Title
THE ROLE OF THE PEGMENT ARRAY IN THE PHOTOCHEMISTRY AND KINETICS OF PHOTOSYNTHESIS

Author
Kelly, Jeffrey John.

Publication Date
1968-06-01

UCRL-18224

University of California

Ernest O. Lawrence Radiation Laboratory

THE ROLE OF THE PIGMENT ARRAY IN THE PHOTOCHEMISTRY AND KINETICS OF PHOTOSYNTHESIS

Jeffrey John Kelly

(Ph. D. Thesis)

June 1968

Berkeley, California

DISCLAIMER

UCRL-18224

UNIVERSITY OF CALIFORNIA

Lawrence Radiation Laboratory
Berkeley, California

AEC Contract No. W-7405-eng-48

THE ROLE OF THE PIGMENT ARRAY IN THE PHOTOCHEMISTRY AND KINETICS OF PHOTOSYNTHESIS

Jeffrey John Kelly

(Ph. D. Thesis)

June 1968

THE ROLE OF THE PIGMENT ARRAY IN THE PHOTOCHEMISTRY AND KINETICS OF PHOTOSYNTHESIS

Jeffrey John Kelly

Lawrence Radiation Laboratory
University of California
Berkeley, California

May 23, 1968

ABSTRACT

The photoreduction of cytochrome c in the presence of intact spinach chloroplasts occurs with a high quantum efficiency, using reduced trimethyl-p-benzoquinone ($TMQH_2$) as reductant and in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). This reaction has a requirement of 2 quanta absorbed per electron transferred to cytochrome c for exciting light in the wavelength region from 620 to 680 mμ. The quantum requirement then falls to 1 quantum per electron at wavelengths greater than 700 mμ. These results confirm the conclusion of Vernon and Shaw [Vernon, L. P., and Shaw, E. R. (1965), Biochemistry 4, 132] that this oxidation-reduction reaction is mediated by chloroplast pigment system I in the presence of DCMU. The quantum requirement of unity observed at long wavelengths shows that the reaction probably occurs with the maximum efficiency obtainable.

The evaluation of the action spectrum for cytochrome c reduction together with that for the chloroplast Hill reaction [Sauer, K., and Park, R. B. (1965), Biochemistry 4, 2791], which is probably photocatalyzed by pigment system II, strongly suggests that there is no appreciable transfer of electronic excitation energy between the two

pigment systems in spinach chloroplasts. The two light reactions apparently interact only at the chemical level of photosynthetic electron transport. A model is presented which rationalizes this conclusion by separating the two pigment systems on opposite sides of the chloroplast lamellar unit.

From studies of photosynthetic partial reactions in flashing light, it has been possible to estimate the concentration, relative to chlorophyll, of the reaction centers or of the pools of intermediates closely associated with photosystems I and II. Relatively long flashes (6-100 msec) of saturating red light indicate a functional unit for cytochrome c reduction by $TMQH_2$ in the presence of DCMU corresponding to 1 equiv/445 chlorophylls for photosystem I. Similar illumination patterns for the Hill reaction using 2,6-dichlorophenolindophenol (DCPIP) and/or ferricyanide led to a maximum reduction of 1 equiv/55 chlorophylls for photosystem II -- about 8 times smaller than the System I unit. These results are consistent with the presence of a pool of endogenous intermediate electron acceptors in the Hill reaction, and suggest that very short (10^{-4} sec) flashes would produce evidence for a primary rate-limiting component in smaller concentration. There is a small difference in the results, depending on whether ferricyanide or DCPIP is used as the Hill oxidant.

The effects of variation of light intensity, flash duration, repetition frequency, chloroplast and substrate concentrations and phosphorylation cofactors and uncouplers support the existence of a relatively simple kinetic scheme--a rapid light activation followed by a slower first-order dark recovery--for each photoreaction. For

long flashes, the rate-limiting step for the dark reaction regenerating System I activity has a first-order rate constant of 13 sec^{-1} at room temperature. That for System II, also first order, is ca. 30 sec^{-1}, but is reduced to one-half this value in the absence of phosphorylation cofactors and the phosphorylation uncoupler, methylamine. Both of these rate-limiting steps appear to result from components endogenous to the broken chloroplasts.

ACKNOWLEDGMENTS

I wish to express my deepest gratitude to Dr. Kenneth Sauer, under whose guidance the work reported in this thesis was conducted. The advice and guidance he has given me have made my years as a graduate student both instructive and fruitful, and I am especially grateful to him for my introduction into the field of biophysical chemistry. I have found his patience and encouragement a continuing source of strength in approaching the problems of this investigation.

It has been a privelege to be associated with the Laboratory of Chemical Biodynamics. The stimulating intellectual environment created by its staff, visitors and students provides a conducive atmosphere in the undertaking of scientific endeavors. I am particularly grateful to Dr. Melvin Calvin for making my residence in his laboratory possible.

I would like to thank Dr. Roderick B. Park for his valuable help during certain aspects of this investigation; Miss Susan Drury for assistance in measuring chloroplast absorption with an integrating sphere; Mr. Alexander Sun for help in preparing the ferredoxin used in parts of this study; and Mr. George Ruben for discussions which have been a great help to me in clarifying certain concepts.

To Mr. Robert Creedy for his knowledge and help in diagnosing the ailments of a Cary 14 spectrophotometer, and to Mrs. Evelyn Litton for her skill in preparing the figures and manuscript, I would also like to extend my thanks.

Most of all, I am grateful to my wife, Kathy, for her many hours of patient listening and her unfailing support. The not inconsiderable amount of time she spent reducing data for the computer, proofreading, and typing is greatly appreciated.

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

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
chl	chlorophyll
cyt	cytochrome
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DCPIP	2,6-dichlorophenolindophenol
$DCPIPH_2$	2,6-dichlorophenolindophenol, reduced form
F_d	ferredoxin
NADP	nicotinamide adenine dinucleotide phosphate
$NADPH_2$	nicotinamide adenine dinucleotide phosphate, reduced form
P_{cy}	plastocyanin
P_{890}	pigment whose absorption changes are associated with the bacterial reaction center
P_i	inorganic phosphate
PMS	phenazine methosulfate
PPNR	photosynthetic pyridine nucleotide reductase
PQ	plastoquinone
P_{700}	pigment whose absorption changes are associated with the photosystem I reaction center
TMPD	N,N,N',N'-tetramethyl-p-phenylene diamine
TMQ	trimethyl-p-benzoquinone
$TMQH_2$	trimethyl-p-benzoquinone, reduced form
Tricine	N-tris(hydroxymethyl)methylglycine
vit. k	vitamin k

I. PHOTOSYNTHESIS - HISTORICAL DEVELOPMENT

Photosynthesis can be thought of as a series of processes whereby living organisms drive biochemical reactions by converting light energy to chemical energy. More specifically, photosynthesis as it occurs in algae and higher plants can be summarized by the equation

$$CO_2\ (g) + H_2O \xrightarrow{h\nu} (CH_2O)_x + 1\ O_2\ (g) \qquad (I\text{-}1)$$

where $(CH_2O)_x$ represents a basic subunit of carbohydrate, the main product of photosynthesis. This equation represents the combined work of many eighteenth and nineteenth century workers who showed that when the chloroplasts of higher plants are illuminated with sunlight, they assimilate carbon dioxide and water and evolve oxygen.

Blackmann (1905, 1911) studied the dependence of green plant photosynthesis on temperature, light intensity and CO_2 concentration. He concluded that photosynthesis exhibits characteristics of both photochemical and chemical processes. The photochemical processes are dependent on the light intensity but not on the temperature or CO_2 concentration, while the chemical processes do not require light but are sensitive to changes in the temperature or the CO_2 concentration.

The discovery of photosynthetic bacteria (Engleman, 1883) which do not evolve oxygen, but which use sunlight to assimilate CO_2, led Van Niel in the early 1930's to formulate a theory of photosynthetic energy conversion based on a comparison of bacterial and green plant

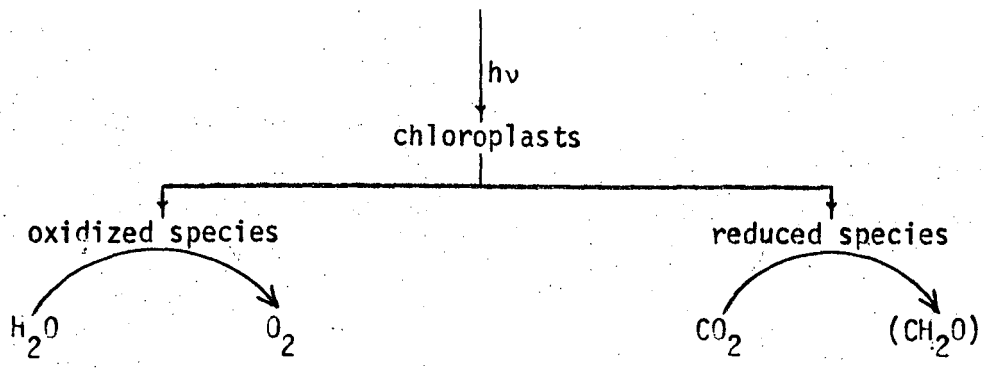
photosynthesis. Van Niel (1931) suggested that the above equation of photosynthesis represented a photolysis of water. In this formulation, the reduced species formed by the photolysis is associated with the mechanism for the reduction of CO_2, while in higher plants the oxidized moiety reacts to form oxygen. Photosynthetic bacteria cannot rearrange the oxidized product to form oxygen, but instead require a suitable oxidizable substance such as H_2S to reduce the oxidized moiety. This is consistent with the evidence of Blackmann suggesting a primary photoact followed by subsequent dark chemical events.

Support for this scheme was provided by Ruben et al. (1941) who showed that water rather than CO_2 is the source of oxygen liberated during photosynthesis. This was accomplished by using ^{18}O-labeled CO_2 or H_2O and noting, by means of mass spectroscopy, that the photosynthetically liberated O_2 contained the tracer only when the water was labeled.

On the other hand, the biochemical pathway involved in the fixation of the reduced moiety was completely explored by Calvin and coworkers during the early 1950's (Bassham and Calvin, 1957). Using radioactive carbon (^{14}C) they were able to map the fate of labeled carbon dioxide during algal photosynthesis. This assimilation process was found to be cyclic, involving sugar phosphates and requiring the energy of 2 $NADPH_2$ and 3 ATP for each CO_2 reduced to the level of carbohydrate. The corresponding mechanism of O_2 evolution from an oxidized moiety has not been elucidated and is the subject of much current research.

Emerson and Arnold (1931, 1932) studied the photosynthetic evolution of oxygen by the alga Chlorella in flashes of light of 10^{-5} sec duration from a neon discharge tube. They found that the maximum oxygen yield per unit of absorbed light energy from flashing light was greatly increased over the maximum yield obtained from continuous illumination. At saturating intensities they found that in a single flash one oxygen molecule was evolved per 2500 chlorophyll molecules. This maximum efficiency of oxygen evolution per flash was found to occur when the light flashes were separated by at least 0.02 sec at 25°C. From these results they proposed the existence of a photosynthetic unit of about 2500 chlorophyll molecules which cooperate in the evolution of one oxygen molecule, and that a slow dark step of 0.02 sec (room temperature) is associated with the function of this unit. A more complete discussion of photosynthetic units can be found in Chapter III.

The experiments discussed so far present a model of photosynthesis that involves three parts. The aspects of this model are illustrated in the diagram below.



First, light quanta are converted to chemical energy by chlorophyll in a cooperative photochemical process which yields oxidized and reduced species. CO_2 is not involved in the photochemical events but

is assimilated by a dark, temperature-sensitive reaction involving the reduced product of the photochemistry. Finally, oxygen is evolved in another dark temperature-dependent process. In Van Niel's formulation, the oxidized and reduced moieties would be the products of the "photolysis" of water catalyzed by higher plant chloroplasts. For bacterial photosynthesis, the water would be replaced by a hydrogen-donor substrate that would be oxidized instead of the water.

Up until the late 1930's most photosynthetic research had been performed on whole plants, leaves, or whole cell algae. A significant step was made by Hill (1939) when he showed that illuminated chloroplasts can evolve O_2 provided that a suitable oxidant is present. This "Hill reaction" is promoted by Fe^{+++}, ferricyanide, quinones and by reducible dyes such as DCPIP. The Hill reaction is a partial reaction, in that CO_2 is replaced by artificial electron acceptors as ultimate recipients of photochemical reducing power. Thus, only part of the overall photosynthetic pathway is utilized.

Hill was unable to demonstrate that CO_2 could function as the oxidant in chloroplasts, but subsequent work by Arnon et al. (1954) showed that indeed, isolated chloroplasts can fix CO_2 and that they are the sites of the light dependent formation of $NADPH_2$ and ATP. Trebst et al. (1958) demonstrated further that the production of ATP and $NADPH_2$ is associated with the chloroplast lamellae, while the process of CO_2 fixation occurs in the chloroplast matrix region, or stroma. Finally, the physiological oxidant, NADP, was found to be effective only when a soluble enzyme, photosynthetic pyridine nucleotide reductase (PPNR) (San Pietro and Lang, 1958) was added back to the photosynthetic membrane.

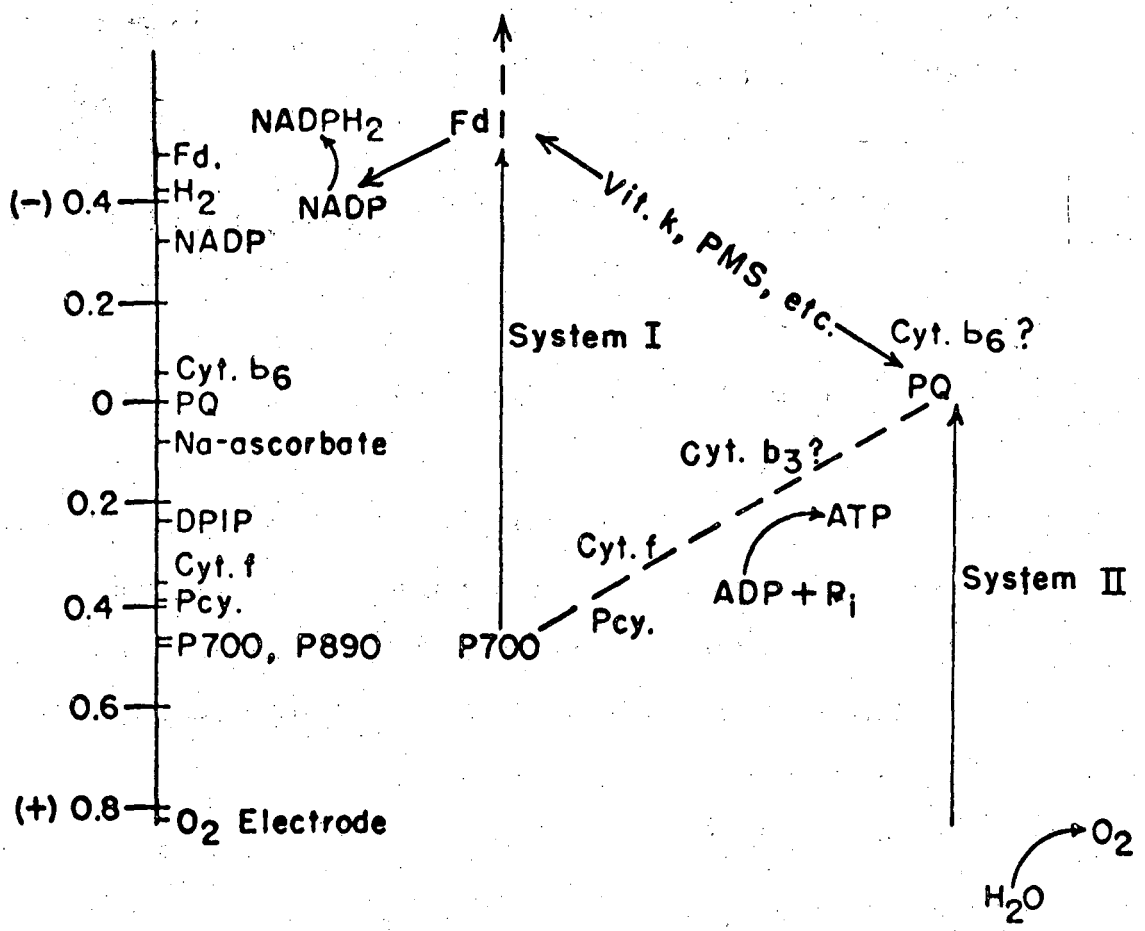
Arnon et al. (1957, 1959) demonstrated that photophosphorylation is coupled to the ferricyanide Hill reaction and to NADP reduction accompanied by oxygen evolution. They also showed that in the presence of phenazine methosulfate or vitamin k, there is another type of photophosphorylation which occurs without oxygen evolution. The latter type has been termed cyclic photophosphorylation and the former, accompanied by oxygen evolution, has been termed non-cyclic photophosphorylation. These processes of oxygen evolution, ATP formation and NADP reduction involve oxidation-reduction reactions that are generally termed photosynthetic electron transport because they involve the photocatalyzed transfer of electrons from water to NADP and because they are similar to the oxidative electron transport processes of mitochondria.

Studies of the quantum efficiency of photosynthesis as a function of wavelength of actinic light by Emerson and Lewis (1943) showed that wavelengths longer than 670 mμ were quite inefficient even though these wavelengths are well within the absorption bands of the photosynthetic pigments. This is known as the red drop phenomenon. Emerson et al. (1957) showed that this low efficiency of far red light could be increased by superimposing light of shorter wavelengths, and this effect has been termed Emerson enhancement. These results indicated that not all light-absorbing pigments are fully effective in promoting photosynthesis, but that cooperation between different sets of these pigments partially alleviated this inefficiency.

The results suggest that there are at least two types of photoreactions and in 1960, Hill and Bendall, considering the possible role of cytochromes f and b_6 in conjunction with their positions in

oxidative electron transport, proposed a scheme involving two photoreactions. Subsequently Duysens et al. (1961) noted by means of a differential absorption spectrophotometer that a c-type cytochrome in Porphyridium cruentum was oxidized by 680 mμ light and reduced by 560 mμ light. The results were interpreted as indicating the interaction of at least two light reactions. These authors labeled the pigments absorbing at 680 mμ and causing the oxidation as System I, while those absorbing at 560 mμ and photocatalyzing the reduction were termed System II. They also showed that oxygen evolution inhibitors such as DCMU prevented the reduction of the cytochrome, indicating that System II is associated with the oxidation of water.

These basic ideas and some subsequent work are illustrated in Figure 1, which is a schematic representation of a current hypothesis of green plant photosynthetic electron transport involving two light reactions operating in series. This scheme incorporates the basic idea of Van Niel that oxidized and reduced species are formed in the photochemical events. The two vertical arrows represent the energy supplied by the two different photoreactions. Light absorbed by System II results in the formation of a strong oxidant, which can oxidize water (bottom of System II arrow), and in the formation of a weak reductant. The dashed line in the figure represents the electron transport chain that connects the weak reductant of System II with the weak oxidant formed by absorption of quanta by System I. The strong reductant formed by System I can then reduce NADP through the enzymes of PPNR. Partial reactions can be envisioned as external substrates interacting directly with components along the electron transport chain, i.e., accepting electrons at the top of



XBL 677-4549-A

Figure 1. Oxidation-reduction diagram of the two light reaction scheme for photosynthetic electron transport. Included are *in vitro* redox potentials for some of the important components and some of the reagents to be used in this study.

System I (replacing NADP), or donating or accepting electrons at a site between the two light reactions. A partial reaction donating electrons instead of H_2O at the bottom of System II has recently been reported by Yamashita and Butler (1968).

Along with Figure 1 are listed various redox components and their *in vitro* oxidation-reduction potentials. Caution must be taken in inferring the *in vivo* ordering of these components from their *in vitro* oxidation-reduction potentials. The ordering presented here is basically dependent on the mutant studies of Gorman and Levine (1965, 1966, and Levine and Gorman, 1966), in which the effect of the absence of one or more of these components has been studied on various partial reactions.

The correlation of structure and function in the higher plant chloroplasts, as in the work of Trebst *et al.* above, has led to electron microscopy studies of the internal chloroplast lamellae which contain the apparatus of photosynthetic quantum conversion and electron transport. Park and Pon (1961), investigating the structure of sonicated chloroplast lamellae, found that the fragments seemed to have a granular appearance and, in some cases, ordered arrays of particles. These particles appear to be in the interior of the membrane and are visible when the outer layer of the membrane is torn away by sonication. The dimensions of the particles are 185 Å x 155 Å x 100 Å thick. A current discussion of chloroplast membrane subunits, particularly as elucidated by the technique of freeze etching, may be found in Park and Shumway (1968).

The lamellar fragments are active in quantum conversion and they support CO_2 fixation in the presence of added stroma (Park and Pon,

1961). Lamellar fragments consisting of about eight of these particles were found to contain DCPIP Hill reaction activity or reduce NADP when PPNR was added. Thus, the integrity of the complete chloroplast lamellae is unnecessary for quantum conversion. Because of this feature that aggregates of these particles can convert light energy to chemical potential, they were named "quantasomes" (Park, 1962). This photoactivity led Park (1962) to suggest that the quantasome may be the morphological equivalent of the physiological photosynthetic unit first suggested by Emerson and Arnold (1932).

The photosynthetic unit is today envisioned as an ordered collection of structural lipid, proteins and pigment molecules which function together to collect and transfer light quanta to reaction centers or traps. Also associated with the photosynthetic unit are the oxidation-reduction components of the electron transport chain. A list of the lipid and protein composition of the chloroplast lamellae can be found in Park and Biggins (1964). They conclude that there are approximately 230 chlorophyll a and b molecules associated with each quantasome unit. The reaction centers appear to contain chlorophyll molecules which are in some way different from the bulk pigments of the photosynthetic unit. The exact nature of this difference is not known but evidence suggests that the reaction center is a chlorophyll associated with other molecules such as quinones or cytochromes; or that the reaction center chlorophylls are unique because of aggregation to form dimers or because of a special interaction with the surrounding lipo-protein matrix. At the reaction center the energy of the absorbed quanta is used to separate oxidized and reduced species resulting in the storage of chemical potential.

Kok (1961) found a pigment which exhibited reversible light induced absorption changes at 430 mμ and 700 mμ. This pigment, termed P_{700}, behaves as a one-electron transferring agent in the dark with an E_o^1 of +0.43 volts. The reduced form of the molecule is stable in the dark, is oxidized upon illumination with far red light (System I) and its reduction by light of short wavelengths is inhibited by DCMU. Witt et al. (1961) found that P_{700} is oxidized by System I light more rapidly than any other component in the electron transport chain, and in all other ways, P_{700} exhibits characteristics consistent with the role of a molecule located in the reaction center. Speculation based on its absorption changes suggest that this molecule is a special chlorophyll molecule and that its oxidation is the first redox reaction promoted by the absorbed quanta. A similar trapping molecule for System II has not been characterized, but the recent report by Döring et al. (1967) suggests that they have found absorption changes which can be attributed to a System II reaction center and that information on the functioning of the System II trap will be forthcoming.

The composition of the bulk pigments which can excite the two photoacts is the subject of some speculation. Because far red light is only efficient in System I, the long wavelength absorbing pigments are thought to be entirely System I pigments. Duysens (1952) showed that quanta absorbed by accessory pigments in algae (those other than chlorophyll a), such as phycocyanin or phycoerythrin, can transfer their excitation energy with very great efficiency (>90%) to cause chlorophyll a fluorescence. In fact, light absorbed by these phycobilins and then transferred to chlorophyll a was found to

be more efficient in promoting chlorophyll a fluorescence than light absorbed by chlorophyll a itself. Subsequent studies (Butler and Bishop, 1963) have shown that almost all chlorophyll fluorescence at room temperature is emitted by System II chlorophyll. Thus, it is thought that accessory pigments, such as phycobilin in algae and chlorophyll b in plants along with part of the chlorophyll a are primarily involved in pigment system II.

The nature of the relationship of the photosynthetic pigments to the electron transport chain of photosynthesis is the major concern of the material presented in this thesis. Chapter II presents a study of the wavelength dependence of the quantum efficiency of a System I photoreaction, in order to characterize further the pigment system responsible for quantum conversion in System I. Comparison of this action spectrum with one for a System II photoreaction may then yield information about the functional relationships between the pigments which absorb for the two photoreactions.

Chapter III describes a study of System I and II partial reactions in flashing light. Through these experiments information can be obtained concerning the photosynthetic unit sizes involved in each of the two photoreactions. Also, kinetic measurements can be made of some of the basic rate-limiting processes of the photosynthetic electron transport chain. A concluding section presents a brief summary of these results and relates them to the overall concept of the photosynthetic quantum conversion process described above.

II. QUANTUM EFFICIENCY MEASUREMENTS OF CHLOROPLASTS

A. Quantum Efficiency of Photosynthesis

The overall reaction of equation I-1, the reduction of CO_2 to (CH_2O) and the evolution of O_2, are processes that involve the transfer of four electrons per molecule. The free energy of one equivalent of (CH_2O), as sugar, is 118 kcals greater than that of one mole of CO_2. However, more energy will be required to drive this photochemical process, due to some energy loss in the evolution of O_2 and the necessity of an energy barrier of some kind to prevent the immediate recombination of the photoproducts. The total energy requirement has been estimated to be >185 kcals (Clayton, 1965). An einstein of 675 mμ quanta has an energy of 42 kcals,* so four einsteins would yield only 168 kcals, even if a 100% efficient photoprocess could be found.

Most of the early quantum efficiency measurements are based on manometric data of O_2 and CO_2 exchange. These studies show a quantum requirement of 6 to 12 $h\nu/CO_2$ except the work of Warburg and coworkers, which have consistently yielded values of 2.5 to 5 $h\nu/CO_2$ (see Rabinowitch [1951] for references and a discussion of this controversy). Repeated efforts to reproduce Warburg's results in other laboratories have failed, and from the above thermodynamic restrictions we can rule out these low values from serious consideration.

---

*For a discussion of the actual free energy of a quantum available to the photosynthetic apparatus, see Ross and Calvin (1967).

It is seen from Figure 1 that a series formulation of a two light reaction scheme would require one quantum as a minimum to transfer an electron through each photoreaction. In this case, the transfer of four electrons from water to NADP would require the sum of eight quanta to drive the four electrons through both photoreactions. This value is quite consistent with the quantum requirement measurements above.

As noted before, action spectra for photosynthesis (O_2 evolution) exhibit a "red drop" or inefficiency in the far red wavelength tail of chlorophyll absorption. Measurements of the action spectrum for the "normal" reduction of NADP by chloroplasts with water as the electron donor (Black et al., 1963; Hoch and Martin, 1963; Govindjee et al., 1962; Sauer and Biggins, 1965) in general confirmed the shape of the red drop action spectrum.

