ion, would change the light saturated portion of the curves, but not the light limiting portion (Rabinowitch, 1951). The incomplete recovery of the rate of photosynthesis under light limiting conditions as seen with reactivated cells (5 hrs), suggests that the recovery of photosynthesis is never 100%, even though these cells appear to have recovered completely at high (saturating) light intensity.

Measurement of the capacity for photoreduction and the quinone Hill reaction demonstrated the photolability of photosystem I (Figure 13). Photoreduction, the indicator of photosystem I activity is impaired in advance of photosynthesis. Photosystem II capacity, as assayed by p-benzoquinone Hill reaction, was not damaged but conversely showed increased rates as dependent upon length of irradiation. This improvement in the mutant was similar to the behavior of photosystem II activity in the wild type strain. The impairment of photoreduction before photosynthesis suggests the possibility that an integral part of this system, which is not absolutely essential for photosynthesis is initially lost. Cyclic photophosphorylation, upon which photoreduction

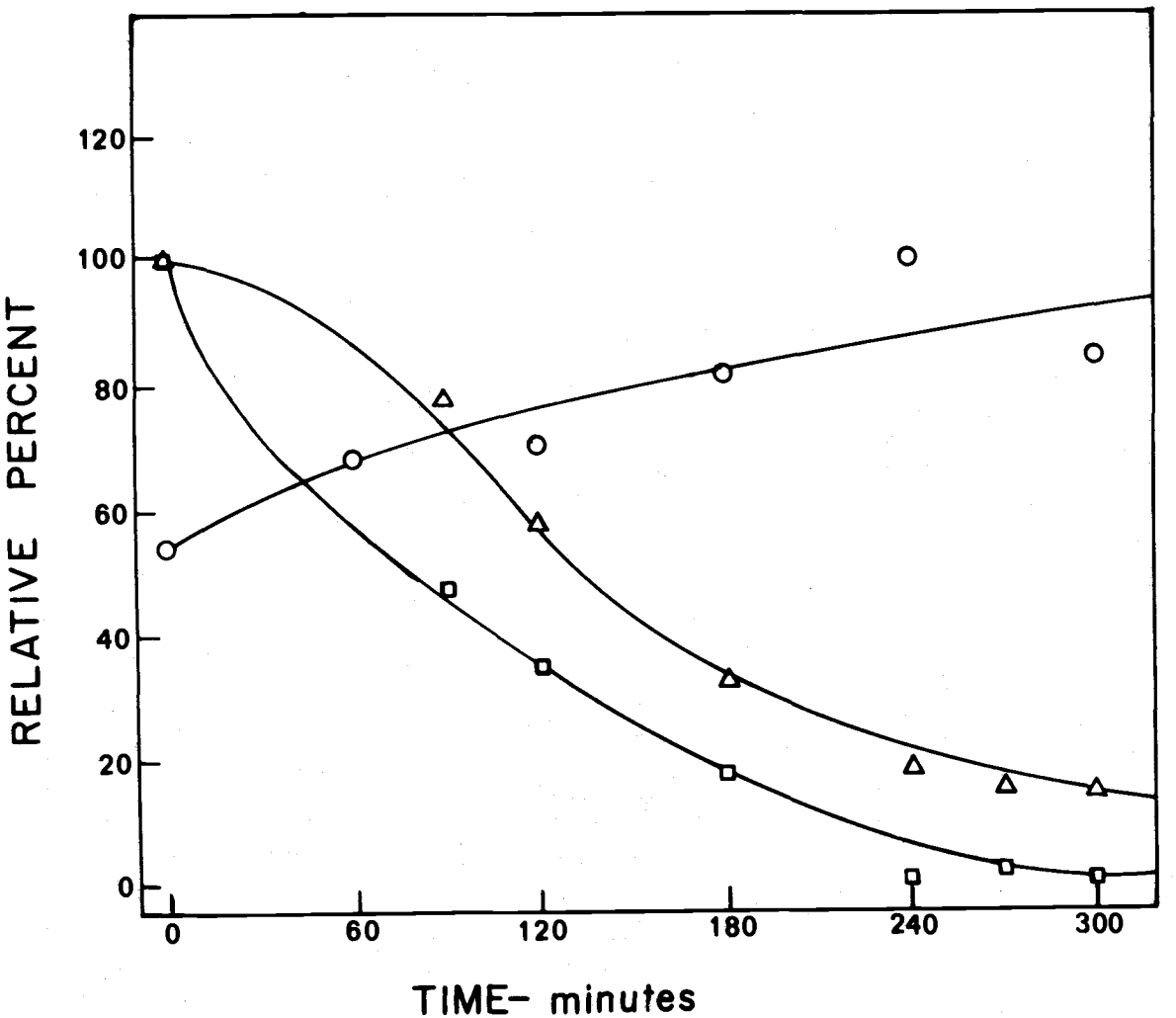


Figure 13. Photosynthesis (Δ-Δ), photoreduction (□-□), and Quinone Hill reaction (o-o) expressed in percent of peak activity when mutant L.S.-4 was subjected to high intensity (1.5×10^6 ergs/sec-cm^2 irradiation.

depends entirely for ATP and which does not provide the entire ATP requirement to photosynthesis, is thus implicated as a possible site of primary damage.

Fluorescence data from the mutant L.S.-4 reinforce these findings. Variable yield fluorescence measured using the techniques developed for mutant L.S.-41 indicates an impairment of photosystem I, with no damage to photosystem II (Figure 14). The 712 nm signal is lost after a slight improvement in its magnitude within the first 30 minutes, while the 650 nm signal gradually improves. The 712 nm light begins to have an effect similar to that caused by 650 nm light. Additionally the base level fluorescence and DCMU induced fluorescence remain nearly constant throughout the photoinactivation and recovery of mutant L.S.-4. The stability of these fluorescence characteristics suggests that little or no alteration of the thylakoids occurs during the photoinactivation process. Thus the photodamage to photosystem I in mutant L.S.-4 appears far more specific than was noted in mutant L.S.-41.

The failure of the 712 nm variable yield component to recover, we interpret as the lack of redevelopment of a tight coupling of the two photosystems in mutant L.S.-4. The 712 nm variable yield signal and the full recovery of the light limiting portion of the light saturation curve both appear dependent upon tight coupling and its important product, high quantum efficiency. These might only be regained in

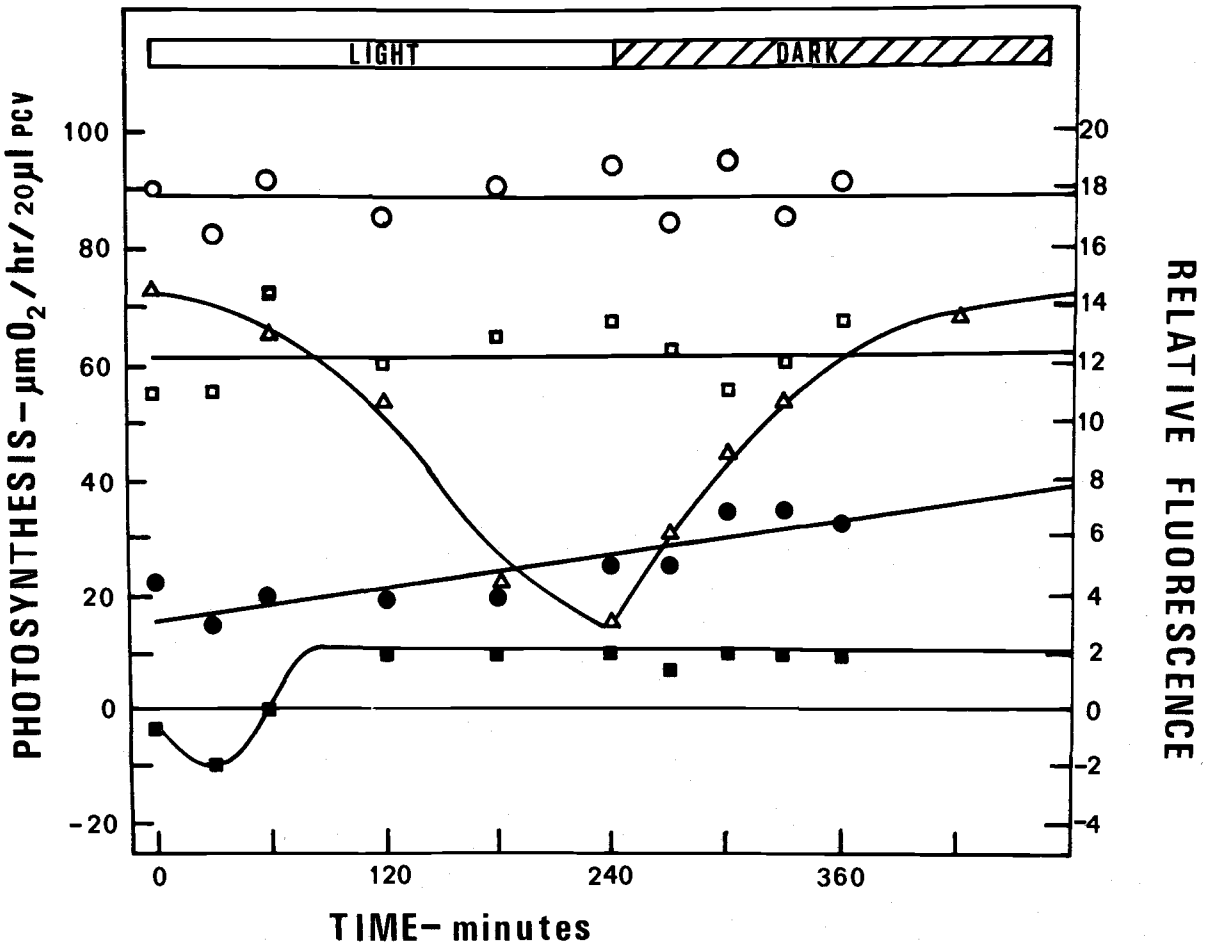


Figure 14. The base fluorescence yield (□-□), DCMU induced fluorescence (o-o), variable yield fluorescence (650 induced change (●-●) and 712 induced change (■-■)), and photosynthesis (Δ-Δ) of mutant L.S.-4 when subjected to high intensity illumination followed by darkness. Measurements were made on 10 μl PCV/3 ml of 0.05 M KH_2-K_2HPO_4 pH 6.5.

mutant L.S.-4 by growth and production of new thylakoids.

