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DELAYED LIGHT STUDIES OF PHOTOSYSTEM II
IN HIGHER PLANT CHLOROPLASTS.

The City University of New York, Ph.D., 1975
Biology

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DELAYED LIGHT STUDIES OF PHOTOSYSTEM II IN HIGHER PLANT CHLOROPLASTS

by

DUNELL E. COHN

A dissertation submitted to the Graduate
Faculty in Biology in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy, The City University
of New York.

1975

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

DELAYED LIGHT STUDIES OF PHOTOSYSTEM II IN HIGHER PLANT CHLOROPLASTS

by

Dunell E. Cohn

Advisor: Walter Bertsch

Delayed light (a delayed fluorescence of chlorophyll a) emitted from spinach chloroplasts from 0.8 to 3.2 msec after the centers of repeating flashes of exciting light has been examined in the absence and in the presence of Hill acceptors under a wide variety of conditions. An attempt is made to characterize the two qualitative parameters of the delayed emission, the intensity and the decay kinetics, in terms of various apsects of energy handling and storage in photosynthesis. In addition, delayed light is used as a qualitative probe of certain aspects of the photosynthetic process in higher plant chloroplasts.

The decay kinetics of the delayed light over the time range examined are interpreted in terms of a kinetic model of photosystem II proposed by H.J. Van Gorkum and M. Donze (Photochem.Photobiol. 17, 333) in which the rapidly decaying components observable in the msec time range represent open reaction centers (capable of separating charge) in which stabilization of separated charge is prevented by the prior oxidation, or by the removal, of the electron donor to photoreaction II. More slowly decaying components of the delayed emission are associated with back reactions from reduced and oxidized components farther removed from the reaction center.

The intensity of the delayed light emission in this time range appears to be stimulated by the formation of the high energy state of photophosphorylation. However, this observation is interpreted as reflecting an effect of the acidic internal thylakoid pH on the exciton yield of the luminescent back reaction itself rather than as an effect of ΔpH on the rates of redox reactions in the vicinity of the reaction center of photosystem II.

The effects of uncouplers of photophosphorylation on the intensity of delayed light in the absence of Hill acceptors have led to an examination of the ΔpH formed under these conditions. The ΔpH formed in the absence of added electron acceptors (as estimated by the quenching of 9-aminoacridine fluorescence) was about 2.5 pH units, as compared to a ΔpH of 3.0 pH units in the presence of methyl viologen. The ΔpH formed in the absence of Hill acceptors was sensitive to DCMU and to dibromothymoquinone, as well as to uncouplers. It appears that the rate of electron flow through photosystem II under acceptorless conditions is too low to account for the observed ΔpH. It is suggested that the proton permeability of the thylakoid membrane may be different under acceptorless conditions than it is in the presence of rapid electron flow to an acceptor.

When chloroplasts are aged for 5 minutes at pH 9.6, or are exposed to uncouplers at pH 8.5 - 9.0, electron flow from water to Hill acceptors is inhibited. Both types of treatment induce rapid msec dark decay of delayed light emission. DCMU-sensitive electron transport through photosystem II can be regenerated in the inhibited chloroplasts by addition of the artificial electron donor, diphenylcarbohydrazide. Neither treatment inhibits electron flow through photosystem I. Uncouplers at alkaline pH, when added in the light, are less effective in producing the inhibition than when added in the dark. These results are discussed in terms of inhibition of the oxygen-evolving apparatus by alkaline intrathylakoid pH.

Conditions that inhibit water from donating electrons to photosystem II lead to a rapid decay of msec delayed light emission in the absence of electron acceptors. This rapid decay is unaffected by electron acceptors. The addition of electron donors to the inhibited chloroplasts slows the decay of the delayed light emission in some cases (i.e., Tris-aged chloroplasts), but not in others (i.e., ultraviolet-irradiated chloroplasts). This difference in the response of the delayed light emission to added donors is duplicated when DCMU is added. Based on the differential effects of donors and of DCMU, it is suggested that there exist at least two sites of inhibition of electron flow between water and photoreaction II.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my thesis advisor, Dr. Walter Bertsch, for several reasons: for encouraging me to enter into graduate studies, for his advice and guidance in the work presented here, and for his instilling in me a sense of independence as a research scientist.

I am especially grateful to Dr. William S. Cohen, a good friend and colleague with whom I have worked closely for several years, for his constant encouragement and particularly for the invaluable contribution to this work of his unbounded enthusiasm for research.

I wish to thank Dr. Shirley Raps and Dr. Ezra Shahn of the Department of Biological Sciences at Hunter College for their help in preparing this manuscript. To all the above, and to Dr. Richard E. McCarty of Cornell University, I am grateful for many stimulating and informative discussions.

A special thank you to my wife, Linda, whose patience, optimism, and love have been a constant source of support during the course of this work.

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ABBREVIATIONS

CCCP	carbonylcyanide-m-chlorophenylhydrazone
CF_1	chloroplast coupling $factor_1$
chl	chlorophyll
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DCPIP	2,6-dichloroindophenol
DLE	delayed light emission
DPC	1,5-diphenylcarbohydrazide
EDTA	ethylenediaminetetraacetate
FCCP	carbonylcyanide-p-trifluoromethoxyphenylhydrazone
FeCy	potassium ferricyanide
FMN	flavin mononucleotide
MES	2-(N-morpholino)-ethanesulfonic acid
MV	methyl viologen
pmf	protonmotive force
PS	photosystem
TES	N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid
Tricine	N-tris(hydroxymethyl)methylglycine
Tris	N-tris(hydroxymethyl)aminomethane
UV	ultraviolet

CHAPTER 1

INTRODUCTION

A. Introductory Statement

This thesis is an investigation, using delayed light emission as a central focus, of certain aspects of the interactions between quantum conversion, electron transport, and the high energy state of photophosphorylation in chloroplasts of higher plants.

Delayed light emission is most simply understood as the emission of light from chlorophyll that has been re-excited by energy stored during the process of photosynthesis. Photosynthetic energy storage in higher plants can be viewed as having several facets: 1) primary photochemical conversion of absorbed light energy into oxidizing and reducing equivalents (quantum conversion), 2) transfer of these oxidizing and reducing equivalents from the primary species to more stable forms (electron transport), 3) conservation of energy from electron transport in a form (high energy state) capable of generating ATP from ADP and P_i (phosphorylation), and 4) the utilization of the stabilized reducing power and ATP to drive synthetic reactions. In the broken chloroplasts used in the present research, the last of these processes is eliminated, and, in the absence of added ADP and P_i, the high energy state is formed, but not utilized. Delayed light emission appears to result from a direct reversal of quantum conversion, but this step itself, and thus the delayed emission, is influenced by the subsequent energy handling processes. The following introductory material is intended to provide historical background for this investigation, justification for the selection of working models to be used in interpretation of our results, and a brief outline of the specific areas of this research.

B. Chemical Aspects of the Overall Reaction of Photosynthesis

In the biological process of photosynthesis, light energy from the sun is stored in a metabolically available form as chemical energy. In algae and higher plants, photosynthesis involves the conversion of carbon dioxide and water into reduced carbon compounds with the concomitant release of molecular oxygen. Our present understanding of photosynthesis as consisting of the generation of oxidizing and reducing power by light comes from the work of C.B. van Niel (1931, 1935, 1941, 1949) comparing higher plant photosynthesis with bacterial photosynthesis. Bacterial photosynthesis achieves the same synthetic ends as that of higher plants, but does not evolve oxygen. Rather, it is dependent on the presence of an oxidizable substrate, such as hydrogen, hydrogen sulfide, or any of a number of simple organic compounds. Van Niel proposed that the two forms of photosynthesis were analogous to each other, and that both could be expressed as

$$CO2 + 2H_2A \xrightarrow[chl]{light} (CH_2O) + H_2O + 2A \qquad (1)$$

where (CH_2O) represents carbohydrate and H_2A an oxidizable substrate. The prediction that the molecular oxygen evolved by higher plants should be derived only from water, rather than from carbon dioxide, was confirmed soon afterwards by the isotopic labelling experiments of Ruben _et_ _al_. (1941).

Van Niel showed that neither the reduction of carbon dioxide nor the oxidation of a substrate were reactions unique to photosynthesis. The essential photosynthetic step was, he suggested, the generation of separated oxidizing and reducing equivalents by a photochemical reaction sensitized by chlorophyll. Van Niel's initial suggestion that water, itself, was the substrate photolyzed in this reaction has since been shown to be wrong. The actual identity of the primary products formed by the photoreaction are still unknown, but it is clear that water is oxidized by the primary donor to the photoreaction only after a number of biochemical steps. These steps are themselves unique to green plant photosynthesis, being the only known metabolic process in which water serves as an oxidizable substrate, i.e., as a source of electrons for the reduction of carbon dioxide.

The findings by Hill (1937, 1939) that illuminated extracts of green plants could reduce artificial electron acceptors with concomitant oxygen evolution, and by Gaffron

(1942) that the green alga, Scenedesmus, could be adapted to oxidize hydrogen instead of water with concomitant reduction of carbon dioxide, provided strong early support for Van Niel's general concept of photosynthesis.

C. Two Photoreactions

A number of observations have led to the concept that green plant photosynthesis involves two photoreactions. Emerson and Lewis (1941, 1943), in examining the quantum yield of photosynthesis in Chlorella, found that while the quantum yield (O_2 evolved/quantum absorbed) was fairly constant over most of the visible spectrum, it dropped sharply at wavelengths longer than 680 nm even though the absorption spectrum of chlorophyll a extended beyond 700 nm. In red algae, Haxo and Blinks (1950) found that light in the green region of the spectrum, which was absorbed by the phycobilins, was more efficient in promoting photosynthesis than was that absorbed by chlorophyll. Duysens (1952) showed that light absorbed by the phycobilins in these red algae was more efficient in sensitizing chlorophyll a fluorescence than was light absorbed by chlorophyll itself. Finally, Emerson and his coworkers found that the low quantum yield of photosynthesis in Chlorella observed at wavelengths longer than 680 nm could be dramatically raised by addition of a weak beam of shorter wavelength light, i.e., 560 nm (Emerson et al., 1957; Brody and Emerson, 1959).

These observations led Emerson and Rabinowitch (1960) to propose that two separate photosystems, each with its own characteristic absorption spectrum, acted together to produce the overall light-dependent processes of photosynthesis in green plants. A more detailed proposal in which the two photosystems interacted via a series of electron transport components was made simultaneously by Hill and Bendall (1960) partly as a result of studies by Hill and coworkers (Hill and Scarisbrick, 1951; Davenport and Hill, 1952) on the cytochrome components of the photosynthetic electron transport chain. This concept of two photosystems connected by electron transport components was proposed independently by Duysens _et_ _al_. (1961) based on his study of the effects of different wavelengths of light on the redox state of cytachrome _f_ in the alga, _Porphyridium_, and by Kok (1961) who observed similar effects on an absorbance change at 700 nm, the pigment responsible being designated P700. A two-photoreaction scheme was also proposed by Losada _et_ _al_. (1961) based on their ability to separate O_2 evolution from the reduction of $NADP^+$ by using a herbicide, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and artificial electron donors.

The two photosystems thus envisaged are assumed to function in series, as follows (see Fig. 1-1). Each photosystem (PS) effects a separation of oxidizing and reducing equivalents. PS II produces a weak reductant (Q^-) and a strong oxidant (Z^+), which is reduced, in a number of steps, ultimately by water, liberating oxygen. PS I generates a strong reductant, X^-, and a weak oxidant (oxidized plastocyanin);

the strong reductant reduces $NADP^+$ after a number of steps. The weak reductant of PS II and the weak oxidant of PS I combine via an electron transport chain connecting the two photosystems.

The chemical identities of Z, Q, and X in the above scheme are still the subject of controversy (see Trebst, 1974, for review). The electron transport chain connecting the two photosystems is also unresolved, although several components have been identified. It does seem clear that there exists a relatively large pool of secondary acceptors from PS II (Amesz, 1973; Witt, 1967) that has traditionally been designated as A, and has been identified as plastoquinone. The commonly used herbicide, DCMU, is believed to inhibit electron flow from Q to the A pool. The electron transport components linking the pool of plastoquinone to PS I are cytochrome f and plastocyanin.

The series formulation, or Z scheme, as outlined above is illustrated schematically in Fig. 1-1. Although this scheme has been challenged on a number of occasions (Arnold and Azzi, 1968; Arnon, 1971) it remains the working hypothesis for most investigators in photosynthesis.

D. The Photosynthetic Unit

Our understanding of the physical nature of the photosystems of photosynthesis derives from the observation of Emerson and Arnold (1932) that the maximum amount of oxygen evolved per flash using short (10^{-5}sec) saturating flashes of light was only one oxygen for every 2500 chlorophyll

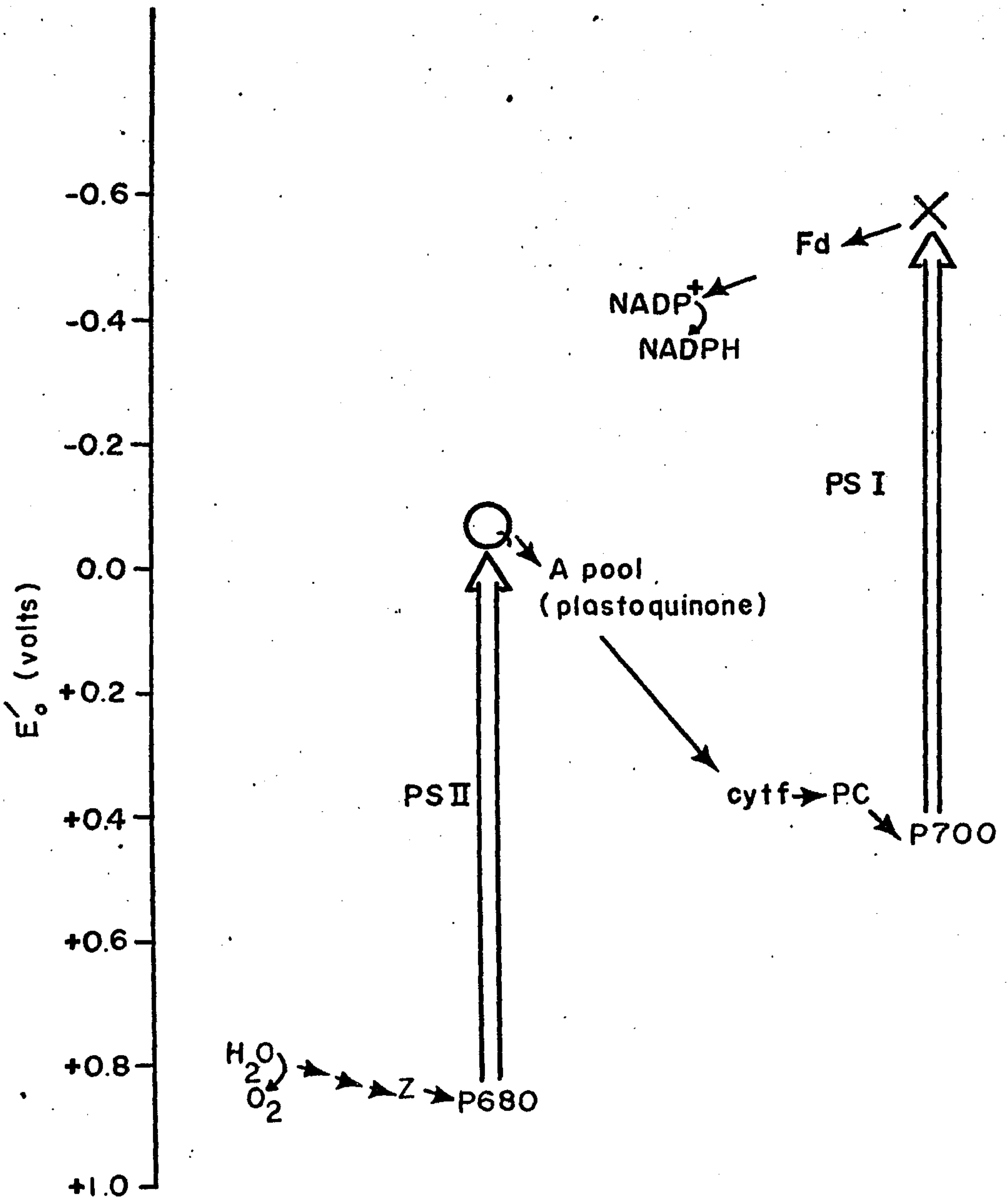


Fig. 1-1. The series model (Z scheme) for electron flow in higher plant photosynthesis.

molecules. Given the estimate of the quantum requirement for oxygen evolution of 8-12 quanta/O_2 (Emerson and Lewis, 1941, 1943), the observation of Emerson and Arnold indicates that a minimum of about 250 chlorophyll molecules must cooperate in the production of one oxygen molecule.

That cooperation between large numbers of chlorophyll molecules must occur was also demonstrated by Gaffron and Wohl (1936) who illuminated cells with such a dim light that years would have been required for any one chlorophyll to accumulate the minimum of four quanta they assumed would be necessary to produce one oxygen molecule from water. However, a steady-state rate of oxygen evolution was achieved after only 30 sec. A mechanism for such cooperation between pigment molecules was provided by Arnold and Oppenheimer (1950) and Duysens (1952) who applied Förster's theory of slow inductive resonance transfer (Förster, 1947, 1949) to photosynthesis.

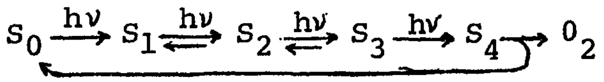
Our current picture of the photosynthetic unit derives from a model proposed by Duysens (1952) in which the bulk of each photosynthetic unit is composed of non-photoreactive ("light-harvesting" or "antennae") molecules, which transfer the energy of absorbed photons to the photoreactive "reaction center" by means of inductive resonance transfer. The probability that excitations will reach the reaction centers is high because the rate of transfer between molecules is very fast ($\geq 10^{12}$ sec^{-1}) compared to the rate of non-photochemical loss of the exciton (10^8 - 10^9 sec^{-1})

(Duysens, 1964; Pearlstein, 1966). In addition, the reaction centers generally have a lower singlet energy level than do the antennae molecules (Clayton, 1964; Hoch and Knox, 1968), which would increase the probability that the transferred energy will localize in the reaction center rather than in the bulk chlorophylls. The reaction centers have been spectroscopially identified and are designated P700 for PS I (Kok, 1961; Rumberg and Witt, 1964) and P680 for PS II (Döring *et al*., 1967; Döring, 1975).

E. The Oxidizing Side of Photosystem II

Although the reactions linking photosystem II to photosystem I are far from completely resolved, elucidation of the reactions between photosystem II and the oxidation of water has been even more refractory. The recent development of two approaches for studying the oxidizing side of photosystem II has begun to yield information about these reactions, which are unique to higher plant photosynthesis.

The first of these approaches has been the study of oxygen production induced by short flashes of light. In the early work of Emerson and Arnold (1932) only the integrated rate of oxygen production was measured. In 1955, Allen and Franck observed that a single flash of light following a dark period resulted in no oxygen evolution, but that oxygen was produced by a pair of flashes. This work has been extended more recently by Kok *et al*. (1970), Forbusch *et al*. (1971) and Joliot *et al*. (1971). Their results indicated that oxygen production was a periodic function of flash number, the maximum yield occurring on the third flash and the cycle repeating with a periodicity of four, presumably reflecting the necessity of accumulating four oxidizing equivalents for the oxidation of two H_2O to form one O_2. As the flash number was increased, the cycle began to damp out and eventually the yield became constant and equivalent to that observed by Emerson and Arnold (1932). Kok et al. (1970) have interpreted this observation as shown in the scheme below:



$$\begin{tikzcd} S_0 \arrow[r, "h\nu"] & S_1 \arrow[r, shift left, "h\nu"] & S_2 \arrow[l, shift left] \arrow[r, shift left, "h\nu"] & S_3 \arrow[l, shift left] \arrow[r, "h\nu"] & S_4 \arrow[r] \arrow[llll, bend left] & O_2 \end{tikzcd}$$

in which the S's represent different oxidation states of some precursor(s) of O_2. From the relative amounts of O_2 produced by successive flashes, Kok and coworkers have found that after prolonged darkness approximately 75% of the S states are in S_1 and 25% in S_0. They have interpreted this as indicating that, while S_1 and S_0 are very stable, S_2 and S_3 revert slowly back to S_1. S_4 is presumed to react rapidly to yield O_2. The rate constants for the return of S_3 to S_2 and for S_2 to S_1 have been estimated by varying the dark time between successive light flashes (Forbusch et al., 1971; Joliot et al., 1971).

Delayed light emission (Barbieri et al., 1970; Joliot et al., 1971; Zankel, 1971) and light emission stimulated by the addition of salts or by pH changes (Mayne and Hobbs, 1971a,b) have also been investigated as a function of the number of flashes of exciting, or preilluminating, light to which the system has been exposed after prolonged darkness. These phenomena were also observed to oscillate with a periodicity of four flashes, and these oscillations have been interpreted as reflecting the state of components on the oxidizing side of photosystem II. The oscillation in the delayed emission was in phase with 0_2 evolution for delayed light in the sub-msec time range (Zankel, 1971), but was out of phase with 0_2 evolution when delayed light was examined at times longer than about 5 msec after the exciting flash (Zankel, 1971; Joliot et al., 1971). This change in phase of delayed light emission relative to oxygen evolution may reflect the rate of some reaction on the oxidizing side of PS II, perhaps the movement of oxidizing equivalents from the donor (Z) of PS II into the "S" states (Van Gorkum and Donze, 1973).

A second recent approach to the oxidizing side of photosystem II began with the discovery by Yamashita and Butler (1968a) that incubation of chloroplasts in high concentrations of Tris buffer at pH 8.0 eliminated electron flow from water to Hill oxidants. DCMU-sensitive electron flow to these Hill reagents was restored by addition of a suitable electron donor. They suggested that the inhibition was due to a lesion in the electron transport reactions between water and photosystem II. In the years since this initial suggestion, a great many treatments have been shown to exhibit similar behavior. From the study of some of these treatments (e.g., heat, NH_2OH incubation, Tris-aging,

Cl^- depletion), it has been suggested that Mn^{2+} (Cheniae and Martin, 1971; Homann, 1968) and Cl^- (Heath and Hind, 1969) are involved in the reactions linking water to the photosystem II reaction center. Mn^{2+}, particularly, has been found to be released from a bound state in normal chloroplasts by several of the oxidizing side treatments and to be oxidized by photosystem II when it is added back to these systems (Lozier et al., 1971). These observations, and the ability of manganese to assume a variety of oxidation states, have made this bound Mn^{2+} an attractive candidate for the accumulation of oxidizing equivalents prior to water oxidation.

F. Photophosphorylation

Light dependent phosphorylation of ADP to form ATP in isolated chloroplasts was first observed by Arnon et al. (1954). This synthesis of ATP was not accompanied by net oxidation or reduction of substrates and was termed cyclic photophosphorylation. Simultaneous reduction of $NADP^+$ and ATP synthesis, noncyclic photophosphorylation, was reported four years later from the same laboratory (Arnon et al., 1958).

Three major hypotheses have been developed for the mechanism by which redox reactions in the electron transport chain are coupled to the synthesis of ATP. In the first of these, the "chemical coupling hypothesis", a high energy chemical compound involving one of the electron transport components is presumed to be the intermediate between electron transport and ATP synthesis. In the other two models, the "chemiosmotic hypothesis" and the "charge distribution hypothesis", the high energy intermediate created by the redox reactions of electron transport is not a chemical compound, but an electrochemical activity difference across or within a membrane. Some of the basic features of these hypotheses are discussed in this section with the view of justifying selection of the chemiosmotic hypothesis as the working model for interpretation of our results.

At the time of the discovery of photophosphorylation, the prevalent hypothesis for the coupling of electron transport to phosphorylation, as expressed, for example, by Chance and Williams (1956), was derived from studies of oxidative phosphorylation in mitochondria. According to this hypothesis (the chemical coupling hypothesis), a covalent bond is formed between a reduced transport component, AH_2, and a species X. Upon oxidation of this complex, the bond to X is elevated to a high energy level, $A{\sim}X$. In a series of reactions in which P_i replaces X and ADP replaces A, ATP is synthesized. This hypothesis appears to require that a single redox reaction in the electron transport pathway provide the energy necessary for ATP synthesis.

Once the two-photoreaction scheme for photosynthesis (Hill and Bendall, 1960) was accepted, it was generally assumed that non-cyclic ATP synthesis was coupled to electron flow only between the two photoreactions, since this region of electron transport had a potential drop of about 0.4 eV and contained a cytochrome, two characteristics that made this region easily adaptable to the chemical coupling hypothesis above. However, recent evidence indicates that the electron transport reactions between photosystem II and water and between photosystem I and $NADP^+$ may also be involved in providing energy for ATP synthesis (Trebst, 1974). While it is difficult for the chemical coupling hypothesis to accomodate the idea that several electron transport reactions may cooperate to produce ATP, this idea is easily incorporated into the chemiosmotic hypothesis (see below).

The chemical coupling hypothesis has been weakened over the years, particularly with respect to photophosphorylation, for several reasons. First, it has no requirement for a closed, membraneous system. While there have been several reports of phosphorylation coupled to electron transport reactions in non-vesicular systems (Cole and Aleem, 1973; Hunter _et_ _al_., 1974), none of these has been upheld upon closer examination. Second, evidence from experiments in which photophosphorylation is separated into a light and dark stage have indicated that the high energy "intermediate" created by electron flow in the light is formed in large amounts and is long-lived (Shen and Shen, 1962; Hind and Jagendorf, 1963). The failure, after extensive efforts, to observe a high energy chemical intermediate (as required by the chemical coupling hypothesis)leads to the speculation that such a compound may not exist. Third, the hypothesis does not require the membrane potentials and ion fluxes that have been associated with the coupling of electron flow to phosphorylation. These latter phenomena have been incorporated into successive modifications of the chemical hypothesis, but their incorporation has made the hypothesis unwieldy. The chemical hypothesis has thus become limited in its usefulness as a model for coupling.

Both of the two other general types of coupling hypotheses focus on the concept that electron transport leads to differences in proton activity and in electrical charge either within (charge distribution hypothesis) or across (chemiosmotic hypothesis) a membrane, and that these differences provide the driving force for the phosphorylation of ADP. The charge distribution model has been most clearly stated by Williams (1969), and the chemiosmotic hypothesis by Mitchell (1961, 1966). The chemiosmotic hypothesis has been extensively tested over the past several years and has been strengthened by the large number of its predictions that have been experimentally confirmed. Therefore, of the available hypotheses, this thesis will use the chemiosmotic approach as a working model in interpreting data relevant to the high energy state of photophosphorylation. Its selection as a working model is not based on a firm conviction that it is correct in all aspectsto the exclusion of other hypotheses. Indeed, it appears likely that in the future, aspects of all three hypotheses mentioned above will form part of a more complete understanding of the coupling of electron flow to phosphorylation.

The basic features of the chemiosmotic hypothesis can be summarized briefly, as follows. Electron flow results in the electrogenic translocation of protons across a closed membrane (enclosing an internal space) that exhibits a low permeability to protons. This translocation is due to alternation in the electron transport chain of electron and hydrogen carriers, transversly oriented across the membrane. There exists a membrane-bound ATPase whose reactions are vectorial across the membrane in such a way as to translocate protons during ATP synthesis in the direction opposite to the electron transport-generated translocation. In this model the high energy intermediate, or "high energy state", between electron transport and ATP synthesis is the electrochemical activity gradient of protons across the closed membrane. This electrochemical gradient for protons has two components, the proton concentration difference (ΔpH) and the electrical charge difference ($\Delta\Psi$) across the membrane. The total electromotive force for protons has been designated the "proton motive force" (pmf), and can be expressed as follows:

$$\mathrm{pmf} = \Delta\Psi + \frac{RT}{F}\Delta\mathrm{pH}$$

where R and T are the molar gas constant and absolute temperature, respectively, and F is the Faraday constant. In chloroplasts (in contrast to mitochondria) the translocation of protons coupled to electron transport is from the outside to the inside of the closed inner membrane of the chloroplast, the thylakoid. The above ΔpH thus represents $pH_o - pH_i$. The $\Delta\psi$ represents excess positive charge inside the thylakoid.

Early work inspired by the chemiosmotic hypothesis and confirming many of its predictions includes the observation of proton uptake from the external medium (Jagendorf and Hind, 1963; Neumann and Jagendorf, 1964), the separation of ATP synthesis into light and dark stages, demonstrating that the high energy intermediate is long-lived (Shen and Shen, 1962; Hind and Jagendorf, 1963), and the synthesis of ATP in the dark upon an abrupt transition from an acid medium to a basic one, thus artificially creating the proposed high energy intermediate (Jagendorf and Uribe, 1966a,b).