Measurements of the action spectra for the DCPIP and NADP Hill reactions (Chen, 1952; Biggins and Sauer, 1964) again showed this inefficiency for wavelengths longer than 670 mμ. Since the Hill reaction is a partial photoreaction involving oxygen evolution, we can see from Figure 1 that it must utilize pigment system II. Studies on a photosynthetic mutant lacking P_{700} (Givan and Levine, 1967) (i.e. lacking a System I trapping center) still carry on appreciable Hill activity (using ferricyanide, DCPIP, or p-benzoquinone as the electron acceptor), indicating that these Hill reagents can accept electrons on the electron transport chain between the two photoacts. This, coupled with the fact that longwave absorption is known to oxidize P_{700}, indicates that both overall photosynthesis and the Hill reaction with the above reagents exhibit action spectra consistent with the pigment

absorption of photosystem II. The quantum requirements of both the ferricyanide (Lumry et al., 1957) and the DCPIP Hill reactions (Sauer and Park, 1965) in monochromatic light in the wavelength region 640-670 mμ decrease with decreasing light intensity and extrapolate at zero light intensity to a value of 2.0 quanta per electron transferred to the oxidant.

Vernon and Zaugg (1960) showed that NADP is reduced even in DCMU-poisoned chloroplasts (inhibition of System II or oxygen evolution) when ascorbate and catalytic amounts of DCPIP are added. This then represents a partial reaction other than a Hill reaction. In this reaction reduced DCPIP acts as an electron donor for System I by reacting directly with a component in the electron transport chain between the two systems. The partial reaction is thus activated only by System I.

Action spectra for the NADP reduction with DCPIP/ascorbate have been obtained by Hoch and Martin (1963) and Sauer and Biggins (1965). These results confirm that far red light (λ >680 mμ) is more efficient in photosystem I as compared with near red light. At wavelengths greater than 700 mμ these workers obtained quantum requirements averaging around 2.0 (quanta/electron transferred to NADP) with values as low as 1.5. If these pigments do indeed transfer excitation energy only to System I, then one would expect values as low as 1 quantum/electron transferred in a System I partial reaction (assuming the validity of an overall quantum requirement of 8 $h\nu/O_2$).

In 1965, Vernon and Shaw demonstrated that the photoreduction of cytochrome c by whole chloroplasts is stimulated by the addition of various hydroquinones, including reduced trimethyl-p-benzoquinone

($TMQH_2$). This stimulation is only partially decreased in the presence of DCMU, an inhibitor of oxygen evolution by chloroplasts. This finding suggested to Vernon and Shaw that these hydroquinones serve as electron donors for the long wavelength pigment system I of chloroplasts. We undertook to determine by means of its action spectrum whether this photoreduction is indeed a System I reaction and, if so, to see if this reaction would exhibit an efficiency of one quantum/electron transferred in the long wavelength region. In addition, information could be obtained about the spectral absorption of pigment system I and perhaps on the functional organization of the bulk pigments.

B. Characteristics of the Cytochrome c/$TMQH_2$ Reaction

Vernon and Shaw reported that in the presence of $TMQH_2$ there is a slow reduction of cytochrome c by chloroplasts in the dark. In order to study a light driven process involving these two components, the characteristics of this dark reaction must be determined to see if any corrections to the light-initiated photosynthetic reactions will be necessary. The dark reaction was found to proceed at about the same rate in the presence or absence of chloroplasts, which suggested a direct interaction between cytochrome c and $TMQH_2$. Below, some aspects of this dark reaction are explored.

Preparation of $TMQH_2$. TMQ obtained from K&K Laboratories, Inc. (Jamaica, New York), is a yellow crystalline material of low melting point which sublimes into long needles. The crystals are waxy at room temperature and not clear or well defined. About 100 mg of TMQ dissolved in 10 ml of diethyl ether is placed in a small separatory

funnel. Small amounts (∿1 ml) of a solution containing 500 mg of sodium dithionite in 20 ml of distilled water are added to the ether solution and shaken. The reduction of the quinone by dithionite causes the solution to bleach noticeably, and dithionite addition is continued until the yellow color is completely removed.

The remainder of the procedure is performed in subdued light in order to decrease the chances of photocatalyzed air oxidation. The water layer is removed and the remaining ether solution evaporated to dryness by gentle heating. The resultant, slightly yellowish solid is then sublimed onto a coldfinger by gentle heating to form long, thin, clear crystals of $TMQH_2$. Sublimation is continued until some of the yellow material also starts to sublime. (An increase in yellowness of the evaporated solid was noticed during resublimation, denoting a partial reoxidation to TMQ in air.) The overall yield of clear crystals with no yellowness was about 35%.

Cytochrome c extinction coefficient. Horse heart cytochrome c was obtained from Sigma Chemical Co., St. Louis. The sample was found to contain a considerable amount of water, and so it was dried to constant weight in a vacuum desiccator over $Mg(ClO_4)_2$. Figure 2 shows the spectra obtained when samples of the dried cytochrome in phosphate solution at pH 7.5 were oxidized with ferricyanide and reduced with dithionite. The values obtained for the extinction coefficients at the maximum of the α-band (549.5 mμ) are given below in Table 1 (in units of 10^{+4} ℓ-mole^{-1} cm^{-1}) which also includes a comparison with literature values. The value $\Delta\varepsilon^{red\text{-}ox}_{549.5\ m\mu} = 1.9 \times 10^4$ is in good agreement with those in the literature, and is used throughout this work.

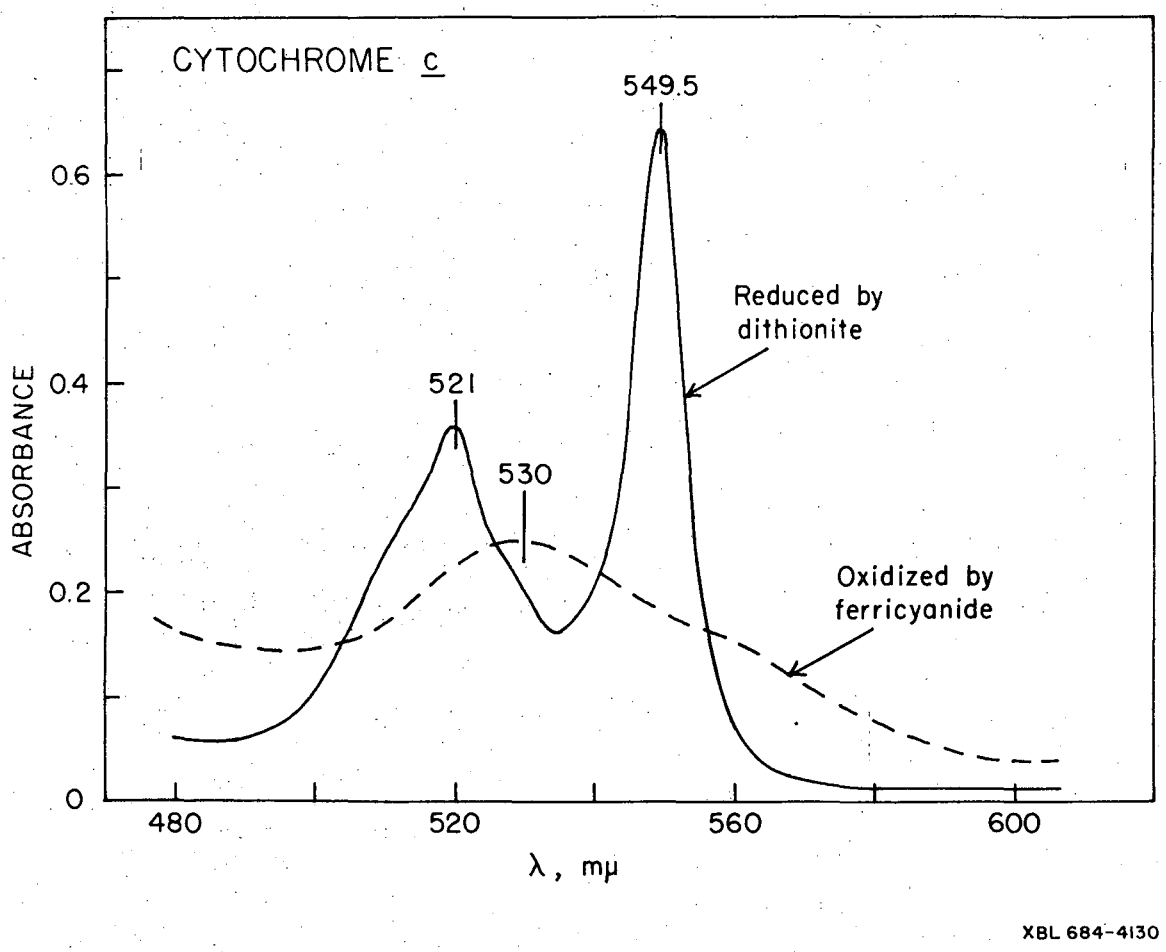


Figure 2. Absorption spectra of oxidized and reduced cytochrome c isolated from horse heart at room temperature.

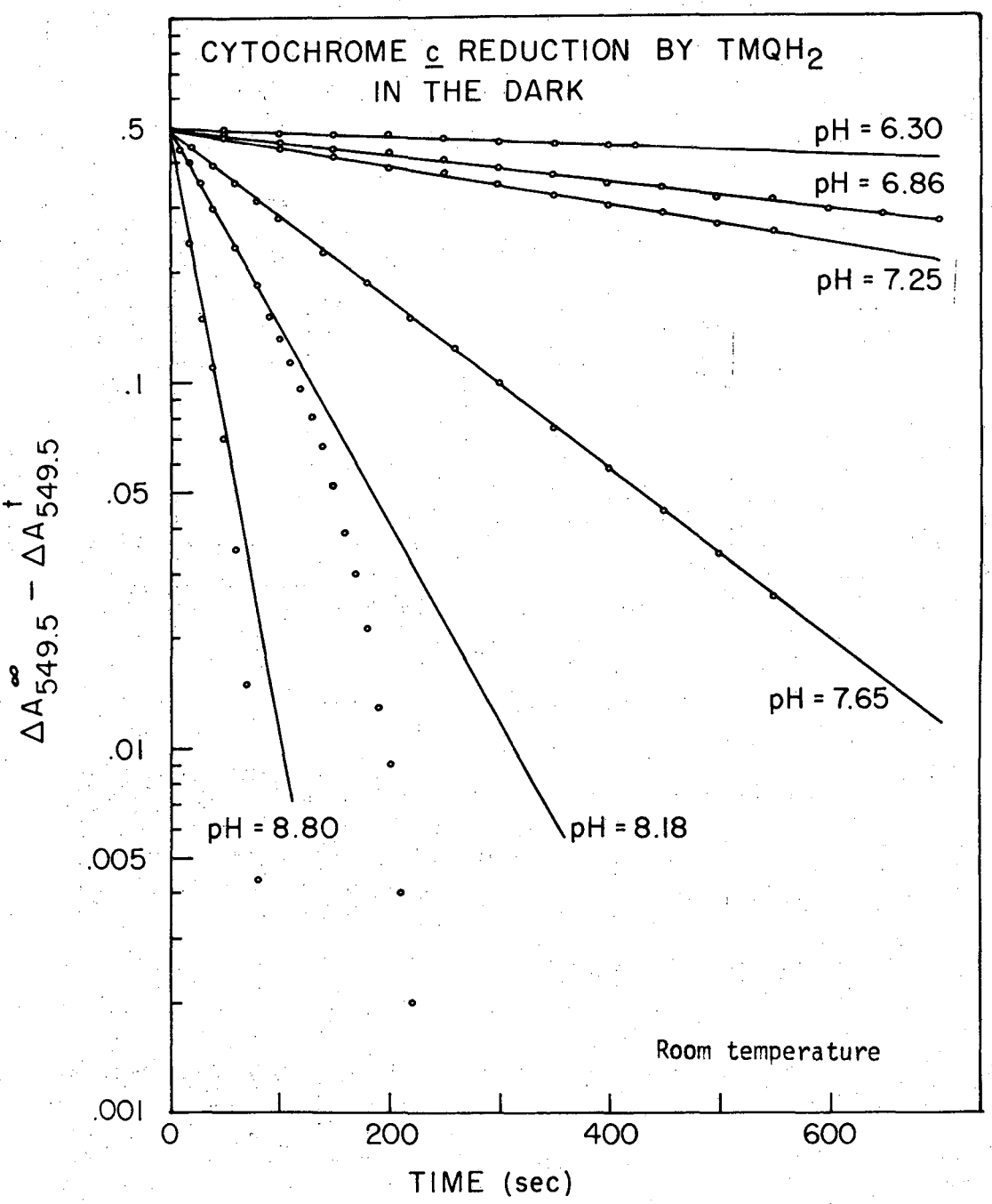
Table 1

Cyt c extinction coefficients	This work	Paléus & Neilands (1950)	Massey (1959)
$\varepsilon^{red}_{549.5\ m\mu}$	2.62	2.7	2.99
$\varepsilon^{ox}_{549.5\ m\mu}$	0.73		
$\Delta\varepsilon^{red-ox}_{549.5\ m\mu}$	1.9	1.9	2.1

Concentration effects on rate of dark reaction. The effects of pH, concentrations of cytochrome c and $TMQH_2$, and of the addition of other substances in the chloroplast reaction mixture were studied to see what factors affect the dark reaction. The final reaction mixture contained about 2.6×10^{-5} M cytochrome c and 4×10^{-5} M $TMQH_2$ unless otherwise noted. The reaction was followed by observing the formation of reduced cytochrome c by its absorption increase at 549.5 mμ in a Cary Model 14 spectrophotometer. The $TMQH_2$ was injected into the cytochrome c solution to start the reaction.

It was found that the reaction rate varied with pH. Three buffers (phosphate, tris, and tricine) were used at 0.10 M concentration to vary the pH from 6.3 to 8.8. Table 2 below shows a summary of the results.

Figure 3 shows the change in cytochrome c absorbance as a function of time for various pH's. From this plot, first order rate constants were calculated by the half-life method. Using the differential method of kinetic order analysis, a plot of the logarithm of the initial velocity versus the logarithm of the OH^- concentration



XBL 684-4131

Figure 3. Rate of cytochrome c reduction with $TMQH_2$ in the dark at various pH's. The ordinate is the logarithm of the quantity (change in absorption at 549.5 mμ at infinite time [$A^{\infty}_{549.5}$] minus the change at time t [$A^{t}_{549.5}$]). Thus the difference on the graph between two points at a given pH represents the absorbance of the amount of cytochrome c reduced during the times associated with the two points.

Table 2

Buffer	pH	Initial Rate (ΔA/min)	k_1 $(sec^{-1})10^3$	$[OH^-]10^7$
Phosphate	6.30	0.0072	0.100	0.20
Tricine	6.86	0.0246	1.08	0.724
Phosphate	7.25	0.0310	1.17	1.78
Tricine	7.65	0.149	5.35	4.47
Tris	8.18	0.300	13.5	15.1
Phosphate	8.80	~1	68	63.1

is given in Figure 4. The reaction is seen from the slope of this plot to be first order in hydroxide ion. Figure 3 also shows that the reaction deviates from first order at pH's greater than about 8, suggesting that another mechanism becomes important at higher OH^- concentration. The fact that the initial velocities at these higher pH's still correspond to a first order reaction, points to a possible activation by one of the products of the slow step.

In order to dissolve $TMQH_2$ in water, it is dissolved first in ethanol or methanol and this solution is injected into the reaction mixture. The final reaction mixture usually contains about 1% of the alcohol. However, even in these two solvents, $TMQH_2$ is only slightly soluble and quite difficult to dissolve. Also, any solution of $TMQH_2$ slowly oxidizes in air to TMQ and turns yellow. This low solubility and air oxidation make it impossible to determine accurately the concentration of $TMQH_2$. Values of the relative concentration of $TMQH_2$ at pH 7.5 of about 2, 4, and 8 x 10^{-5} M indicate that the reaction is also first order in $TMQH_2$.

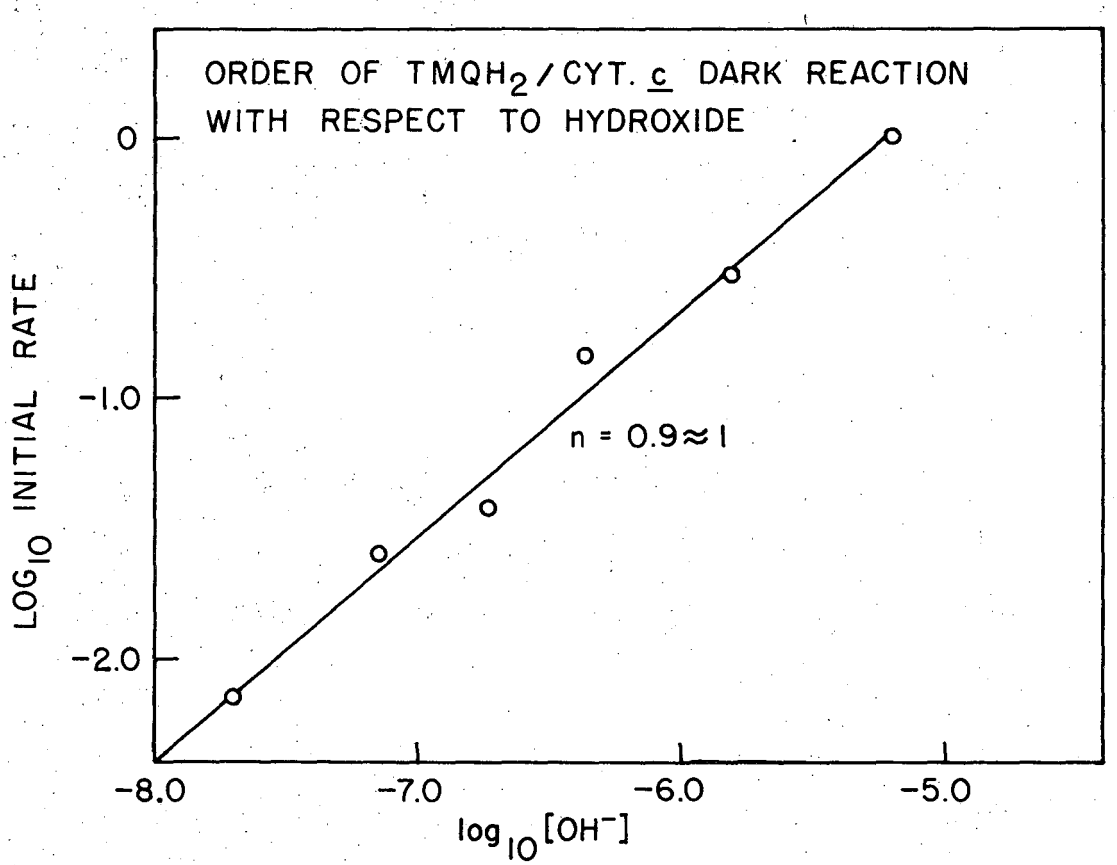


Figure 4. Determination of the kinetic order of the dark reaction with respect to hydroxide by the differential method. The slope of 0.9 suggests that the reaction is first order in hydroxide.

Similar two-fold variations showed that dependence of the reaction on cytochrome c concentration is also first order. Furthermore, the following conditions* had no appreciable effect on the kinetics of the dark reaction at pH 7.5: variation of the ethanol concentration (two-fold); use of different buffers; exclusion of oxygen in the solution (by purging with nitrogen); and the addition of DCMU (10^{-6} M), methylamine (10^{-3} M), fresh chloroplasts (A_{678}^{1cm} 1.0) in the dark, or heated chloroplasts (60°C for 5 minutes) either in the dark or illuminated with broad band red light.

These results suggest that the rate limiting step of the reaction is affected by the concentrations of OH^-, $TMQH_2$ and cytochrome c. The mechanism may involve the abstraction of a hydrogen from $TMQH_2$ by OH^-, perhaps through an equilibrium such as

$$TMQH_2 + OH^- \rightleftharpoons HOH + TMQH^-$$

In any case, the resultant reduced trimethyl quinone species reduces cytochrome c. Of importance to photoreduction experiments with chloroplasts is the fact that none of the materials listed above which are normally present in the reaction mixture affect the rate of the dark reaction.

From Figure 3 it can be estimated that at pH 7.5 it will take about 10 minutes for the dark reaction to reduce all but 0.05 o.d. units of the cytochrome c used in that series of experiments. As the total photoreaction that we normally measure involves 0.05 o.d. units or about 1/10 of the cytochrome c, it would seem that the

*Concentrations given are those to be used in the photoreaction mixture.

photoreaction can be followed for about 10 minutes before the dark reaction will limit the rate of the photo-induced reduction of cytochrome c. There is one other item to consider about the dark reaction, and that is how it will be compensated for when the photoreaction is being observed; but this will be taken into consideration below in Section II-C.

Discussion. In this section we have seen that a dark reaction between $TMQH_2$ and cytochrome c does exist and that it is dependent on the pH of the reaction medium. This dark reaction is quite rapid at physiological pH's (about 7.5) where chloroplasts are most stable. In the set of experiments to follow, which deal with quantum efficiency measurements, a pH of 7.5 was routinely used, allowing about 10 minutes of useful reaction.

It was found after these experiments were performed that chloroplasts, isolated as in Section II-C, would survive a pH of 6.3 for about 15 minutes at full System I activity; and so, in a later set of experiments discussed in Chapter III, this pH was used. The pH 6.3 has the advantage of greatly reducing the rate of the dark reaction and so allowing longer experiments on the same sample and reducing the amount of compensation needed for the back reaction. However, one is limited at this pH by the instability of the chloroplasts.

C. Quantum Requirements of the Cytochrome c/$TMQH_2$ Reaction in the Red Spectral Region of Spinach Chloroplasts

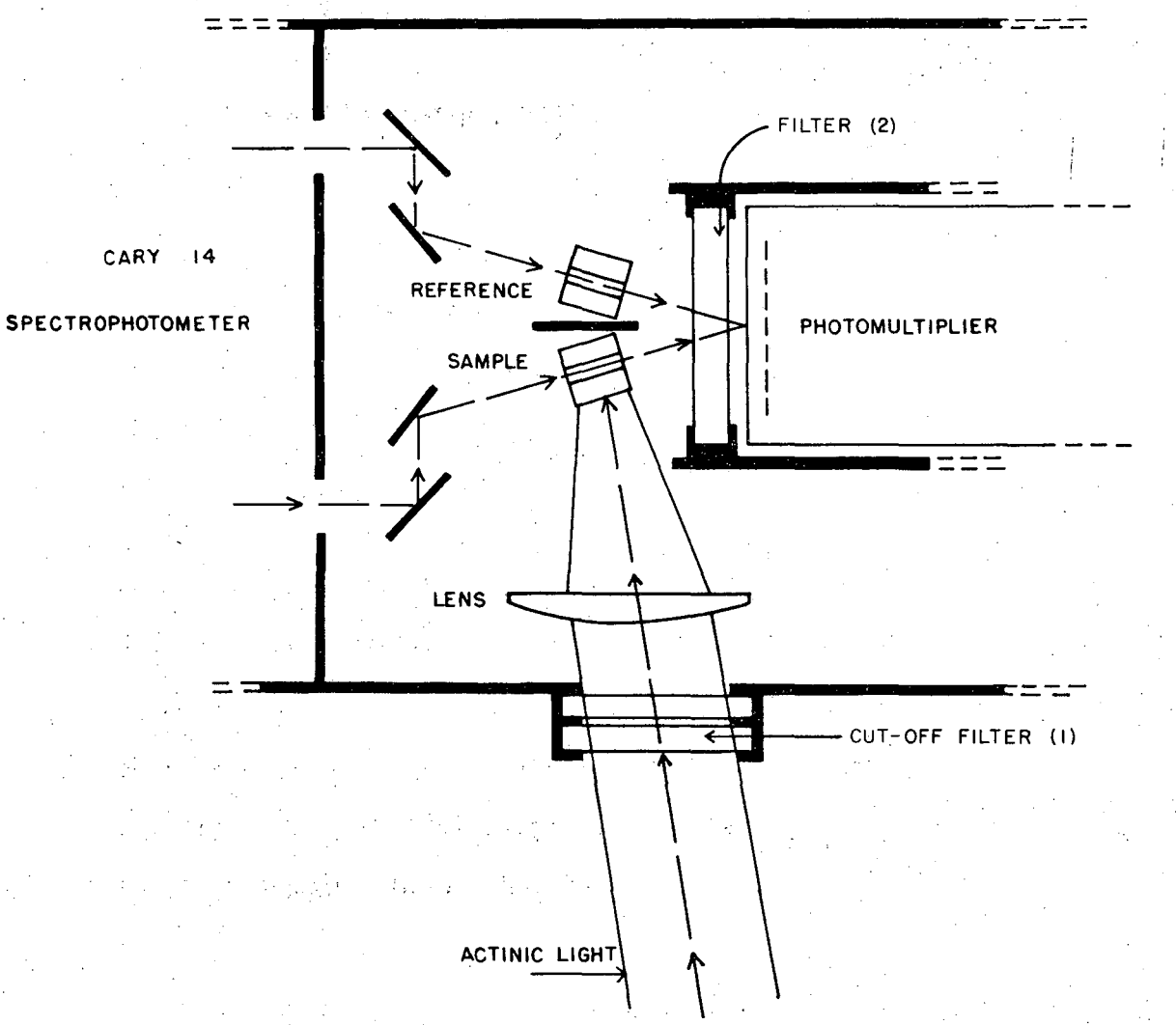
Procedure. The rate of cytochrome c reduction was obtained by continuously monitoring the absorbance of the reaction mixture at

549.5 mμ (the α-band maximum for cytochrome c) while the sample was being irradiated from the side with longer wavelength light. A Cary Model 14 spectrophotometer with a modified Model 1462 scattered transmission accessory was used, as described by Sauer and Biggins (1965). Figure 5 shows a diagram of the modifications performed in order to permit side illumination of the sample cuvette. The cuvettes employed have four clear sides and a rectangular internal cross section of 3 mm x 10 mm. The actinic light is incident at right angles on the sample cuvette and traverses a 3-mm light path in the reaction mixture, while the Cary's measuring beam passes through the 10-mm path length.

The actinic light was obtained from a Bausch and Lomb monochromator (red-blazed grating) with supplementary cut-off filters (1). Monochromator slit widths were routinely set at 3 mm, resulting in an actinic band width of 10 mμ. Table 3 lists the cut-off filters and the wavelength region in which they were utilized.

Table 3

Wavelength Region (mμ)	Corning Cut-off Filter (1)
400 - 474	3-58 + 4-94
475 - 555	3-72 + 4-94
556 - 579	4-97
580 - 649	3-68
650 - 684	2-58
685 - 699	2-64
700 - 719	2-58 + 4-77
720 - 740	2-58 + 7-59
>740	7-69



MUB-2802

Figure 5. Diagram of the sample compartment of a Cary Model 1462 Scattered-transmission Accessory showing modifications allowing side illumination of the sample cuvette. The cut-off filters (1) are used to select the actinic wavelengths, while the filters (2) prevent the actinic light and chloroplast fluorescence from interfering with the spectrophotometer measuring beam.

An opaque barrier prevents the actinic beam from illuminating the reference cuvette. Another filter (2) was attached to the front of the photomultiplier to prevent the actinic light and chloroplast fluorescence from interfering with the spectrophotometer measuring beam. Table 4 lists these filters, photomultipliers, extinction coefficients and measuring wavelengths for the cytochrome c/$TMQH_2$ reaction studied here and for two other photoreactions to be discussed later. The 0 to 0.2 optical density slide wire of the Cary was used and the recording chart speed was varied according to the reaction rate.