Site of the Photolesion within Photosystem I In Vivo Studies

Three in vivo experiments were used to specify the photodamage site within photosystem I. As discussed earlier, photoreduction is impaired sooner in mutant L.S.-4 than photosynthesis; it also recovers slower than photosynthesis (Figure 15). The possibility that cyclic photophosphorylation is initially impaired is suggested. Further investigation of this point involved two in vivo measurements of cyclic photophosphorylation, glucose photoassimilation and the 520 nm light induced absorbency change. Additionally the non-phosphorylation dependent and acyclic electron flow reaction hydrogen photoproduction was examined to determine the involvement of interruption of acyclic electron flow.

Glucose photoassimilation has been demonstrated to be an anaerobic process in green algae. In order for glucose assimilation to occur, the intermediate glucose-6-phosphate has to be formed. The ATP source for glucose phosphorylation in an argon atmosphere is cyclic photophosphorylation (Tanner, Loos and Komer, 1973), which continues to operate even though acyclic electron transport and photophosphorylation are inoperable. Determination of the rate of glucose photoassimilation by L.S.-4 at points along the photoinactivation curve,

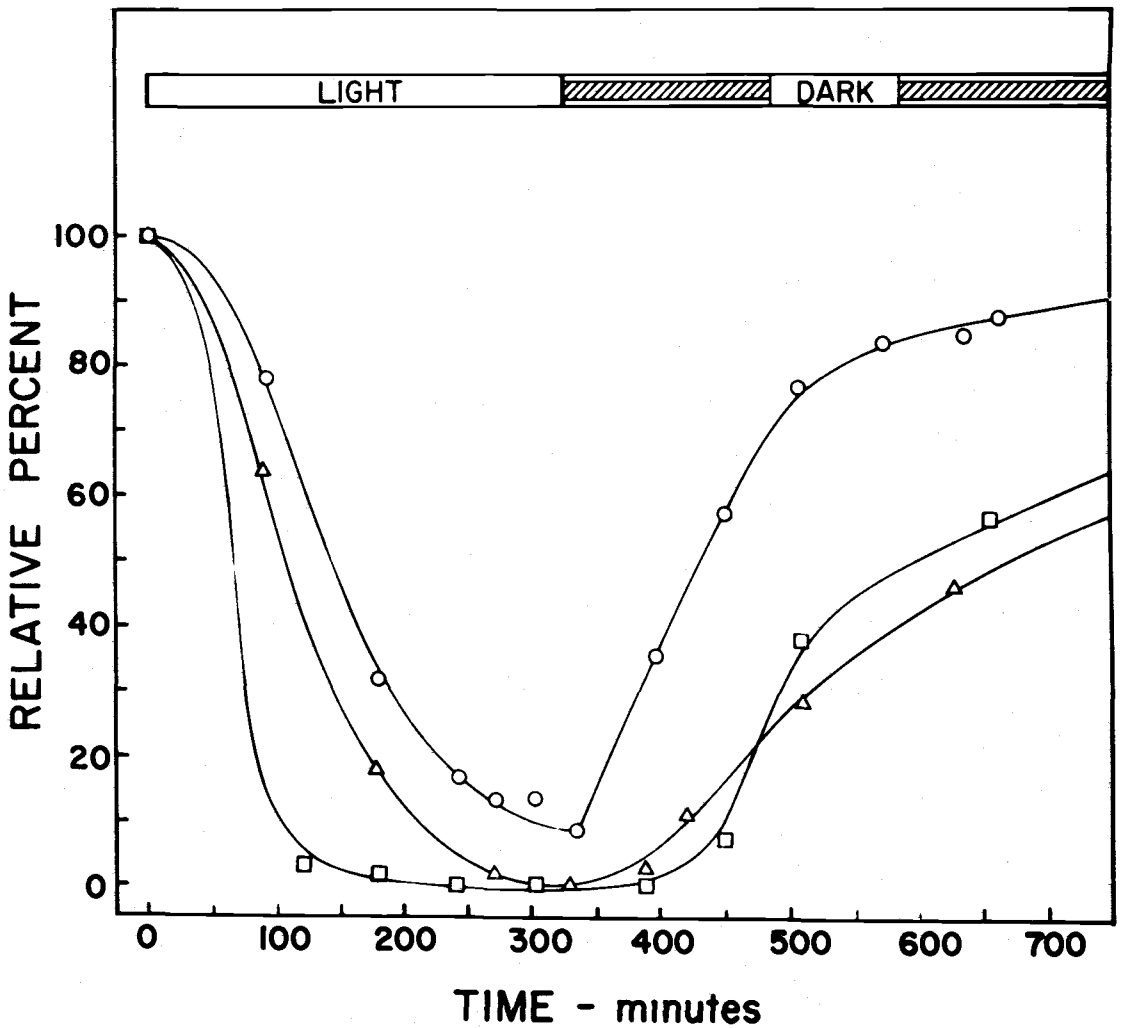


Figure 15. Photosynthesis (o-o), photoreduction (Δ-Δ), and anaerobic glucose photoassimilation (□-□) expressed in percent of peak activity in mutant L.S.-4 subjected to high intensity (1.5×10^6 ergs/sec-cm^2) irradiation followed by darkness.

show that it diminishes more rapidly than photoreduction (Figure 15). The dark restoration of glucose photoassimilation is even more sluggish than photoreduction. Since a small amount of dark glucose uptake occurs in all the samples tested, including those with total impairment of photoassimilation, it is reasonable to assume that transport of glucose and fermentation still occur. Thus we conclude that cyclic photophosphorylation shows very early inhibition upon irradiation of cells of L.S.-4.

Confirmation of these results was sought through measurement of the 520 nm light induced absorbency change. As mentioned earlier, the 520 nm absorbency change is thought by many investigators to be a measure of membrane phosphorylation capability (Fleischman and Clayton, 1968; Baltscheffsky, 1969). Measurement of this light induced absorbency change during the photoinactivation of mutant L.S.-4 demonstrated a decay of the signal with kinetics very similar to those of glucose photoassimilation (Figure 16). The time dependent dark recovery of the absorbency change followed glucose photoassimilation for a time, but soon became slower. In this respect the 520 nm absorbency change responds similarly to the other parameters measured, which depend upon the tight coupling of the two photosystems for their optimal activities. This finding is consistent with the hypothesis that the 520 nm absorbency change is an indicator of photophosphorylation capacity (Fleischman and Clayton, 1968; Baltscheffsky, 1969).

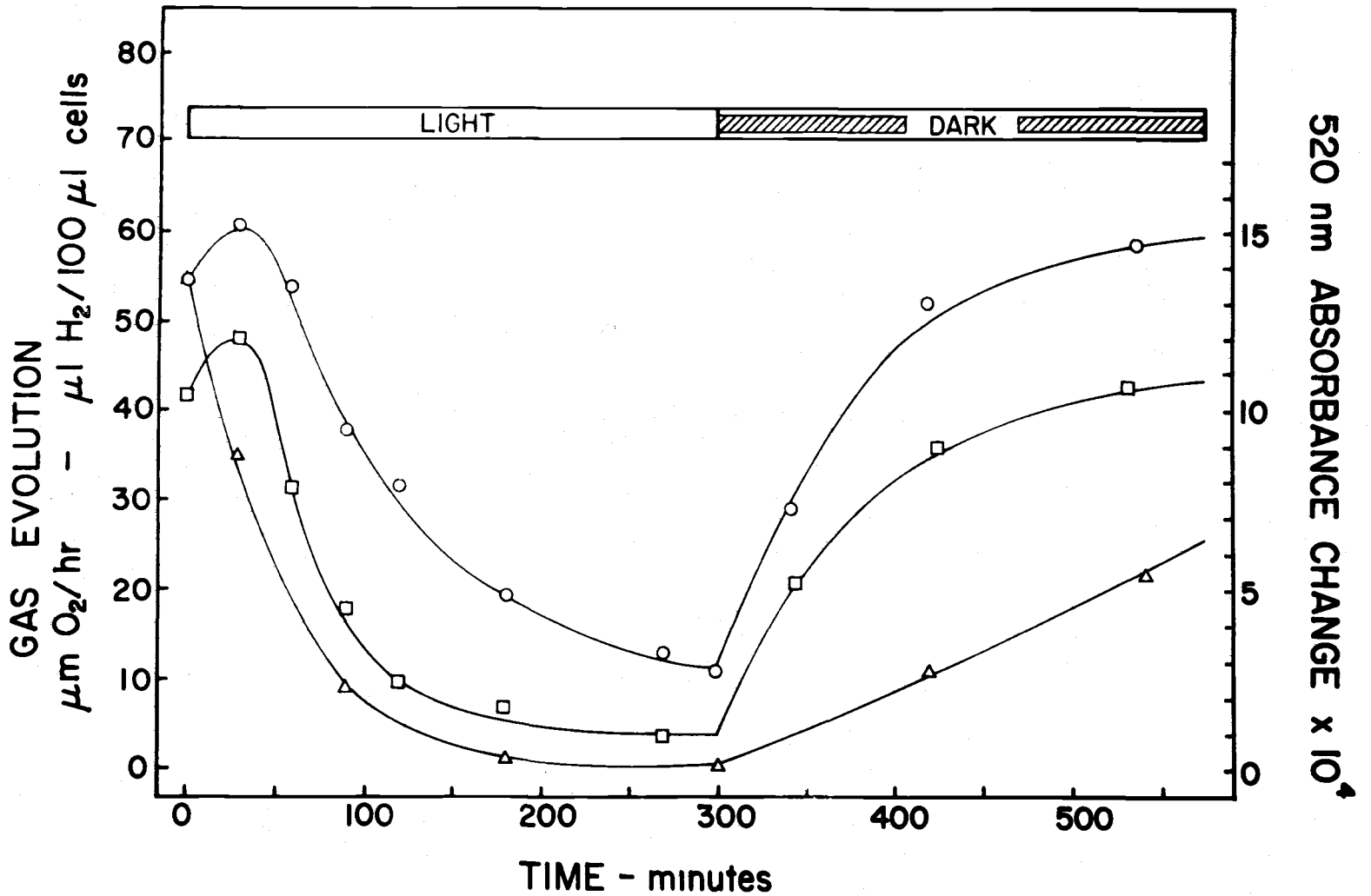


Figure 16. Photosynthesis (o-o), hydrogen photoproduction (□-□), and 520 nm absorbency change (Δ-Δ) in mutant L.S.-4 exposed to high intensity (1.5×10^6 ergs/sec-cm^2) irradiation followed by darkness.

The alternate theory that the 520 nm absorbency change is a measure of triplet carotenoid metastable states (Witt, 1971; Witt and Wolff, 1972) discussed earlier is questionable in view of the amount of carotenoid in this mutant.