The coupling factor of photophosphorylation, CF_1, was isolated independently by Avron (1963) and by Vambutas and Racker (1965) as a large multi-subunit protein. When membrane-bound, the coupling factor has a latent Mg^{2+}-dependent ATPase activity. When this ATPase is activated (Petrack and Lipman, 1961; Petrack _et al._, 1965) the hydrolysis of ATP is coupled to the translocation of protons into the thylakoid (Carmeli, 1970; Carmeli _et al_, 1975), that is, in the direction predicted by the chemiosmotic hypothesis.

Recently, reconstitution experiments have been performed in which purified coupling factors from yeast mitochondria have been incorporated into vesicles formed from soybean phospholipids. When subjected to an artificially imposed proton gradient, ATP is synthesized from ADP and P_i (Ryrie, personal communication). Racker and Stoeckenius (1974) have reported ATP synthesis from reconstituted vesicles containing the oligomycin-sensitive ATPase and coupling factors from bovine heart mitochondria in which a proton gradient is formed across the vesicle membrane by a proton-pumping bacteriorhodopsin from the purple membrane of Halobacterium halobium. These systems appear to contain no electron transport carriers. If this finding is substantiated in further investigations and is extended to photosynthetic systems, such investigations would provide direct evidence against the idea that the high energy intermediate of photophosphorylation is a chemical compound that directly involves an electron transport component.

In chloroplasts, the existence of a net translocation of protons coupled to electron transport from the exterior to an interior region where they remain chemically active was clearly shown by the studies of Gaensslen and McCarty (1971) on the light-induced uptake of ammonia and labelled amines by chloroplasts. The partition of low concentrations of labelled amines between the exterior and interior of the thylakoid has allowed estimation of the magnitude of the pH difference established in the light. These estimates indicate that the transmembrane ΔpH formed in chloroplasts may be in the range of 2.0 to 3.5 units (Rottenberg and Grunwald, 1972; Rottenberg et al., 1972; Portis and

McCarty, 1973).

Studies of the membrane potential ($\Delta\Psi$) developed in the light have mainly relied on using the absorption band shifts of carotenoids and other pigments at about 515 nm as indicators of the membrane potential. The light-induced absorption band shift in bacterial chromatophores was shown to be identical with the absorption shifts seen in the dark upon establishment of diffusion potentials that made the inside of the chromatophore positive with respect to the outside (Jackson and Crofts, 1969). Calibration of the optical changesobserved with the calculated diffusion potentials allowed estimation of the light-induced $\Delta\Psi$. In bacterial chromatophores the magnitude of $\Delta\Psi$ was found to be about 250 mV (inside positive) in the steady-state, following a transient spike of 420 mV (Jackson and Crofts, 1969). Strichartz and Chance (1972) have made similar observations in chloroplasts and found a steady-state $\Delta\Psi$ of 30 mV positive on the inside. Other techniques of estimating $\Delta\Psi$ have led to somewhat higher values for chloroplasts, up to 100 mV (Witt, 1971), but still considerably lower than the $\Delta\Psi$ found in bacterial chromatophores.

The rather large difference between the estimated $\Delta\Psi$ in bacterial chromatophores and that in chloroplasts may be related to the relative importance of the two components of the total pmf in driving ATP synthesis in the two systems. In chloroplasts it appears that $\Delta\Psi$ alone is incapable of driving ATP synthesis, and that in this system the proton concentration gradient, ΔpH, is the major component of the high energy state. Evidence for this comes from the observation that phosphorylation in chloroplasts can be uncoupled by the addition of nigericin (Shavit et al., 1968) or ammonia (McCarty, 1969). The antibiotic nigericin effects an exchange of cations (particularly H^+ for K^+) across the membrane (Chappell and Crofts, 1965), thus reducing the ΔpH and replacing it with a K^+ concentration difference. Since this exchange is electrically neutral, the membrane potential should not be affected. Ammonia, likewise, is believed to reduce ΔpH by being taken up as the neutral amine and becoming protonated inside the thylakoid, but should not affect $\Delta\Psi$, since the protonated form is relatively impermeable to the membrane (Crofts, 1966; McCarty, 1969).

In chromatophores (Jackson et al., 1968; Briller and Gromet-Elhanan, 1970) and in small subchloroplast particles (McCarty, 1969; Hauska et al., 1970), on the other hand, it has been found that both the ΔpH and the $\Delta\Psi$ components of the pmf must be reduced in order for uncoupling of phosphorylation to occur. In the subchloroplast particles, phosphorylation was virtually unaffected even when proton uptake was almost totally inhibited by NH_4Cl or nigericin (McCarty, 1968; Hauska et al., 1970). Uncoupling by these compounds required the addition of valinomycin, an antibiotic that allows K^+ or $NH_4{}^+$ to move across the thylakoid membrane along its electrochemical gradient, thus reducing the membrane potential.

It has been suggested that these differences between chloroplasts and subchloroplast particles may result from differences in their relative permeabilities to Cl^- (McCarty, 1959). Thus, a large membrane potential may not form in chloroplasts because H^+ uptake can be electrically compensated by Cl^- uptake (Deamer and Packer, 1969; Gaennslen and McCarty, 1971). A lower permeability to Cl^- in the subchloroplast particles would lessen the degree to which protonic movements could be electrically compensated, resulting in the development of a larger membrane potential and a smaller ΔpH. The difference in Cl^- permeability of these systems could be intrinsic, or it may be related to their ability to swell under the influence of K^+ or NH_4^+ uptake (Walker, 1975).

The above observations indicate that the driving force for ATP synthesis, the total pmf, may be represented by different contributions of the two components, ΔpH and $\Delta\Psi$, depending on the properties of the system involved. In chloroplasts, under normal conditions, the pmf would appear to be largely in the form of a proton concentration difference.

G. Delayed Light Emission

Delayed light emission (DLE) was first observed by Strehler and Arnold (1951) and was tentatively identified by them as a delayed fluorescence. It has since been found to be a general phenomenon associated with all photosynthetic organisms. The similarity of the delayed emission spectrum to that of prompt fluorescence was confirmed by Arnold and Davidson (1954) and Arnold and Thompson (1956). However, the latter did report a slight shift to longer wavelengths for the delayed emission. The action spectrum for the delayed light emission was found to be that for photosynthesis in the organism concerned (Arnold and Davidson, 1954; Arthur and Strehler, 1957).

In higher plants and algae delayed light emission (DLE) originates from photosystem II. Bertsch *et al*. (1967) found that a mutant of *Scenedesmus* lacking photosystem II emitted approximately 300 times less delayed light than the wild type, but that a mutant lacking photosystem I activity emitted 50 times more. The involvement of photosystem II was also implicated by earlier studies on the effects of interactions between the two photosystems on the emission (Goedheer, 1962; Bertsch *et al*., 1962). A requirement for functional reaction centers for DLE in bacteria was shown by Clayton and Bertsch (1965) using a mutant of *Rhodopseudomonas spheroides* lacking reaction centers. The observation by Arnold and Thompson (1956) that the delayed emission from *Chloropseudomonas ethylicum* occurred at slightly longer wavelengths than prompt fluorescence is now understood in terms of the concept that the excitons responsible for delayed light originate in the reaction center, whereas prompt fluorescence originates primarily in the accessory chlorophylls (Clayton, 1969).

In their first report on the phenomenon of delayed light emission, Strehler and Arnold (1951) suggested that the emission reflected the partial reversability of the early steps of quantum conversion. This view has received widespread support (Ross and Calvin, 1967; Lavorel, 1967, 1968; Bertsch, 1967; Arnold and Azzi, 1968; Clayton, 1969). The discovery that dried films of chloroplasts exhibited several phenomena, such as thermuluminescence (Arnold and Sherwood, 1957, 1959), generally associated with semi-conductors, has led to the development of several solid-state models for quantum conversion (Bertsch, 1969; Arnold and Azzi, 1968). In these models, delayed light may arise from other processes associated with quantum conversion, in addition to the reversal of charge separation. These processes would tend to be of importance mainly in the microsecond time range. Since, in this thesis, delayed light is examined only in the msec time range, the simpler idea that delayed light originates solely from the re-excitation of chlorophyll by separated oxidizing and reducing equivalents (holes and electrons, respectively, in the language often used in photosynthesis, and itself derived from solid-state concepts) will generally be used in the interpretation of our results.

In studies of prompt fluorescence and delayed light emissions under identical conditions, Lavorel (1967, 1968) found that, although the reduced species of the fluorescence quencher, Q^-, was a necessary requirement for delayed emission, it was not sufficient, and a second substrate was required.

He suggested that this second substrate was the species oxidized by the photoreaction, Z^+. He thus suggested that the reexcitation of chlorophyll was due to the direct reversal of the primary charge separation in photosystem II:

$$Z\ chl\ Q \underset{DLE}{\overset{h\nu}{\rightleftharpoons}} Z^+\ chl\ \ Q^-$$

It may be more correct to view the above reaction as a two step process (Duysens, 1971; Van Gorkom and Donze, 1973; Butler, 1973) as below:

$$Z\ P\ Q \underset{DLE}{\overset{h\nu}{\rightleftharpoons}} Z\ P^+\ Q^- \rightleftharpoons Z^+\ P\ Q^-$$

where P is the photoreactive chlorophyll (perhaps P680). In this model, delayed light originates from the recombination of P^+ AND Q^-, and this back reaction is normally prevented by rapid reduction of P^+ by Z.

Delayed light emission has been examined over a time range extending from microseconds to minutes after excitation. Over limited time ranges, various workers have analyzed the decay kinetics of the emission in terms of combinations of several exponential or second order components (Arthur and Strehler, 1957; Ruby, 1968; Clayton, 1969; Bonaventura and Kindergan, 1971; Zankel, 1971). What emerges from these results is that in no case is the process a simple one. The complexity of the process is also indicated by the differential effects of inhibitors (i.e., DCMU) or other agents (i.e., Hill acceptors) on the emission at different times during the decay (Arthur and Strehler, 1957; Bertsch _et al._, 1963; Clayton, 1969). A further complicating factor is that the intensity of exciting light necessary to saturate the delayed light has been found to decrease as the time after excitation at which DLE is measured is increased (Bertsch _et al_., 1963; Arnold, personal communication).

Although delayed light emission has been used in many attempts to understand the mechanism of quantum conversion, the measurement can also be used as a sensitive probe in examining other aspects of the photosynthetic system. Two of these aspects that affect delayed light emission and that will be dealt with in this thesis are 1) the high energy state of phosphorylation and 2) the electron transport reactions between the reaction center of photosystem II and water. Mayne (1967) originally suggested that the intensity of delayed light emission was influenced by the magnitude of the high energy state of phosphorylation. Since then, a large body of literature has developed supporting the concept that the high energy state can provide some of the energy for the back reactions leading to delayed light emission (see Fleischman and Mayne, 1973, for review). It has also been observed that the decay kinetics of msec delayed light emission are affected in a characteristic way by several of the treatments that inhibit electron flow on the oxidizing side of photosystem II (Bertsch and Lurie, 1971; Vernon _et al_., 1971; Lurie, 1972).

H. Present Experiments

Since many of the methods used in the experimental chapters of this thesis (Chapters 3-5) pertain to more than one of the problems investigated, the methods used throughout are presented together in Chapter 2.

In Chapter 3 the effects of various concentrations of uncouplers of photophosphorylation on msec delayed light emission are examined both in the absence and presence of Hill acceptors. These effects are correlated with measurements of the high energy state developed under similar conditions. Our results generally confirm previous observations, but the dependence of delayed light emission intensity on the pH of the medium may imply a larger role for the internal thylakoid pH in modulating the emission than for the ΔpH.

Continuing these studies of the effects of uncouplers on delayed light emission, it is shown in Chapter 4 that raising the pH of the medium in the presence of uncouplers results in inhibition of electron transport on the oxidizing side of photosystem II. These results are interpreted in terms of an effect of the internal pH of the thylakoid on the oxygen-evolving system.

In Chapter 5, the decay kinetics of msec delayed light emission are examined for a number of treatments that inhibit on the oxidizing side of photosystem II. We have found that these treatments appear to fall into two categories with respect to the behavior of the delayed light emission. We suggest that these categories may reflect different sites of action for different treatments, and discuss models that may explain the behavior observed.

Chapter 6 summarizes some of our conclusions about msec delayed light emission and the kind of information it can provide about photosystem II of higher plants.

CHAPTER 2

METHODS AND MATERIALS

A. Chloroplast Preparation

Chloroplasts were isolated from greenhouse-grown Good King Henry (*Chenopodium bonicus henricus*), fresh market spinach (*Spinacea oleracea*) or greenhouse-grown oat (*Avena sativa* var. Garry) seedlings following the method of Jagendorf and Avron (1958). Unless otherwise noted, spinach chloroplasts were used. Fifty grams of leaves were deveined and placed in a Waring Blender jar with 100 ml of ice-cold grinding medium (0.4 M sucrose, 0.05 M Tricine-NaOH, pH 7.8, and 0.01 M NaCl). The leaves were blended for 10 sec at high speed. The resulting suspension was filtered through four layers of cheese cloth, and the filtrate was centrifuged at 1500 x g for one minute to remove cellular debris. The supernatant was then centrifuged at 1500 x g for seven minutes to sediment the chloroplasts, which were washed with approximately 40 ml of fresh grinding medium. After recentrifuging at 6500 x g for five minutes, the pellet was resuspended in 2 to 3 ml of resuspension medium (100mM sucrose, 5 mM TES, pH 7.4, and 20 mM NaCl). The chlorophyll concentration was determined spectrophotometrically according to Arnon (1949) and was then adjusted to 1.0 mg chlorophyll/ml with additional resuspension medium. The chloroplast suspension was then stored on ice until use.

B. Treatments of Chloroplasts

1. EDTA-treatment. EDTA-treated chloroplasts were prepared according to the method of Cohen and Bertsch (1974). The chloroplast pellet (prepared in section A above) was resuspended in 0.1 M sucrose, 5mM TES-NaOH, pH 7.4 and was then centrifuged at 6500 x g for 5 min. The supernatant was discarded and the pellet was resuspended for 5 min on ice in 1 mM EDTA (previously adjusted to pH 7.5 with NaOH) at a chlorophyll concentration of 0.1 mg/ml. This suspension was again centrifuged (27,500 x g for 5 min) and the resulting pellet was resuspended in the normal resuspension medium used in section A above.

2. Aging at Alkaline pH. Chloroplasts were "aged" at alkaline pH by incubation at a chlorophyll concentration of 100 ug/ml in a medium containing 20 mM NaCl, 50 mM Tricine and 2 mM $MgCl_2$, adjusted to pH 9.6. After 5 min incubation in the dark on ice, the suspension was titrated to pH 7.5 and was stored on ice until use. Control chloroplasts in these experiments were treated identically except that the incubation medium was maintained at pH 7.5 at all times.

3. Tris-Aging. Tris-aged chloroplasts were prepared by a procedure modified from that of Yamashita and Butler (1968a). The isolation procedure for normal chloroplasts was followed up to the washing step. Instead of washing in grinding medium, the pellet was resuspended in 0.8 M Tris-HCl (pH 8.0) and allowed to incubate for 15 min on ice.

The suspension was then centriguged at 12,000 x g for 5 min. The resulting pellet was resuspended in the normal resuspension medium. When Tris-aged chloroplasts were prepared, untreated chloroplasts were treated identically except that grinding medium replaced the 0.8 M Tris-HCl.

4. Heat Treatment. One-half ml aliquots of normally prepared chloroplasts were incubated in a constant temperature water bath at 50°C for 2 min at chlorophyll concentration of 1 mg/ml and were then stored on ice until use.

5. Ultraviolet Irradiation. Chloroplasts were resuspended in a medium consisting of 25 mM NaCl, 4 mM $MgCl_2$, and 25 mM TES-NaOH, pH 7.2 at 100 ug chl/ml and were irradiated in a Petri dish. The chloroplast layer was approximately 2 mm thick and was stirred with a magnetic stirrer during the period of irradiation. The temperature was maintained at 4°C using an ice-water bath. The chloroplasts were irradiated for 7 min at a distance of six cm from a G.E. G8T5 8-watt germicidal lamp (2537 Å). Control chloroplasts were treated identically but were not irradiated.

6. Chloride Depletion. Chloroplasts depleted of chloride were prepared according to the EDTA-washing procedure of Izawa _et al_. (1969). Fifty grams of market spinach leaves were deveined and were ground in 150 ml of ice cold medium containing 0.4 M sucrose, 25 mM TES-NaOH, pH 7.4, and 5 mM Na_2SO_4. The homogenate, filtered through four layers of cheese cloth, was centrifuged at 2500 x g for 5 min. The sedimented chloroplasts were resuspended and washed once with

0.1 M sucrose and 0.5 mM TES-NaOH, pH 7.4, twice with 2 mM Tricine and 0.5 mM Na-EDTA at pH 7.8, and finally with 0.1 M sucrose, 5 mM TES-NaOH, pH 7.4, and 5 mM $MgSO_4$. The chloroplasts were then resuspended in a minimum volume of the sucrose-TES-$MgSO_4$ medium above.

7. Other Treatments. Triton X-100, trypsin, and hydroxylamine treatments of chloroplasts were performed according to the methods of Malkin (1971), Selman and Bannister (1971) and Katoh *et al*. (1970), respectively. Whole cells of *Euglena gracilis* strain Z were depleted of manganese as described by Heath *et al*. (1969b)

C. Photochemical Measurements

1. Spectrophotometric Determinations. Photoreductions of the Hill acceptors dichloroindophenol and potassium ferricyanide were measured spectrophotmetrically using an Aminco-Chance Dual Wavelength Spectrophotmeter modified to allow side illumination of the sample by a red actinic beam. The actinic light, provided by a 650 watt tungsten-iodide lamp, was filtered through a red glass filter (Schott RG665), a heat absorbing filter (Schott KG-1), and 7 cm of water. The light intensity incident on the sample was approximately 10^6 erg/cm^2/sec as measured by a Yellow Springs radiometer. Chloroplasts were used at a concentration of 10 ug chlorophyll/ml.

Dichloroindophenol reduction was measured by following the absorbance decrease at 590 nm, using 470 nm as a reference wavelength. The photomultiplier was protected from the red actinic light by two blue glass filters (Corning 4-76 and 4-96) and a green Kodak Wratten filter (#57). The initial concentration of dichloroindophenol was 12 uM. Chloroplasts were used at a concentration of 10 ug chlorophyll/ml.

Photoreduction of ferricyanide was measured following the absorbance change at 420 nm, with 480 nm as a reference wavelength. In this case, a blue glass filter (Corning 4-72) and 1 cm of $CuSO_4$ solution (200 g/l) protected the photomultiplier from stray exciting light. The initial $K_3Fe(CN)_6$ concentration was 0.3 mM. Chloroplasts were used at a concentration of 10 ug chlorophyll/ml.

2. Oxygen Electrode Measurements. Changes in oxygen concentration were measured using a Beckman 39065 electrode mounted in a specially built, thermostatically-controlled lucite chamber. The exicting light, provided by a 650 watt tungsten-iodide lamp, was passed through 8 cm of water and a red glass filter (Corning 2-62). The light intensity incident on the sample was approximately 10^6 erg/cm^2/sec. Chloroplasts were used at a concentration of 20 ug chlorophyll/ml.

The above apparatus was used to follow oxygen evolution coupled to reduction of either dichloroindophenol (0.012 mM) or $K_3Fe(CN)_6$ (0.3 mM). Oxygen consumption associated with the autoxidation of photoreduced methyl viologen was monitored in the same apparatus. For this reaction, methyl viologen was used at a concentration of 0.1 mM, and NaN_3

(0.5 mM) was added to inhibit any catalase activity that may have been present.

D. The High Energy State of Photophosphorylation

1. Quenching of 9-Aminoacridine Flourescence. The quenching of the fluorescence from 9-aminoacridine was used as an indicator of the light-induced transmembrane pH difference (Kraayenhof, 1970; Rottenberg *et al.*, 1971). The fluorescence was measured in a Zeiss Spectrofluorometer (Model ZFM4) modified to allow actinic irradiation of the sample from the top. The red actinic beam was provided by a Unitron 500 watt microscope illuminator, filtered through a heat filter (Schott KG-1) and a red glass filter (Schott RG665). The light intensity incident on the sample was about 8 x 10^5 erg/cm^2/sec. The fluorescence of the acridine dye was excited by broad blue illumination provided by the Hg lamp of the Zeiss Spectrofluorometer, filtered through an Optics Technology short band pass filter, a Corning blue glass filter (5-58) and a 1.0% transmission Balzars neutral density filter to give an incident intensity on the sample of about 10^3 erg/cm^2/sec. The fluorescence emission of the acridine dye was passed through the Zeiss quartz prism monochromator (Model M4 QIII) at 510 nm (2 mm slit width) and 1 cm of $CuSO_4$ solution (200 g/l) which prevented stray light from the actinic beam from reaching the photomultiplier. The output of the spectrofluorometer was fed into a Sargent Model SRG strip recorder. Chloroplasts were used at a concentration of 10 ug chlorophyll/ml and the concentration of the acridine dye was 2 uM. Concentrations of Hill acceptors

used in these experiments were the same as for the photochemical measurements discussed in section C above.

2. Absorbance Change at 515 nm. The light-induced absorbance increase at 515 nm, using 540 nm as the reference wavelength, was measured in the Aminco-Chance Dual Wavelength Spectrophotometer. The red actinic beam was as described in Section C.1 above. The photomultiplier was protected from stray exciting light by a Corning blue glass filter (CS 4-96) and 1 cm of $CuSO_4$ solution (200 g/l). Chloroplasts were used at a concentration of 30ug/ml. Upon illumination, the absorbance at 515 nm rose very rapidly to a maximum and then more slowly declined to a constant value. The initial, rapid on-responses were usually taken for the absorbance changes reported here, but where qualitatively different responses occurred in the steady-state, both the on- and off-responses are reported.

3. Proton Uptake. Light-induced proton uptake was determined in the same chamber and with the same actinic illumination as described for the oxygen electrode apparatus (sec. C above). Changes in pH were detected with an Ingold (Cat. 20371) combination electrode connected to a Corning Model 12 pH meter. The output was displayed on a Sargent Model SRG strip recorder. The pH changes were calibrated after each experiment by the addition of a standard aliquot of HCl.

E. Delayed Light Emission

Delayed light emitted from 0.8 to 3.0 msec after the centers of repeating flashes of white light was measured using a modified Becquerel phosphoroscope as previously described (Bertsch et al., 1963; Bertsch and Lurie, 1971). A schematic diagram of the instrument is shown in Fig. 2-1. The sample was held in a 2 ml cellulose nitrate centrifuge tube between two spinning discs mounted on a common shaft. The discs were mounted in narrow slots in an aluminum housing to provide adequate light baffling. The discs and slots were painted with flat black paint. The sample was illuminated through two oppositely positioned holes in the front disc. The rear disc was cut into the shape of a paddle wheel, the blades of which were aligned with the holes in the front disc, thus protecting the photomultiplier from the exciting flashes and the attendant prompt fluorescence of the sample. The spinning discs were rotated at 125 revolutions per second to provide flashes 250 times per second (4 msec interval between flashes). The signal from the photomultiplier (RCA 8852 Quantacon) was fed into a Tektronix 585A oscilloscope with a type D plug-in amplifier, and the trace on the oscilloscope was photographed to record the data. Due to the width of the paddle wheel sections of the rear disc and the RC time constant of the oscilloscope amplifier, the earliest time that delayed light could be measured was 0.8 msec after the center of the exciting flash.

All delayed light measurements were made at 25°C. The exciting light was provided by a 650 watt tungsten-iodide lamp, filtered through 12 cm of water and focussed onto

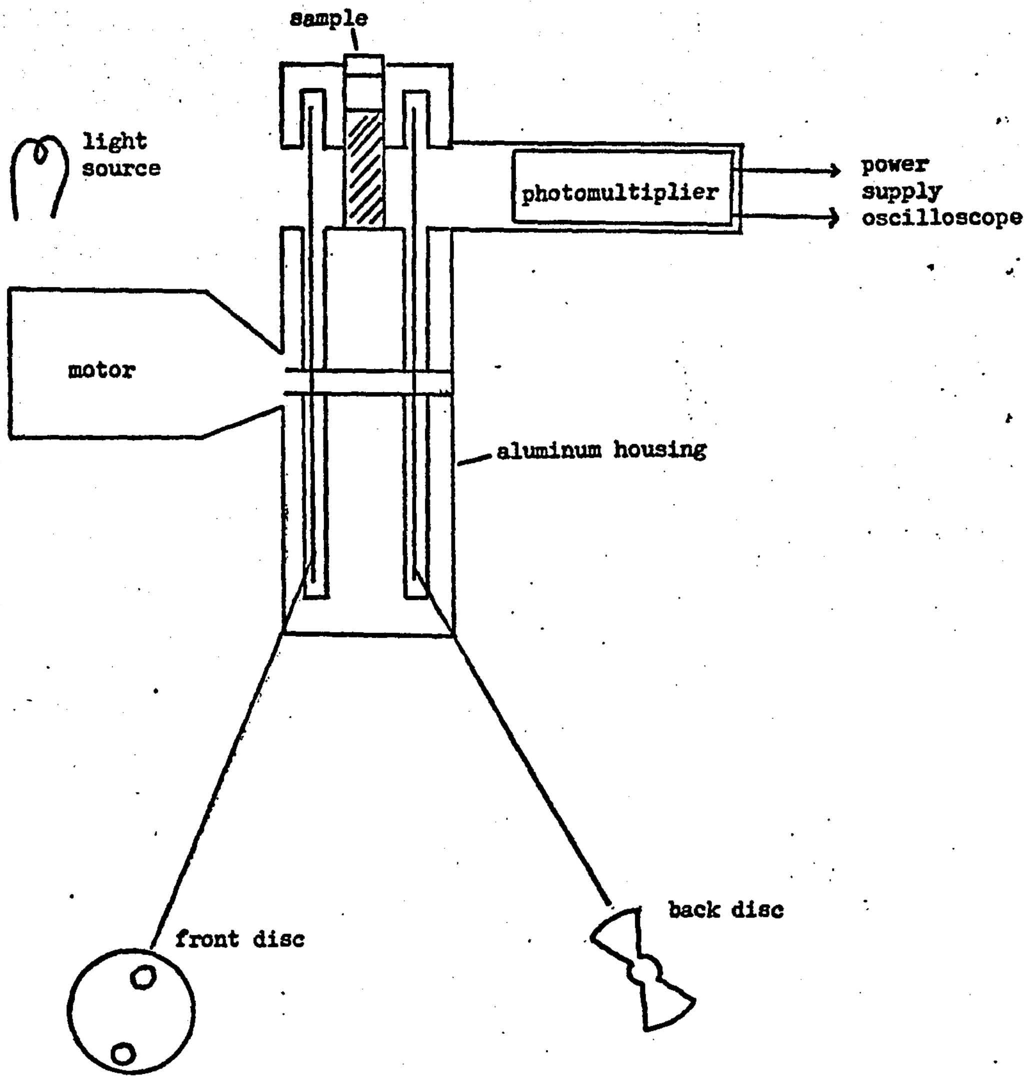


Fig. 2-1. Schematic diagram of the phosphoroscope. See text for description.

the opening of the phosphoroscope. The exciting light intensity incident on the sample could not be measured directly, but Lurie, by measuring rates of DCPIP photoreduction in situ, has estimated it to be close to saturating for photosynthesis (personal communication). In order to eliminate induction transients, all samples were exposed to 30 sec of flashes at 10% of the full intensity before the trace on the oscilloscope screen was photographed. This initial photograph was always taken in the absence of an electron acceptor. Following this, either dichloroindophenol (1 um final concentration) or $K_3Fe(CN)_6$ (0.3 mM final concentration) was injected into the sample with a hypodermic syringe. Again, transient effects were avoided by waiting until the oscilloscope trace was stable (10 sec for DCPIP, 30 sec for ferricyanide) before it was photographed. Chloroplasts were used at a concentration of 10 ug chlorophyll/ml.

F. Reaction Conditions and Reagents

The basic reaction mixture consisted of 20 mM Tricine-NaOH, 20 mM NaCl, and 2 mM $MgCl_2$. KCl replaced NaCl in all experiments using nigericin or valinomycin. Deviations from these conditions will be noted. All reactions were carried out at 25° C.

The nigericin-like antiobiotic X-464 was a gift from Dr. W. E. Scott of Hoffman-LaRoche. DCMU, CCCP, and FCCP were gifts of Dr. P. Heytler of DuPont. Gramicidin and 9-aminoacridine were purchased from K & K Laboratories, valinomycin was purchased from Calbiochem, and the buffers Tris, Tricine, MES and TES were purchased from Sigma Chemical Co. All other chemicals were reagent grade.