Table 4

Reaction	Cut-off Filter (2)	Photo-multiplier	Extinction Coefficient (liters/mole-cm)	Wavelength (mμ)
Cyt c/ $TMQH_2$	Corning 4-96	RCA 6903	19,000	549.5
DCPIP Hill	Corning 4-96	RCA 6903	19,000 (pH 7.5)	580
Ferricyanide Hill	Baird Atomic Broad Band Interference Filter #2	Dumont 7664	1,000	420

Light intensity measurements. Incident light intensities were detected with a silicon solar cell (Hoffman, type 120CG) and measured by a millimicrovoltmeter (Model 149, Keithley Instruments, Cleveland). The wavelength dependence of the efficiency of the silicon cell was determined by comparing its output when illuminated by a Bausch and

Lomb 500 mm grating monochromator (300 watt tungsten source) with that obtained from a Reeder thermopile (Model RBL-500-M, C. M. Reeder Co., Detroit) with the same light source. The thermopile was previously calibrated with a standard lamp (National Bureau of Standards, Washington, D.C.). Intensity measurements were corrected for reflection loss at the cuvette side which was measured to be 5% throughout the wavelength region of actinic light.

Absorbed intensity calculation. The method used to determine the absorbed intensity is the same as that employed by Sauer and Biggins (1965) and for which a complete discussion can be found in Biggins (1965). An outline of the method is given below since an accurate determination of the actual absorbed intensity is necessary for the calculation of the quantum yield of a photochemical reaction.

The difficulty lies in the large contribution of light scattering to the observed absorption spectrum of chloroplasts as measured in a spectrophotometer. Quantasome aggregates, however, exhibit less scattering then chloroplasts. Quantasome scattering was found to be Rayleigh scattering with an inverse fourth power dependence on wavelength, as measured in the wavelength region 760-900 mμ. At these wavelengths, no known pigment absorption is present. Thus, by extrapolating the observed inverse fourth power dependence to shorter wavelengths, a correction can be obtained to remove the contribution of scattering from the observed quantasome spectrum.

To calculate the true absorption spectrum of a chloroplast sample, the absorbance of that sample at 750 mμ is subtracted from that at 678 mμ, the chloroplast absorption maximum. This proves to be a good correction for chloroplast scatter at 678 mμ and yields a value of

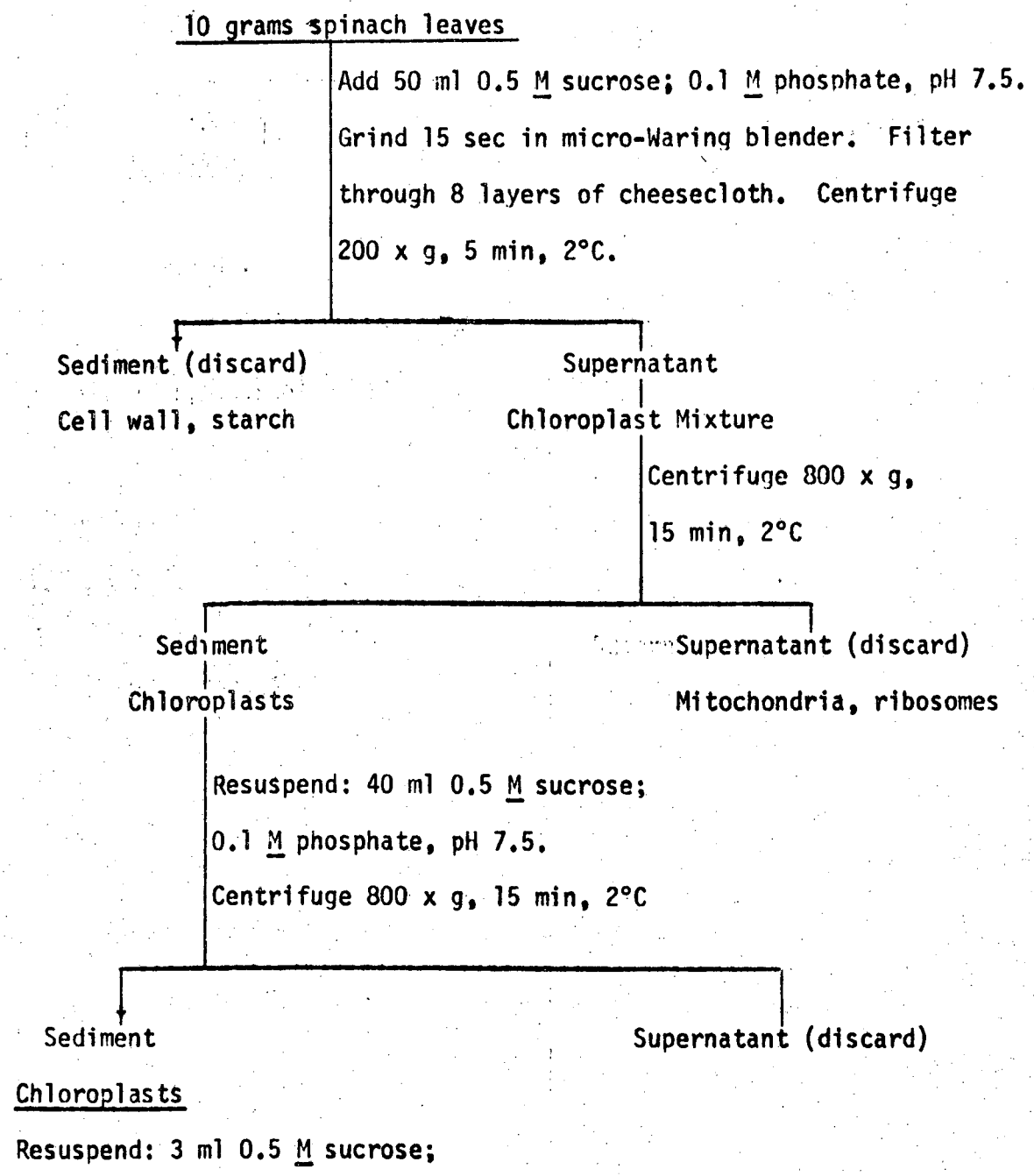
the absorbance at this wavelength which is probably within ± 3% of the true absorbance.* After normalizing the corrected quantasome spectrum to 1.0 at 678 mμ, we can calculate the true absorbance of the chloroplast sample at any wavelength by multiplying the corrected chloroplast absorbance value at 678 mμ with the normalized quantasome value at the desired wavelength. Sauer and Park (1964) discuss the evidence that the true absorption spectra of quantasomes and chloroplasts are identical. Assuming that they are the same, we may then use the above method to determine the actual intensity absorbed by the pigments of the chloroplasts.

Chloroplast preparation. Chloroplasts were at first isolated from fully grown commercial spinach leaves. The results obtained from these chloroplasts, however, are somewhat variable, due probably to varying age and freshness of the leaves. More uniformity and generally higher activity was obtained from chloroplasts isolated from six to eight week-old spinach plants grown from seed in a growth chamber (Sauer and Park, 1965) than was obtained from commercial spinach. Spinacia oleracea L. var. Viroflay or S. oleracea var. early hybrid no. 7 were used.

Following the suggestion of Biggins (1965), the destemmed leaves were washed and refrigerated in a sealed polyethylene bag for an hour in the dark before homogenization. The actual isolation procedure is outlined in Figure 6. The pigmented material is kept cold (0°C) and in the dark as much as possible throughout the isolation procedure to

*As determined by comparison with measurements of chloroplast absorption spectra using the integrating sphere method for scattering correction (Drury and Kelly, 1968).

Figure 6. Isolation procedure for spinach chloroplasts following the method of Park and Pon (1961).



prevent photo-inactivation of the chloroplasts.

Chloroplasts isolated in this manner are similar to Class II chloroplasts or "broken chloroplasts" as defined by Spencer and Unt (1965), since phase microscopy shows these chloroplasts have broken or missing outer membranes. Chloroplasts of this type are used here because we do not want the reaction rate limited by diffusion processes of the chloroplast outer membrane. When dealing with a substrate as large as cytochrome c (M.W. 12,000), this diffusion effect could be quite important.

Chloroplasts isolated in this manner are found to retain 100% of their initial System I or System II photoactivity for four to five hours after isolation. These results are summarized in Table 5.

Table 5

Time after chloroplast isolation (hours)	Cytochrome c/$TMQH_2$ % initial activity	DCPIP Hill
0	100	100
1	98	101
2	100	99
3	99	98
4	99	100
5	99	93
6	96	85
7	85	70

(System I activity was assayed as cytochrome c reduction with $TMQH_2$, using the standard reaction conditions outlined below, and System II

activity was assayed as the DCPIP Hill reaction according to the reaction conditions of Sauer and Park (1965). After isolation, the chloroplasts were stored in the dark at 0°C, in 0.5 M sucrose and 0.05 M phosphate, pH 7.5, until added to the reaction mixture. These results indicate that data taken during the first four to five hours after chloroplast isolation should prove the most reliable. They also show that System I activity as measured under these conditions is more stable than that of System II.

Reaction mixture. The reaction mixture contained potassium phosphate, pH 7.5, 0.05 M; sucrose, 0.5 M; and the following in μmoles/liter: cytochrome c, 50; $TMQH_2$, 85; and DCMU, 0.9. (The $TMQH_2$ and DCMU were made up in ethanol solutions, which were diluted 100- and 200-fold, respectively, in the reaction mixture.) A sufficient amount of the chloroplast preparation was added in the dark at the start of each measurement to give an absorbance of chlorophyll at 678 mμ of 0.3 - 0.7 for a 1-cm path. The dark reaction between cytochrome c and $TMQH_2$ necessitated a fresh reaction mixture (2 ml) being prepared for each wavelength of exciting light studied. All measurements were made in air, except where noted, and at room temperature.

Back reaction. The reaction system exhibits a fairly strong back reaction in the dark, following illumination. The rate of this back reaction is proportional to the percentage conversion of the cytochrome c. Because of the known dark reaction between cytochrome c and $TMQH_2$, this apparent reoxidation of cytochrome c in the dark after illumination is attributed to the difference between the rate of the dark reaction in the reference cuvette and of that in the

reaction cuvette. Since the photoreaction reduces some of the cytochrome c in the reaction cuvette, its dark reaction rate is lower than that of the reference cuvette, which has a higher concentration of oxidized cytochrome c. This difference in rate of dark reaction thus causes an apparent reoxidation of cytochrome c in the reaction cuvette in the dark and leads to the observation that the rate of the back reaction is proportional to the percentage conversion of the cytochrome c. All photochemical rates reported are corrected for the appropriate interpolated dark reaction by adding the average of apparent reoxidation observed before and after illumination to the rate of photoreduction. This correction is made possible by the previous observation that the dark reaction between $TMOH_2$ and cytochrome c is unaffected by illumination or the presence of chloroplasts (heated).

Effect of heating. A reaction mixture in which the chloroplasts had been heated to 65° for three minutes, conditions known to destroy System I activity (Vernon and Zaugg, 1960; Rumberg and Witt, 1964), exhibited no cytochrome c photoreduction when it was illuminated at 680 mμ. This is taken as an indication that the photoreduction requires the integrity of the chloroplast structure and not just the presence of the pigments in solution.

Photophosphorylation. Methylamine is known to uncouple the chloroplast Hill reactions from photophosphorylation and to lower the quantum requirements for the Hill reaction at moderate light intensities (Sauer and Park, 1965). In the case of cytochrome c reduction, however, methylamine (10 μmoles ml^{-1}) had no effect on the rates of either the photoreduction or the back reaction.

The fact that methylamine has no effect on the efficiency of the photoreduction could suggest that the pathway for this reaction does not involve any of the sites of photophosphorylation. However, the reaction mixture contains ∿1.5% ethanol, which is reported by Baltscheffsky (1963) to be an inhibitor of phosphorylation. Thus the above suggestion concerning the sites of photophosphorylation cannot be substantiated.

To check whether phosphorylation does occur under the conditions during which the photoreduction of cytochrome c is measured, a procedure was employed that is a combination of the procedures of Whatley and Arnon (1963) and Kamen (1963). The reaction mixture normally employed was buffered by phosphate, pH 7.5, and the following reagents added in μmole/ml: ADP, 1.0; and $MgCl_2$, 7.5. The reaction was carried out in stoppered vessels in an Aminco Warburg Apparatus (American Instrument Co., Maryland). The intensity of illumination provided by eight photoflood lamps was about 2000 foot-candles at the sample. The reaction was carried out for 10 minutes at 15°C and then stopped by adding trichloroacetic acid. Norit A was then added to adsorb the pyridine nucleotides from the solution. The Norit was centrifuged out, washed twice with water, and then added to scintillation fluid and counted in a liquid scintillation counter.

A comparison was made between the normal cytochrome c reaction mixture containing an absorbance of 1.0 at 678 mμ (1-cm path) of chloroplasts that had been heated to 65°C for five minutes. Both samples were illuminated under the above conditions and assayed for labeled ATP. The two samples were both found to be within 2% of background, indicating that photophosphorylation does not occur under the experimental conditions employed.

Quantum requirement calculations. Quantum requirements were calculated from the reaction rates corrected for the back reaction and from the experimentally determined value of the cytochrome c extinction coefficient (Section II-B). In some cases the inner filter effect (as outlined by Biggins and Sauer, 1964, using the method of Kling et al., 1963) was taken into account in the calculation of absorbed intensities. This correction is necessary for the DCPIP Hill reaction and for the cytochrome c/$TMQH_2$ reaction in the blue absorption region (400-500 mμ). The actual calculations were performed by digital computer.

D. Results

Intensity dependence. The photoreduction was studied as a function of light intensity over a 5- to 30-fold range at each of 24 wavelengths in the region from 620 to 740 mμ. At each wavelength, the calculated quantum requirements were found to increase with increasing incident light intensity, as shown in Figure 7. This behavior has been reported previously by Sauer and Biggins (1965) and Hoch and Martin (1963) for NADP photoreduction with DCPIP/ascorbate/DCMU and by numerous workers for various Hill reactions. A simple mechanism first proposed by Lumry and Rieske (1959) predicts the observed linear relationship. Using the notation of Sauer and Biggins (1965), this relationship can be summarized in the equation:

$$Q = Q_o + \frac{I}{k_D} \qquad \text{(II-1)}$$

which designates the intercept of a plot of quantum requirement, Q, versus intensity, I, as the intrinsic quantum requirement, Q_o, for

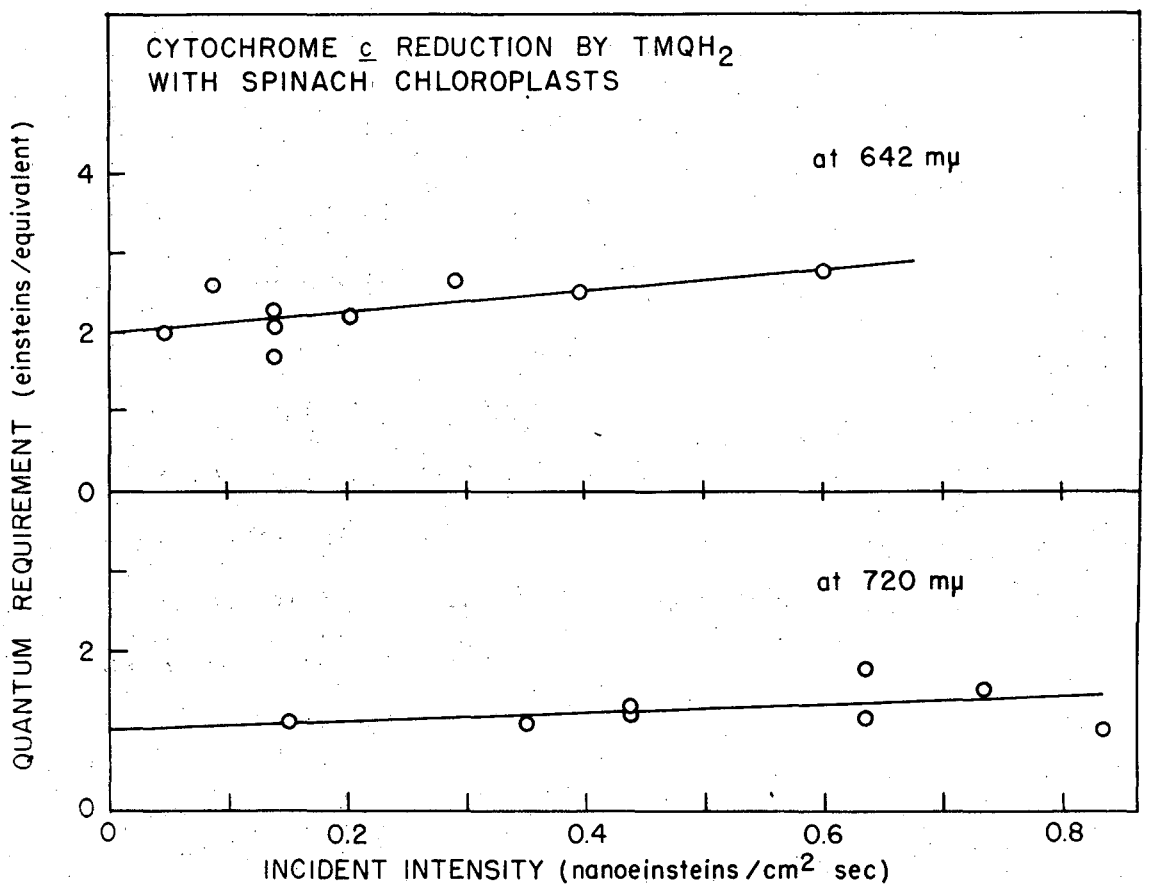


Figure 7. Intensity dependence of the quantum requirement of the cytochrome c/$TMQH_2$ reaction. The example at the top (642 mμ) is at a wavelength where both Systems I and II operate efficiently, while the values at 720 mμ represent a sample in the wavelength region where only System I is efficient.

the reaction at zero intensity, and the slope, $1/k_D$, as a rate parameter of the dark limiting reaction.

The average values for this pseudo zero-order rate constant k_D obtained by Sauer and Biggins were $(1.6 \pm 0.7)\cdot 10^{-7}$ equiv.$\cdot \ell^{-1} \cdot sec^{-1}$ for $NADP^+$ reduction by DCPIP/ascorbate in DCMU-poisoned chloroplasts and $(4.8 \pm 1.7)\cdot 10^{-7}$ for the normal reaction using H_2O as electron donor. As we have noted, the former reaction is photocatalyzed by System I, while the latter uses both Systems I and II. These workers noted that the values indicate the normal reaction has a rate three times faster than the DCMU-poisoned reaction.

Our data for the slopes of the quantum requirements versus absorbed intensity graphs for cytochrome c reduction closely parallel those for the DCMU system of Sauer and Biggins. We find that the observed slopes are independent of wavelength in the entire region studied. When averaged for two separate chloroplast samples, values for k_D of $(1.6 \pm 0.7)\cdot 10^{-7}$ and $(1.5 \pm 1.3)\cdot 10^{-7}$ equiv.$\cdot \ell^{-1} \cdot sec^{-1}$ were obtained. These values are in excellent agreement with those of the System I reaction of Sauer and Biggins and suggests that these two reactions, which both require DCMU, are photocatalyzed by pigment system I and involve the same rate limiting dark reaction.

Cytochrome c/$TMQH_2$ as a System I partial reaction. On the basis of their observation that the photoreduction of cytochrome c by $TMQH_2$ in the presence of chloroplasts is largely DCMU-insensitive, Vernon and Shaw (1965) proposed that in the presence of DCMU the reaction is catalyzed by pigment system I. The action spectrum presented in Figure 8 strongly supports their conclusion. The action spectrum has a fairly constant zero-intensity quantum requirement of 2 quanta

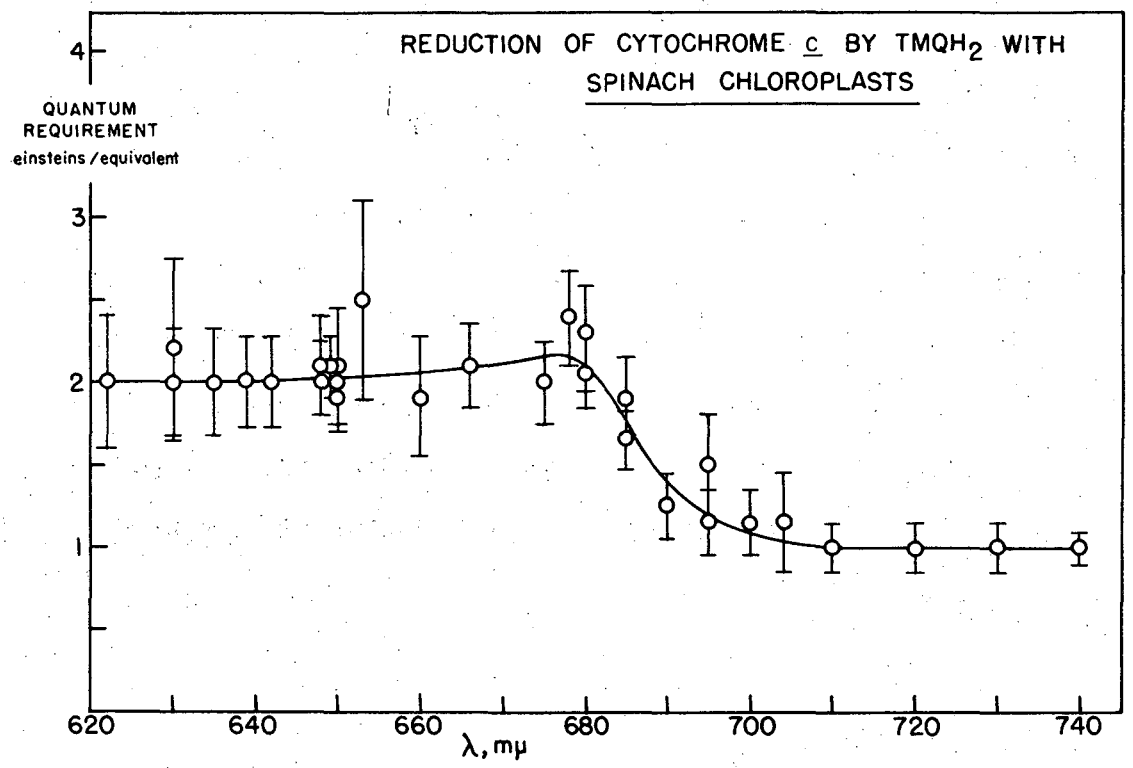


Figure 8. Action spectrum for the reduction of cytochrome c by $TMQH_2$ using spinach chloroplasts (two different preparations). The quantum requirements are values obtained from extrapolations to zero light intensity at each wavelength.

absorbed/equivalent of cytochrome c reduced for wavelengths from 620 mμ to 680 mμ. At longer wavelengths there is a decrease in quantum requirement to 1.0 quantum/equivalent at about 710 mμ, which remains constant to 740 mμ. The very high efficiency (low quantum requirement) at wavelengths longer than 700 mμ is a characteristic feature of System I catalyzed reactions by higher plant chloroplasts. It differs strongly from the action spectrum of the Hill reaction using DCPIP, ferricyanide, or NADP, where the quantum requirement is 2-3 from 640 to 680 mμ and then increases as much as 10-fold at wavelengths longer than 690 mμ (Sauer and Park, 1965; Sauer and Biggins, 1965). The action spectrum for cytochrome c reduction by chloroplasts is similar to those obtained for the chloroplast-catalyzed photoreduction of NADP by ascorbate, coupled with a small amount of $DCPIPH_2$, and in the presence of DCMU, a known System I reaction (Hoch and Martin, 1963; Sauer and Biggins, 1965). Thus the cytochrome c/$TMQH_2$ reaction can be assigned as a System I partial reaction in spinach chloroplasts.

Effect of ferredoxin concentration. One possible route for a System I reduction would be through ferredoxin, which is known to accept electrons from System I (Tagawa and Arnon, 1962). In fact, the Hill reaction using oxidized cytochrome c as the electron acceptor has been shown to require ferredoxin (Davenport and Hill, 1960; Keister and San Pietro, 1963). Chloroplasts prepared as in the procedure of Figure 6 lose most of their ferredoxin during isolation, as evidenced by their low activity in NADP photoreduction without added ferredoxin. The experiments detailed in Table 6 below were performed to determine if spinach ferredoxin is required for the cytochrome c/$TMQH_2$ photoreaction.

Table 6

Cytochrome c Reduction as a Function of Ferredoxin Concentration at Various Light Intensities (broad band red light λ>640 mμ). Rate of Reduction Given in Units of $\Delta A_{549.5}$/min x 10^{-3}

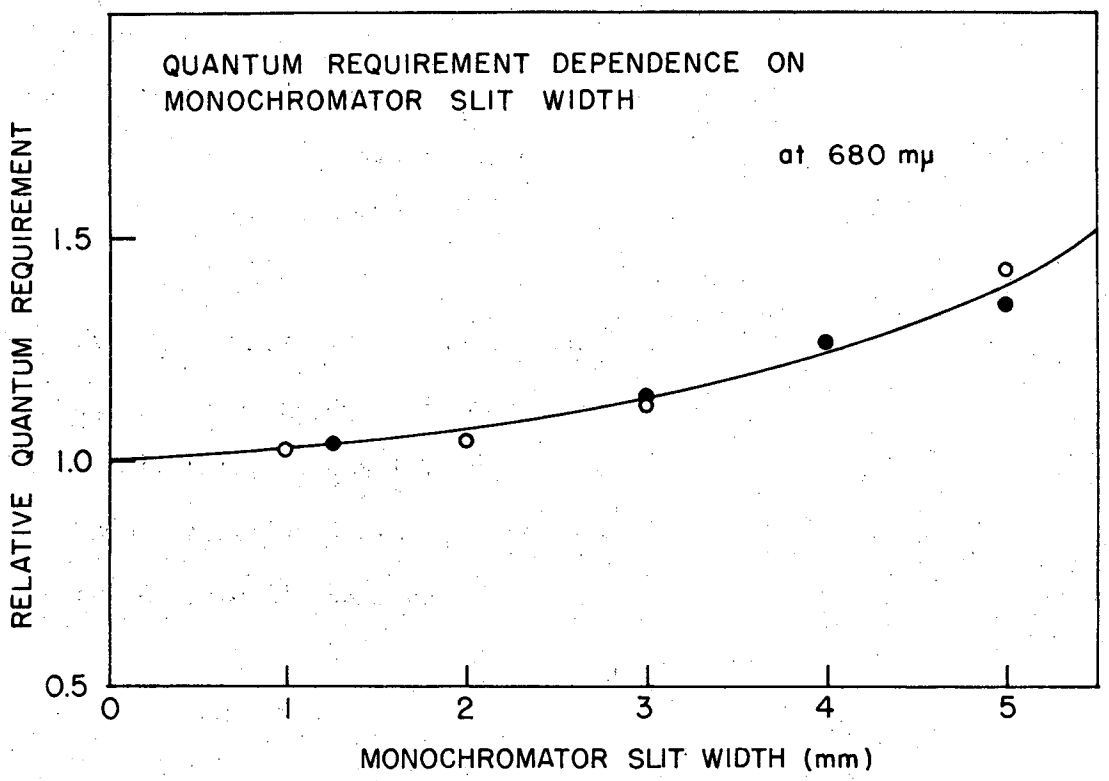
Reaction Mixture Contains:	Relative Intensity		
	40	120	500
a) Chloroplasts as normally prepared	5.1	15.8	26
b) Chloroplasts washed twice in distilled H_2O before suspension in reaction mixture	4.8	16.2	24
c) Chloroplasts as normally prepared + ferredoxin: 50 μg/ml	5.0	15.0	25
d) Chloroplasts as normally prepared + ferredoxin: 100 μg/ml	4.1	11.7	19.7
e) Chloroplasts as normally prepared + ferredoxin: 150 μg/ml	2.5	7.9	11.7
f) Same as e) but solution made anaerobic with N_2	4.3	10.0	18.1

The question of a ferredoxin requirement for the cytochrome c/$TMQH_2$ reaction has been approached from two directions. First, chloroplasts as they were normally prepared were washed twice in distilled water to remove as much of the soluble components as possible. This treatment should remove any residual ferredoxin in the chloroplasts. Table 6 shows that this procedure does not appreciably alter the rate of the cytochrome c photoreduction, indicating that the reaction proceeds in a deficiency of ferredoxin.