To test the results which suggest initial impairment of cyclic photophosphorylation, hydrogen photoproduction was measured in the mutant. Stuart and Gaffron (1971; Gaffron and Stuart, 1972a; 1972b) have demonstrated hydrogen photoproduction to be an acyclic electron flow dependent upon one or both photosystems, but not exhibiting a direct ATP requirement. In the presence of CCCP, an inhibitor of both water photolysis and photophosphorylation, it has been hypothesized that electrons are stripped from organic sources by electron transport of both photosystems. The electrons are transported through photosystem I to hydrogenase which reduces hydrogen ions forming molecular hydrogen. If cyclic photophosphorylation was the only portion of photosystem I impaired, inhibition of this reaction would not be expected. Results of hydrogen photoproduction measurements are presented in Figure 16. The reaction is inactivated during irradiation with kinetics similar to photoreduction inactivation.

This puzzling result is not easily rationalized, yet it may indicate the unknown location of hydrogenase within the photosynthetic apparatus. Since cyclic photophosphorylation, which is damaged earlier, is not necessary for hydrogen photoproduction, and acyclic

electron flow is interrupted only after greater periods of irradiation, which will be shown later, it is apparent that a common component of the two reactions must be damaged. The only component of these two in vivo reactions, which is not common to the other in vivo reactions assayed, is hydrogenase. These results would indicate photodamage to the hydrogenase precursor and predict that hydrogenase donates electrons between the cyclic photophosphorylation site and cytochrome f in the electron transport scheme.

An alternate explanation of hydrogen photoproduction inhibition in mutant L.S.-4 is possible. Although the in vivo reaction is not directly dependent upon ATP, the generation of the organic substrates which donate electrons for hydrogen ion reduction might require ATP. If this indirect ATP requirement does exist, hydrogen photoproduction impairment would be expected. Since both explanations are plausible and the site of hydrogenase electron donation is of interest, further experimentation using mass spectra techniques to monitor not only hydrogen photoproduction but related fermentative reactions is warranted for mutant L.S.-4.

In Vitro Chloroplast Reactions

For further delineation of the specific site of light inhibition in mutant L.S.-4, a number of chloroplast reactions were examined, using chloroplasts isolated from cells at specific points of the

the photoinactivation curve. Two chloroplast reactions in which methyl viologen served as the terminal electron acceptor were developed. For one system water served as the electron donor and consequently, the photoreduction of methyl viologen required both photosystem I and photosystem II. The other system utilized DPIP-ascorbate as the electron donor and required only photosystem I for the reduction of methyl viologen (equivalent to P. 430 (Figure 1)).

The first system depended upon water as the electron source (Kok, Rurainaki and Owens, 1965). Table III demonstrates the reaction is methyl viologen, chloroplast and partially NaN_3 dependent. If DCMU was added the activity was nearly abolished indicating its two

TABLE III

Scenedesmus Chloroplast Catalyzed Methyl Viologen Photoreduction with Water as the Electron Source.

Complete reaction mixture contained 2 ml of standard buffer (20 mM tricine-KOH pH 7.5, 30 mM KCl, 0.4 M sucrose and 1% (W/V) bovine serum albumin) made 0.1 mM methyl viologen and 0.3 mM NaN_3 with chloroplasts (90 μg chlorophyll/sample) added. Methyl viologen photoreduction was measured as oxygen uptake with the Clark Ag-AgCl electrode in association with a Gilson model KM oxygraph when the suspension was illuminated with red wavelengths of an intensity of 2.5×10^5 ergs/sec-cm^2.

	oxygen uptake (μM O_2/hr/μg chlorophyll
complete	0.13
complete without MV	0.04
complete without NaN_3	0.07
complete without chloroplasts	0.00
complete with 2 μM DCMU	0.01
complete with 0.03 μM plastocyanin	0.12

photosystem nature. Plastocyanin in saturating levels (0.03 μM) had no stimulatory effect. This acyclic *in vitro* electron transport reaction was inactivated with nearly the same kinetics as *in vivo* measured photosynthesis (Figure 17). Recovery of activity in the dark again mirrors photosynthesis.

The second system is strictly a photosystem I mediated electron transport utilizing DPIP-ascorbate as an electron source (Izawa *et al.*, 1967). Table IV indicates the DCMU insensitive nature of the system

TABLE IV

Scenedesmus Chloroplast Catalyzed Methyl Viologen Photoreduction with DPIP-Ascorbate as the Electron Source.

Complete reaction mixture contained 2 ml of the standard buffer (20 mM tricine-KOH pH 7.5, 30 mM KCl, 0.4 M sucrose, and 1% (W/V) bovine serum albumin) made 0.1 mM methyl viologen, 0.2 μM DPIP, 20 μM Na ascorbate, 2 μM DCMU 0.03 μM plastocyanin and 50 μg/ml *catalase* with chloroplasts (90 μg chlorophyll/sample) added. Methyl viologen photoreduction was measured with a Clark Ag-AgCl electrode in association with a Gilson model KM oxygraph, when the suspension was illuminated with red wavelengths of an intensity of 2.5×10^5 ergs/sec-cm^2.

	oxygen uptake (μM O_2/hr/μg chlorophyll
complete	0.15
complete without MV	0.05
complete without DPIP	0.02
complete without ascorbate	0.00
complete without chloroplasts	0.00
complete without plastocyanin	0.12
complete without *catalase*	0.15
complete with 0.3 mM NaN_3	0.20

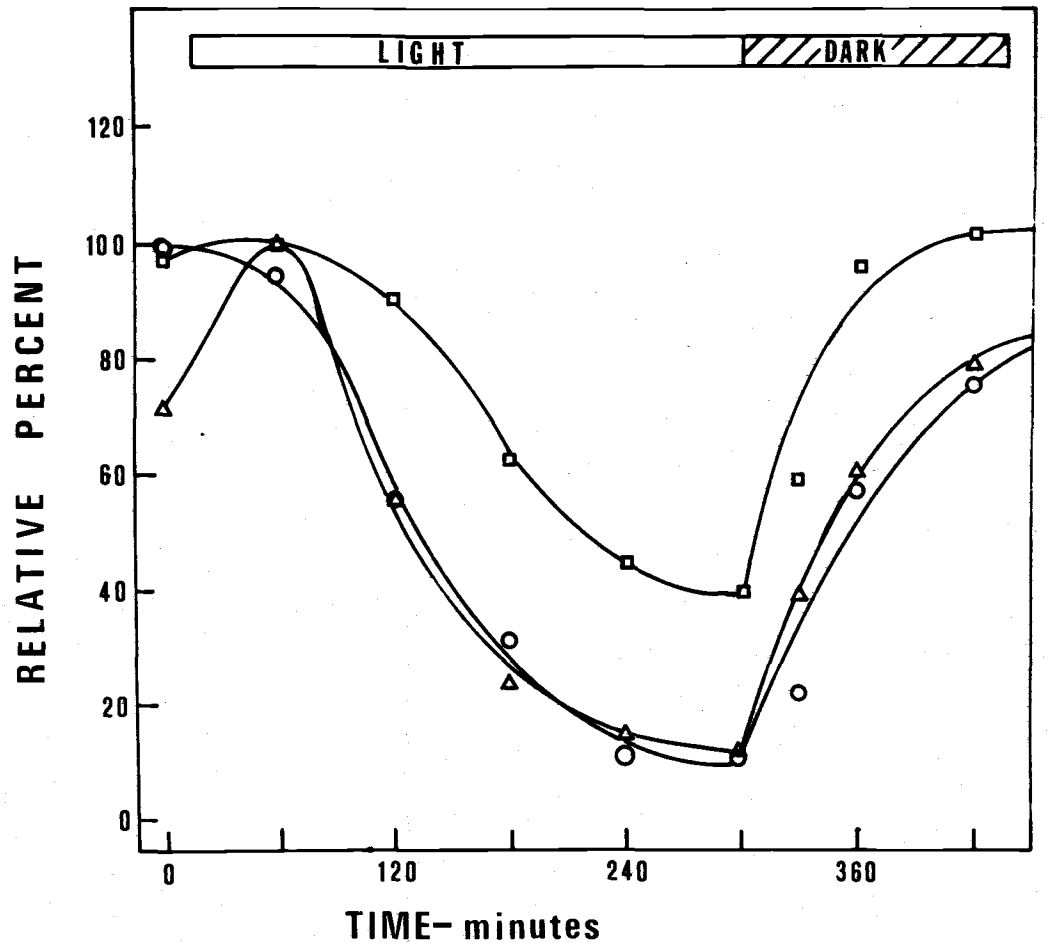


Figure 17. In vivo photosynthesis (Δ-Δ), in vitro methyl viologen photoreduction with water as the electron source (o-o), and in vitro methyl viologen photoreduction with dye-ascorbate as the electron source (□-□) in mutant L.S.-4. For the in vitro measurements chloroplasts were isolated from mutant L.S.-4 at various points during high intensity (1.5×10^6 ergs/sec-cm^2) irradiation and a following dark incubation.

and its dependence on DPIP, ascorbate, methyl viologen and chloroplasts. Since the system is DCMU insensitive and light requiring, it was assumed to be a photosystem I mediated reaction with electrons entering at the site of plastocyanin.

The inactivation curve for the dye-ascorbate methyl viologen system is presented in Figure 17. The photosystem I mediated photoreduction is more resistant than the reaction requiring both photosystems. Recovery of the photosystem I reaction is also more prompt than the other system or photosynthesis. These results indicate the initial damage to acyclic electron flow is on the reducing side of plastocyanin, in photosystem I. A reasonable interpretation of the results would predict cytochrome f as the first member of the acyclic electron transport chain affected.

