LBL-343 c2

THE PHOTOCHEMISTRY AND KINETICS OF ELECTRON TRANSPORT AND THE PIGMENT SYSTEMS IN CHLOROPLASTS

TWO-WEEK LOAN COPY

This is a Library Circulating Copy which may be borrowed for two weeks. For a personal retention copy, call Tech. Info. Division, Ext. 5545

Alexander Shih-Kaung Sun
(Ph. D. Thesis)

July 1971

AEC Contract No. W-7405-eng-48

48

DISCLAIMER

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor the Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or the Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or the Regents of the University of California.

THE PHOTOCHEMISTRY AND KINETICS OF ELECTRON TRANSPORT AND THE PIGMENT SYSTEMS IN CHLOROPLASTS

Contents

Abstract -v-

Acknowledgements -viii-

Abbreviations -x-

Dedication -xi-

I. General Introduction: A Brief Survey of Photosynthesis 1

II. Quantum Requirements for the Two Light Reactions in Spinach Chloroplasts 12

A. Introduction 12

B. Material and Methods 17

C. Results 21

1. Photosystem I: [Ascorbate + $DPIPH_2$ → NADP] reaction with no Pcy or Fp 21

2. Photosystem I: [Ascorbate + $DPIPH_2$ → NADP] reaction using saturated amount of Pcy and Fp 30

3. Photosystem II: [H_2O → DPIP] reaction 35

4. Photosystem (I + II): [H_2O → NADP] reaction 43

D. Discussion 44

Contents (continued)

III. Emerson Enhancement in Broken Spinach Chloroplasts 59

A. Introduction 59

B. Material and Methods 62

C. Results 63

1. [H_2O → NADP] reaction and enhancement studies 63

2. Photosystem II [H_2O → DPIP] reaction and photosystem I [Ascorbate + $DPIPH_2$ → NADP] reaction 88

D. Discussion 89

IV. Concluding Remarks 101

V. References 105

THE PHOTOCHEMISTRY AND KINETICS OF ELECTRON TRANSPORT AND THE PIGMENT SYSTEMS IN CHLOROPLASTS

Alexander Shih-Kaung Sun

Laboratory of Chemical Biodynamics
Lawrence Radiation Laboratory
University of California
Berkeley, California

July 1971

ABSTRACT

From the studies of electron-transport reactions of spinach chloroplasts, we have been able to demonstrate that the quantum requirements are 1.0 ± 0.05 at the wavelengths above 700 nm of actinic light and 1.5 - 2.5 for wavelengths from 620 to 680 nm when the photoreduction of NADP by chloroplasts was measured either aerobically or anaerobically in a 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea (DCMU) poisoned system when ascorbate and 2,6-dichlorophenolindophenol (DPIP) were present as electron donors. The quantum requirements are 1.0 ± 0.05 in the wavelength range from 630 to 660 nm of actinic light when the photoreduction of DPIP by chloroplasts was measured with water as the electron donor. Those of the photoreduction of NADP when H_2O was used as the electron donor are 2.0 ± 0.1 in the wavelength range from 620 to 678 nm of actinic light.

For each reaction, optimal amounts of reagents and cofactors were used, and quantum requirements were extrapolated to zero intensity. The rate of the [ascorbate + $DPIPH_2 \rightarrow$ NADP] reaction can

be increased more than four times when saturating amounts of plastocyanin and ferredoxin-NADP reductase were added. The rate of the DPIP Hill reaction was increased more than three times by added $MgCl_2$ and plastocyanin, but not by ferredoxin or ferredoxin-NADP reductase. The rate of the [$H_2O \rightarrow$ NADP] reaction with saturated ferredoxin can be further increased more than three times when both plastocyanin and ferredoxin-NADP reductase were added.

The effect of antimycin A on the [ascorbate + $DPIPH_2 \rightarrow$ NADP] reaction was studied for mole ratios of antimycin A/chlorophyll from 0.02 to 2.0. It was found that the photoreduction rate of NADP was always slowed down and therefore the quantum requirement of this system was increased, even at the lowest concentration of antimycin A studied. This concentration is much lower than the value of antimycin A/chlorophyll = 0.33, at which the cyclic photophosphorylation is reported to be completely inhibited but non-cyclic photophosphorylation is not affected. We interpret the effect on the efficiency of non-cyclic electron transport of photosystem I to result from a competition with cyclic electron flow, which has become accelerated through an uncoupling action of antimycin A. Ethanol, the most commonly used solvent for inhibitors like antimycin A, at concentrations lower than 1% in the reaction mixture, itself decreases the rate of the photoreduction of NADP at high light intensities but does not change the primary quantum requirement of this system. Ethanol is also found to have an inhibitory effect at concentrations above 1% in the reaction mixture.

The Emerson enhancement effect using red illumination supplemented by far red light is a characteristic phenomenon of photosynthetic oxygen evolution by plants and algae. It has been cited as an important evidence in support of the mechanism of photosynthetic electron transport involving two light reactions operating in series. The present study confirms the occurrence of enhancement in isolated, broken spinach chloroplasts for the photoreduction of $NADP^+$ by water: $[H_2O \rightarrow NADP]$ reaction. Far red light at 700 nm is supplemented optimally with wavelengths of 650 or 670 nm. Divalent cations such as magnesium or manganese are shown to be required for enhancement to occur. The optimum concentrations of added $MgCl_2$ or $MnCl_2$ are about 7.5 mM; at concentrations below 3 mM enhancement is not obtained. The critical dependence on divalent ion concentration is felt to be the reason why the enhancement phenomenon has not been observed in some previous studies using broken chloroplasts. A role for magnesium ion is proposed in which it alters the structure of the active chloroplast membranes in a manner which controls the transfer of electronic excitation between the two photosynthetic pigment systems. These findings favor the series two-light reaction mechanism over the alternative parallel scheme.

ACKNOWLEDGEMENT

I wish to express my deepest gratitude to Dr. Kenneth Sauer, under whose guidance the work reported in this thesis was performed. His advice and originality, his precise and creative approaches to the problem, his patience and encouragement, not only have made my years as a graduate student both instructive and fruitful, but also set the best example for my future career.

I would like to thank Dr. Melvin Calvin for providing me a stimulating multidisciplinary environment, for his constant interest and encouragement, for present the example of an inquiring and original mind.

I would also like to thank Dr. Roderic B. Park for his critical suggestions which have increased the precision of my work.

To Dr. Bud Kramer, Mr. Gerald Babcock, Dr. Helmut Tributsch, for their stimulating discussion and reading part of the manuscript, for their help in isolation of enzymes, for their friendship, all these have become an integral part of my happy years staying in the laboratory.

To Dr. Jeff Kelly for helping me start the first part of this investigation.

To Miss Evie Litton, Mrs. Johanna Onffroy and Mr. Bill McAllister for providing invaluable personal and technical assistance.

To my wife, Lily, for being wife and colleague, for providing stimulating discussion and technical assistance, for sharing with

me my happiness and for sacrificing her own desires and her own work, for numerous little things she did for me, I express my gratitude and my love.

Finally, to my parents, for their unconditional support even when they needed me to take over part of the family responsibility, for continuous inspiration and encouragement. I am particularly in debt to my father, who encouraged me toward science and always told me early in my childhood stories of great devoted scientists and ancient successful men. In his last two years, the excitement in my study became his daily conversation; he had always deeply shared and been involved in the course of this investigation.

This work was supported by the United States Atomic Energy Commission.

ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Chl	Chlorophylls
CP	Chloroplasts
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DCIP	2,6-dichlorophenolindophenol
Fd	Ferredoxin
FMN	Flavin mononucleotide
Fp	Flavoprotein (Fd-NADP reductase)
NADP	Nicotinamide adenine dinucleotide phosphate
$NADPH_2$	Nicotinamide adenine dinucleotide phosphate, reduced form
Pcy	Plastocyanin
P I & P II	Pigment system I and pigment system II
PMS	Phenazine methosulfate
PS I	Photosystem I
PS II	Photosystem II
RC I	Reaction center I
RC II	Reaction center II
Tricine	N-tris (hydroxymethyl) methylglycine
Tris	Tris (hydroxymethyl) amino methane

I. GENERAL INTRODUCTION

Literally, photosynthesis means "synthesis by means of light". This term is usually applied only to the synthesis of organic matter by plants in light. Photosynthesis creates living matter out of lifeless inorganic materials, replenishes the oxygen in the atmosphere, and stores the energy of sunlight to support the life activities of organisms. Its discovery is the most important chapter in the history of science.

Early in this century photosynthesis was usually described by a single overall reaction:

$$CO_2 + H_2O \xrightarrow[\text{(chloroplasts)}]{\text{light}} (CH_2O) + O_2 \qquad \text{(I-1)}$$

In this reaction, CO_2 is absorbed and O_2 is evolved by plants, (CH_2O) represents a basic subunit of carbohydrate, the main product of photosynthesis that is stored in plants. In plants this process occurs in organized subcellular bodies called "chloroplasts". The energy needed for this reaction is obtained from light absorbed by chlorophylls in chloroplasts. In the 1950's, through the efforts of M. Calvin et al. (Bassham and Calvin, 1957), the chemistry of the photosynthetic CO_2 fixation in algae has been almost fully elucidated. Two things are needed for this chemistry: reducing power in the form of $NADPH_2$, and energy in the form of ATP.

In 1939, Hill (Hill, 1939) demonstrated that illuminated chloroplasts can evolve O_2 provided that a suitable electron acceptor is present. The reaction is a light-driven transfer of electrons from H_2O to the electron acceptor, which replaces CO_2 as the ultimate recipient of photochemical reducing power. The main singificance of the Hill reaction is that CO_2 reduction can be separated from the photochemical part of photosynthesis. However, Engleman (1883) found that photosynthetic bacteria could reduce CO_2 at the expense of the light energy, without the evolution of O_2, i.e. the evolution of O_2 can be separated from photosynthesis. This means that there is a central photochemical event in photosynthesis that can be separated from both end products: the evolution of O_2 and the assimilation of CO_2. The discovery of photosynthetic bacteria (Engleman, 1883) which do not evolve oxygen, but still use sunlight to assimilate CO_2, led Van Niel (1931) to formulate photosynthesis as the splitting of H_2O to yield (H) and (OH):

$$H_2O \xrightarrow{2\,h\nu} (H) + (OH) \longrightarrow \frac{1}{2} H_2O + \frac{1}{4} O_2 \qquad \text{(I-2)}$$

He suggested that 2 quanta are needed for a single photolytic act, or 8 quanta for O_2.

In 1954, Arnon et al. (1954, 1957) found that illuminated chloroplasts can reduce NADP to $NADPH_2$ and phosphorylate ADP to ATP; both $NADPH_2$ and ATP are needed for the carbon assimilation in the Calvin cycle. Since then the photoreduction of NADP and the phosphorylation of ADP have been considered as the central photochemical

events in photosynthesis. We call the physical part in chloroplasts, where this photochemical event occurs, the electron transport chain. The mechanism of this electron transport chain and its related reactions have become a major topic in photosynthesis research for the past decade. Physical and chemical approaches to this topic have been so extensively explored that it is beyond one's ability to know all aspects of photosynthesis in detail today. But a scientist in the field should always be acquainted with all the up-to-date developments in this field. After a survey of the literature in the past decade, I feel that the field of photosynthesis today should be classified into the following categories: (1) primary photochemistry and energy transfer, (2) electron transport mechanism, (3) structural aspects, (4) photophosphorylation and ion transport, and (5) oxygen evolution. The research of this thesis is primarily concerned with the primary photochemistry, energy transfer and electron transport mechanism. A brief summary of the present knowledge of photosynthesis is given as follows.

(1) Primary photochemistry and energy transfer

The purpose of research in this area is to understand the mechanism by which photons are first absorbed by chlorophylls, transferred to different locations in the chloroplasts and then transformed to chemically stable forms of energy. Emerson and Arnold (1932) measured the O_2-evolution in Chlorella in response to brief (10^{-5} sec) flashes of light. These experiments showed that maximum yield per flash was about one O_2 evolved for every

2500 Chl molecules present, or about 300 Chl molecules per quantum absorbed (suuposing 8 quanta per one molecule of O_2 evolved, as discussed before). These experiments suggest that Chlorella contains photosynthetic units. Each unit consists of a chemical reaction center served by 300 "antenna" light harvesting Chl molecules. Quanta of energy absorbed anywhere in the light-harvesting Chl's are collected efficiently by the reaction center, where they are converted into the first stable products of photosynthesis. In the last decade, evidence has accumulated which shows that an essential part of the reaction center is a special form of Chl a, or Chl a in a specialized environment associated with a small number of molecules that can be oxidized or reduced (Clayton, 1962; Kok, 1961). In 1961 Kok found a pigment in higher plant chloroplasts which exhibited reversible light-induced absorption changes at 430 nm and 700 nm. This pigment, termed P700, behaves as a one-electron transferring agent in the dark with E_0' of +0.43 volt. The reduced form of the molecule is stable in the dark, is oxidized upon illumination with far-red light (PS I) and its reduction by light of short wavelength is inhibited by DCMU. This is considered as reaction center I (RC I). Kelly and Sauer (1968) demonstrated that there are two effective photosynthetic units of different sizes in spinach chloroplasts; one is a PS I unit containing 445 Chl molecules, the other is a PS II unit associated with 55 Chl molecules. (Please see the definition of PS I and PS II in the following section.) The trapping molecule for PS II has not been fully characterized.

There have also been a number of hypotheses discussing the mechanism of the transfer of the absorbed energy from antenna Chl molecules to the reaction centers. It is generally accepted that the migration of electronic excitation energy is the mechanism by which the energy reaches the reaction center (Franck, 1962; Rabinowitch, 1957). The detailed mechanism of reaction centers and the migration of electronic excitation energy in the photosynthetic unit (PSU) are the subjects of much current research.

(2) Electron transport mechanism

The research in this area is to understand how an electron is derived from H_2O, transferred through the electron-transport chain and finally accepted by NADP. It was shown (Hoch and Martin, 1964; Sauer and Biggins, 1965) that the quantum efficiency for photoreduction of NADP with far-red light when H_2O served as the electron donor was much lower than with red-light. On the contrary, the quantum efficiency for the photoreduction of NADP with far-red light approached one quantum per electron transferred when ascorbate (+ $DPIPH_2$) served as the electron donor. Since far-red light has been shown to be absorbed almost exclusively by photosystem I, but red light absorbed by both PS I and PS II, these experiments suggest that the [$H_2O \rightarrow$ NADP] reaction requires the participation of both PS I and PS II, while the [ascorbate + $DPIPH_2 \rightarrow$ NADP] reaction requires only photosystem I light. Govindjee et al.(1962,1964) demonstrated that the rate of NADP reduction by chloroplasts illuminated simultaneously with red and far-red light was higher than the sum of the rate of the same chloroplasts illuminated separately by red and far-red light. Mathematically, enhancement values higher

$$Rate_{R + FR} > Rate_R + Rate_{FR}$$

than one indicate a cooperation between the two photosystems, and therefore have been interpreted as indicating the participation of both photosystems. However, the absence of an enhancement effect for the [$H_2O \rightarrow$ NADP] reaction has also been reported (Gibbs et al., 1963; McSwain et al., 1968). Together with the evidence that PS II may comprise two rather than one short-wavelength light reactions (Knaff and Arnon, 1969), Knaff and Arnon proposed that there are three light reactions in chloroplasts, two in PS II, i.e. PS IIa and PS IIb and one in PS I. Whether PS I and PS II behave cooperatively and how many light reactions there are in chloroplasts have been among the most controversial topics in the past few years.

(3) Structural aspects of chloroplasts

The photosynthetic unit (Emerson and Arnold, 1932) is a physiological unit of function defined by experiments with intact plants. Thomas et al. (1953) attempted to find the morphological expression of the physiological photosynthetic unit. They found that chloroplast lamellar fragments as small as 100 Å in diameter were still active in the Hill reaction. The shadow-cast preparation of isolated chloroplasts (thylakoids) lamellae under the electron microscope revealed a repeating structure on the inner surface of this membrane. These repeating structures are called quantasomes (Park and Biggins, 1964). The quantasome particle is 180 Å by 160 Å and 100 Å thick, with a molecular weight of 2.1×10^6, and contains

about 230 Chl molecules. It is considered the morphological unit corresponding to the physiological photosynthetic unit.

The light phase of photosynthesis is performed in the internal membranes (thylakoids) of chloroplasts. This means that either PS I and PS II are an integral part of the membranes, or they are structures which are intimately associated with the membranes. Another approach to the understanding of the molecular organization of the photochemical systems is to fragment the membranes selectively so as to release specific components or complexes of the photosynthetic electron transport chain, and to study the properties of the isolated complexes. Using digitonin and differential centrifugation, Boardman and Anderson (1964) fragmented chloroplasts and separated fragments of varying size and with different proportions of Chl a and Chl b. The larger fragments which were sedimented by forces of 1000 g for 10 min (D-1) and 10,000 g for 30 min (D-10) had lower Chl a/Chl b ratios (2.2 - 2.4) than the chloroplasts (Chl a/Chl b = 2.8), whereas the smaller fragments sedimentable by 144,000 g for 60 min (D-144) had a much higher ratio of Chl a/Chl b = 6.0. D-1 and D-10 were active in the Hill reaction with either ferricyanide or trichlorophenolindophenol as oxidant, but rates of reduction of NADP were considerably below those obtained with ferricyanide or trichlorophenolindophenol. D-144 was completely inactive in the Hill reaction, but it reduced NADP at a high rate if supplied with an electron donor "ascorbate + $DCIPH_2$" and with an enzyme preparation containing ferredoxin, and some ferredoxin-NADP reductase and plastocyanin. The small

fragments had the photochemical properties of PS I, whereas the large fragments were enriched in PS II.

To avoid unknown artificial effects introduced by detergent, Sane et al. (1970) used a French press to disrupt spinach chloroplasts by shearing and subsequently fractionating the fragment with fractional centrifugation. The Chl a/Chl b ratio, absorption and difference spectra, cytochrome and Mn distribution, P700 content and photochemical activities of these fractions are remarkably similar to digitonin fractions obtained by Anderson and Boardman (1966). Electron micrographs obtained by thin sectioning, freeze fracturing and deep etching of the French press fractions showed that the PS I fraction originated from stroma lamellae and possibly end grana membranes, whereas the PS II fraction originated from the grana regions. This led them to suggest that there are two kinds of PS I: one present in stroma lamellae and physically separated from PS II, and a second PS I present in grana regions and probably in closer association with PS II. The PS II fraction they obtained has both PS I and PS II activity and the enrichment in PS II activity is merely due to loss of stroma lamella containing only PS I.

The knowledge of the structure of chloroplast lamellae enables us not only to understand the biochemical and photochemical functions of the chloroplast in relation to its structure, but also to formulate a model explaining the interaction between PS I and PS II. Anderson and Boardman (1966) suggested that their results on the digitonin fractionated chloroplast lamellae were in

support of the Hill-Bendall's series scheme of PS I and PS II. But Sane et al. (1971), based on the results from the French-pressed and fractionated chloroplasts lamellae membrane, suggested that PS I in stroma is separate from and operates in parallel with PS II and PS I in the grana region. The stroma PS I and the electron transport chain in grana will be coupled if there is an electron carrier existing between them (Park and Sane, 1971). How this coupling occurs and the property of the electron carrier are the subjects of future research.

(4) Photophosphorylation and ion transport

That light energy absorbed by a photosynthetic system could lead to the esterification of inorganic phosphate into ATP was first demonstrated by Frenkel (1954) with bacterial chromatophore fragments. Arnon et al. (1954) were able to carry out the same reaction with chloroplasts. Two types of photophosphorylation have been discovered in isolated chloroplasts, i.e. cyclic and non-cyclic photophosphorylation. In a cyclic photophosphorylation the absorption of radiant energy generates only ATP and neither a reductant nor an oxidant is produced (Tagawa et al., 1963). It is insensitive to DCMU and proceeds more efficiently in far-red light than in red light. Thus it is generally considered that cyclic photophosphorylation is associated with PS I alone. In non-cyclic photophosphorylation an added electron acceptor is reduced, oxygen is evolved and ATP is formed simultaneously. The electron acceptor, which catalyzes the non-cyclic photophosphorylation, could either be NADP, ferricyanide, DCIP or cytochrome c (Avron, 1967). The

participation of PS I and PS II in the photoreduction of NADP by isolated chloroplasts, but only PS II in the photoreduction of ferricyanide (Avron, 1967), suggested that the site of non-cyclic photophosphorylation might be in between PS I and PS II, as shown in Figure 15.

It has been suggested (Chance, 1956; Slater, 1953, 1966) that phosphorylation in chloroplasts, mitochondria, and bacterial chromatophores is linked to the electron transport chain by a "high energy" chemical intermediate. Ion movements observed with electron transport activity have been considered as secondary or competing events associated with the dissipation of an as yet unidentified high energy chemical intermediate (∿) linking electron transport to phosphorylation. Direct evidence for the existence of this chemical intermediate is lacking. Mitchell (1961, 1966) has proposed an alternative hypothesis involving ion movements directly in phosphorylation. His "chemiosmotic" proposal suggests that electron transport and phosphorylation are coupled indirectly through an electrochemical potential resulting from a gradient in the concentration of protons and other ions established across the membranes involved in the electron transport sequence of organelles such as chloroplasts. Electron transport creates this electrochemical potential which, in turn, is partly consumed in the formation of ATP. In 1963 Hind and Jagendorf (1963, 1965) discovered that H^+ influx occurs in response to the light-induced electron transport of chloroplasts and that a pH gradient induced by acid-base washing of chloroplasts generates ATP in the dark. Their

studies also showed that the kinetics of an indirectly measured intermediate of phosphorylation (X_E), produced upon illumination of chloroplasts, resembles qualitatively the kinetics of H^+ translocation in chloroplasts. At the present stage, the evidence shows that ion movements, and in particular H^+ movements, are directly linked to phosphorylation in that the buildup of the electrochemical potential resulting from H^+ movements across membranes of chloroplasts supply the energy required for the synthesis of ATP.

The major concept of photosynthesis today is primarily generated from the interpretation and hypothesis of the above experimental evidence. There are some other important approaches to the problem of photosynthesis, such as the flash-light studies on the oxygen evolution (Allen and Franck, 1955; Joliot et al., 1969; Kok et al., 1970), analysis of photosynthesis using mutant strains of algae and higher plants (Levine, 1969), isolation of bacteriochlorophyll-protein (Olson et al., 1969), etc. Because these topics are not relevant to the study in this thesis, they are not discussed here.

II. QUANTUM REQUIREMENTS FOR THE TWO LIGHT REACTIONS IN SPINACH CHLOROPLASTS

A. Introduction

The mechanism of the electron transport scheme in photosynthesis has been extensively investigated since the discovery of the Hill reaction (Hill, 1939). In early experiments, Emerson and Lewis (1942, 1943) found a sharp decrease in the efficiency of photosynthesis with actinic light of wavelengths longer than 685 nm for both Chlorella and Chroococcus (the Red Drop in efficiency). By adding a weak background of green light to the far-red light, Emerson et al. found that the combined wavelengths produced higher photosynthetic rates than the sum of those for the two lights used separately. The concept of enhancement and two light reaction (or Z scheme) thus arose from these experiments (Hill and Bendall, 1960). There are two contrasting pictures of the organization and function of the chlorophyll pigment systems, i.e. whether PS I and PS II are operating independently or cooperatively. This problem has been considered both at the level of the electronic excitation energy transfer and at the level of the electron transport mechanism.

The the first of these, Myers and Graham (1963) proposed the "separate package" model, in which each light reaction is thought to be associated with its own array of light-absorbing pigment

molecules, and no communication at the level of electronic excitation transfer is permitted between the two arrays. It is as if the two light reactions, each with associated pigment systems, are physically separated from one another. However, Duysens and Amesz (1962) proposed the "spillover" model, in which the pigment arrays are still distinct, but electronic transfer is permitted between them in order to make up for deficiencies in the activation of one or the other light reactions. The series two-light scheme of Hill and Bendall requires that both light reactions be driven at equal rates for optimum overall photosynthetic efficiency. An extreme modification of the "spillover" hypothesis, in which a major portion of the chlorophyll pigments is common to both photosystems, has been proposed by Avron and Ben-Hayyim (1969). In this common pool one finds all, or nearly all, of the bulk pigments absorbing in the red region; i.e. at wavelengths shorter than about 680 nm. A common feature of all of the models is the assignment of far-red ($\lambda > 700$ nm) absorption almost exclusively to PS I. This is thought to be responsible for the "red-drop" in the quantum yield of photosynthesis at long wavelengths.

At the level of the electron transport mechanism, the question is how many quanta are required to transfer one electron from H_2O to NADP, which is the basic photosynthetic electron transport reaction, as discussed in the first chapter in the text.

Recently Avron (1967) has reviewed that two quanta, one from each of the photosystems I and II, are required to transfer one electron from H_2O to NADP for operating both photosystems I and II in a

cooperative way, and one quantum is required when reduced $DPIPH_2$ (ascorbate + $DPIPH_2$) is the electron donor for operating photosystem I alone. In an alternative hypothesis, Arnon (1967) proposes that only one quantum is required to transfer one electron in each of the above photo-induced electron transport reactions and that electron transfer from H_2O to NADP or ferredoxin is driven by photosystem II alone. His latest hypothesis (Knaff and Arnon, 1969) suggested that there are three light reactions involved in photosynthesis; two are in photosystem II or short-wavelength light reactions, and one is in photosystem I or long-wavelength light reaction. The most direct way to test these hypotheses is to measure quantum requirements and also the action spectra of the various photosynthetic reactions. Unfortunately, the quantum requirement values reported previously were always higher than the values predicted by either of the hypotheses (Schwartz, 1965; McSwain and Arnon, 1968; Sauer and Biggins, 1965; Sauer and Park, 1965). No conclusion could be reached, as it is always possible to argue that the chloroplasts under investigation were partially inactivated during preparation and thus resulted in artificially high quantum requirements. Therefore, we feel that quantum requirement measurements need to be investigated again under improved experimental conditions.