Secondly, ferredoxin was added in order to see if its presence stimulated the rate of cytochrome c reduction. The table shows that high concentrations tend to inhibit cytochrome c reduction. The last experiment shows that this inhibition is removed to some extent when oxygen is excluded from the reaction system. This is consistent with the observations of Black, et al. (1962), that ferredoxin mediates a a photoreduction of oxygen by chloroplasts. Thus it would appear that ferredoxin-mediated reduction of oxygen competes with the process of cytochrome c reaction in aerobic solutions where the ferredoxin concentration is high. However, under the normal reaction conditions added ferredoxin is not a requirement for cytochrome c photoreduction.

Dependence on monochromator slit width. In Figure 8 there appears to be a small peak in the curve of the action spectrum at the wavelength maximum of the chloroplasts, 678 mμ. This apparent inefficiency of chloroplasts in their most absorbent wavelength region was most puzzling until we began to consider possible sources of instrumental error. Figure 9 presents data on the dependence of the observed quantum requirement on the monochromator slit width of the actinic light at 680 mμ.

This figure suggests that the high quantum requirements obtained in this region result from the monochromator half-height band width of 10 mμ routinely used in these and previous studies. In this particular region of the spectrum, the sample is appreciably more transparent to light in the wings of the band of wavelengths incident on it than to those near the center. Measurements were made at several wavelengths in this region to determine the extent of this



XBL 684-4126

Figure 9. Effect of monochromator slit width on the relative quantum requirement with 680 mμ exciting light. The filled circles denote relative quantum requirements obtained with the cytochrome c/ $TMQH_2$ reaction and the unfilled circles denote those obtained using the DCPIP Hill reaction.

error. At the absorption maximum, 678 $m\mu$, the quantum requirement at zero band width (extrapolated as in Figure 9) is about 16% less than the value obtained with 3 mm monochromator slit widths. The effect falls off on either side of the absorption maximum, amounting to a decrease of only about 2% at 660 and 690 $m\mu$.

Discussion. The quantum requirements for the cytochrome c/$TMQH_2$ reaction are uniformly lower than those observed previously for NADP reduction using ascorbate/$DCPIPH_2$, and we feel that the former are more representative of the optimum photochemical potential of pigment system I. The higher quantum requirements for NADP reduction by ascorbate/$DCPIPH_2$ probably result from the presence of a cyclic as well as a noncyclic pathway for this reaction. Cyclic photophosphorylation is mediated both by ferredoxin, which is an essential cofactor for the NADP photoreduction by chloroplasts (Tagawa et al., 1963; Arnon et al., 1964), and by $DCPIPH_2$/ascorbate (Gromet-Elhanen and Avron, 1963; Shen et al., 1963). Thus it is possible that cyclic electron flow may compete with NADP for electrons from System I.

Quantum yields (reciprocals of the quantum requirements) extrapolated to zero light intensity for the cytochrome c reduction and for the DCPIP Hill reaction (Sauer and Park, 1965) are given in Table 7 for the various wavelengths of exciting light. Included in the table are both the observed values and the values when corrected for the effect of monochromator slit width.

Figure 10 shows activation spectra obtained by the technique of Sauer and Park (1965) of multiplying the zero-intensity quantum yields at each wavelength by the normalized total absorbance of spinach chloroplasts. The activation spectra obtained in this way

Table 7

Quantum Yields for Cytochrome c Reduction by $TMQH_2$ and the DCPIP Hill Reaction of Spinach Chloroplasts at Various Wavelengths

Wavelength (mμ)	Observed		Corrected for Slit Width		
	ϕcyt	ϕDCPIP	ϕcyt	ϕDCPIP	ϕcyt + ϕDCPIP
622	0.50		0.50		
630	0.50		0.50		
635	0.50	0.44	0.50	0.44	0.94
639	0.50	0.49	0.50	0.49	0.99
642	0.50	0.50	0.50	0.50	1.00
648	0.50	0.51	0.50	0.51	1.01
650	0.49	0.52	0.49	0.52	1.01
653	0.49	0.51	0.49	0.51	1.00
660	0.48	0.48	0.49	0.49	0.98
666	0.48	0.42	0.50	0.46	0.96
670	0.47	0.39	0.53	0.44	0.97
675	0.46	0.38	0.54	0.43	0.97
678	0.46	0.37	0.55	0.43	0.98
680	0.48	0.36	0.57	0.41	0.98
683	0.53		0.60		
685	0.58	0.30	0.63	0.36	0.99
690	0.72	0.26	0.73	0.28	1.01
695	0.83	0.18	0.83	0.19	1.02
700	0.88	0.13	0.88	0.13	1.01
704	0.94				
710	1.00	0.08	1.00	0.08	1.08
720	1.00	0.08	1.00	0.08	1.08
730	1.00	0.04	1.00	0.04	1.04
740	1.00				

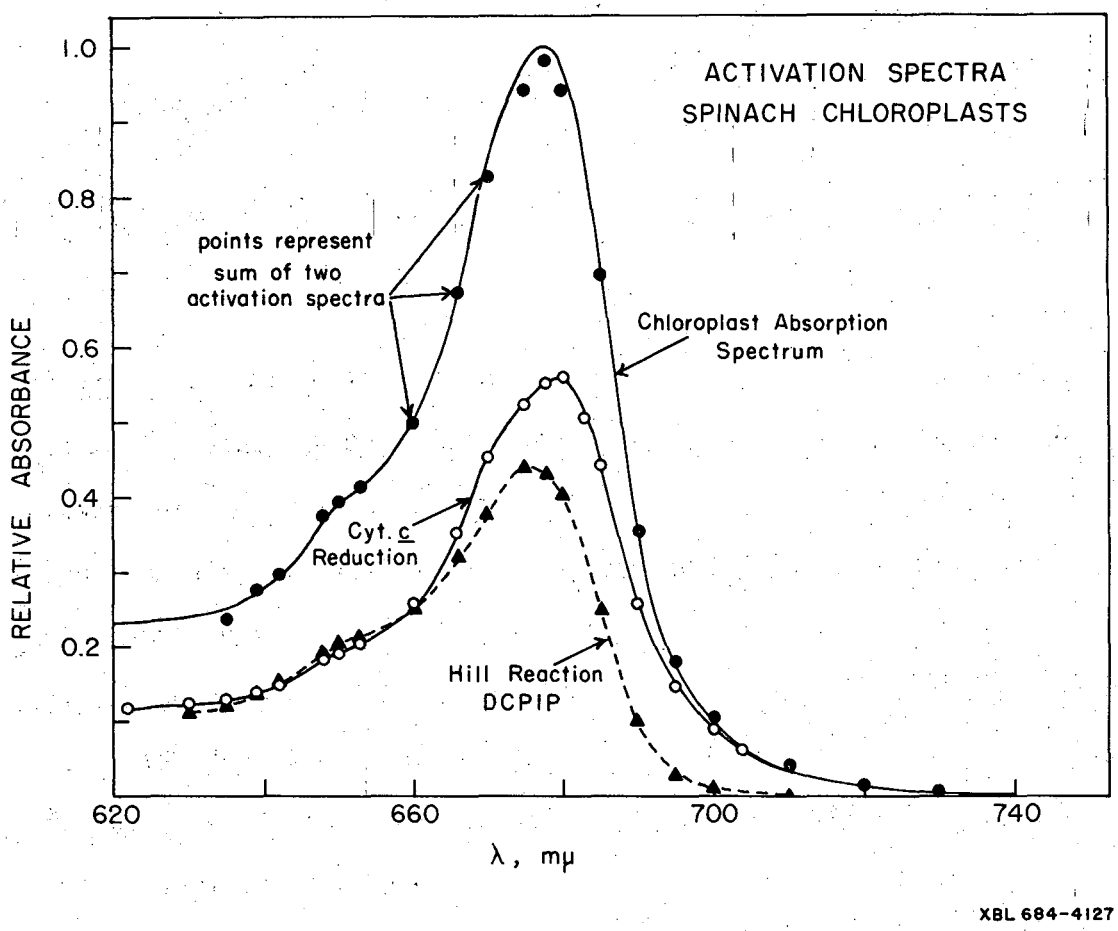


Figure 10. Absorption spectra of pigments responsible for cytochrome c reduction by $TMQH_2$ in the presence of DCMU (open circles, lower solid curve) and for the DCPIP Hill reaction [filled triangles, and the dashed curve; data from Sauer and Park (1965) corrected for slit width dependence] by spinach chloroplasts. Upper solid curve gives the normalized absorption spectrum of spinach chloroplasts, corrected for light scattering (Sauer and Biggins, 1965). The upper filled circles are the sum at each experimental wavelength of the two activation spectra below.

represent the absorption spectra of the "active pigments," i.e., that portion of the total pigments responsible for the sensitization of the particular photoreaction being studied. The maximum of the cytochrome c/$TMQH_2$ activation spectrum (System I) occurs at about 683 mμ and is virtually identical with the normalized absorption spectrum (topmost curve in Figure 10) at longer wavelengths. The maximum is at 675 mμ for the DCPIP Hill reaction and is nearly zero at wavelengths longer than 700 mμ. This reaction has been assigned to System II by Rumberg et al. (1962) on the basis of absorption change measurements. Even if System I participates as well in this reaction, its activation spectrum is probably very close to the absorption spectrum of System II; e.g., see Sauer and Park (1965). The difference between the two activation spectra in Figure 10 in the region of maximal absorption by chlorophyll b (ca. 650 mμ) is slight. It would be hazardous to assign chlorophyll b primarily to pigment system II on the basis of this evidence.

In the last column of Table 7 are given values for the sum of the quantum yields for the two reactions at each experimental wavelength. Points representing the sum of the activation spectra are also presented in Figure 10. This synthesized spectrum is very similar, both in shape and magnitude, to the observed absorption spectrum, giving strong confirmation to the proposal of Sauer and Park (1965) that the activation spectra are really the absorption spectra of the respective pigment systems and that the sum of the activation spectra is simply the over-all measured absorption spectrum. This would not necessarily be the case if there were appreciable transfer of electronic excitation energy from one pigment system to

the other. If electronic energy transfer were possible, the sum of quantum yields could be as high as 2 at some wavelengths, particularly in the wavelength region from 620 to 685 mμ, where both systems appear to absorb comparably. If no electronic energy transfer is possible, then the sum cannot be greater than 1.0 at any wavelength. The data in the last column of Table 7 are quite clear on this point. The values observed are all 1.0 ± 0.1 in the wavelength region from 620 to 740 mμ.

We have every reason to believe that the cytochrome c/$TMOH_2$ reaction is operating at optimum efficiency, since only one absorbed quantum is required for each electron transferred at wavelengths longer than 700 mμ. It is not easy to postulate a simple mechanism whereby this intrinsic quantum yield is then reduced to 0.5 at shorter wavelengths, at the same time permitting efficient electronic energy transfer to occur. We believe therefore, that the simplest explanation lies in the postulate that electronic energy transfer can occur only within each pigment system and not between them, and that the coupling of the two pigment systems occurs only at the chemical level.

E. Quantum Requirements in the Blue Absorption Region of Spinach Chloroplasts

As mentioned in the previous section, the action spectra obtained in the red region of the chloroplast absorption does not give sufficient detail to determine the assignment of chlorophyll b to either of the systems. A possible alternative is to look at quantum requirements in the Soret region where chlorophyll b absorbs a major portion

of the light at 470 mμ. In conjunction with this, both the cytochrome c/$TMQH_2$ and DCPIP Hill (using the reaction conditions of Sauer and Park, 1965) reactions were performed in the blue wavelength region (400 to 500 mμ). At the same time a control at 678 mμ was used to relate the chloroplast activity to that observed in the red region. The experiments exhibited more scatter in the light intensity dependence of the quantum requirements than was observed for the red region, so the results presented in Figure 11 below should be interpreted more as general trends than as accurate action spectra.

The quantum requirement for the cytochrome c/$TMQH_2$ reaction in this region is essentially the same as in the near red. For twelve wavelengths in the region 400 mμ to 500 mμ the experimental points have an average value of 2.0 ± 0.5 quanta/equivalent. The only irregularity appears to be a slight decrease near the absorption maximum at 436 mμ where quantum requirements as low as 1.5 were observed. The control at 678 mμ had a quantum requirement of 2.1, so the chloroplast efficiency found in the near red from 620 to 670 mμ is found in the values in the Soret region.

The DCPIP Hill reaction in the presence of methylamine had higher quantum requirements in this region than in the red region (with values ranging from 2.6 at 480 mμ to 5.0 at 436 mμ). The outstanding feature of the DCPIP Hill action spectrum appears to be a rather broad maximum in the 420 to 460 mμ wavelength region. One of the major points of interest in Figure 11 are the complimentary aspects of the two curves. The fact that the System II Hill reaction appears to be inefficient at the Soret maximum lends credibility to the apparent increased efficiency of the System I reaction. Also of

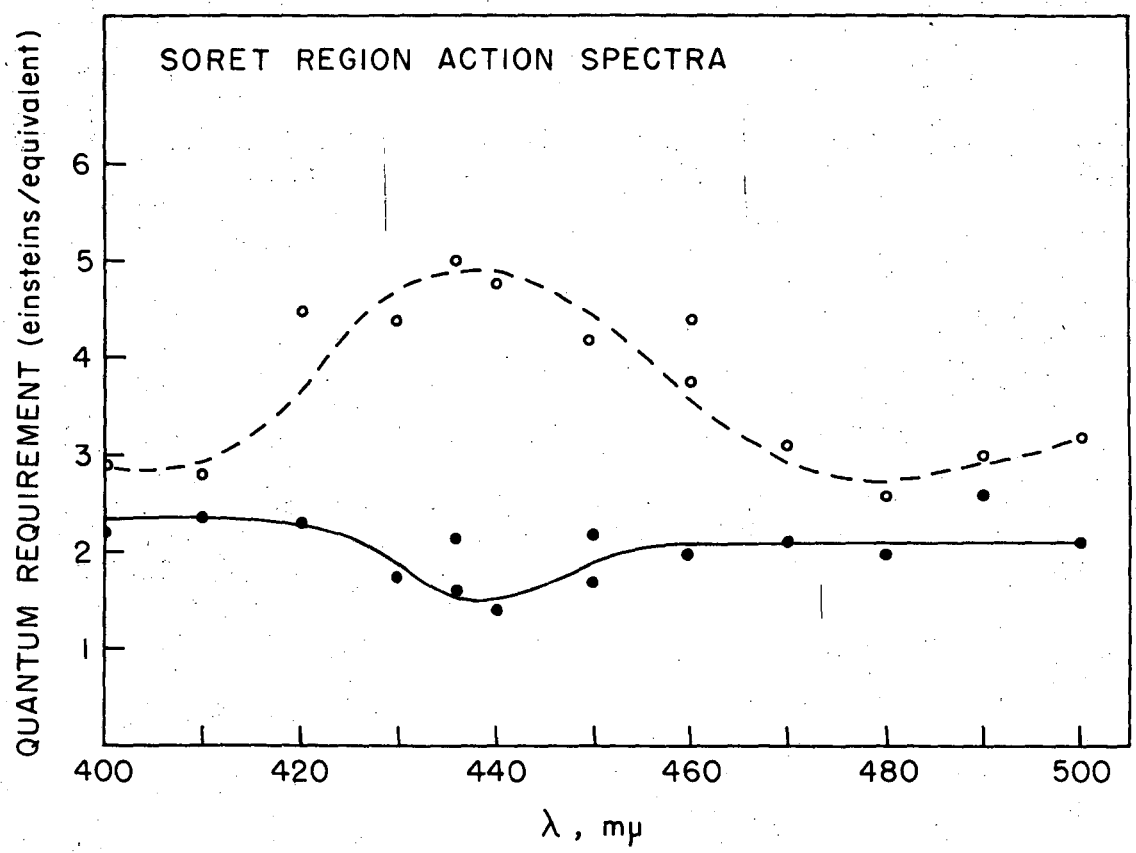


Figure 11. Action spectra for the DCPIP Hill reaction (unfilled cicles and dashed line) and the cytochrome c/$TMQH_2$ reaction (filled circles and solid line) for spinach chloroplasts in the Soret spectral region.

interest is the fact that the quantum yields that would be obtained from the points on the graph do not exceed a sum of 1.0 for the two reactions at any wavelength. The highest value is 0.8 and thus the Soret region also shows no evidence of intra-system energy transfer at the electronic level.

There appears to be a general inefficiency of the blue region in performing the System II Hill reaction. This was also observed by Rieske et al. (1959), who reported that blue light is 35% less efficient than red light in the ferricyanide Hill reaction. It is difficult to assign chlorophyll b to either of the action spectra by means of the data of Figure 11. There does seem to be a general increase in System II efficiency in the wavelength region around 470 and 480 mμ, which suggests the possible role of chlorophyll b and/or β-carotene (480 mμ) in System II.

F. Photoreactions of Barley Chloroplasts

Introduction. Another means of approaching the problem of the distribution of chlorophyll b between the two pigment systems and the question of energy transfer between these systems is through the use of a barley mutant* lacking chlorophyll b. Such a mutant was first reported by Highkin (1950) and some of its properties were reported by Highkin and Frenkel (1962). It is found to contain no chlorophyll b to a limit of detection of less than 1 molecule of chlorophyll b per 1000 molecules of chlorophyll a (Boardman and Highkin, 1966). We hoped to obtain activation spectra of the two

*The mutant was provided to R. B. Park by the courtesy of H. R. Highkin.

photoreaction systems by following the cytochrome c reduction and DCPIP Hill reaction. In addition, the nature of the mutant and its photochemical functions are of interest. Are the photoreaction systems in the mutant as efficient as in the normal barley? Does the mutant show evidence of a complimentary pigment which replaces chlorophyll b? Are there any other detectable differences besides a lack of chlorophyll b?

Experimental. Barley chloroplasts prepared by the method developed for spinach chloroplasts by Park and Pon (1961) were found to have low photoactivity as measured by the DCPIP Hill and cytochrome c/$TMQH_2$ reactions. The conditions of chloroplast isolation were explored and assayed by means of photoactivity and by the appearance of the chloroplasts in light microscopy. The general procedure involved is still that of Park and Pon, using both normal and mutant barley grown from seed in vermiculite and harvested three weeks after planting. It was found that an initial centrifugation of 100 g instead of 200 g was necessary in order not to sediment the majority of the chloroplasts along with the larger fragments. The other important isolation parameters were found to be the pH of isolation and the reaction pH.

Effect of pH. Chloroplasts from normal barley leaves were isolated in 0.5 M sucrose and 0.1 M phosphate at pH 6.4 or 7.3. The isolated chloroplasts were then suspended in a reaction buffer of 0.5 M sucrose and 0.05 M phosphate at pH 6.4, 6.9, 7.3, and 7.8. The effects of these variations in pH on photochemical efficiency are given in Table 8 in terms of the zero intensity quantum requirement obtained with 680 mμ actinic light. The quantum requirements

Table 8

Effect of pH on Quantum Requirements of Normal Barley Chloroplasts at 680 mμ

Reaction	Grinding Buffer pH	Zero Intensity Quantum Requirements (einsteins/equivalent) 6.4	6.75	6.9	7.3	7.8
Cyt. c reduction	6.4	60		3.5	2.0	
	7.3			3.2	1.5	1.6
DCPIP Hill reaction	6.4	10		7.0		
	7.3			25	25	
Ferricyanide Hill reaction	6.4	3,6,9	8		10	
	7.3	8,8	9		15	

Table 9

Effect of Actinic Wavelength on Chloroplast Activity

Reaction	λ(mμ)	Zero Intensity Quantum Requirements (einsteins/equivalent) Normal Barley	Mutant Barley	Spinach
Hill reaction DCPIP	650	11	15	2.0
	680	15	20	2.5
		6	8	
	700	20	20	10
Cyt. c reduction	650	1.8	2.3	2.0
	680	3.2	1.5	2.1
		1.8	1.5	
	700	1.15	1.0	1.15
		1.2	1.0	

for spinach chloroplasts isolated and illuminated at pH 7.3 with the same wavelength were 1.5 quanta per electron for cytochrome c reduction and 3.0 for the DCPIP Hill reaction.

The cytochrome c reduction is seen to be fairly independent of isolation pH but dependent on reaction pH. Isolation and reaction at pH 7.3 give results comparable with those obtained for spinach chloroplasts. The results of the DCPIP Hill reaction are inconclusive, but suggest that preparation and reaction at a lower pH than 7.3 may be advantageous. The same general trend as was found in the DCPIP reaction is evident for the ferricyanide Hill reaction, but here another factor becomes evident. Chloroplasts isolated and reacted at lower pH values, although generally more reactive, were highly unstable and usually lost more than half of their activity upon a few minutes of illumination.

Results. In general the photoactivity of barley chloroplasts prepared in this manner was found to be rather low as compared to that obtained with spinach chloroplasts. The pH of isolation appears to be an important factor, particularly for System II reactions. Also, it is possible that destructive enzymes are released during isolation which decrease activity. Because moderate activity was all that could be obtained from barley chloroplasts, a comparison of the activity in normal and mutant barley will be considered only qualitatively.

Table 9 presents such a comparison along with representative values for spinach chloroplasts. Of primary import is that the mutant barley is seen to contain both Systems I and II, and to be about as efficient as normal barley in utilizing monochromatic light

in the region 650 to 700 mμ. In terms of cytochrome c reduction, the mutant was found to be consistently more efficient than the normal barley. In the Hill reaction with 650 mμ light, the normal barley is apparently more efficient than the mutant. This could reflect the loss of chlorophyll b from System II of the mutant, resulting in fewer quanta being utilized by the System II photoreaction.

Figure 12 shows a difference spectrum obtained between sonicated, washed lamellae of normal and mutant barley. It shows clearly the lack of chlorophyll b in the mutant (650 and 473 mμ) and also the possible existence of a pigment which absorbs maximally at about 687 mμ in the mutant. (The slight dip at 678 mμ is due to the absorbances of the two samples not being exactly the same at the chloroplast maximum.) The trough at 687 mμ could indicate either a different pigment or a larger pool of a similar pigment in the mutant. The amount of this pigment absorption is approximately one-fifth the amount of chlorophyll b in the normal barley, and one-fifteenth of the total chlorophyll a. Attempts to determine if any significant difference in photochemical activity occurred at 687 mμ proved inconclusive, as the activities were not sufficiently high in these chloroplast preparations.

Conclusions. Although in the barley chloroplasts activity sufficient to determine activation spectra was not obtained, the experiments above provide at least one interesting result. Chlorophyll b has been thought to transfer most of its absorbed photons to the photoreaction II trap, and indeed, detergent solubilized chloroplast particles that exhibit mostly System II activity have lower chlorophyll a/chlorophyll b ratios (Anderson and Boardman, 1966;

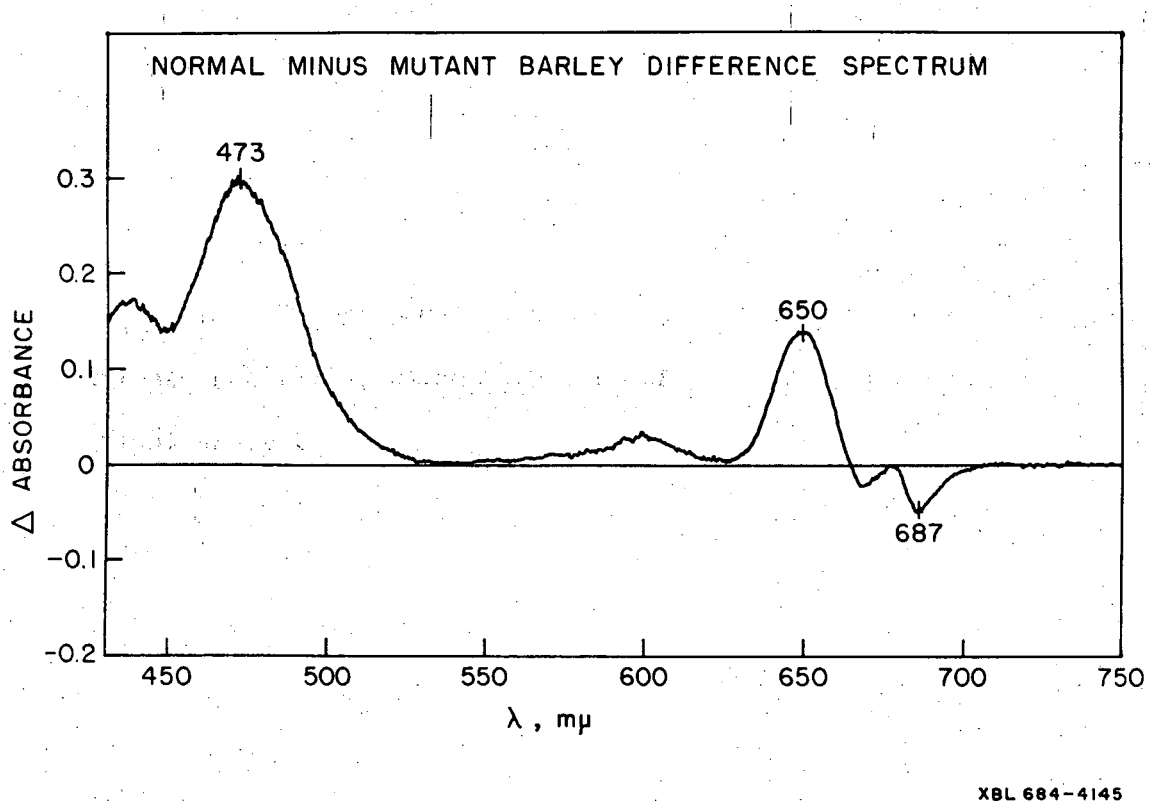


Figure 12. Normal minus mutant barley difference spectrum obtained between sonicated, washed lamellae preparations of normal and mutant barley.

Wessels, 1968). The observation here that the barley mutant contains efficient Systems I and II (essentially these same results have been subsequently reported by Boardman and Highkin, 1966) in spite of the fact that it has no detectable amount of chlorophyll b, indicates that chlorophyll b acts only as an accessory pigment in System II and that its presence is not required for System II activity. This completely rules out the theory advanced by Rumberg (1964) that a chlorophyll b molecule is the System II trap in chloroplasts, or that it is directly involved in the trapping mechanism. Even if this one trap-molecule were the only chlorophyll b present in the mutant, it would have to be in a concentration of about 1/500 chlorophyll a (see Chapter III). Since the chlorophyll b concentration is less than 1/1000 chlorophyll a molecules (if there is any chlorophyll b present at all), chlorophyll b cannot be involved in the trapping site.

