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Title: THE EFFECTS OF A MUTATION WITHIN VITAMIN E BIOSYNTHESIS UPON THE DEVELOPMENT AND FUNCTION OF THE PHOTOSYNTHETIC APPARATUS

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A new photosystem-II mutant of the green alga Scenedesmus obliquus D_3, strain PS-28, has been shown to lack α-tocopherol (vitamin E). The photosynthetic activity of dark grown samples of PS-28 is about 20% of the wild-type control. Culturing the mutant at low light intentisites (10^4 ergs/sec-cm^2) stimulates photosynthetic activity by as much as 3 fold. Mutant PS-28 has a high relative fluorescence which lacks the variable yield component, but the levels of plastoquinone A, cytochrome b-559 (H. P.), and chlorophyll are nearly normal. This evidence suggests that the nature of the mutation in PS-28 is not pleiotropic, but occurs at a specific site, in the vitamin E biosynthetic pathway.

In both mixotrophic and heterotrophic samples of the mutant photosynthesis can be destroyed by exposure of the cells to high intensity irradiation (10^6 ergs/sec-cm^2). This photoinactivation is proportional to the intensity of the irradiation, and does not occur if

treatments are performed anaerobically; thus, the damage to the photosynthetic process occurs via a photodynamic mechanism. α-Tocopherol, α-tocopheryl acetate or synthetic antioxidants, such as nordihydroguaiaretic acid and N, N'-diphenyl-p-phenylenediamine, when added to the growth medium neither stabilize the mutant against photoinactivation nor reverse the mutation syndrome.

The capacities for hydrogen photoreduction, the production of a 518 nm light-induced absorbancy change and PMS-mediated photophosphorylation are only moderately affected by the mutation. Also, the above mentioned processes do not appear to be influenced by exposure of the cells to damaging intensities of white light. Contrarily, the rates of hydrogen photoproduction and anaerobic glucose photoassimilation are below normal in the mutant, and these processes show strong sensitivities to irradiation. The ferricyanide or DCPIP Hill reactions (Photosystem-II) in contrast to the ascorbate-DCPIP to methylviologen photoreduction (Photosystem-I) are not observed in chloroplasts prepared from the mutant. Summarized, these findings indicate that the mutant has a partially impaired photosystem-II which is sensitive to high intensity irradiation treatments, and a fully functional photosystem-I which is stable to irradiation.

The lipid and fatty acid complement in irradiated and unirradiated samples of PS-28 were compared to similar samples of the wild-type, and in no case was any difference noted between the

mutant and the parent strain. Furthermore, several photosystem-II mutants, possessing limited photosynthetic capacities, but having normal levels of α-tocopherol were also found to be susceptible to photoinhibition by high intensity irradiation treatment. These results indicate that α-tocopherol does not function as a general membrane antioxidant for the photosynthetic process.

The levels of vitamin E were analyzed during the greening of mutant C-2A'. In dark grown cells of C-2A' the level of α-tocopherol is equivalent to that of the wild-type. After greening, the level of α-tocopherol in the mutant is equivalent to that of mixotrophic samples of the wild-type. Contrarily, the level of plastoquinone A is at a minimum in dark grown cells of C-2A' and is synthesized in parallel with the onset of photosynthesis during greening. These observations suggest that the role of α-tocopherol in photosynthesis is different than that of plastoquinone A, which is a known electron transport intermediate.

A thorough consideration of the above information rules out a role for vitamin E in photosynthetic electron transfers or phosphorylations. The data do not support the conclusion that vitamin E functions in the chloroplast as a general membrane antioxidant. This suggests that α-tocopherol must either function as a site specific antioxidant or as a structural component in or near the photosystem-II chloroplast subunit.

The Effects of a Mutation Within Vitamin E Biosynthesis Upon the Development and Function of the Photosynthetic Apparatus

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Richard Charles Sicher, Jr.

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Professor of Botany and Plant Pathology
in charge of major

Redacted for Privacy

Chairman of Department of Botany and Plant Pathology

Redacted for Privacy

Dean of Graduate School

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ABBREVIATIONS

ATP	adenosine triphosphate
BHT	butylated hydroxytoluene
Chl.	chlorophyll
cm	10^{-1} meters
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethyl urea
DCPIP	2,6-dichlorophenol-indophenol
DPPD	N,N'-diphenyl-p-phenylenediamine
hr	hour
I. D.	inner diameter
kg	10^{3} grams
l	liter
M	molar
μm	10^{-6} moles (when appearing on a figure or table)
μM	10^{-6} molar
μl	10^{-6} liters
ml	10^{-3} liters
min	minute
$NADP^{+}$(NADPH)	nicotinamide dinucleotide phosphate, oxidized and reduced forms
NDGA	nordihydroguaiaretic acid
nm	10^{-9} meters
PCV	packed cell volume

PMS($PMSH_2$)	phenazine methosulfate, oxidized and reduced forms
RUDP	ribulose-1,5-diphosphate
sec	second
STK	sucrose-tricine-KCl
TCPIP	2,3,6-trichlorophenol-indophenol
TMPD	tetramethyl-p-phenylenediamine
Tris	tris(hydroxymethyl)aminomethane
W	watt

THE EFFECTS OF A MUTATION WITHIN VITAMIN E BIOSYNTHESIS UPON THE DEVELOPMENT AND FUNCTION OF THE PHOTOSYNTHETIC APPARATUS

I. INTRODUCTION

Vitamin E was discovered as a mammalian dietary requirement necessary for reproduction in laboratory rats (Evans and Bishop, 1923), and for this reason has been of interest to chemists, biochemists and nutritionists for several years. It is only recently that the function of vitamin E in green plant parts has come under scrutiny. Vitamin E which is synthesized by the plant, is a component of the chloroplast and thus far has no established functions (Dilley and Crane, 1963). In this thesis a mutant of the green alga Scenedesmus obliquus D_3, strain PS-28, which does not synthesize vitamin E (Bishop and Sicher, 1974), is analyzed in an effort to determine the validity of the current hypotheses concerning the mode of action of vitamin E.

Definition and Role of Photosynthesis

Except in rare instances, all of the vascular plants and algae require the energy of sunlight to maintain their metabolism. Certain species of bacteria also have this capability, but these organisms are distinguished from higher plant forms by their inability to evolve oxygen. Early investigations into the subject of photosynthesis

established that CO_2, H_2O, and light were the substrates, and that carbohydrates were the products (cf., Rabinowitch, 1945). The net biochemical reaction of higher plant photosynthesis was determined by van Niel (1931) to be:

$$CO_2 + 2H_2O \rightarrow CH_2O + O_2 + 1H_2O$$

where CH_2O represents the reduction of CO_2 to stable end products. There is a net energy capture by the plant of 112 kCal for each mole of CO_2 fixed. However, the main significance of the van Niel hypothesis stems from the realization that the oxygen evolved by green plant photosynthesis must be derived from the photolysis of water.

Arnon and his co-workers (1954a) successfully demonstrated that all of the reactions of photosynthesis occurred within the chloroplast. It was shown that the highly pigmented thylakoids were responsible for the light reactions, which include light absorption, exciton transfer, and the generation of electron flow from water. Each photosynthetic unit contains two trapping centers (Photosystems I and II) that function in series to promote electrons from water against a thermodynamic gradient to reduce nicotinamide adenine dinucleotide phosphate ($NADP^+$) (cf., Bishop, 1971a). Additionally, electron transport is coupled to the formation of adenosine triphosphate (ATP), via an as yet unexplained mechanism (Frenkel, 1954; Arnon, et al., 1954b). Therefore, the products of the light reactions

of photosynthesis are reduced $NADP^+$, high energy phosphate in the form of ATP, and oxygen. A diagram of the photosynthetic electron transport system as it is currently viewed by Bishop (1971a), is given below (Figure 1).

The fixation of carbon during photosynthethesis is performed by the enzyme ribulose-1,5-diphosphate (RUDP) carboxylase (Zelitch, 1975), which is loosely attached to the surface of the thylakoid (Howell and Moudrianakis, 1967). However, with the above exception noted, the enzymes of the Calvin cycle occur in the stroma of the chloroplast in a soluble form. The reaction mechanism of RUDP carboxylase calls for the utilization of ATP and reduced $NADP^+$ in the carboxylation of RUDP. The first stable end products formed are two molecules of 3-phosphoglyceric acid (Bassham, 1965). These findings were a verification of an hypothesis advanced by Ruben (1943), who predicted that the products of the light reactions of photosynthesis, except oxygen, would be consumed by the carboxylation reactions. It is currently believed that a stoichiometry of 2 NADPH and 3 ATP consumed per molecule of CO_2 fixed will satisfy the energetic requirements of photosynthetic carboxylation (Myers, 1974).

Kortschack, et al. (1965) and Hatch and Slack (1967, 1968) recently discovered a carboxylation mechanism that was a modification of the normal process, and was common to sugarcane, maize

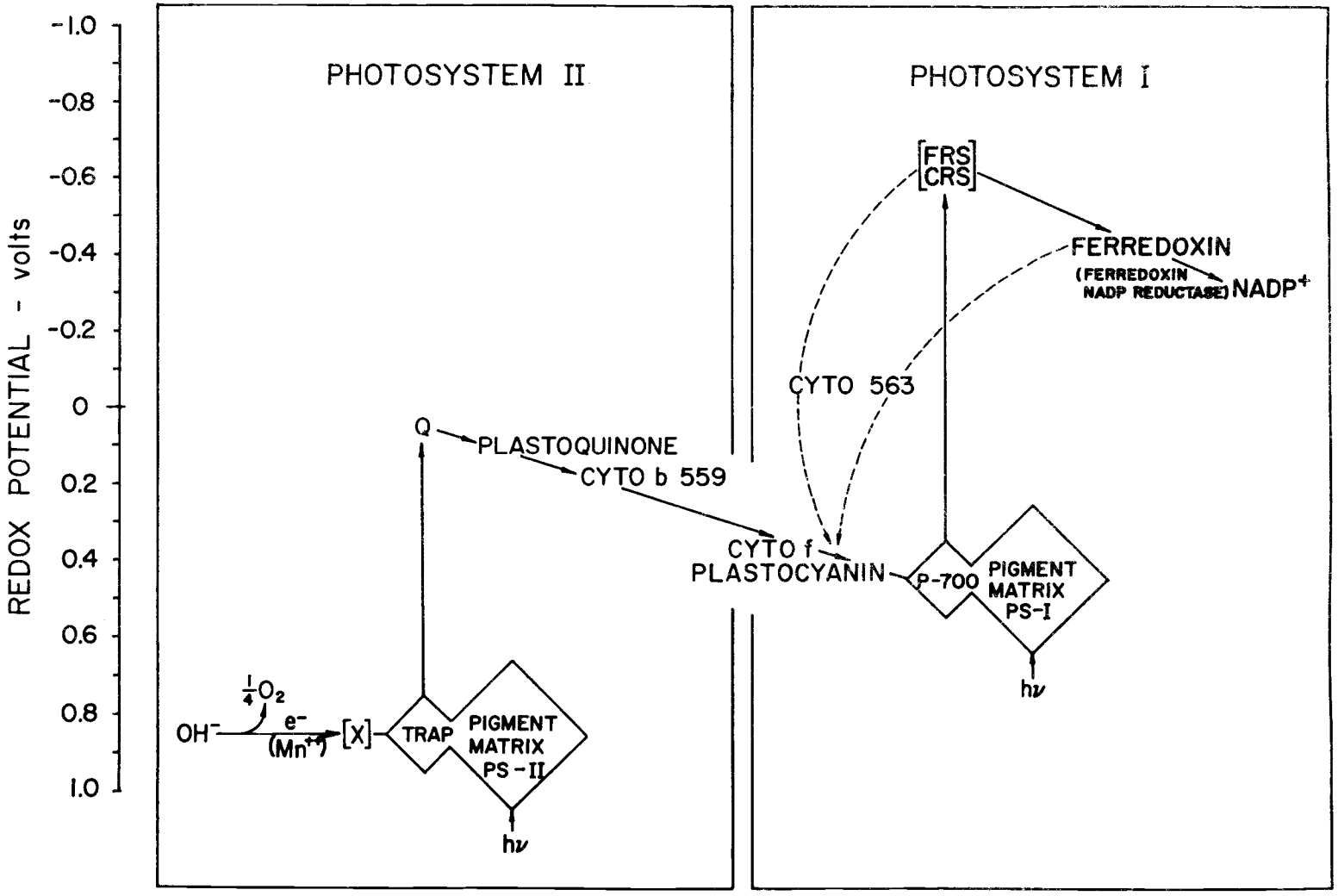


Figure 1. Higher plant photosynthetic electron transport scheme depicting the action of both photosystems.

and other species of tropical grasses. Several characteristics distinguish plants that possess the cy pathway from those that do not. An important difference is that plants which have this pathway rapidly accumulate radiotracer $^{14}CO_2$ into the four carbon dicarboxylic acids, malate and aspartate (Kortschack, et al., 1965). Laetsch (1968) reported that cy plants had a unique leaf anatomy, which involved two types of parenchymal cells, mesophyll cells and bundle sheath cells. It is now certain that C_4 plants have an additional carboxylation reaction catalyzed by the enzyme phosphoenolpyruvate (PEP) carboxylase, which enables increases in the photosynthetic efficiency of those species by several fold (Hatch and Slack, 1970). Most investigators feel that CO_2 is fixed in the mesophyll cells of the leaf of the C_4 plant by PEP carboxylase and that the aspartate or malate thus formed is transported to the bundle sheath cells. There the C_4 compounds are decarboxylated and the released CO_2 is free to enter the Calvin cycle by the usual means. The function of the C_4 pathway is, therefore, to concentrate CO_2 in the bundle sheath cells for entry into the Calvin cycle. This is important because the enzyme RUDP carboxylase has a very high K_M for its substrate, CO_2 (Cooper, et al., 1969).

Studies in recent years (Zelitch, 1966, 1974) established a close relationship between the operation of the Calvin cycle and photorespiration, which is a light induced evolution of CO_2 and uptake of O_2.

Photorespiration, which reduces the efficiency of photosynthesis by up to 50% (Zelitch, 1974), occurs best when O_2 concentration are high and CO_2 concentrations are low. Considerable interest in photorespiration has been generated by the discovery that the enzyme RUDP carboxylase can readily catalyze a reaction between oxygen and its usual substrate, RUDP, leading to the formation of phosphoglycolic acid (Bowes, Ogren and Hageman, 1971). Furthermore, there appears to be a direct relationship between the rates of photorespiration and the levels of glycolic acid found in the leaf. These facts suggest that the enzyme RUDP carboxylase is simultaneously responsible for photosynthesis and photorespiration. In support of this hypothesis is the observation that plants with C_4 photosynthesis, which maintain high CO_2 concentrations at the site of carboxylation in the bundle sheath cells, are known to have very much slower rates of photorespiration than those species which do not have this pathway (Zelitch, 1966). Because photorespiration decreases photosynthetic efficiency it has been researched extensively, but a further discussion of these studies would be beyond the scope of this thesis.

Photosynthetic Components and Lipophilic Quinones of Biological Importance

Studies of certain proteins commonly occurring in either mitochondria or chloroplasts led to the suggestion that these molecules

participate in electron transport phenomena. Several cytochromes, non-heme iron proteins, flavoproteins, and copper containing proteins currently are believed to be involved in the electron transfer pathways of mitochondria and chloroplasts. In photosynthetic organisms these molecules include cytochrome-f, cytochrome b-563, cytochrome b-559 (both high and low potential forms), ferredoxin (a non-heme iron protein), ferredoxin-NADP-oxidoreductase (a flavoprotein), and the copper containing protein, plastocyanin (see Figure 1). The above mentioned proteins are attached to the surfaces of the photosynthetic membrane (Anderson, 1975). The membrane itself is thought to be of the lipid-protein fluid mosaic type as described by Singer (1974). The lipid complement of the membrane is composed of mainly two glycolipids, monogalactosyl diacylglycerol and digalactosyl diacylglycerol, and two anionic lipids, phosphatidylglycerol and plant sulpholipid, sulphoquinovosyl diacylglycerol (Benson, 1963). The majority of the protein of the photosynthetic membrane is associated with the chlorophyll-protein complexes of Thornber, et al., (1967a, 1967b). Chlorophyll-protein complex 1, which represents 28% of the chloroplast membrane protein, contains the P700 reaction center, and chlorophyll-protein complex 2, which represents 50% of the chloroplast membrane protein, contains the light harvesting (chlorophyll a/b, 1:1) pigment protein complex (Thornber, 1975). Other components of the

photosynthetic membranes are the carotenoids, the sterols, and of ultimate importance to the discussion here, the lipophilic quinones.

To date, four major classes of lipophilic quinones have been isolated from biological sources, and these are the substituted ubiquinones, plastoquinones, napthoquinones and tocopheryl quinones. Representatives from these four classes were isolated from photosynthetically active green plant parts (Kegel, et al., 1962; Henninger and Crane, 1963, 1964), and there is a continuing interest among chemists and biochemists alike to establish the individual functions of these compounds. All of the above mentioned quinones are structurally similar to the chlorophyll molecule in that they possess a long hydrophobic side chain attached to an aromatic nucleus. It is currently believed that the terpenoid side chain anchors the quinone to the membrane, leaving the aromatic nucleus free to function on the membrane surface.

Ubiquinone

Ubiquinone or Coenzyme Q is a 2, 3-dimethoxy-5-methyl benzoquinone with an isoprenoid side chain attached to the 6 position of the aromatic ring that can be of varying lengths and degrees of unsaturation. Ubiquinone is characterized by a broad symmetric absorbance band in ethanol with a maximum at 275 nm, and reduction to the ubiquinol can easily be achieved with sodium borohydride

(Crane, et al., 1959; Lawson, et al., 1960). Ubiquinone reacts positively with alkaline-ethyl cyanoacetate, Craven's reagent, which allows its easy detection in the presence of other lipid soluble quinones without interference (Barr and Crane, 1971).

Bacteria, photosynthetic bacteria, plants, fungi, lower animal forms, and vertebrates were shown to possess ubiquinone compounds (Lester and Crane, 1959; Bishop, et al., 1962; Pennock, 1962a, 1962b), and there was evidence that a ubiquinone homolog supports photosynthetic electron transport in certain species of bacterial phototrophs (Lester and Crane, 1959; Bishop, 1959). Crane (1959) found ubiquinone in equivalent amounts in all parts of the plant, and this led Pumphrey and Redfearn (1960), to suggest that it was located in the mitochondria. The observation of endogenous reductions and reoxidations by spectral means firmly established ubiquinone as a functional component in mitochondrial electron transfers (Crane, 1962). There is also evidence that ubiquinone is important in transposing an electrochemical pH gradient across the mitochondrial membrane, and is therefore, important to oxidative phosphorylation (Anderson, et al., 1976).

Napthoquinone

The napthoquinones of biological interest are divided into two main series depending upon the pattern of unsaturation in the

isoprenoid side chain (Brodie, 1965). The vitamin K_2 series is found in microorganisms and animals, whereas plants generally contain napthoquinones of the vitamin K_1 type (Lester and Crane, 1959; Martius, 1961). Although direct evidence for its presence in mammalian tissue is difficult to demonstrate, phylloquinone (vitamin K_1) is an essential blood clotting factor (Green, et al., 1956).

The original observation associating quinones with photosynthesis arose when it was discovered that green leaves satisfy the nutritional requirement for vitamin K in laboratory rats (Almquist, 1937). Dam (1942) demonstrated that the outer green leaves of cabbage possess more vitamin K activity than either the inner leaves or the root, and the fact that most of the activity appeared in the pressed juices of the leaves led him to the conclusion that vitamin K is concentrated in the chloroplast. Kegel and Crane (1962) were the first investigators to isolate vitamin K from plant material, and to prove its existence in the chloroplast by chemical means other than by a bioassay. The suggestion that quinones might participate in electron transport in photosynthesis followed the discovery by Warburg and Luttgens (1944), that certain benzoquinones acted as hydrogen acceptors for the Hill reaction. However, Wessels (1954) virtually ruled out the participation of vitamin K in photosynthetic electron transport, when he found that menadione and phthiocol (two

vitamin K analogs that function in blood clotting) actually inhibited the Hill reaction.

Evidence for the participation of vitamin K in photosynthesis was enhanced by the discovery of photophosphorylation (Frenkel, 1954; Arnon, et al., 1954b). Menadione and other vitamin K analogs were found to be catalysts of photophosphorylation in isolated chloroplasts, and Dicumarol, a vitamin K antagonist, was found to inhibit the reaction. It was later demonstrated that photophosphorylation could be catalyzed by a wide variety of substances, some of which were definitely non-biological (Whatley, et al., 1959). Therefore, evidence for the in vitro participation of vitamin K in chloroplast reactions is still in question.

More recently, Lichtenthaler (1969) fractionated chloroplasts into photosystems-I and II particles, and found an enrichment of vitamin K in the photosystem-I sub-chloroplast fragment. From this evidence, and from the observation that vitamin K biosynthesis parallels increases in chlorophyll content during chloroplast development, he concluded that vitamin K is an electron transfer component in photosystem-I. Hopefully, this work will be re-evaluated in light of Thornber's modification of chloroplast fractionation techniques (see above).

In bacterial and animal systems vitamin K_2 is associated with terminal oxidative metabolism, and in Mycobacterium phlei there is

direct evidence that napthoquinone is involved in oxidative phosphorylation (Brodie, 1965). However, more evidence is required before any generalizations can be made about the role of vitamin K_2 in the phosphorylation mechanisms of other species.

Plastoquinone

The plastoquinones found in plant tissues are 2, 3-dimethyl, 5-solanesyl 1:4 benzoquinones or their related derivatives. Purified plastoquinone is a yellow, crystalline solid which dissolves readily in most organic solvents, and it is characterized by an absorption maximum in ethanol at 255 nm with a shoulder at 263 nm. The plastoquinol is a weakly absorbing compound with a maximum at 290 nm (Redfearn, 1965; Crane, et al., 1960).

Crane and co-workers Kegel, Henninger, and Crane, 1962; Henninger and Crane, 1963, 1964), were the first investigators to isolate more than one species of plastoquinone from the same plant. More than 12 individual plastoquinones now have been isolated, and currently these are segregated into three basic types: plastoquinone A is the predominant form, and has an unsubstituted side chain; the C type plastoquinones have an hydroxyl group in the side chain; and the B type plastoquinones are esterified through an hydroxyl group in the side chain (Threlfall, et al., 1965; Das, et al., 1965; Griffiths, 1966; Barr, et al., 1967). In older literature reference is made to

a plastoquinone D, but under current nomenclature plastoquinone D is a C type plastoquinone.

Plastoquinone A is located exclusively in the chloroplast, and is present in higher plants and algae that use water rather than H_2, H_2S and other substrates as a source of reducing power. This evidence suggested that plastoquinone A might be involved in the reactions leading to the evolution of oxygen. Bishop (1959) demonstrated this when he showed that purified plastoquinone A restored Hill reactivity to petroleum ether extracted lyophilized chloroplasts. This provided the first direct evidence that plastoquinone A was an essential electron transfer component in photosynthesis. Krogmann (1961) extended Bishop's experiments and found that there was a decrease in the Hill reaction and in phenazine methosulfate (PMS) mediated photophosphorylation as plastoquinone A is sequentially extracted with heptane from freeze-dried chloroplasts. Both reactions were restored upon re-addition of purified plastoquinone A. It was later demonstrated by extraction and re-addition experiments that plastoquinone A functions in the photoreduction of $NADP^+$ from water, but not from ascorbate-DCPIP (2, 6-dichlorophenol indophenol) (Arnon and Horton, 1963). The above reports are consistent with the observation that plastoquinone A is involved in the mechanism of oxygen evolution.

Crane, et al., (1960) demonstrated that plastoquinone A was an electron carrier in photosynthesis by observing its light induced reduction in isolated chloroplasts. They (Crane, et al., 1960) illuminated chloroplasts, extracted the lipoquinone fraction, and analyzed for an increase in plastoquinol. More recently, Witt and his colleagues (Witt, et al., 1963; Klingenberg, et al., 1962), observed in situ changes in the plastoquinone pool using the technique of flash photometry.

Because of these findings, most investigators now believe that plastoquinone A functions on the reducing side of photosystem-II, (internal to the site of oxygen evolution), and mediates the flow of electrons into photosystem-I (Bishop, 1971a). The evidence that plastoquinone A is essential for photophosphorylation was interpreted to mean that both cyclic and linear photosynthetic electron flow must pass through the plastoquinone A site (Eck and Trebst, 1963).

Tocopheryl Quinone

Four tocopheryl quinones (α, β, γ, δ) are found in the chloroplast (Dilley and Crane, 1963). The tocopheryl quinones have a fully saturated side-chain with an hydroxyl group attached to the tertiary carbon atom of the first isoprene adduct. Because of this feature cyclization between the side-chain and the quinone nucleus can occur to form a chroman (tocopherol; Smith, et al., 1942).

Tocopherol was first discovered in animal nutrition studies as a fat soluble substance necessary for reproduction in laboratory rats (Evans and Bishop, 1923). The obsolete name "antisterility vitamin" was replaced by the more acceptable names Vitamin E, or tocopherol (from the Greek, tokos, offspring; pherein, to bear; and -ol, alcohol; Sure, 1924). The unsubstituted tocopherol is generally known as tocol, and the α, β, γ, and δ tocopherols are 5,7,8-trimethyl, 5,8-dimethyl, 7,8-dimethyl, and 8-methyl tocol respectively (Karrer and Fritzche, 1938).

D-α-tocopherl is the most prevalent and widely distributed tocol in plants, and it is also the most active form of vitamin E in animal nutrition (Bieri, 1969). The four tocopheryl quinones and four tocopherols were identified as lipid components of the chloroplast, and were found in all aerobic, photosynthetic organisms, except the blue-green algae (Hiroyama, 1967; Carr, et al., 1967; Henninger, et al., 1965).

α-Tocopheryl quinone is characterized by an ultra-violet absorption spectrum in ethanol with a maximum at 262 nm and a shoulder at 269 nm. The β, γ and δ tocopheryl quinones have maxima at 261 nm, 258 nm, and 253 nm, respectively. The tocopherols easily can be detected with the Emerie-Engel reagent (Barr and Crane, 1971), or they can be oxidized to the corresponding quinone with ferric chloride or silver nitrate, and then identified by spectral

analysis (Henninger and Crane, 1964; Baxter, et al., 1943).

Effects of a Vitamin E Deficiency

The dysfunctions caused by a vitamin E deficiency were reviewed by Mason (1954), and more recently by Scott (1969). There are structural and functional failures in several tissue types when vitamin E is deleted from the diet. Histopathies were reported in the reproductive system (testicular degeneration, fetal resorption); musculature (skeletal, cardial and smooth muscle dystrophies); nervous system (encephalomalacia); and the vascular system (exudative diathesis, erythrocyte hemolysis) of rats or chicks on a viatmin E deficient diet.

One of the most important tools in determining the physiological function of vitamin E has been the bioassay, and for any rigorous treatment fetal resorption in the gestating rat has been the assay of choice for measuring relative vitamin E activity. If the activity of α-tocopherol in this test is set at 100, then the activities of β, γ, and δ tocopherol are 40, 8, and 1 respectively (Jaffe and Harris, 1943). In similar studies Issodores and Mattill (1951), found that α-tocopheryl quinone and α-tocopheryl hydroquinone had no activity in the fetal resorption test. This experiment is especially significant because it implies that the rat does not have the ability to synthesize the chroman from either the quinone or hydroquinone forms. Over

one hundred compounds, either naturally occurring or synthetic, were tested for their biological activity in the rat fertility test, and none of these compounds were more effective than α-tocopherol (Bieri, 1969).

Vitamin E in Electron Transport

Whether or not vitamin E is a mitochondrial electron transfer component is still unclear. Olivera, et al. (1969) found that the level of α-tocopherol in horse heart mitochondria is comparable to the level of the individual cytochromes. Therefore, they felt that viatmin E was present in sufficient quantity to accommodate any proposed electron transport activity. In an effort to demonstrate the function of vitamin E in electron transport Nason and Lehman (1956), extracted mitochondrial preparations with isooctane and found that re-addition of α-tocopherol solubilized with bovine serum albumin stimulated the oxidation of reduced pyridine nucleotide. These authors concluded that α-tocopherol functions as an electron transfer component in the respiratory chain just prior to cytochrome c, and that antimycin A is a competitive inhibitor at this site. In analogous experiments, Edwin and Green (1960) found that not only α-tocopheryl quinone, but several lipids could reverse the inhibition of succino-oxidase caused by a factor isolated from Tetrahymena pyriformis. Because of the latter experiments it is currently felt that the

re-activations observed by Nason and Lehman (1956) were purely physical, and were caused by the removal of adsorbed solvent molecules from the surfaces of enzymes. Extraction and re-addition experiments, or observations of in situ redox cyclings were not successful for α-tocopherol or α-tocopheryl quinone in mitochondria (Molenaar, et al., 1972).

The role of vitamin E in photosynthetic electron transfers is equally uncertain. Krogmann and Olivera (1962) found that α-tocopheryl quinone, α-tocopherol, and ubiquinone-6 were ineffective in restoring the TCPIP (2, 3, 6-trichlorophenol indophenol)-Hill reaction to heptane extracted chloroplasts. Shortly thereafter, Trebst (1963), reported that both α-tocopherol, and α-tocopheryl quinone would re-activate the ferricyanide Hill reaction in petroleum ether extracted chloroplasts. From these studies Trebst (1963) concluded that the re-activation of the ferricyanide Hill reaction by various quinones was a non-specific process, and was dependent primarily upon the redox potential of the quinone; the more negative the redox potential, the more effective the quinone was in restoring electron transfers to extracted chloroplasts.