The "separate package" and "spillover" models can also be tested experimentally in a similar way by which they predict different results. One approach is to make careful measurements of quantum yields at different wavelengths for each light reaction

operating in the absence of each other. According to the "separate package" hypothesis, photons absorbed by the pigment system of the functioning light reaction can be used photochemically, and those photons absorbed by the pigments of the non-operative photosystem will necessarily be wasted. The latter will result in fluorescence or heat, but never in any photochemistry. By contrast, in the "spillover" hypothesis, photons absorbed in either pigment system have, by virtue of the possibility of electronic excitation transfer, the potential ability to do photochemistry. As pointed out in a previous publication (Kelly and Sauer, 1965), the "separate package" hypothesis requires that at a given wavelength of activation the sum of the quantum yields for the two light reactions measured separately ($\phi_I + \phi_{II}$) can never exceed 1.0 equivalents-(einsteins absorbed)$^{-1}$. According to the "spillover" hypothesis, the sum of quantum yields may exceed 1.0, and may approach a limiting value of 2.0 in the case of perfectly efficient electronic excitation transfer.

Until recently, such studies of quantum yield spectra have not produced convincing evidence of sums exceeding 1.0 (Kelly and Sauer, 1965; Hoch and Martin, 1963; Sauer and Biggins, 1965). Unfortunately, this result was not conclusive, as it was again possible to argue that the chloroplasts under investigation were partially, and artificially, inactivated. Now Avron and Ben-Hayyim (1969) have presented experimental findings in which the quantum yield at 640 nm for the ferricyanide Hill reaction, probably associated solely with photosystem 2, is 1.0 and that for DCMU-blocked photoreduction of

NADP by ascorbate/$DPIPH_2$, a photosystem I reaction, is about 0.5. With diquat or FMN as the electron acceptor, even higher values were reported. Because these results appeared to be in conflict with findings previously reported from this laboratory, we undertook a new study of this question.

It has been reported that ferredoxin is easily oxidized in the air and that the cyclic, but not the non-cyclic, photophosphorylation is inhibited at low concentrations of antimycin A (Tagawa et al., 1963), desaspidin (Arnon et al., 1965), and 2,4-dinitrophenol (Tagawa et al., 1963). For example, 10 μg of antimycin A per 100 μg of chlorophyll (a + b) in the reaction mixture, or antimycin A/chlorophyll (a + b) = 0.165 inhibits 95% of the cyclic photophosphorylation, but the efficiency of non-cyclic photophosphorylation is not affected even at double this concentration of antimycin A (Arnon et al., 1964). Therefore, we used anaerobic conditions for the [ascorbate + $DPIPH_2$ → NADP] reaction to prevent the leak of electrons to oxygen via ferredoxin. We also expect that antimycin A may increase the quantum efficiency of non-cyclic NADP reduction if it behaves as an inhibitor to block the cyclic electron flow at low concentrations of antimycin A. On the other hand, if antimycin A serves as an uncoupler of cyclic photophosphorylation, it may function as does methylamine (Sauer and Park, 1965) in providing a bypass for electron flow that serves to speed up the rate-limiting step in the cyclic pathway. If the cyclic and non-cyclic pathways compete for photosystem I photons, then the uncoupling action of antimycin A should indirectly cause

a decrease in the rate of non-cyclic electron flow and an increase in quantum requirements should be observed in non-cyclic NADP reduction.

In this chapter, we present the action spectrum of the quantum requirements of the [ascorbate + $DPIPH_2 \rightarrow NADP$], [$H_2O \rightarrow DPIP$] and [$H_2O \rightarrow NADP$] reactions under our modified reaction conditions and the effect of antimycin A and ethanol, which is the most commonly used solvent for inhibitors, on the electron transport chain. An alternative approach to the above questions can be made by investigations of the Emerson enhancement effect on isolated chloroplasts. Results of such studies, which generally confirm the conclusions referred to above, are the subject of the next chapter.

B. Material and Methods

(1) Spinach and preparation of chloroplasts

Spinach (*Spinacia oleracea* var. early hybrid No. 7) grown in vermiculite in a growth chamber under controlled conditions similar to those of Sauer and Park (1965): light intensity approximately 3200 f-c in 10 hr light/14 hr dark cycles, temperature *ca*. 18°C, leaves harvested six to eight weeks after germination.

In order to obtain the maximum photochemical capability, the spinach leaves were picked two to six hours after the start of the illumination cycle in the growth chamber and just before the isolation of chloroplasts. The leaves were rinsed with cold distilled

water and ribs were removed, then stored at -20°C for 10 min. Ten g of leaves were homogenized between 10 to 15 sec in 50 ml of 0.5 M sucrose, 0.1 M tricine, pH 7.6 buffer in a Waring blendor. The resultant homogenate was strained through 8 layers of cheesecloth and centrifuged at 200 g for 1 min. The supernatant was then centrifuged at 1000 g for 10 min and the precipitate was resuspended in 1 ml of 0.5 M sucrose, 0.05 M tricine, pH 7.6, and stored at 0°C. All the above procedures were carried out at 0°C. The best activity of the chloroplasts could be obtained within 6 hours after isolation.

(2) Reagents

NADP was obtained from P-L Biochemical, Inc., Milwaukee, Wisconsin; DPIP and tricine from Sigma Chemical Co., St. Louis, Missouri; ethanol (200 proof) from Commercial Solvents Corp., Terre Haute, Indiana; antimycin A and sodium ascorbate from Calbiochem, Los Angeles, Calif.; sucrose from J. T. Baker Chemical Co., New Jersey; DCMU from duPont de Nemours, Wilmington, Delaware; nitrogen directly from the liquid nitrogen tank obtained from Pacific Oxygen Co., Oakland, Calif.

(3) Apparatus and light intensity measurements

Experiments were carried out using a modified Cary Model 14 spectrophotometer, as described by Sauer and Biggins (1965) for photoreduction of NADP, and by Sauer and Park (1965) for photoreduction of DPIP. Measurements of light intensity were based on secondary standard lamps obtained from the National Bureau of

Standards, Washington, D.C., in the same way as Sauer and Biggins (1965). The quantity of light absorbed by chloroplasts was measured using the same method as Sauer and Biggins (1965).

(4) Preparation of ferredoxin, NADP reductase and plastocyanin

Ferredoxin was prepared from commercial spinach according to the procedures of Tagawa and Arnon (1962). The ratio of absorbances at $\frac{420\ nm}{276\ nm}$ of purified ferredoxin was 0.445. NADP reductase and plastocyanin were isolated in the same preparation as was ferredoxin (Tagawa and Arnon, 1962), but NADP reductase was fractionated from the DEAE cellulose column at the concentration of $(Cl^-) = 0.12$ mole-ℓ^{-1} and plastocyanin at $(Cl^-) = 0.26$ mole-ℓ^{-1}. Both NADP reductase and plastocyanin were used without further purification with ammonium sulfate.

(5) Reaction mixture

The reaction mixture, for the [ascorbate + $DPIPH_2 \rightarrow$ NADP] reaction, contained the following in µmoles/ml: tricine (pH = 7.5), 45; $MgCl_2$, 7.5; DPIP, 0.053; DCMU, 0.01; sodium ascorbate, 5.0; NADP, 0.67; ferredoxin, 100 µg/3 ml; NADP reductase, saturating amount or not added; plastocyanin, saturating amount or not added; antimycin A, varied; ethanol, varied; antimycin A was dissolved in ethanol; chlorophyll, 40 µg/3 ml.

The reaction mixture for the [$H_2O \rightarrow$ DPIP] reaction contained the following in µmoles/ml: tricine (pH = 7.5), 45; $MgCl_2$, 4.5; DPIP, 0.053; plastocyanin, saturating amount; chlorophyll, 40 µg/3 ml.

The reaction mixture for the [$H_2O \rightarrow$ NADP] reaction contained the following in µmoles/ml: tricine (pH = 7.5), 45; $MgCl_2$, 7.5;

ferredoxin, 200 µg/3 ml; NADP, 0.67; NADP reductase, saturating amount; plastocyanin, saturating amount; chlorophyll, 40 µg/3 ml.

(6) Procedures for obtaining anaerobic conditions

Three ml of reaction mixture was prepared in a small test tube (0.5 x 6 cm). The chloroplasts were added to the reaction mixtures in the dark. The test tube was covered with a tight rubber cap and shaken well. The reaction mixture was flushed with nitrogen while shaking for 2 min. The nitrogen was passed through syringe-needles penetrating the rubber cap on the test tube. The reaction mixture was transferred quickly with a nitrogen-flushed syringe into two cuvettes, on which rubber caps had been tied firmly with string and flushed with nitrogen through syringe-needles for 2 min. These procedures were carried out in the dark.

(7) Measurement of chlorophyll concentrations

The measurements of total chlorophyll concentration (Tagawa and Arnon, 1962; Strain and Svec, 1966) and chlorophyll a and b concentrations are according to the following equations, where the chlorophylls are in 80% acetone solutions:

$$\text{Chl } (a + b)\ \mu g/ml = 29 \times (A_{652\ nm}) \quad (1)$$

$$\text{Chl } a\ (\mu g/ml) = 11.63 \times (A_{665\ nm}) - 2.39\ (A_{649\ nm}) \quad (2)$$

$$\text{Chl } b\ (\mu g/ml) = 20.11\ (A_{649\ nm}) - 5.18\ (A_{665\ nm}) \quad (3)$$

$$\text{Total Chl } (\mu g/ml) = 6.45\ (A_{665\ nm}) + 17.72\ (A_{649\ nm}) \quad (4)$$

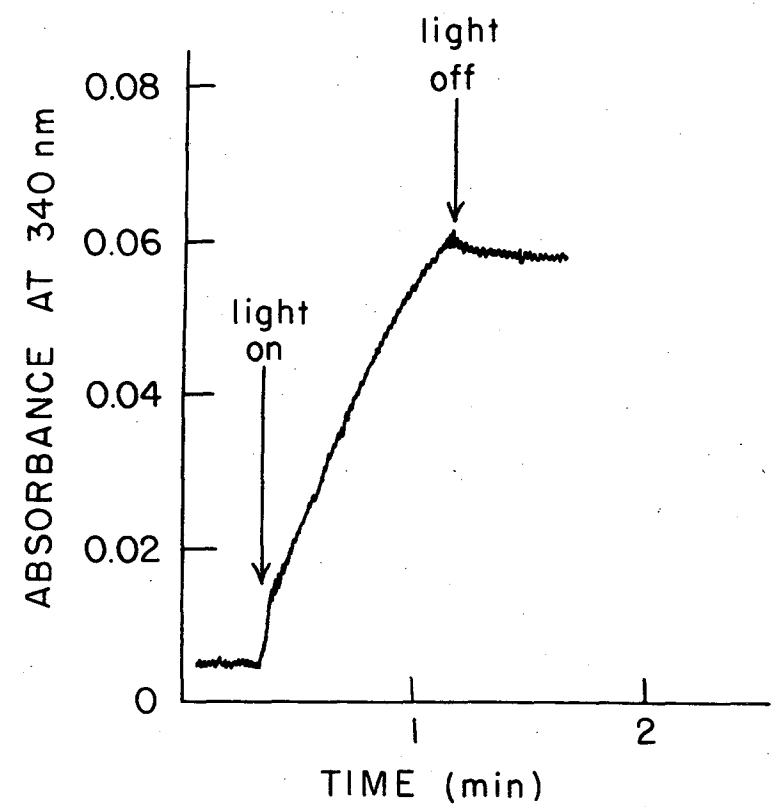
where A_λ = the absorbance of the chlorophyll solution in 80% acetone at a particular wavelength λ.

C. Results

1. Photosystem I: [ascorbate + $DPIPH_2$ → NADP] reaction with no plastocyanin or NADP reductase

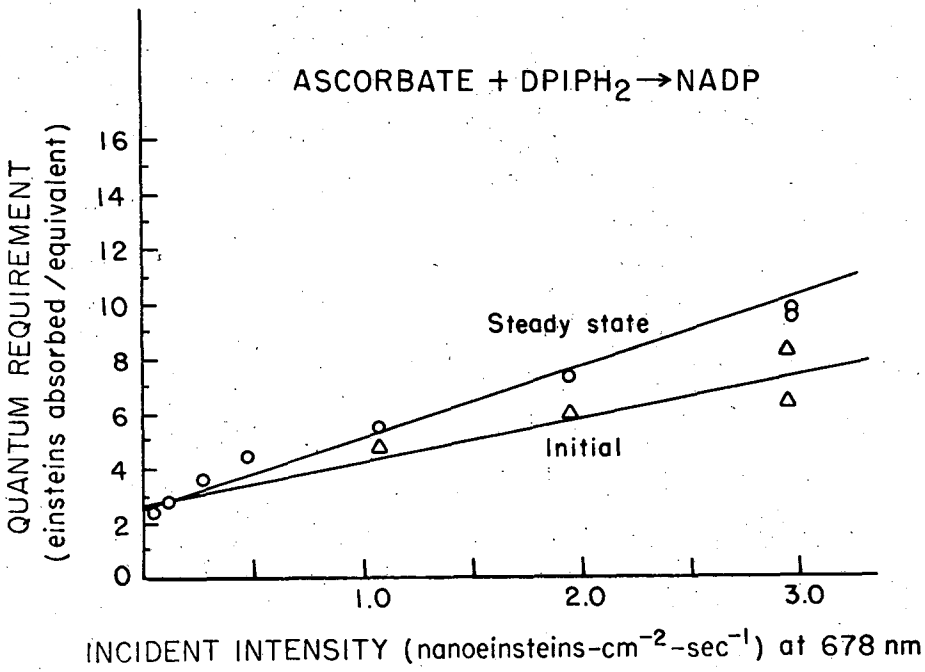
Typical time course for the photoreduction of NADP. The typical time course for the photoreduction of NADP in high light intensity by isolated chloroplasts for the [ascorbate + $DPIPH_2$ → NADP] reaction is shown in Figure 1. The trace shows a rapid initial burst just after turning on the actinic light. This suggests that there is a reservoir of electrons that can be rapidly transferred via photosystem I, and that the rate-limiting step is then the replenishment of this reservoir by the ascorbate/DPIP couple. The second portion of the slope is a straight line, corresponding to the steady-state photoreduction of NADP in this system. There is a rapid small reversal at the time of turning off the exciting light. This may also indicate that the backward oxidation of NADPH in the dark to the reservoir is rapid.

Primary quantum requirement. The relationship between the quantum requirement for the photoreduction of NADP and incident intensities is plotted in Figure 2. The primary quantum requirements are obtained by extrapolation to zero incident intensity. In most cases the quantum requirements obtained from the steady state of the photoreduction of NADP at different intensities lie fairly well on a straight line. The quantum requirements obtained from the rapid initial slope were plotted separately. They also lie on a straight line, but with a smaller slope. The primary quantum



XBL 7012-5490

Figure 1. Typical time course for the photoreduction of NADP by isolated chloroplasts in the [ascorbate + $DPIPH_2$ → NADP] reaction. The reaction mixtures are given in the text, but plastocyanin and NADP reductase were not added. Actinic light at 678 nm; incident intensity 3.0 nanoeinsteins-$cm^{-2}sec^{-1}$; chlorophyll concentration 13 $\mu g\text{-}ml^{-1}$. The molar absorptivity for NADPH at 340 nm is 6.2×10^3 $\ell\text{-}mole^{-1}\text{-}cm^{-1}$.



XBL 7012-5500

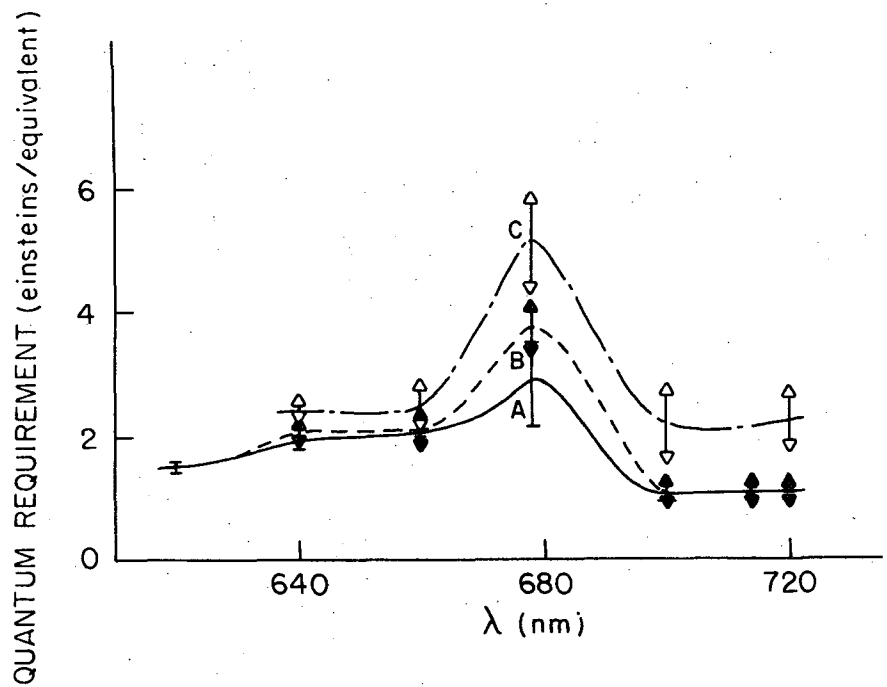
Figure 2. The relationship between the quantum requirement for NADP reduction and incident intensity in the [ascorbate + $DPIPH_2$ → NADP] reaction; no plastocyanin or NADP reductase added. Circles: obtained from the steady-state rate; triangles: obtained from the rapid initial phase. Illumination and chlorophyll concentration as in Figure 1.

requirements at zero intensity, obtained from either initial rates or steady-state rates, are identical.

Action spectrum. The action spectrum of the quantum requirements for the photoreduction of NADP in the [ascorbate + $DPIPH_2$ → NADP] reaction without the addition of any inhibitors or ethanol, is given as curveA in Figure 3. Curve A shows clearly that the quantum requirements at wavelengths above 700 nm are near 1.0. The presence or absence of air does not make any difference in the quantum requirements or in the rate of NADP photoreduction in this system.

Effect of ferredoxin. Ferredoxin is a non-heme protein and has one of the most negative redox potentials in chloroplasts (Arnon, 1966). It is known to be readily water-soluble and essential to the electron transport chain. The rate of photoreduction of NADP was almost zero when no ferredoxin was added to the reaction mixture together with the isolated chloroplasts. If other factors were not rate-limiting, such as light intensity, DPIP and NADP concentrations, etc., the rate of photoreduction of NADP increased from $\Delta A_{340\ nm}$/min = 0 to 0.062 as a linear function of ferredoxin concentrations from zero to 40 μg/3 ml of reaction mixture. The photoreduction rate of NADP saturated at about $\Delta A_{340\ nm}$/min = 0.084 when the ferredoxin concentration was 80 μg/3 ml of reaction mixture or higher. In our reaction mixture 100 μg of ferredoxin in 3 ml of reaction mixture was used.

Effect of ethanol. In general, ethanol at concentrations less than 1% of the reaction mixture decreases the rate of photoreduction of NADP, i.e. higher quantum requirements at higher light intensities,



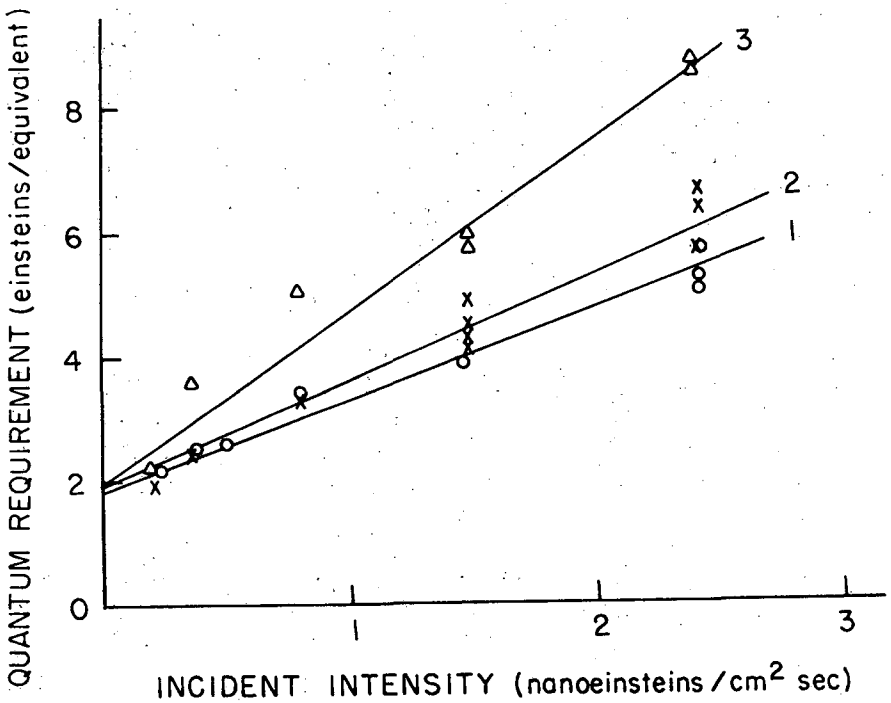
XBL 704-5168

Figure 3. Action spectrum of the zero-intensity quantum requirement of photoreduction of NADP in the [ascorbate + $DPIPH_2 \rightarrow NADP$] reaction, with no plastocyanin or NADP reductase added, with or without ethanol or antimycin A. Curve A: normal sample without adding any inhibitors or ethanol; the limits of deviations and the curve are shown in the graph as —I—. Curve B: samples with added ethanol at concentrations = 1%; the limits of deviation and the curve are shown as -‡-. Curve C: samples with added antimycin A dissolved in ethanol. The concentration is antimycin A/chlorophyll = 0.02; the limits of deviations and the curve are shown as —‡— -.

but it does not change the zero-intensity quantum requirement of the reaction. In Figure 4, for example, curve 1 is the normal sample without the addition of ethanol; curves 2 and 3 are the samples with the addition of 10 μl and 20 μl of ethanol to 30 ml of reaction mixture respectively. These curves exhibit almost the same zero-intensity quantum requirements (from 1.85 to 1.97) within the experimental errors, but the added ethanol increases the quantum requirements at higher intensities and the slopes of the curves increase with the increase of ethanol concentration even when it is 1% or less. For example, the slope of curve 1 is 3.3 but that of curve 3 is 4.8. When the concentration of ethanol is higher than 1% of the reaction mixture, the rate of photoreduction of NADP becomes irregular and thus no reliable data can be obtained.

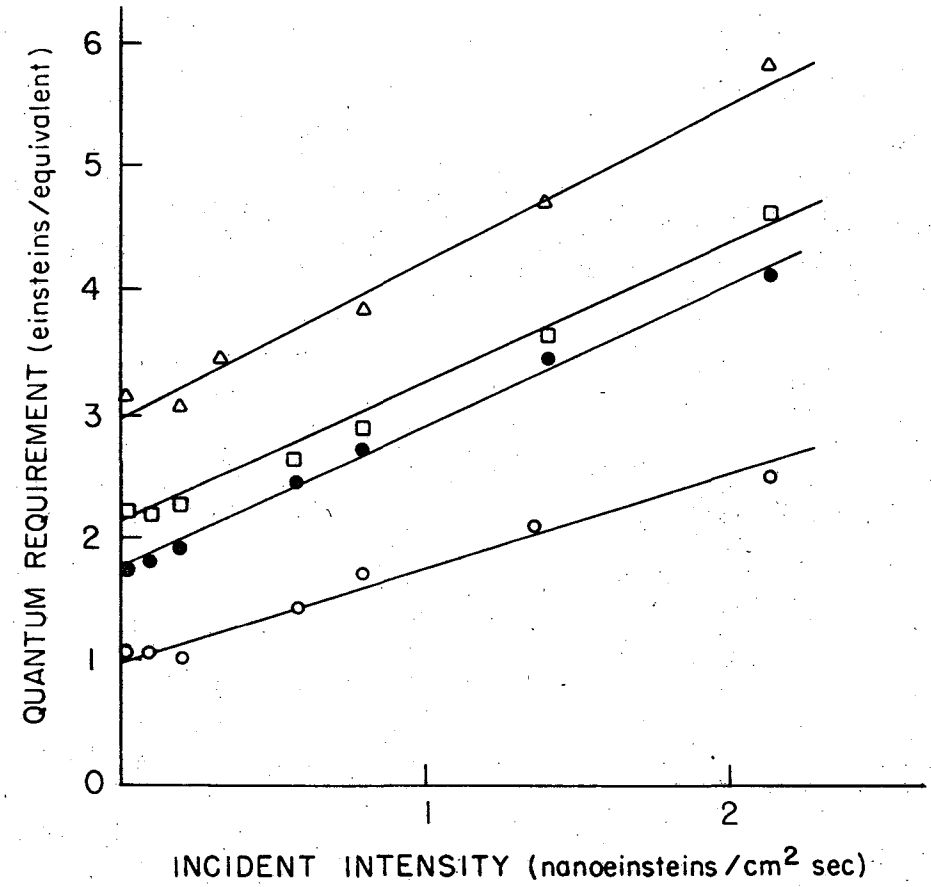
The action spectrum for the zero-intensity quantum requirements of the [ascorbate + $DPIPH_2 \rightarrow NADP$] reaction with 1% or less ethanol added is shown as curve B in Figure 3. Compared with curve A, the quantum requirements at different wavelengths, with or without ethanol, have almost the same values except that there are somewhat larger deviations when ethanol is added to the sample.