G. Conclusion

In this chapter we have seen that a System I reaction can indeed function with a quantum efficiency of one quantum per electron transferred. This rules out the possibility of System I involving a two photon per electron process and supports the conclusion that the minimum quantum requirement for the evolution of one molecule of oxygen would be eight quanta. The data support, but do not prove conclusively, the separation of photosynthetic pigments into either photosystem I or II. Two general conclusions can be made. First, the data obtained with the barley chloroplasts suggest that chlorophyll b may preferentially belong to the System II antenna pigments.

Second, the chlorophyll maximum in chloroplasts at 678 mμ can be resolved by means of the activation spectra in Figure 10 into two functional components, one associated with System II, with a maximum at about 675 mμ, and the other with a maximum at 683 mμ, associated with System I.

Confirmation of this distinction between two sets of chlorophyll a pigments can be found in recent work involving detergent treatment of chloroplasts. Detergents such as digitonin, sodium dodecyl sulfate or Triton X-100 effect the partial separation of the two pigment systems onto particles of different densities (Anderson and Boardman, 1965; Sironval et al., 1965; Briantais, 1967). The photosystem II activity of these particles was assayed with the DCPIP Hill reaction while System I activity was measured by following NADP reduction with DCPIP/ascorbate and DCMU. The particles containing mostly System I activity have a chlorophyll a maximum at 680 mμ, while the particles rich in System II activity have a maximum at 675 mμ. Thus, the partial separation by detergents shifts the absorption maxima in directions consistent with the above activation spectra.

The data of this chapter suggest that the coupling between the two pigment systems occurs only at the chemical level and that electronic excitation transfer cannot occur between them (Kelly and Sauer, 1965). A model of the photosynthetic apparatus in which only chemical communication is allowed between the two systems has been termed "separate-package" by Myers (1963), while he referred to the special case where excess excitation in photosystem II is transferred

to System I as "spill-over". This question of whether energy transfer between photosystems occurs in the photosynthetic apparatus has been considered previously on the basis of quantum yield measurements of O_2 evolution in algae (Myers and Graham, 1963; Franck and Rosenberg, 1964).

The separate-package model predicts that quantum yields of photosynthesis will vary with wavelength if the fractional absorption of the two photosystems varies. This is because the transfer of electrons through both systems will be limited by the rate at which quanta are received by the photocenter of the system with the least absorption. If energy transfer occurs, the excess quanta absorbed in the predominating photosystem will be divided between the two systems so that 50% of the absorbed quanta will be used in each system. Thus, in the case of energy transfer, the quantum yields will be constant throughout the chloroplast absorption region. As an example, consider a wavelength such as the chlorophyll b maximum at 650 mμ where one might expect System II pigments to absorb a majority of the light, say 60%, and System I 40%. In the separate package model a steady state quantum yield maximum of 0.4 per electron transferred through both photosystems will be observed. This value reflects the functional loss of the extra quanta absorbed by System II. If, however, energy transfer were to occur at this wavelength, the excess System II quanta could be divided between the two systems so that 50% of the absorbed quanta would be used in each system. This would lead to a maximum quantum yield of 0.5 per electron transferred.

The observation that quantum yields of photosynthesis decrease rapidly for wavelengths greater than 680 $m\mu$ (the red drop phenomenon--Emerson and Lewis, 1943) coupled with the evidence that the long wavelength chloroplast pigments are efficient in System I, suggests that at least in these long wavelength pigments, energy is not transferred from System I to System II. Thus a model in which excitation can be transferred freely between the two systems must be ruled out. The result that only System I is efficient at long wavelengths suggests that the System II photoreaction requires more energy than System I. It appears that far red light can drive the System I reaction, but does not contain enough energy to promote the photoreaction of System II.

In the near red spectral region (600-680 $m\mu$) where one might expect System II absorption to predominate, the quantum yields are constant (Emerson and Lewis, 1942; 1943). These constant quantum yields require either that the pigment absorption be distributed in a fixed ratio between the two systems for the entire near red wavelength region (separate-package) or that transfer occurs between the two systems (spill-over).

Myers (1963) and Bannister and Vrooman (1963) attempted to get confirmation of the spill-over model from enhancement studies. Consider two light beams of different wavelengths, such as 700 $m\mu$ where System I absorption predominates and 650 $m\mu$ where we could expect System II absorption to predominate. In the 700 $m\mu$ beam the quantum yield of O_2 evolution would be low because the photosynthetic apparatus is limited by the low absorption of System II. In the 650 $m\mu$

beam there are two cases possible. Either photosynthesis would be limited by the absorbance of System I (separate-package) or there would be a perfectly balanced distribution caused by spill-over with half of the absorbed quanta going to each reaction. In the two beams together there would be more than an additive increase in oxygen evolution, because quanta that were previously wasted in the 700 mμ light beam would be offset by quanta from the 650 mμ beam. The extent of this enhancement is related to the fractional absorption of the two systems at the wavelengths in question, and a comparison with the predictions of the two models can be made to determine which is the in vivo situation.* Bannister and Vrooman concluded that their results could be explained on the basis of either model, while Myers' comparison seemed to favor the spill-over model, although the precision of the experimental measurements employed did not allow a definite conclusion.

Recently, Malkin (1967) made a theoretical analysis of the enhancement data for green, blue-green and red algae. The same general approach of obtaining fractional absorption from enhancement data employed by Myers, and Bannister and Vrooman, was used by Malkin. He extended this method, however, by deriving general equations relating enhancement functions with quantum yields. Selected literature data on five different species of algae, when analyzed by means of these equations, strongly support the conclusion that spill-over

---

*For a derivation of this relationship between enhancement and fractional absorption as determined by the two models, and a discussion of the effects of light intensity and saturation of the two beams, see Bannister and Vrooman (1963).

occurs in these algal systems. Malkin notes, however, that there is a general inconsistency with his spill-over analysis if the experimental data is obtained in the presence of the inhibitor DCMU. DCMU is the major means employed to isolate System I activity from that of System II, and it is used in almost all experiments designed to measure directly the activity of System I. This inconsistency with Malkin's spill-over conclusion in the presence of DCMU requires that DCMU not only blocks electron flow through System II, but also that it affects the physical properties of the photosynthetic system in a way that stops energy transfer.

Bishop (1967) studied quantum requirements for CO_2 fixation in normal and hydrogen adapted Scenedesmus obliquus, strain D_3 and two mutant strains, No. 11 and No. a', which lack photosystem II activity. If still-over were to occur during the photoreduction (a System I process using H_2 as the electron donor), its action spectrum should be similar to that for overall photosynthesis since the photoreduction could utilize quanta absorbed by System II as well as by System I. He found, however, that the action spectrum for photoreduction differed significantly from that of overall photosynthesis, suggesting that little or no energy transfer occurs. The same shape of photoreduction action spectrum as for the wild type alga with DCMU was obtained without DCMU for the mutants lacking an active System II. So, a System I reaction in the absence of DCMU, but with an inoperative System II, does not indicate the increased efficiency in the 610-680 $m\mu$ region which would be indicative of spill-over. Thus, direct measurements on a green alga which are not dependent on DCMU

poisoning are also inconsistent with the spill-over model.

Two recent studies also support the separate-package model. Williams (1968) performed a theoretical calculation on the fractional absorption of the pigment systems in the alga Chlorella pyrenoidosa using the experimental data of Gingras et al. (1963) on the action spectrum of O_2 evolution in algae partially poisoned with DCMU. The premise of the calculation is that transfer from System II to System I will be facilitated by DCMU because excitation absorbed by System II will be blocked from getting to the System II trap. The values predicted on the basis of the separate-package model were in much better agreement with the experimental results than those predicted using the spill-over model. Joliot et al. (1968) used an amperometric method to measure System I activity (photoreduction of methyl viologen) or System II activity (O_2 evolution in spinach chloroplasts). They studied the phenomenon of enhancement in steady and flashing light and were able to obtain accurate action spectra for the two photosystems. The action spectrum of System I proved to be independent of the state of the traps in System II. This demonstrates again the absence of electronic excitation transfer from System II to System I. Their System II action spectrum also has a definite shoulder at 650 mμ, whereas the action spectrum for System I does not. This suggests that a majority of the chlorophyll b molecules absorb in photosystem II.

A scheme to account for the separate-package model within the chloroplasts will have to incorporate what is known about the chloroplast pigments in vivo. It is known that electronic excitation can

be transferred over fairly large distances (30-40 Å) between molecules of chlorophyll a in solution (Watson and Livingston, 1950; Weber, 1960). Such processes should occur in vivo as well. If the absorption and emission oscillators of the pigment molecules of the two pigment systems in vivo are oriented unfavorably with respect to one another, then radiative transfer would have a low probability. This seems unlikely in view of the absence of any strong orientation of the bulk of the pigment molecules in chloroplasts or active lamellar fragments isolated from them, using the tests of fluorescence polarization (Arnold and Meek, 1956; Goedheer, 1957) or dichroism (Goedheer, 1955, 1957; Olson et al., 1962; Sauer and Calvin, 1962; Sauer, 1965). A much simpler explanation of the separate-package mechanism is that the two pigment systems are physically separated in vivo by a distance greater than 30-40 Å, and that the medium separating them is one which does not especially facilitate the transfer of electronic excitation energy in competition with chemical processes at room temperature.

A model for pigment ordering within the chloroplast (as proposed in Kelly and Sauer, 1965), consistent both with the requirement of separated pigment systems and with the current picture of chloroplast lamellar structure (Park and Biggins, 1964; Park, 1966) is shown in Figure 13. The model consists of a lamellar array on the order of 100 Å thick made up of a planar assembly of quantasome particles, with the molecules of the two pigment systems imbedded on opposite faces of the planar array and separated by a matrix containing protein and colorless lipid. Separations of at least 30-40 Å would be

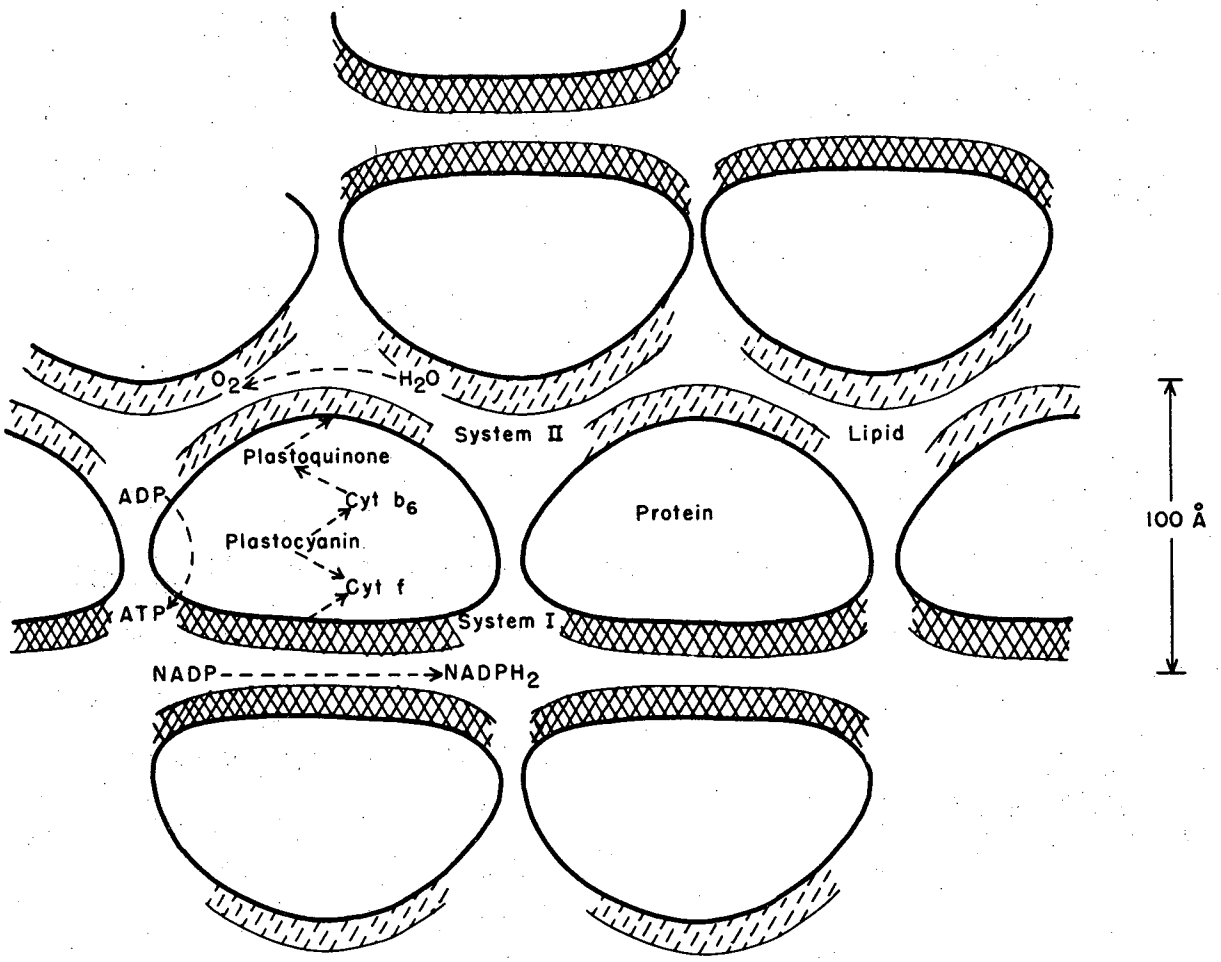


Figure 13. A model of a cross-section of the chloroplast lamellar system, showing a proposed physical separation of the two pigment systems and of the products of their photoreactions. Portions of several identical quantasome units are sketched.

perfectly feasible in such a model. The intervening lipo-protein matrix would contain many of the intermediate cofactors (cytochromes, quinones, plastocyanin, phosphorylation sites, etc.) which couple the two pigment systems at the chemical level. If adjacent lamellae are in an antiparallel arrangement, shown in the model and supported by the electron microscopic studies, then the model has the additional advantage suggested by Robinson (1964) of providing for the physical separation of the powerful reductants (chloroplast ferredoxin, $NADPH_2$) normally produced by pigment system I reactions and the powerful oxidants (molecular oxygen, etc.) which are products of pigment system II reactions.

Other models are possible. For example, if the respective pigment systems were always separately located on quantasomes in different regions of the lamellar array, which is consistent with the views of Olson et al. (1961) and of Gross et al. (1964), the requisite separation in space would be accomplished. We know of no compelling evidence, either morphological or photochemical, in support of this hypothesis. On the other hand, it makes the problem of chemical communication between the two pigment systems that much more difficult.

The antiparallel lamellar hypothesis illustrated in Figure 13, where the pigment systems are on opposite sides of the same quantasome and where the central region provides the principal pathway of chemical communication between them is appealing because it is conceptually simple and compatible with the concept of the chloroplast as an array of photosynthetic units roughly the size of quantasomes. In the next chapter we will report the size of functional

photosynthetic units determined by studying partial reactions in flashing light. The results can then be related to this concept of the quantasome as a fundamental photosynthetic unit.

III. PHOTOSYNTHETIC PARTIAL REACTIONS IN FLASHING LIGHT

A. Introduction

The flash studies of Emerson and Arnold (1931, 1932) (see Chapter I) were the first to indicate the existence of a photosynthetic unit. Their evidence suggested that about 2500 chlorophyll molecules cooperated in the evolution of one oxygen molecule in the green alga Chlorella, with an associated dark step requiring 0.02 sec at room temperature. Subsequent experiments by Arnold and Kohn (1934) showed that the chloroplasts of 6 different plant species exhibit photosynthetic units of from 2000-3000 chlorophyll/O_2 evolved. The same size of photosynthetic unit (2000 chlorophylls) and the same limiting dark time were found by Clendenning and Ehrmantraut (1950) for the quinone Hill reaction using the same flash length.

Kohn (1936), Briggs (1941), Weller and Franck (1941), and Tamiya and Chiba (1949) found greater yields of oxygen (smaller photosynthetic units) and longer dark times when using flash lengths that were long ($\geq$0.6 msec) compared to the 10^{-5} sec flashes of Emerson and Arnold. Ehrmantraut and Rabinowitch (1952), using 10^{-4} sec flashes, reconfirmed the short flash results for overall photosynthesis and the results of Clendenning and Ehrmantraut for the quinone Hill reaction. They were the first to point out the importance of the flash length.

Gilmour et al. (1954) found that long and short flashes give different results in the ferricyanide Hill reaction of sugar beet chloroplasts. They suggested that a reservoir of photosynthetic

products must be filled in order to obtain the increased yield and long dark period characteristic of long flashes, and that a flash of 10^{-5} sec is too short to fill this reservoir, regardless of intensity. Thus, they concluded that there is a pool of photo-reducing power that is operative only at high light intensity and that it is located in the Hill reaction part of photosynthesis. (A comprehensive review of the early photosynthetic work performed in flashing light can be found in Rabinowitch, 1956).

The effect of flash duration was explained by Allen and Franck (1955) in terms of a limiting enzyme (one for every 2000 chlorophylls). If this enzyme has a working period of approximately 10^{-4} sec, then it can turn over many times during a long flash. This mechanism requires the storage of chemical energy between the first enzyme and the slow step, and so is essentially the same as that proposed by Gilmour et al.

Kok (1956) studied photosynthetic oxygen evolution in Chlorella using flashes which varied in length from 0.2 msec to 320 msec. He found that with suitably long dark time, the yield per flash as a function of flash length (t_f) was linear in the region 2 to 320 msec, had a positive slope, and extrapolated to a finite value at zero flash length. For flash durations of less than 2 msec, the yield per flash became progressively smaller than the values expected, falling to 70% of the extrapolated value at t_f = 0.2 msec. These results conform to the theories proposed above and suggest that a saturating flash of only 2 msec duration is sufficient to saturate the slower dark intermediates in photosynthesis.

We have seen in Chapter II that it is possible to distinguish between the arrays of pigment molecules associated with each of the

light reactions from studies of the action spectra of partial reactions. Also, it was noted that the Hill reaction using DCPIP or ferricyanide is associated primarily with pigment system II (Sauer and Park, 1965), whereas the cytochrome c/$TMQH_2$ photoreaction in the presence of DCMU is driven solely by System I. With steady illumination, each of these partial reactions occurs with a high quantum efficiency under light-limiting conditions in broken chloroplasts. Thus, meaningful functional sizes of the pigment array associated with each light reaction can be obtained by studying the respective partial reactions in flashing light.

B. Theoretical

A simple mechanism and kinetic analysis will suffice to develop the results. Consider a rapid light reaction followed by a slow dark reaction to yield stable products

$$Q \xrightarrow[\text{chloroplasts}]{h\nu} Q^* \qquad \text{(III-1)}$$

$$\text{reactants} + Q^* \xrightarrow{k_1} Q + \text{products} \qquad \text{(III-2)}$$

where Q is a trapping site or a rate-limiting intermediate and $Q + Q^* = Q_o$ represents the total pool of these intermediates. The concentration of the reactants can be made sufficiently large so that reaction III-2 is pseudo first-order.

In a single short flash ($t_f << 1/k_1$) of saturating intensity, virtually all of Q is converted to Q^*. If we assume 100% efficiency for the formation of products after the light energy is trapped and an exponential decay of the excited intermediate, then the amount of

product formed in a single flash (P_f) will be Q_o, if the dark time following the flash is sufficiently long for all of the Q^* to return to Q.

During a long flash of duration t_f there will be a recycling of Q to form Q^* so that the total conversion during one flash will be

$$P_f = Q_o + K_1 Q_o t_f = Q_o (1 + k_1 t_f) . \qquad \text{(III-3)}$$

In a train of light flashes separated by dark intervals of duration (t_d), the amount of Q available for excitation by a succeeding flash can be found from

$$\frac{dQ}{dt} = - \frac{dQ^*}{dt} = k_1 Q^* \qquad \text{(III-4)}$$

where the amount of Q^* and Q at t_d after the previous flash will be

$$Q^* = Q_o e^{-k_1 t_d} \qquad \text{(III-5)}$$

and

$$Q = Q_o - Q^* = Q_o (1-e^{-k_1 t_d}) . \qquad \text{(III-6)}$$

Thus the total conversion of material per flash in flashing light will be

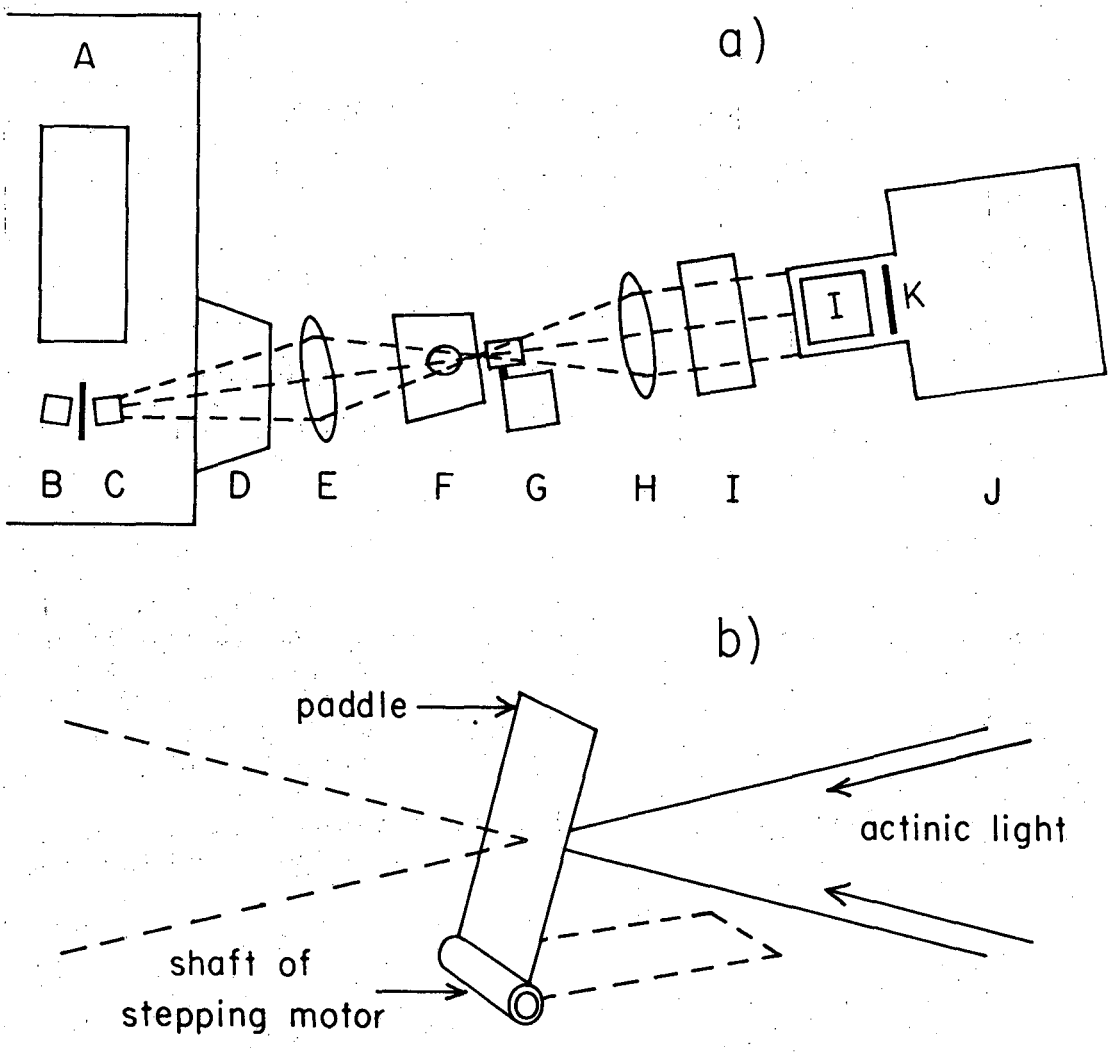
$$P_f = Q_o (1-e^{-k_1 t_d}) + Q_o k_1 t_f . \qquad \text{(III-7)}$$

Equation III-7 predicts that a plot of P_f vs. t_f will be linear (as observed by Kok, 1956), with a slope of $k_1 Q_o$ and an intercept of $Q_o(1-e^{-k_1 t_d})$. For sufficiently long dark times, as in most of the experiments reported here, Eq. III-7 reduces to Eq. III-3. Thus the size of a functional unit (which we define as the number of molecules

of chlorophylls a and b per electron equivalent of intermediate Q produced with saturating flashes) and a value for k_1 can be obtained from a study of flash yield as a function of flash length. It should be pointed out that such functional units measured kinetically need not correspond to actual morphologically distinct units in the chloroplast. The morphological unit may contain any integer multiple of functional units without altering the kinetic behavior.

C. Material and Methods

Flashing light apparatus. Hill oxidant (DCPIP or ferricyanide) or cytochrome c photoreduction was followed spectrophotometrically as described in Section II-C. The experimental apparatus is also similar to that detailed previously, but with side illumination of the reaction cuvette, as illustrated in Figure 14a. Light from a 1000-W projector bulb housed in a Luxtar Model V-1000 strip film projector, with infrared wavelengths filtered out by a Corning 1-60 filter and 7 cm of water, is focused on a paddle connected to the drive of a stepping motor (Model 55-100, Cedar Engineering, Minneapolis, Minn.). In the "up" position, illustrated in Figure 14b, the paddle blocks the light and the sample is in the dark, while in the down position the sample is illuminated. The duration and frequency of the flashes produced by the stepping motor are controlled by commercial pulse generators as described by Kuntz and Calvin (1965). Red wavelengths (λ >635 mμ) are isolated by means of a supplementary Corning 2-58 filter. The sample cuvette has aluminum foil taped to its far side to increase absorption of the exciting light by the chloroplasts, while the reference cuvette has black tape on its



XBL 684-4200

Figure 14. a) Diagram of flashing light experimental apparatus. The labeled components are as follows: A, scattered transmission accessory of Cary 14; B, reference cuvette; C, sample cuvette; D, filter box with Corning 2-58 (transmits 640 mμ); E, collimating lens; F, mask; G, stepping motor and paddle; H, focusing lens; I, water filters; J, 1000 watt slide projector; K, Corning 1-60 infrared filter.

b) Position of the paddle during illumination ("down") and when the sample is in the dark ("up").