Confirmation of the above prediction was sought by monitoring cytochrome f by means of its absorbency change at 552 nm. The action spectrum of this change compares favorably to the absorption spectrum of reduced ferricytochrome f (α band (Figure 18)). Illumination with a photosystem I wavelength (705 nm) caused a decrease in absorbency, while a photosystem II wavelength (663 nm) illumination caused a nearly equivalent increase in absorbency (Figure 19a). These absorbency changes were interpreted as photooxidation and photoreduction of cytochrome f, respectively. The absorbency change remained constant in magnitude during high intensity irradiation of wild-type cells. In

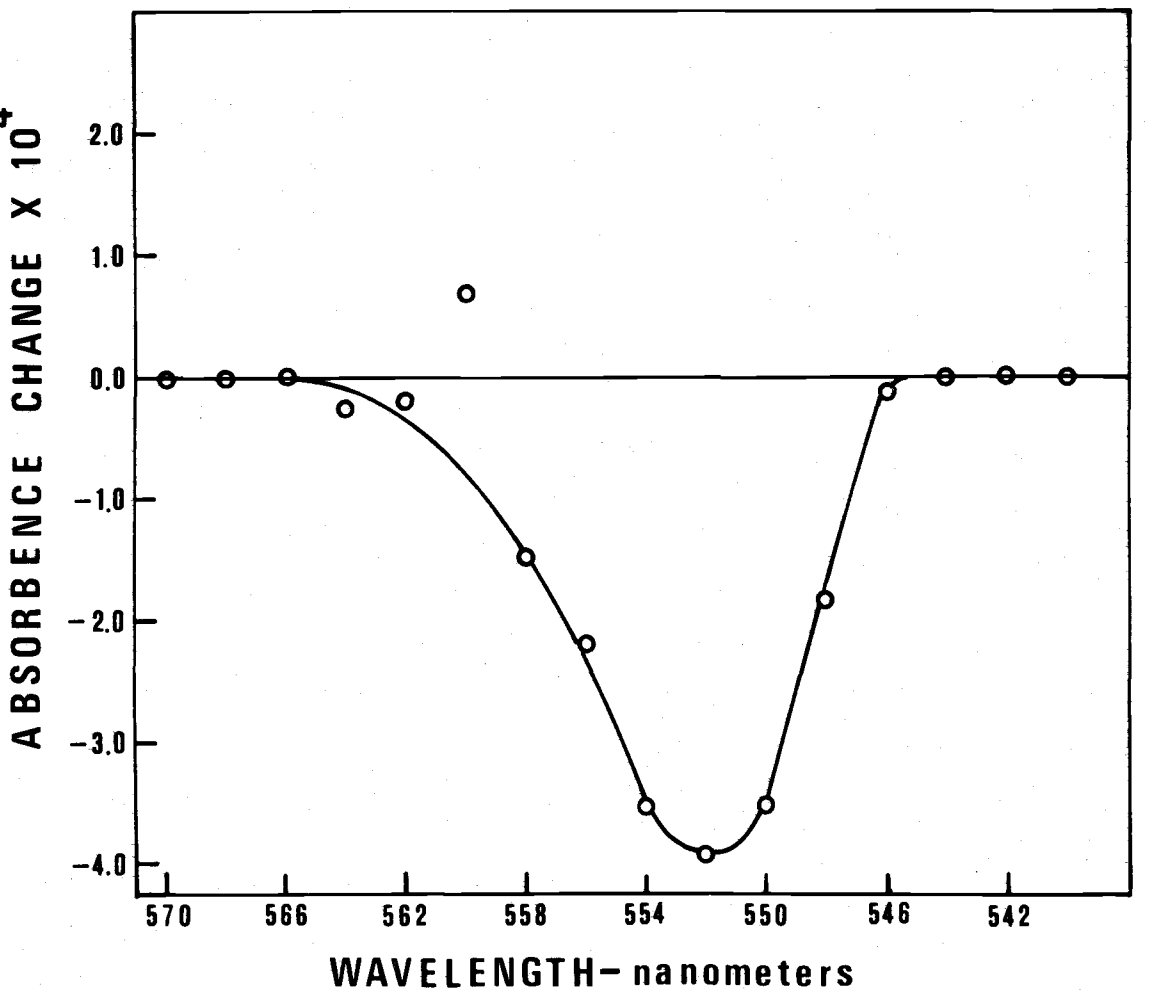


Figure 18. The action spectra of a 552 nm absorbency change constructed with wild-type chloroplasts and non-irradiated chloroplasts of mutant L.S.-4. The effect of 705 nm light alone was measured.

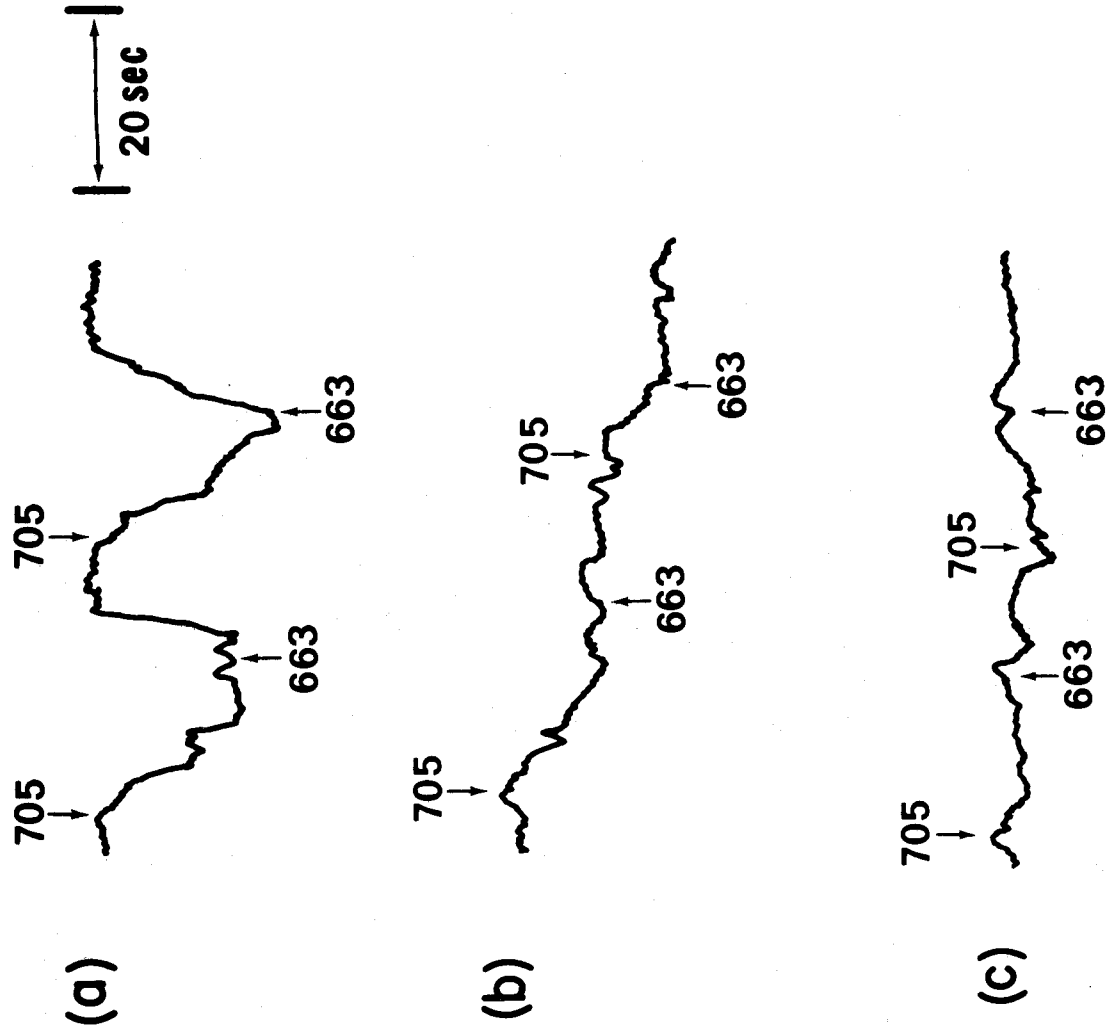


Figure 19. Tracings on the 552 nm absorbency change at zero time (a), 120 minutes (b), and 240 minutes (c) during the high intensity (1.5×10^6 ergs/sec-cm^2) irradiation of mutant L.S.-4. Measurements were made with a time base of 20 seconds/2.54 cm and a sensitivity of 0.005 A.

the mutant, prior to high intensity illumination the same pattern was observed (Figure 19a). After two hours of irradiation, photooxidation of cytochrome f occurred, but the photoreduction was impaired (Figure 19b). After five hours, neither photooxidation nor photoreduction was observed (Figure 19c). Following dark aerobic incubation only partial recovery of the light induced absorbency changes of cytochrome f (Figure 19b) took place. Presumably, the positive absorbency change (photoreduction) depends upon tight coupling of the photosystems. A plot of magnitude of the ΔA at 552 nm against irradiation time indicates that the photoreduction of cytochrome f is inactivated with kinetics similar to that of photosynthesis and of the water-dependent methyl viologen photoreduction (Figure 20).

The in vivo and in vitro observations of mutant L.S.-4 chloroplasts during photodynamic inactivation indicate the initial and subsequent sites of damage. Glucose photoassimilation and 520 nm absorbency change results suggest initial impairment to cyclic photophosphorylation. The damage soon spreads to other portions of the cyclic pathway including the components which become hydrogenase. After a longer period the acyclic electron transport chain is interrupted between the two photosystems at a site immediately on the reducing side of cytochrome f. The damage then moves progressively down the electron transport chain to the trap reaction center of photosystem I, P 700. Aerobic dark conditions allow recovery of acyclic and some