Effect of antimycin A. The effects of antimycin A, from the concentration of antimycin A/chlorophyll = 0.02 to 0.62, on the quantum requirements at different intensities are shown in Figure 5. We found that antimycin A always inhibits this reaction, i.e. an increase in quantum requirement at all concentrations of antimycin A and all intensities of actinic light studied. Even at the concentration of antimycin A/chlorophyll = 0.02 we found antimycin A



XBL 704-5164

Figure 4. The effect of ethanol on the quantum requirement of the photoreduction of NADP in the [ascorbate + $DPIPH_2$ → NADP] reaction, where plastocyanin and NADP reductase were not added. Incident light was at 640 nm. Circles: no ethanol added; X's: 10 λ ethanol/3 ml of reaction mixture; triangles: 20 λ ethanol/3 ml reaction mixture.



XBL 704-5167

Figure 5. Effect of antimycin A on quantum requirements of the [ascorbate + $DPIPH_2$ → NADP] reaction, where no plastocyanin or NADP reductase were added. Actinic light was at 700 nm. Open circles: no antimycin A was added; closed circles: antimycin A/chlorophyll = 0.02; open squares: antimycin A/chlorophyll = 0.2; open triangles: antimycin A/chlorophyll = 0.62.

showed a clear inhibition of this reaction. But it has been reported (Arnon et al., 1964) that non-cyclic photophosphorylation accompanying the [H_2O → ferredoxin/NADP] reaction is not inhibited even at a concentration of antimycin A/chlorophyll = 0.33, which is 16-fold more concentrated than the concentration of antimycin A/chlorophyll = 0.02.

The action spectrum of the zero-intensity quantum requirements for the [ascorbate + $DPIPH_2$ → NADP] reaction is shown as curve C in Figure 3. In most cases the samples with added ethanol have the same primary quantum requirements as the normal samples at the wavelengths from 630 nm to 720 nm. However, antimycin A (plus ethanol) always increases the zero-intensity quantum requirements for the photoreduction of NADP, even at the lowest concentration studied.

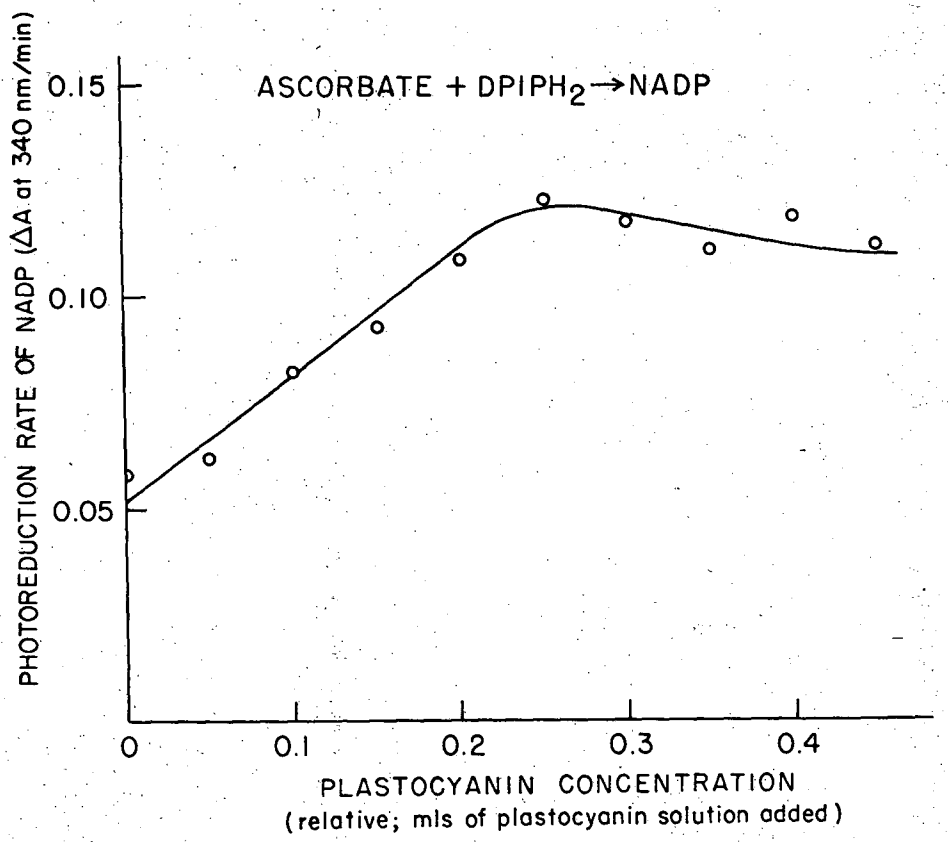
High concentrations of antimycin A, antimycin A/chlorophyll ≥ 2.0, completely inhibits the photoreduction of NADP of the [ascorbate + $DPIPH_2$ → NADP] reaction. No measurable rate of photoreduction of NADP could be obtained at these concentrations.

The effect of oxygen. It was thought that part of the electrons transported by photosystem I in the [ascorbate + $DPIPH_2$ → NADP] reaction would leak to oxygen via ferredoxin. For this reason, the reaction was studied under anaerobic conditions. Experiments showed, on the other hand, that the presence of air had no effect on the rates of reaction or quantum requirements. Apparently the leak of electrons to oxygen in this system is negligible under the experimental conditions.

2. Photosystem I: [ascorbate + $DPIPH_2$ → NADP] reaction using saturating amounts of plastocyanin and NADP reductase

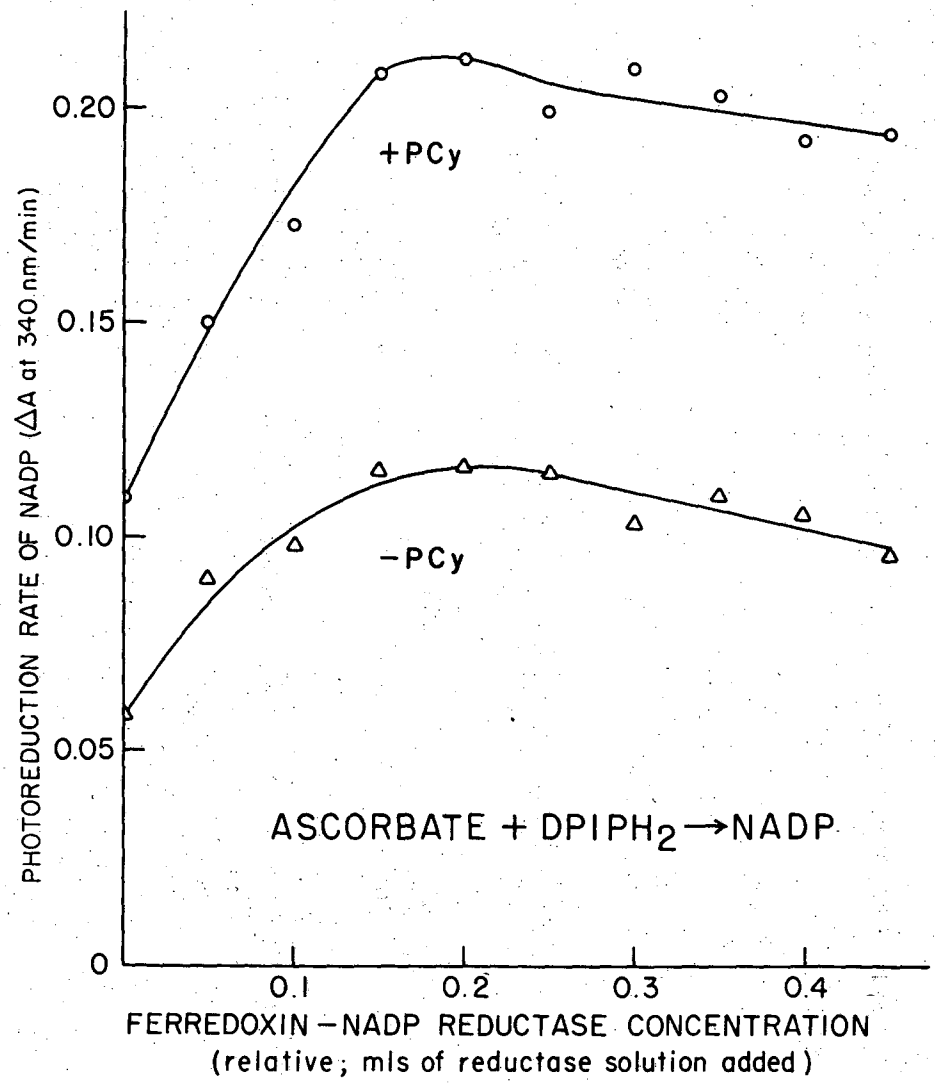
Plastocyanin. Plastocyanin is a copper-containing protein in chloroplasts, and is water soluble. It may serve as the electron carrier between PS I and PS II (Park and Sane, 1971). Adding back plastocyanin to the spinach chloroplasts preparation may restore part of what was lost during the isolation process. If other factors, such as light intensity, ferredoxin concentration, etc., are not rate-limiting, the rate of photoreduction of NADP in the [ascorbate + $DPIPH_2$ → NADP] system can be increased more than two-fold when optimal amounts of plastocyanin are added. The relationship between the rate of photoreduction of NADP by this system and plastocyanin concentrations is shown in Figure 6.

Ferredoxin-NADP reductase. Ferredoxin-NADP reductase is another water soluble protein in chloroplasts and is essential to the electron transport chain in photosystem I (Avron, 1967; Arnon, 1966). With optimal concentrations of ferredoxin and plastocyanin, the rate of photoreduction of NADP can be increased further. The relationship between the rate and the NADP reductase concentrations is shown in Figure 7. The rate of NADP photoreduction (ΔA at 340 nm/min) was 0.16 when no plastocyanin or NADP reductase was added, increased to 0.12 when optimal plastocyanin was added, and to 0.22 when both optimal plastocyanin and NADP reductase were present. The final rate with optimal plastocyanin and NADP reductase is almost four times greater than the rate without these additions.



XBL 7012-5497

Figure 6. The influence of plastocyanin concentrations on the rate of photoreduction of NADP in the [ascorbate + $DPIPH_2$ → NADP] reaction. The reaction mixture is given in the text. Plastocyanin was isolated from spinach following the method given in the text. The absolute concentration of plastocyanin is unknown. Actinic light was at 678 nm.



XBL 7012-4900

Figure 7. The influence of plastocyanin and NADP reductase concentrations on the rate of photoreduction of NADP in the [ascorbate + $DPIPH_2$ → NADP] reaction. Curve 1 shows that the rate of photoreduction of NADP can be increased by adding NADP-reductase alone. Curve 2 shows that the rate can be increased further when both plastocyanin and NADP reductase are added. Plastocyanin and NADP reductase were isolated from spinach following the method given in the text. Both absolute concentrations were unknown. The reaction mixture is given in the text. The actinic light was at 678 nm.

The rate of the photoreduction and primary quantum requirements. If we compare the results in Figure 1 and Figure 7, the rate of photoreduction of NADP in the [ascorbate + $DPIPH_2 \rightarrow NADP$] reaction is approximately ΔA 340 nm = 0.09 min^{-1} at the highest intensity of the actinic light. Under comparable conditions the rate can be increased to 0.42 if saturating plastocyanin and ferredoxin-NADP reductase are both added. However, the increased rate does not give a lower primary quantum requirement at zero intensity of actinic light. Comparing the results shown in Figure 2 and Figure 8, the quantum requirement at the highest incident intensity, i.e. 3.0 nanoeinsteins cm^{-2} sec^{-1} is 9.5 if no plastocyanin or NADP reductase is added to the reaction mixture, but it is 4.2 when the system is saturated with NADP reductase and plastocyanin. Extrapolated to zero intensity of the incident light, they both have the same value of the primary quantum requirements, 2.4 $\pm$ 0.1.

Action spectrum. The primary quantum requirements at zero intensities at the wavelengths from 620 nm to 720 nm for this reaction are almost the same as those without the addition of plastocyanin and NADP reductase, shown as curve A in Figure 3. Therefore, they are not presented here again. The quantum requirement of 1.0, namely, one equivalent of NADP reduced by one photon absorbed, can also be obtained when the actinic light is at 700 nm or longer.

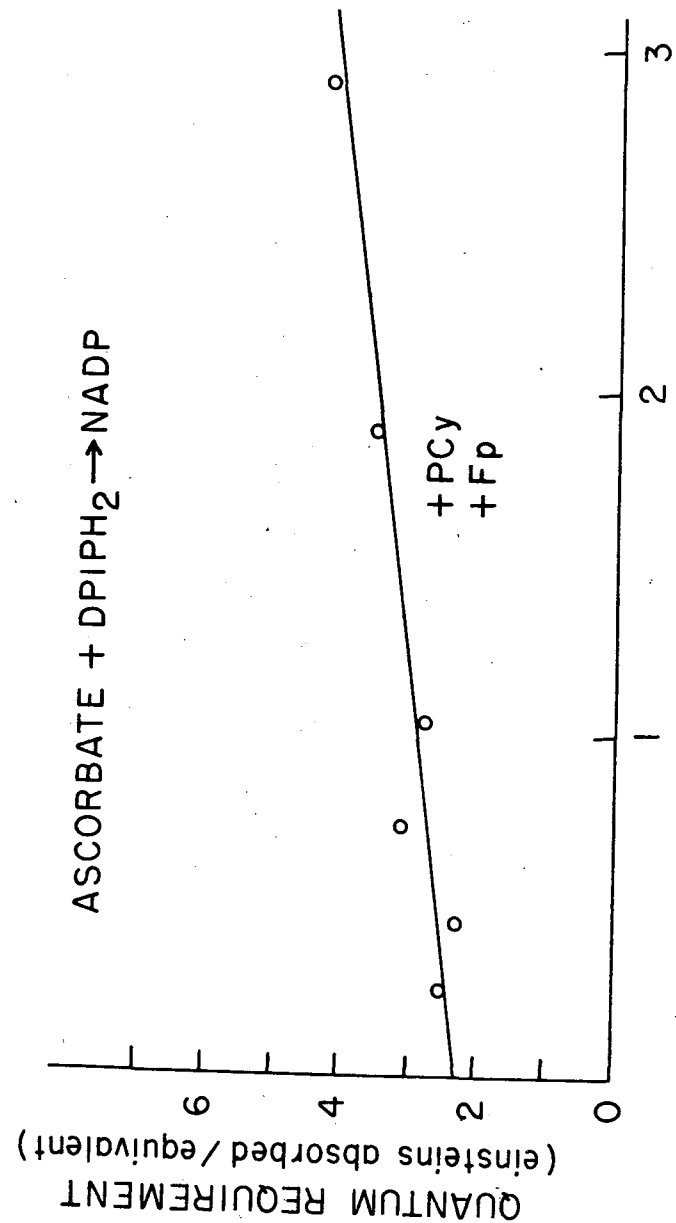
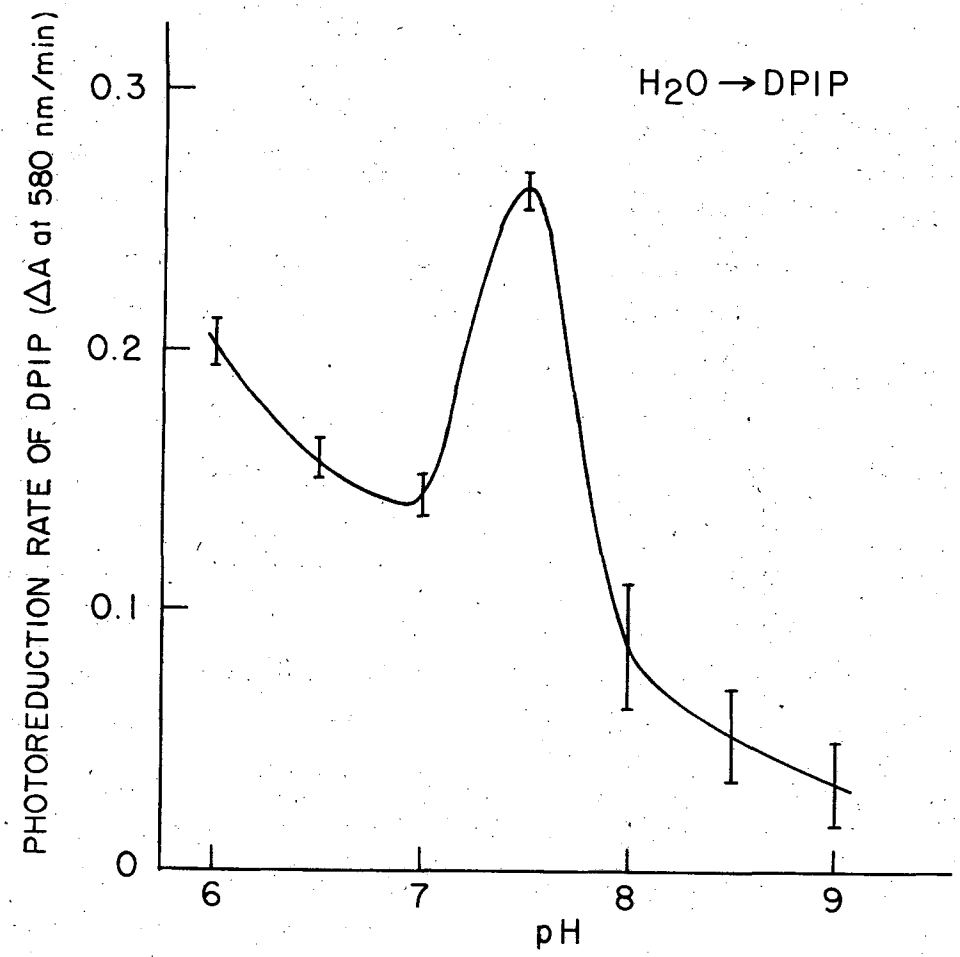


Figure 8. The relationship between the quantum requirement of NADP photoreduction and incident intensities in the [ascorbate + $DPIPH_2 \rightarrow NADP$] reaction with saturating plastocyanin and NADP reductase. Incident light was at 678 nm.
XBL 7012-5491

3. Photosystem II: [$H_2O \rightarrow$ DPIP] reaction

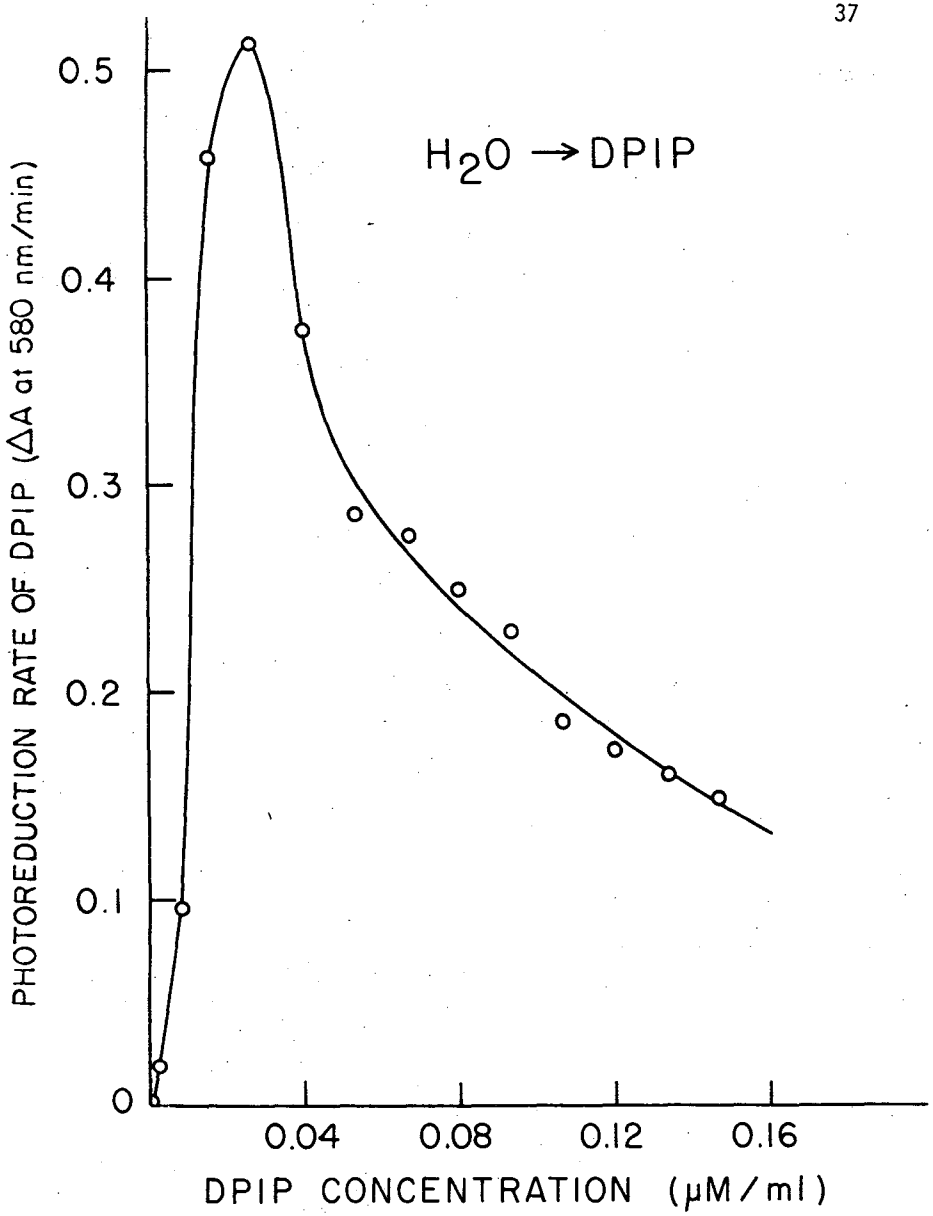
pH Dependence. In order to obtain the optimum rate, the photoreduction of DPIP was studied from pH 6.0 to 9.0, using 45 mM of tricine as the buffer. The dependence of the photoreduction rate of DPIP on the pH is shown in Figure 9. These results were obtained from five to seven independent experiments at each pH. Because the absorbance of DPIP changes with pH (Armstrong, 1964), we compare only the measured rate of change of absorbance at 580 nm. As shown in Figure 9, the optimum rate of photoreduction of DPIP is at pH 7.5.

DPIP Concentration. Since DPIP has an absorption maximum at 600 nm with a broad spectrum in the visible region, it will diminish the effective absorption of chloroplasts. Therefore we tried to find the optimum DPIP concentration, which will not screen the absorption of chloroplasts too much, but will approach saturation as a substrate. The concentration-dependent rate of photoreduction of DPIP is shown in Figure 10. The corrections for the screening by DPIP at different concentrations on the actual absorption of chloroplasts were not made at this point; the measured changes of absorbance at 580 nm were taken directly as the rate of photoreduction of DPIP. As shown in Figure 10, the rate increased almost linearly from ΔA at 580 nm/min = 0 to 0.4 with increase of DPIP concentration from zero to 1.6×10^{-5} mole·ℓ^{-1}. The rate reached a maximum at ΔA at 580 nm/min = 0.5 when DPIP concentration was 2.67×10^{-5} mole·ℓ^{-1}. A similar observation was found previously in this laboratory (Kelly and Sauer, 1968), that the rate of photoreduction of DPIP in flash



XBL 7012-5496

Figure 9. The dependence of photoreduction rate of DPIP on pH. The results were obtained from five to seven independent experiments under comparable conditions. The reaction mixture is given in the text, but no plastocyanin was added. Incident light was at 678 nm with intensity 3.0 nanoeinsteins-cm^{-2}-sec^{-1}.



XBL 7012-5499

Figure 10. The photoreduction rate of DPIP by chloroplasts as a function of DPIP concentration. The reaction mixture is given in the text but no plastocyanin was added. Incident light was at 678 nm.

light experiments was dependent on the DPIP concentration. The maximum flash rate was obtained when the DPIP concentration was 2.7×10^{-5} mole·ℓ^{-1} which was almost the same as we found in this experiment. At higher DPIP concentrations, e.g., 6.0×10^{-5} mole·ℓ^{-1}, the maximum rate remained constant in flashing light experiments but decreased about forty percent in this experiment. This phenomenon might be due to screening of DPIP on the effective absorption of chloroplasts, which was not predominant in flash light experiments, because the light intensity was saturated and not the rate-limiting factor. But under the continuous illumination condition, the photoreduction rate of DPIP, ΔA 580 nm/min = 0.51, which was more than a hundredfold higher than that in flash light experiments, was very much light-intensity dependent. When the DPIP concentration was increased from 3×10^{-5} to 6×10^{-6} mole·ℓ^{-1}, the screening effect of DPIP was also more than doubled. Thus, both the effective absorption of chloroplasts and the rate decreased considerably.