CHAPTER 3

THE EFFECTS OF UNCOUPLERS ON MSEC DELAYED LIGHT EMISSION

A. Introduction

It has been proposed (Mayne, 1967, 1968) that the high energy state of photophosphorylation can provide some of the activation energy necessary for delayed light emission (DLE), and this proposal has received wide support (see Fleishman and Mayne, 1973, for review). In the present work we have investigated the effects of a wide variety of uncouplers of photophosphorylation on steady-state msec DLE, both in the presence and absence of added electron acceptors. We have attempted to relate the effects on DLE intensity to the effects of uncouplers on the high energy state formed under similar conditions. Our estimations of the high energy state are based on the quenching of 9-aminoacridine fluorescence as an indicator of the transmembrane pH difference (ΔpH) and the absorption change at 515 nm as an indicator of the membrane potential ($\Delta\psi$). The variation in msec DLE intensity as a function of the external pH has also been examined and is discussed in terms of the proposed relationship of DLE to the high energy state. The following introductory material is intended to briefly review this proposed relationship and to provide a framework in which our results can be evaluated.

Mayne (1967) reported on the effects of various photosynthesis inhibitors on the intensity of 3 msec delayed light emitted from chloroplasts. His central observations were that when the rate of electron flow was increased by the addition of Hill acceptors (in the absence of uncouplers) DLE intensity was increased, but that when electron flow was accelerated by the addition of uncouplers of photophosphorylation, the DLE intensity was lowered. He concluded that the intensity of DLE was dependent in some way on the presence of the high energy intermediates of phosphorylation.

In view of the above suggestion (although published earlier) and the demonstration of dark "acid-base" ATP synthesis (Jagendorf and Uribe, 1966a), Mayne and Clayton (1966) subjected chloroplasts to an acid-base transition and observed the emission of a flash of light, the spectrum of which was similar to that of DLE and of chlorophyll fluorescence. Mayne (1968) showed that the energy of the emission did not derive, as he had originally suggested, solely from the high energy phosphorylation intermediates created in the acid-base transition. He found that preillumination of the chloroplasts was a necessary requirement for the stimulated emission. The action spectrum for preillumination was that of photosystem II. Mayne suggested that a transmembrane pH gradient, created either artificially by an acid-base transition or as a result of the vectorial translocation of protons by electron transport as proposed by Mitchell (1961), could contribute toward the activation energy for DLE.

In a comparison of acid-base induced luminescence with acid-base ATP synthesis, Miles and Jagendorf (1969) found that conditions for generating the two phenomena were not identical. They differed in their pH optima, the acid incubation times required, and in the kind of organic acids necessary to produce the response. It thus appears that although both phenomena depend on pH changes in the environment, different components of the photosynthetic apparatus may be involved in the two cases.

The possible involvement of the membrane potential component of the high energy state of phosphorylation in stimulating DLE was first suggested by Fleischman (1971) and by Crofts _et al_. (1971) to account for the similarities between DLE and the carotenoid (518 nm) absorption shift seen in purple bacteria (Fleischman and Clayton, 1968). Sherman (1972) demonstrated that DLE intensity from chromatophores of _Rhodopseudomonas spheroides_ was lowered by agents that inhibit the development of a membrane potential, but that the emission was not lowered by agents that dissipate only the proton concentration gradient. The observations that light emission in chloroplasts could be triggered by sudden transitions in salt concentration (Miles and Jagendorf, 1969; Barber and Kraan, 1970) have also been interpreted in terms of a transmembrane potential.

Wraight and Crofts (1971) have observed two components in the dark-to-light induction transient of msec DLE from chloroplasts. Based on their kinetics and their differential sensitivities to valinomycin and to nigericin (see Chapter 1, section F for the presumed mode of action of these antibiotics), the authors concluded that the fast, valinomycin-sensitive component reflected stimulation of DLE intensity by the membrane potential. The slower, nigericin-sensitive component was ascribed to stimulation by the transmembrane pH gradient. Wraight and Crofts (1971) developed a model in which both aspects of the high energy state contribute to the activation energy for the back reactions leading to the production of delayed light. In this model, the separation of electrons and holes by the photoreaction occurs vectorially across the membrane, the electrons to the outside to reduce the primary acceptor, Q, and the holes to the inside, oxidizing the primary donor, Z. This immediately brings the transmembrane electrical potential into equilibrium with the charge separation reactions. To explain the dependence on transmembrane ΔpH they assumed the primary photoproducts to be in equilibrium with secondary pools of hydrogen carriers having pH-dependent midpoint potentials. The pools reduced by Q^- would be in equilibrium with the pH of the external phase, while the pools oxidized by Z^+ would be in equilibrium with the internal pH. The development of a transmembrane pH gradient would thus affect the availability of the substrates for DLE, Z^+ and Q^-, by affecting the equilibria of these species with the secondary pools. A similar proposal was made independently by Kraan _et_ _al_. (1970) in which the primary acceptor and donor themselves are hydrogen carriers with pH-dependent midpoint potentials:

1) $ZH \cdot Q \overset{h\nu}{\rightleftharpoons} ZH^{+} \cdot Q^{-}$

2) $ZH^{+} \rightleftharpoons Z + H^{+}$ (interior phase)

3) $Q^{-} + H^{+} \rightleftharpoons QH$ (exterior phase)

The development of a proton gradient (acid inside) due to vectorial proton transport accompanying electron transport would thus increase the concentrations of ZH^{+} and Q^{-}. Light emission would result from the reversal of equation 1.

One complication in much of the above work is that uncouplers not only dissipate the high energy state, but they also increase the rate of electron transport, another factor that may itself influence the intensity of DLE (Bertsch _et_ _al_., 1969; Clayton, 1969). A clear separation of the effects of the high energy state from those of electron transport was reported by Wraight and Crofts (1971) and by Cohen and Bertsch (1974). These workers observed stimulation of msec DLE intensity from chloroplasts in which electron transport through photosystem II was inhibited by DCMU, but which developed a high energy phosphorylation state by cyclic electron flow involving photosystem I on addition of the cofactor 2,3,5,6-tetramethyl-p-phenylenediamine.

B. Results

1. The effects of uncouplers on msec DLE

The effect of the uncoupler of photophosphorylation, gramicidin, on delayed light emission (DLE) decay from 0.8 to 3.2 msec after repeating flashes of light is shown in Fig. 3-1. The lowering of msec DLE intensity by the uncoupler was apparent both in the presence and in the absence of the electron acceptor, ferricyanide. The decay of the DLE in the presence or absence of ferricyanide, over the time range investigated, was only slightly altered by the addition of gramicidin, even though the rate of electron transport from water to ferricyanide was increased almost three-fold by this concentration of gramicidin (1 uM).

Table I summarizes similar data obtained with a number of uncouplers and with valinomycin. All of the uncouplers examined decreased the intensity of 1 msec DLE observed in the presence of an electron acceptor. Table I also shows that most of the uncouplers (the sole exception being NH_4Cl), as well as valinomycin, had a similar strongly depressing effect on the msec DLE intensity observed in the absence of a Hill acceptor. Valinomycin (lum), which does not uncouple photophosphorylation in chloroplasts or accelerate the rate of electron transport, had a large depressing effect on DLE intensity only in the absence of an electron acceptor. In all cases, activation of electron transport by the addition of $K_3Fe(CN)_6$ resulted in an increase in the 1 msec DLE intensity.

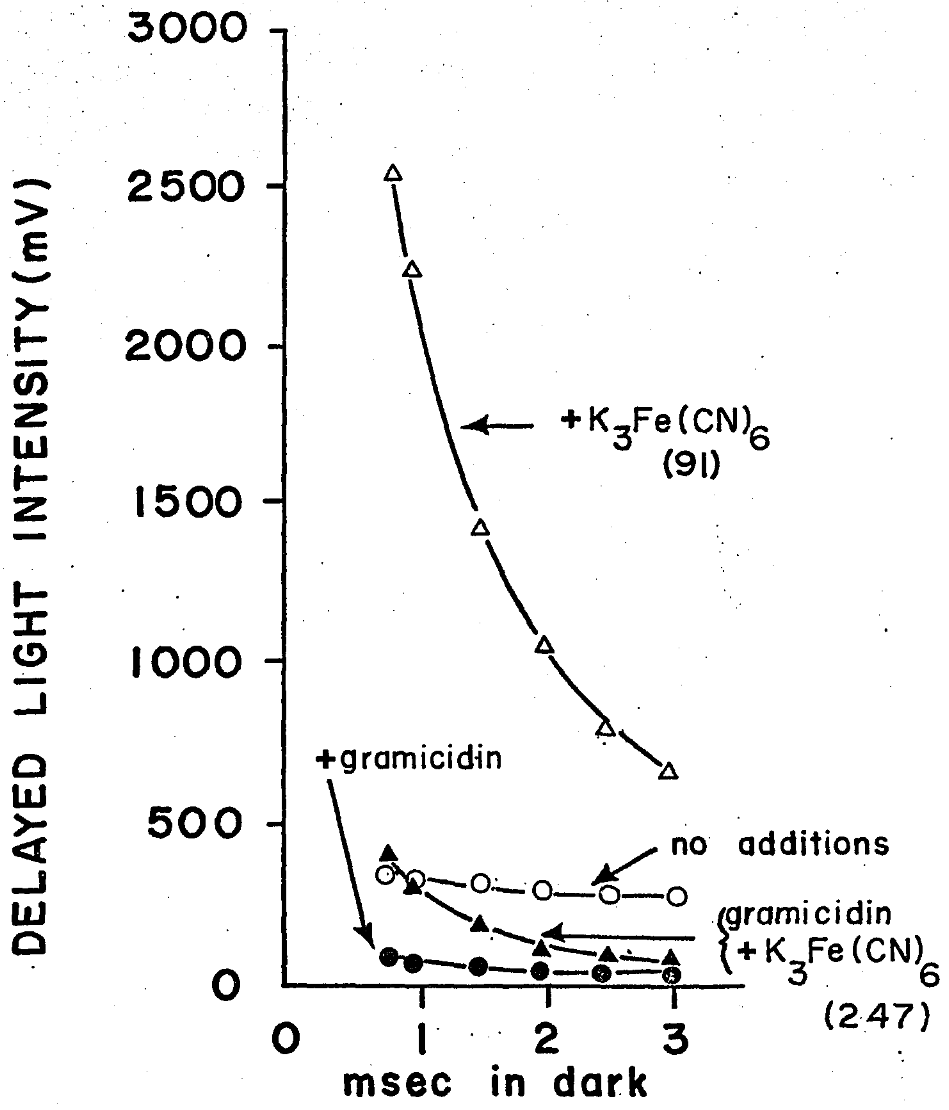


Fig. 3-1. Effect of gramicidin on msec delayed light emission in the absence and presence of $K_3Fe(CN)_6$. The reaction mixture contained: 20 mM KCl, 2mM $MgCl_2$, 20 mM Tricine, pH 7.5 and spinach chloroplasts equivalent to 10 ug chlorophyll/ml. When added, gramicidin was 1 uM and $K_3Fe(CN)_6$ was 0.3 mM. The numbers in parentheses indicate the rate of $K_3Fe(CN)_6$ reduction expressed as ueq/mg chl/hr.

Table 3-I

THE EFFECTS OF UNCOUPLERS ON MSEC DELAYED LIGHT EMISSION IN THE ABSENCE AND PRESENCE OF $K_3Fe(CN)_6$ AND ON ELECTRON TRANSPORT

The reaction mixture contained: 20 mM KCl, 2 mM $MgCl_2$, 25 mM Tricine, pH 7.5, and spinach chloroplasts equivalent to 10 ug chlorophyll/ml. The ratio of DLE at 0.8 msec to that at 2.8 msec after illumination is presented as a crude indication of the decay kinetics of the DLE. When added, $K_3Fe(CN)_6$ was present at an initial concentration of 0.3 mM.

	Intensity of 1 msec DLE (mV)		DLE intensity $\frac{0.8\text{msec}}{2.8\text{msec}}$		Rate of $K_3Fe(CN)_6$ reduction (ueq/mg chl/hr)
Addition	$-K_3Fe(CN)_6$	$+K_3Fe(CN)_6$	$-K_3Fe(CN)_6$	$+K_3Fe(CN)_6$	
None	355	2250	1.22	3.21	91
Gramicidin (1 uM)	80	320	1.67	4.00	247
Nigericin (0.5 um)	80	295	1.60	4.54	280
CCCP (5 uM)	73	307	2.27	4.07	214
FCCP (1 uM)	51	295	2.68	4.36	211
NH_4Cl (10 mM)	450	1000	1.80	3.08	161
Methylamine (40 mM)	135	420	1.82	4.20	204
Hexylamine (5 mM)	77	300	1.40	3.75	210
Chloroquinphosphate (0.1 mM)	95	355	1.36	3.94	222
Valinomycin (1 uM)	95	1800	1.27	3.00	84

We have examined the effects of a number of uncouplers on delayed light intensity and on electron transport rate as a function of the uncoupler concentration. Fig. 3-2 shows the effects of various concentrations of gramicidin on the rate of electron transport and on 1 msec DLE intensity in the presence and absence of the electron acceptor methyl viologen. In order to see more clearly the differential effect of gramicidin on DLE intensity with and without the acceptor, these data are replotted in Fig. 3-3 with the DLE intensities (with and without the acceptor) in the absence of the uncoupler normalized to 1.00. As seen in this Figure, the acceptorless DLE was more sensitive to low concentrations of the uncoupler than was either the DLE in the presence of the electron acceptor or the rate of electron transport. These latter two parameters appeared to show a somewhat similar sensitivity (but opposite response) to gramicidin.

That the DLE intensity in the presence of a Hill acceptor and the rate of electron transport do not always exhibit a similar sensitivity to an uncoupler is shown in Fig. 3-4, where the normalized values of 1 msec DLE intensity and electron transport rate are plotted as functions of the concentration of CCCP. In this case, the DLE intensity was reduced both in the presence and absence of an electron acceptor by concentrations of the uncoupler that had virtually no effect on the rate of electron transport. As with gramicidin, the acceptorless DLE was more sensitive to low concentrations of CCCP than was the DLE in the presence

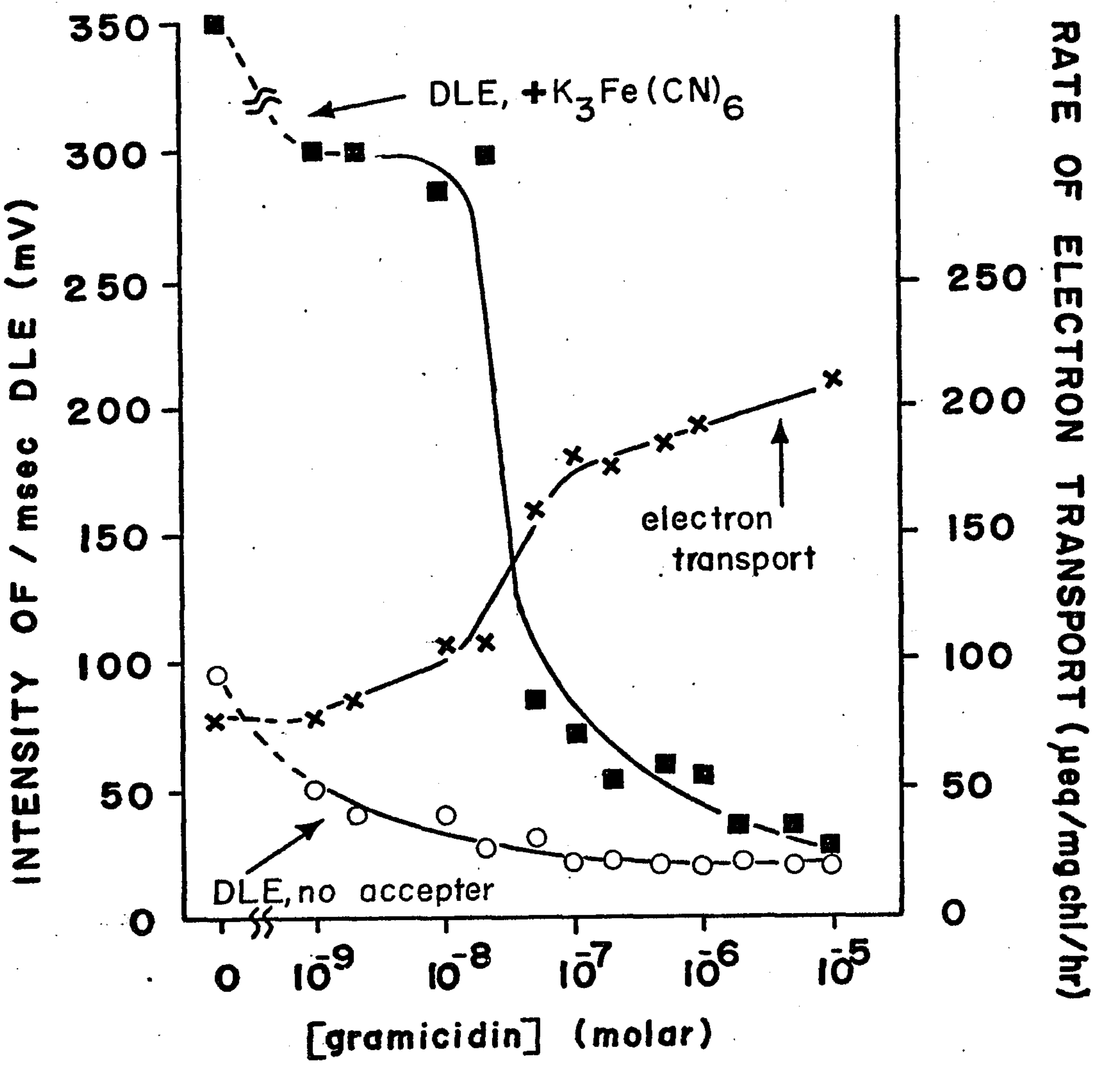


Fig. 3-2. Dependence of 1 msec DLE intensity and $K_3Fe(CN)_6$ reduction on gramicidin concentration. The reaction mixture contained 20 mM NaCl, 2 mM MgCl2, 20 mM Tricine, pH 7.5, and spinach chloroplasts equivalent to 11.4 ug chl/ml. When added, $K_3Fe(CN)_6$ was present at 0.3mM.

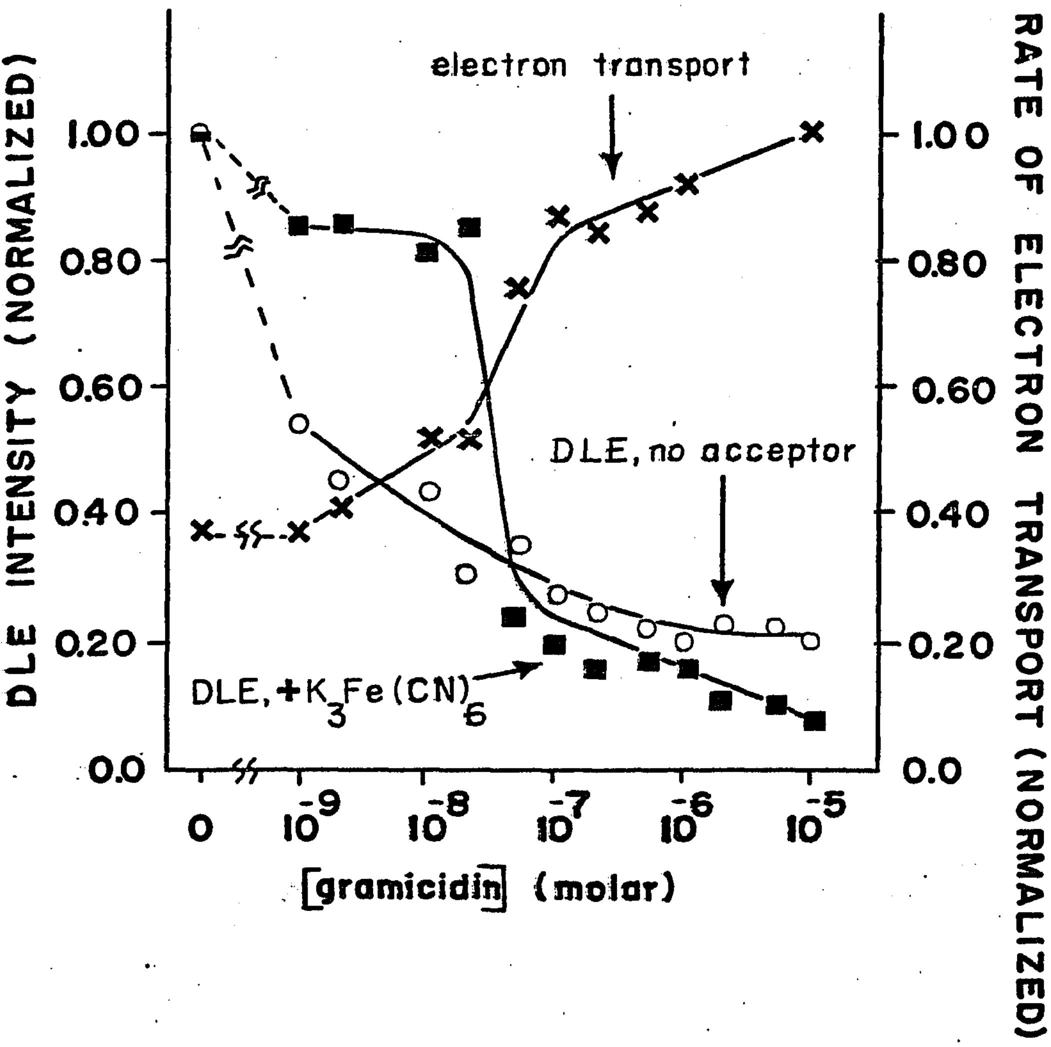


Fig. 3-3. Relative dependence of 1 msec DLE intensity in the absence and presence of $K_3Fe(CN)_6$ on gramicidin concentration. Conditions as in Fig. 3-2. The values in the absence of gramicidin were: DLE $(-K_3Fe(CN)_6)$, 90 mV; DLE$(+K_3Fe(CN)_6)$, 350 mV; $K_3Fe(CN)_6$ reduction, 79 ueq/mg chl/hr.

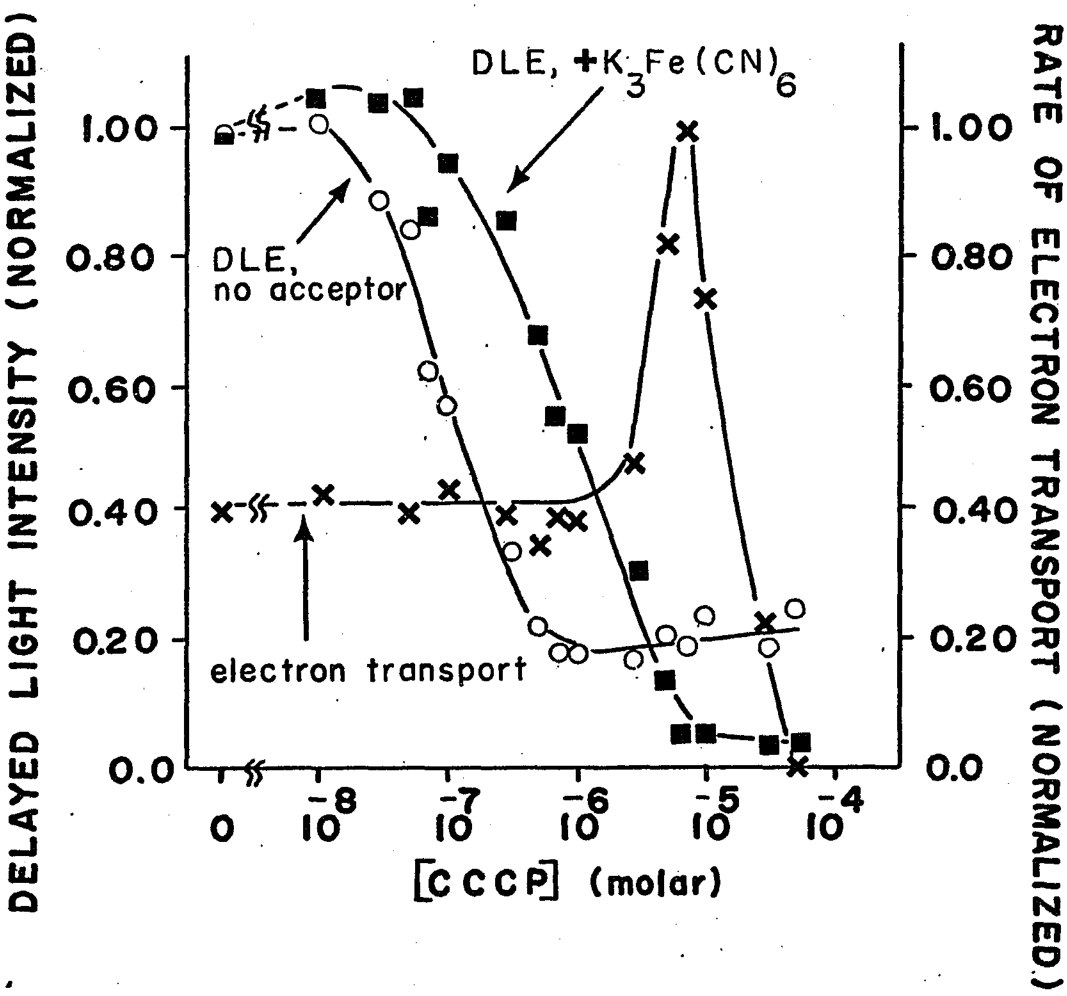


Fig. 3-4. Relative dependence of 1 msec DLE intensity in the absence and presence of $K_3Fe(CN)_6$ on the concentration of CCCP. The reaction mixture contained: 20 mMNaCl, 2 mM $MgCl_2$, 20 mM Tricine, pH 7.5, and spinach chloroplasts equivalent to 10 ug chl/ml. Electron transport was measured as O_2 evolution with $K_3Fe(CN)_6$ (0.3 mM) as the electron acceptor. Control values, in the absence of gramicidin were: DLE (no acceptor), 185 mV; DLE (with acceptor), 1100 mV; O_2 evolution, 24.7 umoles O_2/mg chl/hr.

of the acceptor. The rate of electron transport exhibited a marked peak in stimulation by CCCP over a narrow concentration range, and was severely inhibited at a concentration of the uncoupler only slightly greater than that required for optimal stimulation of electron transport. Several other uncouplers inhibited electron transport at high concentration (see Chapter 4), but CCCP and its analog FCCP were unusual in that severe inhibition set in at concentrations very close to those optimal for stimulation.

Figs. 3-5, -6, and -7 show the concentration dependence of DLE intensity and the rate of electron transport from water to ferricyanide for three amine uncouplers; methylamine, hexylamine, and NH_4Cl, respectively. In all three cases, the lowering of DLE intensity in the absence of an electron acceptor failed to exhibit the heightened sensitivity to low concentrations of the uncoupler that was seen in the case of the ionophores, CCCP or gramicidin.