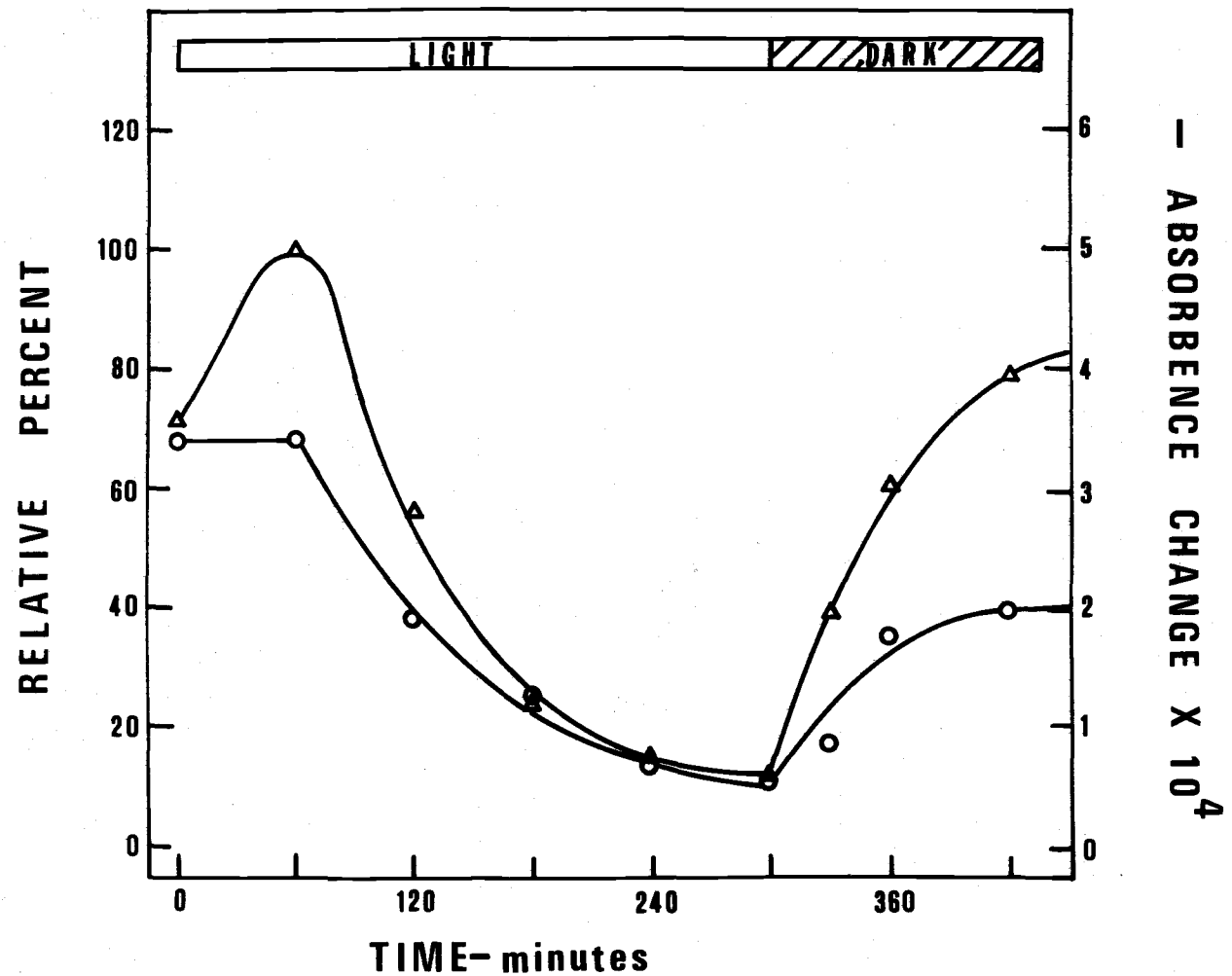


Figure 20. Photosynthesis (Δ-Δ and the 552 nm absorbency change (o-o) in mutant L.S.-4 during high intensity (1.5×10^6 ergs/sec-cm^2) illumination and a following dark incubation.

cyclic electron flow. A certain degree of the cyclic photophosphorylation mechanism is repaired, but tight coupling of the two photosystems was never obtained in recovery experiments performed. Tight coupling of photosystems may only occur through new thylakoid synthesis.

Characteristics of the Restoration of the Mutant L.S.-4 from Photoinactivation

As has been indicated in the previous section the mutant L.S.-4 is capable of photosynthetic capacity recovery in dark aerobic conditions unlike mutant L.S.-41 (Figure 21). The restoration is oxygen dependent. If air - 4% CO_2 is bubbled through the darkened culture vessel following photoinactivation, photosynthesis is revived within 3 hours. When N_2-4% CO_2 is used, no return of photosynthesis is evident (Figure 21). The oxygen requirement suggests that respiration and its product, ATP, are necessary for revitalization of photosynthesis. Since protein synthesis requires large quantities of ATP, the role of this function in reactivation was investigated.

Two classic inhibitors of protein synthesis, cycloheximide and chloroamphenicol, were used to determine if actual synthesis of new protein was requisite for reactivation of photosynthesis. Cycloheximide has been shown to effect the cytoplasmic 80S ribosome translation, while chloroamphenicol blocks translation on the 70S ribosome typical of chloroplast and mitochondria. Cycloheximide, in levels

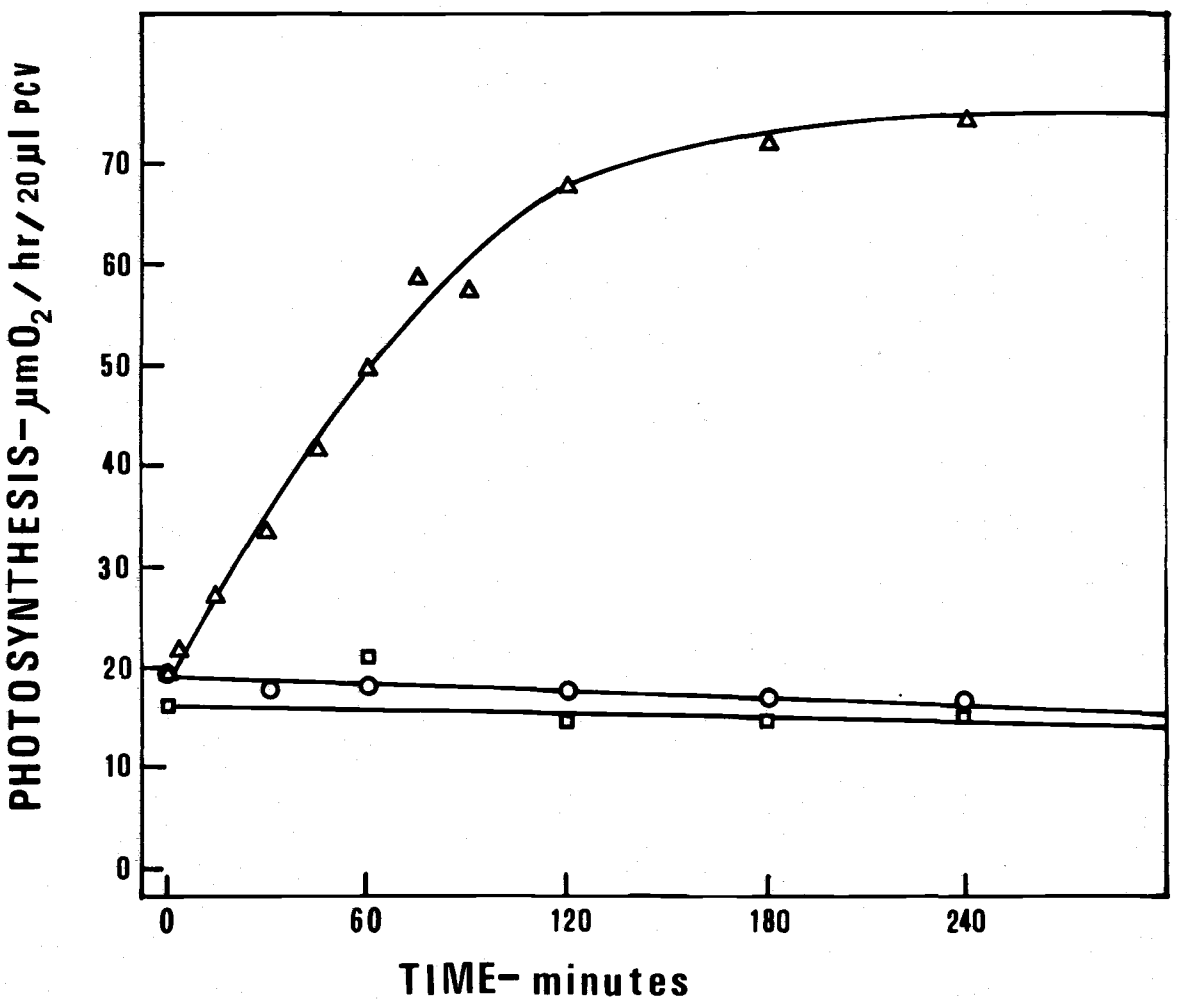


Figure 21. Dark recovery of photosynthetic capacity in mutants L.S.-41 (□-□) and L.S.-4 (△-△) in air - 4% CO_2 and mutant L.S.-4 in N_2 - 4% CO_2 (o-o) following photoinactivation.

demonstrated by Bishop (unpublished results) to be inhibitory to Scenedesmus protein synthesis, has no effect upon the recovery of photosynthetic capacity. However, chloroamphenicol, the 70S ribosome translation inhibitor, affected restitution to varying degrees with varying concentrations (Figure 22); a concentration of 20 μg/ml abolished the revitalization of photosynthesis. These results exhibit the importance of 70S ribosome protein synthesis to the recovery of photosynthetic competance by inactivated chloroplasts of mutant L.S.-4. The 70S ribosome has been shown by Goodenough and Levine (1970) to be integral for proper thylakoid structure, synthesis of cytochrome b559, and of the quencher, Q. Since cytochrome b559 and the quencher function only in photosystem II, which is unaffected in the mutant, it is conceivable that a membrane structural protein is synthesized by the 70S ribosome allowing recovery.

Plastoquinone, α-tocopherol and Carotenoids

Carotenoid levels were measured both before and after irradiation of mutant L.S.-4 for 5 hours (Table V). Again the data reflect the de-epoxidation of violaxanthin and the oxidation of α-carotene to lutein under autotrophic conditions. Although the levels of several of the carotenoids differ slightly between wild-type and the mutant, the major patterns of carotenoid flux from nonirradiated to irradiated cells do not differ.

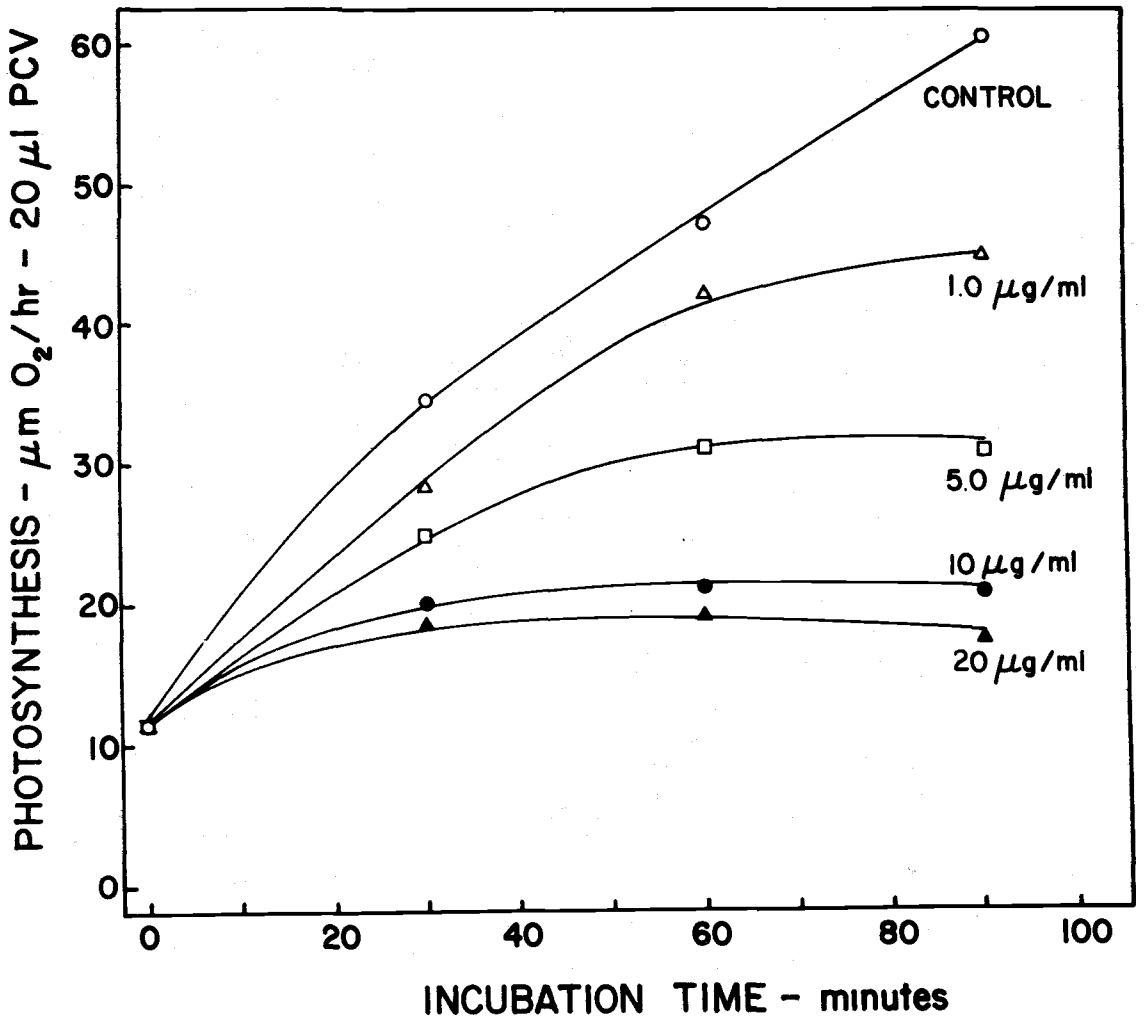


Figure 22. Dark recovery of photosynthetic capacity in mutant L.S.-4 in the presence of various levels of chloroamphenicol.