Magnesium chloride. Both magnesium and chloride ions are essential for photosynthesis. As in other reactions involving the transfer of a phosphate group (McElroy and Nason, 1954), Mg^{++} is required for phosphorylation by chloroplasts (Arnon, 1958). The chloride ion, replaceable by bromide but not by other anions, was found to be essential for oxygen evolution by isolated chloroplasts (Warburg, 1949; Arnon and Whatley, 1949). We found that the rate of the [$H_2O \rightarrow$ DPIP] reaction could be greatly enhanced by adding appropriate amounts of magnesium chloride. The results are shown in Figure 11. The maximum photoreduction rate of DPIP, in the presence

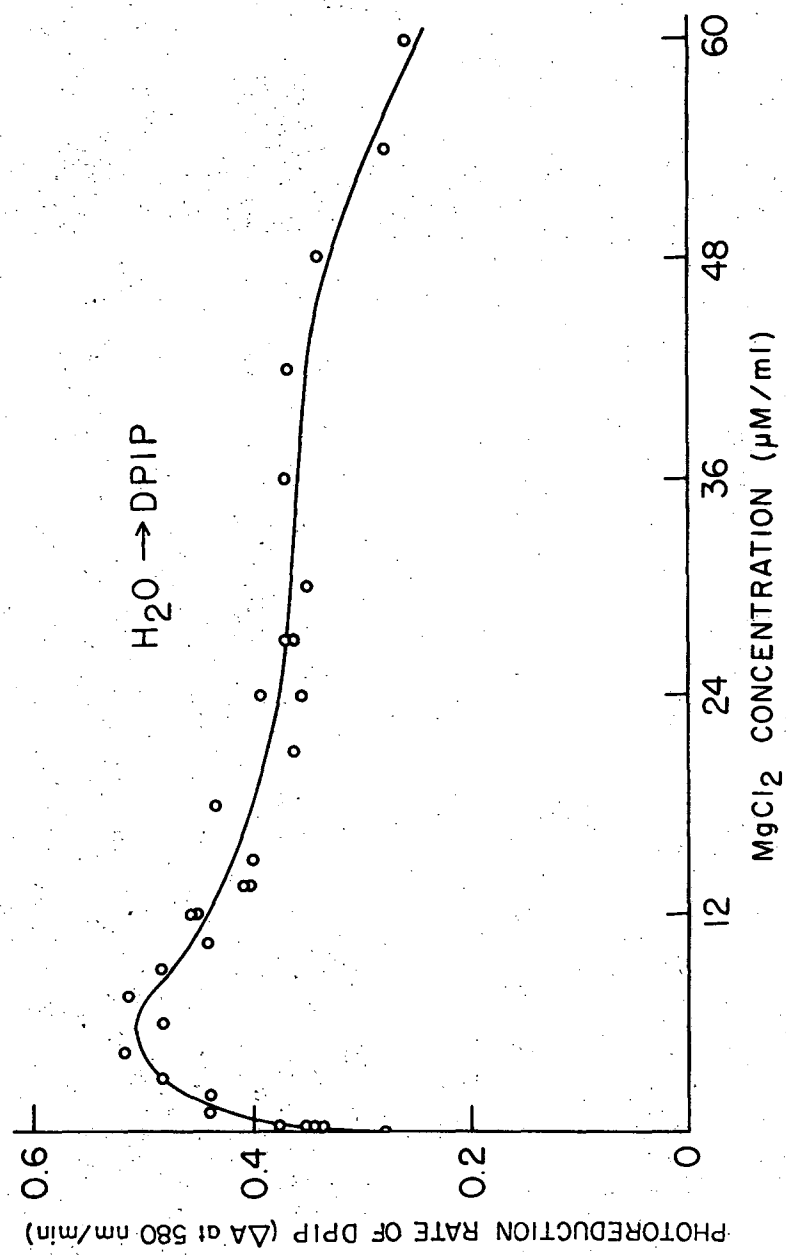


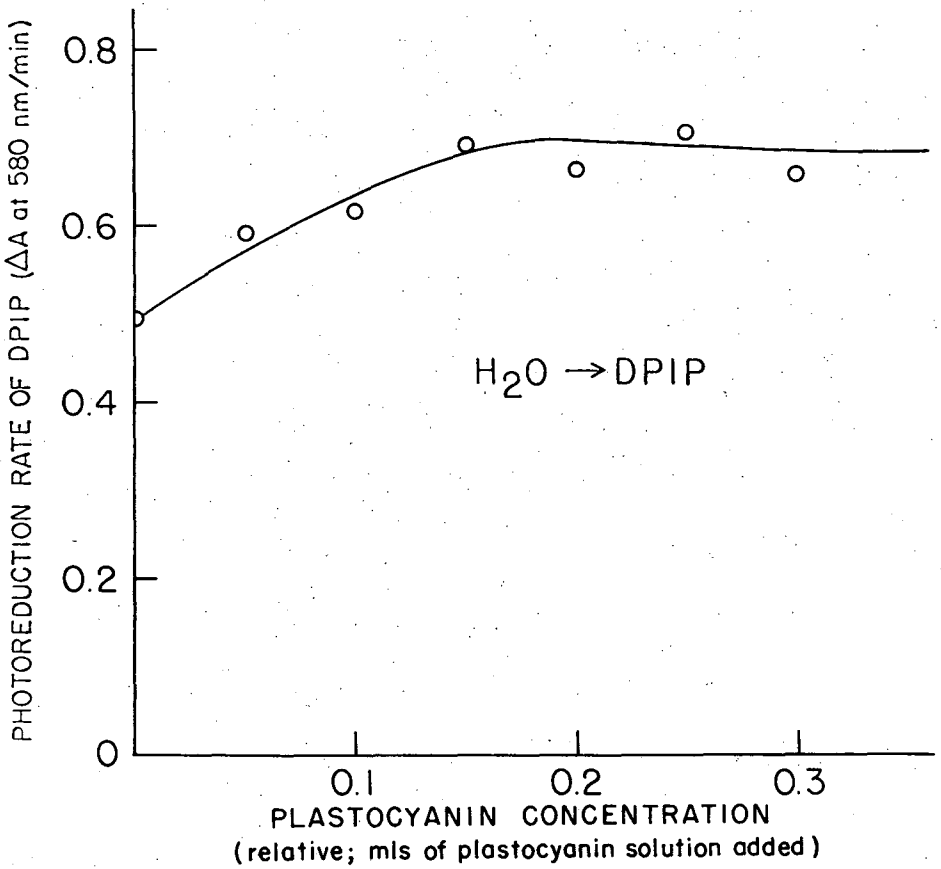
Figure 11. The relationship between the rate of photoreduction of DPIP and $MgCl_2$ concentration. The reaction mixture is given in the text; no plastocyanin added. Incident light was at 678 nm with intensity about 3.0 nanoeinsteins-cm^{-2}-sec^{-1}. XBL 7012-4901

of 6 μmole·ml^{-1} of magnesium chloride, was about sixty percent greater than the rate in the absence of added magnesium chloride.

Plastocyanin. Since plastocyanin has been implicated in non-cyclic electron transport from water to NADP (Katoh et al., 1965; Katoh et al., 1966; Gorman et al., 1966), and also is partly water soluble, we investigated its effect on the rate of photoreduction of DPIP in the [$H_2O \rightarrow$ NADP] reaction. Figure 12 shows that the rate of photoreduction of DPIP is increased more than forty percent when saturated plastocyanin is added. After optimizing all the above conditions, the rate of photoreduction of DPIP was increased more than threefold from ΔA at 580 nm/min = 0.25 to 0.78.

Ferredoxin and NADP reductase. Both ferredoxin and NADP reductase were investigated as to their effects on the photoreduction of DPIP by the chloroplasts. Each of these components was added, separately or together, at different concentrations to the reaction mixture. No significant improvement of the photoreduction rate of DPIP was found when lower concentrations of either or both ferredoxin and NADP reductase were added. A small decrease in the rate occurred when higher concentrations of either or both of them were added. Apparently they are not involved in this part of the electron transport chain.

Action spectrum of the quantum requirement. When the rate of photoreduction of DPIP was optimized, the quantum requirements of this reaction were measured in the same way as those in the [ascorbate + $DPIPH_2 \rightarrow$ NADP] reaction. The action spectrum of the zero-intensity quantum requirements is shown in Figure 13. These are average values from the best five to seven independent experiments



XBL 7012-5498

Figure 12. The relationship between the photoreduction rate of DPIP and plastocyanin concentration. The reaction mixture is given in the text. Plastocyanin was isolated from spinach and its absolute concentration is unknown. Actinic light was at 678 nm.

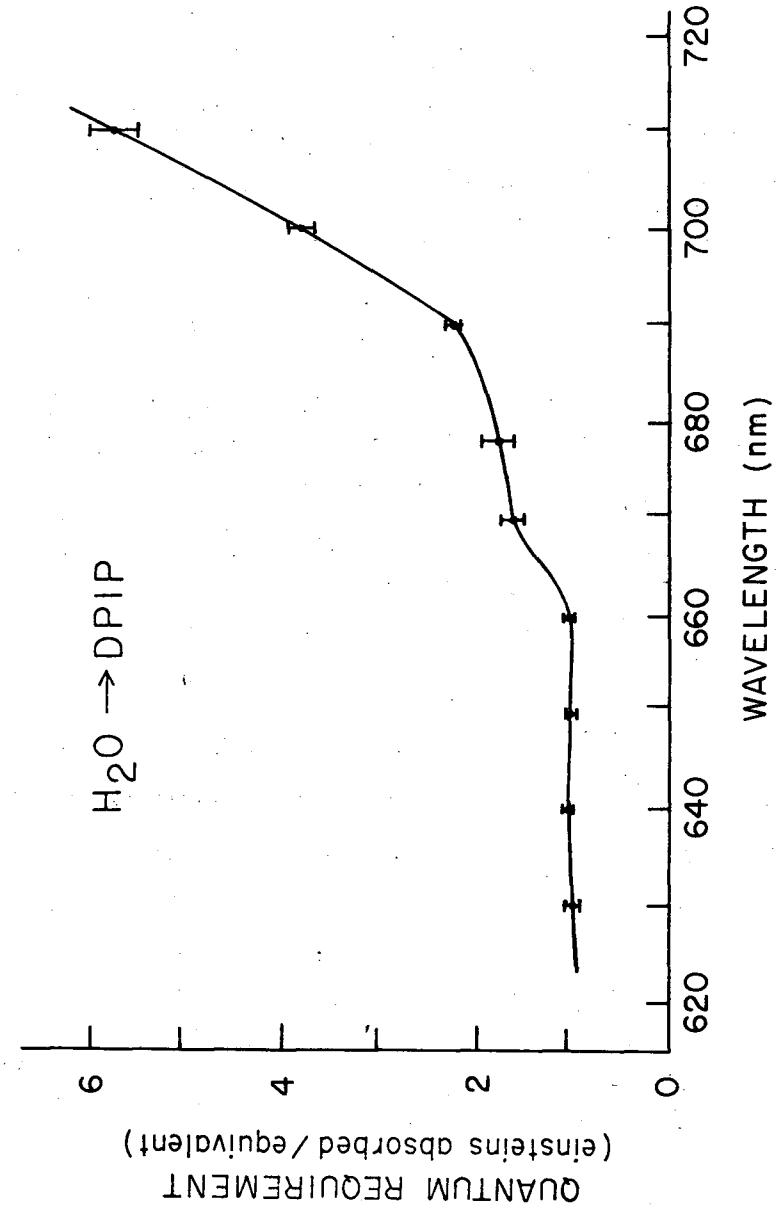


Figure 13. Action spectrum of the quantum requirements of photoreduction of DPIP for the [$H_2O \rightarrow$ DPIP] reaction. The reaction mixture is shown in the text.

XBL 7012-5492

of a total of more than fifteen. Quantum requirements of 1.0 ± 0.05 were obtained from 630 to 660 nm of actinic light. Higher values were obtained when the actinic light was at 670 nm or longer wavelengths.

4. Photosystem (I + II): [$H_2O \rightarrow$ NADP] reaction

Optimum rate of photoreduction of NADP. The rate of photoreduction of NADP in this system, as in the other reactions, is very much dependent on pH, the concentration of magnesium chloride, plastocyanin, ferredoxin, and NADP reductase, method of preparation of chloroplasts and the age of the spinach. Similar experiments have been carried out as those in the [$H_2O \rightarrow$ DPIP] reaction to optimize the reaction rate of the system. The optimum conditions have been obtained and used as the standard reaction mixture given in the Materials and Methods section. Addition of magnesium chloride, ferredoxin, plastocyanin, and NADP reductase are all required for rapid rates in this reaction. The optimum rate of photoreduction of NADP by water was obtained from pH 7.0 to 8.0, with a maximum at pH 7.5, which was used in our experiment. The rate of the reaction when saturating ferredoxin was added was 20-fold more than the rate when no ferredoxin was added. With the optimum concentration of $MgCl_2$, 7.5 $\mu moles \cdot ml^{-1}$, the rate could be increased about 30 percent from ΔA at 340 nm/min = 0.065 to 0.090, over the rate when no $MgCl_2$ was added.

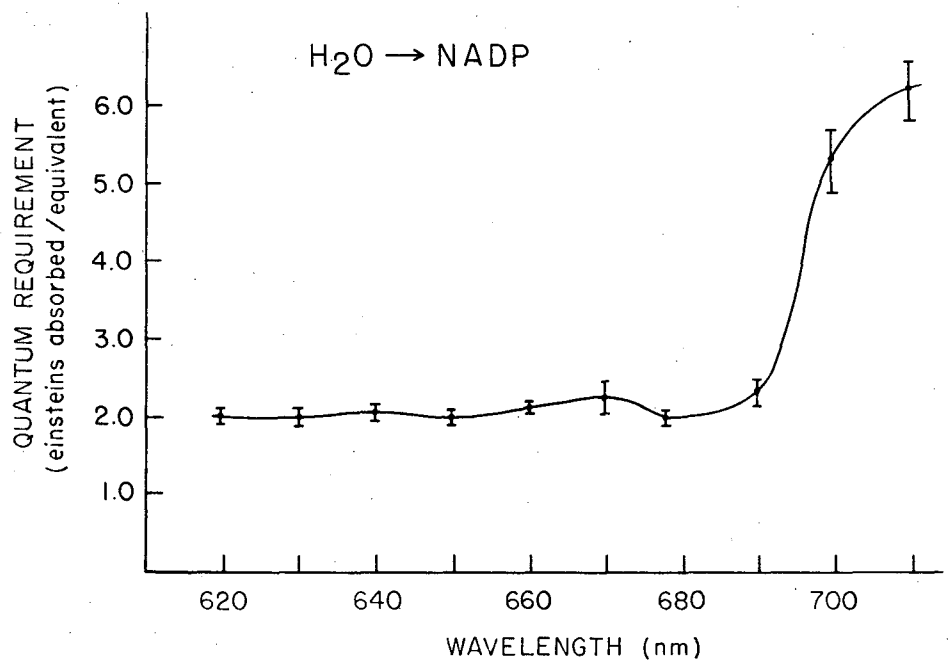
With the optimum concentration of $MgCl_2$ ferredoxin and pH, the photoreduction rate of NADP of the reaction was more than doubled

by adding saturating NADP reductase, and more than tripled by adding both saturating NADP reductase and plastocyanin.

Action spectrum of quantum requirements. The action spectrum of zero-intensity quantum requirements for the [$H_2O \rightarrow$ NADP] reaction is shown in Figure 14. Only the optimized conditions for the reaction have been used for obtaining these quantum requirements. The results shown in Figure 14 were the average values of the best five to seven independent experiments of a total of more than fifteen. Many times when chloroplasts were not very active we were not able to obtain these low quantum requirements. The quantum requirements from 620 to 660 nm and at 678 nm are very close to 2.0 ± 0.1, and they are slightly higher at 670 and 690 nm. The photoreduction of NADP by this reaction becomes very inefficient when the actinic light is at 700 nm or longer wavelengths.

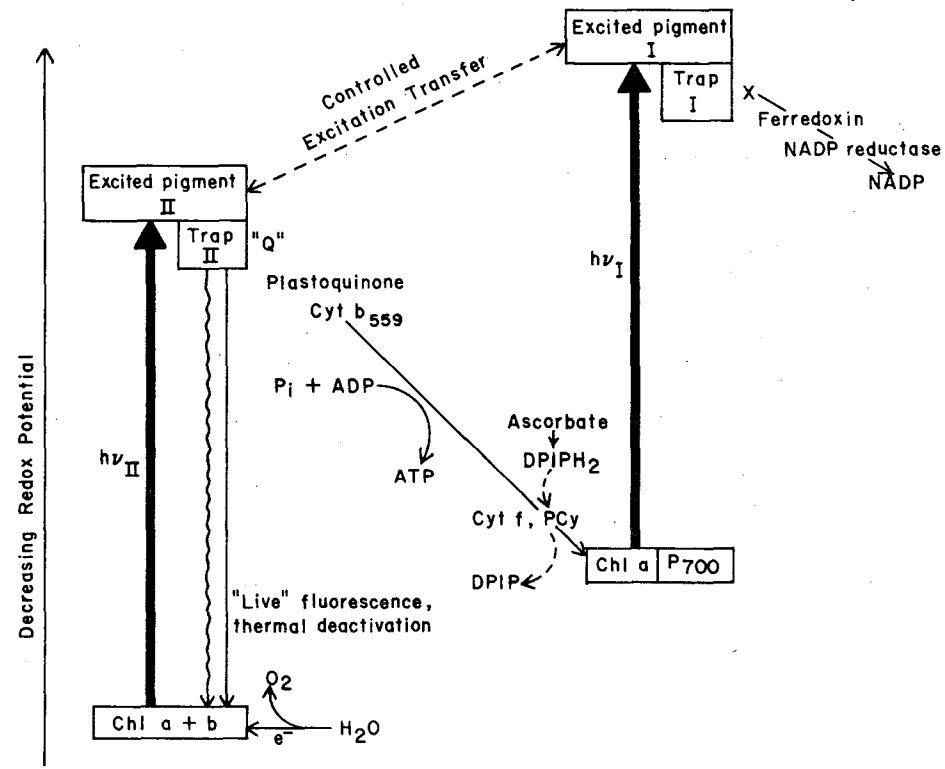
D. Discussion

The normal course of photosynthetic electron transport in higher plant chloroplasts leads to the reduction of NADP to NADPH and the oxidation of water to molecular oxygen. A current version of the mechanism of this process, based on the series two-light reaction scheme originally proposed by Hill and Bendall (1960) and elaborated for the [$H_2O \rightarrow$ NADP] reaction by Losada *et al.* (1961), is shown in Figure 15. DCMU is a potent inhibitor of the reduction of NADP (Bishop, 1958) and this inhibition can be relieved, in part, by the addition of the couple $DPIPH_2$ plus ascorbate (Vernon



XBL 7012-5495

Figure 14. Action spectrum of the quantum requirements of photoreduction of NADPof the [$H_2O \rightarrow NADP$] reaction. The reaction mixture is given in the text.



XBL 7012-5493

Figure 15. Hypothesis of two light reactions in isolated chloroplasts. See text for details.

and Zangg, 1960). Based on the action spectrum of quantum requirements, Hoch and Martin (1963) and Sauer and Biggins (1965) suggested that the photoreduction of $NADP^+$ in the [ascorbate + $DPIPH_2$ → NADP] reaction is associated solely with light reaction I. In the absence of DCMU, the oxidized form of DPIP serves as an electron acceptor for the chloroplast Hill reaction, leading to the photooxidation of water to molecular oxygen. This reaction has been assigned to light reaction II alone, on the basis of action spectra and the absence of enhancement in the presence of long wavelengths of light, but it has not been possible to rule out the possibility that light reaction I participates as well. A detailed discussion of this question was given by Sauer and Park (1965). Therefore, we used the [H_2O → NADP] reaction for the study of photosystem (I + II), the [ascorbate + $DPIPH_2$ → NADP] reaction for photosystem I, and the [H_2O → DPIP] reaction for photosystem II. However, based on the study of cytochrome b_{559} and C_{550} at low temperaure and the absence of enhancement effect, Knaff and Arnon (1969) suggested that there are two light reactions in photosystem II, which is the only energy source needed for the [H_2O → NADP] reaction, and one light reaction in photosystem I, which only carries out cyclic photophosphorylation. These two discrepant hypotheses need careful measurements on quantum requirement and on enhancement effect of the above three reactions. They will be discussed in the next section.

As shown in Figure 3, a quantum requirement value of 1.0 for the transfer of one electron from ascorbate/$DPIPH_2$ to NADP is obtained at the wavelengths above 700 nm of actinic light. We take

this to mean that non-cyclic electron transport from ascorbate to NADP is the only reaction occurring under these conditions, if the hypothesis is correct--namely, one photon is required to transport one electron in photosystem I. This suggests that photosystem I requires only one photon to carry out the photoreaction. Since chlorophyll b probably has negligible absorption above 670 nm, the light absorbed above 700 nm is due to chlorophyll a only. This indicates that photosystem I is strongly associated with chlorophyll a and also suggests that above 700 nm photosystem II has little absorption. Other studies have found that values for the Chl a/Chl b ratio of the photosystem II particles obtained, using digitonin or triton treatments, range from 2.0 to 2.4, and for the photosystem I fraction from 4.5 to 7.0 (Black and San Pietro, 1963; Anderson and Boardman, 1966; Anderson, Fork and Amez, 1966; Schwartz, 1967). Thornber et al. (1967) found that photosystem I particles had a Chl a/Chl b ratio as high as 17. Boardman and Highkin (1966) noted that a barley mutant lacking Chl b had unimpaired ability to reduce NADP. The evidence supports the idea that photosystem I particles contain primarily Chl a.

It is known that NADP/ferredoxin is a physiological electron acceptor and consequently its reaction mechanism must be very specific. It has been demonstrated (Nelson and Neumann, 1969) that NADP, NADPH and ferredoxin can competitively form a complex with NADP reductase, which mediates the electron transfer from reduced ferredoxin to NADP. This specific mechanism permits NADP to receive electrons only from the end of the electron transport

chain that is closely associated with NADP reductase, and thus prevents NADP from receiving electrons directly from photosystem II, of which the electron source is cut completely by DCMU under our experimental conditions. Therefore, the photons absorbed by photosystem II at wavelengths from 620 to 690 nm cannot be utilized and the energy absorbed will be wasted unless there is an electronic excitation transfer from photosystem II to photosystem I. The quantum requirement values between 1.5 and 2.5 of this reaction at wavelengths from 620 690 nm strongly suggest that this electronic excitation transfer from photosystem II to photosystem I is very inefficient.

Arnon et al. (1964) found antimycin A to behave as an inhibitor to the electron transport chain of photosystem I, even at concentrations as low as antimycin A/chlorophyll = 0.02. Cyclic, but not non-cyclic, photophosphorylation is reported to be blocked at this concentration. We interpret the effect on the efficiency of the non-cyclic electron transport of PS I to result from a competition with cyclic electron flow, which has become accelerated through an uncoupling action of antimycin A. Ethanol, a commonly used solvent for reagents like antimycin A, is also found to have an inhibitory effect at concentrations above 1% in the reaction mixture at higher light intensities, but it does not change the zero-intensity quantum requirement for the [ascorbate + $DPIPH_2 \rightarrow NADP$] reaction. The concentration of ethanol, which was used as the solvent for antimycin A on the zero light intensity quantum requirement, should be solely due to antimycin A itself, not due to ethanol.

The quantum requirement of photosystem II, or the [$H_2O \rightarrow DPIP$] reaction, is close to 1.0 at the wavelengths from 630 to 660 nm of actinic light. If the hypothesis, i.e. one quantum required to carry out the photosystem II reaction, is correct, these quantum requirement values strongly suggest that photons absorbed by chloroplasts from 630 to 660 nm can be used by photosystem II very efficiently. The photons originally absorbed by photosystem I in this region should also be transferred to photosystem II, probably by an electronic excitation transfer process. When the actinic wavelengths are 670 or 678 nm, quantum requirement values of 1.7 to 2.3 are obtained. This means that photons absorbed by photosystem I in this region cannot be transferred to photosystem II completely. Therefore part of the photons absorbed by photosystem I is wasted and quantum requirements for the [$H_2O \rightarrow DPIP$] reaction higher than 1.0 are obtained. The quantum requirement of photosystem II becomes much higher when the actinic light is beyond 690 nm, apparently because the photons absorbed in this region have insufficient energy to activate light reaction II. On the basis of the results of the present study, where quantum yields of 1.0 equivalent-(einstein absorbed)$^{-1}$ are observed for the [$H_2O \rightarrow DPIP$] reaction using actinic wavelengths from 630 to 660 nm, we can now unambiguously assign this reaction to light reaction II alone. If both light reactions were involved, then at least two absorbed photons would be required for each electron transferred and the quantum yield would never exceed 0.5 at any actinic wavelength. From the analogous results of Avron and Ben-Hayyim (1969), the

ferricyanide Hill reaction can also be assigned to light reaction II alone. In our studies of the DPIP Hill reaction, the absence of any effect resulting from the addition of ferredoxin and/or ferredoxin-NADP reductase supports the assignment to light reaction II.

Quantum requirement values close to 2.0 for photosystem (I + II), the [H_2O → NADP] reaction, are obtained when the actinic light is between 620 and 678 nm. Because we have optimized the experimental conditions of this reaction in a similar way to those of the other two reactions, and also the chloroplasts activity in this reaction should be as active as in other reactions. Thus the difference in quantum requirement values of these three reactions should be due to the difference in electron donors of the [ascorbate + $DPIPH_2$ → NADP] and the [H_2O → NADP] reaction, and also due to the difference in electron acceptors of the [H_2O → NADP] and the [H_2O → DPIP] reaction. Thus the quantum requirement values here do suggest that two quanta are required to transfer one electron from water to NADP (or PS (I + II) reaction), but only one quantum is required to carry out the [ascorbate + $DPIPH_2$ → NADP] or PS I reaction and the [H_2O → DPIP] or PS II reaction respectively.