NH_4Cl was different from other uncouplers in its effect on DLE intensity (Fig. 3-7) in that it dramatically stimulated acceptorless DLE at concentrations above that necessary to achieve optimal stimulation of the rate of electron transport. In fact, the minimum in acceptorless DLE occurred at concentrations of NH_4Cl lower than those that gave optimal stimulation of electron transport rate. This was also observed with methylamine (Fig. 3-5), although the stimulation of acceptorless DLE by high concentrations was much smaller than with NH_4Cl. The hexylamine concentration curve

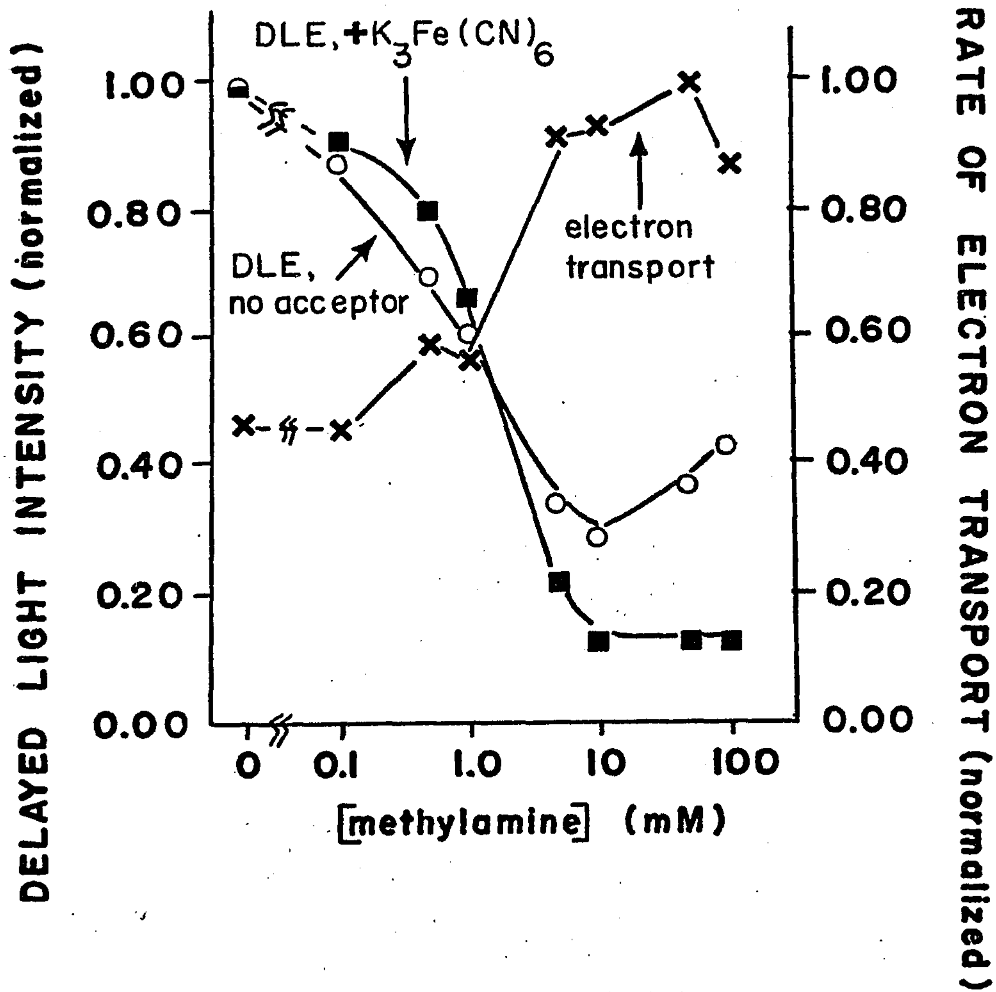


Fig. 3-5. Relative dependence of 1 msec DLE intensity in the absence and presence of $K_3Fe(CN)_6$ on the concentration of methylamine. The reaction mixture contained: 20 mM NaCl, 2 mM $MgCl_2$, 25 mM Tricine, pH 7.5, and Good King Henry chloroplasts equivalent to 10 ug chl/ml. Electron transport rate was measured as $K_3Fe(CN)_6$ reduction. When added, $K_3Fe(CN)_6$ was present at 0.3 mM. Values in the absence of methylamine were: DLE (no acceptor), 407 mV; DLE (with acceptor), 2250; $K_3Fe(CN)_6$ reduction, 77 ueq/mg chl/hr.

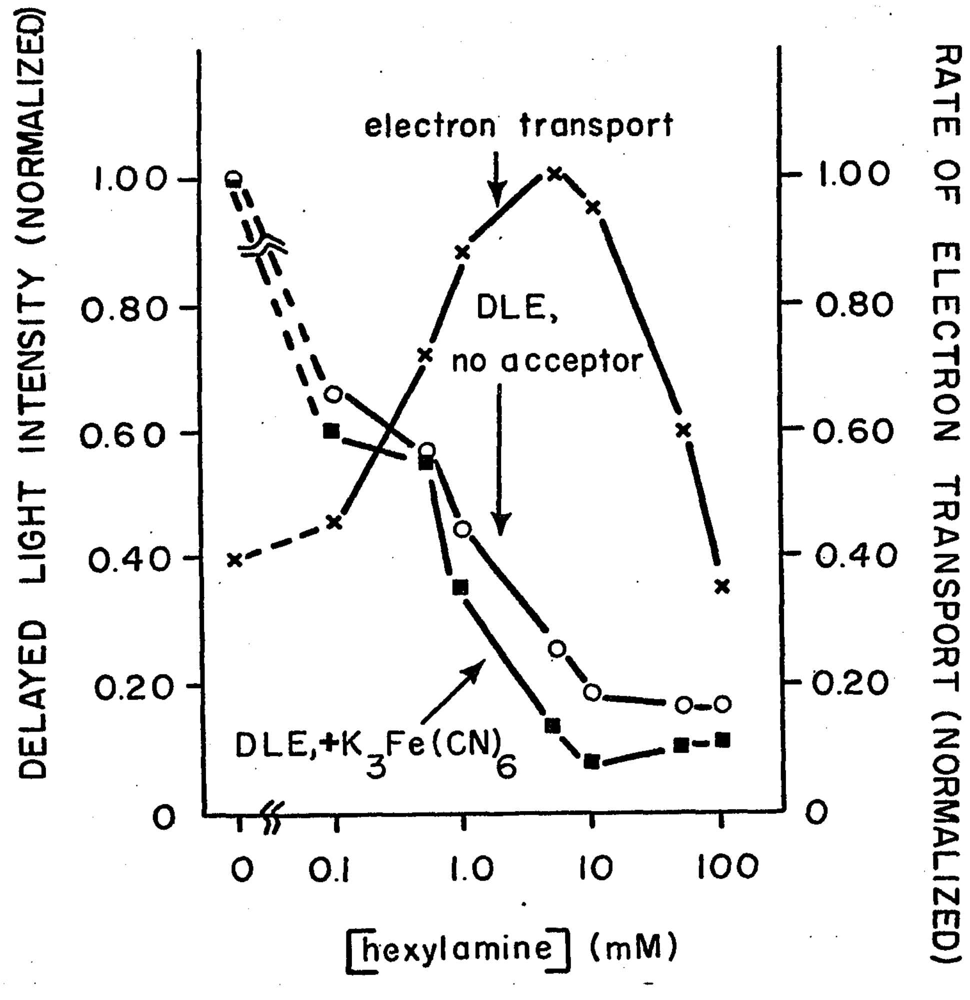


Fig. 3-6. Relative dependence of 1 msec DLE intensity in the absence and presence of $K_3Fe(CN)_6$ on the concentration of hexylamine. Conditions as in Fig. 3-5. For values in the absence of hexylamine, see Fig. 3-5.

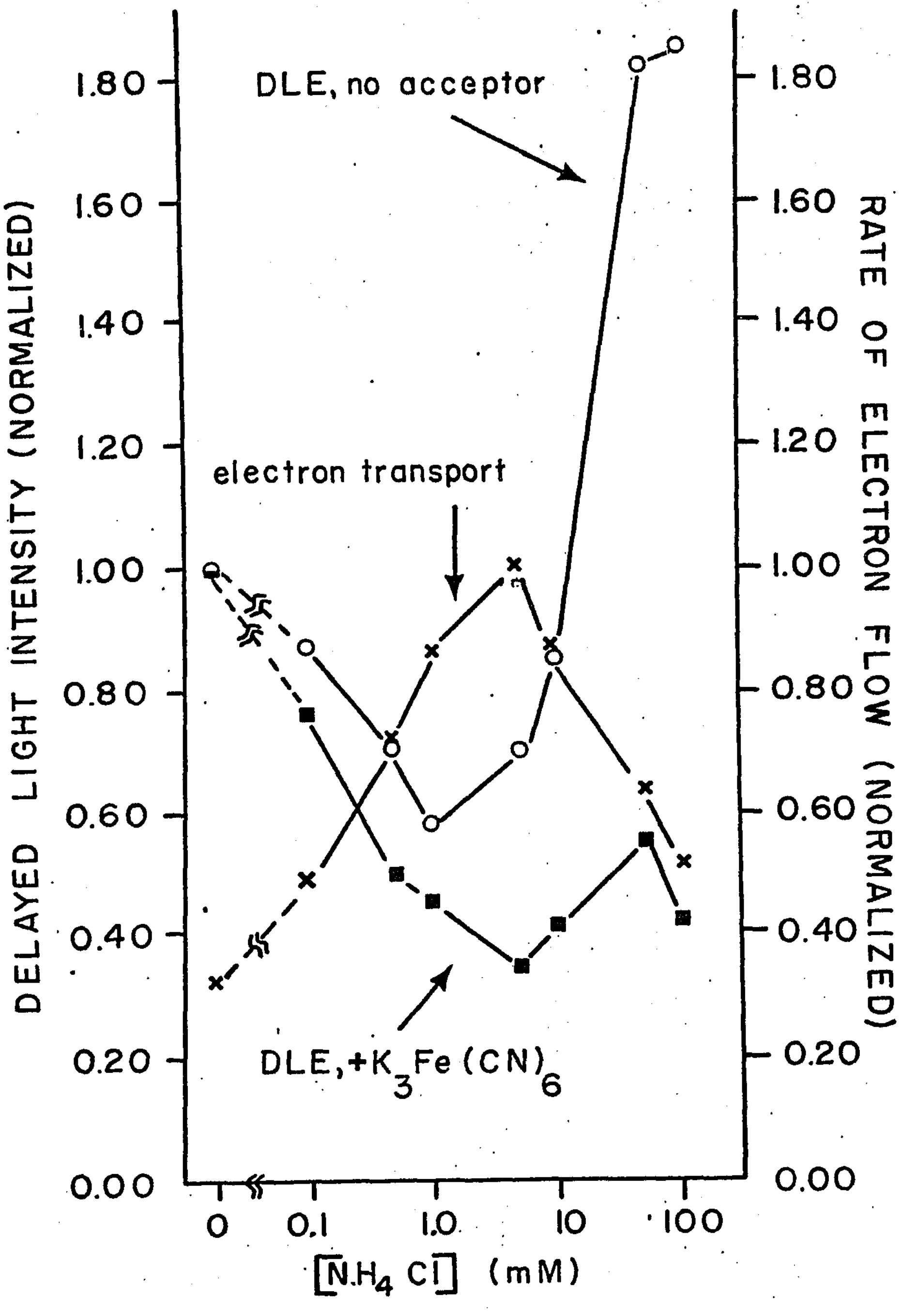


Fig. 3-7. Relative dependence of 1 msec DLE intensity in the absence and presence of $K_3Fe(CN)_6$ on the concentration of NH_4Cl. All conditions were identical to Fig. 3-5. For values in the absence of NH_4Cl, see Fig. 3-5.

did not show a minimum in DLE (Fig. 3-6). In the ammonium chloride case (Fig. 3-7), DLE in the presence or absence of an electron acceptor was not suppressed at any concentration to the degree seen with the other amines or with the inophorous uncouplers (Figs. 3-3 and 3-4).

2. Estimation of the high energy state.

The observation that acceptorless DLE was strongly decreased by uncouplers led us to investigate the magnitude of the high energy state developed in the light by chloroplasts in the absence of added electron acceptors. We used the absorbance change at 515 nm induced by a strong red actinic beam as an indicator of the transmembrane electrical potential ($\Delta\Psi$). The quenching of 9-aminoacridine fluorescence at 510 nm, excited by a weak blue beam, served as an indicator of the transmembrane pH difference (ΔpH) developed with red actinic illumination.

Fig. 3-8 shows that both measurements indicated the development of a sizeable light-induced high energy state in the absence of electron acceptors. The magnitude of the 515 nm absorbance change in the absence of an acceptor was almost as large (80-90%) as that developed in the presence of methyl viologen. The acceptorless acridine fluoresence quenching varied from about 30% to 50% of that seen with an acceptor. These observations are in agreement with data of other workers (Baltascheffsky and Hall, 1974; Kraayenhof *et al.*, 1972; Neumann and Jagendorf, 1964), but the implications of these observations have not been discussed.

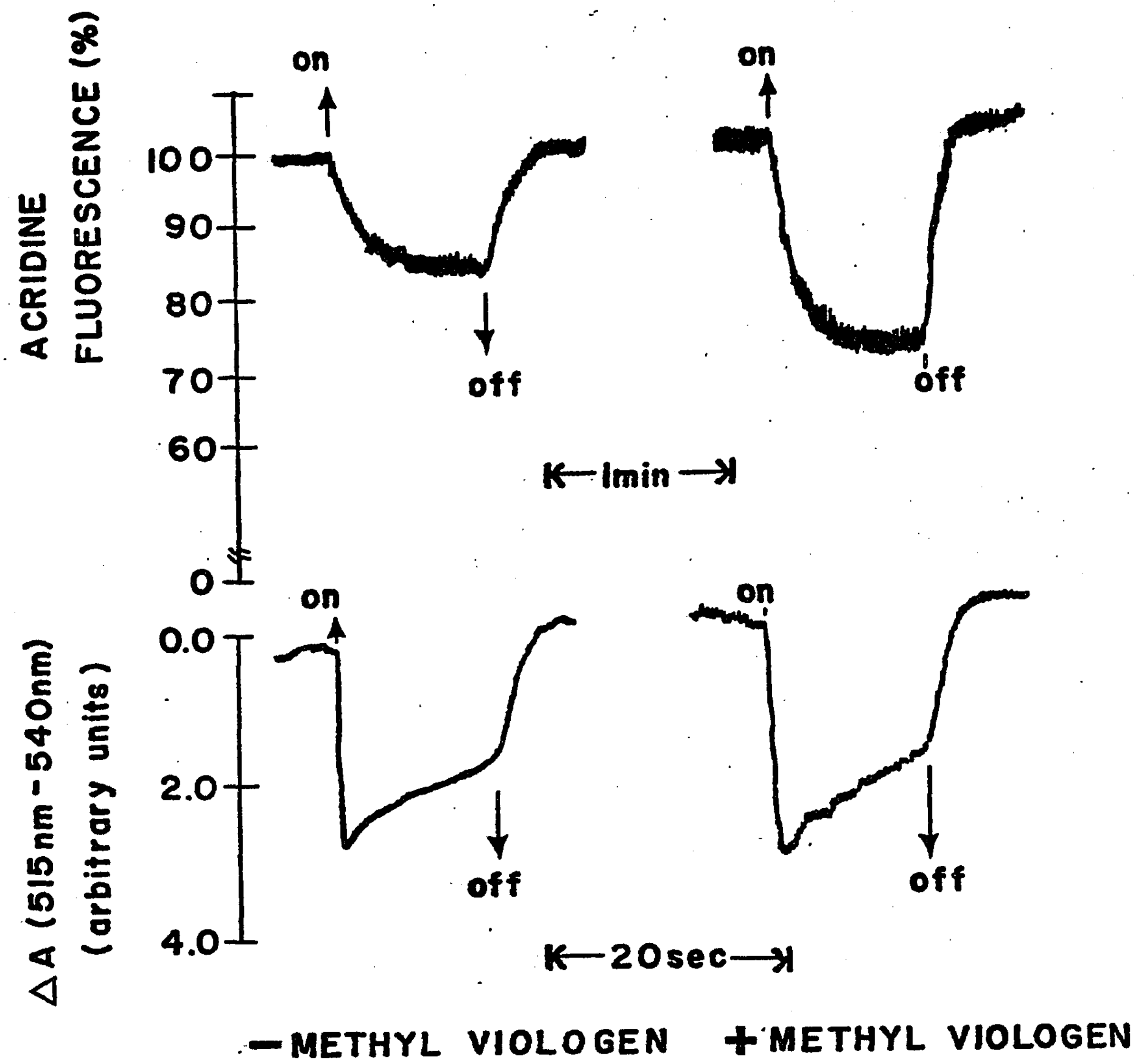


Fig. 3-8. Light-induced quenching of 9-aminoacridine fluorescence and the light-induced absorption change at 515 nm in the absence and presence of methyl viologen. Reaction conditions as in Table 3-II.

The transmembrane pH difference (ΔpH) may be calculated from the quenching of 9-aminoacridine fluorescence by the following equation (Schuldiner *et al.*, 1972):

$$\Delta pH = \log\frac{Q}{100 - Q} + \log\frac{V}{v}$$

where Q represents the percent fluorescence quenching, V is the total volume of the incubation mixture and v is the total osmotically active internal volume. The validity of such a treatment has been supported by the study of Deamer *et al.* (1972) on model liposome systems, but has recently been questioned by Fiolet *et al.* (1974). We have not attempted to measure the internal volume of our chloroplasts. If, however, we assume an internal volume of 40 ul/mg chlorophyll, which is consistent with measurements of other workers (Portis and McCarty, 1973; Schuldiner *et al.*, 1972), then the fluorescence quenchings observed would correspond to a ΔpH of 3.0 - 3.1 pH units in the presence of an acceptor and 2.4 - 2.6 pH units in the absence of added acceptors.

The high energy state (as indicated by our measurements) developed in the absence of added acceptors was not transient in nature, but resembled that seen in the presence of acceptors. Both the fluorescence quenching and the 515 nm absorption change could be generated repeatedly on successive exposures to light, and, as is shown in Table 3-II, they were inhibited by the uncoupler, gramicidin, and by the electron transport inhibitors, DCMU and dibromothymoquinone.

Table 3-II

EFFECTS OF UNCOUPLERS AND ELECTRON TRANSPORT INHIBITORS ON THE QUENCHING OF 9-AMINOACRIDINE FLUORESCENCE, THE ABSORBANCE CHANGE AT 515 nm, AND ON ELECTRON TRANSPORT RATE

The reaction mixture contained: 20 mM NaCl, 2 mM $MgCl_2$, 200 mM sucrose, and 20 mM Tricine, pH 8.0. Chlorophyll concentrations were 10 ug/ml for the fluorescence measurement, 20 ug/ml for the O_2 measurement, and 30 ug/ml for the 515 nm absorption change. When methyl viologen was present, NaN_3 was added at a concentration of 0.5 mM. The concentration of 9-aminoacridine was 2 uM. Due to background noise, the minimum signals detectable were 2% for the fluorescence quenching and 1 unit for the 515 nm absorbance change.

Acceptor	Additions	Quenching acridine fluorescence %	ΔA (515 - 540 nm) (arbitrary units)		O_2 consumed or evolved umoles/mg chl/hr
			on response	off response	
None	None	10.2	9	5.5	0
	Gramicidin (1 uM)	< 2	< 1	< 1	0
	DCMU (4 uM)	< 2	< 1	< 1	-
	Br_2-thymoquinone (0.5 uM)	< 2	4.5	< 1	0
Methyl viologen (0.1 mM)	None	27.3	10.5	6.5	47
	Gramicidin	4.8	< 1	< 1	134
	DCMU (4 uM)	< 2	< 1	< 1	-
	Br_2-thymoquinone (0.5 uM)	< 2	4.5	< 1	0
$K_3Fe(CN)_6$ (0.3 mM)	None	26.0	11	6.5	54
	Gramicidin (1uM)	< 2	< 1	< 1	162
	DCMU (4 uM)	< 2	< 1	< 1	-
	Br_2-thymoquinone (0.5 uM)	10.5	6.5	3.5	30

Disappearance of protons from the external medium was also examined with a glass pH electrode. The amount of H^+ uptake observed in the absence of added acceptors was quite sizeable, usually from 25 to 40% of that observed with electron acceptors, but occasionally as high as 60%. Fig. 3-9 shows the largest amount of proton uptake we have observed in the absence of an electron acceptor. In spite of these indications that electron transport involving photosystem II was taking place in the absence of added Hill reagents, we have been unable to detect any oxygen evolution or consumption under these conditions. We estimate that the minimum rate of O_2 changes measureable with our apparatus (and using 20 ug chlorophyll/ml) is about 2 umoles O_2/mg chl/hr or about 5-10% of that we normally observe in the presence of an acceptor.

Fig. 3-10 shows the effects of gramicidin concentration on DLE intensity, on the rate of electron transport, and on the high-energy state as measured by the 515 nm absorption shift and the quenching of the acridine fluorescence, measured with the same chloroplast preparation. Both aspects of the high-energy state generated in the absence of a Hill acceptor, like DLE, were more sensitive to low concentrations of gramicidin than those generated in the presence of the acceptor.

We have measured the dependence of both the 515 nm absorbance change and acridine fluorescence quenching on the intensity of the exciting light. Fig. 3-11 shows that

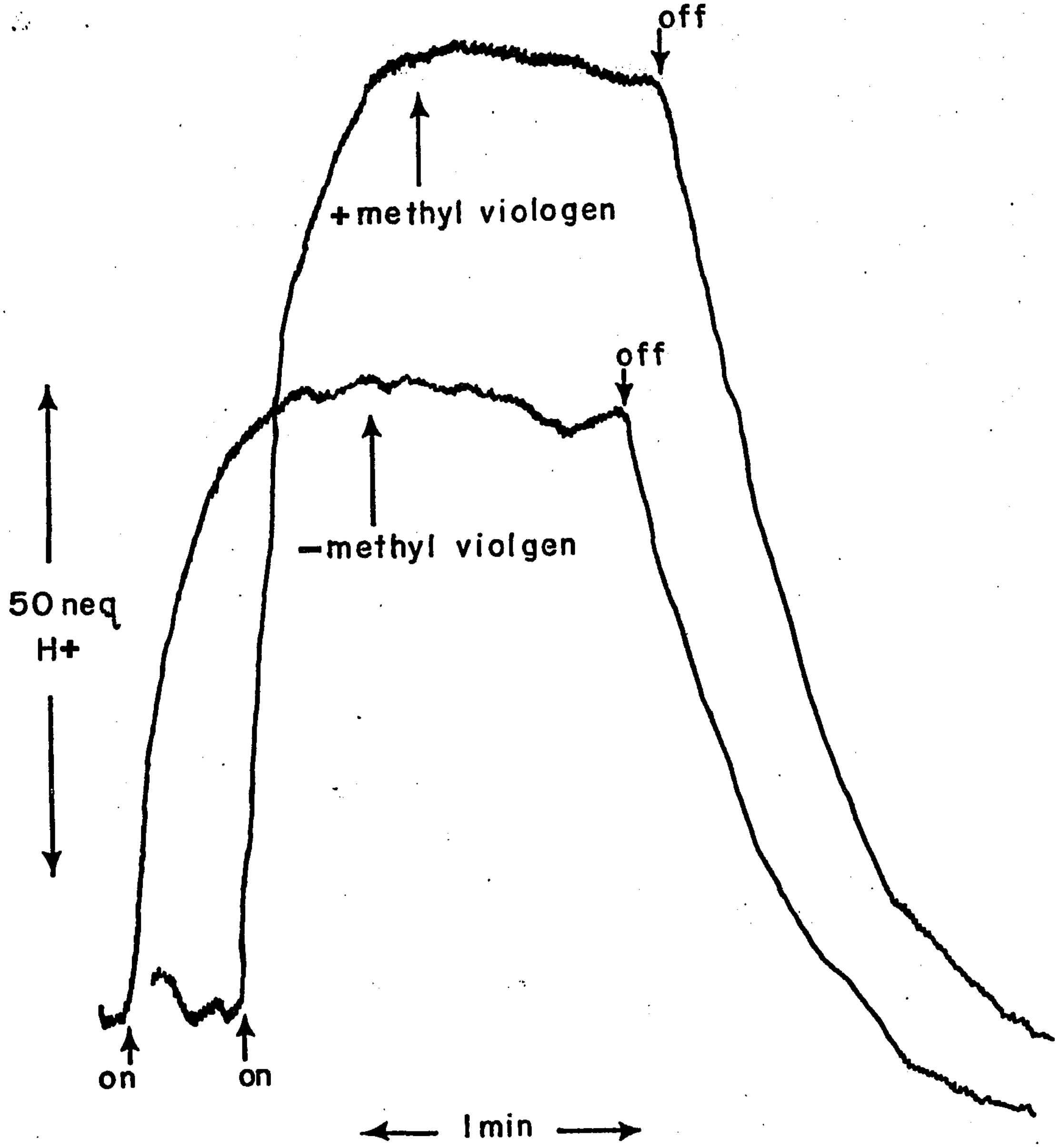


Fig. 3-9. Light-induced uptake of protons in the absence and presence of methyl viologen. Reaction conditions as in Table 3-II, except that Tricine was present at 0.5 mM, pH 8.0. Chloroplasts were equivalent to 30 ug chlorophyll/ ml.

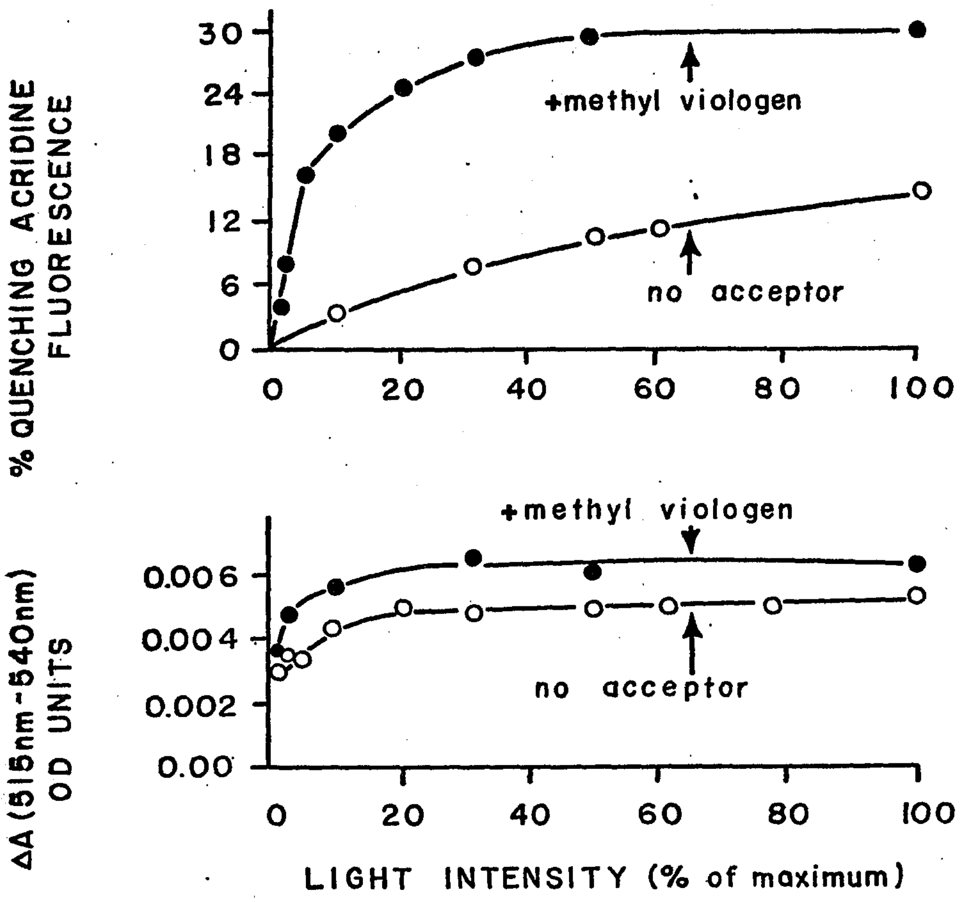


Fig. 3-11. Light intensity dependence of 9-aminoacridine fluorescence quenching and 515 nm absorption change. Reaction conditions as in Table 3-II.

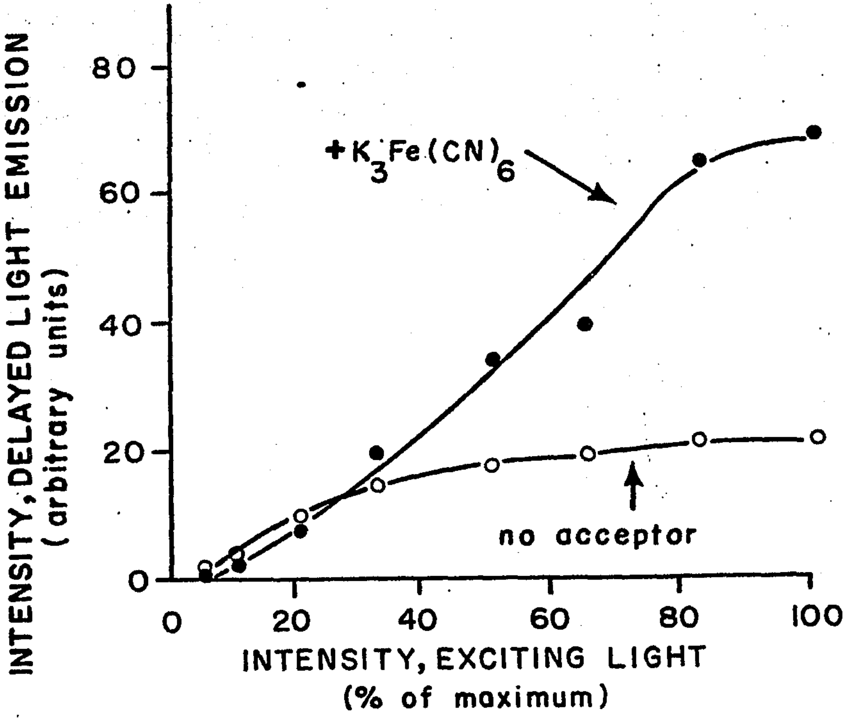


Fig. 3-12. Light intensity dependence of 1 msec DLE intensity. Reaction conditions as in Fig. 3-4.

fluorescence increases over this pH range (Fig. 3-13). In view of the proposed dependence of DLE intensity in chloroplasts on ΔpH, it was surprising to find that the msec DLE intensity decreased considerably over this range of external pH. The effect on DLE intensity, and on electron transport, of varying the external pH is shown in Fig. 3-14, both in the presence and absence of electron acceptors and gramicidin. In the presence of gramicidin, DLE intensity decreased somewhat as the external pH was raised from pH 6.5 to pH 7.0, but above this pH, DLE intensity remained almost constant. In the absence of gramicidin, msec DLE intensity increased slightly between pH 6.5 and pH 7.0, but then dropped sharply as the external pH was raised further. The inhibition of electron transport seen with gramicidin at external pH's above 8.0 will be discussed in Chapter 4.