Dilley and Crane (1963, 1964) found that the levels of α-tocopheryl quinone increased in illuminated chloroplasts. In contrast to the report by Trebst (1963), these workers were unable to demonstrate any activity of α-tocopheryl quinone in restoring the

ferricyanide Hill reaction to acetone extracted chloroplasts. In a different report from the same laboratory it was found that the re-addition of both α-tocopheryl quinone and plastoquinone A to acetone extracted chloroplasts stimulated the oxidation of NADPH (Henninger and Crane, 1963). Crane and co-workers (Dilley, Henninger and Crane, 1963; Henninger and Crane, 1963) concluded that no one quinone could re-activate every partial reaction of photosynthesis, and that there must be several sites in the photosynthetic chain where the various quinones function (Dilley, et al., 1964).

In more recent experiments Brand, Krogmann, and Crane (1971) demonstrated that heptane extraction of lyophilized spinach chloroplasts reduces photosystem-I activity. Plastocyanin and a concentrate of the crude heptane extract partially restored activity to the extracted chloroplasts. In a previous publication Henninger and Crane (1967) reported that plastoquinone C was effective in restoring activity to the extracted chloroplasts. However, in the latest paper in this series Brand, et al., (1971) reported that the substance most responsible for restoring activity to heptane extracted photosystem-I particles was a triglyceride. The above report was viewed with skepticism by Baszynski (1974), who noted that triglycerides are not present in the membranes of the chloroplast. He demonstrated that α-tocopherol was active in reconstituting photosystem-I in heptane extracted chloroplasts.

It is obvious that the above findings are internally inconsistent, and a word of caution concerning extraction and re-addition experiments is in order. Many attempts to determine the specificity of the various quinones in electron transport were irreproducible, and this must be attributed to the very nature of the experiments involved. Virtually all of these studies relied upon lyophilized chloroplasts (or mitochondria), and lyophilization in itself can inactivate the process being studied. Furthermore, extraction with various organic solvents removes several components from the membrane, and surely disrupts the integrity of the membrane. Re-addition of the quinone to the extracted chloroplasts and mitochondria can not be assumed to be quantitative, and it is not known that the quinone has returned to its original site in the membrane. Finally, in many of these experiments the appropriate controls were not tested, and as suggested above artifactual and non-specific reactions may have been observed (Trebst, 1963; Edwin and Green, 1960). Therefore, extraction and re-addition experiments in themselves are not conclusive unless accompanied by the appropriate *in vivo* spectral data.

Vitamin E in Phosphorylation

Despite the lack of direct evidence that vitamin E participated in oxidative phosphorylation, Clark and co-workers (1958), proposed a model reaction to demonstrate its feasibility. Experimental

information was provided by Asano, et al. (1962), who were able to demonstrate the reduction of ferricytochrome c, and the formation of ATP from napthotocopheryl phosphate added to preparations of Mycobacterium phlei.

Corwin (1965) discovered that in mitochondria from vitamin E deficient rats oxalacetic acid accumulated during succinate oxidation, and that this could not be prevented by the in vitro administration of α-tocopherol. Corwin (1965) concluded from this work that vitamin E might function at phosphorylation site I. Carabello, et al. (1971) observed a decline in the P:O ratio of citrate oxidation by isolated mitochondria prepared from vitamin E deficient guinea pigs. An injection of a vitamin E-water emulsion 10 minutes prior to sacrificing the animal restored the P:O ratio to normal. They also noted that the oxidation of ascorbate-TMPD (tetramethyl-para-phenylenediamine), which bypasses phosphorylation site I, was not affected by a vitamin E deficiency. These results were in agreement with those of Corwin (1965).

Schwarz (1962), in studying respiratory decline in liver slices concluded that vitamin E may function as a catalytic agent in intermediary metabolism. However, respiratory decline was not observed in carefully isolated mitochondria from either control or vitamin E deficient rats. If the microsomal fraction were recombined with the mitochondrial fraction in the test system respiratory decline occurred.

Therefore, it was concluded by Schwarz (1962) that respiratory decline in vitamin E deficient liver homogenates was caused by extra-mitochondrial malfunctions.

Working with spinach chloroplasts, Krogmann and Olivera (1962), examined α-tocopherol and α-tocopheryl quinone for possible function in photosynthetic phosphorylation by extraction and re-addition analysis. They found that neither of these compounds would substitute for plastoquinone A in restoring photophosphorylation to heptane extracted chloroplasts.

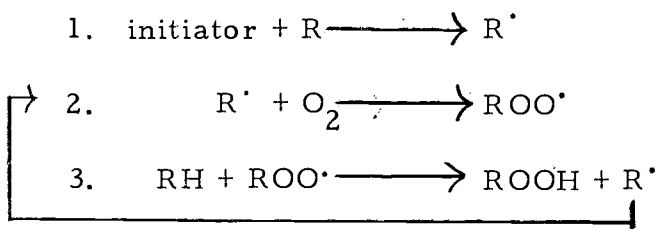
Although α-tocopherol is concentrated in the mitochondria of animal tissue and the chloroplasts of green plant parts, there is still little evidence that it is involved in phosphorylation reactions. There is direct evidence, however, that a vitamin E deficiency has a profound effect on the membrane bound functions of cellular organelles.

Vitamin E as a Membrane Stabilizer

Vitamin E was shown to be the principal antioxygenic substance of several vegetable oils and plant extracts (Mattill, 1931). This observation lead to the early suggestion that vitamin E might function as a biological antioxidant (Olcott, 1935). However, it was not until 1962 that a formal antioxidant theory appeared (Tappel, 1962). In this hypothesis, it is suggested that lipid peroxidation is a continuing,

free radical catalyzed process that damages cellular structure and inhibits normal enzyme function.

It is widely recognized that free radical chain reactions proceed by the following mechanism:



where visible and ultraviolet light, transition metals, and peroxides can act as initiators (Tappel, 1962; Scott, 1965). The di- and tri- unsaturated fatty acids are the major substrates of free-radical peroxidations. These fatty acids are abundant in the thylakoid lipids of the chloroplast, occurring mainly in the monogalactosyl and digalactosyl diacylglycerides. The major effect of lipid peroxidation is the cleavage of unsaturated fatty acids leading to the formation of malonyldialdehyde. This compound is capable of cross-linking reactions with proteins, which causes the loss of enzyme activity. α-Tocopherol is thought to break the free-radical chain reaction by donating an electron or an hydrogen ion to the free-radical (Hilditch, 1950; Zalkin and Tappel, 1960).

According to Green (1969) the lines of evidence providing a basis for the antioxidant theory are as follows:

1. α-tocopherol is a natural in vitro antioxidant.
2. The minimum dietary level of vitamin E is directly related to the quantities of polyunsaturated fatty acids in the diet.
3. Naturally occurring or synthetic antioxidants can in some cases replace the dietary requirement for vitamin E.
4. Certain labile substances (ascorbic acid, ATP, and titratable sulfhydryls) are diminished or destroyed in vitamin E deficient tissue.
5. Vitamin E deficient tissues contain higher levels of the products of lipid peroxidation than do normal tissue.

Bunyan and co-workers (1967) were unable to demonstrate an increase in the level of lipid peroxides in the kidney, liver, testis, and adipose tissue of the rat during the onset of vitamin E deficiency. In similar studies, Lee and Barnes (1969) were unable to identify consistent changes in the polyunsaturated fatty acid composition of rats kept on a diet deficient in vitamin E for 14 months. Carpenter (1966) found that the levels of total lipid, lipid phosphorous, and the level of polyunsaturated fatty acids in vitamin E deficient rat testis remained unchanged until tissue degeneracy was advanced. Green, et al. (1967) observed a concomitant destruction of ^{14}C-α-tocopherol whenever in vitro lipid peroxidation occurred, however, this was in contrast to in vivo results in which no difference in the rates of vitamin E depletion were observed in rats maintained on diets either

high or low in polyunsaturated fatty acids.

The discovery of the dietary requirement for selenium in animal nutrition has strengthened "... the concept that vitamin E may act through its 'antioxidant' or 'antioxygenic' property..." (Hoekstra, 1975). Schwarz and Foltz (1958) discovered that selenium would substitute for vitamin E in preventing dietary liver necrosis in rats, and it was later demonstrated that selenium could prevent other vitamin E related diseases, such as exudative diathesis (Patterson, et al., 1957). However, certain forms of muscular dystrophy, encephalomalacia, and fetal-resorption in the rat are not affected by the introduction of selenium to the diet (Sondegaard, 1967).

The apparent overlap in function of vitamin E and selenium posed a problem in determining the mechanism of action of the two substances. Schwarz (1965) proposed that the two nutrients functioned independently, but at closely related sites in metabolism. On the other hand, Tappel (1965) expanded the biological antioxidant theory to include the role of selenium as a membrane stabilizer.

One of the biological functions of selenium was determined when it was discovered that glutathione peroxidase is a seleno-enzyme (Rotruck, et al., 1973). Mills (1957), and Mills and Randall (1958) discovered gluthathione peroxidase in cattle erythrocytes as an enzyme, other than catalase, that would prevent the oxidative destruction of hemoglobin by reducing H_2O_2 to $2H_2O$ molecules.

The importance of this enzyme was expanded when it was found to be active in bovine liver, lung, and kidney (Mills, 1960). Although glutathione peroxidase is specific for a source of reducing power, (glutathione), it will reduce a wide variety of peroxide substrates including lipid hydroperoxides. The inactivation of lipid hydroperoxides and hydrogen peroxide is thought to be the mechanism of membrane stabilization provided by glutathione peroxidase, and it was this finding that accelerated the acceptance of the antioxidant mechanism as the mode of action for vitamin E (Hoekstra, 1975).

The possible function of vitamin E in the chloroplast as an anti-oxidant has not been investigated thoroughly. In a series of reports, Heath and Packer (1965, 1968a, 1968b), demonstrated free radical formation in isolated chloroplasts. It was shown that light and exogenously supplied linoleic acid stimulated the production of malonyldialdehyde. The effect of light was explained by its role in free radical initiation, or by the generation of photosynthetic peroxides (Heath and Packer, 1968a, 1968b). The damage to the chloroplasts was limited to photosystem-II.

The formation of singlet oxygen during photosynthesis may represent another source of oxidant stress in plants (Krinsky, 1966). The mechanism of singlet oxygen generation and dissipation are given below (Farenholtz, et al., 1974), where reactions 1 and 2

1. $h\nu + R \longrightarrow R^*$
2. $R^* + {}^3O_2 \longrightarrow R + {}^1O_2$
3. $R + {}^1O_2 \longrightarrow RO_2$ $\quad k_{ox}$
4. ${}^1O_2 \longrightarrow {}^3O_2$ $\quad k_d$
5. ${}^1O_2 + Q \longrightarrow {}^3O_2 + Q$ $\quad k_{qp}$
6. ${}^1O_2 + Q \longrightarrow QO_2$

represent the generation of singlet oxygen and reactions 3-6 diagram the mechanisms of singlet oxygen quenching (k_{ox} is oxidative decay, k_d is the rate of dissipation, k_{qp} is the rate of physical quenching, and k_{qd} is the rate of degenerative quenching). The sensitizers of singlet oxygen formation are usually a combination of light, oxygen and either a transition metal, H_2O_2, or an in vivo pigment (primarily chlorophyll) (Foote and Wexler, 1964; Foote, 1968; and Foote and Denny, 1968). Recently, Doledein, et al. (1974) reported that several unsaturated fatty acids were decomposed in vitro by singlet oxygen. Furthermore, they observed a linear correlation between the rate of reactivity with singlet oxygen, and the degree of unsaturation within the fatty acid.

Several reports indicated that α-tocopherol was an in vitro scavenger of singlet oxygen (Fahrenholtz, et al., 1974; Foote, et al., 1974; Stevens, et al., 1974). These reports established α-tocopherol as one of the best scavengers of singlet oxygen yet found, being only

slightly less efficient than β-carotene, where the quenching action is diffusion limited (Fahrenholtz, et al., 1974). Therefore, α-tocopherol, in addition to being an active inhibitor of free radical chain reactions, also is effective as a singlet oxygen scavenger, deactivating up to 120 molecules of $^1O_2$ per molecule α-tocopherol before being destroyed.

STATEMENT OF PURPOSE

The complex nature of photosynthesis, which is characterized by such diverse reactions as water photolysis, separate photoacts, electron transport, photophosphorylation, and carbon dioxide fixation is well suited for mutant analysis. A new algal mutant of Scenedesmus obliquus D_3, PS-28, is described which lacks the ability to synthesize vitamin E (Bishop and Sicher, 1974; Bishop and Wong, 1974). Although vitamin E was among the first substances isolated from the chloroplast, its function, if any, has yet to be determined.

Several functions for vitamin E, such as a role in electron transport, in photophosphorylation, and in the protection of the chloroplast membranes from photodynamic damage, have been postulated. It is the purpose of this study to evaluate each of the above mentioned possible functions for vitamin E, and to explore the interrelationships, either structurally or functionally, between the mode of action of vitamin E and the photosynthetic process.

It is assumed that vitamin E functions in the chloroplast as an electron transport carrier, an intermediate in photophosphorylation, and as a general membrane antioxidant, and it is a purpose of this thesis to determine if these assumptions are correct. It is anticipated that the studies performed below will assist in determining the

nature of the genetic lesion in mutant PS-28, and that this information will contribute to the understanding of the photosynthetic process.

The possible role of vitamin E and related quinones in the developing chloroplast will be analyzed. It is assumed that vitamin E functions as a photoprotective agent during the development of the chloroplast, and it is expected that information obtained from these developmental studies will assist in determining the importance of vitamin E to the chloroplast.

III. MATERIALS AND METHODS

Algal Culture

Scenedesmus obliquus strain D_3 and the mutant strains derived from it were cultured heterotrophically on nitrate medium (Kessler, Arthur, and Brugger, 1957) supplemented with 0.5% glucose and 0.25% yeast extract. The cells were maintained in the dark on 250 ml of media in 500 ml screw cap Erlenmeyer flasks while agitated on a rotary shaker at 28 C. Autotrophic cultures were grown on nonsupplemented nitrate medium in bubble tubes (Senger, 1970), into which a mixture of air-4% CO_2 was introduced from the bottom. Illumination was provided by a fluorescent light bank equipped with soft white and grow-lux elements, and the light intensity was approximately 1.0×10^4 ergs/sec-cm^2. Mixotrophic cultures were grown under conditions identical to autotrophic growth, but in this case the nitrate medium was enriched with glucose and yeast extract. Various inhibitors and other substances were added to the mixotrophic cultures as noted in the appropriate places.

Two-day old cultures were used routinely in all experiments. These cultures were approaching the end of logarithmic growth, and possessed maximal photosynthetic activity (Berzborn and Bishop, 1973).

Greening studies were performed as described by Senger and Bishop (1972a) except that in these studies 2-day old instead of 5-day old cultures were used. Light intensities and inhibitor concentrations are noted where necessary.

The packed cell volume (PCV) of the algal cultures was determined by centrifuging an aliquot of the sample in a cytocrit centrifuge tube (SGA Scientific Inc.) using a Sorvall table top centrifuge (model GLC-1) for 5 min at 300 x g.

Mutant Isolation

The photosynthetic mutants used in this study were induced by x-ray irradiation according to the techniques described by Bishop (1971b). The mutants were identified and isolated by Professor Bishop using the fluorescence methods described by Bennoun and Levine (1967).

Chlorophyll Determinations

Whole cells of Scenedesmus were extracted repeatedly with warm methanol until all of the pigments had been removed. Cellular debris was eliminated from the sample by centrifugation for 5 min at 300 x g. The chlorophyll concentration of chloroplast preparations was determined by diluting aliquots of each sample in methanol and centrifuging out the precipitate. Chlorophyll concentrations were

determined spectrophotometrically using a Zeiss PMQ 2 spectrophotometer. The optical densities were converted to chlorophyll concentrations (mg/l) using the equations developed by Holden (1965, p. 466):

$$\text{chlorophyll (a + b)} = 25.5A_{650} + 4.0A_{665}$$

$$\text{chlorophyll a} = 16.5A_{665} - 8.3A_{650}$$

$$\text{chlorophyll } \underline{b} = 33.8A_{650} - 12.5A_{665}$$

where A_{650} and A_{665} are the absorbancies measured at the respective wavelengths.

The chlorophyll concentrations in mg/l were converted to chlorophyll concentrations in μmoles with the following equation:

$$\mu\text{moles total chlorophyll} = \text{chlorophyll (mg/l)} \times 0.0011$$

where the conversion coefficient was based on an average molecular weight of chlorophyll (a + b) of 906. Appropriate dilution factors were applied where necessary.

High Intensity Irradiations

Cells (600 μl PCV) to be irradiated were suspended in 300 ml of 0.05 M KH_2-K_2HPO_4 buffer, pH 6.5, and were collected in a Kolle culture vessel. Irradiations were performed in a constant temperature bath maintained at 25 C. Illumination was provided by a 1000 W Sylvania tungsten-halogen lamp (DNX) which was focused

through a series of lenses to provide a nearly uniform field of light (1.0×10^6 ergs/sec-cm^2) on the samples. A uniform cell suspension (2 µl per ml) was maintained by bubbling a slow stream of air-4% CO_2 through the sample during irradiation. The light intensities were regulated with copper screens, and were measured with a YSI model 65 radiometer.

Several antioxidants were added to cultures of PS-28 to determine if the mutation could be reversed or if photosynthetic decline during high intensity irradiation could be prevented. The synthetic antioxidant, N, N'-diphenyl-p-phenylenediamine (K and K manufacturers) was dissolved in a trace of acetone and was sterilized by Millipore filtration (0.47 µm Solvinert filter). Sterile cultures (150 µl PCV uniform inoculum of wild-type Scenedesmus and mutant PS-28) were made 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} M with N, N'-diphenyl-p-phenylenediamine and were grown mixotrophically for 2 days. The culture made 10^{-6} M with respect to the antioxidant represented the highest concentration in the dilution series which did not have adverse affects upon the developing cells and was used for photosynthesis measurements and high intensity irradiation experiments.

Nordihydroguaiaretic acid (Aldrich Chemicals), a second synthetic antioxidant, was dissolved in weak base and was sterilized as above. A dilution series was prepared exactly as above and the cultures were grown mixotrophically for 2 days. The culture made

10^{-7} M with nordihydroguaiaretic acid was used for photosynthesis measurements and high intensity irradiation experiments.

α-Tocopherol and α-tocopheryl acetate (Nutritional Biochemical Corporation) were complexed with bovine serum albumin by the method of Nason and Lehman (1956). Twenty ml of 0.2% w/v bovine serum albumin in 0.1 M KH_2-K_2 HPO_4 buffer, pH 7.5, were sterilized by Millipore filtration (0.47 μm), and the filtrate was combined in a sterile 50 ml Erlenmeyer flask with 125 mg of α-tocopherol or α-tocopheryl acetate dissolved in 5 ml of absolute ethanol. Aliquots (2 ml, 1 ml, 0.1 ml) of the bovine serum albumin-vitamin E (or its acetate analog) suspension were pipetted into 200 ml of NGY medium which was uniformly inoculated with either wild-type Scenedesmus or mutant PS-28 (150 μl PCV of cells). The cultures were grown mixotrophically for 2 days in the dark and were then assayed for normal photosynthesis and photosynthesis under high light intensity conditions.

Ultraviolet Irradiations

Ultraviolet irradiations were performed with the thermal jacketed 15 W germicidal lamp (Westinghouse) described by Mantai (1968). Cells for irradiation (500 μl PCV) were suspended in 25 ml of 0.05 M KH_2-K_2HPO_4 buffer, pH 7.5. Samples were assayed for photosynthesis at 3 minute intervals until all activity had ceased.

To insure uniform irradiation the device was continuously shaken and was cooled with running tap water (18 C) to avoid heat build up. The total light intensity emitted from the lamp was estimated to be 3.8×10^4 ergs/sec-cm^2 and 80% of the lamp energy was delivered at 254 nm (Mantai and Bishop, 1967).

Oxygen and Hydrogen Evolution

Photosynthesis and respiration measurements were made with a Clark Ag-AgCl electrode in conjunction with a Gilson model KM oxygraph (Gilson Medical Electronics). The oxygraph was fitted with a projection lamp providing a light intensity of 2.5×10^5 ergs/sec-cm^2, with a spectral distribution composed of wavelengths greater than 580 nm. For analyses the samples (20 µl PCV) were suspended in 2.0 ml of 0.05M KH_2-K_2HPO_4 buffer, pH 6.5, and were tested an average of three light-dark cycles.

Cells (20 µl PCV) to be heat treated were suspended in 2.0 ml of 0.05 M KH_2-K_2HPO_4 buffer, pH 7.5 and were collected in 12 ml conical centrifuge tubes. The samples were heated in a hot water bath (45 C) under gentle agitation. Photosynthesis was measured as described above at given time intervals.

Hydrogen photoproductions were measured with the apparatus described by Jones and Bishop (1976). The instrument consists of a Clark Ag-AgCl electrode adapted for measuring hydrogen. The

circuitry for the hydrogen electrode was described by Wang, Healy, and Myers (1971). Cells (100 µl PCV) were suspended in 10 ml of 0.05 M Sorenson's buffer, pH 6.5, and were gassed under a continuous stream of argon in 50 ml Erlenmeyer flasks. The temperature (25 C) was held constant by immersing the flasks in a temperature bath of a Gilson respiromentor. After the cells had adapted to anaerobiosis for 4 hours, 0.8 ml of the suspension was transferred to the argon flushed sample chamber. The samples were illuminated with white light (3.0×10^3 ergs/sec-cm^2) that had been filtered through a 4 cm solution of dilute copper sulfate.

Photoreduction

Photoreduction was measured at 25 C using a Gilson differential respirometer (Bishop, 1972). Cells (50 µl PCV) were collected by centrifugation and were resuspended in 2.5 ml of 0.05 M Sorenson's buffer, pH 6.5. The cell suspensions were placed in reaction vessels and 0.5 ml of 5×10^{-5} M DCMU was placed in the sidearm of each flask. The samples were adapted overnight in a gas phase of H_2-4% CO_2 in the dark. The DCMU in the side-arm was then added to the cell suspensions and the rates of photoreduction were measured by CO_2 consumption (µl) as the difference between light minus dark samples. The 1000 W Sylvania lamp described above served as the

light source, and the light intensities were varied with copper screens.

Anaerobic Glucose Assimilation

The anaerobic photoassimilation of glucose was measured by the technique reported by Pratt and Bishop (1968a) with the following modifications. Cells (100 µl PCV) were suspended in 9 ml of 0.05 M KH_2-K_2HPO_4 buffer, pH 6.5. The samples were contained in 50 ml Erlenmeyer flasks which were supported in the temperature bath of a Gilson Respirometer (25 C). After the reaction vessels were gassed with argon for 10 min, 1.0 ml of 0.031 M glucose prepared in the above buffer was added to the samples. During the experiment the cells were continuously gassed with argon, under gentle agitation, in a light field of 4.0×10^4 ergs/sec-cm^2. The reactions were stopped at 15 minute intervals by centrifuging down the cells. An aliquot (25 µl) of the supernatant was assayed for glucose content by the Glucose Fast-Pack method (Calbiochem). The amount of photoassimilated glucose was then calculated by taking the difference between the light sample and the appropriate dark control.

Fluorescence

Fluorescence measurements were made with the instrumentation described by Senger and Bishop (1972b). The actinic light source

was a projector lamp maintained at 536 nm with a Bausch and Lomb high intensity monochromator. The light intensity of the actinic beam was about 1.0×10^3 ergs/sec-cm^2 at the sample holder. Variable yield fluorescence was induced with 650 nm and 712 nm wavelengths, each with an incident intensity of 2.0×10^2 ergs/sec-cm^2. The fluorescence of hydrogen adapted cells was measured under a gas phase of H_2-4% CO_2 in a Thunberg cuvette. In these systems stray light contributed less than 1% of the total signal intensity.

518 nm Absorbancy Change

The 518 nm absorbancy change was measured with an Aminco-Chance DW-2 spectrophotometer according to the procedures described by Pratt and Bishop (1968b). The samples (10 µl PCV) were suspended in 3 ml of 0.05 M KH_2-K_2HPO_4 buffer, pH 6.5, and were placed in a fluorescence cuvette (four transparent walls). The spectrophotometer was operated in the dual mode, with 518 nm as the actinic wavelength (540 nm isosbestic). Actinic light was provided by a high pressure Hg arc lamp (150 W), filtered through a combination of a Corning infrared filter (no. 1-69), and a Schott interference filter (663 nm, 11 nm half band width). The light intensity of the actinic beam was about 2.5×10^2 ergs/sec-cm^2. The photomultiplier was protected from stray light by a Balzer K-4 broad band pass

interference filter in conjunction with a Corning green (4-64) filter.

Split Beam Spectral Analyses

Low temperature absorbance spectra (77 K) were obtained with an Aminco-Chance DW-2 spectrophotometer adapted for these studies with the manufacturer's low temperature Dewar and lucite sample chamber. The samples (5 µl PCV) were taken up in 1.0 ml of a solution of 60% glycerol and 20 mM KH_2-K_2HPO_4 buffer, pH 6.5. This solution without cells served as a blank. The baseline was set using 730 nm as a reference wavelength.

Methanol absorption analyses of the various algal strains were performed using a Beckman DK2-A spectrophotomenter. The warm methanol extracts were prepared as above and after appropriate dilutions corresponded to 12 µlPCV per sample in 3 ml.

Chloroplast Isolation and Reactions

Algal chloroplast fragments were prepared according to the method of Berzborn and Bishop (1973). The only modification of this procedure was the substitution of 1.5 mm glass beads for 0.35 mm glass beads. All chloroplast reactions were performed in the standard STK buffer (20 mM tricine-KOH, pH 7.5; 30 mM KCL; 0.4 M sucrose; and 1% w/v bovine serum albumin).

Water to methylviologen and DCPIP-ascorbate to methylviologen photoreductions were performed according to the methodology of Harvey (1974). The water to methylviologen assay of photosynthesis was originally described by Kok, Rurainski, and Owens (1965). Oxygen uptake was measured polarigraphically with the Clark Ag-AgCl electrode as described above.

The ferricyanide Hill reactions were performed according to the procedures of Cheniae and Martin (1968). The reaction mixture contained chlorophyll, 100 μg; ferricyanide, 150 μmoles; and STK buffer to equal 2.0 ml total. The reactions (oxygen evolution) were measured polarigraphically as described above.

In vitro photophosphorylation experiments were performed according to the procedures described by Pratt and Bishop (1968a). Assays were performed in 25 ml Erlenmeyer flasks suspended in the temperature bath of a Gilson respiromenter (25 C). The reaction mixture contained 45 μmoles of Tris-HCl (pH 7.8), 12 μmoles $MgCl_2$, 60 μmoles NaCl, 12 μmoles of potassium phosphate buffer (pH 7.8), 1.2 μmoles of sucrose, 12 μmoles of ADP, 0.09 μmoles of phenazine methosulfate, about 1 μC ^{32}P, 150 μg of chlorophyll, and H_2O to equal 2.0 ml. The reactions were intiated with white light (2.5×10^5 ergs/sec-cm^2), and were terminated at 4 minute intervals with 0.3 ml of 20% trichloroacetic acid. Esterified (^{32}P)-ATP was assayed by the procedure of Avron (1960). Aliquots of the aqueous

phase (inorganic phosphate was removed by partitioning into isobutanol-benzene) were spotted on Whatman 3 MM filter paper discs (2.5 cm), and were placed in counting vials with 5.0 ml of scintillator fluid, 0.4% Omnifluor (New England Nuclear) in toluene. The samples were counted with a Packard Tricarb scintillation counter (model 2425), set to the ^{32}P window. The counting efficiency in these experiments was estimated to be about 49%. The count rates were converted to μmoles of ATP synthesized by the following equation:

$$\mu\text{moles of ATP formed} = (\text{corrected cpm})\ (2.3/1.0)\ (82.6/x)$$

where 1.0 was the dilution factor in preparation of the counting discs, 2.3 was the dilution factor correcting for total sample volume (after TCA addition), 1.0 was the volume of the reaction mixture assayed, 82.6 was the number of μmoles of inorganic phosphate added to the sample, and x was the added cpm of ^{32}P.