The effect of added plastocyanin in increasing the rate of electron transport in the [H_2O → DPIP] reaction at moderate light intensities suggests that the DPIP accepts electrons close to photosystem I in broken chloroplasts. Presumably added plastocyanin is relieving a rate-limiting step in this reaction under conditions where light is not limiting. This observation is

consistent with the finding that methylamine, an uncoupler of non-cyclic photophosphorylation, also serves to accelerate electron flow in the [H_2O → DPIP] reaction (Sauer and Park, 1965; Kelly and Sauer, 1968). The site of non-cyclic photophosphorylation is thought to occur between photosystem II and plastocyanin. We believe that the influence of added plastocyanin on this system II reaction indicates that DPIP accepts electrons either directly from plastocyanin or else from an adjacent electron transport component whose oxidation state is controlled by plastocyanin.

Associated with each of the two photosynthetic reaction centers is an array of light absorbing or antenna pigment molecules (chlorophylls a and b and carotenoids in the case of higher plants). Many models have been proposed to describe the arrangement of these molecules (Duysens and Amesz, 1962; Myers and Graham, 1963; Avron and Ben-Hayyim, 1969). In one model, the "separate package" hypothesis, each pigment molecule is associated with one or the other of the light reactions and no communication, by way of electronic excitation transfer, is permitted between them. As a consequence of this model, photons absorbed by a particular pigment molecule can be transferred only to the reaction center of its own pigment system. If that reaction center happens not to be functioning, then the excitation will be wasted and will appear only as fluorescence or heat. If we measure the quantum yields at low light intensities, where the arrival of photons is rate limiting, for each of the light reactions operating separately, then according to the "separate package" hypothesis the

sum of the quantum yields at each wavelength should not exceed 1.0 (Kelly and Sauer, 1965). In Table I we present the zero intensity quantum yields at several wavelengths between 620 and 720 nm for the [ascorbate + $DPIPH_2$ → NADP] reaction attributed to photosystem I and for the [H_2O → DPIP] reaction attributed to photosystem II. The sums of the two quantum yields at each wavelength are given in column 4. Although the sums are reasonably close to unity at long wavelengths (670 to 710 nm) they exceed unity by 45 to 60% in the shorter wavelength range (630 to 660 nm) of activating light. Light reaction II itself has a quantum yield near unity in this wavelength range, and the quantum yield of light reaction I is around 0.5. These results, together with those reported previously by Avron and Ben-Hayyim (1969), are clearly inconsistent with the "separate package" hypothesis and suggest that it is no longer tenable.

An alternative model, the "spillover" hypothesis, includes the possibility that excitation present in one of the pigment systems may, if it is not needed there, be transferred to the other reaction center and used there. The efficiency of transfer may depend on the wavelength of activation, on the conditions of the reaction and on the rates of competing excitation-loss processes. In an extreme version of the "spillover" hypothesis, proposed by Avron and Ben-Hayyim (1969), the distinction between the two pigment systems disappears (at least at all but the longest wavelengths) and photons absorbed anywhere in the antenna may ultimately reach either type of reaction center. It should be

TABLE I

Quantum yields extrapolated to zero intensity for NADP reduction by [ascorbate + $DPIPH_2$] and by water and for the DPIP Hill reaction of spinach chloroplasts at various wavelengths.

λ (nm)	Quantum yields (equivalents/einstein absorbed)			
	[ascorbate + $DPIPH_2$ → NADP] ϕ_I	[H_2O → DPIP] ϕ_{II}	$\phi_I + \phi_{II}$	[H_2O → NADP] $\phi(I + II)$
620	0.67 ± 0.04	[illegible]	[illegible]	0.50 ± 0.02
630	0.61 ± 0.04	1.00 ± 0.04	1.61 ± 0.08	0.50 ± 0.02
640	0.53 ± 0.04	0.95 ± 0.05	1.48 ± 0.09	0.49 ± 0.02
650	0.51 ± 0.03	1.00 ± 0.04	1.51 ± 0.08	0.50 ± 0.02
660	0.50 ± 0.03	0.96 ± 0.05	1.46 ± 0.08	0.48 ± 0.03
670	0.43 ± 0.03	0.63 ± 0.05	1.06 ± 0.08	0.45 ± 0.05
678	0.38 ± 0.11	0.56 ± 0.06	0.94 ± 0.17	0.50 ± 0.03
690	0.61 ± 0.04	0.44 ± 0.03	1.05 ± 0.07	0.43 ± 0.04
700	1.00 ± 0.05	0.25 ± 0.03	1.25 ± 0.08	0.19 ± 0.03
710	0.96 ± 0.05	0.18 ± 0.03	1.14 ± 0.08	0.16 ± 0.01
720	0.96 ± 0.05			

recognized that proximity of a given antenna pigment molecule to a reaction center may be important in determining the probability of transfer of its excitation, particularly where the time required for all of the necessary transfer steps to occur is comparable with the chlorophyll fluorescence lifetime. These spillover models, in common with most others, assume that the longest wavelengths of activating light involve photons with insufficient energy to activate the reaction center of light reaction II.

If there is a single pigment system absorbing in the red region (630 to 660 nm) and if both types of reaction center can receive excitation from this pigment system, then we might expect the quantum yields for both light reaction I and for light reaction II to be the same for these actinic wavelengths. Our measurements show, however, that the quantum yield of light reaction II is about twice as large as that for light reaction I in the red region. This suggests that the two types of reaction centers are not symmetrically disposed with respect to the antenna pigments. When reaction center I is not operating because of the absence of suitable electron acceptors (NADP and/or ferredoxin) all excitation in the red region is able to be trapped and utilized for light reaction II photochemistry. On the other hand, when light reaction II is blocked by DCMU, only half the red photons absorbed are capable of producing electron transfer in light reaction I. We know that this is not due to inactivation of system I reaction centers, because they are fully effective for long wavelength photons (700 to 720 nm). Also, in order to account for a quantum

yield of 0.5 at short wavelengths for the [$H_2O \rightarrow$ NADP] reaction, where both light reactions operate in series, it is necessary to assume that the photons absorbed are partitioned equally between the two light reactions and that each light reaction is able to use essentially all of the excitation that it receives.

One measure of the fate of excitation not utilized by light reaction I is the fluorescence associated with photosystem II. This fluorescence, which has an emission spectrum that is consistent with its origin in photosystem II pigments, has been interpreted as containing a "live" portion and a "dead" portion (Clayton, 1969). The "live" portion is seen to compete with photochemistry carried out by light reaction II and attains a maximum efficiency when photochemistry is saturated at high light intensities. The "dead" portion, on the other hand, contributes a constant yield of fluorescence that is independent of the state of photosystem II. If this indeed is the case, then it is unlikely that the "dead" fluorescence comes from the same site as is responsible for the "live" fluorescence and for photochemistry. The "dead" fluorescence may come from a non-functional trap or from the antenna pigment molecules themselves. In either case, it represents a constant leak of excitation energy from the chloroplast membranes. The addition of DCMU, which blocks photosystem II light reactions, causes the "live" fluorescence to rise to its maximum value. This suggests that DCMU does not close the traps of light reaction II as energy sinks. In fact they appear to operate just as efficiently in this respect in the presence or in

the absence of DCMU. This observation is difficult to reconcile with our quantum yield measurements, for virtually all of the short wavelength photons are trapped by light reaction II in the [$H_2O \rightarrow$ DPIP] reaction in the absence of DCMU, whereas in the presence of DCMU in the [ascorbate + $DPIPH_2 \rightarrow$ NADP] reaction at least half of these short wavelength photons must reach light reaction I. Yet the maximum yield of "live" fluorescence from photosystem II is the same in both cases.

Perhaps the simplest way around this apparent inconsistency is in the alternative model of Avron and Ben-Hayyim (1969) in which a control mechanism determines the fraction of excitation that reaches each reaction center. This control mechanism is under the influence of factors which differ from one set of reaction conditions to another. The most likely candidates for this factor are the redox potential of the system and the concentrations of added cofactors. Experiments by Murata (1969) suggest that Mg^{++} concentration plays a role in excitation transfer among the antenna pigments; however, the effects observed here could result from a complex interaction among several such components. Additional results reported by Avron and Ben-Hayyim (1969), where flavin mononucleotide (FMN) or diquat are the electron acceptors for the photosystem I reaction, show that in these cases 640 nm photons are fully effective in producing electron transfer. This contrasts sharply with the [ascorbate + $DPIPH_2 \rightarrow$ NADP] reaction and is further evidence that some control mechanism is determining the fraction of red light excitation that is available to photosystem I.

In summary, the results of quantum yield measurements for the separate photosystem I and photosystem II reactions show that s simple separate package model for the antenna pigment molecules is inconsistent with the experimental findings. The variability of the fraction of photons in the red region of the spectrum that is available to each of the reaction centers suggests the presence of a control mechanism that regulates electronic excitation transfer among major portions of the antenna pigment molecules. The investigation and identification of the mechanism of this "controlled spillover" hypothesis will be an important field for future research.

III. EMERSON ENHANCEMENT IN BROKEN SPINACH CHLOROPLASTS

A. Introduction

In Chapter II, we addressed the question of the number of photons required to carry out PS I, PS II or PS (I + II) reactions. In this chapter we report experiments designed to answer the question whether PS I and PS II are both required to transfer electrons in a series fashion in the [$H_2O \rightarrow$ NADP] reaction (Avron, 1967; Sun and Sauer, 1971; Hoch and Martin, 1963; Sauer and Biggins, 1965). An alternative mechanism has been proposed by Knaff and Arnon in which both of the photosystems operate in independent pathways and only PS II (PS IIa + PS IIb) is involved in the [$H_2O \rightarrow$ NADP] reaction (1969). In both reactions electrons are transferred from water to $NADP^+$, leading to oxygen evolution and the reduction of $NADP^+$ to NADPH. The phosphorylation of ADP to ATP can be coupled to the [$H_2O \rightarrow$ NADP] reaction.

In Chapter II we reported that the [ascorbate + $DPIPH_2 \rightarrow$ NADP] reaction, characteristic of PS I, requires only one quantum of light at 700 nm or longer wavelengths in order to transfer one electron from $DPIPH_2$ to $NADP^+$ in the presence of DCMU (Sun and Sauer, 1971). The [$H_2O \rightarrow$ DPIP] Hill reaction is characteristic of PS II and requires only one quantum of 630 to 660 nm light to transfer one electron from water to DPIP, provided that the reaction conditions are adjusted to give efficient spillover of

excitation from P I to P II. The [$H_2O \rightarrow$ NADP] reaction requires two quanta from 620 to 678 nm to transfer one electron from H_2O to NADP under the same conditions favoring spillover (Sun and Sauer, 1971). However, it is impossible on the basis of action spectra and quantum requirement measurements alone to answer the question of whether the [$H_2O \rightarrow$ NADP] reaction is driven by PS (I + II) or PS (IIa + IIb). One way of providing conclusive evidence that the [$H_2O \rightarrow$ NADP] reaction involves PS (I + II) is to demonstrate the occurrence of the Emerson enhancement effect for this reaction in chloroplasts.

Using whole cells of Chlorella and of Chroococcus, Emerson and Lewis (1942, 1943) found a sharp decrease in the efficiency of photosynthesis with actinic light of wavelengths longer than 685 nm (red drop in efficiency). By adding a weak background of green light to the far-red light, Emerson et al. (1957, 1958) found that the combined wavelengths produced higher photosynthetic rates than the sum of the rates for the two lights used separately. The series formulation involving two light reactions gained support from these experiments (Hill and Bendall, 1960). The basic idea was that far-red light, absorbed predominantly by PS I, could be supplemented or "enhanced" by adding light that was preferentially absorbed by PS II. This enhancement has been demonstrated to occur in whole cells or intact leaves in a wide variety of oxygen-evolving organisms (Emerson, 1957, Emerson et al., 1950, 1957; Govindjee and Rabinowitch, 1960; Myers and French, 1960; Gibbs et al., 1963; Mayne and Brown, 1963). Nevertheless, it was argued

that the origin of enhancement in whole cells is not in the primary light-driven electron transport reactions, but is a consequence of feedback loops in the dark reaction involving the requirements of CO_2 fixation for NADPH and ATP. NADPH is produced via non-cyclic electron transport and ATP is produced, at least in part, via cyclic electron flow involving only PS I. In order to resolve the origin of enhancement, it is necessary to find out whether enhancement occurs in isolated broken chloroplasts, where CO_2 fixation is not coupled and only a primary light reaction effect would be observed.

The occurrence of significant enhancement in the [$H_2O \rightarrow$ NADP] reaction by isolated broken chloroplasts has been reported by Govindjee et al. (1962, 1964), Gordon (1963), Joliot et al. (1968), and Avron and Ben-Hayyim (1969). On the other hand, Gibbs et al. (1963) and McSwain and Arnon (1968) studied the reduction of $NADP^+$ (and of ferricyanide) by H_2O in isolated chloroplasts and found no measurable enhancement. The lack of enhancement was interpreted as indicating no cooperation between PS I and PS II. Arnon (1967) proposed that either the [ascorbate + $DPIPH_2 \rightarrow$ NADP] or the [$H_2O \rightarrow$ NADP] reaction is driven by a single light reaction, PS I for the former and PS II for the latter. The hypothesis was that only one photon per electron transferred was required in each of these reactions. It was subsequently modified in the light of the behavior of a new photoreactive chloroplast component C550 (1969). The latest version of this hypothesis (Knaff and Arnon, 1969) suggests that three light reactions are involved in photosynthesis;

two are in PS II and are short-wavelength light reactions, and one is in PS I and is a long-wavelength reaction. The hypothesis also states that there is no direct cooperation between PS I and PS II, and only PS II (a + b) is involved in activating the basic reaction of photosynthesis, i.e., the [$H_2O \rightarrow$ NADP] reaction.

In the present study we present further evidence that Emerson enhancement does occur in broken spinach chloroplasts for the [$H_2O \rightarrow$ NADP] reaction. We have also found what we believe to be the reason why some laboratories have been unable to observe enhancement in isolated chloroplasts despite apparently extensive and painstaking efforts. The discovery of the cause of this variability in turn uncovers an important new phenomenon relevant to photosynthetic control mechanisms.

B. Materials and Methods

(1) Spinach and preparation of chloroplasts: same as described in Chapter II.

(2) Reagents

In addition to those chemicals described in Chapter II, manganous chloride was obtained from J. T. Baker Chemical Co., Phillipsburg, N. J., and trizma base from Sigma Chemical Co., St. Louis, Missouri.

(3) Apparatus and light intensity measurement

The apparatus for monitoring $NADP^+$ reduction, based on a Cary 14 spectrophotometer, was similar to that used in Chapter II.

Actinic lights from two identical monochromators (Bausch and Lomb, 500 mm, red-blaze grating) were brought to approximate focus on the same side of the cuvette in the sample beam. Apart from the converging lenses and an intermediate mirror in one beam, each monochromatic beam was supplemented with appropriate short-wavelength cut-off filters (Sauer and Biggins, 1965). The intensities of the actinic lights were measured as in Chapter II.

(4) Reaction mixture and preparation of ferredoxin, NADP reductase and plastocyanin

The reaction mixture of the [ascorbate + $DPIPH_2$ → NADP] reaction, of the [H_2O → DPIP] reaction and of the [H_2O → NADP] reaction were the same as described in Chapter II except for those stated specifically in the text under special conditions. Saturating amounts of plastocyanin were added to each of the three reaction mixtures and saturating ferredoxin and ferredoxin-NADP reductase were added to the [H_2O → NADP] and the [ascorbate + $DPIPH_2$ → NADP] reactions, except for those cases stated specifically in the text. Ferredoxin, plastocyanin and NADP reductase were prepared from commercial spinach as described in Chapter II.

C. Results

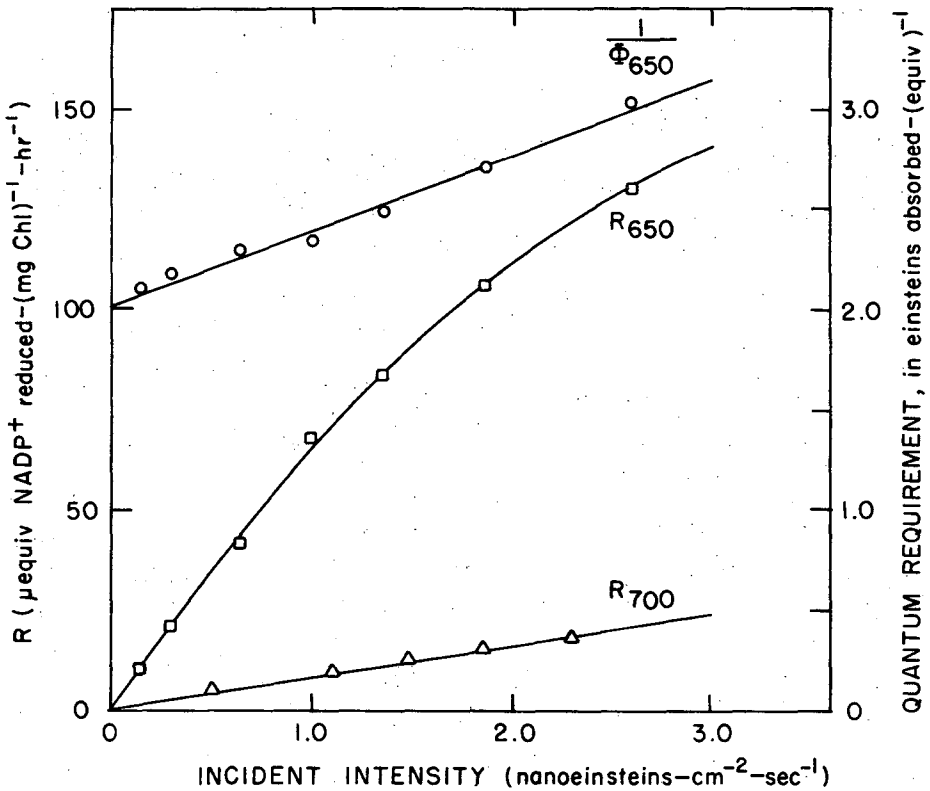
1. [H_2O → NADP] reaction and enhancement studies

The rate of photoreduction of NADP as a function of incident intensity. As described in Chapter II, the quantum requirements for the [H_2O → NADP] reaction increased gradually as a linear

function of the incident light intensity within the range studied. The quantum requirements at 650 nm and the rates of photoreduction of $NADP^+$ at 650 nm and 700 nm within this intensity range are shown in Figure 16. Incident light intensities from zero to approximately 3.0 nanoeinsteins-cm^2-sec^{-1} were used in the study of enhancement effect. Only very active chloroplasts, as in Figure 16, were used.

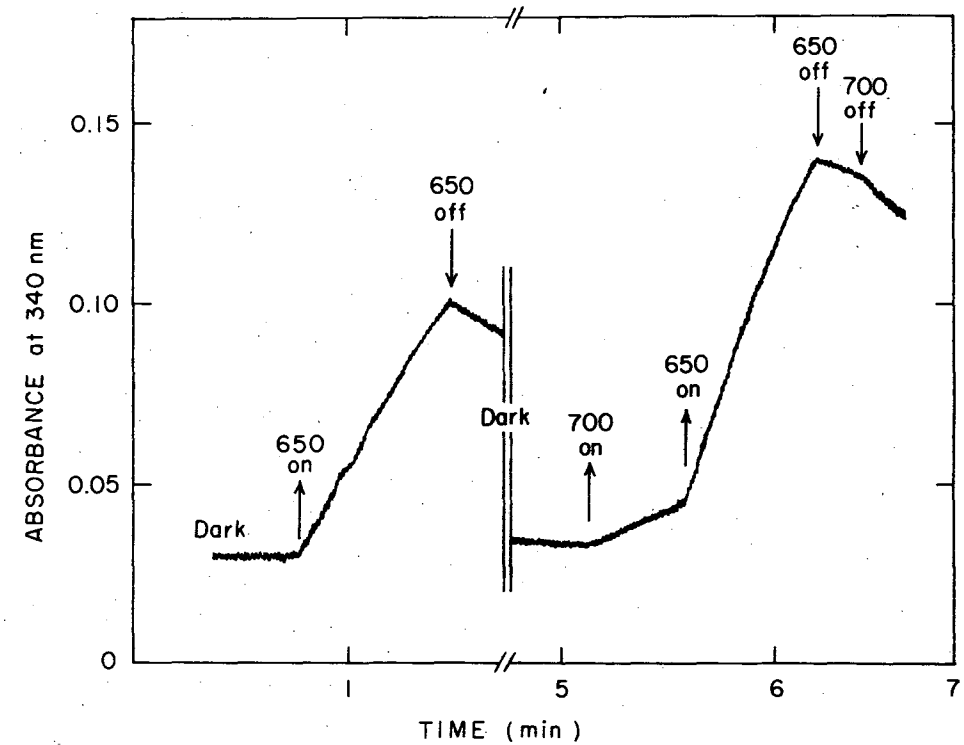
Patterns of sequential presentation of two actinic lights. In order to obviate possible biases, we examined four different sequences of presentation of the actinic lights. (A) First illumination with a red light (650 nm) to obtain the rate of the reaction, R_R, at intensity I_R; dark interval (ca. 3 min) until the rate of the back reaction became constant; illumination with 700 nm light of intensity I_{FR} to obtain a rate R_{FR}; illumination with red light of intensity I_R added to the far-red light, giving the rate R_{FR+R}. This pattern is designated [R_R; R_{FR}, R_{FR+R}]. A typical time course of these rates is shown in Figure 17. (B) Using the notation adopted above, we then modified the actinic illumination to provide the sequence [R_{FR}; R_{FR+R}; R_R]. (C) A third pattern used was [R_{FR}; R_R, R_{R+FR}]. (D) A fourth pattern used was [R_R, R_{R+FR}; R_{FR}]. The objective of using these different patterns was to demonstrate that enhancement can be observed regardless of the order in which the actinic wavelengths are presented.

Several different measures of enhancement are used in the literature on this subject (Delrieu and Kouchkovsky, 1971). In order to facilitate comparisons with other results, we have



XBL716-5250

Figure 16. Quantum requirements at 650 nm (o) and the rates of photoreduction of $NADP^+$ at 650 nm (□) or 700 nm (Δ) in the [$H_2O \rightarrow$ NADP] reaction by broken chloroplasts (CP-suc) as functions of the actinic light intensity. The reaction mixture (Solution A) is given in the text. The rate of $NADP^+$ reduction was measured as the change in absorbance at 340 nm per unit time. Chlorophyll concentration 27 μg/ml.



XBL716-5251

Figure 17. Typical time course for the photoreduction of $NADP^+$ by broken chloroplasts (CP-suc) in the [$H_2O \rightarrow$ NADP] reaction using two actinic wavelengths. The reaction mixture (Solution A) is described in the text. The illumination pattern [R_R: R_{FR}, R_{FR+R}] is illustrated in this experiment; incident intensity of far-red light at 700 nm, 2.3 nanoeinsteins-cm^{-2}-sec^{-1}; incident intensity of red light at 650 nm, 1.4 nanoeinsteins-cm^{-2}-sec^{-1}. Chlorophyll conconetration 27 μg/ml.

calculated enhancement ratios based on a portion of our results in three different ways, according to the following equations:

$$E_1 = \frac{R_{FR+R} - R_R}{R_{FR}} \qquad E_2 = \frac{R_{FR+R} - R_{FR}}{R_R} \qquad E_3 = \frac{R_{FR+R}}{R_{FR} + R_R}$$

where

R_R = the rate of the reaction with red actinic light alone.

R_{FR} = the rate of the reaction with far-red light alone.

R_{FR+R} = the rate of the reaction when both red and far-red lights were incident simultaneously.

The rates of the reaction obtained with a single sample are used to calculate the enhancement values, E_1, E_2, and E_3. The enhancement values shown in Table II were obtained using an incident light intensity, I_R, about 1.4 nanoeinsteins-cm^{-2}-sec^{-1} at 650 nm and I_{FR} about 2.3 nanoeinsteins-cm^{-2}-sec^{-1} at 700 nm. At these intensities, R_R is nearly four times greater than R_{FR}, and the value of E_1 is significantly larger than E_2 or E_3. At lower relative light intensities at 650 nm, when the demoninators become smaller, values of E_2 or E_3. At lower relative light intensities at 650 nm, when the denominators become smaller, values of E_2 and E_3 close to 2.0 are obtained. Because values of E_1, E_2 and E_3 are essentially describing the same enhancement phenomenon, we have chosen E_1 as the preferred parameter to characterize the enhancement effect for the remainder of this study.

In Table III the values of E_1 are shown for the four different sequences of illumination described above. The E_1 values obtained

TABLE II

ENHANCEMENT CALCULATED AS E_1, E_2 or E_3 FOR THE [H_2O -- NADP] REACTION BY CHLOROPLASTS

Spinach chloroplasts (CP-suc) in tricine, 45 mM, pH 7.5; $MgCl_2$, 7.5 mM; plastocyanin, ferredoxin and ferredoxin-NADP$^+$ reductase, added in saturating amounts. Experimental conditions as in Fig. 17. Illumination pattern: [R_R; R_{FR}, R_{FR+R}]. Incident intensities: 1.4 and 2.3 nanoeinsteins-cm^2-sec^{-1} at 650 and 700 nm, respectively. Definitions of E_1, E_2 and E_3 given in the text.