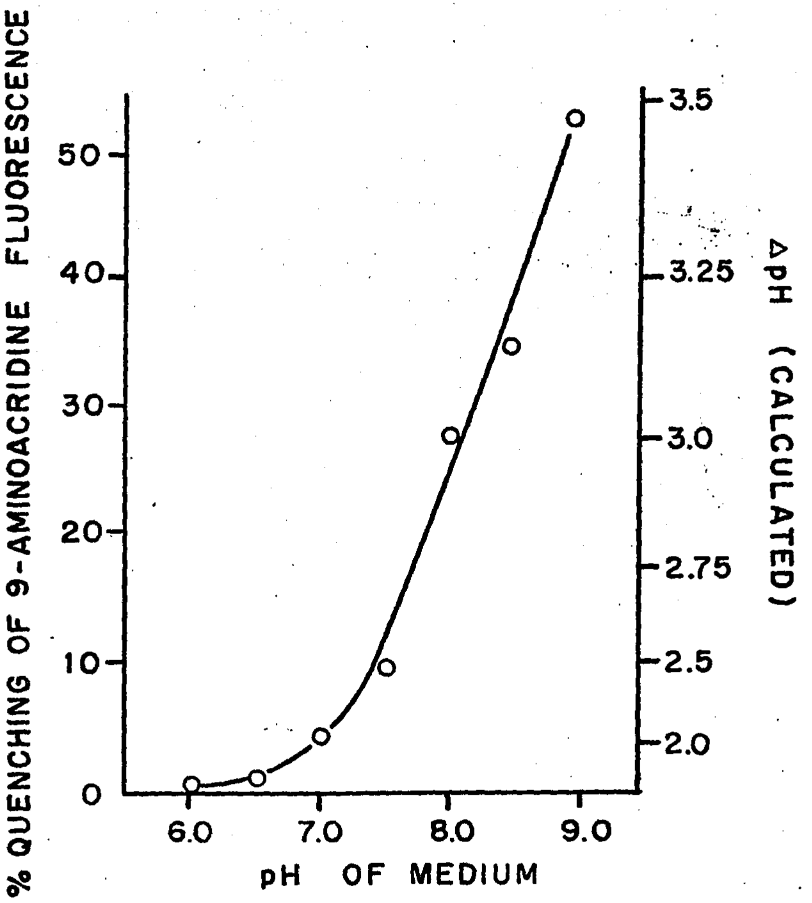


Fig. 3-13. Dependence of 9-aminoacridine fluorescence quenching on the external pH. The reaction mixture contained: 20 mM NaCl, 2 mM $MgCl_2$, 200 mM sucrose, 20 mM of either TES (pH 6 - 7.5) or Tricine (pH 8.0 and above), 0.1 mM methyl viologen, 2 uM 9-aminoacridine, and spinach chloroplasts equivalent to 10 ug chlorophyll/ml. Values of ΔpH were calculated assuming an internal volume of 40 ul/mg chl.

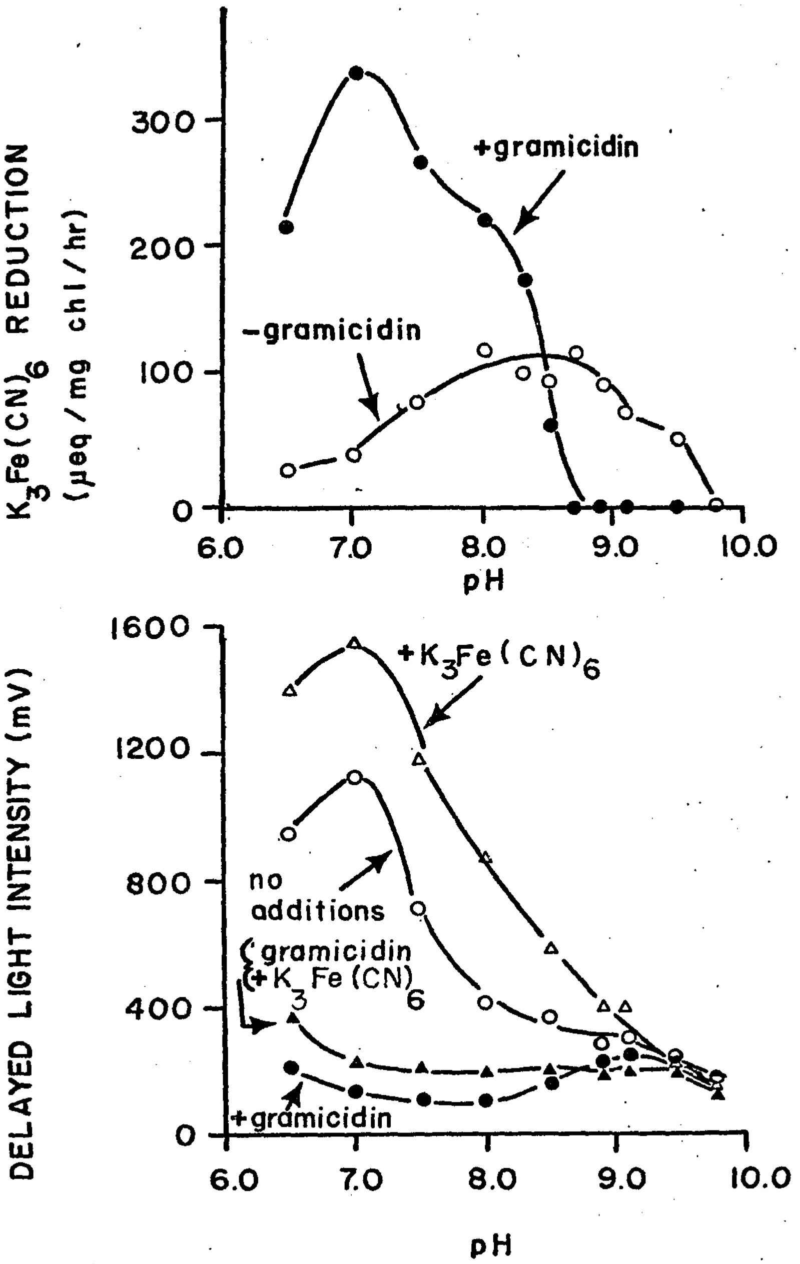


Fig. 3-14. Dependence of electron transport and 1 msec DLE intensity in the presence and absence of gramicidin on the external pH. The reaction mixture contained: 20 mM NaCl, 2 mM $MgCl_2$, 50 mM of either TES (pH 6.5 - 7.5) or Tricine (pH 8.0 and above), and spinach chloroplasts equivalent to 10 ug chlorophyll/ml. When added, $K_3Fe(CN)_6$ was present at a concentration of 0.3 mM and gramicidin at a concentration of 1 uM.

C. Discussion

The initial impetus for the present research was the observation that uncouplers decreased steady-state DLE both in the presence and absence of added electron acceptors. Mayne had previously observed this uncoupler-induced decrease in DLE intensity in the presence of electron acceptors, and this observation was the foundation of his proposal (Mayne, 1967, 1968) that the high energy state could provide activation energy for DLE. Lurie (1972), in our laboratory, found that uncouplers also decreased DLE intensity in the absence of electron acceptors. Based on the assumption that without added acceptors no steady-state high energy state should exist, Lurie interpreted her finding as indicating that the uncoupler-induced decrease in DLE intensity was not related to the high energy state. We have investigated this point of disagreement by combining the DLE observations with parallel measurements of the high energy state, both in absence and presence of electron acceptors. Our observation of an acceptorless high energy state leads to the conclusion that the major effects of uncouplers on DLE operate through the high energy state, in support of Mayne's proposal. In addition to this general conclusion, several other points of interest arose during this research, and these will be discussed in turn.

1. The high energy state in the absence of electron acceptors.

A sizeable level of high energy state, as indicated by the quenching of 9-aminoacridine fluorescence and the absorption change at 515 nm, was developed by our chloroplasts upon illumination in the absence of an added electron acceptor. Because of its sensitivity to DCMU and to dibromothymoquinone (Table II), we presume that development of this high energy state requires electron transport through photosystem II and turnover of plastoquinone. The electron flow would appear to involve more than pool filling, since the high energy state is maintained in the steady-state and can be repeatably generated by subsequent illuminations. These observations imply that there is some continuous turnover of the system in the absence of added electron acceptors.

Our estimations of the ΔpH from the degree of 9-aminoacridine fluorescence quenching indicate that the proton concentration difference formed in the absence of acceptors (2.4 - 2.6 pH units) is about 20 to 40% of that formed in the presence of an acceptor (3.0 - 3.1 pH units). We have also found that the extent of proton uptake in the absence of an acceptor ranges from 25 to 60% of that observed in the presence of an electron acceptor. From these data it would appear that a considerable amount of electron flow is occurring in the absence of an acceptor, perhaps on the order of 20 to 40% of that in the presence of an acceptor if other factors are assumed to remain the same in the two situations. Our inability to observe any oxygen evolution or consumption without electron acceptors makes it unlikely that reactions involving oxygen could be occurring at this rate. Prompt fluorescence studies have shown that the quencher of fluorescence, Q, is largely reduced at high light intensity in the absence of acceptors (Itoh et al., 1971; S. Malkin and Kok, 1966). This would indicate that no rapid turnover of photosystem II is taking place under these conditions.

Some of these difficulties can be overcome by assuming that the ionic permeabilities of the chloroplasts are different in the presence of rapid electron flow to acceptors than in the acceptorless state. Permeability changes could be brought about by the high energy state created by electron flow to acceptors. It has been shown that conformational changes in the membrane-bound coupling factor, CF_1, take place under the influence of the high energy state (Ryrie and Jagendorf, 1971). The state of CF_1 has been linked to the proton permeability of the thylakoid by studies on the effects of adenylates on proton uptake (McCarty et al., 1971; Telfer and Evans, 1972). It thus appears possible that the development of a high energy state could induce conformational changes that result in an increased permeability of the membrane to protons. This would lead to a non-linear relationship between the rate of electron flow and the proton concentration difference generated by that flow. Low rates of electron transport, as may be occurring in the absence of acceptors, might thus result in the development of a larger ΔpH than would be expected from comparison with the ΔpH formed by higher rates of electron flow to acceptors.

There are several possible sources for a low steady-state rate of electron flow in the absence of added electron acceptors. The most likely is a Mehler-type reaction in which reduced intermediates, either between the two photosystems or on the reducing side of photosystem I, react with molecular oxygen. Several investigators have reported rates of oxygen-induced electron transport on the order of 10 ueq/mg chl/hr (Forti and Jagendorf, 1961; S. Malkin, 1968). This rate is close to the minimum detectable with our oxygen electrode. If the proton permeability of the membrane were only a few-fold lower in the absence than in the presence of an acceptor, such an acceptorless rate of electron flow could generate the magnitude of ΔpH that we have estimated.

Another possible source of electron flow that could generate the ΔpH observed under acceptorless conditions could be an endogenous electron transport cycle around photosystem I. The sensitivity of the ΔpH formed under acceptorless conditions to DCMU and to dibromothymoquinone (Table 3-II) may imply that activation of this cycle would require photosystem II turnover, i.e., the cycle might require activation by some reduced species formed by photosystem II. Such a cyclic electron flow could be quite rapid, and thus would not require the assumption of membrane permeability changes to explain the magnitude of the resulting ΔpH.

We have observed that the acceptorless high energy state (and the acceptorless DLE) was more sensitive to low concentrations of gramicidin (Fig. 3-10) or CCCP (Fig. 3-4) than was the high energy state in the presence of an acceptor. This observation may simply reflect a difference in the variables that were limiting the rate of electron flow before the addition of the uncoupler. In situations (case 1) where the rate limitation on the rate of coupled electron flow are independent of the high energy state (i.e., light intensity, substrate availability), the addition of an ionophorous uncoupler would decrease the high energy state, but should have no effect on the rate of electron flow, since the rate limitation is unchanged. In situations (case 2) where the rate of coupled electron flow is limited by some aspect of the high energy state itself (i.e., ΔpH, internal acidity), the addition of an ionophore would relieve the limitation and the rate of electron flow would increase. However, the increased rate of inward proton translocation accompanying this more rapid electron flow could compensate (at least partially) for the increased outward leakage of internal protons induced by the ionophore, and thus the high energy state would be less affected than in case 1. In the above terms, we would place the acceptorless conditions in case 1, and the plus-acceptor conditions in case 2. We have not been able to measure the rate of electron flow under acceptorless conditions, and thus cannot determine how this rate is affected by uncouplers. However, the light saturation curves for the quenching of 9-aminoacridine fluorescence support the idea that the acceptorless electron flow is more light limited than is electron flow to an acceptor (Fig. 3-11).

The above analysis of our finding that the high energy state and DLE intensity were more sensitive to low concentrations of ionophorous uncouplers in the absence than in the presence of acceptors suggests that at low light intensity (case 1), uncouplers should be more effective inhibitors of photophosphorylation than they are at high light intensity (case 2), and that, conversely, the rate of electron flow should be less stimulated by uncouplers at low light than at high light. This differential effect of uncouplers on photophosphorylation as a function of light intensity was reported by Avron and Shavit (1963). We have found the suggested differential effect on the rate of electron transport to also be the case. Addition of 1 uM gramicidin increased the rate of DCPIP photoreduction (ueq/mg chl/hr) from 32 to 35 at low light intensity (1% maximum), while at high light intensity (100% maximum) the rate increased from 65 to 155.

The observation that valinomycin (1 uM) decreased acceptorless DLE intensity to a much greater degree than DLE intensity with an acceptor (Table 3-I) may reflect the greater "sensitivity" of the acceptorless high energy state to ionophores. Although valinomycin is generally regarded as affecting permeability of the membrane to K^+ (or NH_4^+), Telfer and Barber (1974) have shown that at higher concentrations (10 uM), valinomycin may significantly affect proton permeability. They also reported that valinomycin acts as an inhibitor of electron transport, an effect that makes its uncoupling activity difficult to detect as a stimulation of the rate of electron flow. By its effect on K^+ permeability, valinomycin reduces the membrane potential component ($\Delta\Psi$) of the high energy state. Our observation of the differential effect of valinomycin on DLE with and without an acceptor may indicate that $\Delta\Psi$ is a more significant component of the total high energy state in the acceptorless case than it is with an electron acceptor.

At the higher uncoupler concentrations normally used for uncoupling of phosphorylation, DLE intensity in the presence of electron acceptors was reduced by a greater percentage than was acceptorless DLE intensity (Table 3-I). This may indicate a "basal" level of DLE that is independent of the high energy state. Even with these uncoupler concentrations, the activation of rapid electron transport by addition of an acceptor led to an increase in DLE intensity at 1 msec (Table 3-I). This stimulation of the DLE may reflect the formation of a low level of high energy state even in the presence of these concentrations of uncouplers. This suggestion is in agreement with observations (Schuldiner *et al.*, 1972; Heldt *et al.*, 1973) that uncouplers did not completely abolish ΔpH at these concentrations. Alternatively, the increase in DLE intensity at 1 msec upon activation of rapid electron flow to acceptors may reflect an effect of electron transport itself on DLE in addition to its effects on the high energy state. That more rapid withdrawal of charge from the reaction center may lead to an increased intensity of DLE at 1 msec is suggested in the two-quantum model of Bertsch (Bertsch, 1969; Bertsch and Lurie, 1971; Bertsch et al., 1971) and in the model of Van Gorkum and Donze (1973) in which charge accumulation on the oxidizing side of photosystem II is emphasized.

2. The stimulation of DLE intensity by NH_4Cl

A general mechanism for the uncoupling of phosphorylation by ammonia and amines has been proposed by Crofts (1968) and has received considerable support (McCarty, 1969). In this model, the uncharged amine equilibrates across the membrane and is protonated in the acid interior. The cationic species is relatively impermeable to the membrane. Amine uncoupling thus results in the substitution of an amine cation gradient for a proton gradient. The substitution is electrically neutral, and the membrane potential thus should be unaffected (McCarty, 1969).

Delayed light intensity, both in the absence and presence of electron acceptors, was decreased by low concentrations of NH_4Cl (Fig. 3-7). This effect would appear to reflect the dissipation of the proton gradient, an action all uncouplers of chloroplasts have in common. However, at higher NH_4Cl concentrations (above 1 mM) acceptorless DLE was greatly stimulated. A similar, though smaller, effect is seen in the presence of an acceptor at slightly higher NH_4Cl concentrations (above 5mM). Accompanying this minimum in DLE intensity as a function of NH_4Cl concentration, there is a maximum in electron transport rate (Fig. 3-7), i.e., at NH_4Cl concentrations above 5mM, the rate of electron transport is inhibited below the maximum NH_4Cl-stimulated rate. This inhibition of electron transport appears to involve an effect on the oxygen-evolving mechanism of photosystem II (Izawa et al., 1969; Velthuys, 1974; see also Chapter 4).

From the concentration profiles, it would seem reasonable to relate the stimulation of DLE to the inhibitory effect of NH_4Cl on electron flow. However, the dependence of electron transport rate on hexylamine concentration shows a pattern quite similar to that seen with NH_4Cl, but no stimulation of DLE is seen with hexylamine (Fig. 3-6). Of course, it is possible that the mechanisms of inhibition of electron transport are different for the two amines, but in the absence of evidence to this effect, it would appear that the stimulation of DLE intensity by NH_4Cl is not a direct consequence of its inhibitory effect. It seems plausible to ascribe the inhibitory effect of both of these uncouplers on electron transport to a characteristic they have in common. As indicated above, amines, in general, are presumed to uncouple by substitution of an amine cation gradient for a proton gradient. The electron transport inhibition could then be caused by the presence of the amine cations inside the thylakoid, by the uncharged amine species, or, as suggested in Chapter 4, by the elevated internal pH of the thylakoid.

McCarty and Coleman (1970), in a study of the effects of aliphatic amines on photophosphorylation, have indicated that the cationic form of long-chain aliphatic amines may behave somewhat as cationic detergents and that they may have a second effect on phosphorylation in addition to their action as amines in decreasing the proton gradient.

Felker et al. (1974) have also observed the stimulation of DLE intensity by NH_4Cl, and have argued that this stimulation represents an increase in the membrane potential caused by the accumulation of NH_4^+ inside the thylakoid. They reason that if the membrane were less permeable to NH_4^+ than to H^+, then substitution of an NH_4^+ gradient for a proton gradient may lead to a higher membrane potential. In this model, the difference between NH_4Cl and hexylamine would reflect the difference in the membrane permeabilities of the two cationic species. The suggestion (McCarty and Coleman, 1970) that the hexylamine cation may also behave somewhat as a cationic detergent might indicate that this species could have an effect on the permeability of the thylakoid membrane to other ions, and thus might be less able to develop an elevated membrane potential. Our finding that the stimulation of acceptorless DLE by NH_4Cl was much more pronounced, on a relative basis, than was the stimulation of DLE in the presence of an acceptor may, in terms of the hypothesis of Felker et al., indicate that the membrane in the acceptorless state has somewhat different properties than it does in the presence of rapid electron flow.

3. The pH dependence of DLE

Most methods of estimating ΔpH have led to the conclusion that the ΔpH formed in the light by chloroplasts increases as the pH of the external medium is raised from pH 6.5 to pH 9.0 (Rottenberg et al., 1972; Schuldiner et al., 1972). Portis and McCarty (1973) have raised objections to some of these methods, and have reported an almost constant ΔpH over the external pH range of 6.5 to 8.0. In either case the observation (Fig. 3-14) that msec DLE intensity decreased strongly as the external pH (pH_e) was raised over this range did not agree with the proposed dependence of of DLE intensity on ΔpH. Nor did the behavior of the DLE intensity reflect a simple dependence on the rate of electron transport, another factor proposed by several workers (Clayton, 1969; Bertsch et al., 1969) to influence DLE intensity. Particularly in the presence of gramicidin, the rate of electron transport and the intensity of msec DLE showed no simple relationship (Fig. 3-14).

A possible interpretation of these observations is that the internal pH (pH_i) may be a predominant factor modulating msec DLE intensity. Following the suggestions of Kraan et al. (1970) and Wraight and Crofts (1971), an increase in pH_i may increase the availability of holes as substrates for DLE by shifting the midpoint potential of a hydrogen carrier on the oxidizing side of photosystem II (either the primary donor or a secondary donor). A dependence on pH_i could also explain many of the DLE phenomena previously ascribed to ΔpH, since, under most conditions, an increase in ΔpH will be accompanied by a decrease in pH_i. This would be especially true if the external medium were buffered.

However, it is possible that under the conditions of our experiments electrons were relatively plentiful compared to holes as substrates for DLE. This could result from using conditions where the pool of secondary acceptors is relatively filled compared to the pools on the oxidizing side of the photosystem II. Such a situation could give rise to an apparent lack of an effect of the external pH on the availability of electrons for DLE, even though under other conditions the pH_e might exert a strong effect on DLE intensity.

In summary, our observation that a rather large, light-induced, ΔpH was formed in the absence of added electron acceptors appears to reconcile the observed effects of uncouplers on acceptorless DLE with Mayne's proposed relationship (Mayne, 1967, 1968) between the high energy state and DLE intensity. Most of the differences between the response of acceptorless DLE and DLE in the presence of acceptors to various conditions derive, we believe, from the different magnitude of the high energy state formed under these two conditions. The pH dependence of the 1 msec DLE intensity may indicate that the internal pH exerts a stronger influence on DLE than does ΔpH. This possibility is discussed in more detail in Chapter 6.

CHAPTER 4

INHIBITION OF PHOTOSYSTEM II ELECTRON TRANSPORT BY UNCOUPLERS AT ALKALINE pH

A. Introduction

In coupled chloroplasts the pH optimum of the Hill reaction is about pH 9.0. In the presence of uncouplers of photophosphorylation, this pH optimum is shifted to pH 8.0 or below (Avron, 1971; Jagendorf and Smith, 1972). Above a pH of about 8.5, the addition of an uncoupler actually inhibits the Hill reaction, and this inhibition is quite severe by pH 9.0.

In the present study, we have attempted to elucidate the nature of this inhibitory action of uncouplers at alkaline pH. We have found that chloroplasts inhibited in this way exhibit a very rapid decay of msec delayed light emission in the absence of added electron acceptors. This type of rapidly decaying emission has been previously associated with conditions that inhibit electron transport between water and photoreaction II, such as Tris-aging (Bertsch and Lurie, 1971) or heat treatment (Vernon *et al.*, 1971). In addition we have found that DCMU-sensitive photoreduction of Hill acceptors can be restored in the uncoupler-inhibited chloroplasts by the addition of compounds, such 1,5-diphenylcarbohydrazide, that are capable of donating electrons to photoreaction II.

From these results, we conclude that the inhibition of electron transport by uncouplers at alkaline pH occurs between the site of water oxidation and photoreaction II. We suggest that the inhibition may be directly related to the elevation of the internal thylakoid pH that results from the uncoupler-induced decrease in the transmembrane pH gradient. This interpretation is in agreement with a similar suggestion recently made by Harth et al., (1974).

B. Results

Table 4-I shows the effects of 1 uM gramicidin at pH 7.5 and pH 8.8 on the rates of electron transport through both photosystems (electron flow from water to methyl viologen) and through photosystem I alone (electron flow from reduced DCPIP to methyl viologen in the presence of 2 uM DCMU used to inhibit photosystem II activity). Addition of the uncoupler gramicidin at pH 7.5 stimulated electron flow involving both photosystems ($H_2O \longrightarrow MV$) several fold, but this reaction was severely inhibited by the uncoupler at pH 8.8. In contrast, the photosystem I reaction (DCPIP ⟶ MV) was stimulated by the uncoupler both at pH 7.5 and at pH 8.8.

Table 4-II shows similar results for the following uncouplers: NH_4Cl (10 mM), nigericin (0.5 uM), chloroquinphosphate (0.1 mM), and CCCP (5 uM). Table 4-III gives results with three amine uncouplers; methylamine (50 mM), hexylamine (10 mM), and NH_4Cl (10 mM). In each case the indicated concentration of the uncoupler stimulated electron transport

Table 4-I

THE EFFECT OF GRAMICIDIN ON ELECTRON TRANSPORT AT pH 7.5 AND AT pH 8.8

The 5 ml reaction mixture for $H_2O \longrightarrow MV$ contained 20 mM NaCl, 50 mM Tricine (pH 7.5 or 8.8), 2 mM $MgCl_2$, 0.1 mM MV, 0.5 mM NaN_3, and Good King Henry chloroplasts equivalent to 100 ug chlorophyll. For DCPIP ⟶ MV, the following components were added: 0.5 mM neutralized ascorbate, 0.04 mM DCPIP and 2 uM DCMU. Gramicidin, when added, was 1 uM final concentration.

Condition	Rate of Electron Transport (ueq/mg chl/hr)	
	$H_2O \longrightarrow MV$	DCPIP ⟶ MV
pH 7.5	103	111
pH 7.5 + gramicidin	803	480
pH 8.8	332	148
pH 8.8 + gramicidin	69	554

Table 4-II

THE EFFECTS OF UNCOUPLERS ON ELECTRON TRANSPORT AT pH 7.5 AND pH 9.0

The reaction mixtures were as in Table 4-I, except that 20 mM KCl replaced NaCl.

Uncoupler added	Rate of Electron Transport (ueq/mg chl/hr) pH 7.5 $H_2O \rightarrow MV$	 pH 9.0 $H_2O \rightarrow MV$	 $DCPIP \rightarrow MV$
None	85	335	115
Gramicidin (1 uM)	895	99	380
Nigericin (0.5 uM)	1060	70	440
NH_4Cl (10 mM)	880	69	390
Chloroquinphosphate (0.1 mM)	500	172	370
CCCP (5 uM)	580	112	280

Table 4-III

EFFECT OF AMINE CONCENTRATION AND pH ON THE RELATIVE RATE OF $K_3Fe(CN)_6$ REDUCTION

The reaction mixture contained 20 mM NaCl, 2 mM $MgCl_2$, 50 mM Tricine, 0.3 mM $K_3Fe(CN)_6$, and spinach chloroplasts equivalent to 10 ug chlorophyll/ml. Absolute rates of ferricyanide reduction, expressed in ueq/mg chl/hr, in the absence of amines were: pH 7.5, 77; pH 7.9, 108; pH 8.6, 146.

	Relative Rate of Electron Transport								
	NH_4Cl			methylamine			hexylamine		
pH	7.5	7.9	8.6	7.5	7.9	8.6	7.5	7.9	8.6
[Amine] (mM)									
0.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
0.1	1.5	1.6	1.4	1.3	1.2	1.2	1.1	1.1	1.2
1.0	2.6	1.7	1.2	1.6	1.4	1.2	2.2	1.1	1.1
10.0	2.9	1.4	0.5	2.6	1.9	0.6	2.4	1.2	0.0
50.0	2.0	0.9	0.3	2.8	1.3	0.1	1.4	0.0	0.0
100.0	1.6	0.5	0.2	2.4	0.9	0.0	0.8	0.0	0.0

at pH 7.5, but inhibited electron flow from photosystem II at higher pH values (8.5 - 9.0). The pH at which substantial inhibition occurred varied slightly from day to day, but always fell into the range of pH 8.5 to 9.0 with the above uncoupler concentrations. Similar effects were also seen in chloroplasts uncoupled by EDTA-treatment (Table 4-VII).

Table 4-III also shows that with amine uncouplers the inhibition depended both on the uncoupler concentration and on the pH of the medium. In addition, uncouplers were observed to inhibit more effectively after dark incubation with the chloroplasts. Both these effects are illustrated for NH_4Cl in Figure 4-1. As shown in this Figure, a low concentration of NH_4Cl (1 mM) resulted in inhibition of electron transport at pH 8.7 only after the chloroplasts were exposed to the uncoupler in the dark. When the pH was raised to pH 8.9, this concentration of the uncoupler inhibited even when added in the light. At a higher concentration of NH_4Cl (10 mM), the inhibition at pH 8.7 was independent of a dark incubation period. A similar enhancement of the inhibition by dark incubation was observed with other uncouplers as shown for gramicidin in Table 4-IV.