Vitamin C

Ascorbic acid (vitamin C) was determined by the procedure of Roe and Keuther (1943). Cells (2.0 ml PCV) were collected and were re-suspended in about 30 ml of 5% acetic acid. The cell suspensions were disrupted by passage through a French press twice at 24,000 p.s.i. The homogenized samples were brought to a 5%

concentration of trichloroacetic acid with an equal volume of a 10% solution of the acid. Cell debris was removed by centrifugation for 15 min at 5000 x g, and the supernatant was collected in 125 ml Erlenmeyer flasks. One gram of activated charcoal was added to each 10 ml of the acidified extract, and this suspension was shaken vigorously for 5 min. The oxidized extract was filtered through Whatman no. 1 filter paper, and the above treatment with activated charcoal was repeated 5 or 6 times. Activated charcoal is a mild oxidizing agent and its purpose in this procedure is to oxidize ascorbic acid to dehydro-ascorbic acid. A stronger oxidizing agent than activated charcoal introduces the possibility of interference from other cellular carbohydrates in the dinitrophenylhydrazine test. Four ml of the activated charcoal treated filtrate were combined with 1.0 ml of 2,4-dinitrophenylhydrazine (2% dinitrophenylhydrazine in 9N H_2SO_4) and one drop of a thiourea solution (10% thiourea in 50% aqueous ethanol). This solution was allowed to react at 37 C for 3 hr. After the reaction had gone to completion the samples were placed in an ice bath and 1.0 ml of 85% H_2SO_4 was added a drop at a time to each sample. The optical densities of the samples were measured at 540 nm with a Zeiss PMQ-2 spectrophotometer. The concentrations (μmoles) of ascorbic acid in each sample were determined by multiplying the optical densities obtained in the above assay by 0.155 and the appropriate dilution factors to account for

total sample volume. This coefficient was obtained from a standard curve prepared by oxidizing a standardized solution of vitamin C with bromine water and forming the diphenylhydrazone as described above.

Whole Cell Lipid Analyses

The whole cell lipid composition of the algal strains examined here was investigated by the procedures of Allen and Good (1971). Cells (2.0 ml PCV) were collected by centrifugation and resuspended in 30 ml of 1:1 $CHCl_3$-CH_3OH plus 0.01% w/v butylated hydroxytoluene (BHT). All chemicals used in these analyses were reagent grade, or were redistilled prior to use. The samples were extracted by sonication over ice with 3, 1 minute bursts (80 W) of the 0.5 inch probe (Bronwill Scientific). Cell debris was removed by centrifigation for 5 min at 300 x g and the pellets were extracted for 3 more times in the same manner as above. This procedure was repeated if necessary until the pellets were devoid of pigmentation. The combined extracts were evaporated to dryness at room temperature under reduced pressure with a Buchler flash evaporator. The samples were then dissolved in a minimum volume of 9:1 $CHCl_3$-CH_3OH. Aliquots of the samples were spotted on silicic acid thin layer plates, 20 x 20 cm, 0.025 cm thickness, (Silica gel G, Merck), approximately 1 inch from the bottom and 1 inch from the left-hand

side of the plate. The plates were developed by upward migration first in $CHCl_3$: CH_3OH: 7N NH_4OH (97.5: 37.5: 6.0), and afterwards at a right angle to the first direction in $CHCl_3$: CH_3OH: HOAC: H_2O (85: 12.5: 12.5: 2.0). After the plates were dried, the lipids were detected by exposing the plates to iodine vapor (Allen and Good, 1971). Identification of the individual lipid spots was made with Supelco lipid standards chromatographed as above.

Fatty Acid Analyses

Cells (4.0 µl PCV) were extracted with 1:1 $CHCl_3$-CH_3OH plus 0.01% w/v BHT, and dried as above. The samples were resuspended in a minimum volume of 9:1 $CHCl_3$-CH_3OH and streaked on 20 x 20 cm silicic acid (Silica Gel G, Merck) thin layer plates. The plates were developed vertically with $CHCl_3$-CH_3OH-7N NH_4OH (97.5: 37.5: 6). All of the silicic acid above the origin and below the pigmented solvent front was collected, and the lipids were eluted from the adsorbent with 1:1 $CHCl_3$-CH_3OH by filtration. The eluant was taken to dryness under reduced pressure, and then the fatty acids were transesterified in 5 ml of a methanolic boron trifluoride (Supelco), for 3 hours at room temperature. The reaction was terminated by the addition of 3.0 ml of water to the reaction mixture, and the fatty acid methyl esters were gathered by partitioning into redistilled pentane. The combined pentane extracts were

reduced to dryness, and the samples were then dissolved in precisely 1.0 ml of redistilled pentane.

The fatty acid methyl esters were analyzed by isothermal (180 C) gas chromatography. The gas chromatograph (Varian Aerograph 1200) was equipped with a 6 ft x 1/8 in I. D. column packed with 10% SP-2340 on 100/120 Supelcoport B-2412 (Supelco). The nitrogen carrier gas had a flow rate of 25 ml/min and the flame ionization detector was operated at 220 C. The fatty acid methyl esters were identified and quantitated by area comparisons to Supelco standards (RM-2, 4-7021).

Quantitative Analyses of Chloroplast Quinones

Plastoquinone A and vitamin E were analyzed by the procedures of Bishop and Wong (1974). Cells (2.0 ml PCV) were extracted in warm methanol as described above, and were dried under reduced pressure. The dried samples were dissolved in a minimum volume of redistilled $CHCl_3$ and were streaked on 20 x 20 cm silicic acid (Silicia Gel G, Merck) thin layer plates. The chromatograms were developed vertically with benzene-heptane 85:15 (reagent heptane, Eastman Organic Chemicals; reagent benzene, Mallinckrodt, activated charcoal and silicic acid filtered). The individual bands corresponding to plastoquinone A and α-tocopherol were located with 0.01% Rhodamine B in ethanol, by fluorescence quenching under

ultraviolet light. These bands were scraped from the plates and were eluted free of the adsorbent with redistilled $CHCl_3$. The eluant was dried under a vacuum and the compounds were dissolved in ethanol (3 ml) for quantitation.

The plastoquinone A concentrations were calculated from the following equation, based on an a_{mM} of 15:

$$\text{total } \mu\text{moles plastoquinone A} = (A_{255})\ (0.2005)\ (Vt/Va)$$

where A_{255} was the absorbancy of the reduced versus the oxidized absorbance of plastoquinone A at 255 nm, Vt was the total volume of the extract in $CHCl_3$, and Va was the volume of the extract assayed.

Vitamin E was quantitated by the method of Emmerie-Engel (Barr and Crane, 1971), adapted for spectrophotometry. The optical densities obtained for 1 ml of the ethanolic solution of vitamin E were converted to concentration (μmoles) by the following equation:

$$\text{total } \mu\text{moles } \alpha\text{-tocopherol} = (3)\ (0.35)\ (A_{520})$$

where 3 was the dilution factor correcting for the volume of the sample assayed, 0.35 was the conversion coefficient obtained from a standard curve made with authentic α-tocopherol (Nutritional Biochemical Corporation), and A_{520} was the absorbance observed for the sample.

α-Tocopheryl quinone was isolated by the procedures described by Barr and Crane (1971). Cells (2.0 ml PCV) were extracted with warm methanol as above, and the extracts were dried under reduced pressure. The dried samples were taken up in a minimal volume of redistilled petroleum ether and were adsorbed onto a column of acid alumina. The column was eluted sequentially with 100 ml each of 10% and 20% diethylether-petroleum ether. The final elution of the column, 200 ml of 30% diethylether-petroleum ether, contained the α-tocopheryl quinone. This fraction was reduced to 2 ml under vacuum and the sample was chromatographed on thin-layer silicic acid plates (Silica gel G, Merck) with petroleum ether-benzene-ethanol) (8:3:0.7). Quinones present in the chromatogram were detected with 0.01% Rhodamine B as before, and the band corresponding to α-tocopheryl quinone was eluted by filtration with chloroform. The eluant was taken to dryness under reduced pressure and the sample was dissolved in exactly 3 ml of ethanol. The concentrations of α-tocophyl quinone (μmoles) were quantitated with the following equation:

$$\text{total } \mu\text{moles } \alpha\text{-tocopheryl quinone} = (A_{262})\ (0.1658)\ (Vt/Va)$$

where A_{262} was the optical density of the sample in 3 ml (oxidized minus reduced), 0.1658 was a conversion coefficient based on an

extinction coefficient of 17.8 a_{mM}, V_t was the total sample volume, and V_a was the volume of the sample assayed.

IV. RESULTS AND DISCUSSION

General Characteristics, Photosynthesis, and Respiration

A mutant of the alga Scenedesmus obliquus, strain PS-28, was induced by x-ray irradiation, and was isolated with standard techniques (Bishop, 1971b). The mutant strain grows well on nitrate-glucose-yeast extract medium (Kessler, Arthur, and Brugger, 1957), either in the dark or in the light, but is incapable of autotrophic growth on nonsupplemented nitrate medium. These findings indicated that PS-28 could not grow under strictly photosynthetic conditions, therefore, the mutation had affected the photosynthetic apparatus.

Mutant PS-28 does not synthesize α-tocopherol (Bishop and Wong, 1974). To investigate the function of α-tocopherol, in its relation to photosynthesis, several secondary mutants were generated from PS-28 by further x-ray induction. These sub-mutants were identified by pigment deficiencies and were isolated by visual means. The subisolates were designated C-28-2, C-28-3, C-28-4, etc., and data obtained from certain of these are presented in subsequent sections. It was observed that for both mixotrophic and heterotrophic growth, the photosynthetic rate of the mutant was approximately half that of the wild-type (Table 1). As expected the photosynthetic activity of the wild-type improved when cultured

Table 1. Photosynthesis and respiration measurements of wild-type Scenedesmus and mutant strain PS-28.

Comparative rates of photosynthesis and respiration between wild-type Scenedesmus and mutant strain PS-28 are expressed as the average of 5 independent observations. Measurements were performed with a Clark Ag-AgCl electrode in conjunction with a model KM Gilson oxygraph. For experimental details see Materials and Methods.

	wild-type heterotrophic	wild-type mixotrophic	PS-28 heterotrophic	PS-28 mixotrophic
Photosynthesis*	44.6 ± 7.1	56.4 ± 8.0	24.7 ± 9.3	26.7 ± 9.4
Respiration**	21.2 ± 4.8	23.2 ± 2.5	25.2 ± 2.3	22.6 ± 2.2

* Rate of photosynthesis expressed as μmole O_2 evolved/hr-20 μl PCV, as determined at saturating intensities of red light (2.5 x 10^5 ergs/sec-cm^2).

** Rate of respiration expressed as μmoles of O_2 consumed/hr-20 μl PCV.

photohetrotrophically (cf., Harvey, 1974). In contrast to the above, the photosynthetic activity of the mutant was not enhanced upon transfer to mixotrophic growth conditions (Bishop and Wong, 1974). The respiratory rates of both hetertrophic and mixotrophic samples of the mutant and the wild-type were virtually the same upon comparison. It should be noted that there are significant differences between mixotrophic and heterotrophic cultures, and comparisons between samples cultured by the two methods are only relative.

High Light Intensity Experiments

The photosynthetic responses of the mutant and wild-type Scenedemus to high intensity irradiation were different (Figure 2). When 2-day old dark grown cultures of both the mutant and wild-type were exposed to high intensity illumination (10^6 ergs/sec-cm^2), the photosynthetic capacity of the wild-type increased and that of the mutant decreased. The low light intensities utilized for mixotrophic culturing (10^4 ergs/sec-cm^2) did not have a deleterious effect upon the mutant, but at higher light intensities photodynamic damage occurred. The conclusion that the loss of photosynthetic activity in mutant PS-28 during high intensity irradiation was of a photodynamic nature stems from two lines of evidence. Firstly, the response was intensity dependent (data not shown), and secondly, photosynthetic decline did not occur when the irradiations are performed in the absence of oxygen (Figure 3). These findings indicate that a photosensitized form of oxygen (either singlet or free-radical) is responsible for the deleterious effect of high intensity irradiation upon the oxygen evolving apparatus of mutant PS-28.

To date two types of light sensitive mutants of Scenedesmus have been described. The first type loses pigmentation (bleaches) rapidly upon exposure to even weak light (Williams, 1971), and the second type only bleaches after extended exposures to high intensity

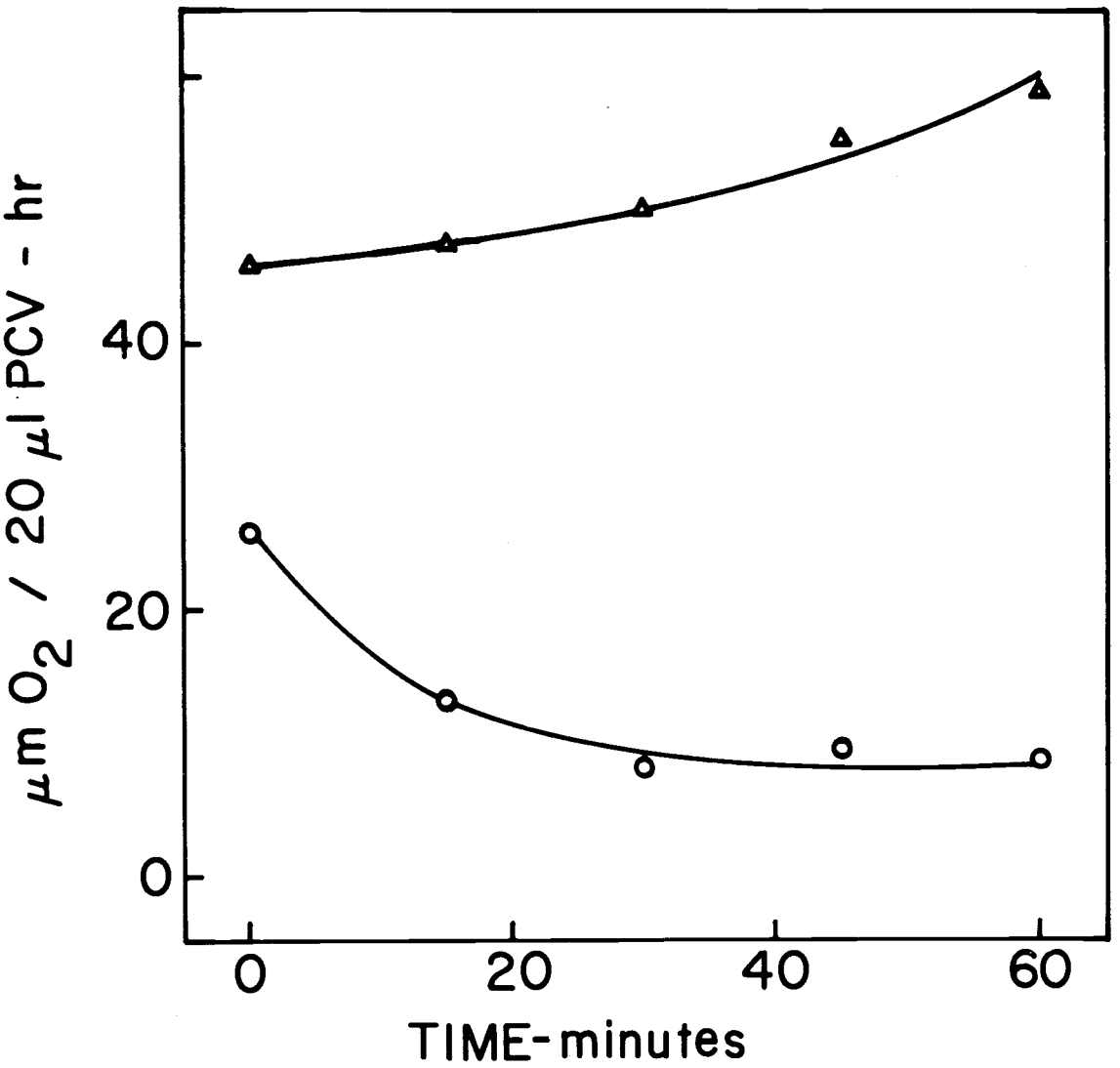


Figure 2. Photosynthetic capacity of heterotrophic wild-type Scenedesmus (△—△) and mutant PS-28 (O—O) during a time course exposure to high intensity white light (1.0×10^6 ergs/sec-cm^2). Data presented above are representative of three independent experiments. For experimental details see Materials and Methods.

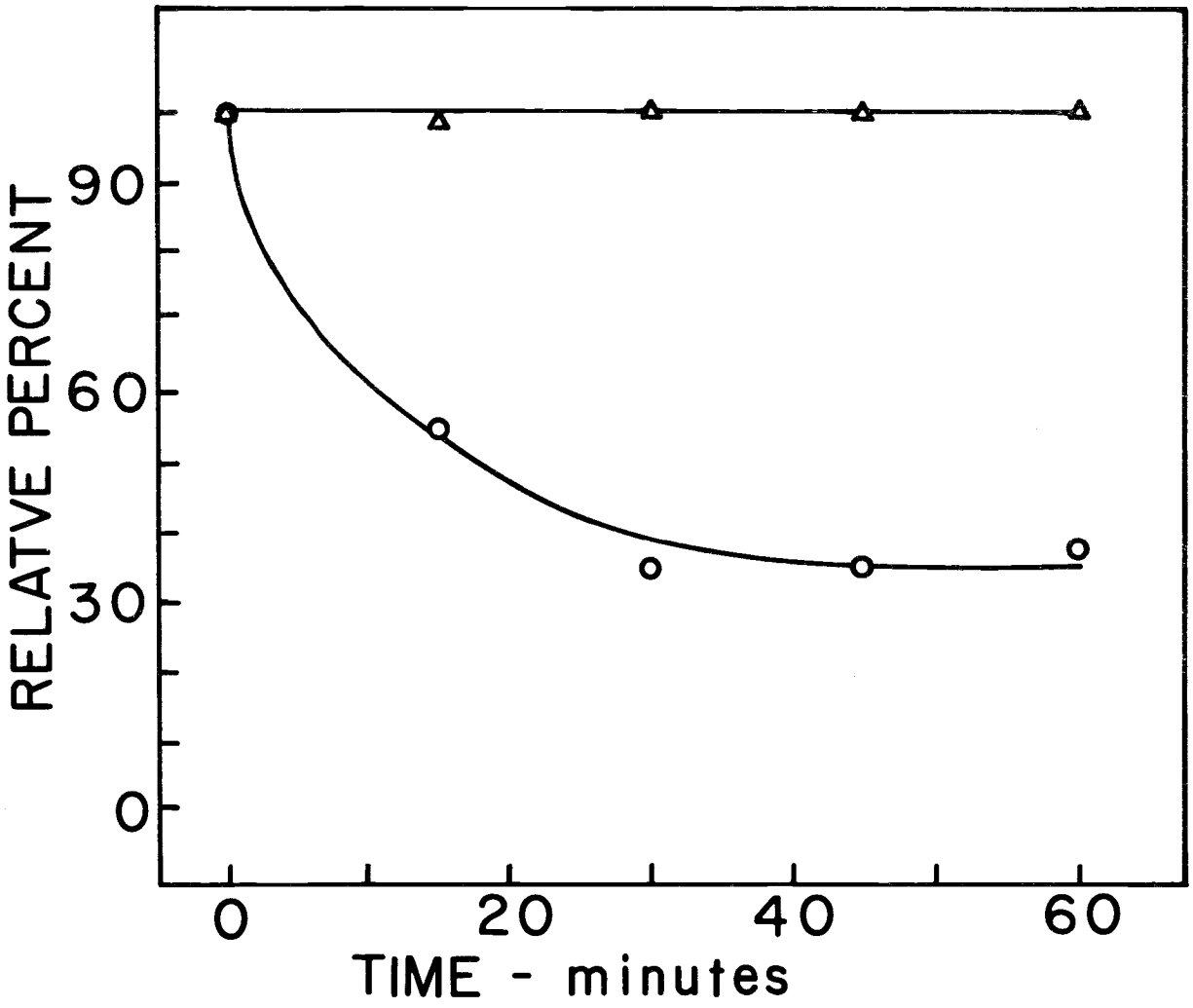


Figure 3. Photosynthetic capacity of heterotrophic mutant PS-28 during a time course exposure to high intensity irradiation (1.0×10^6 ergs/sec-cm^2) under air-4% CO_2 (O—O) or under nitrogen-4% CO_2 (Δ—Δ). For experimental details see Materials and Methods.

irradiation (Harvey, 1974). The chlorophyll concentration of mutant PS-28 remains constant during high intensity light exposures for several hours, therefore, the light sensitive nature of PS-28 resembles that of the latter mutants described above. Photodamage in light sensitive mutants which do not bleach rapidly occurs first in the electron transport chain of the photosystem that has been mutated (Harvey, 1974).

The results of experiments with two synthetic antioxidants, N, N'-diphenyl-p-phenylenediamine (DPPD), and nordihydroguaiaretic acid (NDGA), demonstrated that these two compounds could neither reverse the mutation nor stabilize it against photoinactivation (Figures 4 and 5). These two compounds were used successfully to reverse certain of the effects of a vitamin E deficiency in animal systems (Krisnamurthy and Bieri, 1962; Scott, and Stoewsand, 1961). Additionally, several other substances were added to the growth medium, including α-tocopherol and α-tocopheryl acetate without detectable benefit (Bishop and Wong, 1974). In the latter experiments it was not certain that vitamin E or its acetate derivative had penetrated the cell.

Photoreduction

The photochemical reaction performed by certain species of anaerobically adapted algae, whereby CO_2 is reduced to carbohydrate

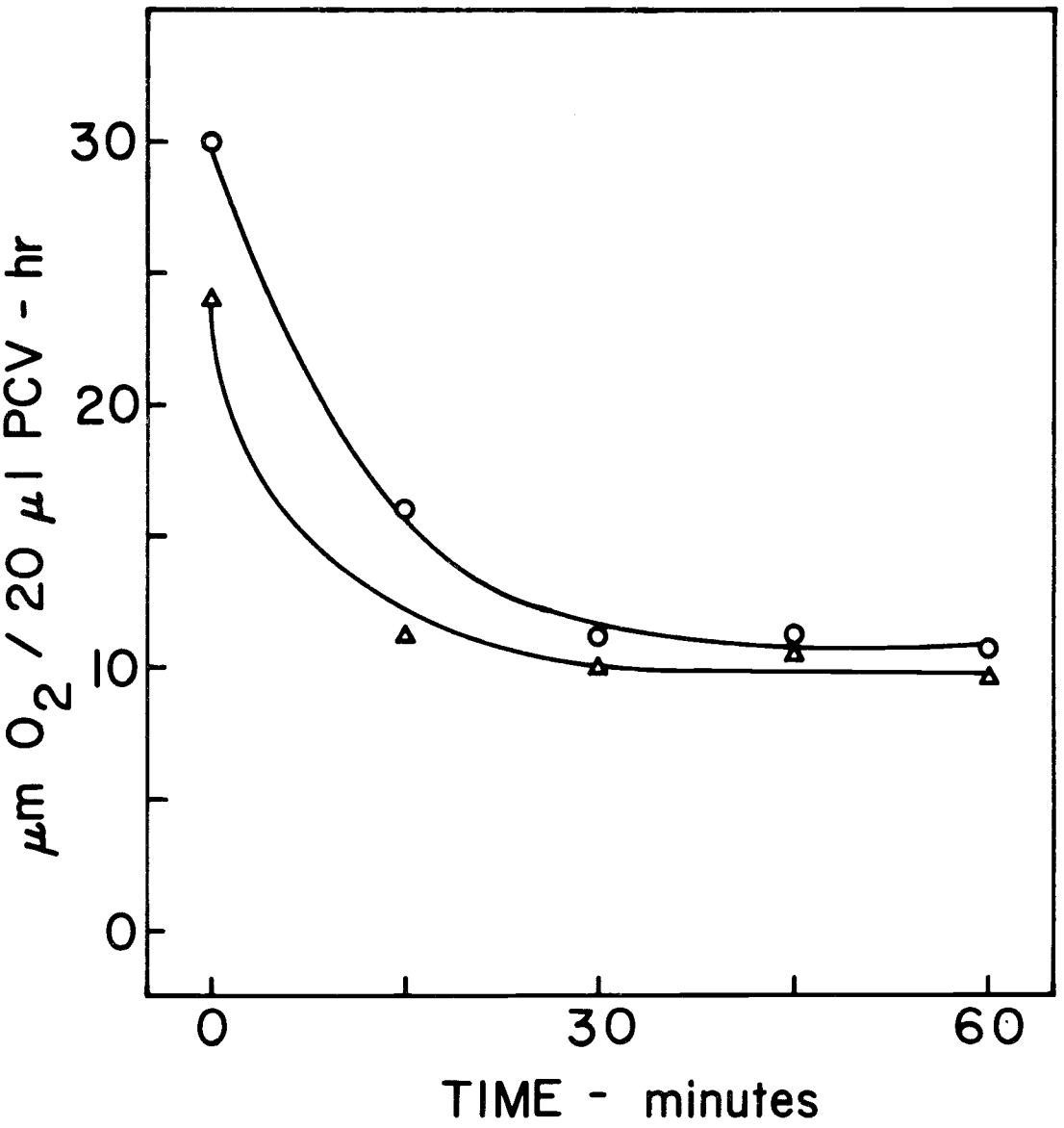


Figure 4. Photosynthetic capacity of mixotrophic mutant PS-28 (O—O) during a time course exposure to high intensity irradiation (1.0×10^6 ergs/sec-cm^2) cultured in the presence of 10^{-5} M nordihydroguaiaretic acid (Δ—Δ). For experimental details see Materials and Methods.

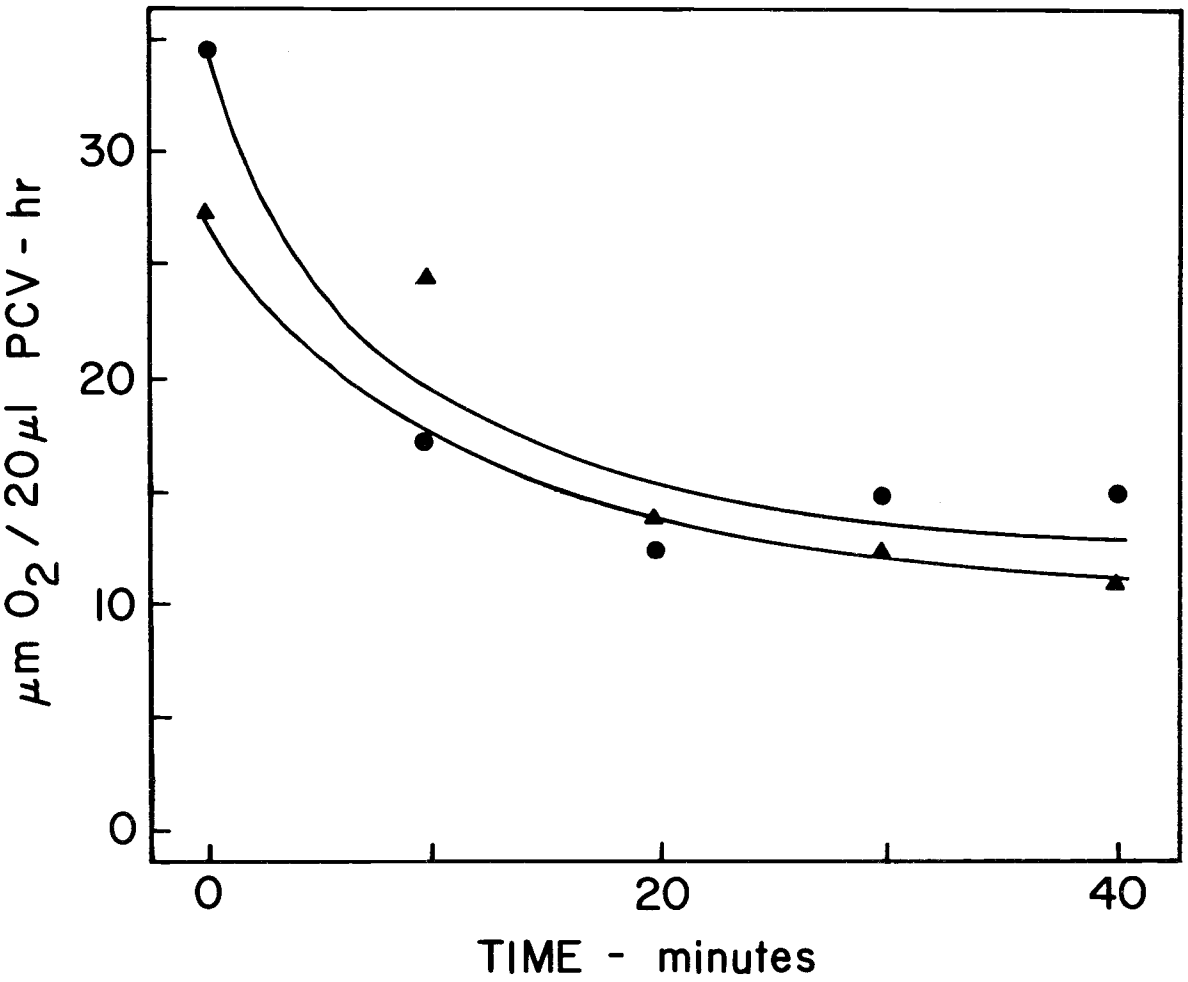


Figure 5. Photosynthetic capacity of mixotrophic mutant PS-28 (O—O) during a time course exposure to high intensity irradiation (1.0×10^6 ergs/sec-cm^2) cultured in the presence of N,N'-diphenyl-p-phenylenediamine (▲—▲). For experimental details see Materials and Methods.

by molecular hydrogen is known as photoreduction (Gaffron, 1940). The reaction is catalyzed by the enzyme hydrogenase, which is completely inhibited by even a trace of oxygen. The comparative ability of the mutant grown heterotrophically to perform this reaction is equal to that of the wild-type (Figure 6). Based upon the hypothesis that photoreduction is strictly a photosystem-I driven reaction (Bishop and Gaffron, 1962, Bishop, 1966) it was concluded that mutant PS-28 has an impaired photosystem-II (because of the low photosynthetic rates), and an intact photosystem-I. These conclusions are supported by data on photoreduction that were obtained with poisoned and non-poisoned cells (Figure 8). The herbicide DCMU is known to be a potent inhibitor of oxygen evolution (Bishop, 1958). It was observed that wild-type Scenedesmus when not treated with DCMU rapidly inhibited the hydrogenase reaction via the production of photosynthetic oxygen. Mutant PS-28 was incapable of evolving enough photosynthetic oxygen to inhibit the hydrogenase reaction, so that the sample that had not been treated with DCMU behaved identically to the wild-type and PS-28 samples that were treated with DCMU (Figure 8).