Sample	Rate of NADP$^+$ Reduction ΔA-min^{-1} at 340 nm			E_1	E_2	E_3
	R_{650}	R_{700}	$R_{700+650}$			
1	0.117	0.0306	0.191	2.43	1.38	1.30
2	0.107	0.0305	0.182	2.46	1.42	1.32
3	0.099	0.0240	0.162	2.66	1.41	1.33

TABLE III

ENHANCEMENT OBTAINED USING DIFFERENT PATTERNS OF ILLUMINATION FOR THE [$H_2O \rightarrow NADP$] REACTION BY CHLOROPLASTS

Reaction conditions as in Table II, but with different illumination patterns, as indicated. R, rate of $NADP^+$ reduction, $\Delta A\text{-min}^{-1}$ at 340 nm.

Sample	Illumination Pattern							
	(A) [R_R; R_{FR}, R_{FR+R}]				(B) [R_{FR}, R_{FR+R}; R_R]			
	R_{650}	R_{700}	$R_{700+650}$	E_1	R_{700}	$R_{700+650}$	R_{650}	E_1
1	0.110	0.0288	0.186	2.64	0.0288	0.186	0.105	2.84
2	0.105	0.0276	0.179	2.71	0.0284	0.189	0.109	2.82
3	0.100	0.0282	0.172	2.58	0.0284	0.182	0.105	2.74

Sample	Illumination Pattern							
	(C) [R_{FR}; R_R, R_{R+FR}]				(D) [R_R, R_{R+FR}; R_{FR}]			
	R_{700}	R_{650}	$R_{650+700}$	E_1	R_{650}	$R_{650+700}$	R_{700}	E_1
1	0.0284	0.092	0.181	3.13	0.092	0.181	0.0294	3.02
2	0.0278	0.090	0.181	3.27	0.101	0.181	0.0284	2.83
3	0.0279	0.095	0.186	3.28	0.109	0.187	0.0280	2.81

in the illumination sequences (A) and (B) are very similar, but E_1 of (A) is always slightly smaller than that of (B). The E_1 values obtained in the illumination sequences (C) or (D) are always larger than those obtained in (A) or (B). Therefore, the illumination order (A) is the most conservative way to measure the enhancement effect among the four. We use this as the standard illumination sequence in our subsequent experiments.

Absence of enhancement with two actinic lights at 650 nm. In order to confirm the absence of unsuspected contributing effects in the enhancement study, we carried out the following control experiment: We first illuminated the sample with actinic light I at 700 nm and actinic light II at 650 nm, to obtain the E_1 value, as described in Table II. Then we changed the wavelength of the actinic light I monochromator from 700 to 650 nm and carefully adjusted its intensity to about 0.3 nanoeinsteins-cm^{-2}-sec^{-1}, which gave the same rate of $NADP^+$ reduction as when the actinic light I at 700 nm was 2.3 nanoeinsteins cm^{-2}-sec^{-1}. We then repeated the experiment and obtained E_1 in the same way as described before. The only difference is that actinic light I in the first case is at 700 nm and in the second case is at 650 nm. The results are shown in Table IV. The enhancement value E_1 obtained with actinic light I at 700 nm and II at 650 nm is 2.4 to 2.6. But E_1 is less than 1.0 when both actinic lights I and II are at 650 nm. Apparently there is an enhancement effect in the former but no such effect in the latter experiment. Because the rate of the reaction is not quite a linear function of the incident light

TABLE IV

ENHANCEMENT E_1 WITH TWO ACTINIC LIGHTS AT DIFFERENT OR AT THE SAME WAVELENGTH FOR THE [H_2O -- NADP] REACTION BY CHLOROPLASTS

Reaction conditions as in Table II. Wavelengths of illumination are indicated in the table. Three chloroplast samples were measured and twelve measurements on each were made successively in the order of presentation. R, rate of $NADP^+$ reduction, $\Delta A\text{-min}^{-1}$ at 340 nm.

Sample	R_{650}	R_{700}	$R_{700+650}$	E_1	R_{650}	R'_{650}	$R_{650+650}$	E_1
1	0.095	0.0307	0.174	2.59	0.099	0.0321	0.130	0.90
2	-	-	-	-	0.099	0.0314	0.128	0.91
3	0.095	0.0288	0.168	2.53	0.091	0.0302	0.117	0.86
1	0.092	0.0287	0.165	2.54	0.090	0.0311	0.118	0.88
2	0.093	0.0286	0.162	2.42	0.090	0.0301	0.115	0.84
3	0.089	0.0274	0.157	2.46	0.090	0.0296	0.113	0.78

intensity, as shown in Figure 16, the "enhancement" ratio is found to be somewhat less than 1.0 when the two actinic lights are both at 650 nm.

Repeatability of the enhancement effect in the [$H_2O \rightarrow$ NADP] reaction. The rate of the reaction and the red/far-red enhancement effect are closely correlated with the activity of the chloroplasts. When the rate of the reaction is within 20% of the rate shown in Figure 16 under identical experimental conditions, the enhancement values, E_1, are very reproducible, 2.4 $\pm$ 0.3. The rate of the reaction decreases steadily, but slowly, when the same sample is illuminated repeatedly. For Sample 1 in Table IV the rate of the reaction is $\Delta A_{340\ nm}$/min = 0.095 at the first illumination; it decreased to 0.090 at the 10th illumination. High light intensities and long illuminations tend to decrease the rate of the reaction more rapidly than do low light intensities and short illuminations. Chloroplasts studied immediately following isolation tend to retain their activity better than do those that have been standing in the dark at 0°C for 6 hr. In our experience when the chloroplasts are fresh and their activity is high, the rate of the reaction does not decrease more than 20% of the rate at the first illumination during the course of an experiment lasting 30 min. A typical example is shown in Table V. The rate of the reaction was $\Delta A_{340\ nm}$/min = 0.117 at the first illumination; it decreased to 0.095 at the 10th illumination. Nevertheless, E_1 at the first illumination is 2.43, and it is 2.33 at the 13th. When the rate of the reaction decreased to $\Delta A_{340\ nm}$/min = 0.086 at the 16th

TABLE V

REPEATABILITY OF THE ENHANCEMENT EFFECT FOR THE [H_2O -- NADP] REACTION BY CHLOROPLASTS

Reaction conditions as in Table II. The order of the 18 measurements on a single chloroplast sample was top to bottom in each column, then left to right. R, rate of reduction of $NADP^+$, $\Delta A \cdot min^{-1}$ at 340 nm. Illumination patterns as described in the text.

Illumination Pattern		Measurement Index Number					
		1-3	4-6	7-9	10-12	13-15	16-18
	R_{650}	0.117	0.111	0.107	0.095	0.088	0.086
	R_{700}	0.0305	0.0305	0.0237	0.0253	0.0230	0.0247
	$R_{700+650}$	0.191	0.188	0.159	0.154	0.137	0.128
(A)	E_1	2.43	2.33	2.22	2.33	2.13	1.70
(B)	E_1	-	2.63	2.45	2.73	2.60	2.20

illumination, E_1 decreased to 1.70. We disregard the result when the rate of the reaction decreases below $\Delta A_{340\ nm}/min$ = 0.095; i.e., 20% below the rate at the first illumination.

Comparison of the enhancement effect with different chloroplasts and reaction mixtures. There are two major differences between our experiments and those in which the enhancement effect fails to occur (Gibbs et al., 1963; McSwain and Arnon, 1968). Chloroplasts isolated in NaCl (CP-NaCl) were used in those studies, whereas we used chloroplasts isolated in sucrose (CP-suc) in our initial studies (Sun and Sauer, 1971). The second difference is in the reaction mixtures. Therefore, we investigated the enhancement effect with different reaction mixtures and using both CP-suc and CP-NaCl. We call our standard reaction mixture Solution A. The second reaction mixture, Solution B, is after McSwain and Arnon (1968). The three major differences between Solutions A and B are:

Solution A: Tricine buffer, 45 mM, pH 7.5; $MgCl_2$, 7.5 mM; (ADP+Pi) not added

Solution B: Tricine buffer, 33 mM, pH 8.2; $MgCl_2$, 1.7 mM; (ADP+Pi), 3.3 mM

We examined the effect on the enhancement for each chloroplast preparation and for each reaction mixture in turn. The results are shown in Table VI. We find that CP-suc and CP-NaCl were equally active in Solution A with saturating plastocyanin (PCy) and

TABLE VI

ENHANCEMENT FOR THE [$H_2O \rightarrow NADP$] REACTION BY CHLOROPLASTS IN DIFFERENT REACTION MIXTURES

Chloroplasts in Solution A [Tricine buffer, 45 mM, pH 7.5; $MgCl_2$, 7.5 mM; $NADP^+$, 0.67 mM] or Solution B [Tricine buffer, 33 mM, pH 8.2; $MgCl_2$, 1.7 mM; ADP + Pi, 3.3 mM; $NADP^+$, 3.3 mM], and for both solutions, ferredoxin, saturating; plastocyanin (PCy), saturating or not added; ferredoxin-$NADP^+$ reductase (Fp), saturating or not added. Enhancement values determined for reactions in "normal" solutions A and B are given in columns (1) and (5), respectively. Alterations of the "normal" solutions, as indicated in columns (2), (3), (4), and (6), (7), (8) were done singly, not compounded. Enhancement values E_1 are tabulated; illumination pattern (A).

Chloroplast Preparation	PCy + Fp	Solution A				Solution B			
		Normal (1)	pH 8.2 (2)	+(ADP+Pi) (3)	$MgCl_2$ 1.67 mM (4)	Normal (5)	pH 7.5 (6)	−(ADP+Pi) (7)	$MgCl_2$ 7.5 mM (8)
CP-suc	+	2.32	1.62	1.80	0.92	−0.24	0.63	0.34	2.09
		2.14	1.39	1.44	0.84	0.25	0.26	0.27	2.23
		2.26	1.59	1.62	0.74	0.36	0.32	0.36	2.14
	−	1.49	−0.43	1.37	0.34	0.37	0.24	0.42	1.63
		1.54	−0.20	1.20	0.36	−0.21	0.63	0.53	1.37
		1.36	0.14	1.14	0.68	0.32	−0.16	0.96	1.41
CP-NaCl	+	2.43	1.84	1.80	0.98	0.98	0.86	1.08	2.03
		2.13	1.75	1.26	0.84	0.74	0.68	0.84	1.98
		2.36	1.78	1.54	1.02	0.63	0.62	0.86	1.86
	−	2.12	1.57	1.68	0.93	0.55	0.74	1.10	2.26
		2.59	1.72	1.52	1.06	0.64	0.62	0.84	1.88
		2.34	1.81	1.57	0.86	0.76	0.64	0.53	1.94

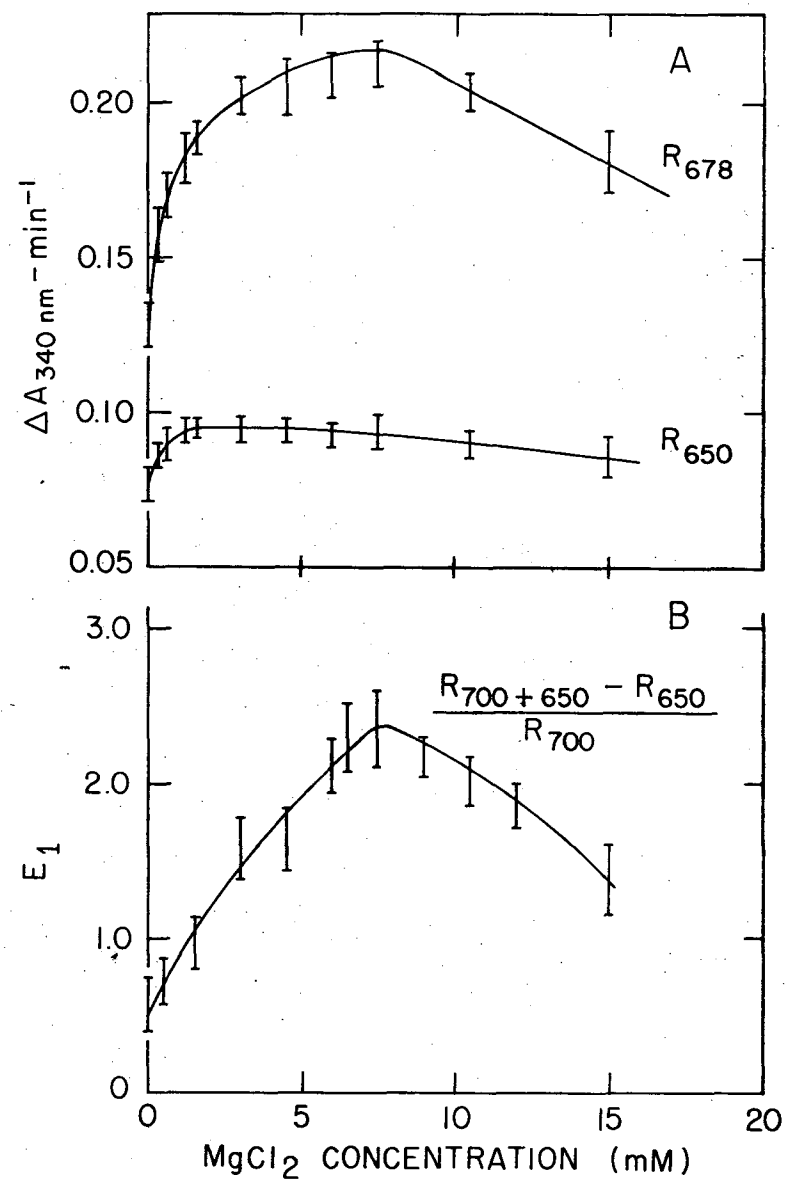
ferredoxin-NADP reductase (Fp). The enhancement values E_1 are all in the range 2.3 ± 0.2, as shown in column (1) of Table VI. When Solution A is not supplemented with PCy or Fp, the CP-suc are not so active as in the former condition (Sun and Sauer, 1971), and E_1 was ca. 1.5. However, CP-NaCl are as active in both conditions, and E_1 values are 2.4 ± 0.3. It is possible that the NaCl isolation process produces better retention of endogenous PCy and/or Fp. Because the enhancement effect could be demonstrated with both CP-suc and CP-NaCl, the discrepancy in the enhancement results cannot be due solely to the difference in the chloroplast preparations.

Next, we examined the enhancement effect using CP-suc or CP-NaCl in either Solution A or B. The results are shown in columns (1) and (5) of Table VI. We find that both types of chloroplasts exhibit enhancement in Solution A with or without PCy and Fp, but no enhancement is obtained when the same CP-suc or CP-NaCl preparations are used in Solution B either with or without PCy and Fp. These results clearly slow that the critical factors controlling enhancement reside in the three differences between Solutions A and B. When we change the pH from 7.5 to 8.2 or add (ADP+Pi) in Solution A [columns (2) and (3)], the values of E_1 decrease to 1.4 - 1.9 but enhancement is still evident. When we change the pH from 8.2 to 7.5 or do not add the (ADP+Pi) in Solution B [columns (6) and (7)], the E_1 values were below 1.0, and no enhancement could be observed. When we changed the $MgCl_2$ concentration from 7.5 mM to 1.67 mM in Solution A [column (4)], we find

that all E_1 values decrease to 1.0 or below. On the other hand, when we change the $MgCl_2$ concentration from 1.67 mM to 7.5 mM in Solution B [column (8)], the E_1 values increased from below 1.0 to about 2.0. These results clearly show that a higher concentration of $MgCl_2$, 7.5 mM, is necessary for the enhancement effect in the [$H_2O \rightarrow$ NADP] reaction using broken spinach chloroplasts.

Dependence of enhancement on the $MgCl_2$ concentration. We reported in Chapter II that the rate of photoreduction of $NADP^+$ in the [$H_2O \rightarrow$ NADP] reaction is dependent upon the $MgCl_2$ concentration. Further studies now indicate a complex relationship between the optimal $MgCl_2$ concentration and the wavelength and intensity of the actinic light. The results in Figure 18 (upper curves) show that the rate of the [$H_2O \rightarrow$ NADP] reaction reaches its maximum at 1.5 mM $MgCl_2$ when the incident actinic light at 650 nm is 1.40 nanoeinsteins-cm^{-2}-sec^{-1}, but the maximum rate of the reaction occurs at 7.5 mM $MgCl_2$ when the actinic light at 678 nm is 2.4 nanoeinsteins-cm^{-2}-sec^{-1}.

The enhancement effect of far-red (700 nm) light on red (650 nm) light for the [$H_2O \rightarrow$ NADP] reaction of broken chloroplasts is also a function of the $MgCl_2$ concentration (Figure 18, lower curve). No significant enhancement is observed when $MgCl_2$ is below 3.0 mM. Enhancement reaches a maximum at 7.5 mM $MgCl_2$ ($E_1 = 2.4 \pm 0.3$), which is the same concentration of $MgCl_2$ that gives a maximum rate, R_{678}, using 678 nm light alone. At higher $MgCl_2$ concentrations (up to 15 mM) both the rate of the [$H_2O \rightarrow$ NADP] reaction at 678 nm and the enhancement value decrease. The similar dependence on $MgCl_2$ concentration of both the rate and the enhancement effect



XBL716-5254

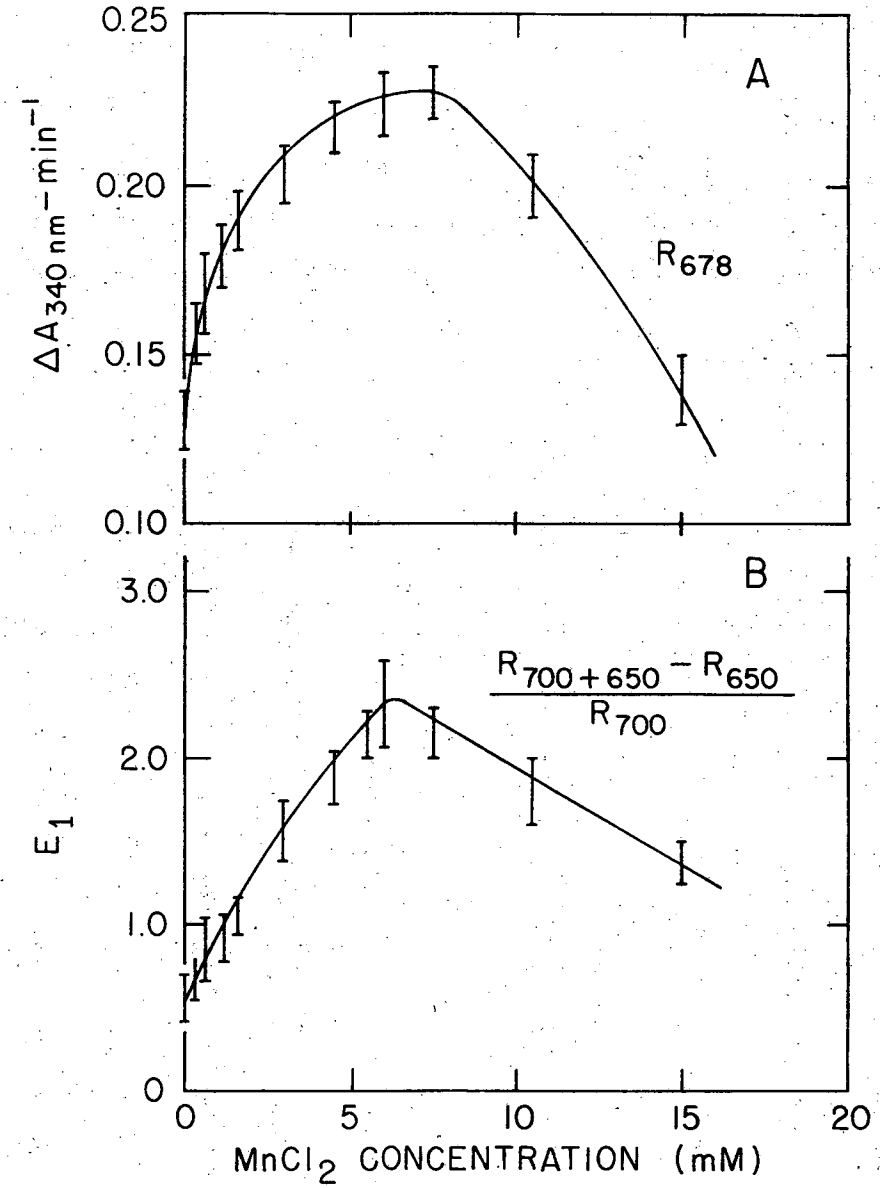
Figure 18. Effect of the $MgCl_2$ concentration on the rate (A, upper curves) and on the enhancement E_1 (B, lower curve) of the [$H_2O \rightarrow$ NADP] reaction by broken chloroplasts (CP-suc). Experimental conditions as described in Figure 17, except $MgCl_2$ concentration varied; incident intensity of 678 nm light, 2.4 nanoeinsteins-cm^{-2}-sec^{-1}. Vertical bars show standard deviations of replicate measurements.

suggest that these two features arise from a common origin affecting the state of the broken chloroplasts.

Dependence of enhancement on $MnCl_2$, NaCl and sucrose. Because of the well known ability of Mn^{2+} to replace Mg^{2+} in enzymatic reactions (Dixon and Webb, 1964), we investigated the effect of $MgCl_2$ (as a replacement for $MgCl_2$) on the rate and the enhancement effect for the [$H_2O \rightarrow$ NADP] reaction. As shown in Figure 19, we find that $MnCl_2$ duplicates the behavior of $MgCl_2$ (Figure 18) in both respects.

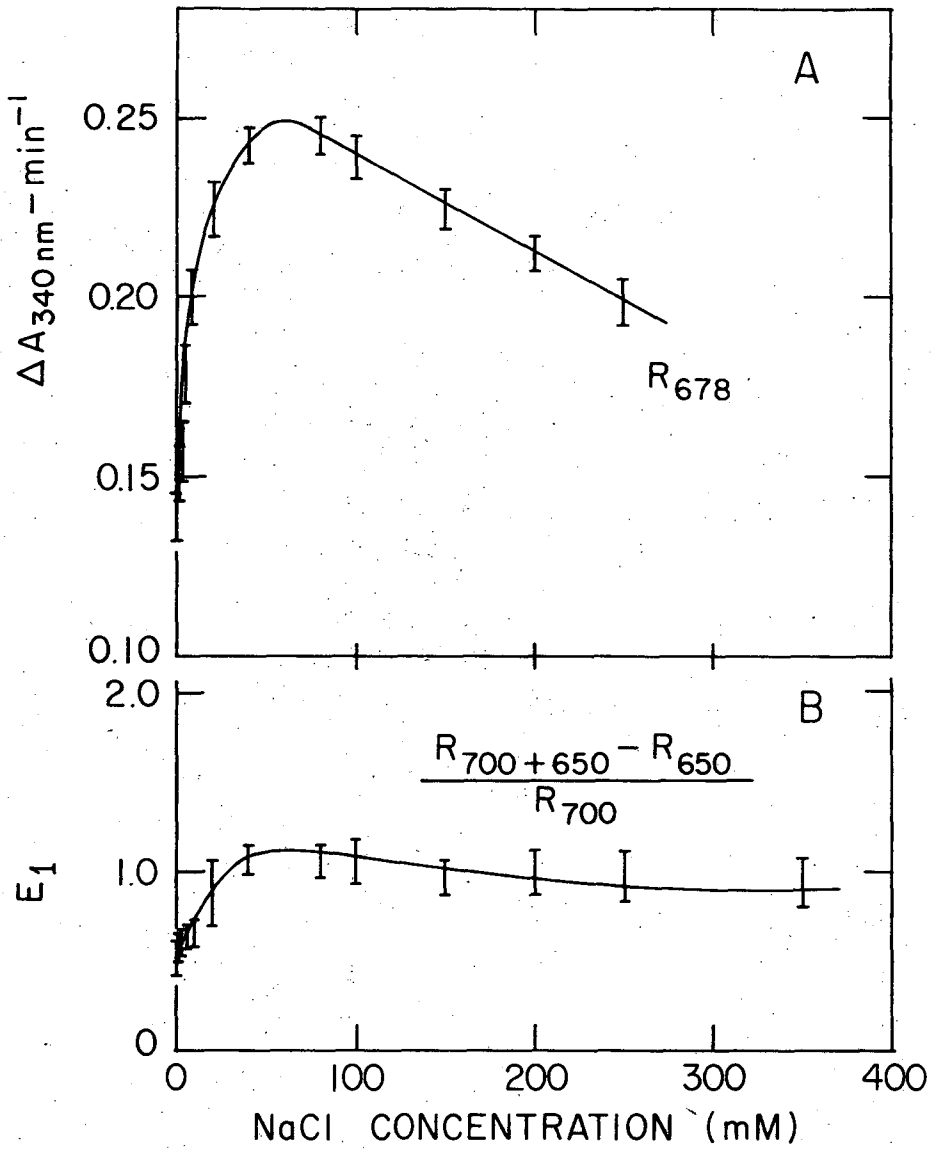
The effect of NaCl on the rate and enhancement of the [$H_2O \rightarrow$ NADP] reaction is shown in Figure 20. The rate increases from $\Delta A_{340\ nm}$/min = 0.14 at zero concentration to the maximum $\Delta A_{340\ nm}$/min = 0.25 at 75 mM NaCl, then decreases at higher concentrations. The enhancement ratio E_1 also increases from 0.5 $\pm$ 0.1 at zero concentration to 1.1 $\pm$ 0.1 at 75 mM NaCl and decreases at higher concentrations. Although both the rate and the enhancement are affected by NaCl concentration, no enhancement significantly greater than unity could be observed throughout the range 0 to 350 mM.