Figure 4-2 compares the pH dependence of the ferricyanide and DCPIP Hill reactions in the presence of gramicidin. The pH dependencies of the coupled reactions (no uncoupler, not shown here) were very similar to one another and were comparable to those observed previously (Bamberger *et al.*,

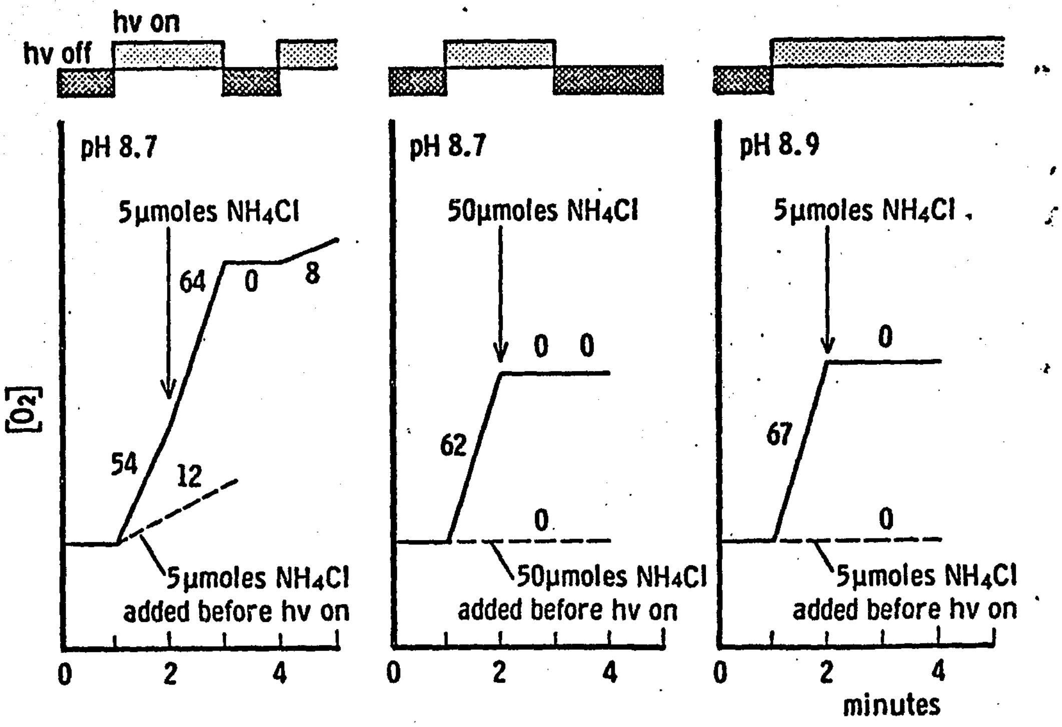


Figure 4-1. Effect on O_2 evolution of NH_4Cl concentration, pH, and time of uncoupler addition. The reaction mixture contained, in 5 ml: 20 mM NaCl, 50 mM tricine (pH 8.7 or 8.9), 2 mM $MgCl_2$, 0.4 mM $K_3Fe(CN)_6$ and spinach chloroplasts equivalent to 100 ug of chlorophyll. The numbers above the traces indicate the rates of O_2 evolution in umoles/mg chl/hr. The dashed lines indicate experiments in which the uncoupler was added just before turning the light on.

Table 4-IV

EFFECT OF DARK INCUBATION ON THE INHIBITION OF O_2 EVOLUTION BY GRAMICIDIN AT ALKALINE pH

The reaction mixture contained 20 mM NaCl, 2 mM $MgCl_2$, 50 mM Tricine, 0.3 mM potassium ferricyanide and spinach chloroplasts equivalent to 20 ug chlorophyll/ml. Each condition was maintained for 1 minute before the next addition, or change, was made.

Sequence of Addition	Rate of O_2 Evolution (ueq/mg chl/hr)
Sample 1: pH 8.6	
Dark	0
Light on	231
+ 1 uM gramicidin	577
Light off	0
Light on	63
Sample 2: pH 8.6	
Dark	0
+ 1 um gramicidin	0
Light on	30
Sample 3: pH 8.8	
Dark	0
Light on	222
+ 1 um gramicidin	151
Light off	0
Light on	0

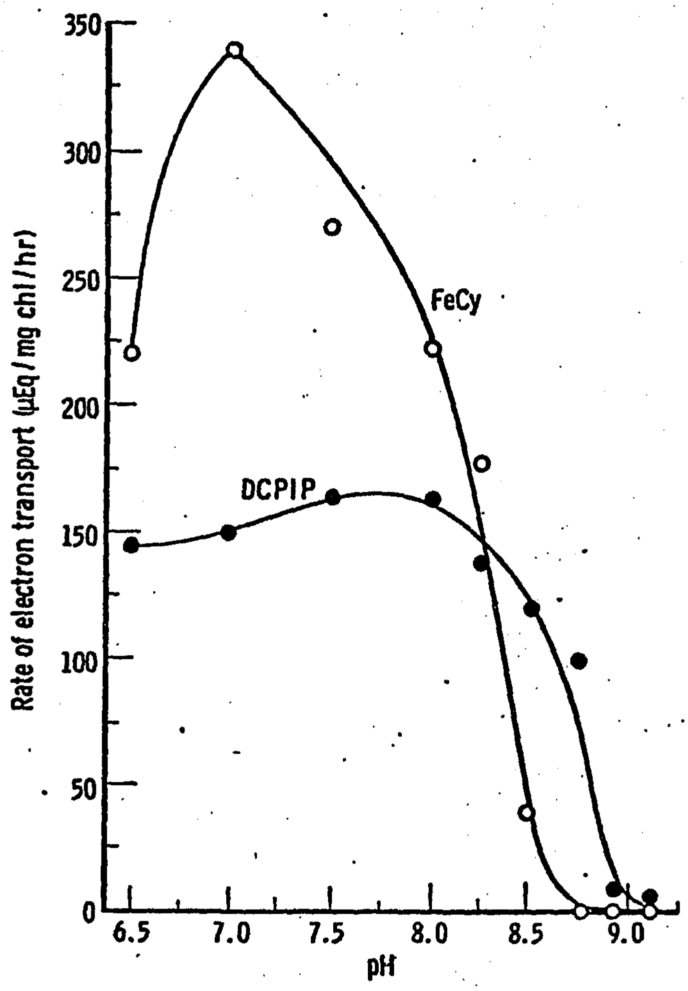


Figure 4-2. pH dependence of electron transport in the presence of 1 uM gramicidin. Reaction mixtures contained, in 5 ml: 20 mM NaCl, 2 mM $MgCl_2$, spinach chloroplasts equivalent to 50 ug chlorophyll, either 50 mM TES (pH 6.5 - 7.5) or 50 mM Tricine (pH 8.0 - 9.5), and either 0.3 mM potassium ferricyanide (FeCy) or 12uM DCPIP. Rates were determined spectrophotometrically.

1973; see also Fig. 3-14). Both uncoupled Hill reactions were inhibited at alkaline pH, but the pH at which this inhibition occurred was consistently slightly lower for ferricyanide than for DCPIP. Inhibition of Hill reactions using methyl viologen or FMN as acceptors showed a pH dependence similar to that of the DCPIP Hill reaction as shown in Table 4-V.

Figure 4-3 shows the effects of gramicidin and of NH_4Cl on msec delayed light emission (DLE) and on electron transport from chloroplasts at pH 8.8. The effects of uncouplers at this pH were quite different from those observed at pH 7.5 (Wells et al., 1971; Felker et al., 1974). At pH 7.5, uncouplers lower the intensity of the emission without causing a large change in the kinetics of the dark decay regardless of presence or absence of acceptors (see Fig 3-1 and Table 3-I). At pH 8.8, on the contrary, uncouplers caused a marked increase in the DLE decay kinetics in the absence of an electron acceptor. This rapid dark decay was accompanied by an increase in the intensity of the emission at 0.8 msec after illumination (Fig. 4-3). Addition of a Hill acceptor, in this case, had little or no effect, whereas at pH 7.5, Hill acceptors induce an increase in the msec DLE intensity and increase the rate of the decay of the emission.

Figure 4-3 also shows that at pH 8.8, gramicidin and NH_4Cl had somewhat different effects on delayed light emission. Gramicidin at high pH caused the emission to decay

Table 4-V

EFFECT OF GRAMICIDIN ON RATES OF ELECTRON TRANSPORT WITH FOUR ELECTRON ACCEPTORS AS A FUNCTION OF pH

The reaction mixture contained: 20 mM NaCl, 2 mM $MgCl_2$, 50 mM Tricine and spinach chloroplasts equivalent to 20 ug chlorophyll/ml. When added, gramicidin was present at 1 uM, potassium ferricyanide at 0.3 mM, DCPIP at 0.04 mM, methyl viologen at 0.2 mM, and flavin mononucleotide (FMN) at 0.1 mM. Rates of electron transport were measured by O_2 evolution or consumption. When methyl viologen or FMN were used as acceptors, NaN_3 was added at a concentration of 0.5 mM.

Additions	Rate of electron transport (ueq/mg chl/hr)		Rate $\frac{+\text{ gramicidin}}{-\text{ gramicidin}}$
	- gramicidin	+ gramicidin	
pH 7.5			
$K_3Fe(CN)_6$	169	831	4.9
DCPIP	250	723	2.9
Methyl viologen	85	923	10.8
FMN	169	1046	6.2
pH 8.6			
$K_3Fe(CN)_6$	346	87	0.25
DCPIP	310	373	1.2
Methyl viologen	327	423	1.3
FMN	427	473	1.1
pH 8.8			
$K_3Fe(CN)_6$	335	0	0.0
DCPIP	238	27	0.1
Methyl viologen	338	100	0.3
FMN	353	96	0.3

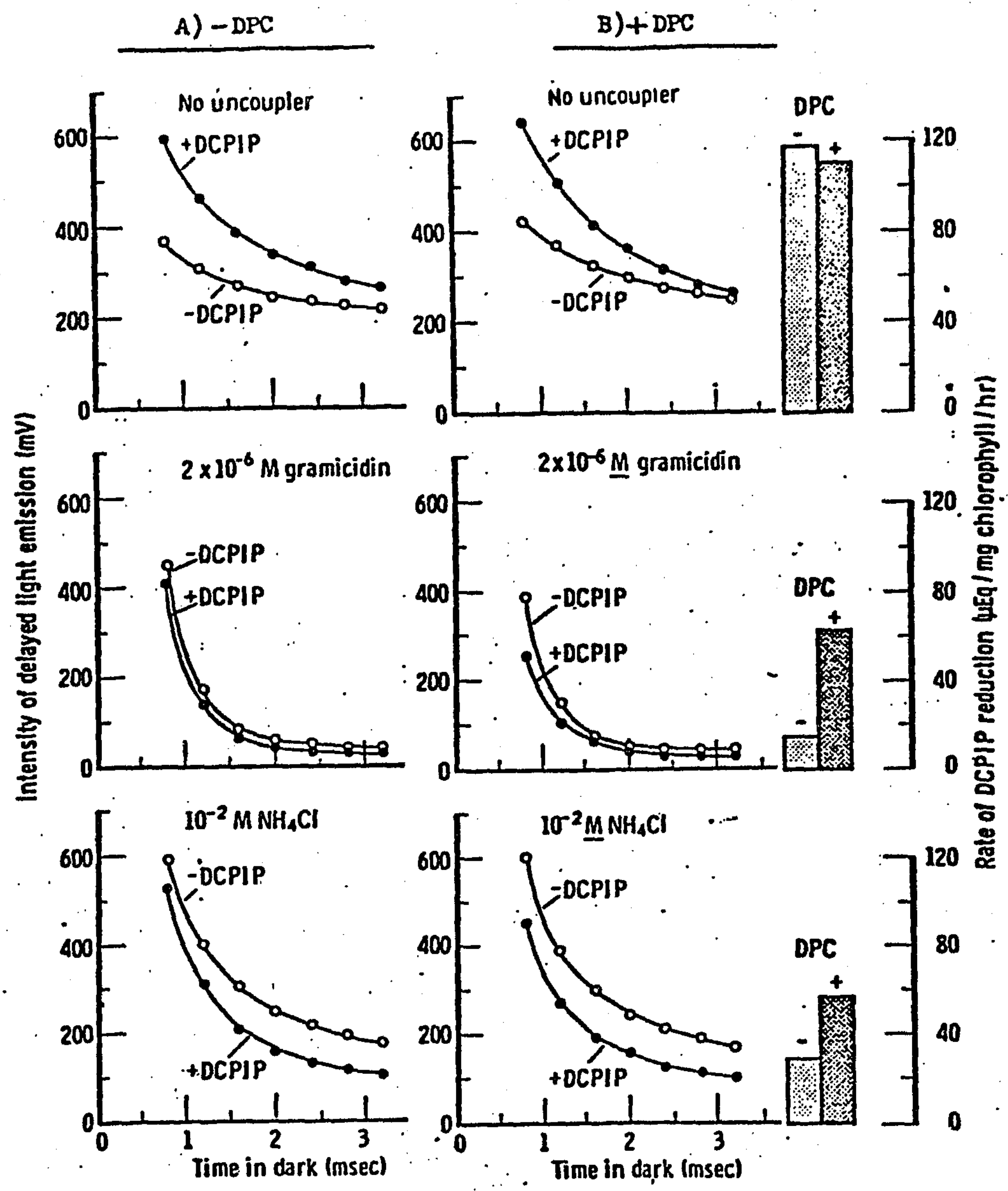


Figure 4-3. Effects of gramicidin and NH_4Cl at pH 8.8 in the absence (A) and presence (B) of DPC on msec delayed light emission and on electron transport. Reaction mixtures contained, in 2 ml: 20 mM NaCl, 50 mM tricine (pH 8.8), 2 mM $MgCl_2$, and spinach chloroplasts equivalent to 20 ug chlorophyll. When added, DCPIP was present at lum and DPC at 0.5 mM.

extremely rapidly. This was also the case when nigericin was used as the uncoupler or when chloroplasts were uncoupled by EDTA treatment. With NH_4Cl the dark decay was not so fast, but the emission intensity in the absence of an acceptor was increased at 0.8 msec by NH_4Cl.

Inhibition of the maximal NH_4Cl-stimulated rate of electron transport through photosystem II was observed at pH 7.5 when the concentration of NH_4Cl was increased to 100 mM (Table 4-III, see also Fig. 3.7). In this case the intensity of acceptorless msec DLE was also stimulated considerably, but there was relatively little effect on the decay kinetics of the emission. Under these conditions, as with inhibition by uncouplers at alkaline pH, electron transport to ferricyanide was more easily inhibited than was electron transport to DCPIP. That is, at pH 7.5, lower concentrations of NH_4Cl were necessary to inhibit ferricyanide reduction than DCPIP reduction.

We also examined the effect of alkaline pH in the absence of uncouplers. Ageing chloroplasts at pH 9.6 (5 min incubation at pH 9.6, then titrated to pH 7.5) severely inhibited electron transport involving photosystem II (see Table 4-VIII), but did not inhibit electron flow through photosystem I. Msec delayed light emission from chloroplasts aged pH 9.6 had characteristics similar to those shown in Figure 4-3 for gramicidin at pH 8.8.

The rapid decay of the delayed light emission observed in the absence of a Hill acceptor at pH 8.8 with gramicidin, or observed following alkaline incubation, is similar to that observed in chloroplasts which have been Tris-aged (Bertsch and Lurie, 1971) or heated (Vernon et al., 1971). In these systems electron transport from photosystem II can be restored by the addition of exogenous electron donors. Figure 4-3 shows the effect of the electron donor 1,5-diphenylcarbohydrazide (DPC) on the photoreduction of DCPIP, and on the delayed light emission, from chloroplasts at pH 8.8 in the presence of NH_4Cl and gramicidin. Although the delayed light emission was hardly affected by addition of DPC, the rate of DCPIP photoreduction in the inhibited chloroplasts was stimulated by this electron donor. Tables 4-VI and 4-VII show the restoration of photosystem II electron transport by addition of DPC to chloroplasts inhibited at alkaline pH by uncouplers or by EDTA-treatment. Electron transport could also be restored by the addition of other electron donors, and such restored electron transport was sensitive to DCMU as is shown in Table 4-VIII for chloroplasts inhibited by ageing at pH 9.6.

The intensity of prompt fluorescence in the absence of an electron acceptor was attenuated when chloroplasts at alkaline pH were exposed to either gramicidin or NH_4Cl, as shown in Table 4-IX. Addition of the electron donor DPC had little or no effect on the level of prompt fluorescence in the uncoupler-inhibited chloroplasts, but addition of DCMU restored the fluorescence intensity to that of the untreated chloroplasts.

TABLE 4-VI

RESTORATION OF PS II ELECTRON TRANSPORT IN CHLOROPLASTS INHIBITED BY UNCOUPLERS AT pH 9.0

The 5 ml reaction mixture contained 20 mM KCl, 2mM $MgCl_2$, 50 mM Tricine, pH 9.0, 12 uM DCPIP and Good King Henry chloroplasts equivalent to 50 ug chlorophyll. When added, DPC was 0.5 mM final concentration. Rates are expressed as ueq DCPIP reduced/mg chl/hr.

Uncoupler	Rate of Electron Transport	
	-DPC	+DPC
None	98	102
Gramicidin (1 uM)	4.6	69
Nigericin (0.5 uM)	4.0	66
NH_4Cl (10 mM)	18.8	68
CCCP (5 uM)	0.0	60

Table 4-VII

INHIBITION OF ELECTRON TRANSPORT IN EDTA-TREATED CHLOROPLASTS BY ALKALINE pH AND ITS RESTORATION BY 1,5-DIPHENYLCARBOHYDRAZIDE

The 5 ml reaction mixture contained 20 mM NaCl, 2 mM $MgCl_2$, 50 mM Tricine (pH 7.5 or 9.0), 12 uM DCPIP, and spinach chloroplasts (treated or untreated) equivalent to 50 ug chlorophyll. When added, DPC was present at 0.5 mM final concentration. Rates of DCPIP reduction were measured spectrophotometrically and are expressed in arbitrary units.

Condition	Rate of Electron Transport	
	-DPC	+DPC
Untreated, pH 7.5	20	22
EDTA-treated, pH 7.5	109	104
Untreated, pH 9.0	55	58
EDTA-treated, pH 9.0	9	32

Table 4-VIII

EFFECTS OF SEVERAL ELECTRON DONORS ON PS II ELECTRON TRANSPORT INHIBITED BY AGEING AT pH 9.6

Spinach chloroplasts were aged at pH 9.6 as described in Chapter 2. Residual photosystem II activity was determined at pH 7.5. The reaction mixture contained 20 mM NaCl, 2 mM $MgCl_2$, 25 mM Tricine, pH 7.5, 12 uM DCPIP and spinach chloroplasts equivalent to 10 ug chlorophyll/ml. When added, DPC was present at 0.5 mM, $MnCl_2$ at 2 mM, NH_2OH at 10 mM, and DCMU at 2 uM. Rates of DCPIP reduction were determined spectrophotometrically and are expressed in arbitrary units.

Condition	Rate of Electron Transport	
	-DCMU	DCMU
Control chloroplasts	80	3
Alkaline-aged chloroplasts	6	0
Alkaline-aged chloroplasts, DPC	60	1
Alkaline-aged chloroplasts, $MnCl_2$	40	0
Alkaline-aged chloroplasts, NH_2OH	40	0

Table 4-IX

EFFECTS OF UNCOUPLERS, DCMU, AND ELECTRON DONORS ON PROMPT FLUORESCENCE INTENSITY AT pH 7.5 AND pH 8.6

The reaction mixture contained 20 mM NaCl, 2 mM $MgCl_2$, 50 mM Tricine (pH 7.5 or 8.6) and spinach chloroplasts equivalent to 20 ug chlorophyll/ml. When added, DPC was present at 0.5 mM.

Additions	Fluorescence Intensity (arbitrary units)			
	pH 7.5		pH 8.6	
	-DPC	+DPC	-DPC	+DPC
None	56	58	53	49
NH_4Cl (10 mM)	58	54	32	26
Gramicidin (1 uM)	62	65	34	24
DCMU (1uM)	60	60	55	-
Gramicidin + DCMU	-	-	55	--
NH_4Cl + DCMU	-	-	55	-

Discussion

Exposure of chloroplasts to uncouplers of photophosphorylation at alkaline pH (8.5 - 9.0) or incubation of chloroplasts at pH 9.6, resulted in inhibition of electron transport reactions involving photosystem II, but not those which involve only photosystem I. Associated with the inhibition of electron transport, a rapid decay of msec delayed light emission in the absence of an electron acceptor was observed (Fig. 4-3). In addition, prompt fluorescence was lowered when chloroplasts were exposed to NH_4Cl or gramicidin at alkaline pH (Table 4-IX). These characteristics of delayed light emission (Bertsch and Lurie), 1971) and fluorescence (Yamashita and Butler, 1968a,b; Lozier _et_ _al_., 1971) have been associated with treatments that damage the O_2-evolving apparatus of photosystem II. The suggestion that the alkaline inhibitions we have investigated likewise act on the O_2-evolving side of photosystem II is supported by restoration of DCMU-sensitive electron transport upon the addition of the artificial electron donor, DPC.

However, the addition of DPC to chloroplasts inhibited by uncouplers at high pH does not restore the normal decay of the delayed light (Fig. 4-3) nor does it increase the level of the variable fluorescence (Table 4-IX), effects that are observed when electron donors are added to Tris-aged chloroplasts (Yamashita and Butler, 1968a; Bertsch and Lurie, 1971; Vernon _et_ _al_., 1971). The failure of donors to affect prompt or delayed fluorescence has been noted in chloroplasts in which the O_2-evolving apparatus has been inactivated by UV irradiation or NH_2OH treatment (Yamashita and Butler, 1968b; Katoh et al., 1970). This may suggest that different sites in the O_2-evolving apparatus may be affected by these treatments than are affected by Tris-ageing. This possibility is discussed more fully in Chapter 5.

It has previously been suggested that the rate of electron transport is partially dependent on the internal pH (designated pH_i) of the thylakoid (Bamberger et al., 1973; Rottenberg et al., 1971). Our results support this idea and indicate that one site that is sensitive to pH_i involves electron transport from water to photoreaction II, in agreement with the recent suggestion of Harth et al. (1974). Uncouplers reduce the ΔpH established in the light (Bamberger et al., 1973; Shavit et al., 1970) and thus lead to an elevation of the internal pH. Incubation of chloroplasts at pH 9.6 for 5 minutes in the absence of uncouplers may also lead to an elevation of the internal pH to a level sufficient to inactivate O_2 evolution.

It has been suggested by a number of workers that the O_2-evolving apparatus is located on the inner side of the thylakoid membrane (Arntzen et al., 1969; Kraan et al., 1970; Junge and Ausländer, 1974). This suggestion is consistent with our hypothesis that some step in O_2-evolving reactions is sensitive to the pH of the intrathylakoid space.

The inhibition of electron transport caused by NH_4Cl at alkaline pH has previously been interpreted as being due to the free amine (Izawa et al., 1969). This is consistent with the fact that the inhibition can be achieved at lower pH by a higher concentration of NH_4Cl (Table 4-III). However, these data are also consistent with the idea that the internal pH of the thylakoid is the critical factor. According to the model of amine uncoupling developed by Crofts (1968), amines uncouple by equilibration of the uncharged species across the thylakoid membrane, followed by protonation of the amine in the more acidic internal space. The membrane is assumed to be relatively impermeable to the charged amine cation, which thus accumulates inside (Crofts, 1968; Gaensslen and McCarty, 1971). This results in the substitution of an amine cation gradient for the proton gradient. The degree to which this occurs would depend on the concentration of the permeable, uncharged amine in the external medium (Hind and Whittingham, 1963). Thus, the decrease in ΔpH (and the accompanying increase in pH_i) would be enhanced by raising the NH_4Cl concentration or by raising the external pH.

The observation that the delayed light emission from NH_4Cl-inhibited chloroplasts differed from that observed from chloroplasts inhibited by gramicidin, nigericin, etc. may reflect some action of the amine in addition to its effect on ΔpH, and may be related to the accumulation of NH_4^+ by the chloroplast. This accumulation may, according to Felker et al. (1974), result in an increased membrane potential if the membrane is less permeable to NH_4^+ than to H^+. In this case, the unusual effects of NH_4Cl on delayed light emission would reflect its action on this component of the high energy state. On the other hand, the effects of NH_4Cl on the delayed light emission might be related to the swelling of the chloroplasts that accompanies NH_4^+ accumulation (Gaensslen and McCarty, 1971) or perhaps to an effect of the amine cation itself.

The observation that the effect of an uncoupler at alkaline pH depended on whether it was introduced in the light or in the dark (Fig. 4-1) may reflect the interaction of electron transport, ΔpH and pH_i. When chloroplasts are illuminated in the presence of an electron acceptor, the pH_i would fall as protons are pumped into the thylakoid. Addition of an uncoupler in the light would increase the rate of dissipation of the accumulated protons, thus raising pH_i. However, if this rate of dissipation were not too high compared to the rate of proton accumulation, the pH_i would not rise enough to inhibit oxygen evolution. This could result in an increase in the rate of electron transport by reducing the ΔpH against which protons must be pumped and/or by bringing the new pH_i closer to the optimal pH_i for electron transport. In contrast, in the dark, an uncoupler at alkaline pH would alkalinize the interior to a much greater extent, causing inhibition of the water-oxidizing apparatus. If the concentration of uncoupler were raised, or if the external pH were too alkaline, addition of the uncoupler even in the light might result in the pH_i being raised to a level which inhibits the oxygen-evolving side of photoreaction II.

The rate of electron transport in the presence of an uncoupler exhibits a rather abrupt decrease as the external pH is raised (Fig. 4-2). This would result from the fact that although the rate of electron transport is influenced by pH_i and/or ΔpH, it is electron flow that is responsible for the injection of protons into the thylakoid space. Thus, if the pH_i is initially only somewhat above that optimal for electron transport, then, upon illumination, the resulting electron transport will act to lower the pH_i. This would result in an increased rate of electron transport which would further lower the pH_i, etc. Only at sufficiently high external pH is a point reached where inhibition of the oxygen-evolving apparatus by high pH_i causes such low initial rates of electron flow that the light-induced proton pump can no longer significantly compete with the rate of dissipation of protons caused by the uncoupler. The observation (Fig. 4-2) that electron transport to ferricyanide and to DCPIP in the presence of gramicidin are inhibited at slightly different pH's may be due to a difference between the two Hill reactions in the rate of proton translocation into the thylakoid.

In conclusion, the inhibition of electron transport by uncouplers at alkaline pH is seen to be a direct result of the presumed mode of action of uncouplers in dissipating the proton gradient across the thylakoid membrane, together with an inhibitory effect of the resulting internal thylakoid pH on the oxygen-evolving apparatus of photosystem II.

CHAPTER 5

TREATMENTS THAT INHIBIT ON THE OXIDIZING SIDE OF PHOTOSYSTEM II

A. Introduction

In this chapter, we have examined the decay kinetics of msec delayed light emitted by chloroplasts in which electron flow between water and photoreaction II has been inhibited by a wide variety of treatments. All the treatments examined resulted in a rapid decay of the DLE in the time range measured (0.8 to 3.2 msec after repeating flashes of exciting light). The effects of added donors of electrons to photosystem II and of DCMU on this rapidly decaying DLE were not the same for all treatments, and appeared to indicate separation of the treatments into two categories. We suggest that the two groups of treatments affect at least two different sites between the reaction center of photosystem II and the site of water oxidation. Two different general models for the effects of treatments on electron flow are discussed. The following introductory material is intended as a brief review of some of the characteristics of these treatments.

The initial identification of an inhibition of electron flow on the oxidizing side of photosystem II was reported by Yamashita and Butler (1968a) for chloroplasts incubated in high concentrations of Tris buffer (0.8 M, pH 8.0). Since then, a wide variety of treatments or conditions have been developed that specifically act on this region of the electron transport chain. These include: heat treatment (Yamashita and Butler, 1968b), ultraviolet irradiation (Yamashita and Butler, 1968b), incubation in Triton X-100 (Malkin, 1971), trypsin inactivation (Selman and Bannister, 1971), hydroxylamine extraction (Katoh _et_ _al_., 1970), exposure to uncouplers at alkaline pH (Harth _et_ _al_., 1974; see also Chapter 4), chloride depletion (Izawa et al., 1969), and manganese depletion (Heath and Hind, 1969b).

The interpretation that the inhibition caused by these treatments involves the oxygen-evolving side of photosystem II rests primarily on the ability of the treated chloroplasts to carry out DCMU-sensitive electron flow to Hill acceptors when supplied with artificial electron donors. Electron donors that have been found to be effective in restoring photosystem II electron flow in many of the treated systems include phenylenediamine (ascorbate), hydroquinone (ascorbate), diphenylcarbohydrazide, and Mn^{2+}.

A second characteristic that all of the above treatments have in common is a lowering of the yield of variable fluorescence, i.e., the time varying component of prompt fluorescence that develops upon illumination and which has been associated with the redox state of the primary acceptor (Q) of photosystem II (Duysens, 1963). This suggests that, upon illumination, Q does not become as reduced in the treated systems as it does in normal chloroplasts.

Many of the oxidizing-side treatments result in the loss of some of the chloroplast manganese. Heat treatment or hydroxylamine extraction result in the loss of most or all of the chloroplast manganese (Kimimura and Katoh, 1972; Cheniae and Martin, 1971; Homann, 1968), while Tris-aging results in loss of about two-thirds of the total manganese pool (Cheniae and Martin, 1970; Blankenship and Sauer, 1974).