Mutant C-2A' followed a typical light saturation response of photoreduction (Bishop and Senger, 1972a), whereas the light intensity curve for mutant C-28-21 exhibited nonsaturating kinetics (Figure 7). This was an indication that the capacity of the mutant

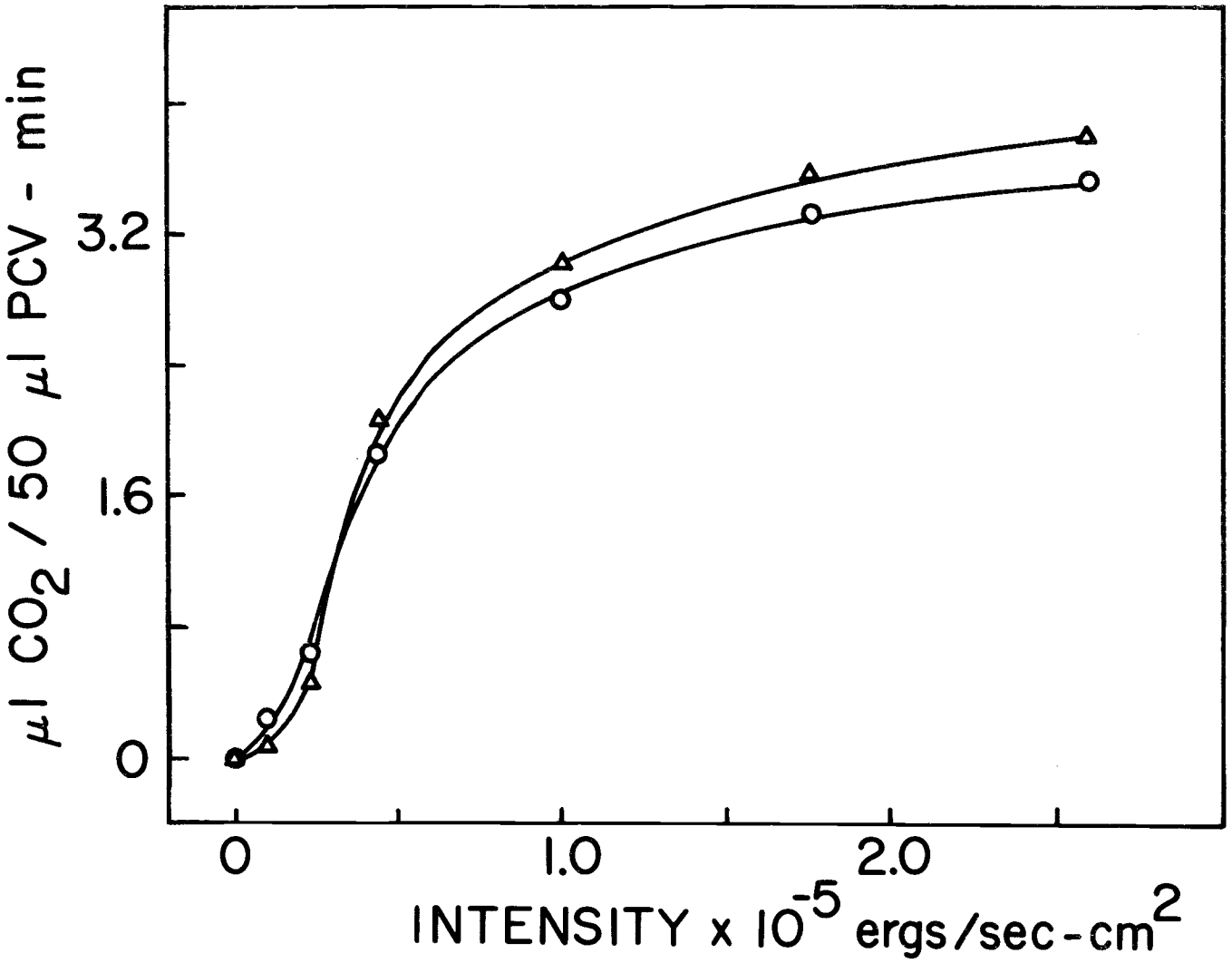


Figure 6. Light intensity responses of photoreduction for heterotrophic wild-type Scenedesmus (Δ—Δ) and mutant strain PS-28 (O—O). For experimental details see Materials and Methods.

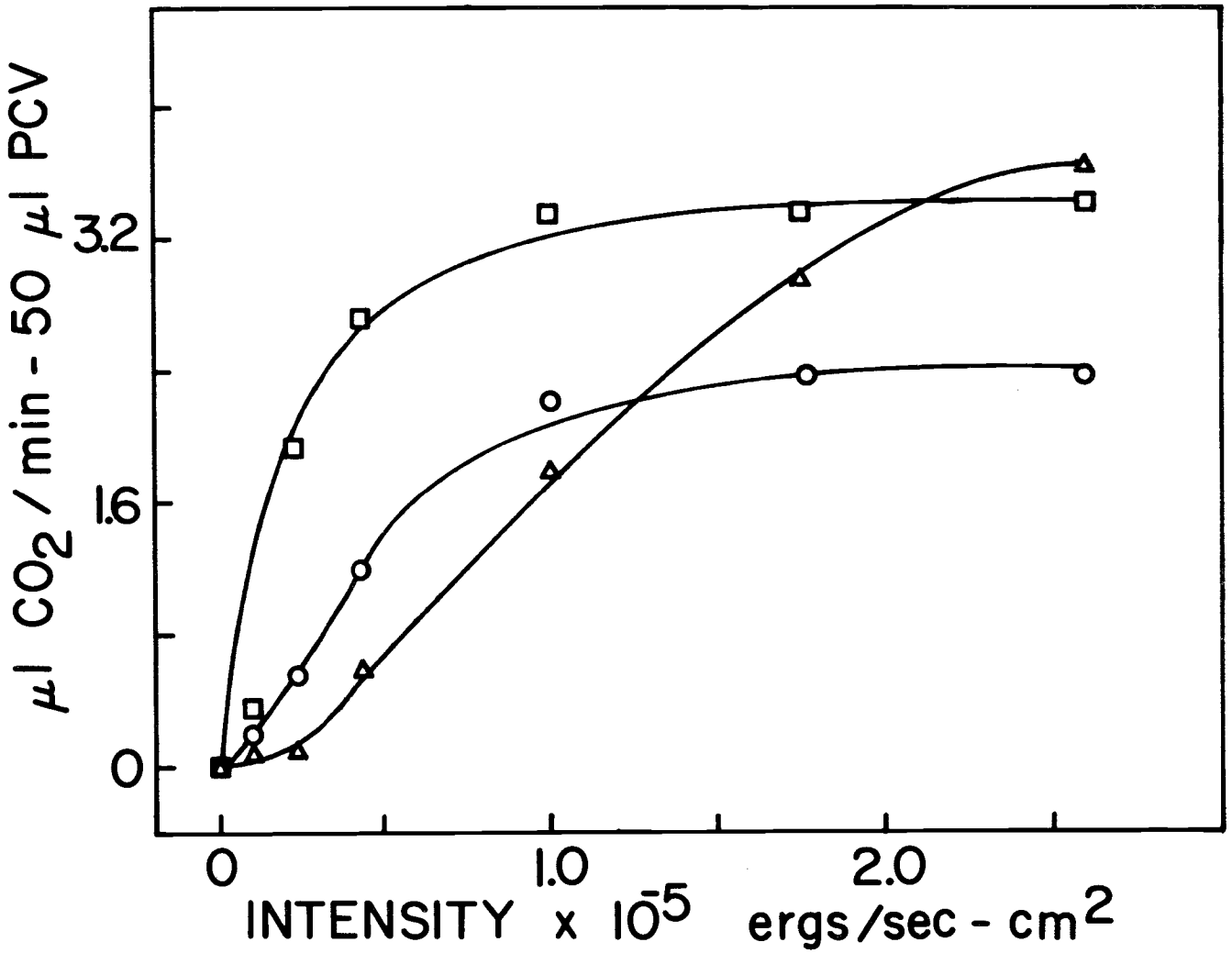


Figure 7. Light intensity responses of photoreduction for mixotrophic Scenedesmus mutants C-2A' (■—■), C-28-21 (Δ—Δ), and C-28-18 (O—O). For experimental details see Materials and Methods.

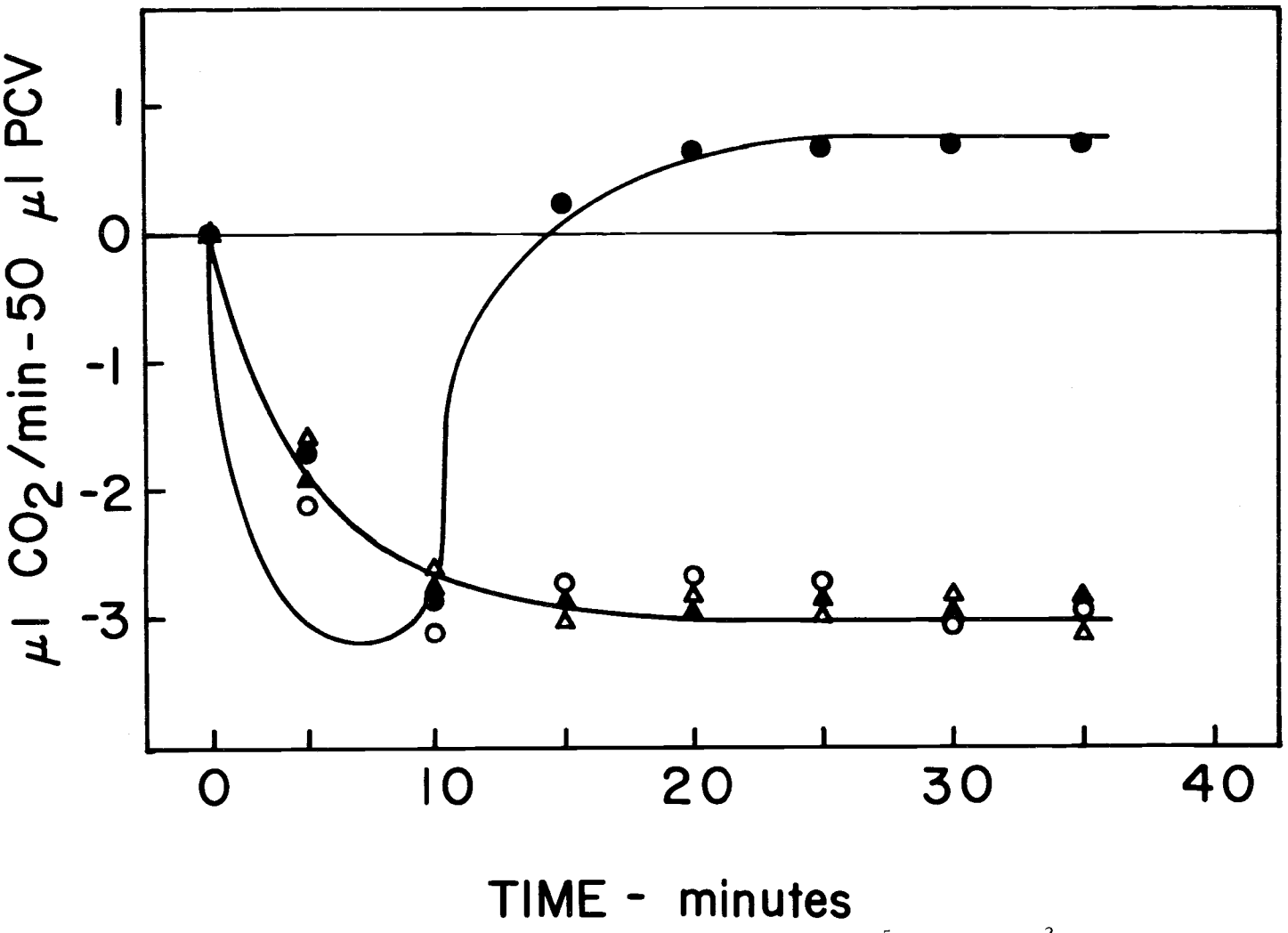


Figure 8. Time course of photoreduction in white light (2.5×10^5 ergs/sec-cm^2) for wild-type Scenedesmus with DCMU (O—O), without DCMU (●—●), and for mutant strain PS-28 with DCMU (△—△), without DCMU (▲—▲). For experimental details see Materials and Methods.

for light absorption was impaired (Williams, 1971). The light saturation curve of photoreduction for mutant C-28-18 indicated that the mutant was partially blocked in photosystem-I (Figure 7). When the saturated rates of photoreduction were corrected on a chlorophyll basis (wild-type, 13.3 μl CO_2/min-μmole Chl.; C-28-18, 44.4 μl CO_2/min-μmole Chl.), the overall efficiency of mutant C-28-18 exceeded that of even the wild-type. Similar results were observed by Williams (1971) for Scenedesmus mutant C-6E.

It was of interest to determine whether or not mutant PS-28 lost photosystem-I during high intensity irradiation. After 1 hour of irradiation the photosynthetic ability of the mutant had been reduced to 30% of the untreated control, but the ability for hydrogen photoreduction remained equal to that of the control. The photosynthetic rate of the irradiated sample that had been incubated under hydrogen-4% CO_2 for 4 hours remained at 30% of the control, indicating that dark repair had not occurred during anaerobiosis. These results suggested that photosystem-I in mutant PS-28 was not damaged by high intensity irradiation. Similar results were reported elsewhere (Bishop and Wong, 1974).

Heat Treatment and Ultraviolet Irradiation

Although it is clear that the effects of ultraviolet irradiation result in the destruction of plastoquinone, it is less certain that the

loss of plastoquinone is responsible for photosynthetic decline (Mantai, Wong, and Bishop, 1970). Lichtenthaler and Tevini (1969) demonstrated that ultraviolet irradiation also resulted in the rapid reduction of α-tocopherol and neoxanthin. It is currently believed that the effect of ultraviolet light on photosystem-II is general rather than site specific (Jones and Kok, 1966a, 1966bα Yamashita and Butler, 1968).

It was observed that the time necessary for photosynthesis to be destroyed by ultraviolet irradiation was about 20 minutes for both the mutant and the wild-type (Figure 9). These results indicated that the inactivation of water photolysis by ultraviolet irradiation was not influenced by the presence or absence of α-tocopherol.

Similar results were obtained in experiments on the heat treatment of whole cells (Figure 10). The rate of photosynthetic decline during the thermal inactivation of wild-type Scenedesmus and PS-28 was virtually equivalent. This result was unexpected because it was assumed that the absence of vitamin E from the photosynthetic lamellae would perhaps make them less stable to thermal inactivation. Whether or not photosystem-II is the site of thermal inactivation in these experiments remains to be determined; however, in chloroplast particles of Scenedesmus it is well established that photosystem-II is inhibited by brief exposures to 35 C temperatures (Stuart, 1971).

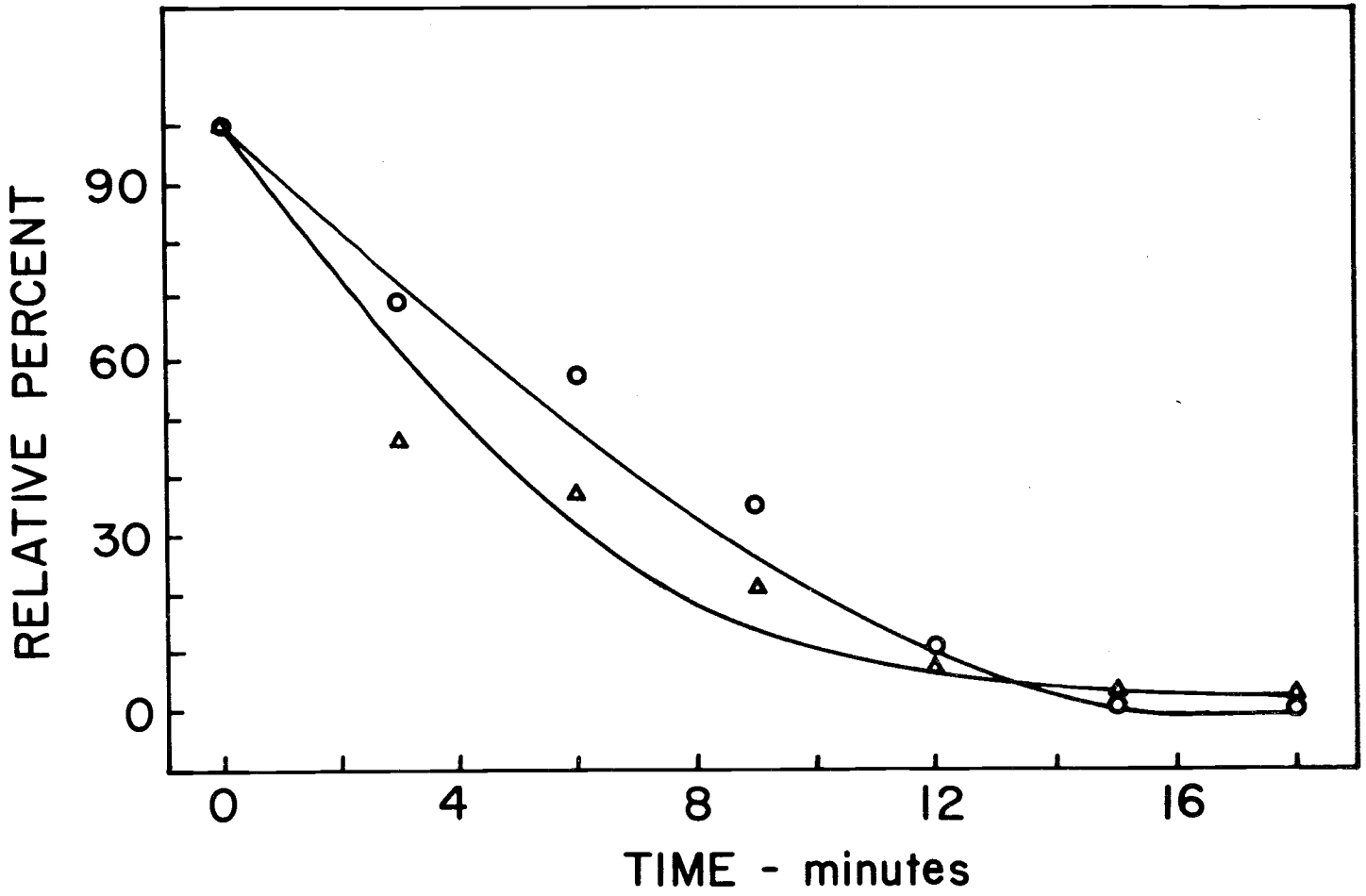


Figure 9. Photosynthesis of heterotrophic wild-type Scenedesmus (Δ—Δ) and mutant PS-28 (O—O) during a time course exposure to ultraviolet irradiation. For experimental details see Materials and Methods.

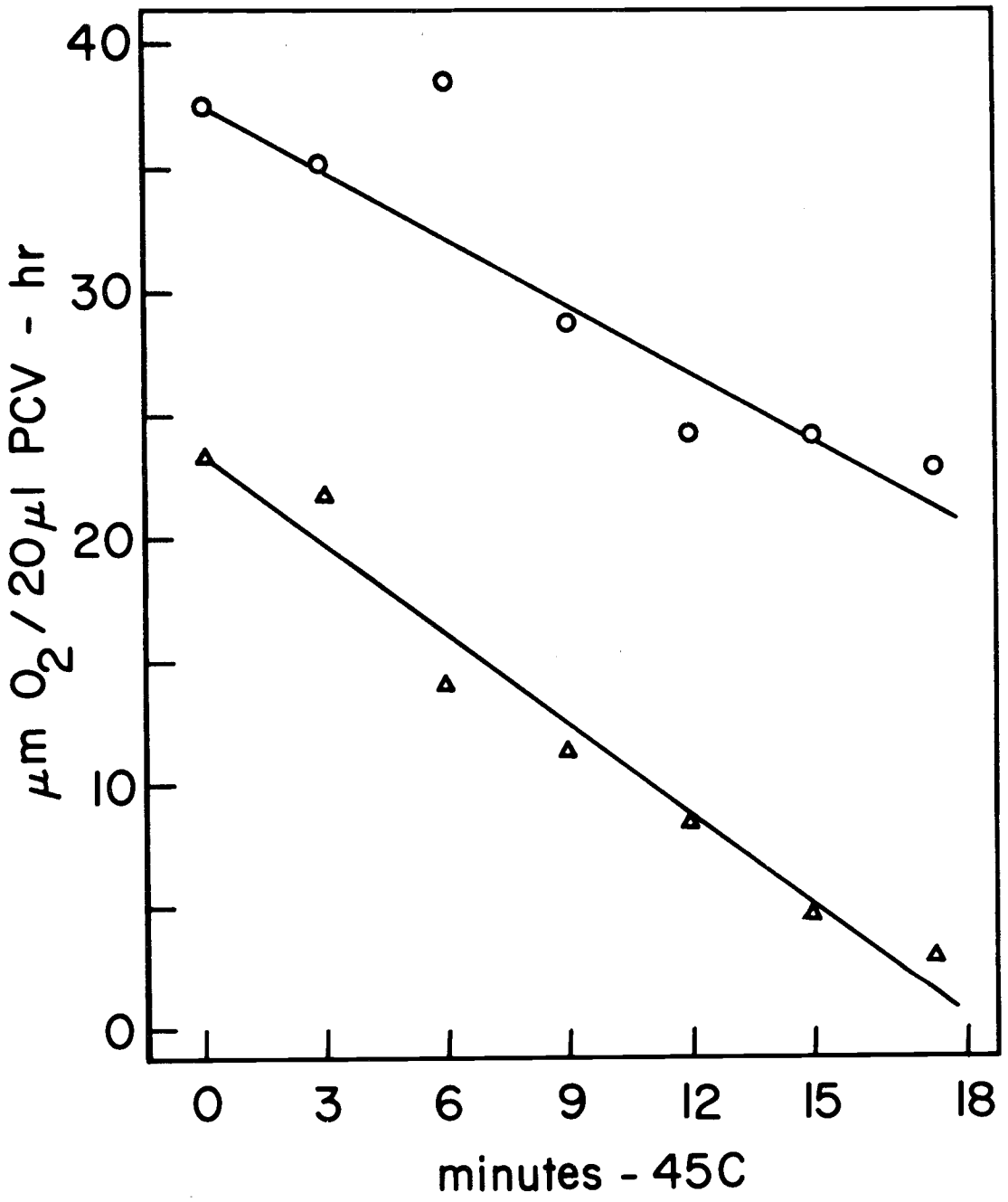


Figure 10. Photosynthesis of heterotrophic wild-type Scenedesmus (O—O) and mutant PS-28 (Δ—Δ) during a time course exposure to elevated temperatures (45 C). For experimental details see Materials and Methods.

Hydrogen Photoproduction

A second light-driven reaction that is common to several species of anaerobically adapted algae is the evolution of hydrogen (Gaffron and Rubin, 1942). Generally after exposure of the cells to long periods of low oxygen tension hydrogen is evolved slowly in the dark and quite rapidly in the light. Spruit (1958) suggested that the hydrogen photoproduced by Chlorella was derived from the photolysis of water. Later work by Kaltwasser, Stuart, and Gaffron (1969), and Stuart and Kaltwasser (1970) suggested that hydrogen photoproduction was a strict photosystem-I response, and that electron flow originated from an organic carbon pool, not from water. However, Stuart and Gaffron (1971; 1972) after observing the drastic inhibition of hydrogen photoproduction by DCMU amended their prior hypothesis to include at least a partial contribution of photosystem-II to the overall process. Basically, the photoevolution of hydrogen is dependent upon respiratory carbon, photosystem-I and photosystem-II, and the presence of the enzyme hydrogenase.

Hydrogen photoevolution by mutant PS-28 was greatly impaired in comparison to the wild-type (Figure 11). This observation coupled with the results obtained from experiments on hydrogen photoreduction suggested that mutant PS-28 had a functional photosystem-I, and a functional hydrogenase, but was impaired in photosystem-II. This

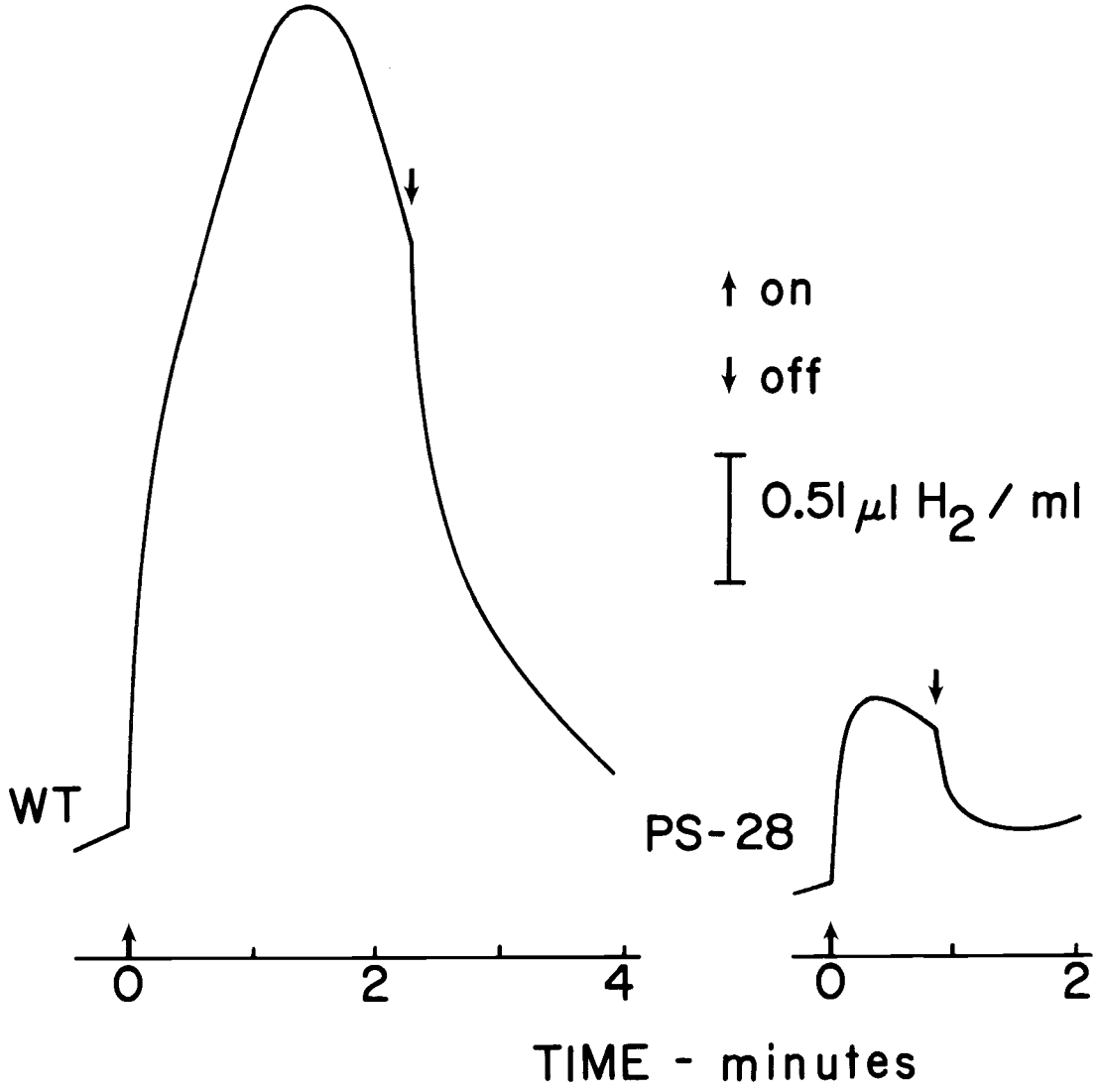


Figure 11. Hydrogen photoproduction by wild-type Scenedesmus and mutant strain PS-28. For experimental details see Materials and Methods.

conclusion is consistent with the evidence presented by Bishop, Frick, and Jones (1975), who demonstrated that both photosystems are required for maximal photoproduction in Scenedesmus.

Anaerobic Glucose Photoassimilation

The anaerobic photoassimilation of glucose is thought to be a process which permits the in vivo measurement of photosystem-I driven cyclic photophosphorylation (Tanner, Loos, and Kandler, 1966). Theoretically, the assimilation of one mole of glucose into a starch polymer (Pratt and Bishop, 1968a) corresponds to the utilization of three moles of ATP, and it is believed that no reducing power is consumed by the reaction (Taylor, 1960). Senger (1970) reported that DCMU did not inhibit anaerobic glucose photoassimilation in unicellular green algae, but there is ample evidence to the contrary (Tanner, Daschel, and Kandler, 1965).