We also studied the effect of sucrose, in lieu of $MgCl_2$, on the rate and the enhancement of the [$H_2O \rightarrow$ NADP] reaction. The rate of the reaction is about $\Delta A_{340\ nm}$/min = 0.17 $\pm$ 0.02, under the experimental conditions described in Figure 20, at sucrose concentrations up to 125 mM. No enhancement effect ($E_1 \leq 1.0$) can be observed in this sucrose concentration range. At sucrose



XBL 716-5255

Figure 19. Effect of the $MnCl_2$ concentration on the rate (A, upper curve) and on the enhancement E_1 (B, lower curve) of the [$H_2O \rightarrow$ NADP] reaction by broken chloroplasts. Experimental conditions as in Figure 18 except $MnCl_2$ in place of $MgCl_2$.



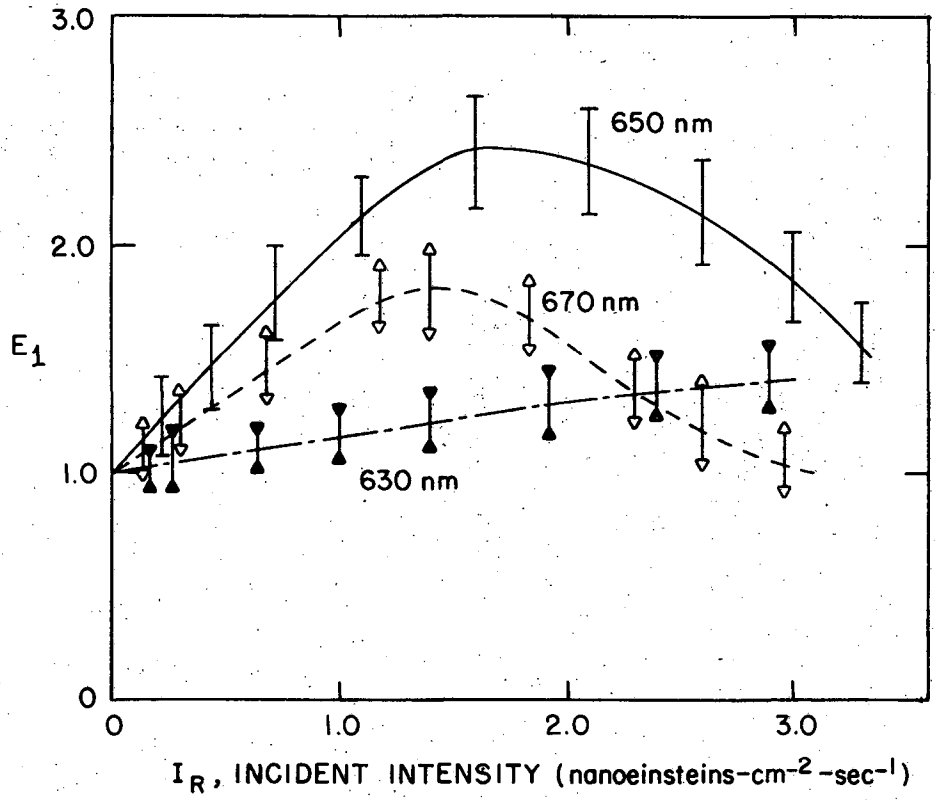
XBL716-5256

Figure 20. Effect of the NaCl concentration on the rate (A, upper curve) and on the enhancement E_1 (B, lower curve) of the [$H_2O \rightarrow$ NADP] reaction by broken chloroplasts. Experimental conditions as in Figure 18, except NaCl in place of $MgCl_2$.

concentrations higher than 250 mM the rate as well as the enhancement ratio decrease markedly.

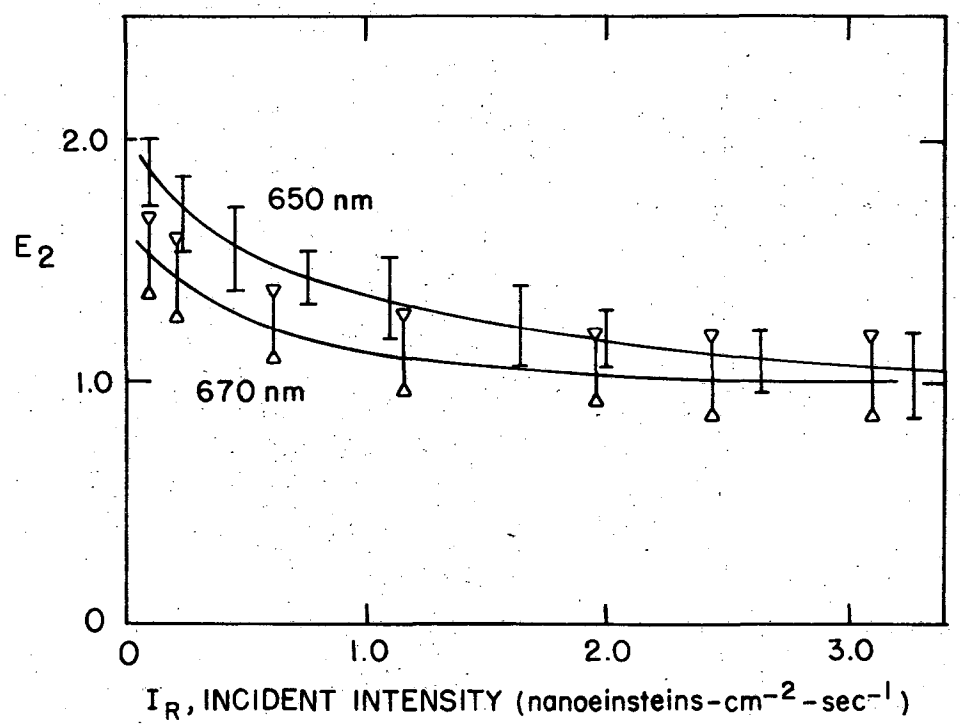
Dependence of enhancement on light intensity. The effect of the intensity of red light (630, 650, or 670 nm) added to a fixed intensity of far-red (700 nm) light in producing enhancement is shown in Figure 21. With reference to the intensity dependence of the rate of reaction at 650 nm shown in Figure 16, the enhancement value increases as long as the rate of the reaction is in the relatively linear region of intensity dependence. The enhancement value starts to decrease as the rate of the reaction approaches light saturation at higher intensities. The enhancement effect reaches its maximum, $E_1 = 2.4 \pm 0.3$, when the red light incident at 650 nm is 1.4 - 1.8 nanoeinsteins-cm^{-2}-sec^{-1}. When the red light at 670 nm is 1.2 - 1.4 nanoeinsteins-cm^{-2}-sec^{-1}, the maximum is $E_1 = 1.8 \pm 0.2$. When the red light is at 630 nm, the maximum enhancement effect cannot be reached, as shown in Figure 21. It presumably occurs at higher incident light intensities than those we studied.

Figure 22 shows the alternative enhancement ratio, E_2 as a function of the incident light intensity, I_R, at 650 and 670 nm. At high intensities both R_{FR+R} and R_R are very large compared with R_{FR}, and the enhancement ratio E_2 is close to 1.0. As the actinic light intensity, I_R, is lowered, the denominator decreases faster than the numerator and the enhancement E_2 increases. We observe limiting values for E_2 of 2.0 and 1.6 at low light intensities when the red actinic light is at 650 nm and 670 nm, respectively.



XBL 716-5247

Figure 21. Dependence of the enhancement E_1 on actinic light intensity of red light for the [$H_2O \rightarrow$ NADP] reaction by broken chloroplasts. $E_1 = (R_{700+R} - R_R)/R_{700}$. Experimental conditions as in Figure 17, except the incident red light intensities I_R are varied from zero to 3.2 nanoeinsteins-cm^{-2}-sec^{-1}. The three curves are for red light at 630, 650 and 670 nm, respectively, as indicated.



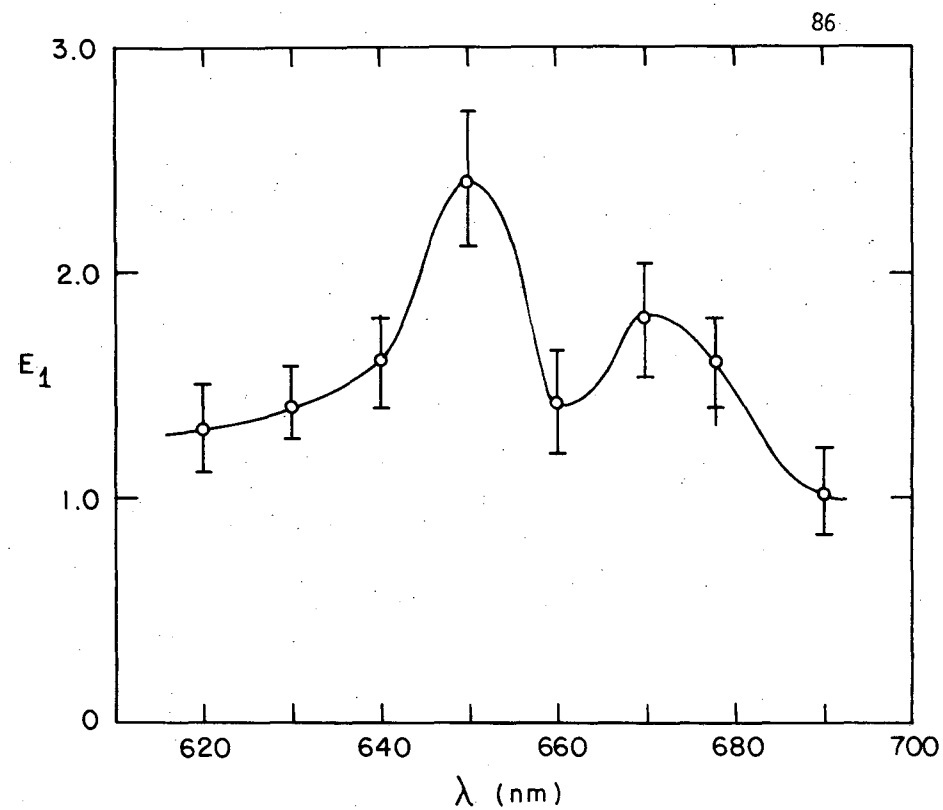
XBL 716-5248

Figure 22. Dependence of the enhancement E_2 on actinic light intensity of red light for the [$H_2O \rightarrow$ NADP] reaction by broken chloroplasts. $E_2 = (R_{700+R} - R_{700})/R_R$. Experimental conditions as in Figure 21.

Action spectrum of enhancement. We find positive enhancement when red light at any wavelength from 620 to 678 nm is coupled with far-red light at 700 nm, but the intensity dependence is different at each wavelength. Figure 23 is a plot of E_1 values measured under conditions of approximately equal absorbed intensities at several wavelengths from 620 to 690 nm. We find optimal enhancement at 650 and 670 nm, where the values are $E_1 = 2.4 \pm 0.3$ and 1.8 ± 0.25, respectively. A minimum occurs near 660 nm ($E_1 = 1.4 \pm 0.2$).

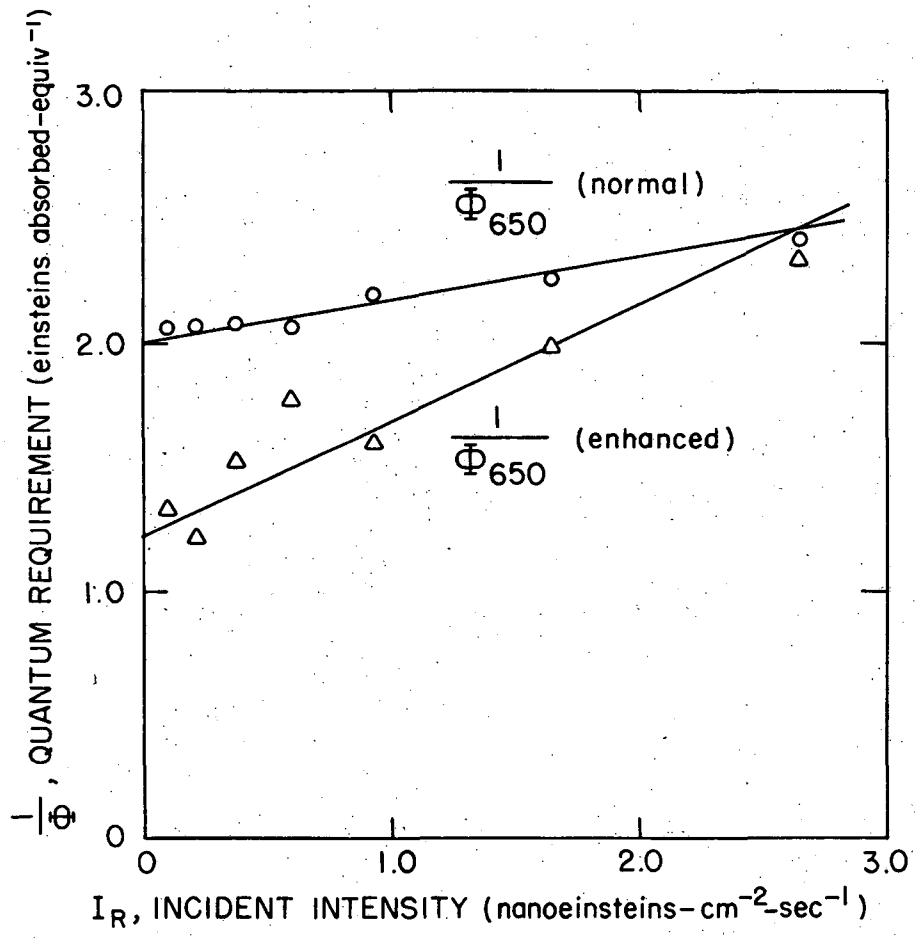
Enhancement viewed as an effect on the quantum requirement for red light. In the traditional scheme for interpreting the two light requirement of electron transport in chloroplasts as a PS (I + II) reaction, enhancement can be viewed as resulting from a deficiency of photons entering PS I when only a single wavelength of actinic light in the region 620 to 678 nm is used. Throughout this wavelength range we observed zero-intensity quantum requirements for the [$H_2O \rightarrow$ NADP] reaction close to 2.0 photons absorbed per electron transferred (Sun and Sauer, 1971). When far-red light, which activates primarily PS I, is added at sufficient intensity that PS I is no longer strongly rate limiting, we expect to observe a corresponding decrease in the quantum requirement for the utilization of red light. The quantum requirement under enhanced conditions can be expressed by the ratio $I_R(\text{absorbed})/(R_{FR+R} - R_{FR})$.

A comparison of the quantum requirements under normal conditions (650 nm light alone) with those under enhanced conditions (650 + 700 nm light), as defined above, is shown in Figure 24 over



XBL 716-5249

Figure 23. Activation spectrum of the enhancement E_1 for the [$H_2O \rightarrow$ NADP] reaction by broken chloroplasts. $E_1 = (R_{700+R} - R_R)/R_{700}$. Measurements made at approximately equal absorbed intensities (1.15 ± 0.15) nanoeinsteins-cm^{-3}-sec^{-1} of red light at wavelengths from 620 to 690 nm. Absorbed intensity 0.37 nanoeinsteins cm^{-3}-sec^{-1} at 700 nm for all measurements. Other reaction conditions as in Figure 17.



XBL 716-5253

Figure 24. Quantum requirements under normal (o) and under enhanced (Δ) conditions for the [H_2O → NADP] reaction by broken chloroplasts. Reaction conditions as in Figure 21. Definitions: $1/\phi_{650}$ (normal) = I_{650}(absorbed)/R_{650}; and I/ϕ_{650}(enhanced) = I_{650}(absorbed)/($R_{700+650}$ - R_{700}).

a range of incident intensities I_R. The intensity of far-red light used (2.3 nanoeinsteins-cm^{-2}-sec^{-1} incident) did not saturate the [H_2O → NADP] reaction by itself (Figure 16); it was sufficient to achieve only 15% of the saturation rate for the [ascorbate + $DPIPH_2$ → NADP] reaction, which does not involve the participation of PS II.

At low intensities of red light, a significant decrease in the quantum requirement of the [H_2O → NADP] reaction occurs in the enhanced versus the normal condition (Figure 24). The quantum requirement under enhanced conditions approaches a value of 1.2 ± 0.2 einsteins absorbed-equivalent^{-1} at zero intensity of red (650 nm) light. This apparent increase in efficiency for red light is due to the utilization of the far-red light in PS I. At higher intensities of red light, the difference between the two quantum requirements disappears. The explanation for this disappearance is probably the same as that for the behavior of E_2 shown in Figure 22.

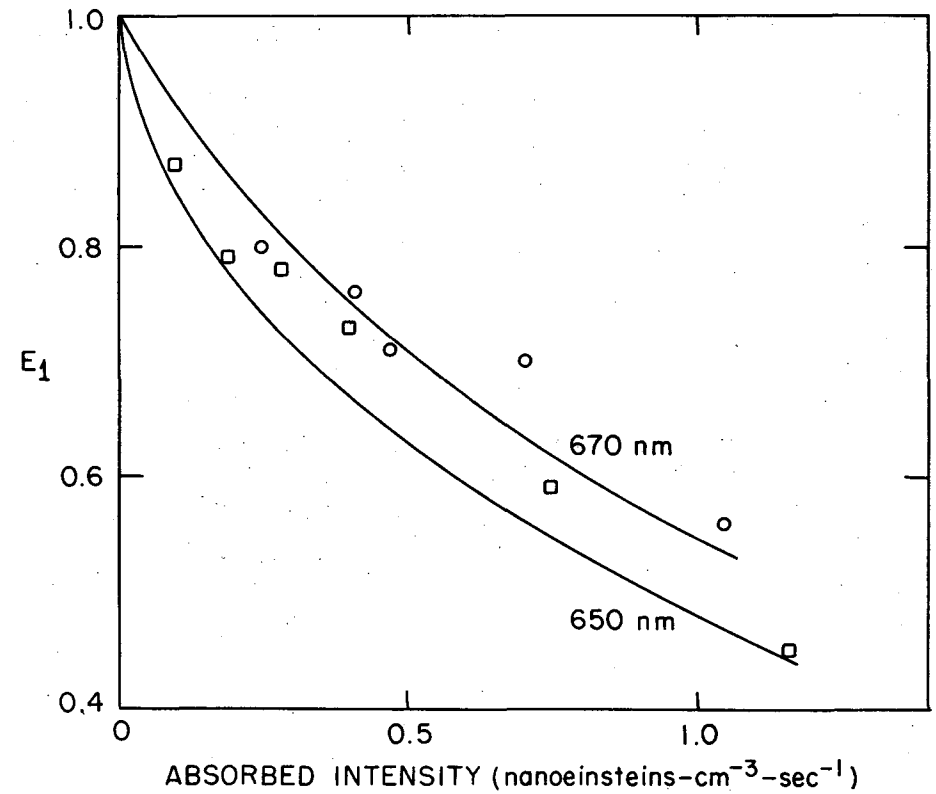
2. Photosystem II [H_2O → DPIP] reaction and photosystem I [ascorbate + $DPIPH_2$ → NADP] reaction

The enhancement values, E_1 or E_2, in the [H_2O → DPIP] reaction are 1.01 ± 0.05 from 620 to 690 nm at various incident light intensities with which a background light at 700 nm at 2.3 nanoeinsteins-cm^{-2}-sec^{-1} was coupled. Thus, no appreciable enhancement effect nor any difference between the normal and differential quantum requirements is observed for the photoreduction of DPIP, in confirmation of previous studies in our laboratory (Sauer and Park, 1965).

For the [ascorbate + $DPIPH_2$ → NADP] reaction run in the presence of DCMU the observed enhancement ratio is always less than unity. The values are a strong function of the intensity of the red light, as seen for 650 (squares) and 670 (circles) in Figure 25. This behavior can be shown to be a result of the approach toward saturation of the rate of the reaction with increasing intensities of red light alone. Assuming that 650 nm photons, which are partitioned about equally between the two photosystems under these reaction conditions, are only half as effective as 700 nm photons and utilizing the linear dependence of the quantum requirement of the reaction as a function of intensity of red light (Sun and Sauer, 1971), it is possible to calculate E_1 ratios which take into account the approach to saturation for the two wavelengths together. The agreement between the experimental points and the calculated curves (Figure 25) is good evidence that, within experimental uncertainties, there is no two-wavelength enhancement for this reaction.

D. Discussion

The occurrence of the Emerson red - far-red enhancement effect in isolated chloroplasts has been the subject of repeated studies (Myers, 1971). For the [H_2O → NADP] reaction, which is the principal focus of the present study, definite enhancement has been reported by Govindjee et al. (1962, 1964), by Joliot et al. (1968), and by Avron and Ben-Hayyim (1969). On the other hand, Gibbs et al.



XBL 716-5252

Figure 25. The enhancement ratio E_1 as a function of intensity of red light absorbed for the [ascorbate + $DPIPH_2$ → NADP] reaction. Measured values using red light at 650 nm (□) or 670 nm (o) at various intensities supplemented by far-red light at 700 nm and constant absorbed intensity (0.43 nanoeinsteins-cm^{-3}-sec^{-1}). The curves are calculated assuming no actual enhancement, but taking account of the approach to saturation of the reaction by red light alone. Chlorophyll concentration 13 μg/ml.

(1963) and McSwain and Arnon (1968) reported no measurable enhancement for the same reaction. Our studies suggest that the concentration of divalent cations (e.g., Mg^{2+}) in the reaction mixture is the principal controlling factor in determining whether enhancement can be observed.

The results summarized in Table VI show that enhancement values can be affected by (1) alterations in the chloroplast preparation procedure, (2) the addition of plastocyanin and ferredoxin-$NADP^+$ reductase, (3) the pH of the reaction mixture, and (4) the addition of ADP and inorganic phosphate. Nevertheless, each of these factors can be overcome and enhancement can always be restored in the presence of 7.5 mM $MgCl_2$. By contrast, we have been unable to find any set of reaction conditions which will give enhancement when the divalent cation concentration is below about 3 mM. In retrospect, the lack of agreement in the literature reports on chloroplast enhancement can be understood largely on this basis. Govindjee et al. (1962, 1964) and Avron and Ben-Hayyim (1969) were able to observe enhancement using Mg^{2+} concentrations of 7.5 and 27 mM, respectively. No enhancement was observed by Gibbs et al. (1963) or by McSwain and Arnon (1968) using Mg^{2+} concentrations of 2 and 1.7 mM, respectively. The results of Joliot et al. (1968), who did observe enhancement in the presence of only 1 mM Mg^{2+}, are the only ones that do not correlate in this way. It may be that there is some synergistic effect involving the high concentration of other salts (0.05 M phosphate buffer + 0.1 M KCl) that distinguishes the reaction conditions of Joliot et al. (1968) from

those of Gibbs et al. (1963) and McSwain and Arnon (1968). This possibility remains to be investigated.

As shown in Figures 18 and 19, added divalent cations produce an optimum not only in the enhancement effect, but also in the velocity of the [$H_2O \rightarrow NADP$] reaction in red light alone. It might be argued that the absence of divalent cations serves only to slow down the rate-limiting step that results in light intensity saturation. In this view, the absence of enhancement at zero added divalent ion would be the fortuitous result of a compensatory decrease in E_1 because of the closer approach to light saturation in the absence of added divalent cations. Figure 21 shows examples of the decrease of E_1 as saturating light intensities are approached. The results of McSwain and Arnon (1968) argue against this interpretation of the divalent cation effect, however. At low (1.7 mM) concentrations of added $MgCl_2$ they found no enhancement to occur over a wide range of incident light intensities, such that the overall rate, $R_{650+700}$, varied by as much as 9-fold. Furthermore, added NaCl is able to increase the rate of the [$H_2O \rightarrow NADP$] reaction (Figure 20) even somewhat more effectively than added $MgCl_2$ or $MnCl_2$, but no enhancement values significantly greater than 1.0 are observed using NaCl. It is apparent from these results and those using added sucrose that the occurrence of enhancement depends on something more specific than the ionic or osmotic strength of the medium.

A special role for divalent cations has been proposed by Murata (1969). He observed that relatively low concentrations

(2-3 mM) of Mg^{2+}, Ca^{2+} or Mn^{2+} served markedly to increase the yield of chlorophyll fluorescence from chloroplasts at room temperature. Added Mg^{2+} (3 mM) also served to decrease the quantum yield (extrapolated to zero incident intensity) of the [ascorbate + $DPIPH_2$ → NADP] reaction activated at 480 nm, and to increase the quantum yield of the [H_2O → DPIP] reaction--slightly at 480 nm, but markedly when activated at 695 nm. It should be noted, however, that the highest quantum yields reported by Murata are less than half those reported for the same reactions in Chapter II of this report. Murata concluded on the basis of his findings that the role of Mg^{2+} and other divalent ions is to suppress the spillover of excitation energy from pigment system II to pigment system I.

The alternative view, namely that added Mg^{2+} enables excitation transfer between the two pigment systems, is supported by the observations of Avron and Ben-Hayyim (1969) and of Rurainski et al. (1971) that added $MgCl_2$ serves to increase significantly the quantum yield of the [H_2O → NADP] reaction extrapolated to zero light intensity. Under the assumption that excitation transfer (spillover) between the two pigment systems tends to equalize the rates of the two photoreactions, the increased quantum yields for the PS (I + II) reaction can be explained most readily if spillover occurs in the presence of $MgCl_2$ rather than in its absence. Murata's own experimental findings can be rationalized satisfactorily using this alternative view of the role of Mg^{2+}. Spillover from P I to P II in the presence of divalent cations

can account for (1) increased fluorescence yield, (2) increased quantum yield for the [H_2O → DPIP] reaction, and (3) decreased quantum yield for the [ascorbate + $DPIPH_2$ → NADP] reaction. Spillover from P I to P II will be efficient only for excitation resulting from red light, where there is no energy barrier to reaching the PS II trap. Spillover in this direction might seem to be an unlikely process in competition with trapping within PS I. Nevertheless, such spillover must occur in order to account for the observed quantum yields of 1.0 for the [H_2O → ferricyanide] (Avron and Ben-Hayyim, 1969) and the [H_2O → DPIP] (Sun and Sauer, 1971) reactions using red actinic wavelengths.