Babcock and Sauer (1975a,b) have recently reported the appearance of a rapid, light-induced transient in the electron paramagnetic resonance signal associated with photosystem II following inhibition of electron transport by Tris-aging or heat. They have suggested that this signal may originate from the oxidized physiological donor (Z^+) to the reaction center of photosystem II, a species whose life-time they assume to be too short to detect in untreated chloroplasts.

Döring and coworkers (Döring _et_ _al_., 1961; Govindjee _et_ _al_., 1970; Döring, 1975) have reported that the light-induced absorption change at about 685 nm that has been associated with the reaction center chlorophyll of photosystem II (chl a_{II}, P680) remains active following inhibition of electron transport by Tris-aging or heat. Döring (1975) has interpreted this finding as indicating the continuous separation of charge by the reaction center in the inhibited systems. He has proposed a model for the action of the treatments in which the separated oxidizing and reducing equivalents formed by photosystem II wastefully recombine in a cyclic reaction that operates when water is no longer available to donate electrons to the reaction center.

The induction of cyclic side reactions around photosystem II by the treatments has also been proposed by Izawa *et al*. (1969), Rosenberg *et al*. (1972) and by Butler (1972). These proposals were devised primarily as an explanation for the low yield of variable fluorescence observed in the treated systems, and for the restoration of the fluorescence yield by DCMU and by added electron donors. This restoration of the fluorescence yield by DCMU or by electron donors has been reported for Tris-aged and heated chloroplasts (Yamashita and Butler, 1968a,b), for chloride-depleted chloroplasts (Izawa *et al*., 1969) and for chloroplasts partially depleted of manganese (Homann, 1968). However, similar restoration of the fluorescence yield by DCMU or by electron donors does not occur in ultraviolet-irradiated chloroplasts (Yamashita and Butler, 1968b), trypsin-treated chloroplasts (Selman and Bannister, 1971) or in chloroplasts totally depleted of manganese (Homann, 1968), even though electron transport in these systems is restorable upon addition of electron donors. No interpretation of the difference in the response of the fluorescence yield of the two groups of treatments has been offered by these investigators.

Bertsch and Lurie (1971) have independently proposed that a cyclic pathway of electron flow is induced by the oxidizing side treatments. Their proposal was based on the observation that treated chloroplasts in the absence of Hill acceptors exhibited a rapid decay of delayed light emission in the msec time range after illumination. Based on the observation that the rapid decay of the msec DLE following some of the treatments was slowed by electron donors, but that following other treatments the DLE decay was not affected by donors, Lurie (1972) proposed that different cyclic pathways were established by different treatments. The present work is an extension of these investigations of msec DLE in treated chloroplasts.

An alternate possibility for the effect of the oxidizing-side treatments on electron transport is that these treatments simply block electron flow from water to the reaction center of photosystem II (Yamashita and Butler, 1968a) with no induction of cyclic electron flow. Mohanty et al. (1972) supported this interpretation and argued that lack of access of electrons from water would result in the maintainence of Q, the primary acceptor of photosystem II, in its oxidized form, and thus would lead to the observed low yield of variable fluorescence. Addition of DCMU, they suggested, would result in the reduction of Q, since it could no longer be oxidized by the pool of secondary acceptors, and thus would lead to an increased fluorescence yield. Again, no mention was made of the lack of an effect of DCMU on the fluorescence yield that has been reported for several of the oxidizing-side treatments.

B. Results

Fig. 5-1 shows the effects of incubating oat chloroplasts for 15 min in 0.8 M Tris-HCl (pH 8.2) on msec DLE and on the rate of reduction of DCPIP. As previously reported (Bertsch and Lurie, 1971; Lurie, 1972) the Tris-aged chloroplasts are characterized by rapidly decaying DLE in the absence of electron acceptors. Addition of the electron acceptor DCPIP had little or no effect on the decay kinetics in the Tris-treated chloroplasts, but lowered the overall intensity of the DLE slightly. Addition of DCPIP to untreated chloroplasts resulted in a more than 2-fold increase in the emission intensity at 1 msec and a more rapid decay of the DLE as previously reported (Bertsch et al., 1971). Fig. 5-1 also shows that the DLE decay kinetics from Tris-aged chloroplasts were slowed by addition of the electron donor diphenylcarbohydrazide (which restored DCPIP reduction to 60% of the control rate). Diphenylcarbohydrazide (DPC) had little or no effect on the DLE or the rates of electron transport in untreated chloroplasts (see Table 5-I). In Tris-aged chloroplasts (Fig. 5-1) DCMU slowed the decay of the DLE in the absence of an acceptor in a manner similar to DPC, but resulted in a considerable lowering of the overall emission intensity. Addition of DCMU to untreated chloroplasts lowered the overall intensity of the emission and prevented any effect of the acceptor DCPIP on the DLE (see Fig. 5-4, left panel).

Fig. 5-2 shows the effects on msec DLE of ultraviolet

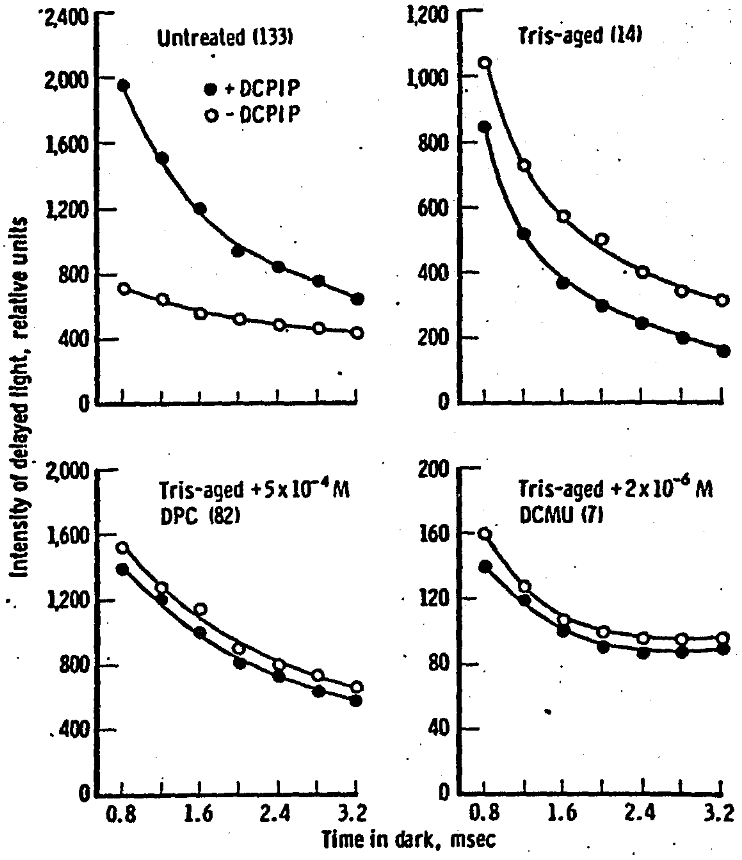


Fig. 5-1. Effect of DPC and of DCMU on msec DLE from Tris-aged oat chloroplasts. The reaction mixtures contained; 20 mM NaCl, 25 mM Tricine-NaOH (pH 7.8), 5 mM $MgCl_2$, either 12 uM DCPIP (photoreduction) or 1 uM DCPIP (DLE), and chloroplasts equivalent to 10 ug chlorophyll/ml. The numbers in parentheses beside each condition indicate the rate of DCPIP photoreduction expressed as ueq/mg chl/hr.

irradiation. The DLE decay kinetics in the UV-irradiated chloroplasts were similar to those observed with Tris-aged chloroplasts. However, the rapid decay of the acceptorless DLE was not affected by the addition of either DCMU or of DPC (which restored reduction of the acceptor DCPIP to 58% of the control). The lack of an effect of DCMU on the intensity of the delayed emission from UV-irradiated chloroplasts was unusual, and may be related to the fact that this treatment itself results in a considerable lowering of the DLE intensity. The other treatments we have examined (see Table 5-I) result in an increase in the DLE intensity at 1 msec when compared to untreated chloroplasts. In these other treatments, DCMU lowered the overall emission intensity whether or not it affected the kinetics of the decay of the emission.

Fig. 5-3 shows that in chloroplasts inactivated by exposure to gramicidin at pH 8.9 (see Chapter 4), addition of DPC restored electron flow, but had no effect on the rapid decay of the acceptorless DLE. On the other hand, addition of DCMU did slow the DLE decay in the gramicidin-inhibited chloroplasts. At pH 8.9 (in the absence of gramicidin) DCMU increased the decay of acceptorless DLE, in marked contrast to its effects on acceptorless DLE at pH 7.5. This difference in the effects of DCMU at the two pH's is shown at Fig. 5-4.

Table 5-I summarizes the data for the three treatments described above, as well as for similar experiments with

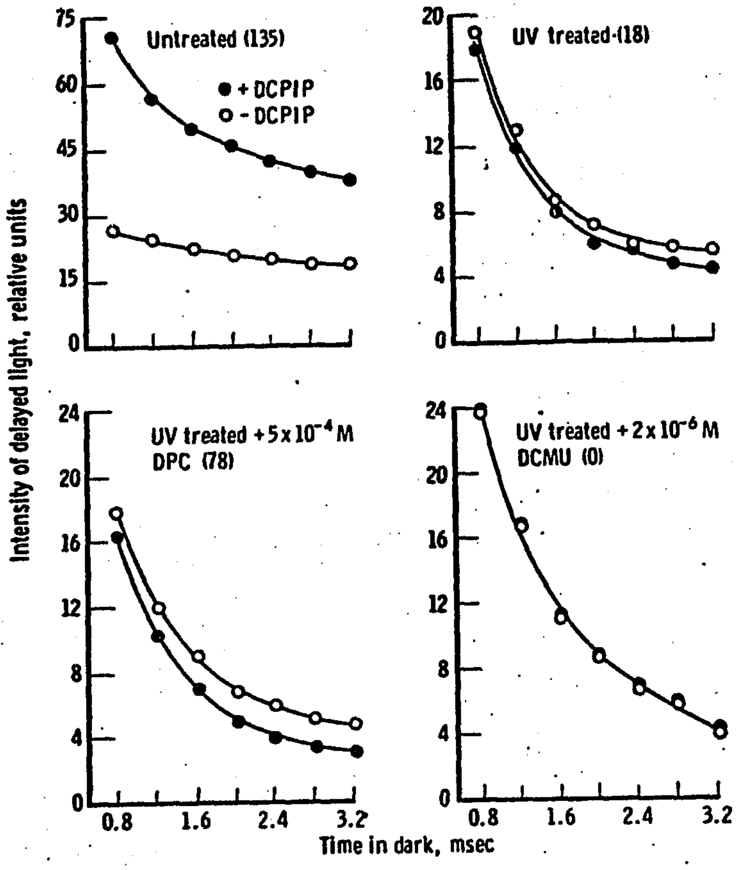


Fig 5-2. Effect of DPC and of DCMU on msec DLE from ultraviolet-irradiated spinach chloroplasts. The reaction mixtures were as in Fig. 5-1. The numbers in parentheses beside each condition indicate the rate of DCPIP photoreduction expressed as ueq/mg chl/hr.

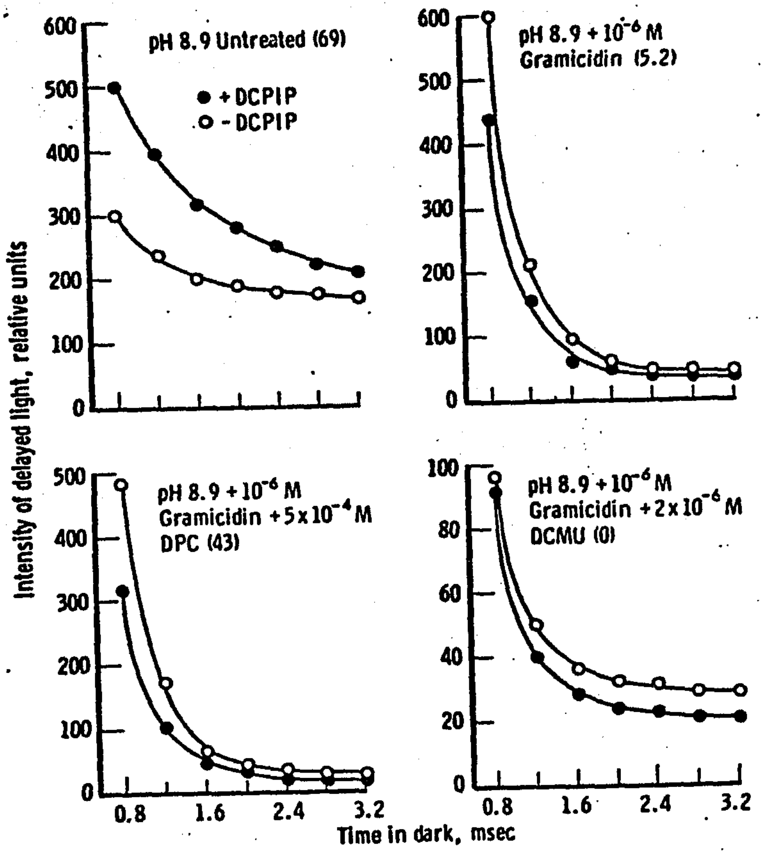


Fig. 5-3. Effect of DPC and of DCMU on msec DLE from gramicidin-inhibited oat chloroplasts. The reaction mixtures were as in Fig. 5-1, except that the buffer was 50 mM Tricine-NaOH (pH 8.9). The numbers in parentheses indicate the rate of DCPIP photoreduction expressed as umoles/mg chl/hr.

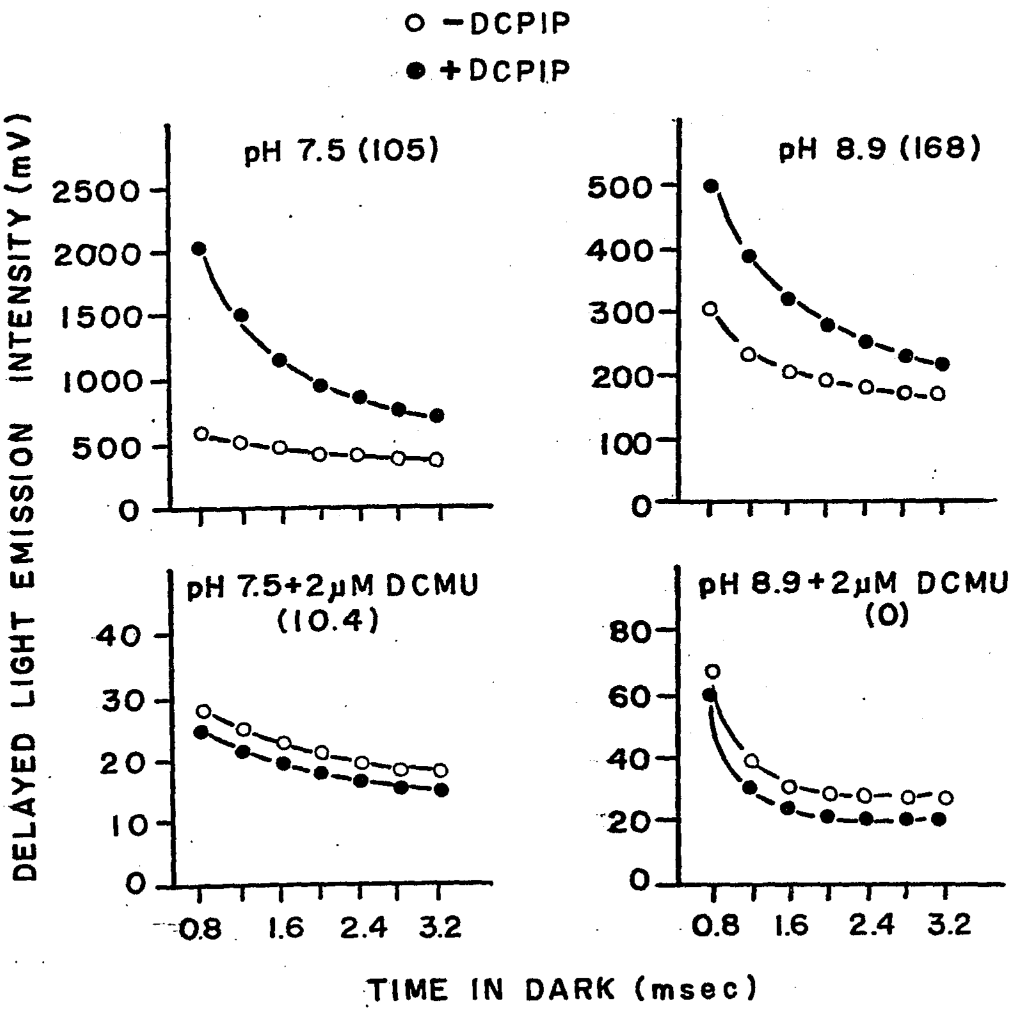


Fig. 5-4. Effect of DCMU on msec DLE from oat chloroplasts at pH 7.5 and at pH 8.9. The reaction mixtures were as in Fig. 5-1 except that the buffer was 50 mM Tricine-NaOH. The numbers in parentheses indicate the rate of DCPIP photoreduction expressed as ueq/mg chl/hr.

chloroplasts and algae inactivated by a variety of other treatments. In this table, the ratio of the DLE intensity at 0.8 msec to that at 2.8 msec is presented as a crude measure of the decay of the emission in the time range examined in our experiments. Data is only presented for DLE in the absence of an electron acceptor (except for the experiment with whole algae) since the effects of the treatments on DLE are more obvious in this case. With the exception of gramicidin-inhibited chloroplasts, the treatments can be grouped into two major classes: those in which the rapidly decaying acceptorless DLE of the treated system is slowed both by donors and by DCMU (Class "A"), and those in which the rapidly decaying DLE associated with the treatment remains rapid in the presence of either donors or DCMU (Class "B"). Chloroplasts inactivated by gramicidin at pH 8.9 is put in a separate class ("C") since it exhibits "mixed" behavior.

Table 5-I

EFFECT OF ELECTRON DONORS AND OF DCMU ON DECAY KINETICS OF MSEC DELAYED LIGHT EMISSION FROM UNTREATED AND TREATED CHLOROPLASTS

All DLE data was measured in the abscence of an electron acceptor. DPC (0.5mM) was used as the electron donor, except in the following cases: Cl^--depleted algae were reactivated by the addition of 10mM NaCl, Mn^{2+}-depleted algae were reactivated by growth for 4 hr in the presence of 16mM $MnSO_4.H_2O$, and 10mM NH_2OH served as the electron donor to chloroplasts inactivated by extraction with 1mM NH_2OH. When added, DCMU was 2uM. Rates of DCPIP photoreduction (ueq/mgchl/hr) in untreated chloroplasts varied from 70 in coupled preparations to 400 in the uncoupled preparations used for chloride-depletion experiments. The whole cells of *Euglena gracilis* used as controls for the manganese-deficiency experiments evolved O_2 at 25 umoles/mgchl/hr.

	DLE intensity $\frac{0.8 \text{ msec}}{2.8 \text{ msec}}$			Rate of Electron Transport (% of control)	
	No Additions	+Donor	+DCMU	-Donor	+Donor
Untreated chloroplasts	1.58-1.70	1.58-1.79	1.35	100	90-110
Class "A" treatments					
Tris-ageing	3.00	2.06	1.78	10	60
Cl^--depletion	3.90	2.13	2.06	23	64
Mn^{2+}-depletion*	4.37	2.19	-	2	92
Class "B" treatments					
UV-irradiation	3.80	3.80	4.0	13	58
Heat	12.7	9.33	10.5	27	63
pH 9.6 ageing	5.25	5.75	4.0	39	93
Trypsin	5.77	5.50	-	5	57
Triton X-100	5.00	4.00	5.50	11	48
NH_2OH	15.0	10.0	9.50	19	36
Class "C" treatments					
Gramicidin/pH 8.9	15.3	20.0	3.42	8	62

*Untreated *Euglena* cells used as control had 0.8/2.8 ratio of 1.81.

C. Discussion

As mentioned in the introduction to this chapter, the wide variety of treatments that inhibit electron transport on the oxidizing side of photosystem II have been found to have two characteristics in common: 1) electron transport through photosystem II is restoreable by addition of a suitable donor of electrons, and 2) the yield of variable fluorescence is attenuated in the treated systems. A third common characteristic indicated by the work of Lurie (1972) and supported by our results is that the treatments induce a rapid decay of the msec delayed light emission measured in the absence of electron acceptors. Although many of the treatments involve conditions that may uncouple photophosphorylation, the effects of the treatments on the decay of the DLE were quite different from those seen in uncoupled chloroplasts (see Chapter 3, Fig. 3-1).

In contrast to the common characteristics outlined above, there have been several reports that these treatments are not identical with respect to certain other parameters. Thus, after some treatments, the low variable fluorescence induced by the treatment is restored by addition of an electron donor, while after other treatments it is unaffected (Yamashita and Butler, 1968a,b). Similarly, the addition of DCMU partially restores the variable fluorescence after some treatments, but not after others (Yamashita and Butler, 1968a,b; Homann, 1968).

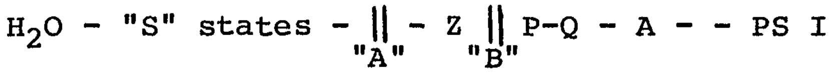
The present investigation of DLE from the treated systems shows a similar pattern. With one group of treatments (Class "A") the addition of an electron donor significantly slowed the decay of the acceptorless DLE (see Table I), making the decay kinetics under these conditions quite similar to those seen in untreated chloroplasts. In a second group of treatments (Class "B") the rapid decay of the DLE induced by the treatments remained rapid upon addition of a donor.

The effect of DCMU on the DLE decay kinetics were similar to the effect of the donors with one exception. After Class "A" treatments, the rapid decay of the DLE was markedly slowed by DCMU. Following Class "B" treatments, the decay kinetics of the DLE remained rapid in the presence of DCMU. The single exception was found in chloroplasts exposed to gramicidin at pH 8.9 (Class "C"). In these chloroplasts, the decay of acceptorless DLE was unaffected by electron donors, but was slowed by DCMU.

The groupings that we have proposed appear to correlate rather well with the reported effects of donors or of DCMU on prompt fluorescence, i.e., the low fluorescence yields of the treated systems we have called Class "A" have been reported to be enhanced upon addition of DCMU or donors, while the fluorescence yields of Class "B" treatments have reported to remain low in the presence of these compounds. An exception to the above statement is the case of heat treatment. This discrepancy between the reported fluorescence effects (Yamashita and Butler, 1968b) and our delayed light findings with heat-inactivation may reside in slight differences in the method of inactivating the chloroplasts. The observation of Homann (1968) that the ability of the fluorescence yield to be restored by DCMU depended on the degree of manganese extraction makes this possibility reasonable.

As outlined in the introduction, one possible interpretation of the effects of the treatments is that they simply block linear electron flow from water to the reaction center of photosystem II. In discussing this possibility, we will use a model of the reaction center of photosystem II elaborated by Van Gorkum and Donze (1973). In their model, all delayed light would arise from the recombination of P^+ and Q^-, the primary oxidized and reduced species formed in the process of charge separation. This back reaction is proposed to have a half-time of 200 usec, and is normally prevented by the more rapid reduction of P^+ by the physiological donor, Z (half-time, 35 usec). Slower components of DLE emission would arise from the regeneration of the P^+Q^- pair by the reversal of subsequent electron transport reactions. In the time range of our measurements, the 35 usec component would not be observable, but we assume that a "tail" of the 200 usec component could be detected.

On the basis of the above model, the two broad classes of behavior of the msec DLE shown by the treatments could result from blocks in the electron transport chain at the two sites between water and the reaction center shown below.



$$H_2O - \text{"S" states} - \underset{\text{"A"}}{\|} - Z \underset{\text{"B"}}{\|} P\text{-}Q - A - - \text{PS I}$$

In class "B" treatments the interaction between Z and P would be inhibited, leading to the recombination of the P^+ and Q^- generated in the light. This recombination should yield the rapidly decaying DLE observed in the early msec time range. Added electron donors are assumed to reduce P^+ more slowly than would Q^-, and thus they should not affect the decay kinetics of the DLE to a large extent. DCMU, which is presumed to inhibit electron transport between Q and A, would not be expected to influence the DLE decay kinetics in such a system, since the reduction of A by Q^- is also quite slow compared to the recombination of Q^- with P^+.

In Class "A" treatments the site of inhibition is further removed from the primary photoreaction. In this case the initial charge separation would be stabilized by the reduction of P^+ by Z, yielding the state $\|Z^+P\ Q^-$. In the dark time between flashes (4 msec), Q^- would be reoxidized by secondary acceptors, yielding reaction centers in the state $\|Z^+P\ Q$. Subsequent charge separations could not be stabilized due to the presence of the already oxidized Z^+, and the rapid component of msec DLE observed in this class of treatments would result. In this case, an electron donor should have a large effect on the DLE since the accumulated Z^+ would be reduced by the donor in the dark period preceding the next flash of exciting light, thus returning the reaction center to the state $\| Z\ P\ Q$. DCMU in such blocked systems would lead to an accumulation of closed traps in the state $\| Z^+P\ Q^- \|$ after a flash of exciting light. These reaction centers could not further separate charge and would yield DLE by the slow back reaction of Z^+ with P. The location of the block in electron transport for this group of treatments (Class "A") does not have to be only one step removed from the primary photoreaction, but could be placed anywhere between water and Z.

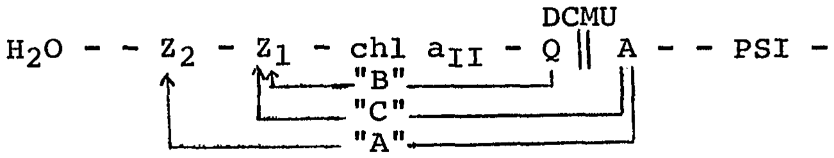
In the case of chloroplasts exposed to gramicidin at pH 8.9, the addition of an electron donor had little effect on the decay of the DLE, whereas DCMU slowed this decay considerably. Such "mixed" behavior is not predicted by the simple model presented above. This treatment was, however, the only one that did not fit into the simple classification. Furthermore, such "mixed" behavior was not shown when chloroplasts were aged at pH 9.6 for 5 minutes and then titrated to pH 7.5 for storage, a treatment we believe inactivates the chloroplasts in a way similar to exposure to gramicidin at pH 8.9 (see Chapter 4).

We feel that the "mixed" behavior shown by the gramicidin-treated chloroplasts may result from the fact that this is the only treated system assayed at a high pH. We have observed (Fig. 5-4) that at pH 8.9, in the absence of gramicidin, the addition of DCMU causes the decay kinetics of DLE in the absence of an acceptor to become significantly <u>more rapid</u>, making it similar to that seen in the presence of

DCMU in the gramicidin-inhibited system (Fig. 5-3). Thus, it appears that at pH 8.9 DCMU itself has an effect on the reaction center of photosystem II which may obscure the effects of the gramicidin treatment. Possibly DCMU at pH 8.9 causes the withdrawal of oxidizing equivalents from P^+ to some endogenous reductant other than Z. Such an effect of DCMU could result in the unusual slowing of the DLE decay kinetics by DCMU that is observed in the gramicidin-inhibited system. In any event, the behavior of this single treatment, while not predicted by the simple model, may be a special case.

A second possible model for the general effects on variable fluorescence and on DLE decay kinetics of the treatments that act on the oxidizing side of photosystem II has been proposed in which wasteful cyclic side reactions, induced by the treatments, cause oxidizing and reducing equivalents to recombine in a non-luminescent fashion (Bertsch and Lurie, 1971; Rosenberg, 1972; Döring, 1975). In this case, there are two possible mechanisms for the rapid decay of the msec DLE: 1) the rapid decay could result simply from the rapid withdrawal by the cycle of the substrates necessary for DLE, or 2) the rapid decay could represent a non-thermal component of DLE that is formed only when light is absorbed by empty reaction centers in the two-sided, solid-state model of the reaction center of Bertsch (1969). Since, in either case the rapidly decaying DLE observed following a treatment would reflect the withdrawal of charge from the reaction center by a cycle, we will not discuss this point of difference.

In a cyclic model, the different responses of the DLE from the treated systems to the presence of electron donors or DCMU could result from the induction of different cyclic pathways by the various treatments as shown below:



If the reduction of Z_1^+ by an added electron donor is assumed to be slower than the cyclic reactions that reduce Z_1^+, then the rapid DLE decay induced by the treatments of Classes "B" and "C" would not be significantly altered in the presence of an electron donor. Similarly, if it is assumed that Z_2^+ is more rapidly reduced by an added reductant, then the flow of charge through the cycle, and thus the DLE decay kinetics, would be modified by an electron donor. Only those cycles involving electron transport component A (Classes "A" and "C") would be affected by the presence of DCMU. This model also allows for the possibility of a cycle between Q^- and Z_2^+, in which case the addition of an electron donor, but not of DCMU, would be expected to slow the decay of the DLE. We have not found any treatment that exhibits this kind of behavior of the DLE.