The rate of anaerobic glucose uptake by mutant PS-28 is one-third that of the wild-type (Figure 12), and the rates reported here are comparable to those obtained by Pratt and Bishop (1968a). Interestingly, the relative loss of glucose photoassimilation caused by the mutation in PS-28 is very similar to the relative decline in photosynthesis (see above). Wild-type cells exposed to high intensity irradiation have an increased rate of glucose uptake (about 25%), whereas the process is virtually eliminated in irradiated cells of

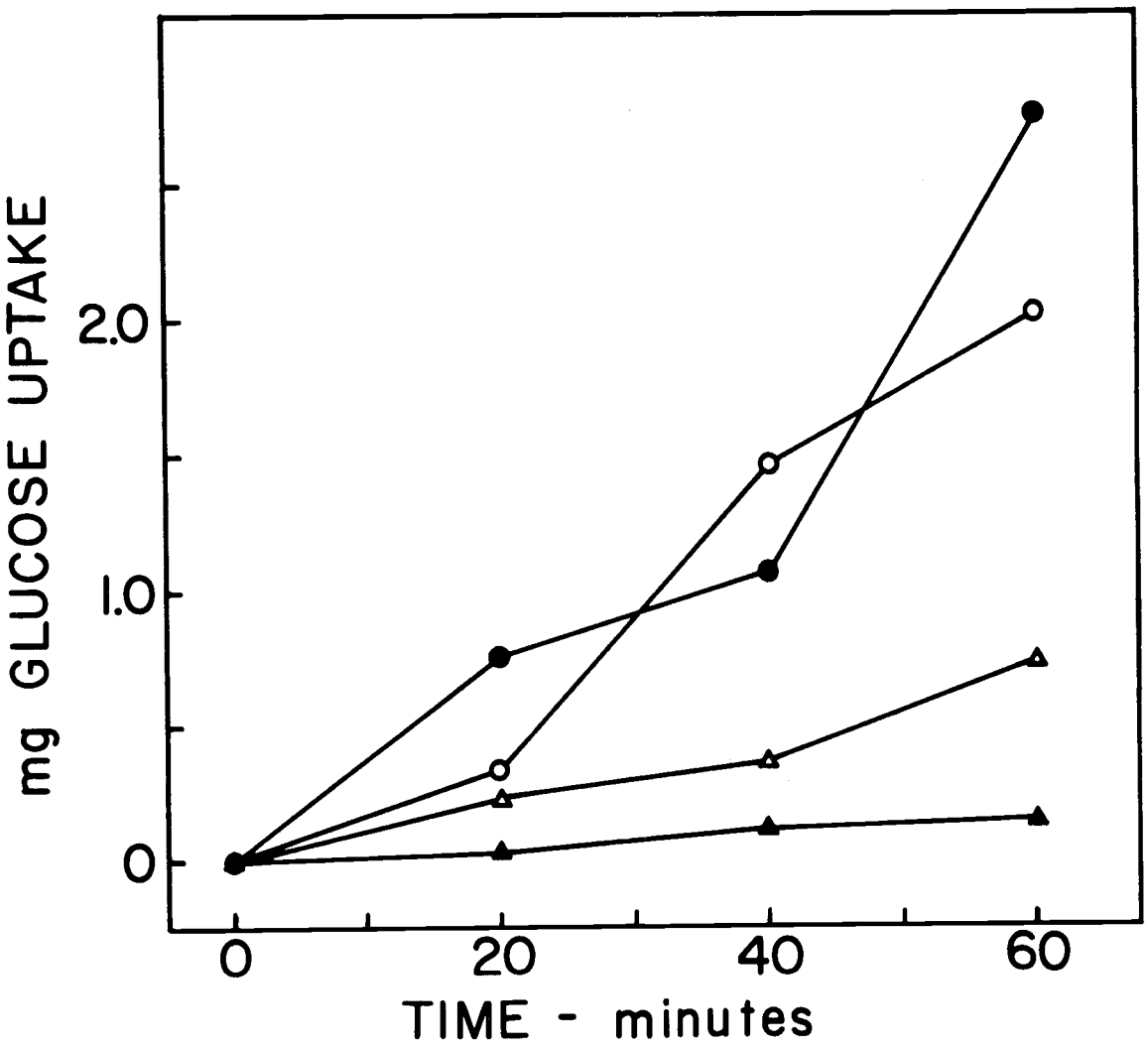


Figure 12. Anaerobic glucose photoassimilation by heterotrophic wild-type Scenedesmus (O—O), mutant PS-28 (Δ—Δ), and irradiated samples of wild-type (●—●) and mutant PS-28 (▲—▲). For experimental details see Materials and Methods.

mutant PS-28 (Figure 12). These results suggest that anaerobic glucose photoassimilation in Scenedemus is in part dependent upon a functional photosystem-II. Evidence in support of this was provided by the analysis of DCMU inhibition. Concentrations of DCMU greater than 10^{-7} M inhibited glucose uptake by greater than 70% (Figure 13). These data suggest that photosystem-II participates in the overall process, but it remains necessary to demonstrate that DCMU does not exert inhibitory effects on Photosystem-I. It already was suggested that higher concentrations of DCMU inhibit Photosystem-I (Tanner and Kandler, 1967), but this restriction does not apply to this study.

In Vitro Photophosphorylation

Phenazine methosulfate (PMS) catalyzed ATP formation in isolated chloroplasts is generally considered to be a photosystem-I reaction (cf., Hauska, McCarty, and Racker, 1970). Chloroplast particles from both wild-type and mutant PS-28 strains of Scenedemus perform cell-free cyclic photophosphorylation, and they possess similar rates of overall activity (Figure 14). The rates of PMS mediated photophysphorylation obtained in these experiments are similar to the rates (15.0-20.0 μmoles/hr-mg Chl.) obtained by Pratt and Bishop (1968a), but are extremely low when compared to the rate of 600 μmoles/hr-mg Chl. obtained by Kamientzky and

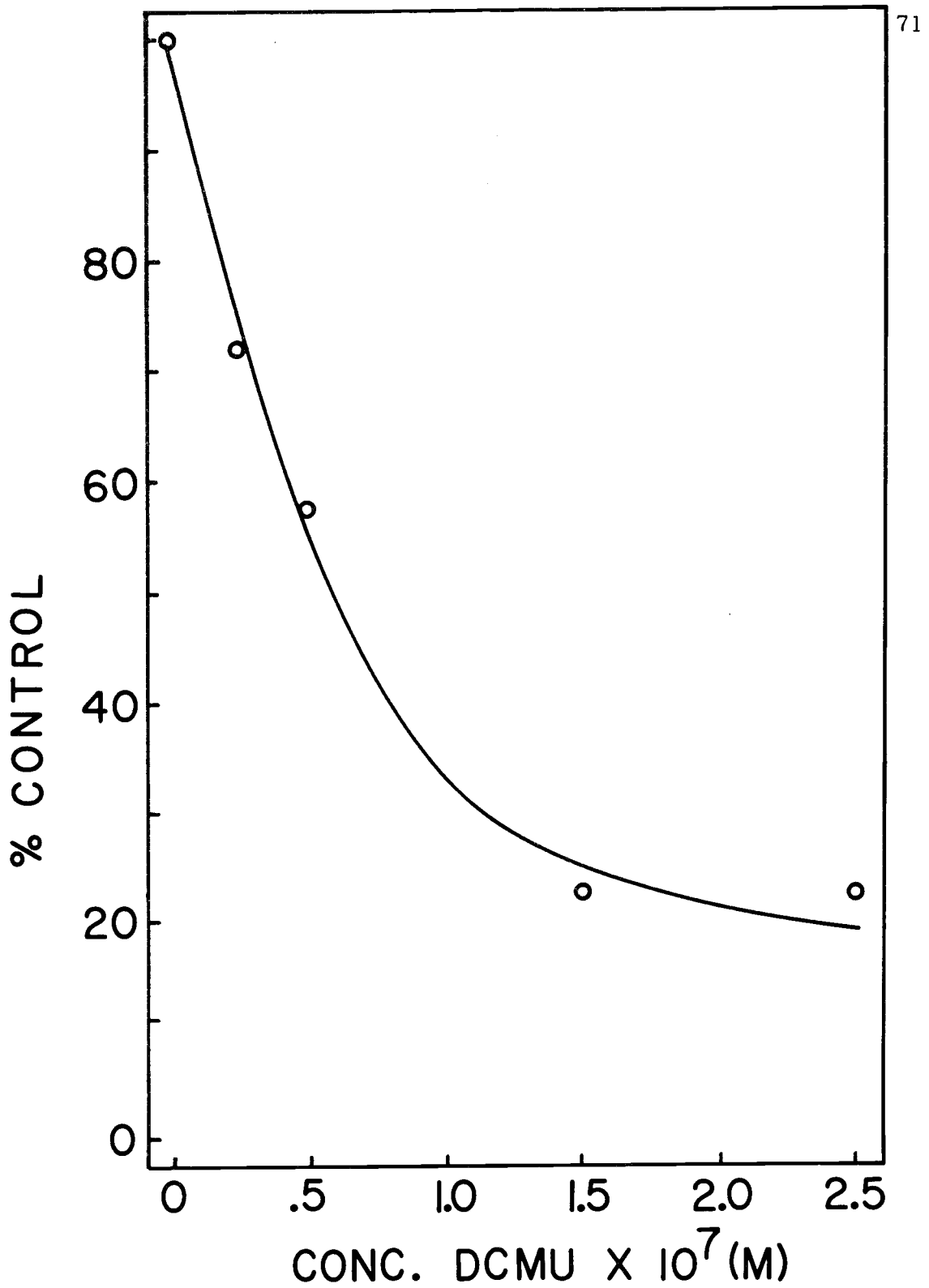


Figure 13. Inhibition of anaerobic glucose photoassimilation by wild-type Scenedesmus with increasing concentrations of of DCMU. For experimental details see Materials and Methods.

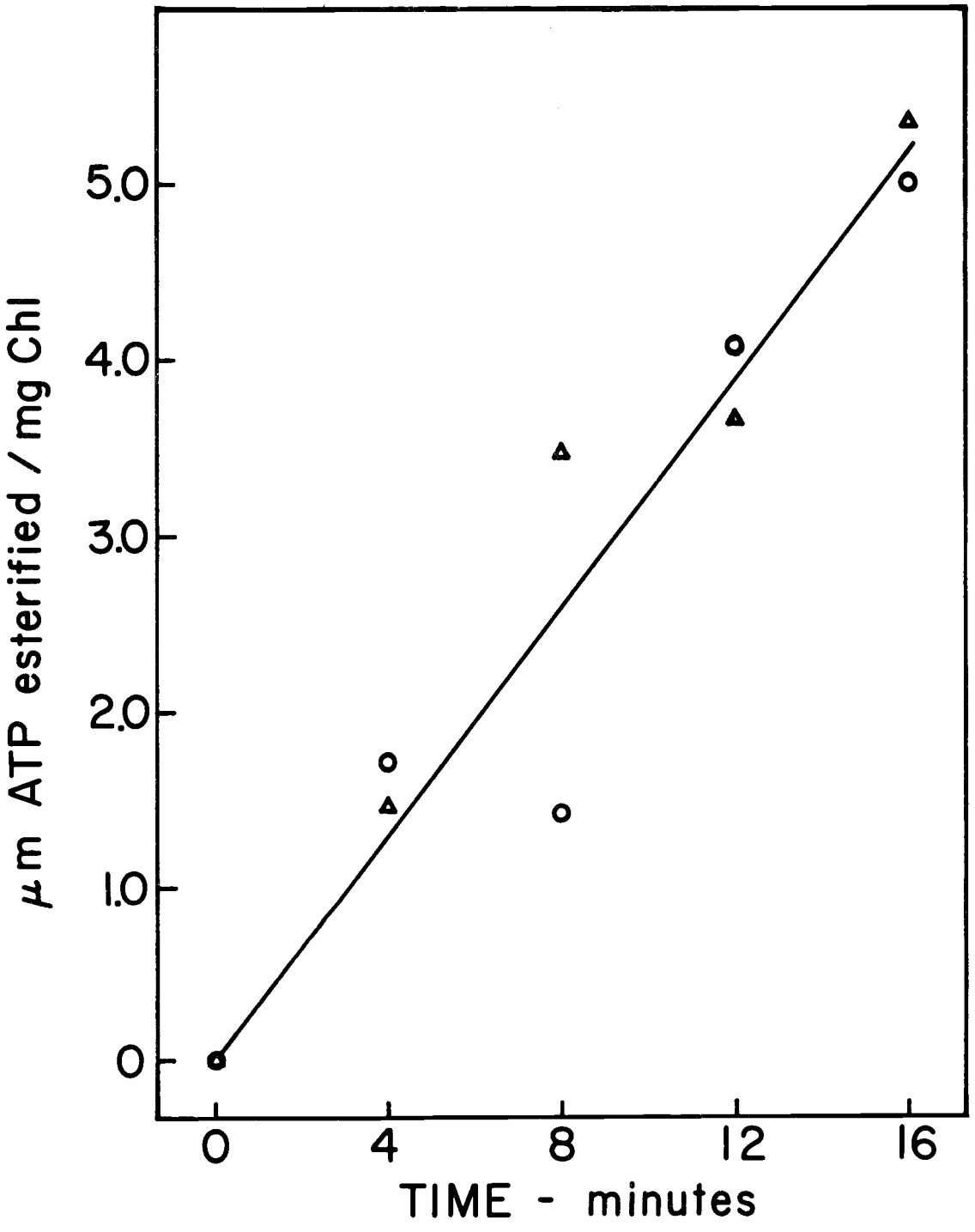


Figure 14. Time course rates of in vitro photophosphorylation by chloroplasts prepared from wild-type Scenedesmus (O—O) and mutant PS-28 (Δ—Δ). For experimental details see Materials and Methods.

Nelson (1975) using whole lettuce chloroplasts.

The results of in vitro photophosphorylation experiments indicate that phosphorylation was intact in the mutant and that the lower rates of anaerobic glucose photoassimilation observed for mutant PS-28 (see above) were not caused by an impairment in the cyclic photophosphorylation mechanism. It is likely that the loss of photosystem-II has influenced the decrease in glucose uptake. Mutant 11, a photosystem-II mutant, was shown to possess a light dependent glucose uptake equal to about one-half the wild-type rate (Tanner, Zinecker, and Kandler, 1967). These data rule out any function of vitamin E in photophosphorylation, which are in agreement with the results obtained by Krogmann and Olivera (1962).

Fluorescence

The primary component of fluorescence when measured at room temperature is of a constant yield, and probably emanates from bulk absorbing chlorophyll. The second component of the fluorescence yield is affected by changes in photosynthetic efficiency, and is believed to be directly related to the photosystem-II trapping center. Duysens and Sweers (1963) proposed a mechanism to explain the variable component of the fluorescence yield. In this scheme the fluorescence is quenched by an hypothetical compound, Q, which lies in the electron transport system between the photosystem-II and

photosystem-I trapping centers. When Q is in the oxidized form fluorescence is quenched and when Q is reduced it becomes a non-quencher. Therefore, when light of photosystem-II wavelengths is absorbed electrons are stripped from water, Q is reduced, and the fluorescence yield is increased. When light of photosystem-I wavelengths is absorbed QH is re-oxidized by P700, and the fluorescence yield is quenched.

In contrast to the wild-type, mutant PS-28 has a very high steady state fluorescence similar to the patterns observed for photosystem-II mutants (Bishop and Wong, 1971). Neither photosystem-I wavelengths (713 nm), nor photosystem-II wavelengths (650 nm) induced a variable yield fluorescence, although the mutant exhibited an as yet unexplained 650 nm light-off transient (Figure 15). The fluorescence data indicate that mutant PS-28 is very dissimilar to the wild-type, and behaves like a typical photosystem-II mutant. Further evidence in support of this conclusion is that the mutant lacks the 697 nm low temperature fluorescence emission band which has been attributed to the photosystem-II trapping center (Bishop and Wong, 1974).

Kessler (1966) demonstrated that the fluorescence of hydrogen adapted algae increased over that of normal aerobic cells, however, if the cells were manganese deficient, then a decrease in fluorescence was observed. Cheniae and Martin (1970) identified the site of manganese function in photosynthesis to be on the oxidizing

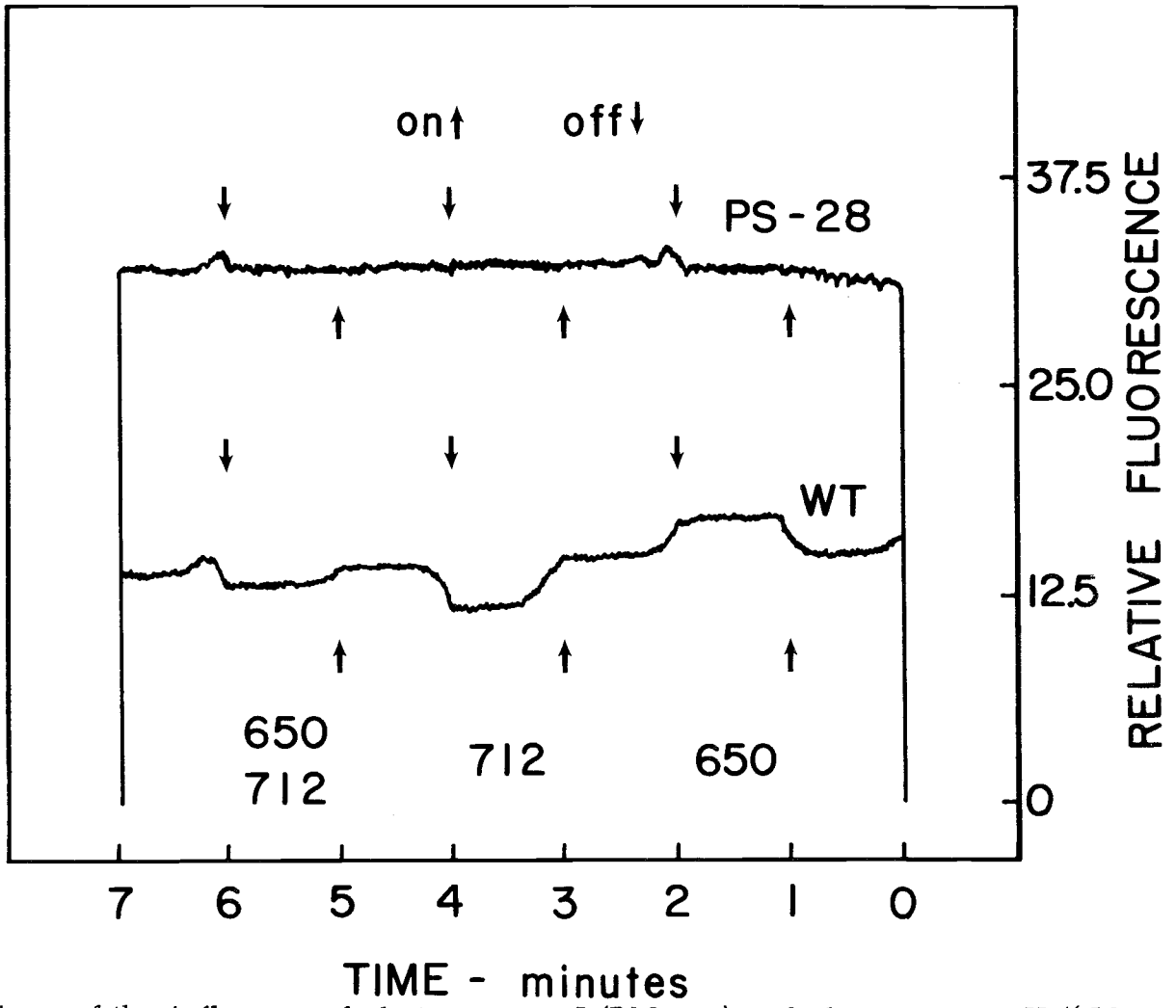


Figure 15. Comparison of the influence of photosystem-I (712 nm) and photosystem-II (650 nm) wavelengths of light on the variable yield fluorescence of wild-type Scenedesmus and mutant PS-28. For experimental details see Materials and Methods.

side of photosystem-II. Therefore, if the steady state fluorescence of mutant PS-28 decreases during hydrogen adaptation it might be concluded that the block in photosystem-II is on the oxidizing side.

The fluorescence level of hydrogen adapted wild-type cells is about 40% higher than the aerobic control (Table 2). When air is vigorously introduced to the sample the fluorescence level rapidly returns to normal (data not shown). Adaptation to an hydrogen environment does not increase or decrease the steady state fluorescence of mutant PS-28 (Table 2). The fluorescence behavior described here was interpreted by Schreiber, Bauer, and Franck (1971). The fluorescence quencher, Q, becomes reduced under hydrogen adapted anaerobic conditions causing an increase in fluorescence. The mutants of Scenedesmus which are blocked on the reducing side of photosystem-II are unable to re-oxidize QH so that fluorescence is maximal at all times (non-quenched). Exposing photosystem-II mutants to DCMU or to reducing conditions (an hydrogen environment) has no effect upon the steady state fluorescence. Therefore, with this interpretation it was concluded that mutant PS-28 is blocked on the reducing side of photosystem-II.

The relative fluorescence of mutant PS-28 chloroplast particles exceeded that of the wild-type (1.27/1.00), but the two to three fold difference observed for whole cells no longer holds true (see Figure 15, and Table 2). These findings indicated that the preparation of

Table 2. Relative fluorescence measurements of aerobic and hydrogen adapted cells of wild-type Scenedesmus and mutant strain PS-28.

Relative fluorescence is expressed in arbitrary units and the results presented here are similar to those obtained in 5 independent experiments. Two-day old heterotrophic samples were gassed with either air-4% or hydrogen-4% CO_2 for four hours prior to measurement. For experimental details see Materials and Methods.

	Wild-type aerobic	Wild-type H_2 adapted	PS-28 aerobic	PS-28 H_2 adapted
Relative Fluorescence	14.8	21.6	36.2	36.6

chloroplasts from wild-type Scenedesmus lowered the coupling efficiency between the two photosystems, thus increasing the fluorescence yield. The relative fluorescence of both wild-type and PS-28 chloroplast particles was reduced upon the addition of potassium ferricyanide and was increased upon the addition of sodium dithionite (Table 3). This finding established that the fluorescence quencher was present in the mutant and that it was functional upon exposure to external oxidants and reductants. This also was evidence that mutant PS-28 was blocked on the reducing side of photosystem-II, otherwide the responses observed in Table 3 would not be expected.

The effect of PMS (or $PMSH_2$) on fluorescence may be explained by two principal mechanisms. The quenching of fluorescence may be caused by membrane conformational changes (Hauska, et al.,

Table 3. Relative fluorescence measurements of wild-type Scenedesmus and mutant PS-28 chloroplast particles.

The relative fluorescence of wild-type Scenedesmus and mutant PS-28 chloroplast particles was expressed in arbitrary units, and is presented here in ratios as designated below. These results were similar to those obtained in 1 other experiment. For experimental details see Materials and Methods.

	Relative[1] Fluorescence	plus[2] H_2O	plus[3] NaS_2O_4	plus[4] $K_3Fe(CN)_6$	plus[5] PMS
PS-28 treated / WT treated	1.27	1.20	1.52	0.54	1.14
PS-28 treated / PS-28 control	1.00	0.95	1.20	0.46	0.89
WT treated / WT control	1.00	1.02	1.36	0.43	0.80

[1] Ratio of control to control. Reaction mixture contained 3 ml of standard reaction mixture (20 mM tricine-KOH, pH 7.5; 30 mM KCl; 0.4 M sucrose; and 1% w/v bovine serum albumin) and chloroplasts, 25 μg chlorophyll.

[2] Same as in (1), but add 0.1 ml of distilled water.

[3] Same as in (1), but add trace amounts of $Na_2S_2O_4$.

[4] Same as in (1), but add 0.1 ml of 25 mM $K_3Fe(CN)_6$.

[5] Same as in (1), but add 0.1 ml of 0.1 mM phenazine methosulfate.

1970), or by a direct chlorophyll-PMS interaction (Homann, 1976). The steady state fluorescence of both mutant PS-28 and wild-type Scenedesmus was quenched by PMS (Table 3). A detailed interpretation of the PMS quenching phenomenon is necessary before the significance of these experiments will be realized; however, it is interesting that a photosystem-II mutant is sensitive to the presence of PMS.

Chloroplast Photoreductions

There are three classes of chloroplast reactions that are of importance to photosynthetic electron transport: 1) those that require only photosystem-II activity; 2) those that require only photosystem-I activity; and 3) those that require the activities of both photosystems. Class (2) photoreductions do not use water as a source of electrons, and are therefore different from the other two classes. Bishop and Wong (1971), and Pratt and Bishop (1968a) demonstrated that it was possible to categorize photosystem-I and photosystem-II mutations of Scenedesmus through the application of in vitro chloroplast reactions.

Water to ferricyanide is a class (1) photoreduction, and the wild-type exhibits a typically high rate (Pratt and Bishop, 1968a). The capacity of the mutant for this reaction is greatly diminished (Table 4). (Negligible rates of potassium ferricyanide reduction are

Table 4. Chloroplast photoreductions of wild-type Scenedesmus and and mutant strain PS-28.

Oxygen measurements were obtained with a Clark Ag-AgCl electrode in association with a Gilson model KM$_5$ oxygraph. Reactions were initiated with red light (2.5×10^5 ergs/sec-cm^2). Data are given as an average of 3 independent observations. For experimental details see Materials and Methods.

	$H_2O \rightarrow MV$[1]	DCPIP-ascorbate $\rightarrow MV$[2]	$H_2O \rightarrow K_3Fe(CN)_6$
wild-type	107.6	270.8	70.9
mutant PS-28	86.7	246.1	5.0

[1] Water to methylviologen. 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) with 0.1 mM methyl-viologen, 0.3 mM NaN_3, and chloroplasts (90 μg of chlorophyll per sample). Data are expressed as μmoles of O_2 consumed/hr-mg Chl.

[2] DCPIP-ascorbate to methylviologen. Reaction mixture contained same as in (1), but with 0.2 μm DCPIP, 20 μM Na^+ascorbate, 2.0 μM DCMU, and 50 μg/ml catalase in place of NaN_3. Data are expressed as μmoles O_2 consumed/hr-mg Chl.

[3] Water to ferricyanide. Reaction mixture contained the same as in (1), but 3.0 mM $K_3Fe(CN)_6$ is substituted for methylviologen and NaN_3. Data are expressed as μmoles O_2 evolved/hr-mg Chl.

expressed as < 5 μmoles per mg chlorophyll per hour because such low rates are difficult to distinguish from background noise and light independent changes in oxygen evolution.) Similar results were obtained with a second class (2) photoreduction, the DCPIP (2,6-dichlorophenol-indophenol)-Hill reaction, corroborating the fact that chloroplast particles prepared from mutant PS-28 are completely lacking detectable photosystem-II activity (data not shown). The DCPIP and ferricyanide Hill reactions also are inactive when electrons are provided to photosystem-II by the exogenous donor, diphenylcarbizide.

The DCPIP-ascorbate to methylviologen chloroplast reaction is a class (2) photoreduction, and the results indicate that photosystem-I in both the mutant and the wild-type is highly functional (Table 4). The rates observed here are comparable to the rates observed by Harvey (1974). The data also are in agreement with *in vivo* analyses which indicate that mutant PS-28 has a functional photosystem-I, and an impaired photosystem-II (see above).

The results of the water to methylviologen photoreduction indicate that both the mutant and the wild-type have coupled photosystems (Table 4). This result was unexpected and was at odds with the class (1) photoreductions which indicate that mutant PS-28 chloroplast particles cannot utilize water as an electron donor. However, similar results were obtained by Cheniae and Martin (1970)

using chloroplast particles that were inactivated with tris-washing. Because of these results it seems likely that substances other than water can serve as an electron donor for the water to methylviologen photoreduction. Therefore, data obtained from this chloroplast reaction should not be weighted heavily.

518 nm Absorbancy Change

The light-induced spectral shift at 518 nm, which has been variously attributed to carotenoids, chlorophyll b, and semi-reduced quinones, was first described by Duysens (1954). Recent interest in the 518 nm absorbancy change stems from the possible interrelationship between it and the potential difference that is established in the light across the chloroplast membrane (Junge and Witt, 1968). Baltscheffsky (1969) presented evidence to support a direct relationship between these spectral shifts in chromatophores of Rhodopseudomonas spheroides and the membrane potential.