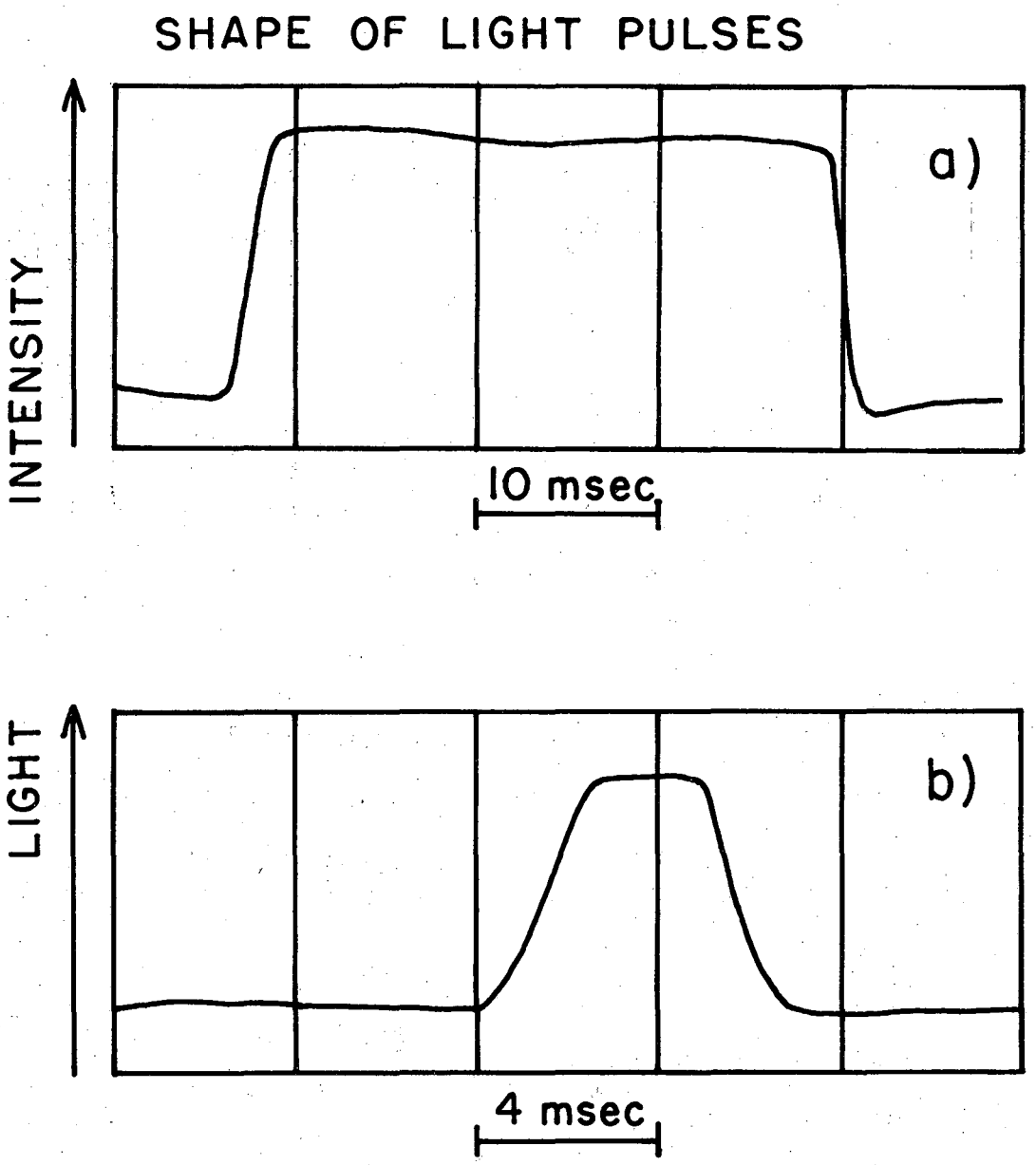
adjacent side so that it is not exposed to the actinic light.

Figure 15 shows traces of the light pulses, measured using a fast-response photodiode (Edgerton, Germeshausen and Grier, Cambridge, Mass., type 5D-100, response time $<1.5 \times 10^{-8}$ sec), whose output is displayed on an oscilloscope (Tektronix 545A) and photographed. The light pulses have rise and fall times of ca. 2 msec. Defining the flash time (t_f) as the pulse width at half-maximum intensity, we find that reasonably square pulses are obtained in this fashion down to t_f = 6 msec, and thus a range of 6-100 msec is available for t_f. The time between flashes is adjustable between 6 msec and many seconds.

Variable light intensities, obtained by altering the voltage of the projector lamp, are measured on a relative scale using a silicon photocell (Hoffman CG-120). If we take the incident red light to have an average wavelength of 700 mμ, then the energy of the incident radiation at the sample cuvette is ca. 70 mW cm^{-2} at maximum lamp voltage (referred to below as maximum intensity).

Reagents. Spinach was grown in vermiculite in the oper air (Jensen and Bassham, 1966) or in a growth chamber as in Section II-C. Chloroplasts were prepared as described previously except that in the Hill reaction studies, tricine buffer (Good, 1962) (General Biochemicals, Chagrin Falls, Ohio), pH 7.4 or 7.7, was used instead of phosphate in the isolation and storage of chloroplasts. Methylamine hydrochloride was dissolved in distilled water and titrated to pH 7.7 with dilute KOH.

Reaction mixtures. The reaction mixture for cytochrome c photoreduction by $TMQH_2$ in the presence of spinach chloroplasts contained: potassium phosphate (pH 6.0), 0.05 M; sucrose, 0.35 M; and the



XBL 679-6175

Figure 15. Traces of the light pulse shape. The flash duration is measured at half-maximum intensity giving (a) 32, and (b) 6 msec. Rise and decay times are about 2 msec.

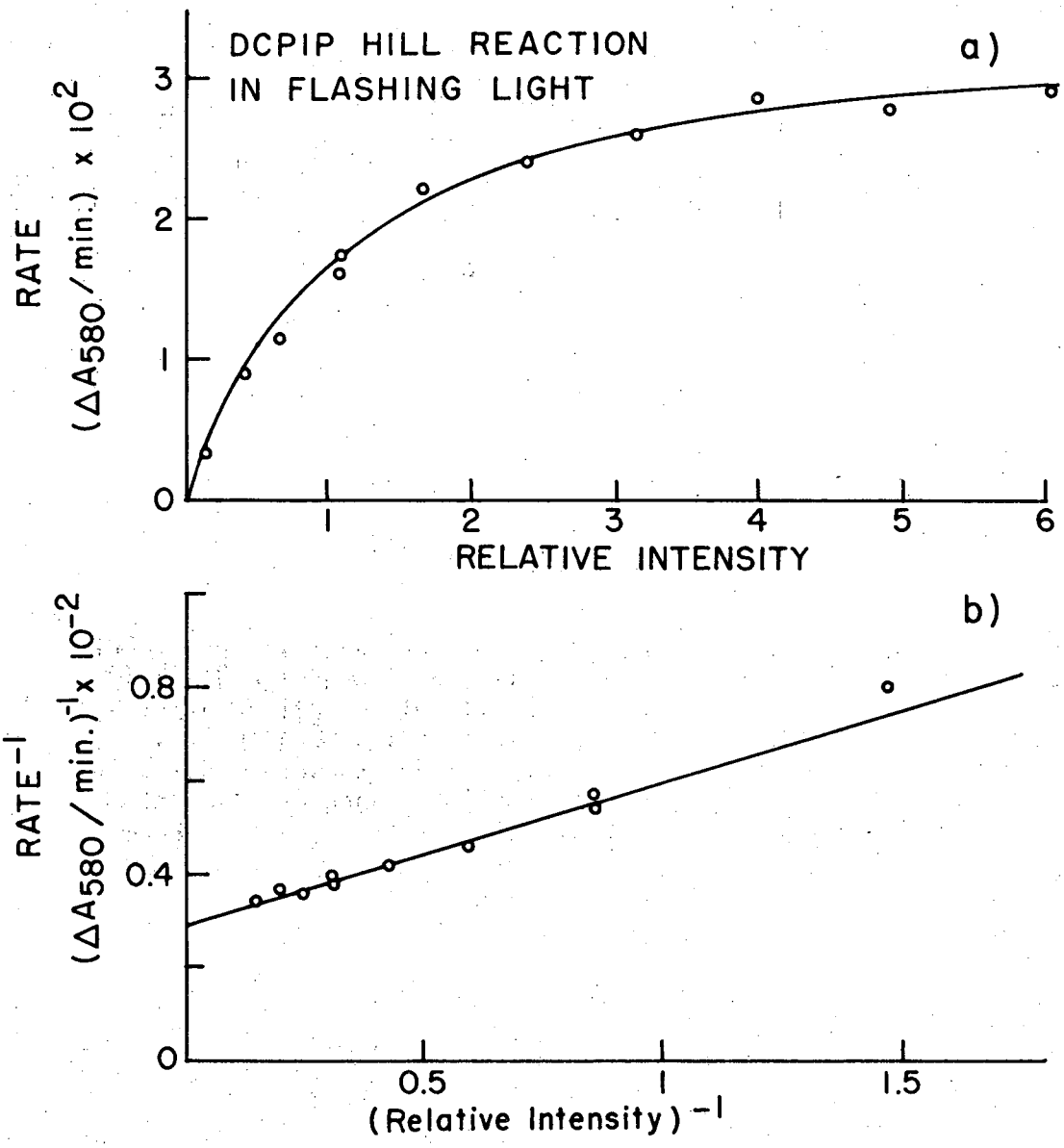
following in micromoles per milliliter: cytochrome c, 0.050; $TMOH_2$, 0.055; and DCMU, 0.020. The stock solutions of $TMOH_2$ in ethanol and DCMU in methanol were diluted 100-fold, respectively, in the final reaction mixture.

For the ferricyanide Hill reaction, the standard reaction mixture consisted of the following in micromoles per milliliter: tricine (pH 7.4), 35; sucrose, 350; potassium ferricyanide, 0.26; and potassium ferrocyanide, 0.26. For the DCPIP Hill reaction, varying amounts of DCPIP were used in place of the ferricyanide and ferrocyanide. In those samples so indicated, methylamine was added to a final concentration of 10.0 μmoles/ml.

Chlorophyll concentrations. Chlorophyll a and b concentrations were measured in 80% acetone using the extinction coefficients of Mackinney (1941). Chlorophyll a:b ratios varied from 2.78 to 3.25 for the spinach used. Sufficient chloroplast preparation was added in the dark at the start of each measurement to give an absorbance at 678 mμ of 0.2 - 0.5 (1-cm path length).

D. DCPIP Hill Reaction

t_f, t_d, and light saturation. Figure 16a shows a typical light saturation curve obtained when the rate of DCPIP reduction in the sample cuvette is monitored at 580 mμ as a function of exciting flash intensity. The figure illustrates that with the apparatus employed here, saturation is just being approached for 19 msec flashes at maximum intensity. This effect increases as the flash length shortens, with a 6 msec flash being only 75% of saturation at maximum intensity. From the intercept of a double reciprocal plot of these



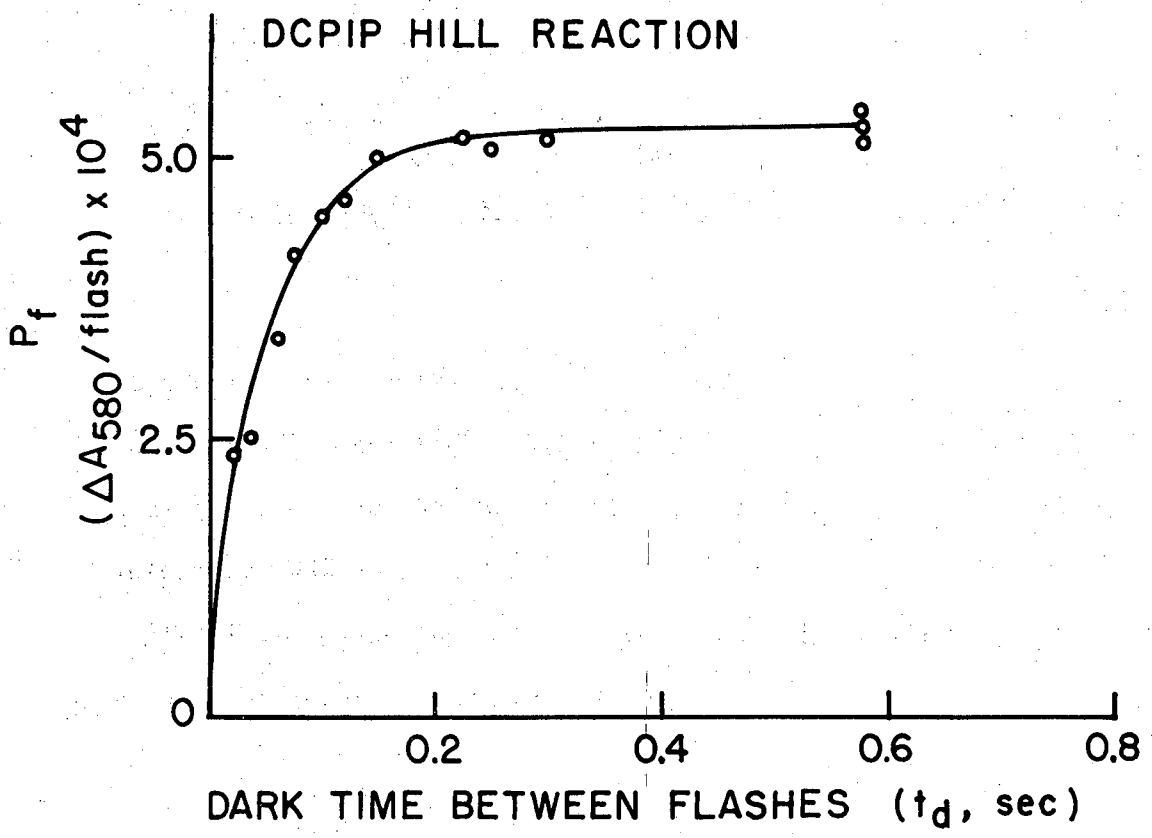
XBL 679-6174

Figure 16. Intensity dependence studies. a) Intensity dependence of the DCPIP Hill reaction (without methylamine) by spinach chloroplasts in flashing light. The flash duration is 19 msec, flash periodicity 2 sec, and A_{678}^{1cm} of the chloroplasts 0.240. The curve is calculated from the reciprocal plot in part b. b) Double reciprocal plot of the data in part a. The intercept represents the reciprocal of the maximum rate (R_∞^{-1}) which would occur at infinite light intensity.

data, (Figure 16b), the maximum rate (R_∞) which would occur at infinite intensity can be calculated. By means of such a correction, values of R_∞ can be obtained even for small t_f where saturation cannot be obtained. The extent of this saturation correction will also be dependent on the chloroplast concentration in the reaction mixture. Thus, in order to obtain values for the reduction per flash as a function of flash length, a study of the intensity dependence at each t_f must be made for each chloroplast preparation.

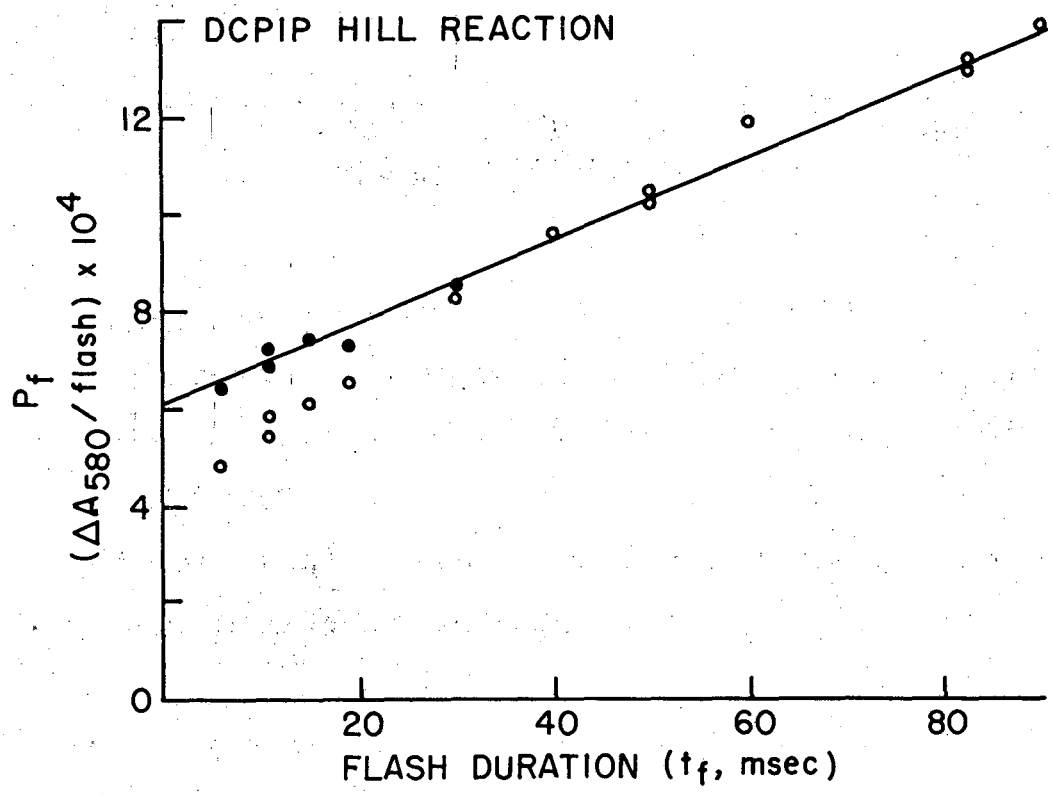
Figure 17 illustrates the effect of the dark time on DCPIP photoreduction per flash. To obtain maximum yield, a dark time of at least 0.2 sec is required. In most of the studies described below, a time of 2.0 sec between flashes was employed to insure that all of the reductants produced in one flash had reacted before a following flash was triggered. Figure 18 shows the effect of the flash length on the reduction per flash. When the short flash results are corrected to light saturation by means of reciprocal plots (filled circles) the yield per flash is observed to be linear in flash duration for the range of 6-100 msec.

Concentration dependence. The rate of DCPIP photoreduction is linearly dependent upon chloroplast concentration, as was observed for the Hill reaction under continuous illumination. It was found, however, that a somewhat higher DCPIP concentration than that used by Sauer and Park (1965) in their steady illumination studies is necessary to obtain the maximum yield per flash. Figure 19a is a saturation curve in terms of DCPIP concentration, and Figure 19b is its reciprocal plot. Values were obtained for several different chloroplast samples at different concentrations. These results



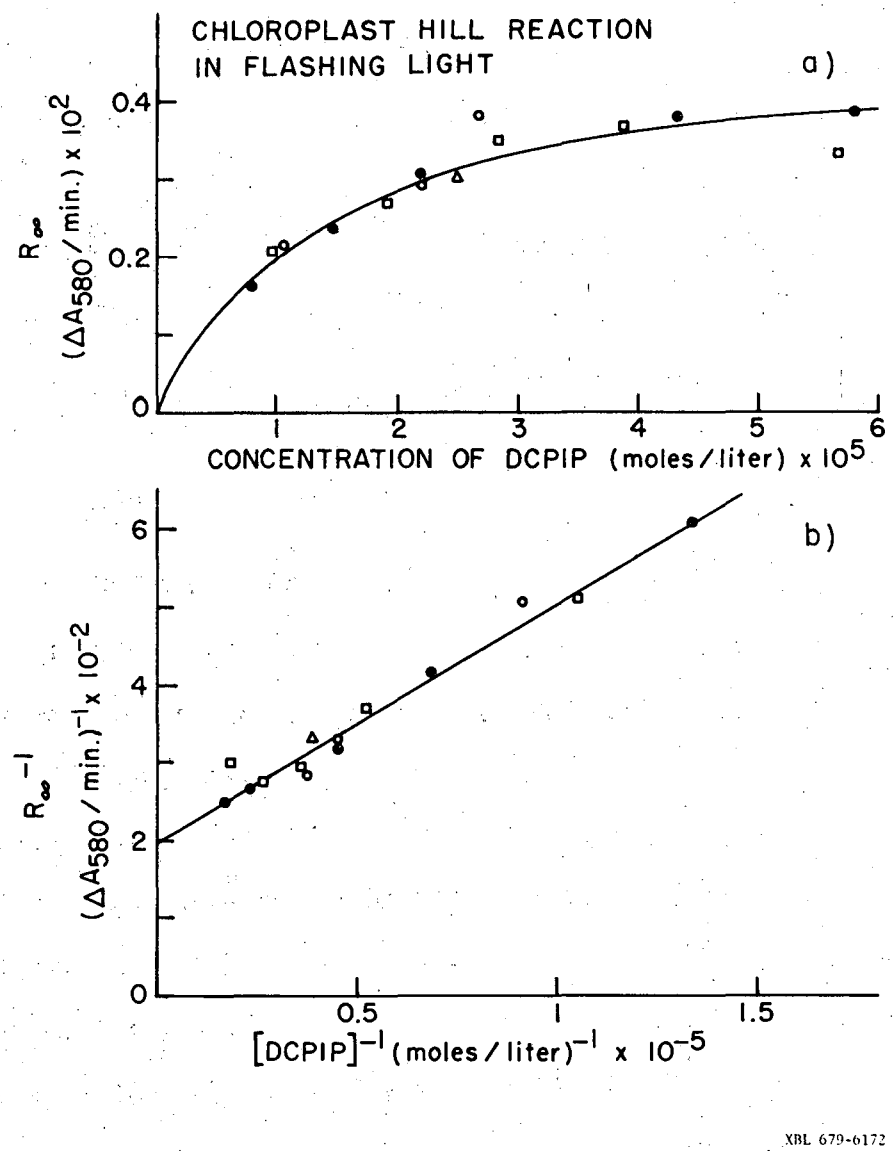
XBL 679-6179

Figure 17. Flash yield dependence on dark time between flashes for the DCPIP Hill reaction (without methylamine) by spinach chloroplasts. The reaction is run with t_f = 19 msec at maximum obtainable intensity (90% of saturation) and with the A_{678}^{1cm} 0.270 for the chloroplast suspension. The curve is calculated from eq. III-7 using experimentally derived parameters.



XBL 679-6173

Figure 18. Flash yield dependence on flash duration for the DCPIP Hill reaction (without methylamine) by spinach chloroplasts. The flashes are at maximum obtainable intensity with a repetition period of 2.0 sec, chloroplast A_{678}^{1cm} 0.270, and DCPIP = 3.82 x 10^{-5} M. The filled points are points corrected to light intensity saturation by means of reciprocal plots such as in Figure 17.



XBL 679-6172

Figure 19. R_∞ dependence on [DCPIP] and its reciprocal plot. a) Maximum flash rate (R_∞) dependence on DCPIP concentration for the DCPIP Hill reaction ± methylamine by spinach chloroplasts. The flash duration is 50 msec, flash periodicity 2 sec, and A_{678}^{1cm} 0.270 for the chloroplasts. The different symbols denote separate chloroplast preparations. The curve is calculated from the reciprocal plot in part b. b) Reciprocal plot of the data in part a. The intercept represents the reciprocal of the rate under saturating light conditions and where the DCPIP concentration is no longer limiting.

could be effectively standardized by means of the photoreduction's linear dependence on the chloroplast concentration. The figure shows that a concentration of 6×10^{-5} M of DCPIP in the reaction mixture gives about 80% of the maximum attainable rate. Since DCPIP absorbs in the red region of the spectrum, a high concentration of DCPIP reduces the effective intensity available to the chloroplasts. Also, the precision of the spectrophotometric analytical technique employed decreases when the DCPIP concentration is much greater than 5×10^{-5} M. For these reasons, experiments were performed at concentrations between 2 and 4×10^{-5} M DCPIP and corrected to saturating concentration by means of Figure 19b.

DCPIP functional unit. Since the relatively long flashes used here completely saturate the dark intermediates of the DCPIP Hill reaction, the slope of the curve in Figure 18 represents the rate of photoreduction at saturation under continuous illumination. This rate is both temperature dependent (Clendenning and Ehrmantraut, 1950), as it depends on the rates of dark enzymatic reactions, and dependent on DCPIP concentration when the latter is less than saturating. The intercept of Figure 18 represents the amount of DCPIP photoreduced by a pool of rate-limiting intermediates, Q_o, under the conditions where each of these intermediates has been activated once (Eq. III-3). It should be noted that this intercept is obtained by extrapolation of results using relatively long flashes, and it may not be the same as would be observed for submillisecond flashes. By converting the intercept into molecules of DCPIP reduced (after correcting to saturation for DCPIP concentration) and dividing by the total number of chlorophyll molecules present in an equal

volume of the reaction mixture, an experimental value for the size of the functional unit of the DCPIP Hill reaction can be obtained.

Table 10 presents a summary of calculations of the size of the functional unit and of the dark reaction velocity constant in the presence and absence of the phosphorylation uncoupler, methylamine. Methylamine affects the value of k_1 but not the size of the functional unit. Addition of phosphorylation cofactors (ADP, 1.0; $MgCl_2$, 7.5; and potassium phosphate (pH 7.7), 50; all in micromoles per milliliter) instead of methylamine, yields a functional unit and rate constant identical with that found in the presence of methylamine.

E. Ferricyanide Hill Reaction

The effect of the concentration of ferricyanide/ferrocyanide (equimolar, as recommended by the studies of Brewer and Jagendorf, 1965) on the rate of the Hill reaction in flashing light (t_f = 100 msec, t_d = 300 msec) is essentially the same as that observed by Sauer and Park (1965). An optimum concentration is ca. 2.5×10^{-4} M, with rates about 15% less at either one-half or twice this concentration. Because of the low molar extinction coefficient of ferricyanide, high chloroplast concentrations and short times between the flashes are used in order to provide large changes in optical density with time.

The dependence on the dark time between flashes is similar to that observed for DCPIP, but 0.400 sec between flashes was routinely used in the ferricyanide studies in order to give greater over-all rates. The higher chloroplast concentrations necessitated a larger

Table 10

Functional Unit Size and Dark Reaction Velocity Constant

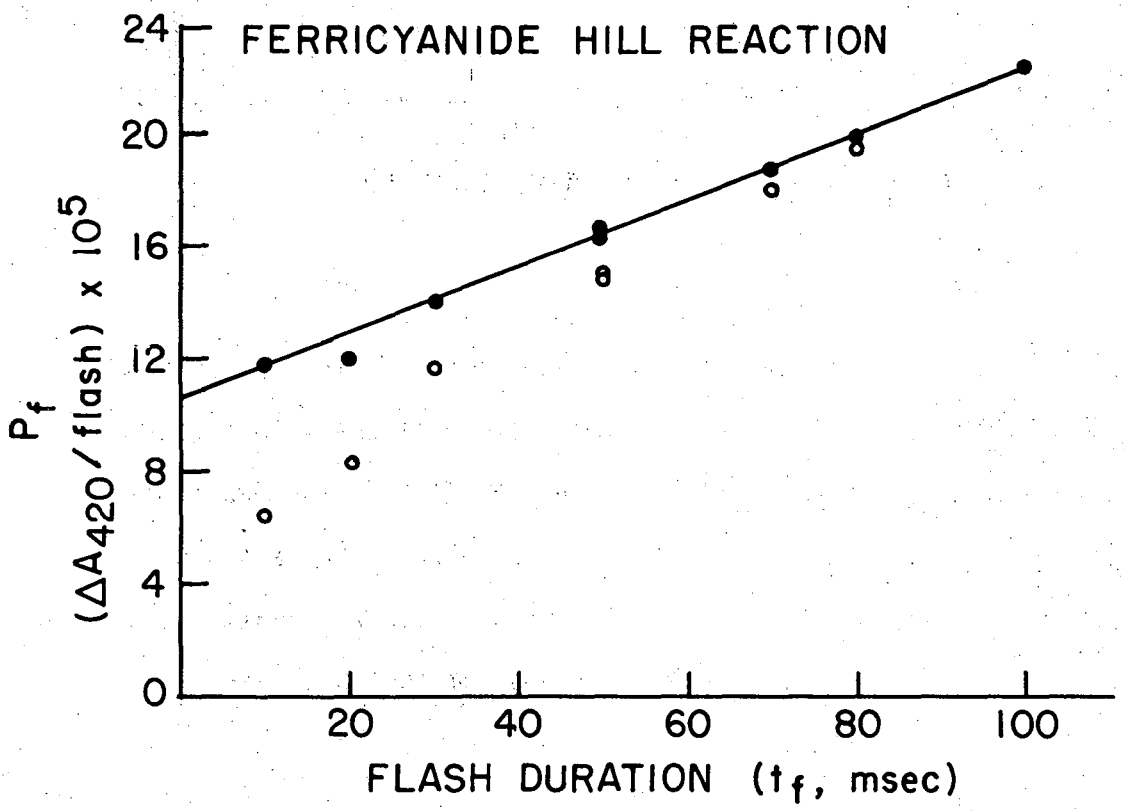
Reaction		Determinations	Functional Unit: Chlorophyll (a+b) / electron transferred	Dark Reaction Velocity Constant (sec^{-1})(22±2°)
DCPIP Reduction	+MA	14	56 ± 10	33.3 ± 6.9
	-MA	14	54 ± 7.4	19.5 ± 3.1
	+ADP, Mg^{2+}, P_i	1	58	32.7
Ferricyanide Reduction	+MA	10	70 ± 10	28.0 ± 8
	-MA	6	73 ± 9	16.6 ± 3
	+MA + catalytic DCPIP	2	62 ± 10	31.8 ± 9.2
Ferricyanide Reduction	+MA	2[a]	74 ± 11	29.6 ± 7.1
	+MA + catalytic DCPIP	2[a]	62 ± 10	31.8 ± 9.2
Cytochrome c/$TMQH_2$	+DCMU	5	445 ± 40	13.2 ± 4

a) The effect of catalytic amounts of DCPIP on a single chloroplast preparation.

correction to light intensity saturation than was needed for the DCPIP experiments. The experimental and corrected values in Figure 20 illustrate the extent of the light intensity corrections for a typical chloroplast preparation. This figure also shows that the ferricyanide Hill reaction exhibits a linear relationship between yield per flash and t_f over the millisecond region, as was observed for DCPIP. Table 10 includes a summary of functional unit size and k_1 values for the ferricyanide Hill reaction measured using ten chloroplast preparations. The addition of a catalytic amount of DCPIP (0.006 μmole/ml) to the ferricyanide reaction mixture containing methylamine resulted in a small, but probably real, decrease in the size of the functional unit and an increase in the dark reaction velocity constant to the value obtained for the DCPIP Hill reaction.