TABLE V

Carotenoid levels of Scenedesmus obliquus wild-type and mutant L.S.-4.

Carotenoid levels of the major components of Scenedesmus wild-type and mutant L.S.-4 expressed as a mole ratio of chlorophyll per carotenoid. Untreated cells were grown heterotrophically for two days, while treated cells were grown in a like manner and subsequently subjected to a five hour irradiation period in a white light field of 1.5×10^6 ergs/sec-cm^2.

Experimental	Chlorophyll	Chlorophyll/ α-carotene	Chlorophyll/ β-carotene	Chlorophyll/ lutein	Chlorophyll/ zeaxanthin	Chlorophyll/ violaxanthin
untreated						
W.T.	2.924	57:1	44:1	18:1	25:1	102:1
L.S.-4	3.247	72:1	93:1	7:1	14:1	17:1
treated						
W.T.	2.974	220:1	58:1	7:1	36:1	331:1
L.S.-4	3.172	389:1	63:1	4:1	30:1	435:1

Plastoquinone A and α-tocopherol levels in wild-type and the mutant are presented in Table VI. Comparable levels of the two components are present in nonirradiated and irradiated cells. Unlike irradiated samples of mutant L.S.-41, which loses α-tocopherol, plastoquinone A, and photosystem II activity, the mutant L.S.-4 loses none of these properties.

TABLE VI

Plastoquinone A and α-tocopherol levels of Scenedesmus wild-type and L.S.-4.

Plastoquinone A and α-tocopherol levels in Scenedesmus wild-type and mutant L.S.-4. Plastoquinone A and α-tocopherol are presented in both µmoles per 2 ml PCV and as a mole ratio of component per chlorophyll. Untreated cells were grown heterotrophically for two days, while treated cells were grown in a like manner and subsequently subjected to a five hour irradiation period in a white light field of 1.5×10^6 ergs/sec-cm^2.

	Chlorophyll	Plastoquinone		α-Tocopherol	
	(µmoles/2 ml PCV)	(µmoles/2 ml PCV)	Plastoquinone/ Chlorophyll	(µmoles/2 ml PCV)	α-tocopherol/ Chlorophyll
ScD_3-W.T.					
untreated	15.668	0.1323	0.0084	0.2075	0.0132
treated	17.079	0.1410	0.0083	0.2060	0.0121
ScD_3-L.S.-4					
untreated	11.276	0.1014	0.0089	0.1329	0.0118
treated	11.111	0.0907	0.0082	0.2340	0.0211

V. CONCLUSIONS

Several conclusions which are listed below and expanded in subsequent sections, may be drawn from the results obtained during the investigation of light-sensitive mutants 4 and 41. New perspective is provided regarding photoprotective agents (both chemical and structural). The sequence and sites of photodynamic damage within the photosynthetic electron transport system of a photosynthetic organism has been achieved. A mutant system has been developed, which is of utility for study of photosystem II. The evidence obtained also permits the development of a strong case for designation of the location of electron donation into photosystem I by hydrogenase.

Photoprotective Agents

Carotenoids have been construed as the chief photoprotective agents of both photosynthetic and non-photosynthetic biological systems. Evidence supporting this hypothesis is based upon carotenogenic mutant investigations (Griffiths, Sistrom, Cohen-Bazire and Stainier, 1955; Krinsky, 1968), in vitro experiments indicating the protective action of carotene against chlorophyll photooxidation (Aronoff and Mackinney, 1943; Fujimori and Livingston, 1957) and recent work demonstrating that carotenoid provides photoprotection to artificial membranes (Anderson and Krinsky, 1973). Until the isolation of mutants L.S.-4

and L.S.-41, all light sensitive mutants previously reported on were carotenogenic (unable to synthesize either cyclized carotenoids or the C-40 polyene proper). However, the two mutants reported herein are clearly not carotenogenic in the traditional sense. Although the possibility that minor differences in the carotenoid content exist which might cause a breakdown in protection has not been eliminated, our observations on these mutants suggest the existence of either other photoprotective mechanisms or photolabile sites that in the normal algal cell are preserved by some alternate mechanism. A protective mechanism other than that provided by carotenoids has been uncovered by this work and that of Bishop (1974), which involves the action of α-tocopherol. The existence of mechanisms which protect the photosynthetic machinery against the detrimental action of excessive quantum absorption is paramount. Thus one would expect a number of protective systems reinforcing each other in a synergistic manner. In addition to carotenoid, α-tocopherol, ascorbic acid and possibly plastoquinone acting in chemical photoprotection, the membrane structure (Papageorgiou, Isoakidou and Argoudelis, 1972) and chlorophyll fluorescence are alternate systems which most likely contribute to the safe dissipation of excess quanta. Inherent in many of these systems would be mechanisms for the non-deleterious dismutation of radical oxygen forms.

Some of these notions find confirmation in investigation of

mutants L.S.-4 and L.S.-41. The role of α-tocopherol in the protection of photosystem II has been demonstrated earlier by Bishop (1974). Mutant L.S.-41 exhibits a secondary photodestruction of photosystem II in association with the total loss of α-tocopherol. Since the mutant L.S.-4 retains a fully active photosystem II and loses no α-tocopherol, it offers additional support to the finding that α-tocopherol, and possibly plastoquinone, function to interrupt free radical lipid peroxidations initiated by excess quanta. Since lipid peroxidation is suppressed subsequent detrimental conformational changes do not occur in the membrane. Thus activity is maintained and the membrane is not subject to further damage.

A major problem with the carotenoid theory of photoprotection has been the observation that all the carotenogenic mutants studied possess aberrant thylakoid structure (Sagar and Zalokar, 1958; Williams, 1971). The work performed on mutant L.S.-41 suggests the importance of proper chloroplast thylakoid structure. Fluorescence data indicate a derangement of the thylakoids and release of more chlorophyll to fluorescence from the pigment systems. Such events, interpretable as alteration of chloroplast ultrastructure are not observed in wild-type or the mutant L.S.-4. However, in mutant L.S.-4, which suffers a very specific photolesion, recovery is possible only if protein synthesis by the 70S ribosome occurs. The 70S ribosome has been shown to contribute, at least in part, the proper

structural components of the thylakoid (Goodenough and Levine, 1970). Thus our results suggest structural damage is a part of photodamage and structural damage culminates in photolability. Further insight into the importance of thylakoid structure in photoprotection might be provided by electron micrographs of chloroplasts in the mutants.

Location of Photodamage in Mutants L.S.-4 and L.S.-41

The sites of photodamage within the photosynthetic electron transport system have been found in both mutants. Photodestruction to mutant L.S.-41 is more general than damage to L.S.-4. Both the photosynthetic electron transfer chain and respiration are impaired in mutant L.S.-41. The initial discernible damage occurs in photosystem I, resulting in its immediate inhibition. This damage appears to halt photosynthesis. Prolonged irradiation causes secondary damage to photosystem II and a general disruption of the pigment systems. Respiration decreases during irradiation but details of either the primary or secondary character of this damage were not investigated. It is known that the photodestruction in mutant L.S.-41 is not reversible during the restoration periods provided. The impairment of respiration and the general nature of the photodamage most likely account for this fact. The possibility that revitalization of photosynthesis or cell growth occurs in heterotrophic media after photoinactivation was not investigated.

Mutant L.S.-4 suffers a very specific photodamage. Results demonstrate cyclic photophosphorylation's initial impairment in photosystem I. Subsequent damage occurs at the reducing side of cytochrome f, which halts acyclic electron transfer and thus photosystem I activity and photosynthesis. Secondary damage may occur at the sites of plastocyanin and P700. No general thylakoid damage occurs and respiration remains functional. Thus recovery from the photodamage is possible at least in part through the respiration dependent function of 70S ribosome protein synthesis.

It is interesting that two reports have been made concerning the photolability of photosystem I in isolated chloroplasts (Forti and Jagendorf, 1960; Krendeleva et al., 1972). One of these cases was demonstrated to be photodynamic (Krendeleva et al., 1972) but the other was nonphotodynamic (Forti and Jagendorf, 1972). Both systems exhibited an initial loss of cyclic photophosphorylation. The findings on the light sensitivity of photosystem I in mutants L.S.-4 and L.S.-41 are the first report of similar photodamage in vivo.

Mutant L.S.-4 as a Tool for Futher Study of Photosystem II

The property of specific inhibition of photosystem I in mutant L.S.-4 without damage to other portions of photosynthesis is of value in the further study of the photosynthetic mechanism. Few methods

exist for the specific inhibition of photosystem I while several inhibitors of photosystem II are known. These methods used to inhibit photosystem I have employed rather harsh irreversible chemical means (Katch and Kimimura, 1972; San Pietro, Brand and Mayne, 1972). By the input of high intensity light into L.S.-4 the inactivation of photosystem I without any damage to photosystem II is achieved. Unlike other agents, high intensity light has little effect on other constituents of the cells. The inhibition is at least partially reversible under dark aerobic conditions. Thus mutant L.S.-4 could be used for studies of photosystem II alone in vivo when the interfering effects of photosystem I were not desired. If full photosynthesis or the action of photosystem I was then required; they could be regained merely by dark aerobic incubation.