Shavit and Avron have reported divalent cation-dependent shrinking and light scattering changes by illuminated broken chloroplasts (1971). Similar results have been observed by Murakami and Packer (1971). It is reasonable to suppose that these conformational changes induced by divalent cations are the basis for the effects on excitation transfer.

Models which account for the enhancement effect in the [H_2O → NADP] reaction and for the dependence on divalent cations can be constructed using either Murata's interpretation (Model A) or its converse (Model B). The models differ in the role assigned to the divalent cation and in the restrictions placed on the relative intrinsic absorptions (i.e., in the absence of spillover) of P I and P II in the red region from 620 to 680 nm. Table VII gives a listing of the postulates of the two models.

TABLE VII

ALTERNATIVE MODELS FOR THE ROLE OF DIVALENT CATIONS IN ENABLING RED - FAR-RED ENHANCEMENT FOR THE [$H_2O \rightarrow$ NADP] REACTION BY BROKEN CHLOROPLASTS

Model A	Model B	Model C
1. Electron transfer between P II and P I occurs in the absence of divalent cations; not in their presence.	1. Electron transfer between P I and P II occurs in the presence of divalent cations; not in their absence.	1. Same as B.
2. Intrinsic absorption of P II is greater than that of P I in the region 620 to 680 nm.	2. Intrinsic absorption of P II is equal to that of P I in the region 620 to 680 nm.	2. Same as A.
All three models:		
3. Intrinsic absorption of P I is greater than that of P II at wavelengths longer than 690 nm.		
4. Excitation transfer, when it is allowed, will occur predominantly in the direction which will enhance the activation of the reaction center that would otherwise be rate limiting.		

In Model A enhancement occurs in the absence of spillover because P II absorbs more than half the photons in the red region of the spectrum, and P I absorbs preferentially in the far-red. This is the traditional view basic to the detailed mathematical analyses of Bannister and Vrooman (1964), Malkin (1967), Williams (1968), and Delrieu and de Kouchkovsky (1971). In the presence of spillover red light is equilibrated between the two reaction centers, and there is no deficiency to be remedied by supplementary far-red light.

In Model B no enhancement occurs in the absence of spillover because the intrinsic absorptions of the two pigment systems are postulated to be identical in the red region of the spectrum. There is no imbalance to be rectified by far-red light. Addition of divalent cations, which enables spillover in this model, provides conditions favoring enhancement. The distribution of red-photon excitation between the two reaction centers, which is equal for red light alone, is altered in the presence of far-red light via the spillover of some of the red excitation from P I to P II. This is in keeping with Postulate 4 of Table VII and results in the observed enhancement. That spillover is an efficient process in the enhancement studies (regardless of the model considered) is demonstrated by the results shown in Figure 24, where red photons approach unit efficiency at low intensities and in the presence of supplementary far-red light.

Neither of the two models described above is entirely satisfactory, and whichever proves to be closest to the truth will

require further modifications as more is learned about the related phenomena. We have already mentioned the apparent inconsistencies between Murata's interpretation of the role of divalent cations (Murata, 1969) (incorporated into Model A) and the effect of $MgCl_2$ on the quantum yield of the [$H_2O \rightarrow$ NADP] reaction (Avron and Ben-Hayyim, 1969; Rurainski et al., 1971). In addition, it is not clear why enhancement should not also be observed in the absence of divalent cations under the postulates of Model A. Spillover should permit a redistribution of red photons in the presence of far-red light, according to Postulate 4 of Table VII, in which case some enhancement would be expected. None is observed. Neither the quantum yield of unity in red light for the [$H_2O \rightarrow$ NADP] reaction in the presence of 4.5 mM $MgCl_2$ (see Chapter II) nor the decrease in the quantum yield in the red in going from 0 to 27 mM $MgCl_2$ for the [ascorbate + $DPIPH_2 \rightarrow$ NADP] reaction (Avron and Ben-Hayyim, 1969) can be reconciled with Model A. The alternative view of Model B also has its drawbacks. The assignment of equal intrinsic absorbances in the red to P I and P II appears to be quite arbitrary and difficult to reconcile with the different absorption spectra of physically separated PS I and PS II fractions (Anderson and Boardman, 1966; Michel et al., 1968). Both the pronounced dependence of enhancement on the wavelength of red light (Figure 23) and the increase in quantum yield of the [$H_2O \rightarrow$ NADP] reaction upon addition of $MgCl_2$ (Avron and Ben-Hayyim, 1969; Rurainski et al., 1971) are difficult to reconcile with Postulate 2 of Model B. Neither model can account for the observation of Avron and Ben-Hayyim

(1969) that the transfer of electrons from ascorbate to diquat or FMN occurs with a quantum yield of 1.0 in either red or far-red light and is unaffected by added $MgCl_2$.

In the third model (C), red light is assumed to be distributed equally between P I and P II, and the [$H_2O \rightarrow$ NADP] reaction proceeds at an optimal rate in the presence of spillover (here Mg^{2+}). The observed quantum efficiencies of 0.5 $\pm$ 0.01 at wavelengths from 620 to 660 nm in the presence of 7.5 mM of Mg^{2+} are consistent with this picture, at least within experimental uncertainties. In the absence of Mg^{2+}, not only is the spillover unfavorable, but the biochemical rate-limiting step between two photosystems may not be optimal and so slow that both absorbed red and far-red lights become largely wasted. Thus no enhancement effect could be observed. Added Mg^{2+} is now considered to enable spillover to occur and allow efficient biochemical reactions. In red light alone this should lead to an increase in rate, since both photosystems were being excited optimally in the presence of spillover and the biochemical reaction is optimal. In the presence of supplementary far-red light, the additional photons are predominantly available to RC I. This produces a limitation at the point of excitation arriving at RC II, which can be relieved via the spillover of some of the red photons previously entering PS I. Thus, spillover from P I to P II in the presence of divalent ions would account for the enhanced rate of the reaction in comparison with the sum of the rates using the two lights separately. In like fastion one can invoke Mg^{2+} stimulated spillover from P I to P II to account for

Murata's observations of (1) increased fluorescence by PS II, (2) increased efficiency of the [$H_2O \rightarrow NADP$] reaction, and (3) decreased efficiency of the [ascorbate + $DPIPH_2 \rightarrow NADP$] reaction, all resulting from added Mg^{2+}.

It seems clear that further experimental results are required before all of these difficulties can be resolved. Recent reports that PS I activity may occur in two kinetically distinct locations in broken chloroplasts (Park and Sane, 1971) need to be considered in future models of enhancement and excitation transfer.

Our findings are more conclusive with respect to the parallel two photosystem hypothesis of Arnon et al. (1965) and as modified by Knaff and Arnon (1969). The parallel hypothesis, by contrast with the traditional Z scheme where the two light reactions operate in series, cannot be reconciled with the observation of red - far-red enhancement using isolated broken chloroplasts for the [$H_2O \rightarrow NADP$] reaction. The failure of McSwain and Arnon (1968) to observe enhancement for this system, an observation which was an essential part of the justification of the parallel mechanism, is now seen to be probably the consequence of the low $MgCl_2$ concentration used in their experiments. Under their conditions, we do not observe enhancement either. Our system contained broken chloroplasts, had no added carbon source, and did not require the components of phosphorylation in order for enhancement to be observed. Thus, it cannot be argued that enhancement occurs only

in relief of an imbalance of cyclic and non-cyclic electron flow with respect to the requirements of the carbon reduction pathway for ATP and reductant. The simplest explanation of the results presented in this paper is that non-cyclic electron transport from H_2O to $NADP^+$ proceeds via two different light reactions characterized as PS I and PS II operating in series.

IV. CONCLUDING REMARKS

There have been numerous important discoveries in photosynthesis that have arisen from the study of cell-free systems in this field. However, the process of isolation of cell-free chloroplasts has also introduced many unknown artificial effects to the system. Since the standard procedures used in various laboratories are quite different, many contradictory results due to different experimental conditions have been reported. One of the most vivid examples is shown in Chapter II of this investigation. Reports of the presence or absence of the enhancement effect in the [$H_2O \rightarrow$ NADP] reaction have caused a great confusion regarding the mechanism of how PS I and PS II are operating. But these contradictory results are merely due to the $MgCl_2$ concentration difference. The discovery of the role of $MgCl_2$ itself may be important to understand the mechanisms of excitation energy transfer, chloroplast membrane conformation, and the relation between PS I and PS II. But it also calls our attention to re-examining all present data with various experimental conditions, especially those data where contradictions lie.

The role of $MgCl_2$ in the chloroplast membrane has become increasingly important since the discovery of its effects on (1) the increase of quantum efficiency at low light intensities (Rurainski, Randles and Hoch, 1971), (2) the increase of PS II

fluorescence yield (Murata, 1969), and (3) the control of the enhancement effect, as was discussed in Chapter III. We have proposed two hypotheses, Model B and Model C in Table VII, on the excitation energy transfer between PS I and PS II as well as on the enhancement effect. There are several ways to check our hypotheses. The first is to measure the PS II fluorescence yield in the presence and absence of different cyclic photophosphorylation and PS I reactions at different $MgCl_2$ concentrations. If the presence of $MgCl_2$ prevents the excitation energy transfer from PS II to PS I (Murata, 1969), we should expect that the PS II fluorescence yield of chloroplasts does not decrease even if the cyclic photophosphorylation or PS I reaction is accelerated by adding cofactors or electron donors such as PMS, FMN or [ascorbate + $DPIPH_2$]. If the PS II fluorescence yield does decrease, the presence of $MgCl_2$ must allow the excitation energy transfer between PS I and PS II; since the acceleration of cyclic photophosphorylation or PS I reaction will then allow effective competition for the excitation energy with PS II fluorescnece, we would expect to see the decrease of the PS II fluorescence yield.

The second way is to measure the enhancement effect of the [$H_2O \rightarrow$ NADP] reaction at different $MgCl_2$ concentrations with different red and far-red light intensities. The difference between Model B and C is that Model B suggests that the intrinsic absorptions of PS I and PS II in the red light region are equal, but Model C suggests it is not. In the absence of $MgCl_2$ when the

excitation energy transfer is not allowed as proposed in both Model B and C, Model C predicts that there is an enhancement effect but Model B predicts no such effect. Our data showed that no enhancement effect could be observed in the absence of $MgCl_2$, but these experiments were done only under one constant strong far-red light intensity with various red light intensities. When the rate of the reaction of the [$H_2O \rightarrow NADP$] reaction is low at zero $MgCl_2$ concentration, as shown in Figure 18, the intensity we used in the study may become saturating, which hinders the enhancement effect. McSwain and Arnon (1969) also showed no appreciable enhancement effect of the [$H_2O \rightarrow NADP$] reaction could be observed in the presence of 1.67 mM $MgCl_2$ under a wide range of far-red and red light intensities, but they never varied the total light intensity with a constant R_{650}/R_{704} ratio in the range from 4 to 6, which is the optimal ratio to obtain the maximum enhancement effect. The solution to this problem is to examine whether the enhancement effect of the [$H_2O \rightarrow NADP$] reaction could occur at low or zero $MgCl_2$ concentration at low total (far-red + red) light intensity with the constant ratio $R_{650}/R_{700} \cong 4 \sim 6$, under which the light intensity is not saturating and the enhancement effect is optimal.

The third way is to measure the zero light intensity quantum requirements of the [$H_2O \rightarrow NADP$] reaction in the absence of $MgCl_2$ in the red light region. We have obtained quantum requirement values of 2.0 $\pm$ 0.1 for the [$H_2O \rightarrow NADP$] reaction in the presence of $MgCl_2$, which allows the efficient excitation energy transfer

of any unbalanced energy between PS I and PS II, as proposed in both Model B and C. If the intrinsic absorption of PS I and PS II are equal in the red light region as proposed in Model B, we should observe the zero light intensity quantum requirements also to be equal to 2.0 in the absence of $MgCl_2$ at any wavelength from 620 to 678 nm. If the intrinsic absorption of PS I and PS II are not equal, and excitation energy transfer is not efficient in the absence of $MgCl_2$, as proposed in Model C, the extra energy absorbed either by PS I or PS II cannot be transferred from one to another and thus becomes wasted. We should expect to see higher quantum requirement values of the [$H_2O \rightarrow NADP$] reaction in the absence of $MgCl_2$. Model A also predicts that the zero light intensity quantum requirements of the [$H_2O \rightarrow NADP$] reaction should be 2.0 in the absence of $MgCl_2$, under which the efficient excitation energy transfer is allowed. But it also predicts that the quantum requirement values should be higher than 2.0 in the red light region in the presence of $MgCl_2$, which is supposed to prevent excitation transfer in this model. Our data on quantum requirements of the [$H_2O \rightarrow NADP$] reaction (= 2.0 $\pm$ 0.1) have already excluded this possibility. Rurainski _et al_. (1971) found that the quantum efficiency of the [$H_2O \rightarrow NADP$] reaction with red-band actinic light did decrease at low and zero $MgCl_2$ concentrations. It is still worthwhile to examine the quantum requirement of the [$H_2O \rightarrow NADP$] reaction at low or zero $MgCl_2$ concentration with monochromatic or actinic light from 620 to 678 nm.

V. REFERENCES

Allen, F. L. and Franck, J. (1955), Arch. Biochem. Biophys. 58, 124.

Anderson, J. M. and Boardman, N. K. (1966), Biochim. Biophys. Acta 112, 403.

Anderson, J. M., Fork, D. C., and Amesz, J. (1966), Biochem. Biophys. Res. Commun. 23, 874.

Armstrong, J. McD. (1964), Biochim. Biophys. Acta 86, 194.

Arnon, D. I. and Whatley, F. R. (1949), Science 110, 554.

Arnon, D. I., Allen, M. B. and Whatley, F. R. (1954), Nature 174, 394.

Arnon, D. I., Allen, M. B. and Whatley, F. R. (1956), Biochim. Biophys. Acta 20, 449.

Arnon, D. I. (1958), in The Photochemical Apparatus, Its Structure and Function, Brookhaven Symposia in Biology, No. 11, p. 181.

Arnon, D. I., Tsujimoto, H. Y., and McSwain, B. D. (1964), Proc. Nat. Acad. Sci. U.S. 51, 1274.

Arnon, D. I., Tsujimoto, H. Y., and McSwain, B. D. (1965), Nature (London) 207, 1367.

Arnon, D. I. (1966), Experientia 22, 273.

Arnon, D. I. (1967), Physiol. Rev. 47, No. 3, 317.

Avron, M. (1967), Current Topics in Bioenergetics, Vol. II, Academic Press, p. 1.

Avron, M. and Ben-Hayyim, G. (1969), Prog. Photosynthesis Res. III, Ed. H. Metzner, p. 1185.

Avron, M. and Newmann, J. (1968), Ann. Rev. Plant Physiol. 19, 137.

Bannister, T. T. and Vrooman, M. J. (1964), Plant Physiol. 39, 622.

Bassham, J. A. and Calvin, M. (1957), The Path of Carbon in Photosynthesis, Prentice-Hall, Englewood Cliffs, New Jersey.

Bassham, J. A. (1967), 7th International Biochemical Congress, Tokyo, Bioenergetics Symposium, Vol. II, p. 337.

Bishop, N. I. (1958), Biochim. Biophys. Acta 27, 205.

Black, C. C. and San Pietro, A. (1963), in Photosynthetic Mechanisms of Green Plants, Publ. 1145, Natl. Acad. Sci.- Natl. Res. Council, pp. 228-234.

Boardman, N. K. and Highkin, H. R. (1966), Biochim. Biophys. Acta 126, 189.

Brody, S. S. and Brody, M. (1961), Arch. Biochem. Biophys. 95, 521.

Brody, S. S. (1968), Biophys. J. 8, 210.

Cederstrand, C. N., Rabinowitch, E., and Govindjee (1966), Biochim. Biophys. Acta 126, 1.

Chance, B. and Williams, G. R. (1956), Advan. Enzymol. 17, 65.

Clayton, R. K. (1962), Photochem. Photobiol. 1, 201.

Clayton, R. K. (1969), Biophys. J. 9, 60.

Delrieu, M.-J. and de Kouchkovsky, Y. (1971), Biochim. Biophys. Acta 226, 409.

Dixon, M. and Webb, E. C. (1964), Enzymes, Academic Press, N. Y., 2nd Ed., p. 422.

Dratz, E. A., Schultz, A. J., and Sauer, K., Brookhaven Symp. in Biology (1966), Biol. Dept., Brookhaven Natl. Lab., Upton, N. Y., p. 303.

Duysens, L. N. M. and Amesz, J. (1962), Biochim. Biophys. Acta 64, 243.

Emerson, R. and Arnold, W. (1932), J. Gen. Physiol. 16, 191.

Emerson, R. and Lewis, C. M. (1942), J. Gen. Physiol. 25, 579.

Emerson, R. and Lewis, C. M. (1943), Am. J. Bot. 30, 165.

Emerson, R., Chalmers, R. V., and Cederstrand, C. (1957), Proc. Nat. Acad. Sci., U.S. 43, 133.

Emerson, R. (1957), Science 125, 746.

Engleman, T. W. (1883), Arch. Ges. Physiol. (Pfluger's) 30, 95.

Fork, D. C. (1963), Plant Physiol. 38, 323.

French, C. S., Myers, J., and McCloud, G. C. (1960), in Comp. Biochem. of Photoreactive Systems, Ed. M. B. Allen, Academic Press, New York, p. 361.

Frenkel, A. W. (1954), J. Am. Chem. Soc. 76, 5568.

Frank, J., Rosenberg, J. L., and Weiss, C. Jr. (1962), in Luminescence of Organic and Inorganic Materials, Eds. H. P. Kallman and G. H. Spruch, John Wiley & Sons, New York, pp. 11-29.

Gibbs, M., Fewson, C. A., and Schulman, M. D. (1963), Carnegie Institution of Washington Yearbook 62, 362.

Gordon, S. A. (1963), Plant Physiol. 38, 153.

Gorman, D. S. and Levine, R. P. (1966), Plant Physiol. 41, 1648.

Govindjee and Rabinowitch, E. (1960), Biophys. J. 1, 73.

Govindjee, R., Govindjee, and Hoch, G. (1962), Biochem. Biophys. Res. Commun. 9, 222.

Govindjee, R., Govindjee, and Hoch, G. (1964), Plant Physiol. 39, 10.

Govindjee, R., Rabinowitch, E. and Govindjee, Biochim. Biophys. Acta 162, 539.

Hill, R. (1939), Proc. Roy. Soc. (London), Series B, No. 847, 127.

Hill, R. and Bendall, F. (1960), Nature 186, 136.

Hind, G. and Jagendorf, A. T. (1963), Proc. Nat. Acad. Sci. 49, 715.

Hind, G. and Jagendorf, A. T. (1965), J. Biol. Chem. 240, 3195.

Hoch, G. and Martin, I. (1963), Arch. Biochem. Biophys. 102, 430.

Joliot, P., Joliot, A., and Kok, B. (1968), Biochim. Biophys. Acta 153, 635.

Joliot, P., Barbieri, G., and Chabaud, R. (1969), Photochem. Photobiol. 10, 309.

Katoh, S. and Takamiya, A. (1965), Biochim. Biophys. Acta 99, 156.

Katoh, S. and San Pietro, A. (1966), in The Biochemistry of Copper, Eds. J. Peisach, P. Aisen, and W. E. Blumberg, Academic Press, New York, p. 407.

Kelly, J. and Sauer, K. (1965), Biochemistry 4, 2798.

Kelly, J. and Sauer, K. (1968), Biochemistry 7, 882.

Knaff, D. B. and Arnon, D. I. (1969), Proc. Natl. Acad. Sci., U.S. 64, 715.

Huzisige, H., Usiyama, H., Takao, K., and Azi, T. (1969), Plant Cell Physiol. 10, 441.

Kok, B. (1961), Biochim. Biophys. Acta 48, 527.

Kok, B., Forbush, B., and McGloin, M. (1970), Photochem. Phobobiol. 11, 457.

Levine, R. P. (1969), Ann. Rev. Plant Physiol. 20, 523.

Losada, M., Whatley, F. R., and Arnon, D. I. (1961), Nature 190, 806.

Malkin, S. and Kok, B. (1966), Biochim. Biophys. Acta 126, 413.

Malkin, S. (1967), Biophys. J. 7, 629.

Mayne, B. C. and Brown, A. H. (1963), Studies on Microalgae and Photosynthetic Bacteria, Univ. of Tokyo Press, Tokyo, p. 347.

McElroy, W. D. and Nason, A. (1954), Ann. Rev. Plant Physiol. 5, 1.

McSwain, B. D. and Arnon, D. I. (1969), Proc. Natl. Acad. Sci., U.S. 20, 523.

Menke, W. (1962), Ann. Rev. Plant Physiol. 13, 27.

Michel, J.-M. and Michel-Wolwertz, M.-R. (1968), Carnegie Inst. of Washington Yearbook 67, 508.

Mitchell, P. (1961), Nature 191, 144.

Mitchell, P. (1966), Biol. Rev. 41, 445.

Myers, J. and French, C. S. (1960), J. Gen. Physiol. 43, 723.

Myers, J. and Graham, J. (1963), Plant Physiol. 38, 105.

Myers, J. (1971), Ann. Rev. Plant Physiol. 22, 289.

Murakami, S. and Packer, L. (1971), Arch. Biochem. Biophys., in Press.

Murata, N. (1969), Biochim. Biophys. Acta 189, 171.

Nelson, N. and Neumann, J. (1969), J. Biol. Chem. 244, 1926.

Nelson, N. and Neumann, J. (1969), J. Biol. Chem. 244, 1932.

Olson, J. M., Koenig, D. F., and Ledbetter, M. C. (1969), Arch. Biochem. Biophys. 129, 30.

Olson, J. M., Thornber, J. P., Koenig, D. F., Ledbetter, M. C., Olson, R. A., and Jennings, W. H. (1969), in Progress in Photosynthesis Research, Ed. H. Metzner, Vol. I, Tübingen, p. 217.

Park, R. B. and Biggins, J. (1964), Science 144, 1009.

Park, R. B. and Sane, P. V. (1971), Ann. Rev. Plant Physiol. 22, 395.

Rabinowitch, E. (1957), J. Phys. Chem. 61, 870.

Rabinowitch, E. and Govindjee (1969), in Photosynthesis, John Wiley & Sons, Inc., New York, p. 121.

Ross, R. T. (1966), J. Chem. Phys. 45, 1.

Ross, R. T. (1967), J. Chem. Phys. 46, 4590.

Ross, R. T. and Calvin, M. (1967), Biophys. J. 7, 595.

Rurainski, H. J., Randles, J., and Hoch, G. E. (1971), FEBS Letters 13, 98.

Rurainski, H. J. and Hoch, G. E. (1971), Proc. 2nd Internat. Congr. on Photosyn. Res., Stresa, Italy.

Sane, P. V., Goodchild, D. J., and Park, R. B. (1970), Biochim. Biophys. Acta 216, 162.

Sauer, K. and Biggins, J. (1965), Biochim. Biophys. Acta 102, 55.

Sauer, K. and Park, R. B. (1965), Biochemistry 4, 2791.

Schwartz, M. (1965), Biochim. Biophys. Acta 102, 361.

Schwartz, M. (1967), Nature 213, 1187.

Shavit, N. and Avron, M. (1967), Biochim. Biophys. Acta 131, 516.

Slater, E. C. (1953), Nature 17, 975.

Slater, E. C. (1966), Comp. Biochem. 14, 327.

Strain, H. H. and Svec, W. A. (1966), The Chlorophylls, Eds. L. P. Vernon and G. R. Seely, Academic Press, New York, p. 51.

Sun, A. S. K. and Sauer, K. (1971), Biochim. Biophys. Acta 234, 399.

Tagawa, K. and Arnon, D. I. (1962), Nature 195, 537.

Tagawa, K., Tsujimoto, H. Y., and Arnon, D. I. (1963), Proc. Natl. Acad. Sci., U.S. 49, 567, and 50, 544.

Thomas, J. B., Blaauw, O. H., and Duysens, L. N. M. (1953), Biochim. Biophys. Acta 10, 2302.

Thornber, J. P., Gregory, R. P. F., Smith, C. A., and Bailey, J. L. (1967), Biochemistry 6, 391.

Van Niel, C. B. (1931), Arch. Mikrobiol. 3, 1.

Vernon, L. P. and Zaugg, W. S. (1960), J. Biol. Chem. 235, 2728.

Vernon, L. P. and Avron, M. (1965), Ann. Rev. Biochem. 34, 269.

Warburg, O. (1949), in Heavy Metal Prosthetic Groups and Enzyme Action, Clarendon Press, Oxford, p. 213.

Wessels, J. S. C. (1962), Biochim. Biophys. Acta 65, 561.

Wessels, J. S. C. (1963), Proc. Roy. Soc. (London), Series B, 157, 345.

Wessels, J. S. C. (1966), in Currents in Photosynthesis, Eds. J. B. Thomas and J. C. Goedheer, Ad. Donker, Rotterdam, p. 129.

Williams, W. P. (1968), Biochim. Biophys. Acta 153, 484.

LEGAL NOTICE

This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Atomic Energy Commission, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights.

TECHNICAL INFORMATION DIVISION
LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720