Shavit and Avron (1967) have reported that the rate of the ferricyanide Hill reaction is higher in a reaction medium containing NaCl than in one containing sucrose. This effect is attributed to a conformational change in the chloroplast lamellae caused by the use of an ionic osmoticum instead of sucrose. Below, in Table 11, is presented a summary of experiments designed to determine if this rate increase correlates with the difference in measured functional unit sizes for the DCPIP and ferricyanide Hill reactions.

The results do not indicate any significant difference in functional unit sizes caused by using NaCl instead of sucrose in the reaction mixture. Thus, it would seem that the proposed conformational differences between chloroplasts suspended in sucrose and NaCl is not the source of the discrepancy between the



XBL 679-6170

Figure 20. Flash yield dependence on flash duration for the ferricyanide Hill reaction (with methylamine) by spinach chloroplasts. The flash periodicity is 0.4 sec, using maximum intensity and with chloroplast A_{678}^{1cm} 0.420. The filled circles are calculated from experimental values by correcting to light intensity saturation using reciprocal plots such as in Figure 17.

Table 11

Ferricyanide Hill Reaction Functional Unit Size (Chl a + b/electron transferred) under Various Experimental Conditions

Chloroplasts Isolated In:	Reaction Mixture Contains:	
	0.35 M Sucrose	0.3 M NaCl
a) Sucrose 0.5 M (DCPIP reaction control)	67 (59)	72 (60)
b) NaCl 0.5 M	85	102
c) Sucrose 0.25 M and NaCl 0.25 M	75	75

ferricyanide and DCPIP functional unit sizes.

F. Cytochrome c Photoreduction with $TMQH_2$

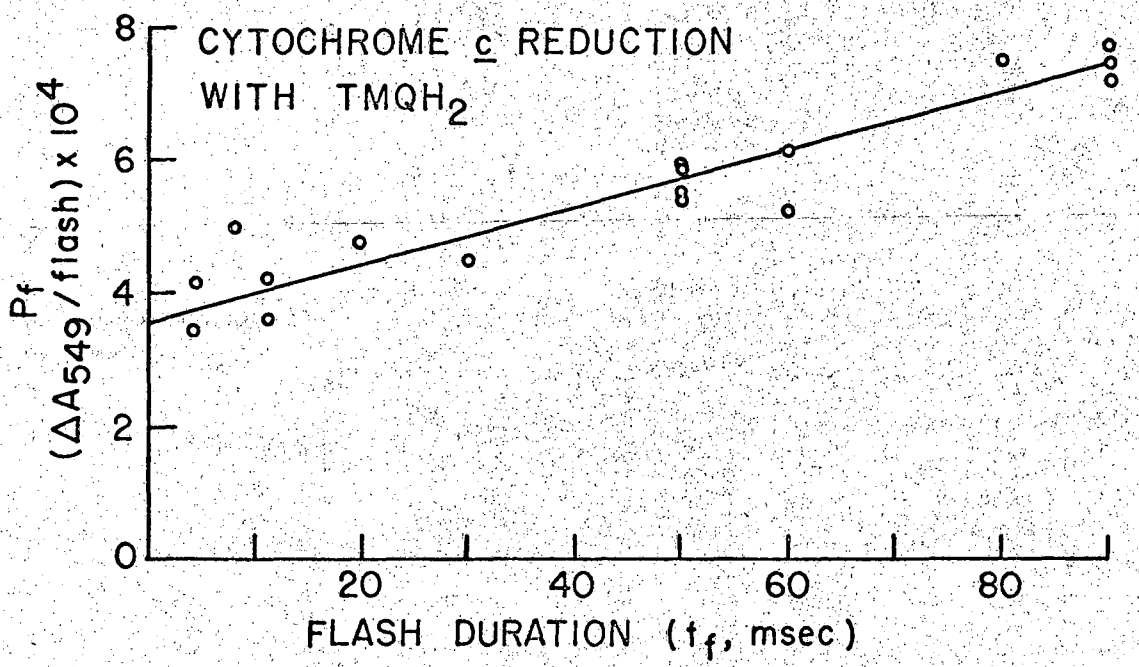
Light intensity saturation curves similar to that of Figure 16a occur for cytochrome c photoreduction, but a substantially lower light intensity is sufficient to reach saturation. Saturation for a 19 msec flash with a 2 sec time between flashes is obtained at about 1.0 units on the relative intensity scale of Figure 16a. This suggests that a larger pool of chlorophyll molecules is associated with each rate-limiting electron-transfer site for this reaction. The dark reaction reaches completion in about 0.5 sec, and intervals of 2 sec between saturating flashes were used to give the maximum yield per flash. A two-fold variation of the cytochrome c or $TMQH_2$ concentrations in either direction gives the same results, indicating that the concentrations generally employed are saturating.

Figure 21 shows the variation of cytochrome c reduction per flash as a function of flash length, and again the dependence is linear in t_f for the long-flash region that was examined. For this reaction, saturation could be obtained for the shortest light pulses used. The size of the functional unit and the dark reaction velocity constant are summarized in Table 10.

In Section II-D it was noted that catalytic amounts of ferredoxin do not affect the rate of cytochrome c reduction under steady illumination. Figure 22 shows the effect of added ferredoxin on the cytochrome c yield per flash as a function of flash length. The results indicate that ferredoxin at low concentration also has no effect on the photoreduction in flashing light.

G. Discussion

Functional units. The chloroplast-catalyzed photoreduction of cytochrome c has been shown in Chapter II to reflect solely System I activity. Thus, the flashing light studies summarized in Table 10 for the cytochrome c/$TMQH_2$ partial reaction indicate that the System I functional unit contains 445 chlorophyll molecules. This number is quite similar to the ratio of chlorophyll to P_{700}, the presumed reaction center of System I, as estimated for spinach chloroplasts by Kok and Hoch (1961). Subsequent measurements by Anderson et al. (1966) of the chlorophyll (a + b) : P_{700} ratio, also for spinach chloroplasts, yielded a value of 440. The agreement of the measured size of the functional unit for the cytochrome c reduction with the ratio of chlorophyll:P_{700} suggests that



XBL 679-6171

Figure 21. Flash yield dependence on flash duration for cytochrome c photoreduction with $TMQH_2$ in the presence of spinach chloroplasts and DCMU. The flash periodicity is 2.0 sec with maximum intensity and with chloroplast A_{678}^{1cm} 0.489.

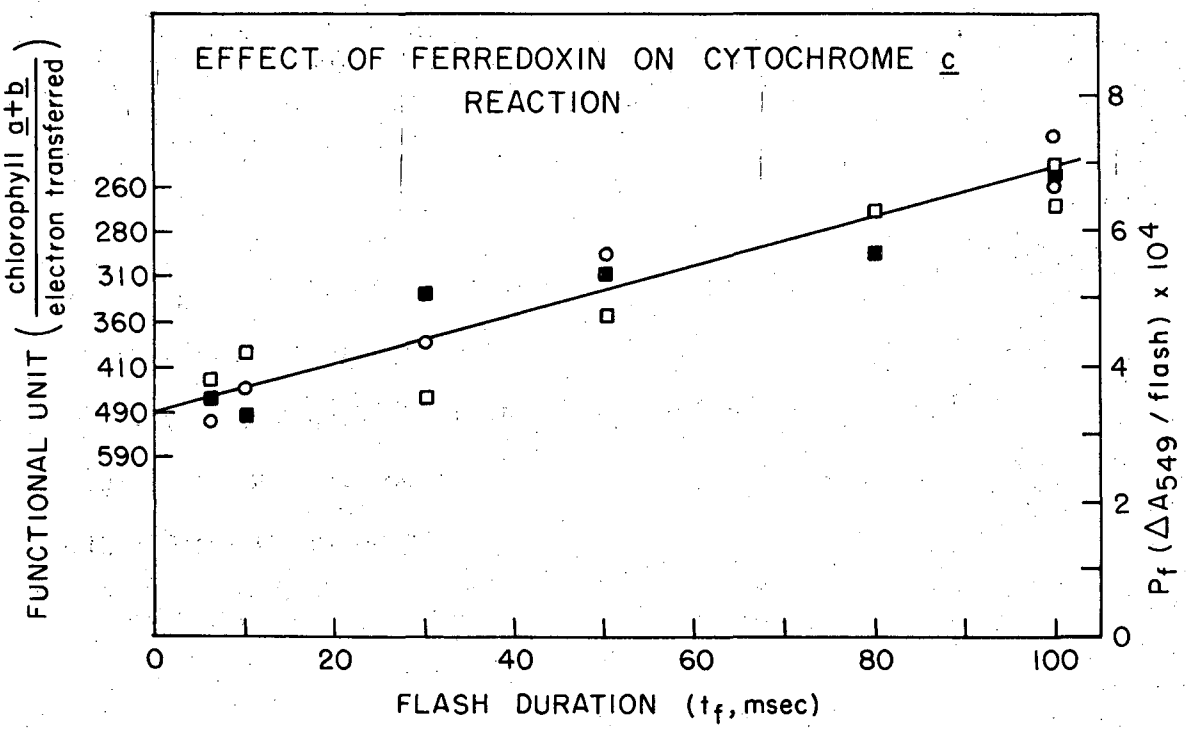


Figure 22. Effect of ferredoxin on the flash length dependence of the ferricyanide Hill reaction. Open circles indicate data for a normally prepared chloroplast sample with no added ferredoxin; closed squares for one with 50 mg/ml ferredoxin; and open squares for one with 100 mg/ml ferredoxin.

System I is limited in efficiency by the turn-over of one trapping site (P_{700} or some stoichiometrically equivalent molecule) per 440 chlorophyll molecules. It must be remembered that a functional unit is a measure of the total amount of chlorophyll a and b to electrons transferred in a partial reaction. Thus, a value for a System I functional unit is not a measure of the number of pigments in a System I photounit, but a ratio of total chlorophyll to a rate limiting component associated with a System I photoreaction.

We have noted before that the DCPIP Hill reaction involves pigment system II, as indicated by its sensitivity to DCMU and its very different action spectrum (Sauer and Park, 1965). For the non-cyclic electron flow leading to oxygen evolution, the best evidence at present suggests that both DCPIP and ferricyanide Hill oxidants utilize only System II. Experiments designed to detect the presence of an Emerson enhancement stimulated by supplementary far red light, indicative of cooperative action between System I and System II, have demonstrated the absence of such an effect both for the DCPIP (Sauer and Park, 1965) and for the ferricyanide (Avron, 1966) Hill reactions. The work of Forbush on the Scenedesmus mutant, cited below, further supports the conclusion that System I activity is not required for the net reduction of DCPIP (Kok et al., 1967).

For the DCPIP Hill reaction, we find a functional unit containing 55 ± 10 chlorophyll (a + b) molecules for light flashes from 6 to 100 msec in duration. Since this is 1/8 of the 440 chlorophylls per P_{700}, there seem to be about 8 System II functional

units for each P_{700} in chloroplasts. These results can be explained in two ways: either System II has many excitation energy trapping sites (8 for each P_{700} of System I), or alternatively, there is a small number of System II trapping sites (perhaps stoichiometric with those of System I) which transfer energy to a pool of intermediates, as suggested initially by Gilmour et al. (1954). The flashing light experiments of Kok (1956) indicate that for shorter saturating flashes than were used here, a pool of intermediates smaller than 8 equivalents/P_{700} becomes rate limiting and a larger System II functional unit than that of the DCPIP Hill reaction would be obtained.

Kok and Cheniae (1966), in their recent review of the oxygen evolution step of photosynthesis, cite experiments on spinach chloroplasts by Forbush in their laboratory, in which a brief (4 μsec), strong flash produced only 1 equivalent of DCPIP net reduction per 1000 chlorophylls. They did observe a rapid initial transient reduction corresponding to 1 equivalent per 500 chlorophylls which, however, reversed completely in 5 msec. Whereas the net reduction was completely inhibited by DCMU, the initial transient was unaffected by this inhibitor. The assignment of the transient to reduction by photosystem I was substantiated by its absence in a Scenedesmus mutant which lacks this photosystem. In a much slower reaction, requiring several seconds for completion, the Scenedesmus mutant is capable of reducing 1 equivalent of DCPIP per 1000 chlorophylls. Kok and Cheniae tentatively proposed chlorophyll arrays of 500 and 1000 chlorophylls for the

primary trapping center of photosystems I and II, respectively, from this short flash data. Thus, very short flashes of light provide evidence for a System II functional unit that is much larger than our observed value for the DCPIP Hill reaction, supporting the existence of a pool of intermediates that is filled by long flashes.

In other experiments on the same Scenedesmus mutant in which a long (5 sec) illumination period was used, Kok et al. (1967) observed an additional slow reduction of DCPIP occurring during the following dark period. The pool size of intermediates (secondary traps) responsible for this latent reduction corresponds to 1 equivalent per 70 chlorophylls. This number is commensurate with and is probably more comparable to our results for "long" flashes. Filling the pool of eight equivalents by long flashes compared with 1 equivalent for short flashes would tentatively seem to be associated with a rate-limiting step having a time constant between 4 μsec and 6 msec; however, care must be used in comparing results obtained with two appreciably different organisms. It is quite possible that the decrease in yield of O_2 per flash from Chlorella for flash lengths shorter than 2 msec observed by Kok (1956) results from this same rate-limiting step.

The ferricyanide Hill reaction of spinach chloroplasts exhibits a functional unit of 70 $\pm$ 10 chlorophyll molecules for long flashes. This unit is about 30% larger than that for the DCPIP Hill reaction, and the difference appears to be significant. Quantum yield measurements throughout the red region of the

spectrum by Sauer and Park (1965) showed the efficiency of ferricyanide reduction to be 20 to 40% lower than that for DCPIP reduction. Furthermore, Biggins and Sauer (1964) found that the addition of catalytic amounts of DCPIP to a ferricyanide Hill reaction mixture increased the efficiency of the reaction by about 20%, although the wavelength dependence of the action spectrum for the reaction did not change. As shown in Table 10, we find that a similar addition of catalytic amounts of DCPIP gives nearly a 20% decrease in the functional unit size for the ferricyanide Hill reaction in the presence of methylamine. These results appear to be entirely consistent with one another. It is possible that ferricyanide participates in a cyclic electron flow, resulting in no net ferricyanide reduction, in competition with the non-cyclic flow.

Pool sizes of System II intermediates. The pool sizes of electron transport intermediates between System I and System II have been measured by several kinetic methods. A summary of these studies is presented in Table 12. Witt et al. (1966), from studies of light-induced absorption changes of endogenous components of Chlorella, concluded that there exists a pool of ca. 12 equivalents per P_{700}, capable of receiving electrons from System II. From the wavelength dependence of the absorption changes, these intermediates appear to be plastoquinone. An additional pool of electron acceptors, containing 6 equivalents per P_{700} and lying closer to System I, rapidly reoxidizes half the molecules in the first pool. The acceptors associated with System II can again be reduced if the

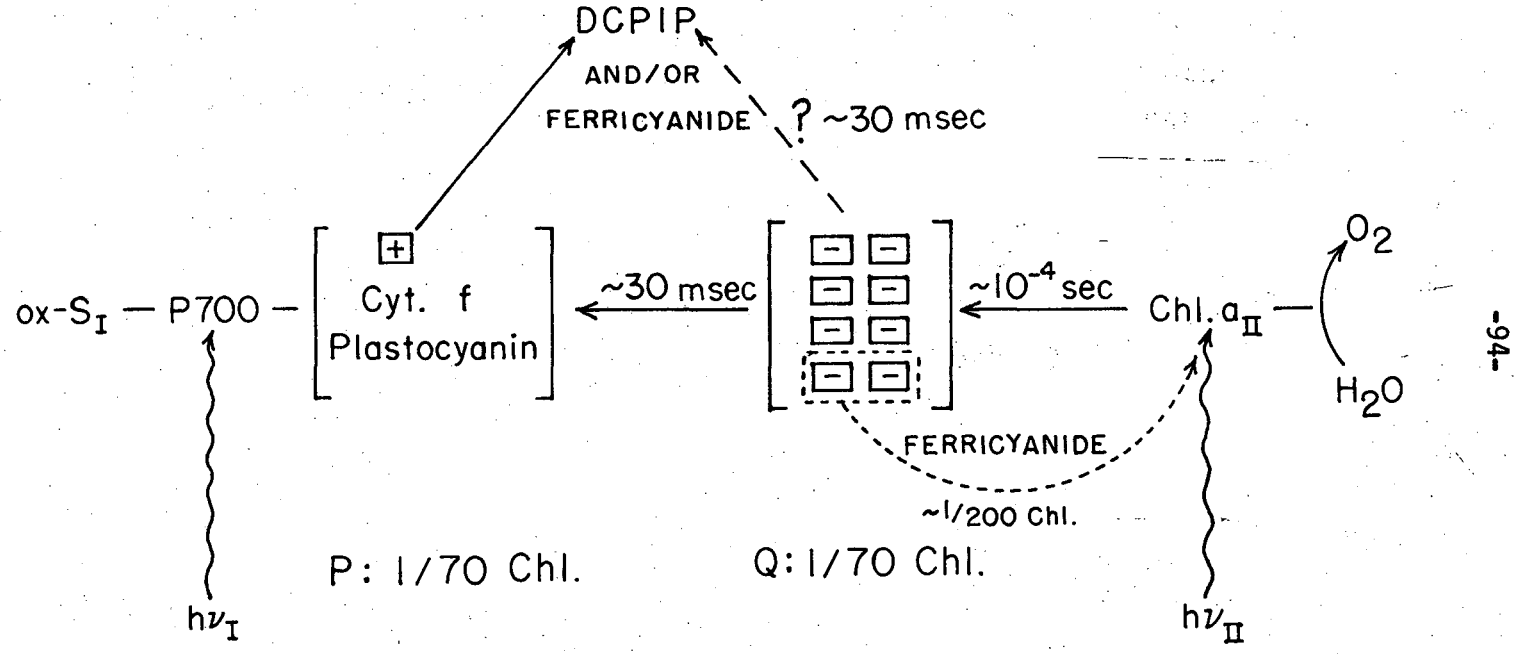
Table 12. Summary of Recent System II Intermediate Pool Size Studies

Reference	Method	Material	Author's Symbol	Equiv of Electron Acceptor/ Chl.(a+b)
Joliot (1961)	O_2 burst after dark adaptation	Chlorella	A	1/35
	O_2 burst after dark adaptation, short flash of light (10^{-4} sec)	Chlorella	E	1/350
Duysens & Sweens (1963)	Fluorescence quenching	Chlorella	Q	1/300
Joliot (1965)	Kinetics of O_2 evolution	Chlorella	A_1[a]	1/100
			A_2[a]	1/35
Witt et al. (1966)	Light-induced absorption changes of endogenous quinones	Chlorella		~1/35[b]
Malkin & Kok (1966)	Fluorescence induction	Spinach chloroplasts	Q[c]	1/70
Malkin (1966)			P[c]	1/70
Kok et al. (1967)	Dark yield of DCPIP reduction on cessation of saturating illumination	Scenedesmus mutant lacking P700		1/70
de Kouchkovsky & Joliot (1967)	Kinetics of O_2 evolution	Zea mays chloroplasts	A	1/70
			E[d]	1/2800

a) Evidence suggested that the earlier (Joliot, 1961) pool A had two kinetically distinguishable components; A_1 (regeneration rate constant 70 sec^{-1}) and A_2 (7 sec^{-1}). b) This value depends upon assumptions about the values of extinction coefficients of P700 and endogenous quinones. c) The thermal reaction in which electrons are transferred from reduced Q to the pool of P has a rate constant of 30-40 sec^{-1}. d) The low concentration of E, relative to that in Chlorella, was attributed to inactivation of some of the system II reaction centers during chloroplast isolation.

flash is sufficiently long (>10^{-2} sec). The removal of the electrons from these intermediates by the System I photoreaction is relatively slow (>0.12 sec). It would appear that, if all of these intermediates were to operate between light reaction II and the site of reduction of DCPIP or ferricyanide, the functional unit observed for the Hill reaction would be 18 times smaller than that for System I. Our observed ratio of 8 is significantly smaller than this, suggesting that the exogenous oxidants such as DCPIP and ferricyanide receive electrons from only a portion of the combined pools of Witt et al. Although this qualitative conclusion is probably correct, precise comparisons of pool sizes reported by different experimenters may be misleading. For example, the calculations based on observed light-induced absorption changes by Witt et al. (1966) and by Anderson et al. (1966) invoke assumed values for the extinction coefficients of P_{700} that differ by 20%. The close agreement of our value for the functional unit for cytochrome c reduction to the chlorophyll/P_{700} ratio of Anderson et al. causes us to favor these workers' results; however, this introduces additional assumptions of identity which are presently unproven.

The results obtained in this study appear to be largely consistent with those from the laboratories of Kok, Witt and Joliot (vide infra), apart from differences attributable to the photosynthetic organisms examined. The scheme (proposed by Kelly and Sauer, 1968) presented below (Figure 23) combines the notations of Witt and of Kok and is used to relate this study to theirs. Light absorbed by photosystem II very rapidly (10^{-4} sec) converts

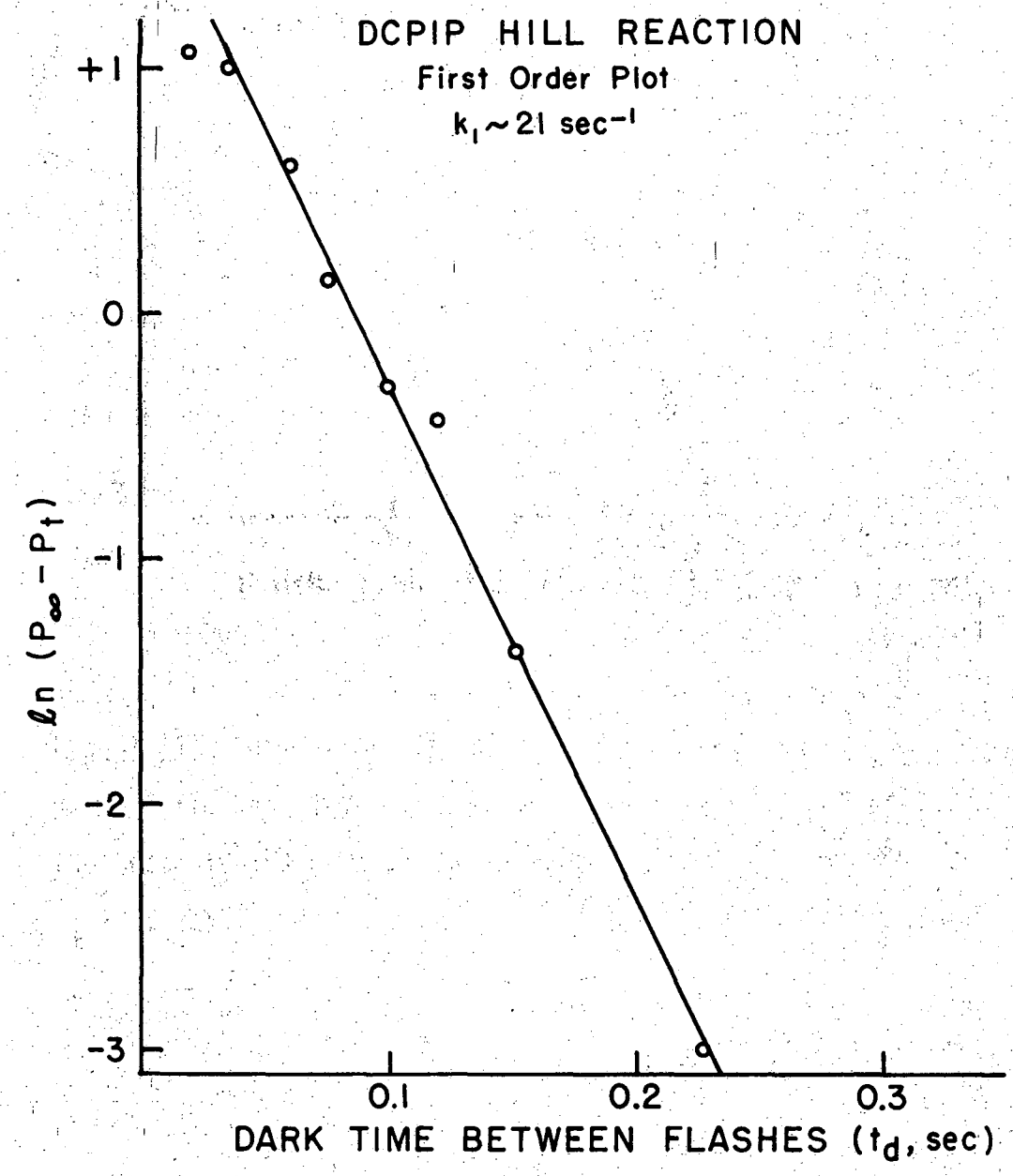


XBL 679-6196

Figure 23. Two light reaction scheme for the interpretation of flashing light results.

chlorophyll a_{II} or a closely associated compound (Joliot's E or Duysens' Q) to an activated (reduced) state (Joliot, 1961; Duysens and Sweers, 1963). This reduced primary compound rapidly transfers an electron to a molecule of Q (Kok's notation and the one adopted here), and during a long flash (2 msec) this process is repeated until all of the pool of Q is reduced. During this interval roughly half the electrons acquired by Q are passed on to a third pool of intermediates [the P (1/70 chlorophyll) of Malkin and Kok (1966), the A_2 (1/70 chlorophyll) of Joliot (1965), which are perhaps identical to the electron acceptors associated with System I (plastocyanin, cytochrome f and P_{700}) of Witt et al. (1966)].