Site of the Hydrogenase Precursor in Scenedesmus Photosynthetic Electron Transport

The enzyme hydrogenase is adapted in some green algae, following anaerobic conditions. Hydrogenase is known to feed electrons better to photosystem I in the case of photoreduction and to receive electrons from photosystem I for hydrogen photoproduction; both of these reactions are characteristic of hydrogenase activated systems. The adaption of hydrogenase does not necessitate protein synthesis (Stiller and Lee, 1964), thus the hydrogenase precursor must be

present and, for the purposes of our study, impairable during high intensity irradiation of mutant L.S.-4.

Results presented above demonstrate initial photodamage occurs near the cyclic photophosphorylation site of mutant L.S.-4 between cytochrome b 563 and cytochrome f 552. Subsequently the acyclic electron transfer chain is interrupted on the reducing side of cytochrome f 552. Interjected between these two events is the inactivation of photoreduction and the potential for hydrogen photoproduction. Both reactions depend upon the common component, hydrogenase, but do not possess another component which is not common to the other in vivo and in vitro reactions used. These results lead to the conclusion that the hydrogenase precursor and thus hydrogenase is located within the cyclic electron transport pathway between cytochrome b 563 and cytochrome f 552. From this position hydrogenase donates electrons to P700 via cytochrome f and plastocyanin for photoreduction and receives electrons through P 430 and cytochrome b 563 for hydrogen photoproduction.

LITERATURE CITED

Abeliovich, A. and M. Shilo. 1972. Photooxidation reactions of C-phycocyanin. Biochimica et Biophysica Acta 283: 483-491.

Allen, J. F. and D. O. Hall. 1973. Superoxide reduction as a mechanism of ascorbate stimulated oxygen uptake by isolated chloroplasts. Biochemical and Biophysical Research Communications 52: 856-862.

Andersen, I. C. and R. C. Fuller. 1958. Suppression of carotenoid synthesis and its effect on the activity of photosynthetic bacterial chromatophores. Nature 181: 252-254.

Andersen, R. and M. J. Kasperbauer. 1973. Chemical composition of tobacco leaves altered by near-ultraviolet and intense visible light. Plant Physiology 51: 723-726.

Anderson, I. C. and D. S. Robertson. 1959. Carotenoid protection of porphyrins from photodestruction. In: Christensen, R. C. and B. Buchmann. Progress in Photobiology. Amsterdam, Elsevier Publishing. p. 477-479.

Anderson, S. M. and N. I. Krinsky. 1973. Protective action of carotenoid pigments against photodynamic damage to liposomes. Journal of Photochemistry and Photobiology 18: 403-408.

Arnold, A. 1931. Der verlauf der assimilation von Helodea canadensis unter konstanten aussenbedingungen zugleich ein beitrag zur kritik der blasenzahlmethode. Planta (Berlin) 13: 529-574.

Aronoff, S. and G. Mackinney. 1943. The photooxidation of chlorophyll. Journal of the American Chemical Society 65: 956-958.

Asada, K., M. Urano and M. Takahashi. 1973. Subcellular location of superoxide dismutase in spinach leaves and preparation and properties of crystalline spinach superoxide dismutase. European Journal of Biochemistry 36: 257-266.

Baltscheffsky, M. 1969. Energy conversion linked changes of carotenoid absorbence in Rhodospirillum rubrum chromatophores. Archives of Biochemistry and Biophysics 130: 646-652.

Bassham, J. A. 1965. Photosynthesis: the path of carbon. In: Bonner, J. and J. E. Varner. Plant Biochemistry. New York, Academic Press. p. 875-902.

Bennoun, P. and R. P. Levine. 1967. Detecting mutants that have impaired photosynthesis by their increase in fluorescence level. Plant Physiology 42: 1284-1287.

Berzborn, R. J. and N. I. Bishop. 1973. Isolation and properties of chloroplast particles of Scenedesmus obliquus D_3 with high photochemical activity. Biochimica et Biophysica Acta 27: 700-714.

Bishop, N. I. 1958. The influence of the herbicide, DCMU on the oxygen-evolving system of photosynthesis. Biochimica et Biophysica Acta 27: 205-206.

_____. 1971. Preparation and properties of mutants: Scenedesmus. In: San Pietro, A. Methods in Enzymology. vol. 23. New York, Academic Press. p. 130-143.

_____. 1972. Whole cell and chloroplast reactions of algal mutants deficient in cytochrome f (552). In: Forti, G., M. Avron and A. Melandri. Proceedings of the IInd International Congress on Photosynthesis, Stresa, 1971. vol. 1. The Hague, Dr. W. Junk N. V. Publishers. p. 459-468.

_____. 1973. Professor, Oregon State University, Department of Botany and Plant Pathology. Personal Communication. Corvallis, Oregon. July 1973.

Bishop, N. I. and J. Wong. 1971. Observations on photosystem II mutants of Scenedesmus: pigments and proteinaceous components of the chloroplasts. Biochimica et Biophysica Acta 234: 433-445.

_____. 1974. Photochemical characteristics of a vitamin E deficient mutant of Scenedesmus obliquus. 1974. (in preparation)

Boardman, N. K. 1971. The photochemical systems in C_3 and C_4 plants. In: Hatch, M. D., C. B. Osmond and R. O. Slayter. Photosynthesis and Photorespiration. New York, Wiley-Interscience. p. 309-322.

Brill, C. 1963. Studies on bacterial chromatophores II: energy transfer and photooxidative bleaching of bacteriochlorophyll in relation to structure in carotenoid depleted Chromatium. Biochimica et Biophysica Acta 66: 50-60.

Butler, W. L. 1966. Fluorescence yield in photosynthetic systems and its relation to electron transfer. In: Sandi, D. R. Current Topics in Bioenergetics vol. 1. New York, Academic Press. p. 49-71.

Claes, V. H. 1954. Analysis of the biochemical synthesis of carotenoid with the help of Chlorella mutants. Zeitschrift fur Naturforschung, ser. B, 9: 461-469.

Demopoulos, H. B. 1973. Control of free radicals in biologic systems. Federation Proceedings 32: 1903-1908.

Dworkin, M. 1959. Function of carotenoids in photosynthetic bacteria. Nature 184: 1891-1892.

_____. 1960. The effect of dark aerobic growth on the photosensitivity of carotenoidless Rhodopseudomonas spheroides. Biochimica et Biophysica Acta 39: 88-92.

Emerson, R. 1935. The effect of intense light on the assimilatory mechanism of green plants and its bearing on the carbon dioxide factor. Cold Spring Harbor Symposia on Quantitative Biology 3: 128-137.

Ewart, A. J. 1898. The action of cold and of sunlight upon aquatic plants. Annals of Botany 12: 363-397.

Faludi-Daniel, A. and A. H. Nagy. 1972. Lability of photosystem II in chloroplasts of carotenoid deficient maize leaves. In: Forti, G., M. Avron and A. Melandri. Proceedings of the IInd International Congress on Photosynthesis Research, Stresa, 1971. vol. 2. The Hague, Dr. W. Junk N. V. Publishers. p. 1653-1658.

Fleischman, D. E. and R. K. Clayton. 1968. The effect of phosphorylation uncouplers and electron transport inhibitors upon spectral shifts and delayed light emission of photosynthetic bacteria. Journal of Photochemistry and Photobiology 8: 287-298.

Foote, C. S. 1968. Photosensitized oxygenations and the role of singlet oxygen. Accounts of Chemical Research 1: 104-110.

Foote, C. S. and R. W. Denny. 1968. Chemistry of singlet oxygen quenching by β-carotene. Journal of the American Chemical Society 90: 6233-6235.

Foote, C. S. and S. Wexler. 1964. Singlet oxygen a probable intermediate in photosensitized autooxidations. Journal of the American Chemical Society 86: 3880-3881.

Forti, G. and A. T. Jagendorf. 1960. Inactivation by light of the phosphorylative activity of chloroplasts. Biochimica et Biophysica Acta 44: 34-40.

Franck, J. and C. S. French. 1941. Photooxidation processes in plants. Journal of General Physiology 25: 309-324.

Fujimori, E. and R. Livingston. 1957. Interaction of chlorophyll in its triplet state with oxygen, carotene etc. Nature 180: 1036-1038.

Gaffron, H. 1940. Carbon dioxide reduction with molecular hydrogen in green algae. American Journal of Botany 27: 273-283.

Gaffron, H. and T. S. Stuart. 1972a. The mechanism of hydrogen photoproduction by several algae. I: the effect of inhibitors of photophosphorylation. Planta (Berlin) 106: 91-100.

_____. 1972b. The mechanism of hydrogen photoproduction by several algae. II: the contribution of photosystem II. Planta (Berlin) 106: 101-112.

Gimmler, H. and M. Avron. 1971. On the mechanism of benzoquinone penetration and photoreduction by whole cells. Zeitschrift fur Naturforschung, ser. B, 26: 585-588.

Gollnick, K. 1968. Type II photooxygenation reactions. Advances in Photochemistry 6: 1-122.

Goodenough, U. W. and R. P. Levine. 1970. Chloroplast structure and function in AC-20, a mutant of Chlamydomonas reinhardi III. Chloroplast ribosome and membrane organization. Journal of Cell Biology 44: 547-561.

Griffiths, M., W. R. Sistrom, G. Cohen-Bazire and R. Y. Stainier. 1955. Function of carotenoids in photosynthesis. Nature 176: 1211-1214.

Griffiths, M. and R. W. Stainier. 1956. Some mutational changes in photosynthetic pigment systems of Rhodopseudomonas spheroides. Journal of General Microbiology 14: 698-715.

Hatch, M. D. and C. R. Slack. 1970. Photosynthetic CO_2 fixation pathways. Annual Review of Plant Physiology 21: 141-162.

Heath, R. L. and L. Packer. 1965. Effect of light on lipid peroxidation in chloroplasts. Biochemical and Biophysical Research Communications 19: 716-720.

_____. 1968a. Photoperoxidation in isolated chloroplasts. I: kinetics and stoichiometry of fatty acid peroxidation. Archives of Biochemistry and Biophysics 125: 189-198.

_____. 1968b. Photoperoxidation in isolated chloroplasts. II: role of electron transport. Archives of Biochemistry and Biophysics 125: 850-857.

Hockstein, P. and L. Ernster. 1963. ADP-activated lipid peroxidation coupled to the TPNH oxidase system of microsomes. Biochemical and Biophysical Research Communications 12: 388-394.

Holden, M. 1965. Chlorophylls. In: Goodwin, T. W. Chemistry and biochemistry of plant pigments. New York, Academic Press. p. 461-488.

Izawa, S., D. G. Winget, T. N. Connolly and N. E. Good. 1967. Inhibition and uncoupling of photophosphorylation in chloroplasts. In: Energy conversion by the photosynthetic apparatus. Upton, New York, Brookhaven National Laboratory. p. 169-187.