There was little difference in the magnitude of the 518 nm absorbancy band obtained for both the wild-type and mutant PS-28 (Figure 16). This indicated that the respective ability of the mutant to transpose a potential difference across the photosynthetic membrane was not impaired by the loss of vitamin E. Pratt and Bishop (1968b) observed similar results for a series of photosystem-II mutants of Scenedesmus in a detailed analysis of the 518 nm

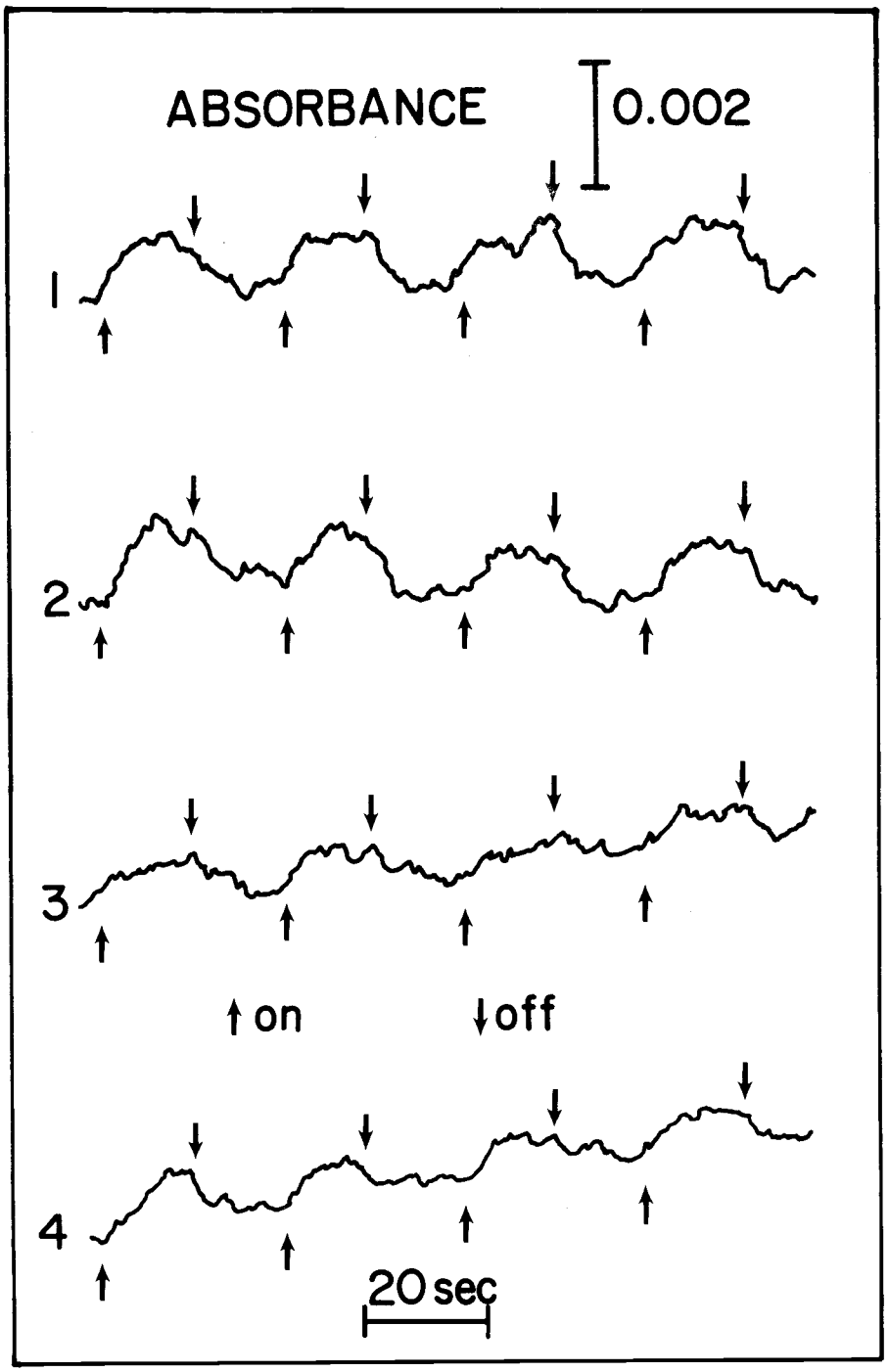


Figure 16. Light-induced absorbancy changes at 518 nm in cells of (1) wild-type Scenedesmus, (2) mutant PS-28, (3) irradiated wild-type, and (4) irradiated PS-28. Irradiations were performed for 1 hr in a field of white light (1.0×10^6 ergs/sec-cm^2). For experimental details see Materials and Methods.

absorbancy change. They demonstrated detectable differences in the kinetics of the 518 nm absorbancy change between photosystem-II mutants and the wild-type, but the instrumentation employed in this study was not capable of such high resolution.

Upon inactivating mutant PS-28 with high intensity irradiation, the 518 nm electrochromic shift was dampened (Figure 16). According to the findings of Pratt and Bishop (1968b) this would indicate that photosystem-I was inactivated by the high intensity treatment. However, the wild-type behaved similarly under the same treatment, and it was observed that the magnitude of the 518 nm absorbancy change was diminished in mixotrophically grown cells of Scenedesmus (data not shown). These findings do not support the concept that high intensity illumination adversely affects photosystem-I in the mutant. The decrease in signal intensity of the 518 nm absorbancy change in samples exposed to light has not been explained.

Methanolic Absorption Spectra

The comparative absorption spectra of methanolic extracts of the wild-type and Scenedesmus mutants PS-28, C-28-21, C-28-18, C-2A', and C-6D are presented below (Figures 17a, and 17b). The latter four mutants possess less than one-tenth of the chlorophyll content of the wild-type when cultured heterotrophically, but rapidly

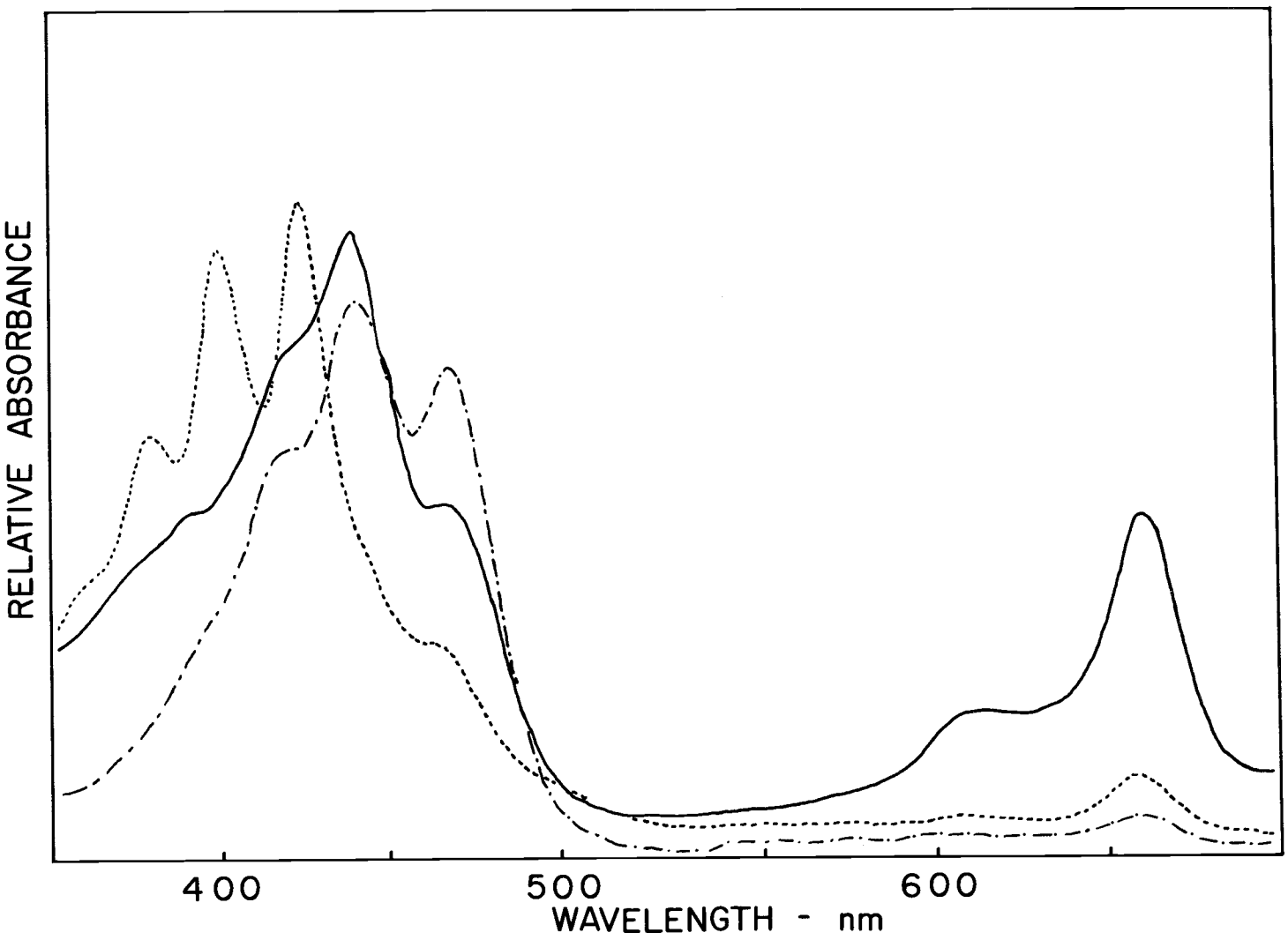


Figure 17a. Comparative absorbancy spectra of methanolic extracts of heterotrophic wild-type Scenedesmus (——), mutant C-2A' (— -—), and C-6D (----). For experimental details see Materials and Methods.

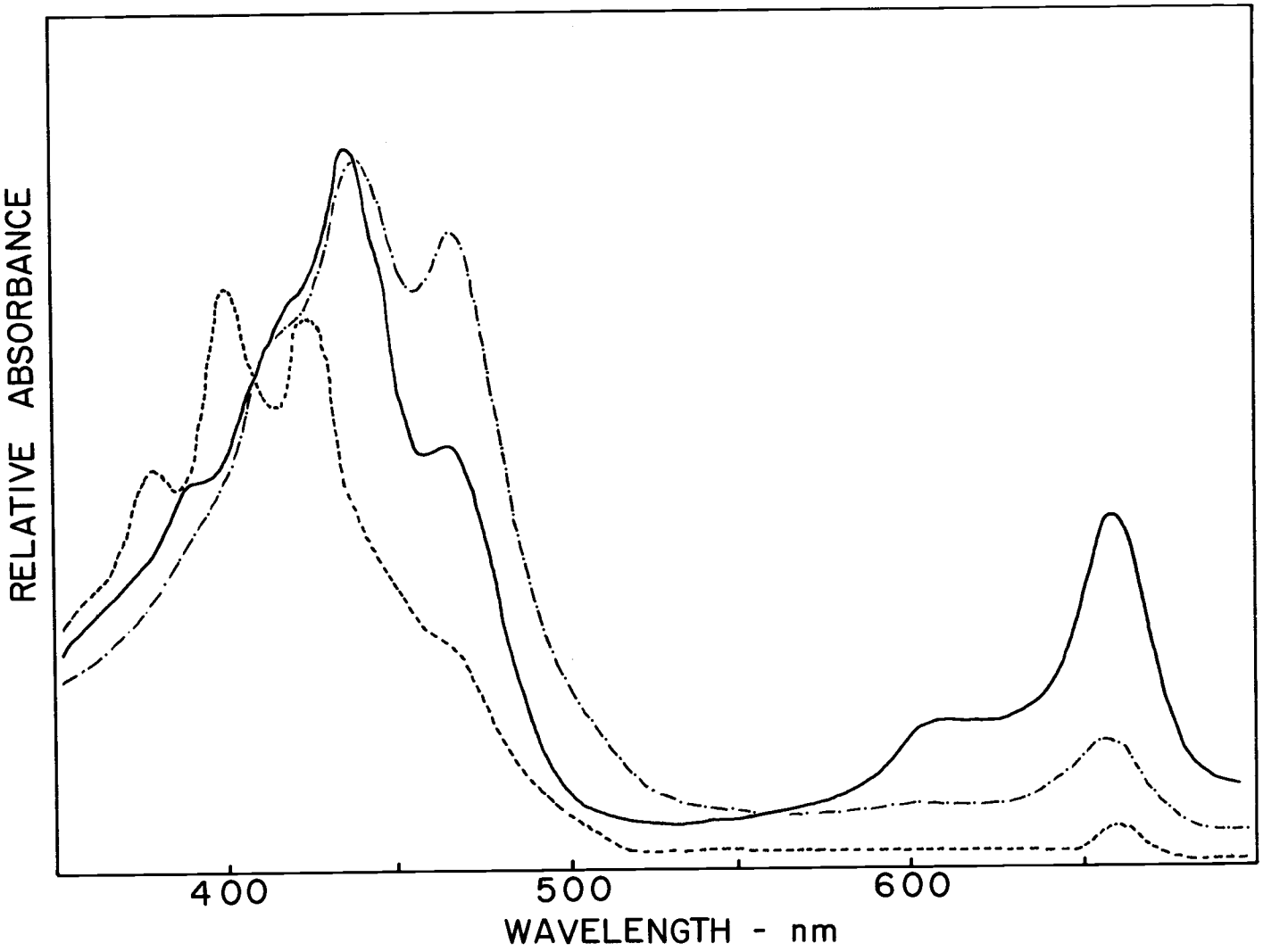


Figure 17b. Comparative absorbancy spectra of methanolic extracts of heterotrophic Scenedesmus mutants PS-28 (—), C-28-21 (— - —), and C-28-18 (----). For experimental details see Materials and Methods.

accumulate chlorophyll when transferred into the light. Williams (1971) analyzed the carotenoid composition of mutants C-6D and C-2A'. Her data indicated that mutant C-6D when cultured in the dark is characterized by a preponderance of acyclic carotenoids (phytoene, phytofluene, and neurosporene) and had only a trace of cyclic carotenoids. The block in carotenoid biosynthesis in mutant C-6D resulted in a number of unusual peaks between 350 nm and 422 nm in the methanol absorption spectrum. In this study it was concluded that mutant C-28-18 also was blocked in carotenoid biosysthesis (Figure 17b). Williams (1971) demonstrated that the carotenoid composition of mutant C-2A' was identical to the wild-type, and that the mutation involved a block in chlorophyll biosynthesis. Again by spectral comparison (Figure 17b) it was concluded that the carotenoid composition of mutant C-28-21 was identical to that of mutant C-2A'. By analogy the mutation in C-28-21 is also involved in chlorophyll biosynthesis. Having an analogous series of greening mutants with and without vitamin E allowed a study of the influence of vitamin E on the greening process.

Greening Studies

After 24 hours of continuous illumination the amounts of chlorophyll in mutant C-2A' has approached the normal wild-type level (Figure 18). The maximum chlorophyll level of greening

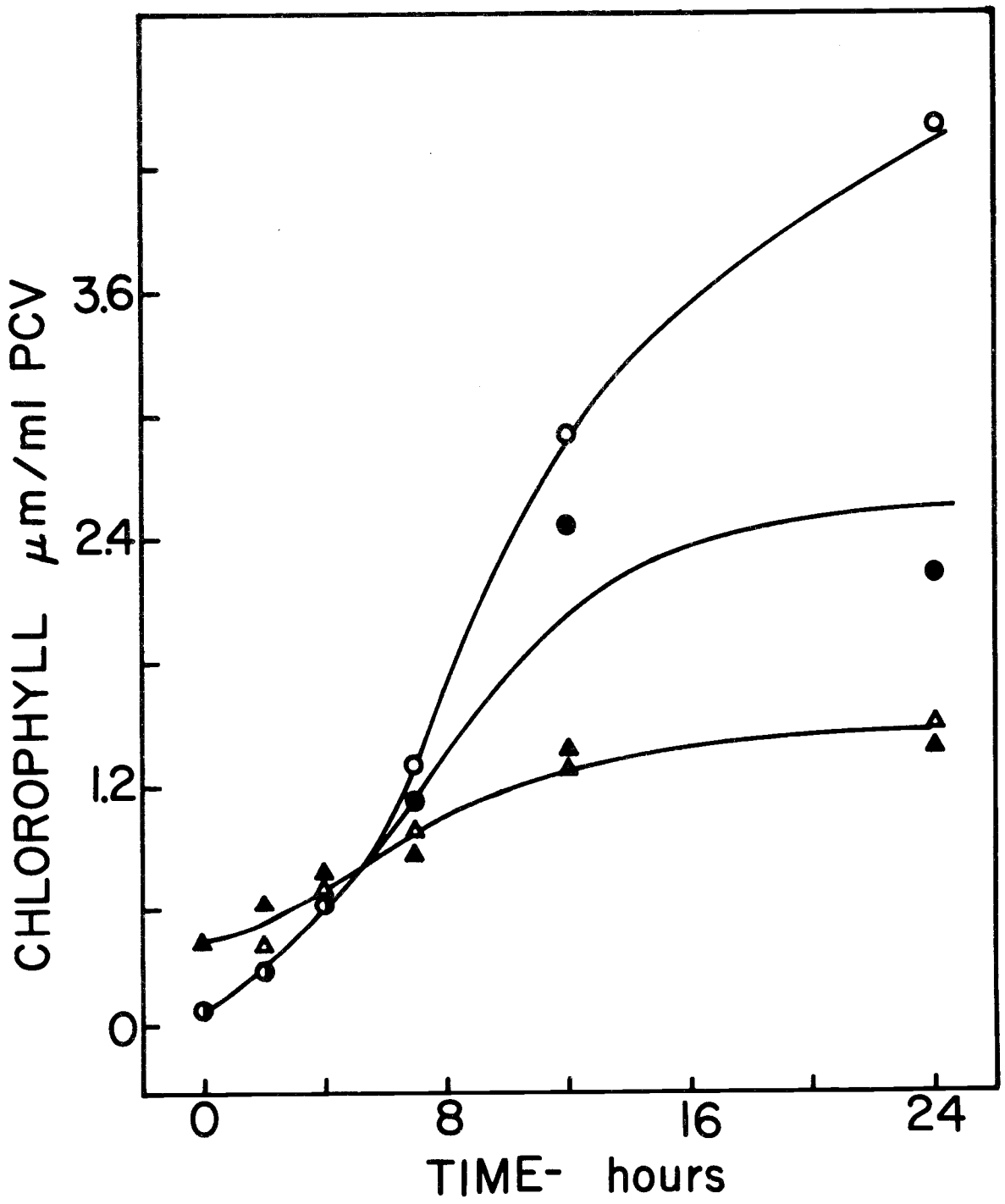


Figure 18. Comparative chlorophyll synthesis during the greening of 2-day old Scenedesmus mutants C-2A' (O—O), C-2A' plus µM DCMU (●—●), C-28-21 (Δ—Δ), and C-28-21 plus µM DCMU (▲—▲). For experimental details see Materials and Methods.

cultures of C-28-21 is about one-third that of the wild-type. Reasons for this will be presented below.

Cells of mutant C-28-21 were insensitive to the presence of μm DCMU in the culture medium during greening, but cells of mutant C-2A' that had been poisoned with DCMU stopped greening after 8-12 hours into the light period (Figure 18). The fact that the greening curve of mutant C-2A' did not exhibit an inhibition by DCMU until the 8th to 12th hour into the light period indicates that the greening process in this mutant is divided into two phases. The first phase is independent of photosynthesis, and the second phase is dependent on photosynthesis (see below). Because mutant C-28-21 is a sub-mutant of PS-28 it also is blocked in photosystem-II, and DCMU which is a photosystem-II inhibitor has no influence upon the pattern of greening (Figure 18).

The onset of photosynthesis during the greening process for both mutants C-2A' and C-28-21 is given in Figure 19. The oxygen evolving apparatus of mutant C-2A' is fully functional at 4 hours into the light period, even though the chlorophyll level is virtually the same as in dark grown cells. The photosynthetic rate of mutant C-28-21 is about one-third that of the wild-type, and the maximum rate is not achieved until after 8 hours of development (Figure 19). The greening pattern of both mutants C-2A' and C-28-21 was altered by light intensities greater than 10^4 ergs/sec-cm^2

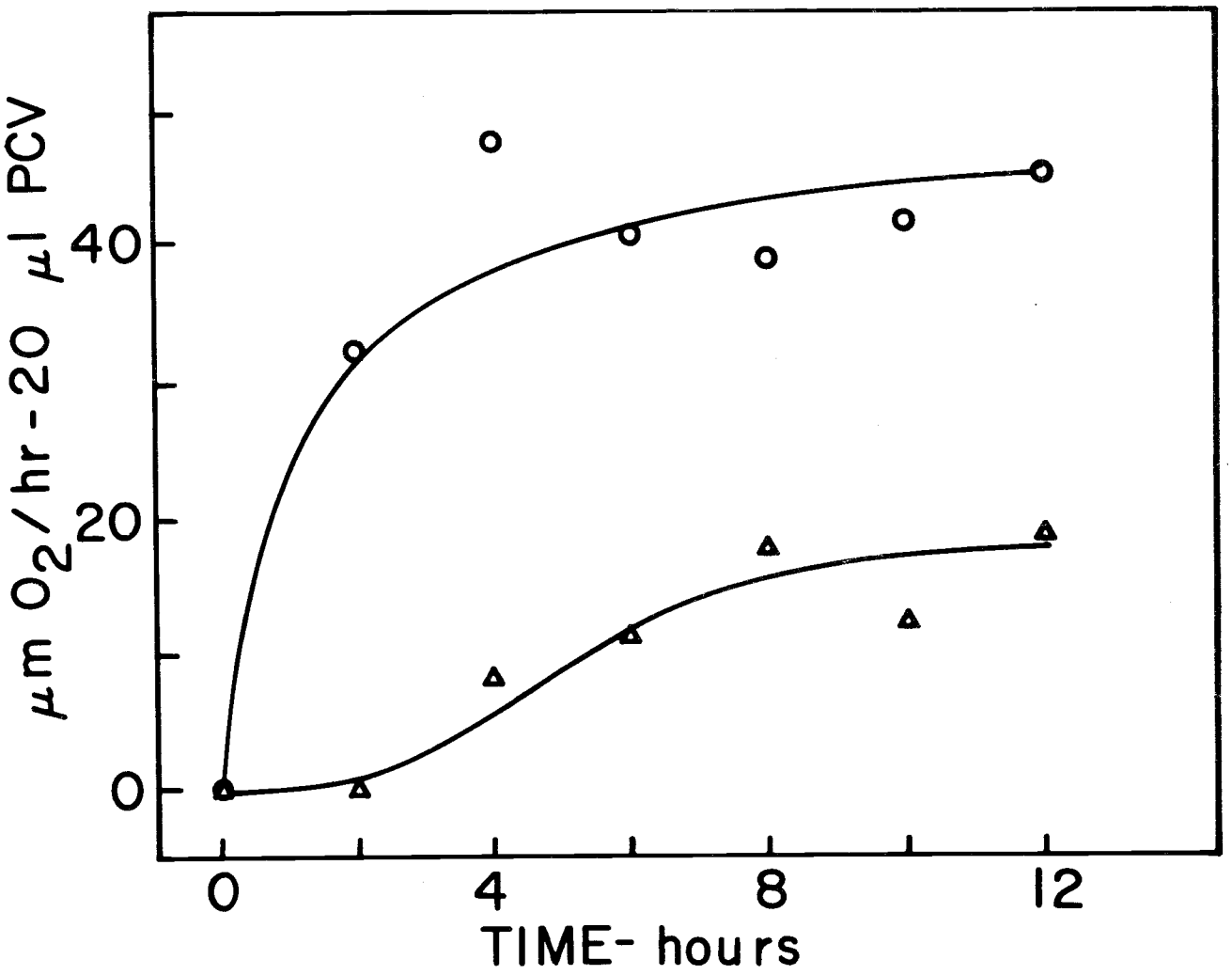


Figure 19. Comparative development of photosynthesis during the greening of 2-day old Scenedesmus mutants C-2A' (O—O), and C-28-21 (Δ—Δ). For experimental details see Materials and Methods.

(Figure 20a and 20b). The amount of chlorophyll synthesized in 12 hours by mutant C-28-21 can be doubled over the normal level by maintaining the light intensity below 10^3 ergs/sec-cm^2 throughout the course of the experiment (Figure 20b). The specific influence of light upon the early stages of the greening process in mutant C-2A' has been discussed by Oh-hama and Senger (1975). They observed that the controlling action of light in this first phase of greening was complex but resulted in one principal thing, maintaining levels of ALA (δ-aminolevuinic acid) high enough to support chlorophyll biosynthesis. The removal of light from the system arrested the greening process at that point. Higher levels of light detrimental to chlorophyll accumulation (Figure 20a and 20b), indicated that mutants C-2A' and C-28-21 are photosensitive for at least up to the 12th hour of the light period. Further evidence in support of this observation was that mutant C-2A' did not green, and in fact bleached in a light field of 10^6 ergs/sec-cm^2 (Senger and Bishop, 1972a).

The antibiotics chloramphenicol and cycloheximide interfere with chloroplast development and chlorophyll accumulation. Cycloheximide is an inhibitor of protein synthesis by 80s ribosomes (cytoplasmic protein synthesis), and chloramphenicol inhibits protein synthesis by 70s ribosomes (plastid protein synthesis; cf., Smillie, et al., 1970). The greening patterns of mutants, C-2A' and C-28-21, in the presence of these two inhibitors are presented below (Figure

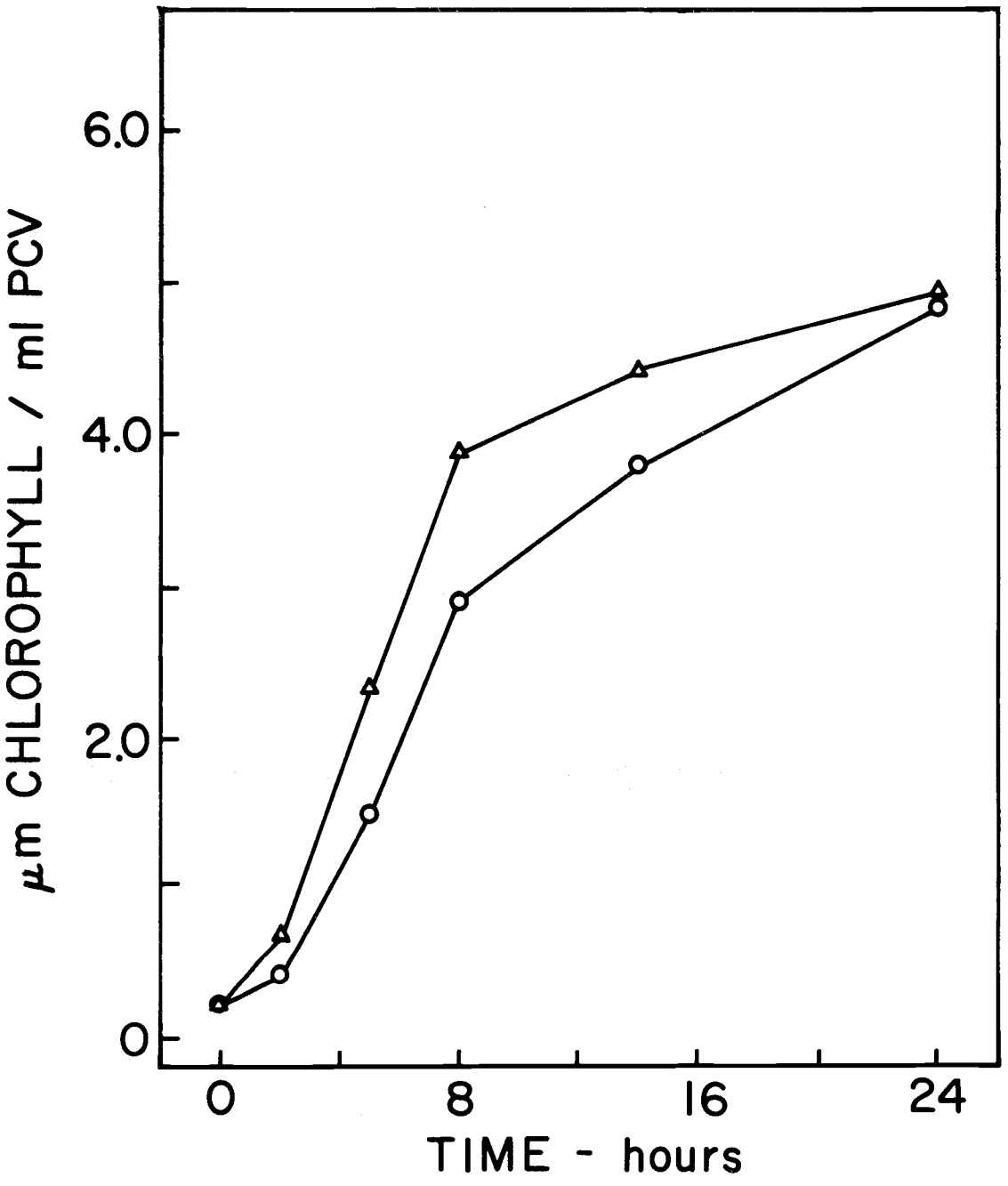


Figure 20a. Chlorophyll synthesis during the greening of 2-day old samples of Scenedesmus mutant C-2A' at two different light intensities: 1.2×10^4 (O—O) and 2.4×10^3 (Δ—Δ) ergs/sec-cm^2. Data are representative of five independent experiments. For experimental details see Materials and Methods.