We do not feel, as yet, that it is possible to distinguish between the linear and the cyclic models presented above. The two models are not mutually exclusive, and their general predictions are quite similar. In either case, the observation that the treatments fall into two classes of behavior (with one arguable exception) suggests that the treatments induce at least two different types of lesions in the photosynthetic apparatus.

CHAPTER 6: CONCLUSIONS AND SPECULATIONS

A. General Conclusions

In this thesis, delayed light emitted in the msec time range after repeating flashes of exciting light has been examined under a variety of conditions. In the quasi-steady-state maintained by the repeating flash regime, we are necessarily dealing with a complex, mixed population of "states" of photosystem II, each of which may contribute several kinetic components to the total DLE. Under these circumstances, it is unlikely that DLE can be used as a quantitative tool in understanding photosystem II.

Our results appear to indicate that the two qualitative aspects of the emission, the intensity and the decay kinetics, may respond to different characteristics of the system. These two aspects of DLE will be discussed in more detail in the following two sections, but, in general terms, the decay kinetics appear to reflect the accumulation of separated charge in the vicinity of the reaction center of photosystem II, while the intensity of the emission appears to respond strongly to some aspect of the high energy state of photophosphorylation. These two parameters are not independent, which again makes quantitation difficult, but we believe that the fact that DLE responds to both parameters makes it a useful qualitative probe of the photosynthetic apparatus.

DLE may be unique in being able to provide so much information about both electron transport and the high energy state in a single, rapid measurement. For instance, a single DLE measurement can often indicate whether a low rate of electron flow caused by an added compound (or a mutation) is due to 1) an inhibition of redox reactions on the reducing side of photosystem II, 2) an inhibition of redox reactions on the oxidizing side of photosystem II, or 3) an inhibition resulting from an effect on the high energy state (e.g., as seen with added ATP). DLE may also indicate secondary effects of various agents that may not be apparent otherwise. For instance, concentrations of CCCP (1 - 10 uM) that are used to uncouple photophysphorylation induce a more rapid decay of acceptorless DLE than do other uncouplers, which may indicate the onset of an inhibition of electron flow from water to photoreaction II. Although such conclusions from an examination of DLE alone may not be unassailable, they can be very useful in indicating possibilities to be further investigated. DLE can also be used to examine photosynthetic systems at many levels of organization, from whole leaves and cells to reaction center preparations.

B. The Decay of msec Delayed Light Emission

Our observations of the decay kinetics of msec DLE under a variety of conditions are most easily understood in terms of the model of the reaction center of photosystem II elaborated by Van Gorkum and Donze (1973). The basic features of this model have been outlined in Chapter 5. In this model, the rapidly decaying component of DLE observable in the early msec time range is ascribed to the back reaction (200 usec half-time) of the primary photoproducts, P^+ and Q^-, to form P*, the excited state of the photoreactive chlorophyll. This back reaction is normally prevented by the rapid reduction of P^+ by the donor Z. Slower components of msec DLE represent the formations of P^+Q^- by back reactions (reverse electron flow) from reduced and oxidized species further removed from the reaction center. The rapid component above would be observable following an exciting flash only when the reaction center pair are in the "open" form, PQ, i.e., are capable of separating charge at the time of the flash, and when the separated charge formed by the flash can not be stabilized by the rapid reduction of P^+, either because Z is already oxidized or is absent. Conditions that increase the proportion of reaction centers in the state, Z^+PQ, would increase the rapidly decaying component of DLE seen in the early msec time range, and would thus accelerate the overall decay of the DLE. More rapid decay kinetics of msec DLE would thus result from conditions that either 1) accelerate the rate of oxidation of Q^- by secondary acceptors, creating more "open" reaction centers, e.g., addition of an electron acceptor, or 2) inhibit the movement of oxidizing equivalents from Z^+ to secondary donors, e.g., treatments that inhibit electron flow on the oxidizing side of photoreaction II. Inhibition of electron transport on the reducing side of photosystem II (e.g., addition of DCMU or dibromothymoquinone) would "close" the photosystem II reaction centers, and slow the decay seen in the presence of an acceptor. The above model provides a rather simple qualitative explanation of the decay of msec DLE under a variety of conditions.

In Chapter 5, we used the model presented above in a qualitative way to interpret the results of our examination of the effects of electron donors and of DCMU on the msec DLE from chloroplasts in which electron flow from water to photoreaction II had been inhibited by a variety of treatments. However, as shown in Table 5-I, when one treatment is compared to another, the decay kinetics of the msec DLE do not correlate with the degree of inhibition of electron transport observed, as might be expected from the model. Of course, many of the treatments (e.g., heat, ultraviolet irradiation, trypsin) involve conditions that might be expected to affect more than one aspect of the photosynthetic apparatus, and thus it may not be too surprising that such a correlation does not exist from one treatment to another. It has been observed (W.S. Cohen, personal communication) that within a single treatment (heat), the DLE decay kinetics do appear to correlate with the degree of inhibition of electron flow when the latter is varied by changing the inhibitory conditions (time of heating).

C. The Intensity of DLE and the High Energy State

Mayne (1967, 1968) originally proposed that the intensity of delayed light emission was stimulated by the high energy state of photophosphorylation. In terms of the chemiosmotic hypothesis (Mitchell, 1961) the high energy state is the protonmotive force (pmf), i.e., the electrochemical potential difference for protons across the thylakoid membrane. In chloroplasts in the steady state, the major component of the pmf appears to be the chemical concentration term, ΔpH (Shavit et al., 1968; McCarty, 1969). Most of our discussion will therefore be in terms of the relationship between delayed light emission and the proton concentrations of the internal (i) and external (e) environments of the thylakoid. As discussed in Chapter 3, the results of our study on the the effects of uncouplers on msec DLE intensity are in general agreement with the proposal that some aspect of the high energy state stimulates DLE, but we find ourselves in some disagreement with the model proposed by Wraight and Crofts (1971) to explain this effect.

The model of Wraight and Crofts (1971) is predicated on the concept that charge separation in photosystem II occurs vectorially across the membrane with the reducing equivalents being formed towards the outer surface and the oxidizing equivalents towards the inner surface of the membrane. This picture of the reaction center was originally proposed to account for the rapid kinetics of the onset of the 515 nm absorption change (Junge and Witt, 1968), and has since received considerable support (see Trebst, 1974, for review). Our interpretation of the inhibition of electron flow through photosystem II by uncouplers at alkaline pH (Chapter 4) also supports the concept that the oxidizing side of photosystem II is exposed to the internal phase of the thylakoid.

In considering the effects on DLE of the chemical concentration term (ΔpH) of the pmf, Wraight and Crofts (1971) reasoned that if the secondary pools of acceptors and donors were redox couples of the hydrogen-carrier type with pH-dependent midpoint potentials, and if the acceptor pools were in equilibrium with the external pH and the donor pools were in equilibrium with the internal pH, then the development of a ΔpH (inside acid) would increase the steady-state concentrations of the reduced primary acceptor (Q^-) and oxidized donor (Z^+). This would result in an increased intensity of DLE. In essence, their model appears to argue that an increase in ΔpH can promote reverse electron flow on both sides of the photosystem. The fact that the addition of uncouplers, or of ADP plus P_i, stimulates electron flow from water to acceptors such as ferricyanide or methyl viologen, while ATP inhibits such electron flow, supports the idea that the high energy state can exert "back pressure" on electron flow. However, these effects are only seen when the overall reaction involves electron flow from plastoquinone to photosystem I. When electron flow from plastoquinone to photosystem I is inhibited by dibromothymoquinone, or by KCN, photosystem II-driven electron transport from water to acceptors such as 2,5-dimethyl-p-benzoquinone or oxidized phenylenediamine can still take place. This electron flow is coupled to phosphorylation, the required ΔpH being generated at least partially by the liberation of protons inside the thylakoid upon oxidation of water (Gould and Ort, 1973; Junge and Ausländer, 1974). However, no stimulation of such electron flow is observed upon addition of uncouplers or of ADP plus P_i (Gould and Ort, 1973; Cohen et al., 1975). We conclude from these findings that the redox reactions between water oxidation and photoreaction II are not subject to "back pressure" by the pmf, and that the supposition of Wraight and Crofts (1971) that an increase in internal acidity would drive reverse electron flow on the oxidizing side of photosystem II may be incorrect.

Another argument that reverse electron flow on the oxidizing side of photosystem II does not occur in response to an increase in internal acidity is based on the discussion in the previous section (B above) of the factors that affect the decay of msec DLE. The model of Wraight and Crofts (1971) predicts that an increase in the steady-state concentration of Z^+ should accompany an increased internal acidity. If our analysis of the decay of msec DLE is correct, this should promote a more rapid decay of the DLE. Although we do find that stimulation of electron flow by addition of an acceptor leads to a more rapid decay of msec DLE, we would ascribe this change in the decay kinetics to the acceleration of electron flow and not to the high energy state created by the electron flow. This interpretation is supported by our observation (Chapter 3) that a decrease in the high energy state (decreasing internal acidity) induced by uncouplers did not slow the decay kinetics of the DLE, as predicted by the Wraight and Crofts model, but resulted in a slightly more rapid decay of the

DLE. This increased rate of decay most likely reflects the increased rate of electron transport induced by the uncoupler. Because of the effect of the rate of electron flow on the DLE decay kinetics, our own results are not unequivocal. Cohen and Bertsch (1974) attempted to eliminate this complication by inhibiting electron flow through photosystem II with DCMU and observing the effects on msec DLE of the high energy state generated by a photosystem I-driven cyclic electron flow. They found the intensity of the DLE was stimulated by the high energy state, but that the decay kinetics were unaffected. It would thus appear that the high energy state does not directly influence the decay kinetics of msec DLE, but only the intensity of the emission.

As indicated in Chapter 3, the observation that the intensity of msec DLE decreased considerably as the external pH was raised from pH 7.0 to pH 8.5 is at variance with the proposed dependence of DLE intensity on ΔpH, since ΔpH appears to remain relatively constant (Portis and McCarty, 1973) or to increase (Rottenberg _et_ _al_., 1972; Schuldiner _et_ _al_., 1972) over this range of external pH. This result led us to consider the possibility that the internal pH, which increases as the external pH is raised, was the major factor modulating DLE intensity.

Our results suggest that DLE intensity is stimulated by increasing the internal acidity (decreasing pH_i) and that such stimulation does not involve changes in the redox equilibria of components on the oxidizing side of photosystem

II. If it is indeed true that all components of the DLE are affected identically by changes in the internal acidity, as would appear to be indicated by the lack of an effect of changes in the high energy state on the decay kinetics of msec DLE, then it would appear that the internal acidity must affect the luminescent reaction itself. One possibility would be that protonation of some component exposed to the internal phase of the thylakoid would increase the proportion of the P^+Q^- back reactions that form an excited chlorophyll (P*) as opposed to the ground state of the photoreactive chlorophyll (P). In this model, the pH_i-sensitive component need not be a redox carrier, although it could be if it is further assumed that the protonation of the component does not affect its midpoint potential. The hypothetical component should be physically in proximity to the reaction center, and should be able to interact with the internal thylakoid pH. This model predicts that large changes in the DLE intensity should only be observed when the changes in pH_i are within a pH unit or so of the pK_a of the hypothetical component. Our finding that DLE in the presence of gramicidin was relatively unaffected by changes in the external pH from pH 7.0 to pH 8.5 (Fig. 3-14) is consistent with this aspect of our proposed model if one assumes the pK_a of the hypothetical component to be approximately 5.0. A quantitative examination of our proposal or of that of Wraight and Crofts (1971) would appear to be extremely difficult given the complexities of the interactions among pH_i, ΔpH, the rate of electron flow, and DLE. Even the qualitative evaluation of these two models will undoubtedly be subject to interpretative differences given the state of our understanding of the interactions noted above.

REFERENCES

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

Amesz, J. (1973) Biochim. Biophys. Acta 301, 35.

Arnold, W. and Azzi, J.R. (1968) Proc. Nat. Acad. Sci. U.S. 61, 29.

Arnold, W. and Davidson, J.B. (1954) J. Gen. Physiol. 37, 677.

Arnold, W. and Oppenheimer, J.R. (1950) J. Gen. Physiol. 33, 423.

Arnold, W. and Sherwood, H. (1957) Proc. Nat. Acad. Sci. U.S. 43, 105.

Arnold, W. and Sherwood, H. (1959) J. Phys. Chem. 63, 2.

Arnold, W. and Thompson, J. (1956) J. Gen. Physiol. 39, 311.

Arnon, D.I. (1949) Plant Physiol. 24, 1.

Arnon, D.I., Whatley, F.R. and Allen, M.B. (1954) J. Am. Chem. Soc. 76, 6324.

Arnon, D.I., Whatley, F.R. and Allen, M.B. (1958) 127, 1026.

Arnon, D.I., Knaff, D.B., McSwain, B.D., Chain, R.K. and Tsujimoto, H.Y. (1971) Photochem. Photobiol. 14, 397.

Arntzen, C.J., Dilley, R.A. and Crane, F.L. (1969) J. Cell. Biol. 43, 16.

Arthur, W.E. and Strehler, B.L. (1957) Arch. Biochem. Biophys. 70, 507.

Ausländer, W., Heathcote, P. and Junge, W. (1974) FEBS Lett. 47, 229.

Avron, M. (1963) Biochim. Biophys. Acta 77, 699.

Avron, M. (1971) Proc. 2nd Int. Congr. on Photosyn. Res., Vol. II, p. 861.

Avron, M. and Shavit, N. (1963) in "Photosynthetic Mechanisms of Green Plants", Nat. Acad. Sci. - Nat. Res. Counc. Publ. 1145, p. 611.

Babcock, G.T. and Sauer, K. (1975a) Biochim. Biophys. Acta 376, 315.

Babcock, G.T. and Sauer, K. (1975b) Biochim. Biophys. Acta 376, 329.

Baltascheffsky, M. and Hall, D.O. (1974) FEBS Lett. 39, 345.

Bamberger, E.S., Rottenberg, H. and Avron, M. (1973) Eur. J. Biochem. 34, 557.

Barber, J. and Kraan, G.P.B. (1970) Biochim. Biophys. Acta 197, 49.

Barbieri, R., Delosme, R. and Joliot, P. (1970) Photochem. Photobiol. 12, 197.

Bertsch, W.F. (1962) Proc. Nat. Acad. Sci. U.S. 48, 2000.

Bertsch, W. (1969) in "Progress in Photosynthesis Research" (H. Metzner, ed.), p. 996, H. Laupp, Tübingen.

Bertsch, W.F. and Lurie, S. (1971) Photochem. Photobiol. 14, 251.

Bertsch, W.F., Davidson, J.B. and Azzi, J.R. (1963) in "Photosynthetic Mechanisms of Green Plants", Nat. Acad. Sci. - Nat. Res. Counc. Publ. 1145, 701.

Bertsch, W.F., Azzi, J.R. and Davidson, J.B. (1967) Biochim. Biophys. Acta 143, 129.

Bertsch, W., West, J. and Hill, R. (1969) Biochim. Biophys. Acta 172, 525.

Bertsch, W., West, J. and Hill, R. (1971) Photochem. Photobiol. 14, 241.

Blankenship. R.E. and Sauer, K. (1974) Biochim. Biophys. Acta 357, 252.

Bonaventura, C. and Kindergan, M. (1971) Biochim. Biophys. Acta 234, 249.

Briller, S. and Gromet-Elhanan, Z. (1970) Biochim. Biophys. Acta 205, 263.

Brody, M. and Emerson, R. (1959) J. Gen. Physiol. 43, 251.

Butler, W.L. (1972) Biophys. J. 12, 851.

Butler, W.L. (1973) Accounts of Chem. Res. 6, 177.

Carmeli, C. (1970) FEBS Lett. 7, 297.

Carmeli, C., Lifshitz, Y. and Gepshtein, A. (1975) Biochim. Biophys. Acta 376, 249.

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

Chappell, J.B. and Crofts, A.R. (1965) Biochem. J. 95, 393.

Cheniae, G.M. and Martin, I.F. (1970) Biochim. Biophys. Acta 197, 219.

Cheniae, G.M. and Martin, I.F. (1971) Plant Physiol. 47, 568.

Clayton, R.K. (1964) Photophysiology (A.C. Giese, ed.) 1, 155.

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

Clayton, R.K. and Bertsch, W.F. (1965) Biochem. Biophys. Res. Comm. 18, 415.

Cohen, W.S. and Bertsch, W.F. (1974) Biochim. Biophys. Acta 347, 371.

Cohen, W.S., Cohn, D.E. and Bertsch, W. (1975) FEBS Lett. 49, 350.

Cole, J.S. and Aleem, M.I.H. (1973) Proc. Nat. Acad. Sci. U.S. 70, 3571.

Crofts, A.R. (1966) Biochem. Biophys. Res. Comm. 24, 127.

Crofts, A.R. (1968) in "Regulatory Functions of Biological Membranes" (ed. J. Jarnefelt) p. 247.

Crofts, A.R., Wraight, C.A. and Fleischman, D.E. (1971) FEBS Lett. 15, 89.

Davenport, H. and Hill, R. (1952) Proc. Roy. Soc. B139, 327.

Deamer, D.L. and Packer, L. (1969) Biochim. Biophys. Acta 172, 539.

Deamer, D.W., Prince, R.C. and Crofts, A.R. (1972) Biochim. Biophys. Acta 274, 323.

Döring, G. (1975) Biochim. Biophys. Acta 376, 274.

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

Döring, G., Renger, G., Vater, J. and Witt, H.T. (1969) Z. Naturforsch. 24b, 1139.

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

Duysens, L.M.N. (1963) in "Photosynthetic Mechanisms of Green Plants", Nat. Acad. Sci.-Nat.-Res. Counc. Publ. 1145.

Duysens, L.M.N. (1964) Progress in Biophysics and Molecular Biology 14, 44.

Duysens, L.N.M. (1971) Proc. Eur. Biophys. Congr., Vol. IV, p. 13.

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

Emerson, R. and Arnold, W. (1932) J. Gen. Physiol. 15, 391.

Emerson, R. and Lewis, C.M. (1941) Am. J. Botany 28, 789.

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

Emerson, R. and Rabinowitch, E. (1960) Plant Physiol. 35, 477.

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

Fiolet, J.W.T., Bakker, E.P. and VanDam, K. (1974) Biochim. Biophys. Acta 368, 432.

Fleischman, D.E. (1971) Photochem. Photobiol. 14, 277.

Fleischman, D.E. and Clayton, R.K. (1968) Photochem. Photobiol. 8, 287.

Fleischman, D.E. and Mayne, B.C. (1973) Curr. Topics in Bioenerg. 4, 77.

Forbusch, G., Kok, B. and McGloin, M.P. (1971) Photochem. Photobiol. 14, 307.

Förster, T. (1947) Z. Elektrochemie 53, 93.

Förster, T. (1948) Ann. Physik 2, 55.

Gaensslen, R.E. and McCarty, R.E. (1971) Arch. Biochem. Biophys. 147, 55.

Gaffron, H. (1942) J. Gen. Physiol. 26, 195.

Gaffron, H. and Wohl, K. (1936) Naturwissenschaften 24, 81.

Goedheer, J.C. (1962) Biochim. Biophys. Acta 64, 294.

Gould, J.M. and Izawa, S. (1974) Biochim. Biophys. Acta 333, 509.

Gould, J.M. and Ort, D.R. (1973) Biochim. Biophys. Acta 325, 157.

Govindjee, Döring, G. and Govindjee, R. (1970) Biochim. Biophys. Acta 205, 303.

Harth, E., Reimer, S. and Trebst, A. (1974) FEBS Lett. 42, 165.

Hauska, G.A., McCarty, R.E. and Olson, J.S. (1970) FEBS Lett. 7, 151.

Haxo, F.T. and Blinks, L.R. (1950) J. Gen. Physiol. 33, 389.

Heath, R.L. and Hind, G. (1969a) Biochim. Biophys. Acta 172, 290.

Heath, R. and Hind, G. (1969b) Biochim. Biophys. Acta 189, 222.

Heldt, H.W., Werdan, K., Milovancev, M. and Geller, G. (1973) Biochim. Biophys. Acta 314, 224.

Hill, R. (1937) Nature 139, 881.

Hill, R. (1939) Proc. Roy. Soc. London B. 127, 192.

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

Hill, R. and Scarisbrick, R. (1951) New Phytologist, 50, 98.

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

Hind, G. and Whittingham, C.P. (1963) Biochim. Biophys. Acta 75, 194.

Hoch, G. and Knox, R.S. (1968) Photophysiology (A.C. Giese, ed) 3, 225.

Homann, P. (1968) Biochem. Biophys. Res. Comm. 33, 229.

Hunter, D.R., Komai, H. and Haworth, R.A. (1974) Biochem. Biophys. Res. Comm. 56, 647.

Izawa, S., Heath, R.L. and Hind, G. (1969) Biochim, Biophys. Acta 180, 388.

Jackson, J.B. and Crofts, A.R. (1969) FEBS Lett. 4, 185.

Jackson, J.B., Crofts, A.R. and vanStedingk, L.V. (1968) Eur. J. Biochem. 6, 41.

Jagendorf, A.T. and Avron, M. (1958) Arch. Biochem. Biophys. 80, 246.

Jagendorf, A.T. and Hind, G. (1963) Nat. Acad. Sci. - Nat. Res. Counc. Publ. 1145, 599.

Jagendorf, A.T. and Smith, M. (1962) Plant Physiol. 37, 135.

Jagendorf, A.T. and Uribe, E. (1966a) Brookhaven Symp. Biol. 19, 215.

Jagendorf, A.T. and Uribe, E. (1966b) Proc. Nat. Acad. Sci. U.S. 55, 170.

Joliot, P., Joliot, A., Bouges, B. and Barbieri, R. (1971) Photochem. Photobiol. 14, 287.

Junge, W. and Ausländer, W. (1974) Biochim. Biophys. Acta 333, 59.

Junge, W. and Witt, H.T. (1968) Z. Naturforsch. 23b, 244.

Katoh, S., Ikegami, I. and Takamiya, A. (1970) Arch. Biochem. Biophys. 141, 207.

Kimimura, M. and Katoh, S. (1972) Plant Cell Physiol. 13, 287.

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

Kok, B., Forbusch, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457.

Kraan, G.P.B., Amesz, J., Velthuys, B.R. and Steemers, R.G. (1970) Biochim. Biophys. Acta 223, 129.

Kraayenhof, R. (1970) FEBS Lett. 6, 161.

Kraayenhof, R., Izawa, S. and Chance, B. (1972) Plant Physiol. 50, 713.

Lavorel, J. (1967) in "Progress in Photosynthesis Research" (H. Metzner, ed.), p. 883, H. Laupp, Tübingen.

Lavorel, J. (1968) Biochim. Biophys. Acta 153, 727.

Losada, M., Whatley, R.F. and Arnon, D.I. (1961), Nature 190, 606.

Lozier, R., Baginsky, M. and Butler, W.L. (1971) Photochem. Photobiol. 14, 323.

Lurie, S. (1972) Doctoral Dissertation, City University of New York.

Malkin, R. (1971) Biochim. Biophys. Acta 253, 421.

Mayne, B.C. (1967) Photochem. Photobiol. 6, 189.

Mayne, B.C. (1968) Photochem. Photobiol. 8, 107.

Mayne, B.C. and Clayton, R.K. (1966) Proc. Nat. Acad. Sci. U.S. 55, 494.

Mayne, B.C. and Hobbs, L.J. (1971a) Abstr. Biophys. Soc., 15th Annu. Meet. TPMD16.

Mayne, B.C. and Hobbs, L.J. (1971b) Abstr. Amer. Soc. Plant Physiol. Meet., p. 31.

McCarty, R.E. (1968) Biochem. Biophys. Res. Comm. 32, 37.

McCarty, R.E. (1969) J. Biol. Chem. 244, 4292.

McCarty, R.E. and Racker, E. (1966) Brookhaven Symp. Biol. 19, 202.

Miles, C.D. and Jagendorf, A.T. (1969) Arch. Biochem. Biophys. 129, 711.

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

Mitchell, P. (1966) Biol. Rev. Cambridge Phil. Soc. 41, 445.

Mohanty, P., Braun, B.Z. and Govindjee (1972) FEBS Lett. 20, 273.

Nelson, N., Drechsler, Z. and Neumann, J. (1970) J. Biol. Chem. 245, 143.

Neumann, J. and Jagendorf, A.T. (1964) Arch. Biochem. Biophys. 107, 109.

Niel, C.B. van (1931) Ark. Mikrobiol. 3, 1.

Niel, C.B. van (1935) Cold Spring Harbor Symposia Quant. Biol. 3, 138.

Niel, C.B. van (1941) Adv. in Enzymology 1, 263.

Niel, C.B. van (1949) in "Photosynthesis in Plants", (J. Franck and F.W. Loomis, eds.) p. 143, Iowa State Univ. Press, Ames, Iowa.

Pearlstein, R.M. (1966) in "Energy Conversion by the Photosynthetic Apparatus", Brookhaven Symposia in Biology 19, 8.

Petrack, B. and Lipman, F. (1961) in "Light and Life" (W.D. McElroy and H.B. Glass, eds.) p. 621.

Petrack, B., Craston, A., Sheppy, F. and Farron, F. (1965) J. Biol. Chem. 240, 906.

Portis, A.R., Jr. and McCarty, R.E. (1973) Arch. Biochem. Biophys. 156, 621.

Racker, E. and Stoeckenius, W. (1974) J. Biol. Chem. 249, 662.

Rosenberg, J.L., Sahu, S. and Bigat, T.K. (1972) Biophys. J. 12, 839.

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

Rottenberg, H. and Grunwald, T. (1972) Eur. J. Biochem. 25, 71.

Rottenberg, H., Grunwald, T. and Avron, M. (1971) FEBS Lett. 13, 41.

Rottenberg, H., Grunwald, T., Schuldiner, S. and Avron, M. (1971) Proceedings 2nd Int. Congr. on Photosyn., (G. Forti, M. Avron, A. Melandri, eds.) Vol. 2, p. 1035.

Rottenberg, H., Grunwald, T. and Avron, M. (1972) Eur. J. Biochem. 25, 54.

Ruben, S., Randall, M., Kamen, M. and Hyde, J.L. (1941) J. Am. Chem. Soc. 63, 877.

Ruby, R.H. (1968) Photochem. Photobiol. 8, 299.

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

Ryrie, I. and Jagendorf, A.T. (1971) J. Biol. Chem. 246, 3771.

Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64.

Selman,B. and Bannister, T. (1971) Biochim. Biophys. Acta 253, 428.

Shavit, N., Dilley, R.A. and San Pietro, A. (1968) Biochemistry 7, 2356.

Shavit, N., Degani, H. and San Pietro, A. (1970) Biochim. Biophys. Acta 216, 208.

Shen, Y.K. and Shen, G.M. (1962) Sci. Sinica 11, 1097.

Sherman, L.A. (1972) Biochim. Biophys. Acta 203, 67.

Strehler, B.O. and Arnold, W. (1951) J. Gen. Physiol. 34, 809.

Strichartz, C.R. and Chance, B. (1972) Biochim. Biophys. Acta 256, 71.

Telfer, A. and Barber, J. (1974) Biochim. Biophys. Acta 333, 343.

Trebst, A. (1974) Ann. Rev. Plant Physiol. 25, 423.

Vambutas, V.K. and Racker, E. (1965) J. Biol. Chem. 240, 2660.

Van Gorkom, H.J. and Donze, M. (1973) Photochem. Photobiol. 17, 333.

Velthuys, B.R. (1974) in Proc. 3rd Int. Congr. on Photosyn. (ed. M. Avron), p. 93.

Vernon, L.P., Klein, S., White, F.G., Shaw, E.R. and Mayne, B.C. (1971) Proc. 2nd Int. Congr. on Photosyn. Res., Vol. I, p. 801.

Walker, N.A. (1975) FEBS Lett. 50, 98.

Wells, R., Bertsch, W. and Cohen, W.S. (1971) Proc. 2nd Int. Congr. on Photosyn. Res., Vol. I, p. 207.

Williams. R.J.P. (1969) Current Top. Bioenerg. 3, 79.

Witt, H.T. (1967) Nobel Symposium No. 5, (ed. S. Claesson), p. 261, Interscience.

Witt, H.T. (1971) Quart. Rev. Biophys. 4, 365.

Wraight, C.A. and Crofts, A.R. (1971) Eur. J. Biochem. 19, 386.

Yamashita, T. and Butler, W.L. (1968a) Plant Physiol. 43, 1978.

Yamashita, T. and Butler, W.L. (1968b) Plant Physiol. 43, 2037.

Zankel, K.L. (1971) Biochim. Biophys. Acta 245, 373.