In our studies, where DCPIP and/or ferricyanide are present in the reaction mixture, the remaining pool of reduced Q then proceeds to reduce these terminal acceptors. This hypothesis is supported by kinetic analysis of the dependence of the yield per flash on the duration of the dark interval between flashes, as shown in Figure 17. When the same data are plotted in semi-logarithmic fashion, as in Figure 24, they are found to result from a single first-order process. The rate constant for this dark decay process at room temperature (22 ± 2°C) is ca. 32 sec^{-1} in the presence of methylamine or phosphorylation cofactors, but it is about half this value when these compounds are missing (e.g. for the experiment described in Figures 17 and 24. The dependence of the rate constant on the presence of phosphorylation cofactors or of an uncoupler suggests that it is measuring the rate-limiting step at or near the site of coupling to non-cyclic phosphorylation



XBL 679-6180

Figure 24. Semilogarithmic plot of P_f as a function of dark time between flashes. The data are the same as that for Figure 17, and the graph indicates that the process involved is first order. $k_1 = 21\ sec^{-1}$.

in the Hill reaction. The observation of a simple first-order behavior points to a single pool of electron transport intermediates as the origin of the terminal step in the reduction of the Hill reagents.

Another possible relationship between the functioning of the System I and II traps has been put forward. In that proposal there are as many System II traps as Q molecules and many System II units funnel their electrons to the same P_{700}. The kinetics analysis of Malkin (1966) favors this proposal, but it is felt that experiments using very short flashes (Clendenning and Ehrmantraut, 1950; Gilmour et al., 1954; Kok, 1956; Kok and Cheniae, 1966) present evidence that there are more Q than chlorophyll a_{II}, thus ruling out this scheme.

A model sufficient to account for the cytochrome c reduction by $TMQH_2$ can be constructed in much simpler fashion. In this case the component responsible for the rate-limiting step for saturating light flashes is stoichiometric with P_{700} and may be identical with it. A second reasonable candidate for this component is cytochrome f, which is found to have the same concentration in vivo as P_{700} (Boardman and Anderson, 1967). Since the cytochrome c reduction reaction does not require ferredoxin, as was shown in Section II-D, ferredoxin cannot be rate-limiting for this reaction. In all probability we are measuring directly the ultimate size of photosystem I, which is a particularly nice feature of using this reaction. Although cytochrome c is a large molecule and might be expected to have restricted access to the sites of photochemical activity in

the chloroplast lamellae, relatively low concentrations of cytochrome c (0.025 μmoles/ml) appear to be saturating. This saturation may result from a prior binding of the oxidized cytochrome c at a site close to the System I reaction center during the 75 msec interval required to reactivate the light reaction. The binding is not the rate-limiting step, however.

Kinetic measurements related to pool sizes. Further information about the pools of intermediates associated with photosystem II has been reported by Joliot (1961). For dark adapted Chlorella pyrenoidosa a burst of oxygen corresponding to one O_2 per 140 chlorophylls (1 equivalent per 35 chlorophylls) occurs during the first second of illumination by intense light. He attributes this burst to the accumulation during the prior dark period of a pool of an oxidized compound, designated A, of equivalent size. Joliot's compound A closely resembles the Q of Kok in its properties. For very short flashes (0.1 msec) of saturating light, on the other hand, the oxygen produced per flash is only 1/10 of this amount. This originates from a second, smaller pool of intermediates (1 equivalent per 350 chlorophylls) which he designates compound E and which may be chlorophyll a_{II} in the notation of Witt. For such short flashes it is necessary to initiate the process (in Joliot's scheme, to generate the active form of E) by a prior flash which produces no oxygen or by a steady background illumination of low intensity. During longer periods of illumination the active form of E can be regenerated during the light pulse. From studies of the yield per flash as a function of flash duration

in the range 10^{-4} to 0.2 sec the regeneration of A appears to be biphasic, with rate constants of about 70 and 7 sec^{-1}, respectively (Joliot, 1965). This is interpreted as giving evidence of the existance of two kinetically distinguishable components, A_1 and A_2, in the pool A. In Chlorella the amount of A_1, the rapid component, appears to be one-quarter of the total pool of A, the latter which is estimated to constitute 1 equivalent per 25 chlorophylls from these experiments. Following the cessation of illumination, there is a slow (40 sec) phase of extra oxygen uptake (apart from that attributable to respiration) associated with the formation of a reduced product, PH, by System I and estimated to correspond to 1 equivalent per 140 chlorophylls.

When isolated chloroplasts, instead of whole Chlorella cells, were examined by de Kouchkovsky and Joliot (1967), evidence was again found for two intermediate compounds in photosystem II. The one corresponding to A was present at a concentration of 1 equivalent per 70 chlorophylls, and that corresponding to E at 1 per 2800 chlorophylls. The low concentration of E, relative to that in Chlorella, was attributed to inactivation of some of the System II reaction centers during chloroplast isolation.

The fluorescence induction studies of Malkin and Kok (1966) and their detailed kinetic analysis by Malkin (1966) provide another measure of pool sizes. The complementary relationship between fluorescence and photochemistry in the utilization of absorbed light quanta permits the estimation of pool sizes of intermediates from the kinetics of the fluorescence increase upon

illumination. In this manner Malkin and Kok determined the presence of two intermediates, designated P and Q, associated with photosystem II in isolated chloroplasts. Upon illumination with saturating light the pool of Q (1 equivalent per 70 chlorophylls) rapidly becomes reduced. This is followed by a thermal reaction (k_1 = 30-40 sec^{-1}) in which electrons are transferred from reduced Q to the pool of P (also 1 equivalent per 70 chlorophylls). Each of these pools is identical in size to the pool of A observed by de Kouchkovsky and Joliot (1967) from oxygen burst measurements on isolated chloroplasts, and is very close to that calculated from our results on Hill oxidant utilization. It is reasonable to conclude that the same pools of photosystem II intermediates are involved in each of these studies.

The rate constant for the dark decay process in the Hill reaction at room temperature as measured here is ca. 30 sec^{-1} in the presence of the cofactors of phosphorylation or of the phosphorylation uncoupler methylamine. This rate-limiting regeneration step is responsible for the dependence of the yield per flash on the duration of the dark interval between flashes, as shown in Figures 17 and 24. No evidence is seen of the biphasic character observed by Joliot (1965) for the oxygen burst for Chlorella, where only endogenous oxidants associated with photosystem II are involved. On the basis of this observation, and noting that our pool size of intermediates is about one-half that observed by Joliot (compound A), we propose that DCPIP and/or ferricyanide react with only a single, small pool of photosystem II intermediates

(1 per 60 to 70 chlorophylls) in isolated chloroplasts. The oxygen burst studies on chloroplasts by de Kouchkovsky and Joliot (1967) also demonstrated a small pool of intermediates (1 per 70 chlorophylls). They did not report on the kinetics of the decay for the chloroplast system, however.

It is possible that the terminal reduction occurs through the transfer of electrons through a second pool (Joliot's A_2 or Kok's P) of endogenous molecules, but the associated steps leading to substrate reduction must all be rapid. The close similarity of the rate constant of 30-40 sec^{-1} at room temperature for the transfer from reduced Q to P, as measured by Malkin and Kok (1966), to our values for the DCPIP and ferricyanide Hill reaction rate-limiting steps makes this hypothesis appealing. This pathway appears in our kinetic scheme by way of substrate coupling through the pool of electron acceptors associated with System I, although other formulations are possible. The transfer of electrons to the terminal Hill acceptor, if sufficiently rapid, would prevent the pool of P from filling and, therefore, in the kinetic analysis one would observe only the pool of reduced intermediates prior to the rate-limiting step.

CONCLUDING REMARKS

From a study of the cytochrome c/$TMQH_2$ photoreaction in broken spinach chloroplasts, we have obtained an action spectrum for photosystem I (Figure 8). In the wavelength regions 400-500 mμ and 620-680 mμ this photoreaction is seen to require about 2 quanta per electron transferred to cytochrome c when the intensity dependence of the quantum requirement is extrapolated to zero light intensity. For wavelengths greater than 700 mμ, the quantum requirement drops to 1 quantum per electron, showing that this reaction probably occurs with the maximum efficiency obtainable. Thus the points on the action spectrum illustrate the maximum efficiency of System I at a given wavelength.

Attempts were made to use the quantum requirement measurements to determine the role of chlorophyll b in the two photosystems. The data for spinach chloroplasts proved inconclusive because the activation spectrum for System II did not show any significant increase in the region of chlorophyll b absorption. However, normal barley appears to be more efficient than a barley mutant lacking chlorophyll b at 650 mμ, the chlorophyll b maximum. Thus, the barley studies support the contention that chlorophyll b absorption is used primarily by photosystem II.

From the quantum yields (reciprocals of the quantum requirements) for the cytochrome c photoreductions, the wavelength dependence of the fractional absorption of photosystem I was determined. A similar

evaluation of the DCPIP Hill reaction data of Sauer and Park (1965) yielded the wavelength dependence of the System II pigments. Comparison of the two fractions indicates that their sum equals the total chloroplast absorption and does not reveal the presence of any pigment absorption which is utilized by both of the systems. Thus, the conclusion of this study is that energy transfer does not occur between the pigments of the two photosystems.

Using partial reactions associated with photosystem I (cytochrome c/$TMQH_2$) and photosystem II (DCPIP or ferricyanide Hill reaction) respectively, we have been able to estimate the concentration, relative to chlorophyll, of the reaction centers or pools of intermediates closely associated with these photoreactions in broken spinach chloroplasts. Relatively long flashes (6-100 msec) of saturating red light produce photoreaction yields corresponding to 1 equivalent per 445 chlorophylls for System I and 1 equivalent per 55 chlorophylls for System II. From the other published results (noted in Chapter III) it appears that the larger System II unit results from a secondary pool of intermediates and that very short flashes (10^{-4} sec) would produce evidence for a primary rate-limiting component in smaller concentration.

The rate-limiting step for the dark reaction regenerating System I activity has a first-order rate constant of 13 sec^{-1} at room temperature. That for System II, also first-order, is ca. 30 sec^{-1}, but is reduced to ca. 20 sec^{-1} in the absence of phosphorylation cofactors or the phosphorylation uncoupler, methylamine. These rate constants are independent of the concentrations of added electron acceptors and donors, indicating that both of these rate-limiting

steps appear to result from components endogenous to the broken chloroplasts.

An interpretation of the results of these investigations has been presented in each of the concluding sections of Chapters II and III. These interpretations can best be summarized by means of a model for the photosynthetic apparatus which incorporates the ideas of Figures 13 and 23. The only major change required in such a model would be the expansion of the primary photosynthetic unit* in Figure 13 to correspond to the 445 chlorophyll System I functional unit measurement of Chapter II. We have noted in Chapter I that electron microscopy provides evidence for a fundamental particle that is found in the chloroplast lamellae and that associated with this particle are about 230 chlorophyll molecules (Park and Biggins, 1964). It is interesting that this is one-half the size of the System I functional unit, suggesting that the morphological photosynthetic unit would consist of two of these particles in vivo. This result would affect the scheme proposed in Figure 14 by requiring that the electron transport chain be associated with two quantasomes rather than one.

One might speculate that in a "two-quantasome photosynthetic unit", one of the particles would be associated with System I activity and the other with that of System II. However, recent electron micrographs of freeze-etched chloroplasts by Branton and Park (1967), indicate that the chlorophyll-containing lamellae may be made up of at least two classes of subunits. The larger particles (of 175 Å

---

*It is possible that several of these units might cooperate in a succeeding process such as O_2 evolution.

cross section, and probably equivalent to the quantasome) are found in both higher plants and bacteria, while the smaller subunits (ca. 110 Å cross section) are associated only with oxygen evolving systems (R. B. Park, private communication). Thus, it is tempting to suggest that the large quantasome particles are associated with System I activity (since a photoreaction leading to carbon fixation is common to both bacteria and higher plants), and that the smaller subunits are associated with the oxygen evolving System II found only in alga and higher plants. The small particles appear to be located on one side of the thylakoid membrane and the large particles on the other. This arrangement could result in the required separation of the pigments associated with the two photosystems so that energy transfer does not occur. Another feature of such an arrangement would be the possibility of a lipid layer between the two types of particles which could contain an intermediate pool of quinones.

However, even this model may be oversimplified. Homann and Schmid (1967) in a study of photosynthetic activity in various mutants of Nicotiana tabacum, found that full System I activity can be associated with single, unfolded thylakoids, while the complete photosynthetic electron transport system, including the oxygen evolving apparatus of System II, appears to require a close packing of at least two thylakoids. If this requirement also applies to the normal chloroplast, then a model of overall electron transport and O_2 evolution would require more than one thylakoid.

Definite correlations between morphology and function must await a deeper understanding of the freeze-etch process and interpretation of the resultant micrographs. Also of importance is the development

of improved techniques in isolating homogeneous fractions of chloroplast particles or particle aggregates. The photoactivities of such preparations would then indicate which is the actual morphological photosynthetic unit. Use of chromatographic supports such as polyacrylamide could provide a means of isolating particles of different size from one another, but a better method of lamellae fragmentation or a refinement of the sonication procedure currently used is needed in order to obtain a good yield of small particles.

One consequence of the model proposed above is the possibility that the intermediate pool between the two systems is a quinone pool that can be reduced by more than one System II photocenter. A way to test this hypothesis would be to see how much of the quinone pool is reduced when part of the System II photoreactions have been blocked by DCMU. The addition of DCMU increases fluorescence yields, indicating an inability of electrons to flow from the System II trap to P_{700} (Yamashita and Butler, 1968). In the absence of DCMU, the fluorescence yield should be low and a large pool of quinone reduced, resulting in a large change in quinone absorption at 260 mμ. At a 50% inhibitory concentration of DCMU, the fluorescence yield should increase to a value half way between the fluorescence yield with no DCMU and that observed for 100% inhibitory DCMU. At this point either one-half of the quinone pool will be reduced, indicating that only one-half of the quinones are connected to operative System II centers; or almost all of the quinones will be reduced, indicating that the unpoisoned photocenters can reduce the entire quinone pool.

One of the questions raised in Chapter III concerned the size of the System II functional unit relative to that of System I.

Although we noted that DCPIP reduction in very short flashes performed by other workers suggests that the System II size is similar to that of System I, it would be beneficial to extend the study of the DCPIP Hill reaction to shorter flash lengths in order to obtain a direct measurement of the pigment to trapping center ratio for System II in spinach chloroplasts. These experiments would also yield information about the rate of the process which fills the intermediate pool associated with this Hill reaction.

The experimental techniques developed in Chapter III could also be used beneficially on other photosynthetic partial reactions. System I reactions such as NADP reduction with DCPIP/ascorbate or TMPD/ascorbate as electron donors, which are thought to have different points of entry from each other (Henninger and Crane, 1967), and the new System II reaction in which p-phenylene diamine substitutes for water as the electron donor are likely candidates for such a study. Differences in functional unit sizes or in kinetics, between these partial reactions and those observed in this study, could be attributed to different rate limiting steps and may provide evidence of different intermediate pools. This may lead to information on the point of entry of these electron donors into the photosynthetic electron transport chain.

Although our knowledge of higher plant photosynthetic electron transport has increased tremendously due to the application of spectroscopic techniques to the study of partial reactions and light induced absorption changes of internal components, there are still many important aspects of the primary photosynthetic processes that

need to be more fully explored. Some evidence has been presented (Henninger and Crane, 1967) that the electron transport chain is split into two pathways between the two light reactions. Experiments need to be performed, perhaps with mutants lacking specific electron transport components, to determine if the chain is actually split or if evidence for such a splitting is due only to varying experimental conditions.

We have seen in this study how some of the properties of the bulk photosynthetic pigments are related to the energy conversion and electron transport processes of higher plant photosynthesis. The answers to many related questions, such as the nature of the photoreaction centers, the mechanism of O_2 evolution, the mode of coupling between electron transport and phosphorylation, and the primary oxidants and reductants for both light reactions are unknown and are primary goals for future work in photosynthesis.

REFERENCES

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

Anderson, J. M. and N. K. Boardman (1965) Biochim. Biophys. Acta 112, 403.

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

Arnold, W. and H. Kohn (1934) J. Gen. Phys. 18, 109.

Arnold, W. and E. S. Meek (1956) Arch. Biochem. Biophys. 60, 82.

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

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

Arnon, D. I., F. R. Whatley and M. B. Allen (1957) Nature 180, 182.

Arnon, D. I., F. R. Whatley and M. B. Allen (1959) Biochim. Biophys. Acta 32, 47.

Avron, M. (1966) Israel J. Chem. 4, 73.

Baltscheffsky, H. (1963) Acta Chemica Scandinavica 17, S308.

Bannister, T. T. and J. Vrooman (1963) in Photosynthetic Mechanisms of Green Plants, Nat. Acad. Sci. -- Nat. Res. Council Publ. 1145, 391.

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

Biggins, J. (1965) Ph.D. Thesis, University of California, Berkeley, 91.

Biggins, J. and K. Sauer (1964) Biochim. Biophys. Acta 88, 655.

Bishop, N. I. (1967) Photochem. and Photobiol. 6, 621.

Black, C. C., C. A. Fewson, M. Gibbs and S. A. Gordon (1963) J. Biol. Chem. 238, 3802.

Black, C. C., C. A. Fewson, M. Gibbs, D. L. Keister and A. San Pietro (1962) Federation Proc. 21, 398.

Blackman, F. F. (1905) Ann. Bot. 19, 281.

Blackman, F. F. and A. M. Smith (1911) Proc. Roy. Soc. London B 83, 389.

Boardman, N. K. and J. M. Anderson (1967) Biochim. Biophys. Acta 143, 187.

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

Branton, D. and R. B. Park (1967) J. Ultrastruct. Res. 19, 283.

Brewer, J. M. and A. T. Jagendorf (1965) Plant Physiol. 40, 303.

Briantais, J. (1967) Biochim. Biophys. Acta 143, 650.

Briggs, G. (1941) Proc. Roy. Soc. (London) B 130, 24.

Butler, W. L. and N. I. Bishop (1963) Nat. Acad. Sci. -- Nat. Res. Council, Publ. 1145, 91.

Chen, S. L. (1952) Plant Physiol. 27, 35.

Clayton, R. K. (1965) in Molecular Physics in Photosynthesis, Blaisdell, New York, 41.

Clendenning, K. and H. Ehrmantraut (1950) Arch. Biochem. Biophys. 29, 387.

Davenport, H. E. and R. Hill (1960) Biochem. J. 74, 493.

de Kouchkovsky, Y. and P. Joliot (1967) Photochem. and Photobiol. 6, 567.

Doring, G., H. H. Stiehl and H. T. Witt (1967) Z. Naturforsch. 22b, 639.

Drury, S. and J. Kelly (1968) unpublished results.

Duysens, L. N. M. (1952) Ph.D. Thesis, University of Utrecht, The Netherlands.

Duysens, L. N. M. and H. E. Sweers (1963) in Studies on Microalgae and Photosynthetic Bacteria, Japanese Society of Plant Physiologists, Ed., University of Tokyo Press, Tokyo, 353.

Duysens, L. N. M., J. Amesz and B. M. Kamp (1961) Nature 190, 510.

Ehrmantraut, H. and E. Rabinowitch (1952) Arch. Biochem. Biophys. 38, 67.

Emerson, R. and W. Arnold (1931) J. Gen. Physiol. 15, 391.

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

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

Emerson, R. and C. M. Lewis (1942) J. Gen. Physiol. 25, 519.

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

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

Franck, J. and J. L. R. Rosenberg (1964) J. Theoret. Biol. 7, 276.

Gilmour, H., R. Lumry, J. Spikes and H. Eyring (1954) Nature 173, 31.

Gingras, G., C. Lemasson and D. C. Fork (1963) Biochim. Biophys. Acta 69, 438.

Givan, A. L. and R. P. Levine (1967) Plant Physiol. 42, 1264.

Goedheer, J. C. (1955) Biochim. Biophys. Acta 16, 471.

Goedheer, J. C. (1957) Ph.D. Thesis, University of Utrecht, the Netherlands.

Good, N. E. (1962) Arch. Biochem. Biophys. 96, 653.

Gorman, D. S. and R. P. Levine (1965) Proc. Nat. Acad. Sci. 54, 1665.

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

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

Gromet-Elhanen, Z. and M. Avron (1963) Biochemistry 3, 365.

Gross, J. A., M. J. Becker and A. M. Shefner (1964) Nature 203, 1263.

Henninger, M. and F. Crane (1967) J. Biol. Chem. 242, 1155.

Highkin, H. R. (1950) Plant Physiol. 25, 294.

Highkin, H. R. and A. W. Frenkel (1962) Plant Physiol. 37, 814.

Hill, R. (1939) Proc. Roy. Soc. London B 124, 192.

Hill, R. and F. Bendall (1960) Nature 186, 137.

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

Homann, P. H. and G. H. Schmid (1967) Plant Physiol. 42, 1619.

Jensen, R. G. and J. A. Bassham (1966) Proc. Nat. Acad. Sci. U.S. 56, 1095.

Joliot, P. (1961) J. Chim. Phys. 58, 570, 584.

Joliot, P. (1965) Biochim. Biophys. Acta 102, 116.

Joliot, P., A. Joliot and B. Kok (1968) Biochim. Biophys. Acta, submitted for publication.

Kamen, M. D. (1963) in Methods in Enzymology, Vol. VI, ed. S. P. Colowick and N. O. Kaplan, Academic Press, N. Y. and London, p. 313.

Keister, D. L. and A. San Pietro (1963) Arch. Biochem. Biophys. 103, 45.

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

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

Kling, O., E. Nikolaiski and H. L. Schläfer (1963) Ber. Bunsenges Physikal Chem. 67, 883.

Kohn, H. (1936) Nature 137, 706.

Kok, B. (1956) Biochim. Biophys. Acta 21, 245.

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

Kok, B. and G. M. Cheniae (1966) in Current Topics in Bioenergetics, Vol. 1, D. R. Sanadi, ed., Academic Press, New York, p. 1.

Kok, B. and G. Hoch (1961) in Light and Life, W. D. McElroy and B. Glass, ed., Johns Hopkins Press, Baltimore, Md., 397.

Kok, B., S. Malkin, O. Owens and B. Forbush (1967), Brookhaven Symp. Biol. 19, 446.

Kuntz, I. D., Jr. and M. Calvin (1965) Photochem. and Photobiol. 4, 537.

Levine, R. P. and D. S. Gorman (1966) Plant Physiol. 41, 1293.

Lumry, R. and J. S. Rieske (1959) Plant Physiol. 34, 301.

Lumry, R., R. E. Wayrynen and J. D. Spikes (1957) Arch. Biochem. Biophys. 67, 453.

Mackinney, G. (1941) J. Biol. Chem. 140, 315.

Malkin, S. (1966) Biochim. Biophys. Acta 126, 433.

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

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

Massey, V. (1959) Biochim. Biophys. Acta 34, 255.

Myers, J. (1963) in Photosynthetic Mechanisms of Green Plants, Nat. Acad. Sci. -- Nat. Res. Council Publ. 1145, 301.

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

Olson, R. A., W. L. Butler and W. H. Jennings (1961) Biochim. Biophys. Acta 54, 615.

Olson, R. A., W. L. Butler and W. H. Jennings (1962) Biochim. Biophys. Acta 58, 144.

Paléus, S. and J. B. Neilands (1950) Acta Chem. Scand. 4, 1024.

Park, R. B. (1962) J. Chem. Ed. 39, 424.

Park, R. B. (1966) in International Review of Cytology, Vol. 20, Academic Press, New York, 67.

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

Park, R. B. and N. G. Pon (1961) J. Mol. Biol. 3, 1.

Park, R. B. and L. K. Shumway (1968) in Comparative Biochemistry and Biophysics of Photosynthesis, K. Shibata, A. Takamiya, A. T. Jagendorf and R. C. Fuller, eds; University of Tokyo Press, Tokyo, 57.

Rabinowitch, E. I. (1951) Photosynthesis and Related Processes, Interscience Publishers, New York, Vol. II, Part 1, Chapter 29.

Rabinowitch, E. I. (1956) Photosynthesis and Related Processes, Interscience Publishers, New York, Vol. II, Part 2, Chapter 34.

Rieske, S. J., R. Lumry and J. D. Spikes (1959) Plant Physiol. 34, 293.

Robinson, G. W. (1964) Ann. Rev. Phys. Chem. 15, 311.

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

Ruben, S., M. Randall, M. Kamen and J. L. Hyde (1941) J. Am. Chem. Soc. 63, 879.

Rumberg, B. (1964) Nature 204, 860.

Rumberg, B. and H. T. Witt (1964) Z. Naturforsch. 19b, 693.

Rumberg, B., A. Müller and H. T. Witt (1962) Nature 194, 854.

San Pietro, A. and H. M. Lang (1958) J. Biol. Chem. 231, 211.

Sauer, K. (1965) Biophys. J. 5, 337.

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

Sauer, K. and M. Calvin (1962) J. Mol. Biol. 4, 451.

Sauer, K. and R. B. Park (1964) Biochim. Biophys. Acta 79, 476.

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

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

Shen, G. M., S. Y. Yang, Y. K. Shen and H. C. Yin (1963) Sci. Sinica 12, 685.

Sironval, C., H. Clysters, J. Michel, R. Bronchart and R. Michel-Walwertz (1965) in Currents in Photosynthesis, J. B. Thomas and J. C. Goedheer, eds., Ad. Donker, Rotterdam, The Netherlands, 111.

Spencer, D. and H. Unt (1965) Australian J. Biol. Sci. 18, 197.

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

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

Tamiya, H. and Y. Chiba (1949) Studies Tokugawa Inst. 6, Part 1.

Trebst, A. V., H. Y. Tsujimoto and D. I. Arnon (1958) Nature 182, 351.

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

Vernon, L. P. and E. R. Shaw (1965) Biochemistry 4, 132.

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

Watson, A. and R. L. Livingstone (1950) J. Chem. Phys. 18, 802.

Weber, G. (1960) Symp. Comp. Biol. Kaiser Found. Res. Inst. 1, 395.

Weller, S. and J. Franck (1941) J. Phys. 45, 1359.

Wessels, J. S. C. (1968) Biochim. Biophys. Acta 153, 497.

Whatley, F. R. and D. I. Arnon (1963) in Methods in Enzymology, Vol. VI, S. P. Colowick and N. O. Kaplan, eds; Academic Press, New York and London, p. 308.

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

Witt, H. T., A. Müller and B. Rumberg (1961) Nature 192, 967.

Witt, H. T., G. Döring, B. Rumberg, P. Schmidt-Mende, V. Siggel and H. H. Stiehl (1966) Brookhaven Symp. Biol. 19, 161.

Yamashita, T. and W. L. Butler (1968) in Comparative Biochemistry and Biophysics of Photosynthesis, K. Shibata, A. Takamiya, A. T. Jagenforf and R. C. Fuller, eds., University of Tokyo Press, Yokyo, p. 179.