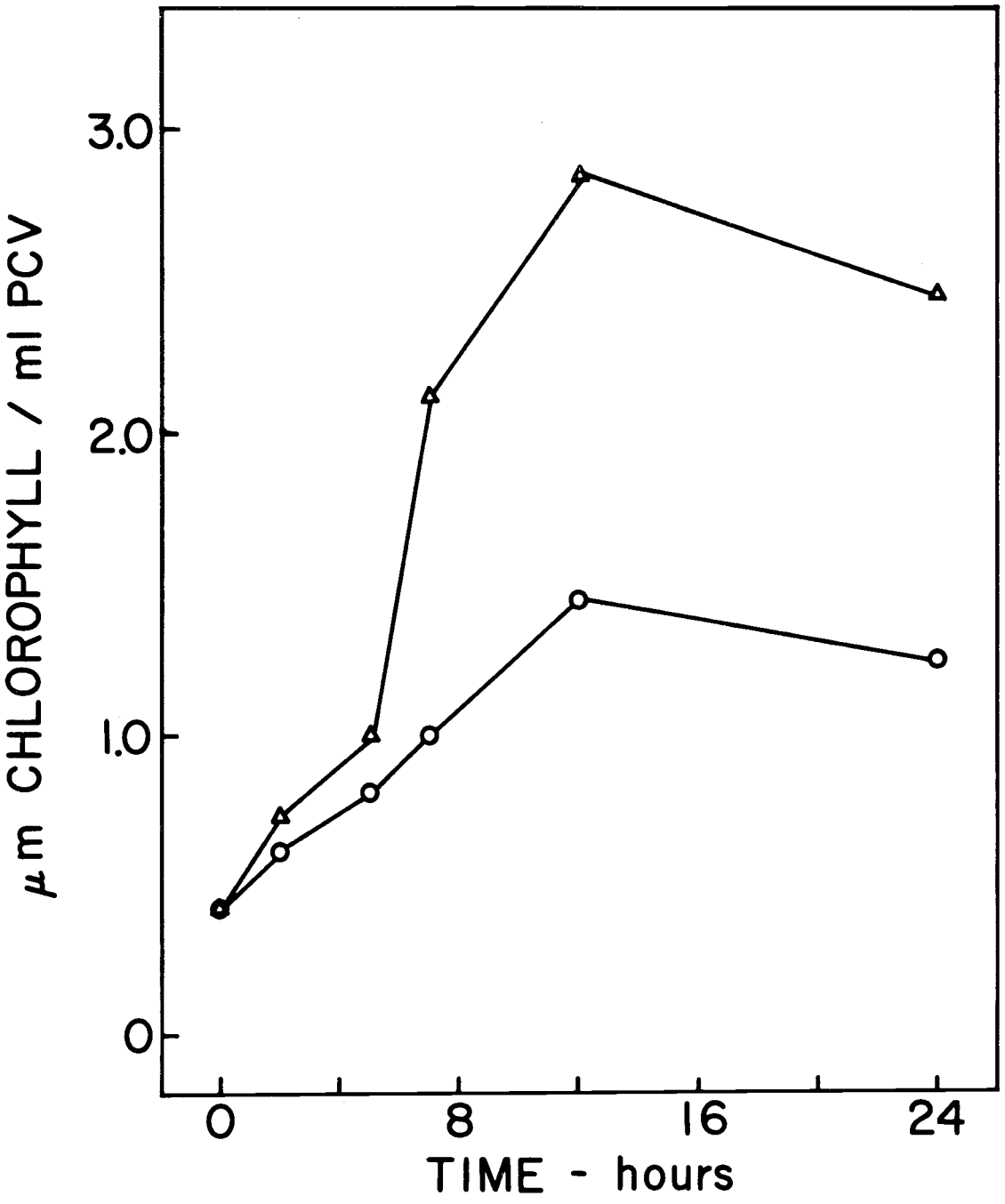


Figure 20b. Chlorophyll synthesis during the greening of 2-day old samples of Scenedesmus mutant C-28-21 at two different light intensities: 1.2×10^4 (O—O) and 2.4×10^3 (Δ—Δ) ergs/sec-cm^2. Data are representative of three independent experiments. For experimental details see Materials and Methods.

21a and 21b). Chloramphenicol (1 mg/ml) only partially inhibits the greening of either mutant during mixotrophic development (Figure 21a). Cycloheximide (1 μg/ml) thoroughly inhibits chlorophyll accumulation in either mutant (Figure 21b). Smillie, et al. 1970 and Kirk and Allen (1965) have described the effects of these two antibiotics on chloroplast development, and the results of their studies using Euglena were similar to the results presented here. It was determined that the lack of vitamin E and the loss of photosystem-II in C-28-21 did not influence the effect of the two protein synthesis inhibitors on greening.

The greening data presented here for C-2A' and C-28-21 fully supported the conclusions of previous authors (Bishop and Senger, 1972a; Senger and Bishop, 1972; Oh-hama and Senger, 1975). The first stage of the greening process, which lasted up to 12 hours, was dependent upon the mobilization of stored carbohydrate as an energy source. The second stage of greening was dependent upon photosynthesis (hence, light intensity) to support the energy requirements of the cell. These observations explained the following: 1) The greening of C-2A' was affected by DCMU poisoning in a biphasic manner. This was not observed for C-28-21. 2) Low light intensities were sufficient to trigger chlorophyll biosynthesis during greening, but were not strong enough to cause photodamage to the chloroplast in the early stages of development. 3) The inhibition of

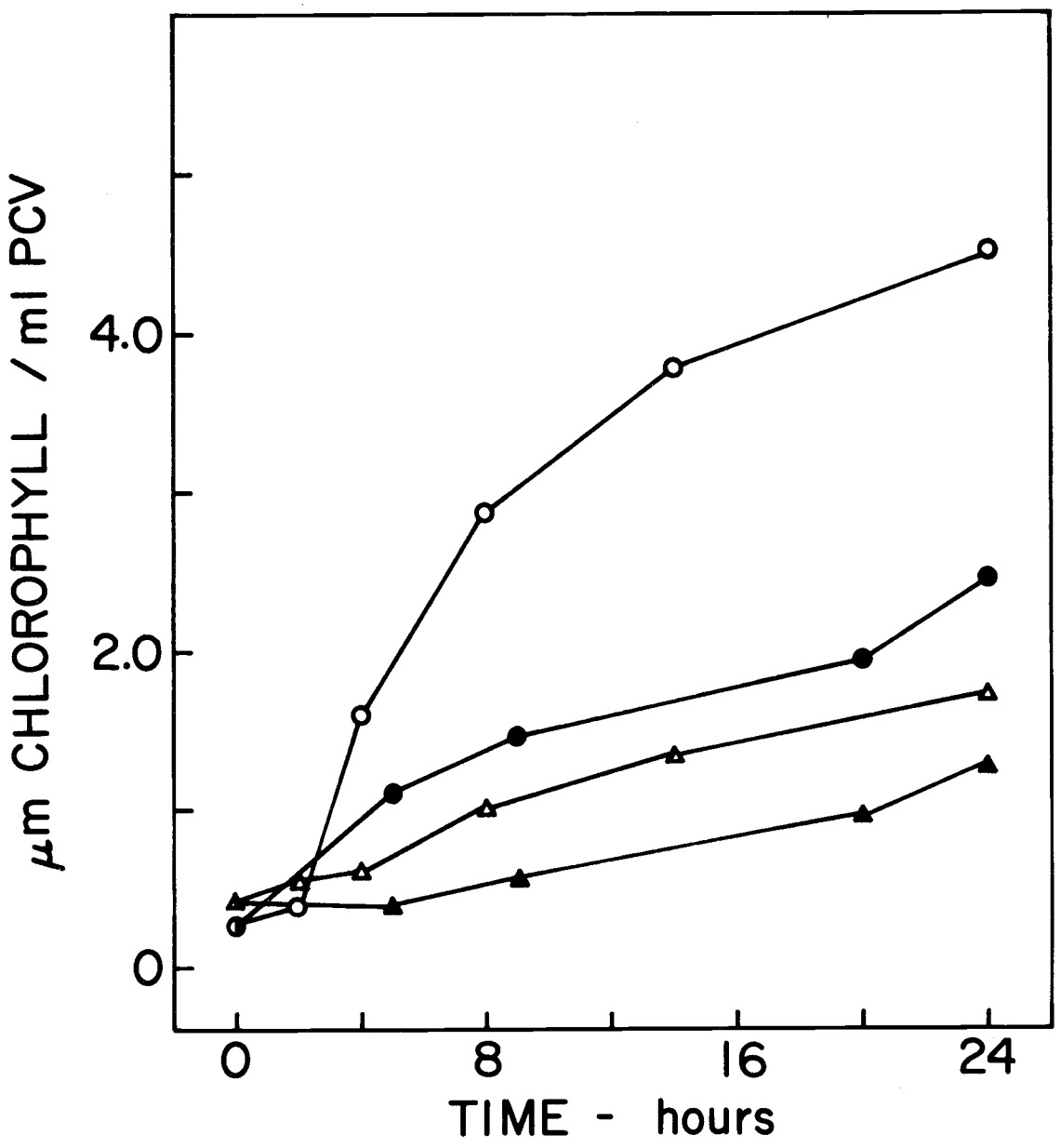


Figure 21a. Comparative chlorophyll synthesis during the greening of 2-day old Scenedesmus mutants C-2A' (O—O), C-2A' plus 1 mg/ml chloramphenicol (●—●), C-28-21 (Δ—Δ), and C-28-21 plus 1 mg/ml chloramphenicol (▲—▲). For experimental details see Materials and Methods.

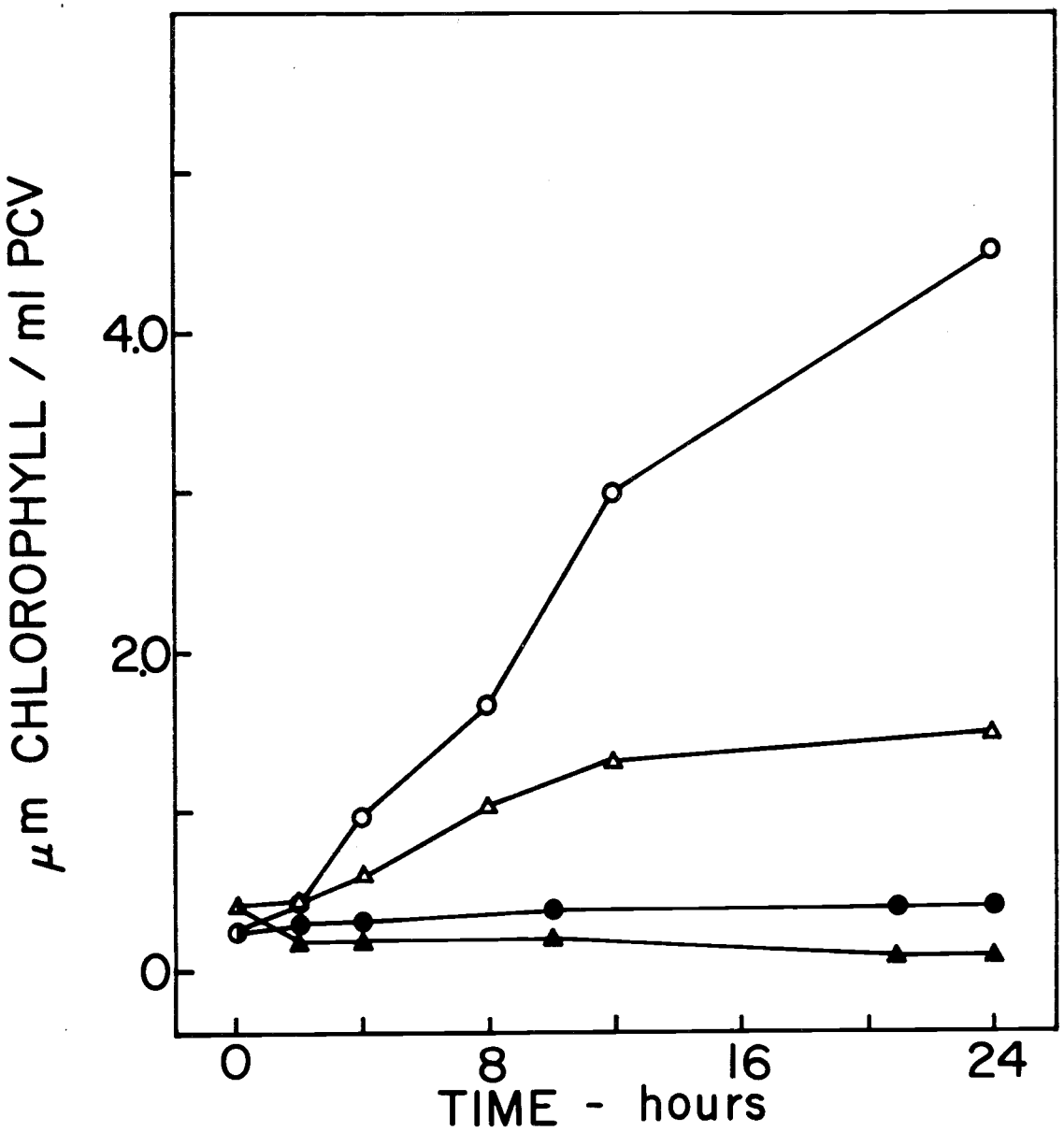


Figure 21b. Comparative chlorophyll synthesis during the greening of 2-day old Scenedesmus mutants C-2A' (O—O), C-2A' plus 1 mg/ml cycloheximide (●—●), C-28-21 (Δ—Δ), and C-28-21 plus 1 mg/ml cycloheximide (▲—▲). For experimental details see Materials and Methods.

protein synthesis prevented the mobilization of stored carbohydrates and prevented greening in both early and late stages. The effect of the total absence of vitamin E in C-28-21 only was apparent because of the loss of photosystem-II activity.

Low Temperature Absorbance Studies

Cooling samples of algae to liquid nitrogen temperature (77 K) allows resolution of the chlorophyll absorbance bands in the red region of the spectrum ordinarily not possible at room temperature. The low temperature spectrum of the wild-type reveals three peaks at 677 nm, 670 nm, and 650 nm (Figure 22a). In a comparable spectrum, the mutant, C-28-21, lacks the 650 nm absorbance band (Figure 22b), which was attributed to chlorophyll b (Cho and Govindjee, 1970). From this information it was concluded that mutant C-28-21 cultured mixotrophically can not synthesize chlorophyll b. This finding explained why the maximum chlorophyll levels of greening samples of the mutant were lower than normal (see Figure 18), and why the light intensity response of photoreduction was unusual (see Figure 7). The absence of chlorophyll b in mutant C-28-21 might indicate that there is a genetic lesion in the manufacturer of the light-harvesting-pigment-protein. This protein, which is the attachment site for all of the chlorophyll b in the chloroplast, is virtually universal in higher plants, but is not essential for

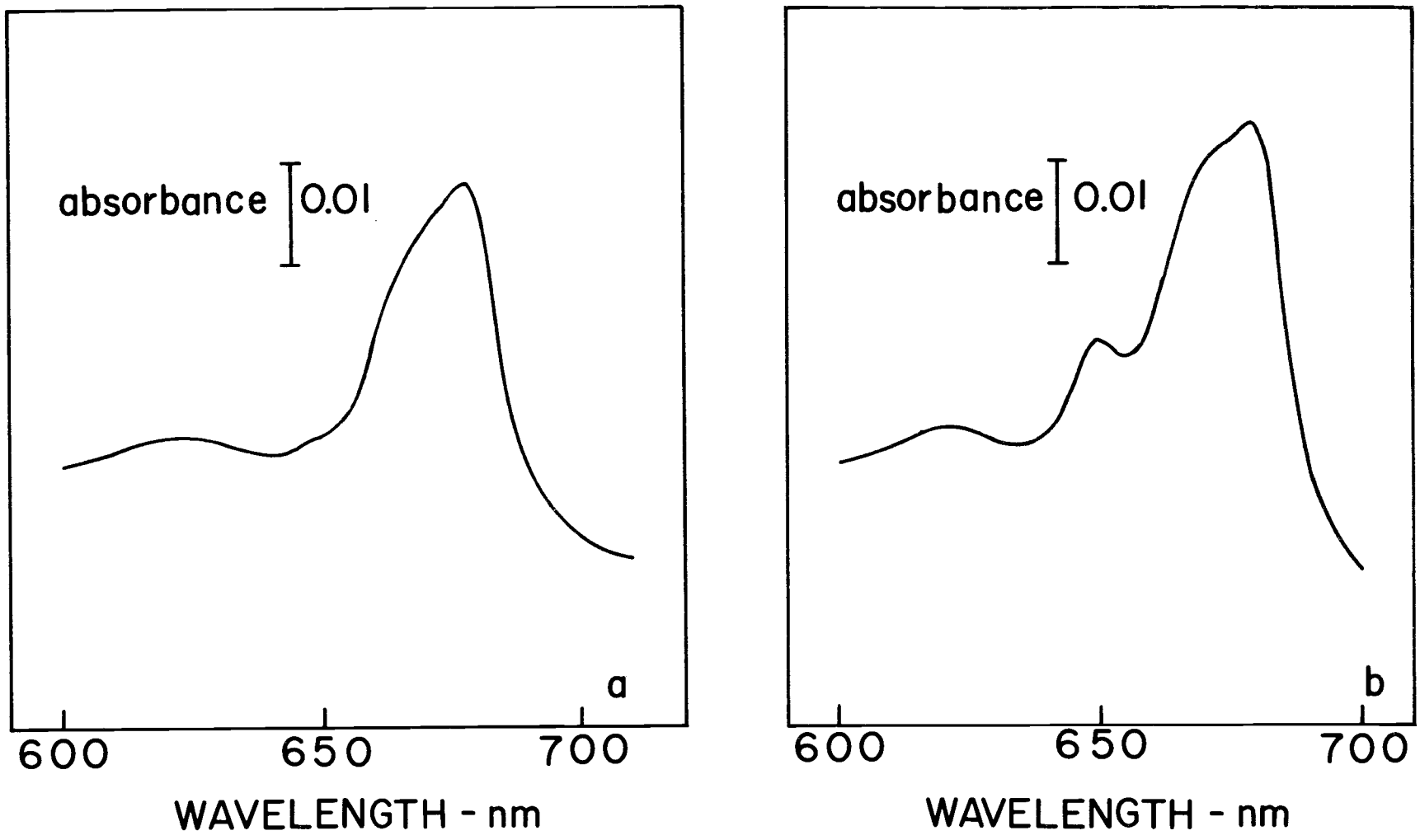


Figure 22. Low temperature absorbancy spectra of (a) mutant C-28-21, and (b) wild-type Scenedesmus. For experimental details see Materials and Methods.

photochemical activity (Thornber and Highkin, 1974). Research is currently being performed in our laboratory to demonstrate the presence or absence of this pigment protein in mutant C-28-21.

Plastoquinone A and Vitamin E

Concentrations of α-tocopherol, plastoquinone A, and chlorophyll were measured in wild-type Scenedesmus and mutant PS-28 under different patterns of growth, and the results are presented in Table 5. Evidence that mutant PS-28 lacked vitamin E was reported elsewhere (Bishop and Sicher, 1974; Sicher and Bishop, 1975), and the chromatographic techniques and identification methodology have been reported in detail by Bishop and Wong (1974).

Photosynthetic membranes are sensitive to changes in the physiological status of the cell; therefore, an analysis of the quinone complement of the thylakoids was performed on normal wild-type Scenedesmus under different patterns of growth. A comparison of the vitamin E levels (μmoles α-tocopherol/ml PCV) grown heterotrophically and mixotrophically indicated that the cultures maintained in the light had lower concentrations of vitamin E (Table 5). The level of vitamin E (μmoles α-tocopherol/ml PCV) measurable for autotrophically grown cultures of wild-type Scenedesmus was at least double the mixotrophic value (Table 5). These results were of interest because they suggested that the presence of glucose in light

Table 5. Plastoquinone A and α-tocopherol values of wild-type Scenedesmus and mutant strain PS-28.

Plastoquinone A and α-tocopherol values are expressed as a ratio to chlorophyll (μmole/μmole) or on a μmole per PCV basis. Values presented in parenthesis indicate the reciprocal of the respective compound to chlorophyll. Two-day old cultures were used as indicated below. For further experimental details see Materials and Methods.

	chlorophyll ml PCV	plastoquinone A ml PCV	α-tocopherol ml PCV	plastoquinone A chlorophyll	α-tocopherol chlorophyll	n*
wild-type mixotrophic	5.1	0.066	0.085	0.013 (75.1)	0.018 (55.7)	6
wild-type heterotrophic	5.4	0.069	0.129	0.013 (77.5)	0.023 (42.8)	6
wild-type autotrophic	3.4	0.041	0.240	0.012 (84.5)	0.071 (14.1)	2
wild-type irradiated	5.0	0.060	0.101	0.012 (83.4)	0.020 (50.1)	1
PS-28 mixotrophic	5.9	0.071	ND**	0.013 (75.2)	ND**	4
PS-28 heterotrophic	5.1	0.057	ND	0.012 (81.4)	ND	4
PS-28 irradiated	5.3	0.030	ND	0.006 (157.4)	ND	2

*n indicates number of samples assayed, and values in table are averaged over the given number of determinations.

**ND indicates substance not detected.

grown cultures of Scenedesmus could substitute partially for the function of vitamin E. Comparisons between cultures grown either autotrophically, heterotrophically, or mixotrophically are only relative because the chlorophyll concentration, cell volume, and rates of cell division were different in each instance. Despite the physiological differences between autotrophic and mixotrophic cultures, it is still considered that cells grown by the former method have the highest vitamin E levels (Hanigk and Lichtenthaler, 1975).

The concentration of plastoquinone A was equivalent in heterotrophic and mixotrophic cultures of both mutant PS-28 and wild-type Scenedesmus (Table 5). This was a surprising result because it was established previously that photosystem-II mutants in general were characterized by low plastoquinone levels (Bishop and Wong, 1971; Smillie and Levine, 1963). This made PS-28 unique as far as typical photosystem-II mutants were concerned. When the mutant was exposed to high intensity irradiation the level of plastoquinone A decreased in parallel with the loss of photosystem-II activity (Table 5). The photodynamic destruction of plastoquinone A also occurred in mutant LS-41 (Harvey, 1974; Table 5).

As expected the chlorophyll to plastoquinone A and chlorophyll to α-tocopherol values were at a minimum when measured in dark grown cultures of mutant C-2A', but after 24 hours of greening the two ratios approximated the normal mixotrophic values obtained for

the wild-type (Figure 23). The chlorophyll to plastoquinone A data presented below for greening cultures of mutant C-2A' were similar to the results presented by Bishop and Senger (1972a).

When the plastoquinone A and α-tocopherol data were expressed on a packed cell volume (PCV) rather than on a chlorophyll basis, a very different pattern was observed (Figure 24). The dark grown cultures of mutant C-2A' possessed normal hetrotrophic values of α-tocopherol, even in the virtual absence of chlorophyll. This situation appeared to be true for dark grown tissues or samples that have etioplasts, but was not evident in those systems with proplastids (Threlfall and Griffiths, 1967). After exposure of the cells to light for 24 hours the vitamin E levels(μmoles α-tocopherol/ml PCV) decrease to a value very near the normal mixotrophic level obtained for the wild-type Scenedesmus (Figure 24). Lichtenthaler and Grumbach (1975) observed similar changes in both the plastoquinone A and vitamin E levels when 8-day old etiolated barley seedlings were illuminated. Contrarily, Threlfall and Griffiths (1967) reported that the levels of α-tocopherol remained constant in etiolated maize shoots after exposure to light. Their (Threlfall and Griffiths, 1967) plastoquinone A data were similar to the results presented below. A more detailed analysis of the changes of vitamin E levels during the development of the chloroplast would be highly desirable.

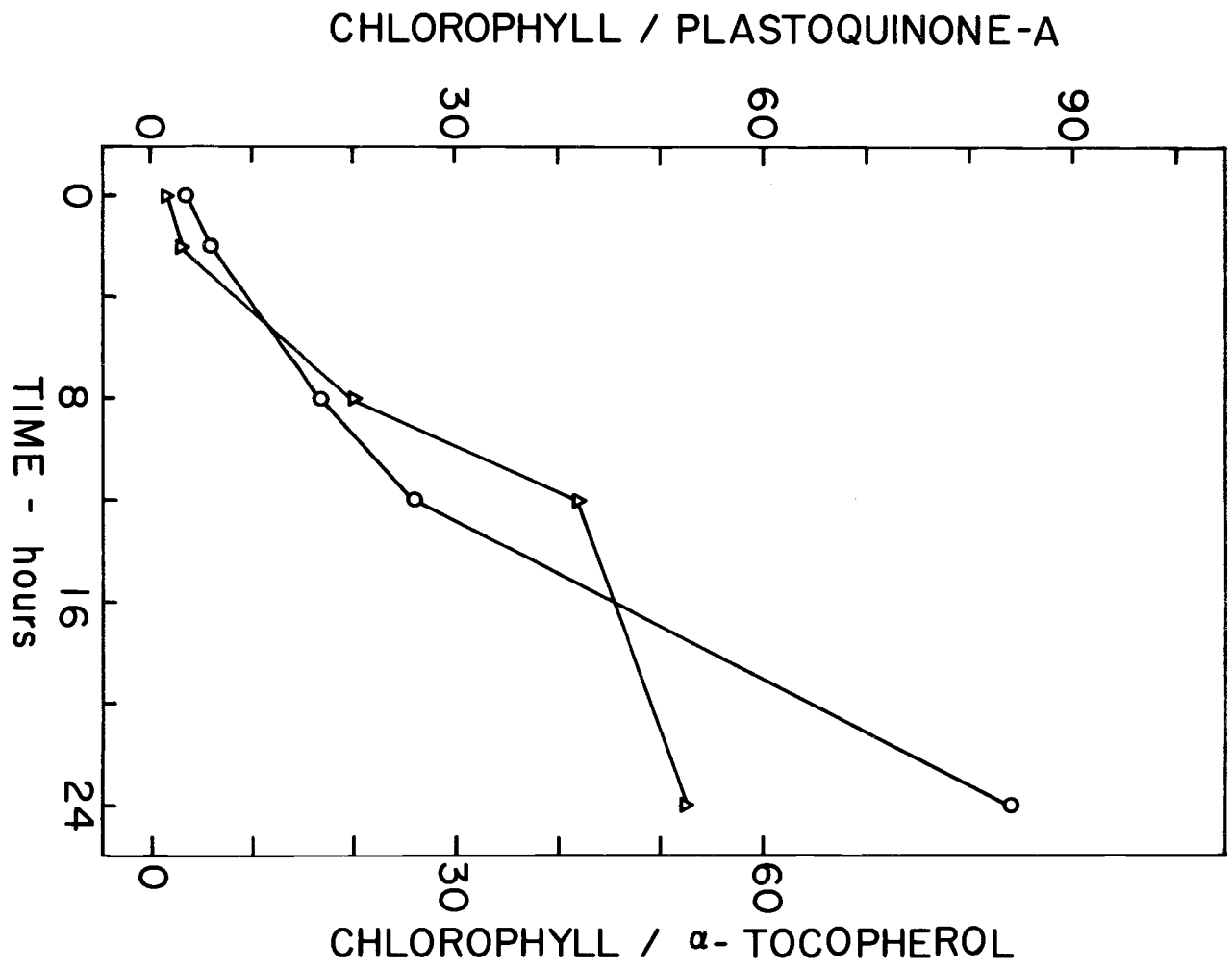


Figure 23. Chlorophyll to plastoquinone A and chlorophyll to α-tocopherol ratios (μmole/μmole of Scenedesmus mutant C-2A' at different stages of greening. Chlorophyll (a + b)/plastoquinone A (O—O); chlorophyll (a + b)/α-tocopherol (Δ—Δ). For experimental details see Materials and Methods.

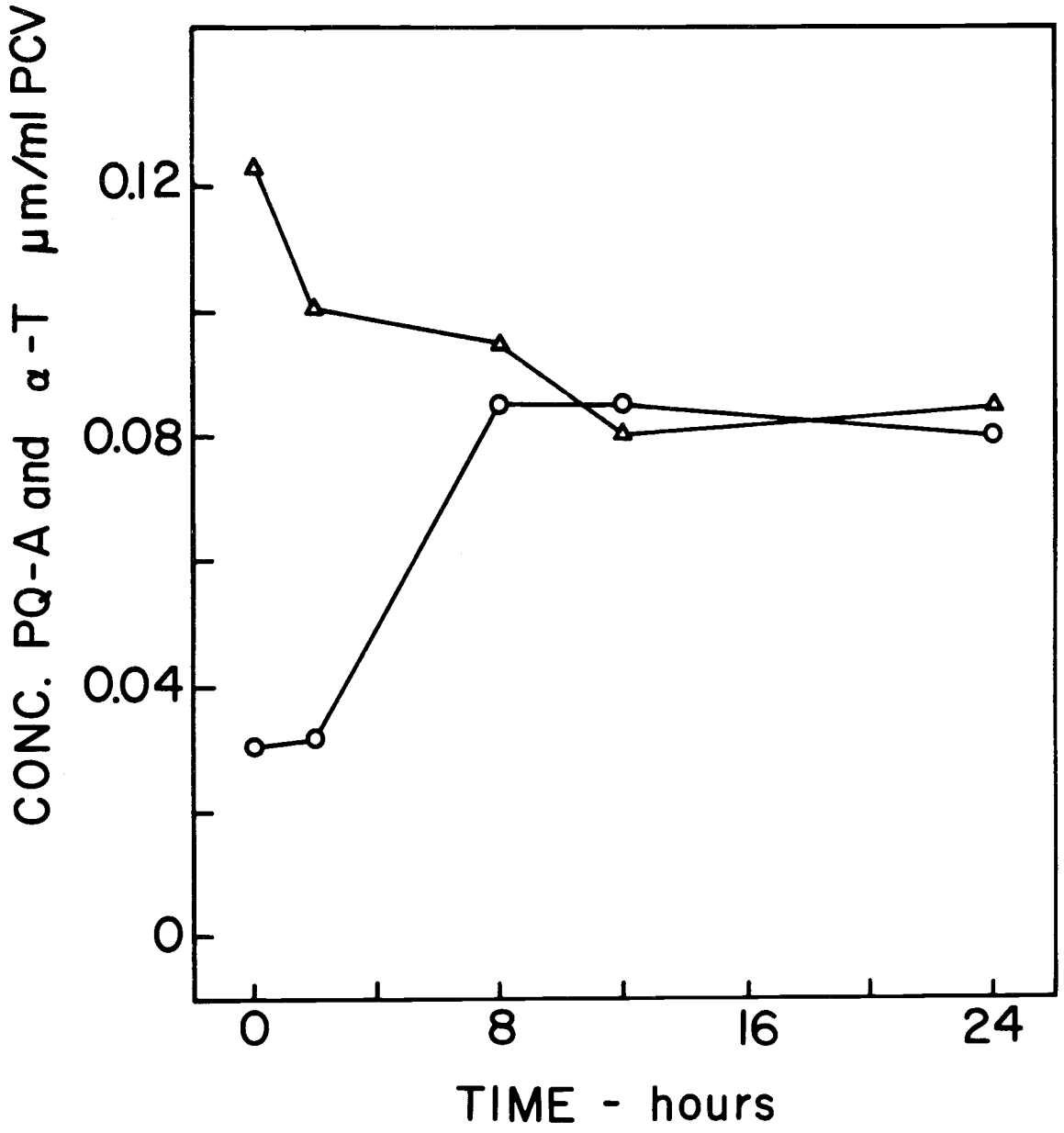


Figure 24. Concentrations of plastoquinone A (μmole/ml PCV) and α-tocopherol (μmole/ml PCV) of Scenedesmus mutant C-2A' at different stages of greening. plastoquinone A (O—O) and α-tocopherol (Δ—Δ). For experimental details see Materials and Methods.