Izawa, S. 1968. Effect of Hill reaction inhibitors on photosystem I. In: Shibata, K., A. Takamaya, A. T. Jagendorf and R. C. Fuller. Comparative biochemistry and biophysics of photosynthesis. Tokyo, University of Tokyo Press. p. 140-147.

Kandler, O. and F. Schötz. 1956. Untersuchungen über die photooxydative farbstoffzerstörung und stoffwechselhemmung bei chlorellamutanten und panaschiesten oenotheren. Zeitschrift für Naturforschung, ser. B, 11: 708-718.

Kandler, O. and C. Sironval. 1958. Photooxidation processes in normal green Chlorella cells I: the bleaching process. Biochimica et Biophysica Acta 29: 359-368.

_____. 1959. Photooxidation processes in normal green Chlorella cells II: effects on metabolism. Biochimica et Biophysica Acta 33: 207-215.

Katoh, S. and M. Kimimura. 1972. Studies of electron transport associated with photosystem I. I Functional site of plastocyanin: inhibitory effects of $HgCl_2$ on electron transport and plastocyanin in chloroplasts. Biochimica et Biophysica Acta 283: 279-292.

Keele, B. B., J. M. McCord and I. Fridovich. 1970. Superoxide dismutase from Escherichia coli B; a new manganese containing enzyme. Journal of Biological Chemistry 245: 6176-6181.

Kessler, E. W. Arthur and J. E. Brugger. 1957. The influence of manganese and phosphate on delayed light emission fluorescence, photo-reduction and photosynthesis in algae. Archives of Biochemistry and Biophysics 71: 326-335.

Kok, B. 1956. On the inhibition of photosynthesis by intense light. Biochimica et Biophysica Acta 21: 234-244.

_____. 1965. Photosynthesis: the path of energy. In: Bonner, J. and J. E. Varner. Plant Biochemistry. New York, Academic Press. p. 875-960.

Kok, B., E. B. Gassner and H. J. Rurainski. 1965. Photoinhibition of chloroplast reactions. Journal of Photochemistry and Photobiology 4: 215-227.

Kok, B., H. J. Rurainski and O. U. H. Owens. 1965. The reducing power generated in photoact I of photosynthesis. Biochimica et Biophysica Acta 109: 347-356.

Kornhauser, A., N. I. Krinsky, P. K. C. Huang and D. C. Clagett. 1973. A comparative study of photodynamic oxidation and radiofrequency-discharge generated $^1O_2$ oxidation of quanosine. Journal of Photochemistry and Photobiology 18: 63-69.

Krendeleva, T. E., N. V. Nizovshaya, A. V. Ivanov and L. B. Rubin. 1972. Selective damage of photosystem I of isolated pea chloroplasts after treatment with ruby laser radiation. Nature New Biology 240: 223-224.

Krinsky, N. I. 1966. The role of carotenoids as protective agents against photosensitized oxidations in chloroplasts. In: Goodwin, T. W. Biochemistry of Chloroplasts. vol. 1. New York. Academic Press. p. 423-430.

_____. 1968. The protective function of carotenoid pigments. Photophysiology 3: 123-195.

_____. 1971. Carotenoid function. In: Isler, O. Carotenoids. Stuttgart, Birkhäuser Verlag Basel. p. 669-716.

Laetsch, W. M. 1971. Chloroplast structural relationships in leaves of C_4 plants. In: Hatch, M. D., C. B. Osmond and R. O. Slayter. Photosynthesis and Photorespiration. New York, Wiley-Interscience. p. 323-349.

Lavelle, F., A. M. Michelson and L. Dimitrijevic. 1973. Biological protection by superoxide dismutase. Biochemical and Biophysical Research Communications 55: 350-357.

MacKinney, G. 1941. Absorption of light by chlorophyll solutions. Journal of Biological Chemistry 140: 315-322.

Mathis, P. and J. Kleo. 1973. The triplet state of β-carotene and of analog polyenes of different lengths. Journal of Photochemistry and Photobiology 18: 343-346.

McCord, J. M. and I. Fridovich. 1969. Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein). Journal of Biological Chemistry 244: 6049-6055.

Mehler, A. H. 1951a. Studies on reactions of illuminated chloroplasts I: Mechanism of the reduction of oxygen and other Hill reagents. Archives of Biochemistry and Biophysics 33: 65-77.

_____. 1951b. Studies of illuminated chloroplasts. II. Stimulation and inhibition of reaction with molecular oxygen. Archives of Biochemistry and Biophysics 34: 339-351.

Myers, J. and G. O. Burr. 1940. Studies on photosynthesis: Some effects of light of high intensity on Chlorella. Journal of General Physiology 24: 45-67.

Noack, K. 1925. Photochemische wirkungen des chlorophylls und ihre bedentung für die kohlensäureassimilation. Zeitschrift für Botany 17: 481-548.

Papageorgiou, G., J. Isoakidou and C. Argoudelis. 1972. Structure dependent control of chlorophyll a excitation density: the role of oxygen. Federation of European Biochemical Societies Letters 25: 139-142.

Pratt, Lee Hubert. Light-induced absorbence changes and partial reactions of photosynthesis in mutants of Scenedesmus obliquus strain D3. Doctoral dissertation. Corvallis, Oregon State University, 1968. 111 numb. leaves.

Pratt, L. H. and N. I. Bishop. 1968. Chloroplast reactions of photosynthetic mutants of Scenedesmus obliquus. Biochimica et Biophysica Acta 153: 664-674.

Prebble, J. and A. S. Huda. 1973. Sensitivity of the electron transport chain of pigmented and non-pigmented Sarcina membranes to photodynamic action. Journal of Photochemistry and Photobiology 17: 255-264.

Pringsheim, N. 1879-1881. 149 p. (Cited in: Emerson, R. 1935. The effect of intense light on the assimilatory mechanism of green plants and its bearing on the carbon dioxide factor. Cold Spring Harbor Symposia on Quantitative Biology 3: 128-137.)

Rabinowitch, Eugene I. 1951. Photosynthesis, vol. 2. New York, Interscience Publishers. p. 858-883.

Sager, R. and M. Zalokar. 1958. Pigments and photosynthesis in a carotenoid deficient mutant of Chlamydomonas. Nature 182: 98-100.

San Pietro, A., J. Brand and B. C. Mayne. 1972. Site of polylysine inhibition of photosystem I in spinach chloroplasts. Archives of Biochemistry and Biophysics 152: 429-431.

Schenk, G. O. 1954. Zur reaktion photochemisch angeregter molekeln mit O_2. Die Naturwissenschaften 41: 452-453.

Schultz, G. 1973. Reversible changes in the ratios of chlorophylls and carotenoids in Vaccinium myrtillus. Zeitschrift für Pflanzenphysiologie 69: 91-93.

Scott, Gerald. Atmospheric oxidation and anti-oxidants. Amsterdam, Elsevier, 1965. 528 p.

Senger, H. 1970. Charakterisierung einer synchronkultur von Scenedesmus obliquus, ihrer potentiellen photosyntheseleistung und des photosyntheses-quotienten während des entwicklungscyclus. Planta (Berlin) 90: 243-266.

Senger, H. and N. I. Bishop. 1972. Changes in fluorescence and absorbence during synchronous growth of Scenedesmus. In: Forti, G., M. Avron and A. Melandri. Proceedings of the IInd International Congress on Photosynthesis Research, Stresa 1971. vol. 1. The Hague, Dr. W. Junk N. V. Publishers. p. 677-687.

Sistrom, W. R., M. Griffiths and R. Y. Stainier. 1956. The biology of a photosynthetic bacterium which lacks colored carotenoids. Journal of Cellular and Comparative Physiology 48: 473-515.

Smith, J. H. C. and V. M. Koski. 1951. Chlorophyll formation in a mutant, white-seedling-3. Archives of Biochemistry and Biophysics 34: 189-195.

Smith, J. H. C., L. J. Durham and C. F. Wurster. 1959. Formation and bleaching of chlorophyll in albino corn seedlings. Plant Physiology 34: 340-345.

Spikes, J. D. 1968. Photodynamic action. Photophysiology 3: 33-64.

Spikes, J. D. and R. Straight. 1967. Sensitized photochemical processes in biological systems. Annual Review of Physical Chemistry 18: 409-436.

Stiller, M. and J. K. H. Lee. 1964. Hydrogenase mediated reactions in Chlorella. Plant Physiology Supplement 39: XV.

Stuart, T. S. 1971. Hydrogen production by photosystem I of Scenedesmus: effect of heat and salicylaldoxime on electron transport and photophosphorylation. Planta (Berlin) 96: 81-92.

Stuart, T. S. and H. Gaffron. 1971. The kinetics of hydrogen photoproduction by adapted Scenedesmus. Planta (Berlin) 100: 228-243.

Tanner, W., E. Loos and E. Komer. 1973. A confirmation of the proposed model for the hexose uptake system of Chlorella vulgaris - anaerobic studies in the light and in the dark. Journal of Membrane Biology 12: 89-99.

Tappel, A. L. 1973. Lipid peroxidation damage to cell compounds. Federation Proceedings 32: 1870-1874.

Ursprung, A. 1917. Über die Stärkebildung im spektrum. Berichte der Deutschen Botanischen Gesellschaft 35: 44-69.

Wallace, R. H. and A. E. Schwarting. 1954. A study of chlorophyll in a white mutant strain of Helianthus annuus. Plant Physiology 29: 431-436.

Williams, Janet Lynn. A study on the carotenoids of wild-type and mutant strains of the alga Scenedesmus obliquus. Master's thesis. Corvallis, Oregon State University. 1971. 66 numb. leaves.

Williams, J. L., M. Frick and N. I. Bishop. 1974. Pigments and photosynthetic characteristics of carotenogenic mutants of Scenedesmus. (in preparation)

Witt, H. T. 1971. Coupling of quanta, electrons, fields, ions and phosphorylation in the functional membrane of photosynthesis. Quarterly Reviews of Biophysics 4: 365-477.

Witt, H. T. and C. Wolff. 1972. On metastable states of carotenoids in the primary events of photosynthesis. In: Forti, G., M. Avron and A. Melandri. Proceedings of the IInd International Congress on Photosynthesis Research, Stresa, 1971. vol. 2. The Hague, Dr. W. Junk N. V. Publishers. p. 931-936.

Yamashita, T., J. Tsugi and G. Tomita. 1971. Reactivation of the Hill reaction of tris-washed chloroplasts. The Journal of Plant and Cell Physiology 12: 117-126.

Zalkin, H. and A. L. Tappel. 1960. Studies of the mechanism of vitamin E action. III Lipide peroxidation in the vitamin E-deficient rabbit. Archives of Biochemistry and Biophysics 88: 113-117.