The results presented in Figure 24 indicate that plastoquinone A and α-tocopherol are synthesized via independent pathways. If the two lipophilic benzoquinones shared a common precursor, as has been suggested by Eck and Trebst (1963), the curves in Figure 24 would be expected to follow similar kinetics. Incorporation studies using ^{14}C-mevalonate and $^{14}CO_2$ supported the independent pathway tenet (Threlfall and Griffiths, 1967).

The molar ratio of chlorophyll to α-tocopheryl quinone in hetrotrophic wild-type samples was 166.6. No trace of this substance was detected in comparable samples of the mutant.

Ascorbic Acid (Vitamin C)

Values of vitamin C were obtained for both mutant PS-28 and wild-type Scenedesmus cultured either heterotrophically or mixotrophically (Table 6). In both circumstances the ascorbic acid values for the mutant lacking vitamin E were equivalent to those of the wild-type. Additionally, an increase in the ascorbic acid values of light grown versus dark grown cultures confirmed the now classic observation that vitamin C levels were affected by light, carbon dioxide, and photosynthesis (Moldtmann, 1939). These results were interesting because following the observation by Dam, et al. (1948) that large doses of vitamin C prevented encephalomalacia and exudative diathesis in the vitamin E deficient chick, Caputto, et al., (1958) reported that

Table 6. Ascorbic acid levels of wild-type Scenedesmus and mutant strain PS-28.

Chlorophyll and ascorbic acid levels are expressed as either a ratio or on a μmole per PCV basis. Values presented in parenthesis indicate the reciprocal of the abscorbic acid to chlorophyll ratio. Two-day old cells were used and were cultured as indicated. Data presented below are similar to those obtained in 1 other experiment. For experimental details see Materials and Methods.

	chlorophyll (a + b) / ml PCV	ascorbic acid / ml PCV	ascorbic acid / chlorophyll (a + b)
wild-type mixotrophic	5.0	0.40	0.080 (12.5)
PS-28 mixotrophic	5.3	0.43	0.082 (12.3)
wild-type heterotrophic	5.3	0.23	0.044 (22.5)
PS-28 heterotrophic	4.5	0.26	0.058 (17.1)

vitamin C was not synthesized in rats fed a diet deficient in α-tocopherol. Furthermore, Tappel (1962) predicted a synergistic relationship between the presence of vitamin C in the cell, and the function of vitamin E in preventing free-radical membrane damage. Noguchi, Cantor, and Scott (1973) pursued this line of investigation and demonstrated that one reason for the lack of detectable ascorbic acid in vitamin E deficient as compared to normal liver tissue, was that reduced compounds such as ascorbic acid, gluthathionione, and reduced $NADP^+$ were oxidized in vitro by enzymes of the microsomes

and mitochondria. The oxidation of vitamin C by hepatic liver cells deficient in vitamin E lead to the formation of free-radicals, and malonyldialdehyde, an end product of free-radical lipid peroxidation. They (Noguchi, Cantor and Scott, 1974) demonstrated that α-tocopherol and the enzyme glutathione peroxidase would prevent the oxidation of ascorbic acid by vitamin E deficient chicks.

Because the ascorbic acid levels in mutant PS-28 were not depleted it was assumed that the complete absence of vitamin E in Scenedesmus grown either in the dark or in the light did not lead to free-radical damage (and the subsequent disappearance of vitamin C). This observation indicated one of two situations exist in mutant PS-28, either there is a mechanism in Scenedesmus other than vitamin E that eliminates or prevents free-radical damage, or more likely, the vitamin C oxidizing system observed in hepatic cells does not occur in Scenedesmus.

Lipids and Fatty Acids

Several species of the Cyanophyceae are known to lack polyenoic fatty acids (Holton, et al., 1968), and vitamin E (Hirayama, 1967). The photosynthetic lamellae of higher plants have very high concentrations of polyunsaturated fatty acids and vitamin E (Lichtenthaler and Park, 1963). These facts suggested a relationship

between the fatty acid composition of the photosynthetic membrane and the occurrence of vitamin E.

Two dimensional thin layer chromatographic analyses were performed on whole cell lipid extracts of heterotrophic PS-28 and wild-type Scenedesmus (Figures 25a and 25b). The lipid composition of the two algal strains was determined to be qualitatively equivalent. These results implied that even in the complete absence of vitamin E, the lipid component of the photosynthetic membrane of dark grown cells was not under stress due to free-radical damage.

The above conclusion was tested further via an in depth analysis of the fatty acid compositions of the two algal strains. The fatty acid composition of heterotrophic and mixotrophic samples of the wild-type and mutant were examined, and compared to like samples from these cultures that were treated with high intensity irradiation (Table 7). In each instance the fatty acid composition of mutant PS-28 was comparable to that of the wild-type, and furthermore, the fatty acid profile presented below for mixotrophic cells closely correlated with the results presented by Klenk, et al. (1963) for autotrophic samples of Scenedesmus. The photo-heterotrophic cultures of both the wild-type and mutant exhibited large increases in the size of the octadecatrienoic (18:3) and octadecadienoic (18:2) fatty acid pools over the dark grown cultures. These fatty acids are the predominant acyl esters of lipids which are abundant in the

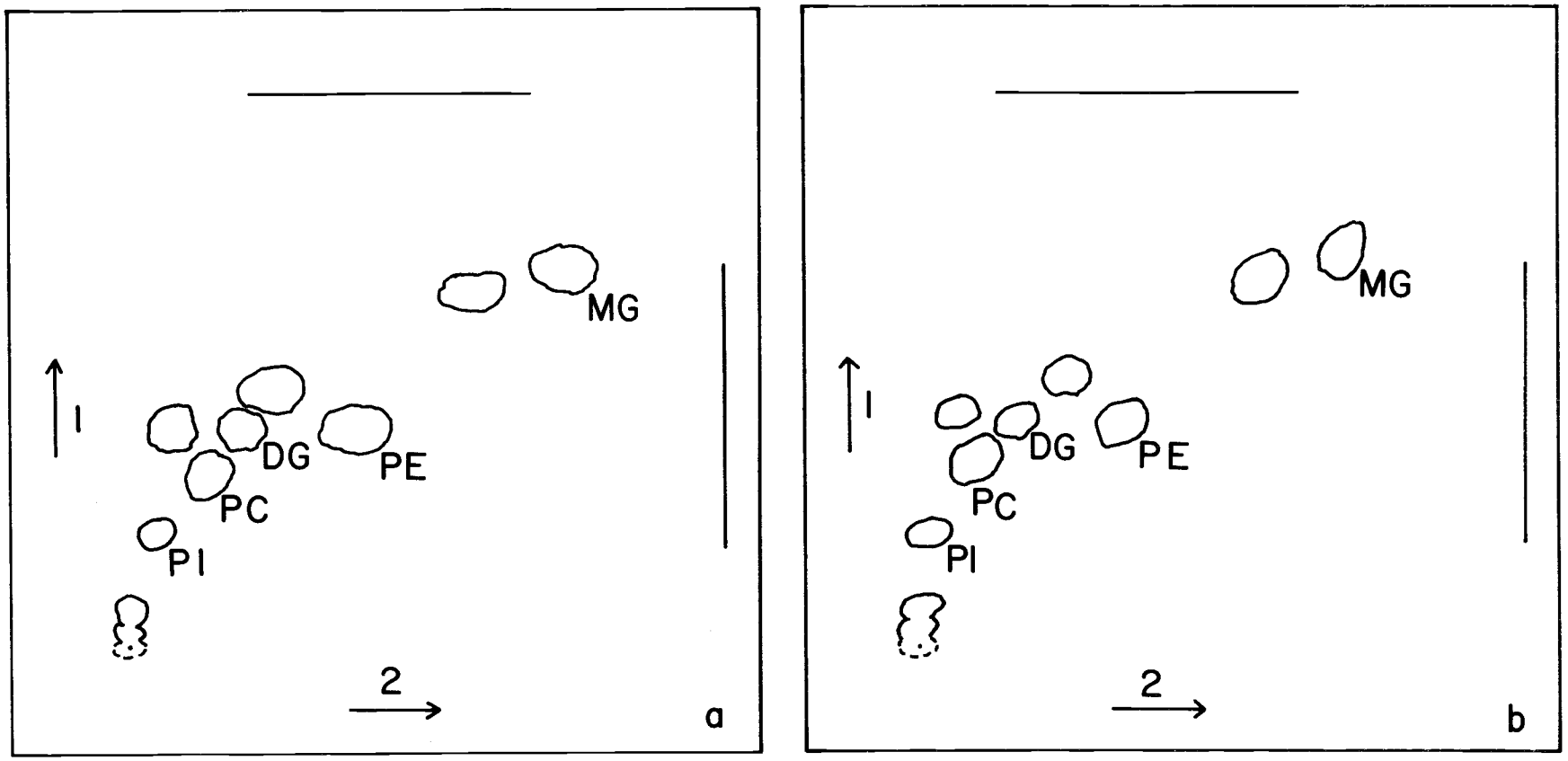


Figure 25. Two dimensional thin layer chromatographic analysis of a (1:1) $CHCl_3$-CH_3OH extract of (a) mutant PS-28, and (b) wild-type Scenedesmus. Solvent direction 1 consisted of $CHCl_3$-CH_3OH-7 N NH_4OH (97.5:37:6), and solvent direction 2 consisted of $CHCl_3$-CH_3OH-CH_3CO_2-H_2O (85:12.5:12.5:2). Lipids were detected with iodine vapor and were identified with Supelco standards. MG-monogalactosyl diglyceride, DG-digalactosyl diglyceride, PE-phosphatidyl ethanolamine, PC-Phosphatidyl choline, PI-phosphatidyl inositol. Lipid degradation spots and pigmented regions have been deleted. For experimental details see Materials and Methods.

Table 7. Fatty acid levels of wild-type Scenedesmus and mutant PS-28.

Fatty acid levels of wild-type Scenedesmus and mutant PS-28 are expressed as a weight percent of the total fatty acid methyl esters detected. Two-day old samples were cultured as indicated, and irradiated samples were exposed to a field of white light (1.0×10^6 ergs/sec-cm^2) for 1 hr. Data presented below are similar to those obtained in 2 other experiments. For further experimental details see Materials and Methods.

Heterotrophic Samples

FAME[1]	WT	WTI[3]	PS-28	PS-28I[3]
16:0	21.0	17.0	19.5	17.1
16:1	10.0	8.0	6.4	3.6
18:0	0.3	3.4	0.9	5.5
18:1	51.0	40.4	49.9	42.7
16:4[2]	3.0	4.4	4.4	3.6
18:2	0.3	8.8	3.7	10.4
18:3	14.0	17.8	15.2	16.9
C20	tr[4]	tr	tr	tr
total	99.6	99.8	100.0	99.8

Mixotrophic Samples

FAME[1]	WT	WTI[3]	PS-28	PS-28I[3]
16:0	16.2	18.4	19.8	20.7
16:1	1.0	1.1	1.1	0.9
18:0	1.9	4.6	3.3	3.8
18:1	20.9	18.4	17.6	15.1
16:4[2]	2.8	2.3	2.2	1.1
18:2	24.8	25.3	27.5	32.0
18:3	32.4	29.9	28.6	26.4
C20	tr[4]	tr	tr	tr
total	100.0	100.0	100.1	100.0

[1] FAME - fatty acid methyl ester.

[2] Substance tentatively identified as hexadecatetranenoic acid methyl ester.

[3] WTI and PS-28I indicates that these samples have been treated with irradiation.

[4] tr indicates that a trace of this substance was detected.

membranes of the chloroplast, and their increases upon exposure to light are common in the green algae (Hulanicka, Erwin, and Block, 1964; Nichols and Appleby, 1969).

When heterotrophic samples of the wild-type or mutant were irradiated for one hour with high intensity light, a slight increase in the 18:2 and 18:3 fatty acids occurred. When mixotrophic samples of the wild-type or the mutant were treated similarly no noticeable alterations in the fatty acid patterns were observed.

The above results indicated that the photosynthetic membranes of the mutant were not under stress (free-radical attack) because of the absence of α-tocopherol. If free-radical catalyzed lipid peroxidation had occurred a decrease in the polyenoic fatty acids should have been observed. Therefore, it was likely that free-radical damage to chloroplast lipids did not occur in irradiated samples of mutant PS-28.

V. CONCLUSIONS

Reducing Side Mutants

Electron transport, which stems directly from water photolysis, can be represented as follows:

1) $Chla_{II} \longrightarrow Q \longrightarrow$ cytochrome b-559 $\longrightarrow$ plastoquinone A

P680 C550 (high potential)

2) $H_2O \longrightarrow \begin{matrix} Mn^{2+} \\ Cl^- \end{matrix} \longrightarrow Z \longrightarrow Chla_{II}$ P680

where reaction sequence (1) represents the reducing side of photosystem-II, and where reaction sequence (2) represents the oxidizing side of photosystem-II (Bishop, 1973).

Significant progress has been made in recent years towards understanding the electron transport processes of photosynthesis; however, several facets of this subject have resisted analysis. The mechanism of water photolysis during higher plant photosynthesis for one, has been a very difficult problem to examine because it is labile when under mechanical or chemical stress.

Several algal mutants of Scenedesmus and Chlamydomonas were isolated which lack photosystem-II activity (Levine, 1969; Bishop and Wong, 1971). Of the several dozen algal mutants examined, those blocked on the reducing side of photosystem-II were

characterized by the following well defined features: 1) Photosynthesis was absent, or was far below normal (Bishop and Wong, 1971). 2) Fluorescence levels were 2-3 times higher than normal, and the variable yield component was destroyed (Bishop and Wong, 1971). 3) The slow component of the EPR signal was absent (Weaver and Bishop, 1963). 4) The major component of the delayed light emission is not detectable (Bertsch, et al., 1967). 5) The Hill reaction using $NADP^+$, DCPIP, ferricyanide or p-benzoquinone as oxidants was not evident (Bishop and Wong, 1971). 6) The levels of chlorophyll and plastoquinone A were below normal (Bishop and Wong, 1971; Smillie and Levine, 1963). 7) There is recent evidence that high potential cytochrome b-559 is not observable in photosystem-II mutants (Epel and Butler, 1972). Because all reducing side photosystem-II mutants of Scenedesmus and Chlamydomonas share the above described features, it was suggested that these mutants are not blocked in a gene coding for a specific factor, but that the nature of the mutation is pleiotropic, one that effects the levels of several photosynthetic factors (Lavorel and Levine, 1968). Bishop (1973) has suggested that the genetic lesion common to reducing side mutants of photosystem-II, may have occurred in a structural gene. This structural gene would normally code for an essential polypeptide of the photosystem-II chloroplast subunit, and would be responsible for the assemblage

and incorporation of the photosystem-II unit (or a portion of the photosystem-II unit) into the thylakoid. However, at present there is no evidence to support this hypothesis (Thornber, 1975). A further possibility would be that when an essential electron transport protein functioning in photosystem-II is not synthesized, the entire subunit is not assembled. Finally, it is possible that the mutation leading to reducing side photosystem-II mutants has occurred in the translation process (probably at the ribosomal level).

Oxidizing Side Mutants

Mutant strains of Chlamydomonas were described recently that were blocked on the oxidizing side of photosystem-II (Butler, Epel and Levine, 1972; Epel and Butler, 1972). These mutants did not perform photosynthesis and have a low fluorescence level which lacks the variable yield component. Chloroplasts prepared from these mutants photoreduced $NADP^+$, and have a variable yield fluorescence if electrons were artificially supplied to photosystem-II with diphenylcarbizide. Butler, Epel and Levine (1972) reported that mutants blocked on the oxidizing side of photosystem-II lacked approximately one-half of the high potential cytochrome b-559 pool. No information has been published about the plastoquinone levels of these mutants. A more detailed analysis of mutants of this type would be highly desirable.

Mutants PS-28

Several lines of evidence point to the fact that PS-28 is a photosystem-II mutant. The mutant has impaired rates of photosynthesis (Table 1), hydrogen photoevolution (Figure 11), anaerobic glucose photoassimilation (Figure 12), ferricyanide and DCPIP photoreduction (Table 4), and a high relative fluorescence level without a variable yield component (Figure 15). These features are similar to the characteristics described for mutants blocked on the reducing side of photosystem-II; however, there are other lines of evidence which do not support this conclusion. In contrast to the other reducing side photosystem-II mutants of Scenedesmus, PS-28 has normal heterotrophic levels of plastoquinone A and chlorophyll (Table 5). Furthermore, Bishop and Wong (1974) demonstrated that there were detectable levels of high potential cytochrome b-559 in chloroplast preparations of PS-28.

An additional feature unique to PS-28 is the absence of detectable levels of α-tocopherol and α-tocopheryl quinone in whole cell and chloroplast lipid extracts (Table 5). Bishop and Sicher (1974) and Sicher and Bishop (1975) suggested that there was a direct relationship between the lack of α-tocopherol and the loss of photosystem-II activity in mutant PS-28. Upon interpretation this means that PS-28 is not a typical reducing side photosystem-II mutant

demonstrating pleiotropism, but has a genetic block in a specific gene coding for a single factor, vitamin E. Unfortunately, it is not possible to establish this point unequivocably because of the difficulties in manipulating the sexual stages of Scenedesmus (Levine, 1969).

The light saturation kinetics of hydrogen photoreduction were identical for both mutant PS-28 and wild-type Scenedesmus (Figure 6). This observation provided the initial evidence that mutant PS-28 had a functional photosystem-I. Additional evidence supporting this finding came from studies of PMS-mediated photophosphorylation (Figure 14), the 518 nm absorbancy change (Figure 16), and the DCPIP-ascorbate to methylviologen photoreduction (Table 4). All of these photochemical events are dependent upon an active photosystem-I, and are found to be fully functional in PS-28. There are no indications that photosystem-I in the mutant is damaged by high intensity irradiation treatments.

Vitamin E and Greening Studies

The cellular level of α-tocopherol was equivalent in dark grown cultures of mutant C-2A' and wild-type Scenedesmus (Figure 23). This was a surprising result because the chloroplast in heterotrophic samples of C-2A' has only completed partial development, and the levels of chlorophyll and photosynthesis were minimal. Therefore,

the synthesis of α-tocopherol in contrast to chlorophyll and plastoquinone A did not follow the development of photosynthesis in greening cultures of C-2A' (Figure 23). This finding suggested that α-tocopherol did not function in photosynthesis in the same manner as plastoquinone A.

One effort to explain why etiolated plant systems have normal levels of α-tocopherol was advanced by Lichtenthaler (1969). He believes that the reduced compounds α-tocopherol and plastohydroquinone are present in the stroma of the chloroplast in lipophilic globules (the plastoglobuli). The oxidized forms of these compounds, α-tocopheryl quinone and plastoquinone, are not in the plastoglobuli but function in photosynthetic electron transport and are found in the thylakoids. Unpublished observations from our laboratory did not support the interpretations of Lichtenthaler (1969). Firstly, the presence of plastohydroquinone could not be demonstrated in dark grown cells of wild-type Scenedesmus. Secondly, early log phase cultures of wild-type Scenedesmus do not form appreciable amounts of plastoglobuli as observed in electron micrographs or by isolation techniques. Attempts to measure the levels of plastoquinone A and α-tocopherol in lipid extracts of wild-type chloroplast preparations (which are free of plastoglobuli) were unsuccessful because these compounds virtually disappeared either because of their auto-oxidizability during cell homogenization or due to enzymatic

degradation. A further analysis of this problem in plant material from which whole chloroplasts can be isolated would be desirable.

Function of Vitamin E as an Antioxidant

In either mixotrophic or hetrotrophic samples of the mutant photosynthesis can be destroyed by exposure of the cells to high intensity irradiation (10^6 ergs/sec-cm^2). This photoinhibition is proportional to the incident light intensity and only occurs in the presence of oxygen (Figure 3). These observations suggest that destruction of photosynthesis by high intensity irradiation proceeds by a photodynamic process, involving either a singlet or free-radical oxygen mechanism. Bishop and Sicher (1974) and Sicher and Bishop (1975) suggested that the loss of photosynthesis during high intensity light treatment in PS-28 was caused by the absence of α-tocopherol; therefore, the function of α-tocopherol in the chloroplast would be antioxygenic (to either scavenge free-radicals or to quench singlet oxygen; see Introduction). However, evidence presented in this thesis did not support this conclusion. Firstly, the lipid and fatty acid compositions of the mutant were normal (Figure 25a and 25b) and the level of polyunsaturated fatty acids were stable to extended treatments of high intensity irradiation (Table 7). Secondly, α-tocopherol, α-tocopheryl acetate and the two synthetic antioxidants, N,N'-diphenyl-p-phenylenediamine and nordihydroguaiaretic acid, were

not effective in reversing the mutation or in protecting the mutant from photoinactivation (Figure 4, and 5). Thirdly, the vitamin C levels of the mutant were normal in both dark and light grown cultures (Table 6). Further evidence that vitamin E did not function as a general lipid antioxidant during photosynthesis was obtained from light sensitivity studies on several photosystem-II mutants which were only partially blocked in photosynthesis. These algal strains possess normal levels of vitamin E and were all susceptible to photoinhibition by high intensity irradiation (unpublished observations). It was concluded from these lines of evidence that the absence of α-tocopherol in mutant PS-28 was not directly responsible for the destruction of photosynthesis by high intensity light treatment. At present the evidence does not support the hypothesis that vitamin E functions during photosynthesis as a general membrane antioxidant. However, it is still possible that vitamin E functions as a site specific antioxidant, but there are no data available from Scenedesmus or other green plant species on this subject.

Other Possible Functions of Vitamin E

There is no direct evidence to suggest that vitamin E is an electron transport carrier during photosynthesis. Mixotrophic cultures of PS-28 assayed in early log phase demonstrate rates of oxygen evolution as high as 60% of normal. This evidence did not

support the contention that α-tocopherol or α-tocopheryl quinone (which were never observed in the mutant) participated in photosynthetic electron transport processes. Additionally, the spectral evidence obtained by Stiehl and Witt (1968) using sensitive ultraviolet flash photometric techniques indicated that a single chloroplast quinone participated in photosynthetic electron transport. The in vivo spectrum obtained by Stiehl and Witt (1968) has a peak at 263 nm. This closely matched the in vitro spectrum of the plastoquinones and the tocopheryl quinones. Because the in vivo spectrum lacked fine structure, and because of pool size measurements, Stiehl and Witt (1968) contended that the light-dark difference spectrum arises from plastoquinone A.

A further observation that argues against the participation of vitamin E in photosynthetic electron transport, stemed from the finding that the blue-green algae, lack α-tocopherol (Hirayama, 1967). This is in spite of their primitive procaryotic nature, possess photosynthetic activities and a chemical composition similar to the other classes green plants. In work performed in this laboratory, no vitamin E was detected in 16 species of Anabaena, and in 3 other Anabaena species only a trace of α-tocopherol was found. The tocopherols also were not observed in the bacteria (Lester and Crane, 1959). Therefore, it is probable that the distribution of vitamin E is restricted to the eucaryotes.

The chloroplast ultrastructure of the blue-green algae (Menke, 1961) differs considerably from that of the higher plants (Weier, et al., 1963). The principle difference being that higher plant chloroplasts form grana, regularly ordered, stacked discs. The lamellar systems of the blue-green algae do not form grana. Therefore, it is possible that α-tocopherol is important in the stacking of chloroplast membranes. Preliminary ultrastructural evidence obtained from studies on PS-28 indicate that this concept may be correct.

The principle evidence suggesting that vitamin E and related substances function in photosynthesis as electron transport carriers comes from extraction and re-addition experiments (Bishop, 1959; Trebst, 1963). It was found that freeze-dried chloroplasts extracted with various organic solvents lost their capacity for the Hill reaction, however, partial restoration of photochemical activity was obtained upon re-addition of the crude extract, or by addition of plastoquinone.

Subsequent and more detailed analyses have produced far more complicated results. It was found that photosystem-I activity could be inactivated by extraction, and activity could be restored with different quinones (Henninger and Crane, 1963). In one instance photochemical activity was restored to freeze-dried chloroplast that had been extracted with acetone, a procedure that removed 90% of the chlorophyll among other substances, with mixtures of β-tocopherol

quinone and α-tocopheryl quinone or plastoquinone A. These mixtures were more efficient in restoring the Hill reaction to acetone extracted chloroplasts than plastoquinone A or B alone, and the overall rate of activity obtained exceeded that of the unextracted freeze-dried chloroplasts, (Dilley, Henninger, and Crane, 1963).

These results were interpreted as evidence that plastoquinones and possibly other quinones function at specific sites in the electron transport chain. However, this interpretation was not supported by spectrophotometric evidence (Stiehl and Witt, 1968), and because of the non-specific nature of extraction and re-addition experiments it was not known that the quinone has returned to its original site in the membrane. Perhaps the re-addition of an isoprenoid quinone to the extracted membranes partially restores a structural requirement, thus allowing photochemical activity.

Data obtained from PS-28 did not support the suggestion that vitamin E participates in photophosphorylation. Hydrogen photoreduction, which is an ATP requiring reaction, is normal in the mutant (Figure 7). Secondly, the rates of PMS-mediated photophosphorylation obtained with chloroplasts prepared from PS-28 and wild-type Scenedesmus were comparable (Figure 14). Although the rates of anaerobic glucose photoassimilation were decreased in PS-28 (Figure 13), evidence presented here, Figure 14, and elsewhere (Tanner, Daschel and Kandler, 1965), suggested that this was

due to impaired activities of photosystem-II and not of photophosphorylation.

It is possible that α-tocopherol is a structural lipid. The lipids of the photosynthetic membrane are divided into two specific types according to the lipoprotein fluid mosaic model of Singer (1974). The first type, boundary lipids, are closely associated with the surfaces of globular membrane proteins. The second type, matrix lipids, occur in the membrane in molecular bilayers and are freely translational (hence, membrane fluidity). This feature allows the membrane to change conformations and still retain attachment sites for affiliated proteins. α-Tocopherol does not appear to function as a general lipid antioxidant, and for this reason it probably performs a more specific function, i.e., as a boundary lipid.

There are three major polypeptide groupings in the chloroplast, and these are associated with the photosystem-I complex, the photosystem-II complex, and the light-harvesting-pigment-protein complex. Photosystem-I functions normally in PS-28, therefore, it is not likely that it has any connection with vitamin E. This finding contradicts the proposals of Baszynski (1974) who demonstrated that α-tocopherol restored photosystem-I activity to acetone extracted freeze-dried spinach chloroplasts.

Several chloroplast dissociation procedures demonstrated that two main subchloroplast particles are obtained by fractionation

(Anderson, 1975). The first subchloroplast particle was enriched in photosystem-I and the second was enriched in both photosystem-II and the light-harvesting-pigment-protein complex. In fact, prior to the recognition of the existence of the chlorophyll a/b, 1:1, light-harvesting-pigment-protein complex it was believed that chlorophyll b was associated with photosystem-II. There is every indication that the light-harvesting-pigment-protein complex in vivo is closely associated with photosystem-II. Therefore it is likely that α-tocopherol is associated with either the photosystem-II complex, the light-harvesting-pigment-protein complex, or with the resultant subchloroplast aggregate. In a brief and early investigation Lichtenthler (1969) found that vitamin E was enriched in the photosystem-II complex upon chloroplast fractionation but that the photosystem-I particles contained a small portion of the compound. In the light of the recent advances in this field a more detailed and modern investigation of this subject would be desirable.

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