AN ABSTRACT OF THE THESIS OF

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Title: THE EFFECTS OF ULTRAVIOLET IRRADIATION ON THE PHOTOSYNTHETIC APPARATUS

Abstract approved: ____________________________

Norman I. Bishop

The effects of ultraviolet (UV) irradiation on photosynthesis and various partial reactions involving the individual photoreactions of photosynthesis were examined. These reactions included photoreduction in adapted green algae, Hill reaction, cyclic photophosphorylation, the 520 nm absorbance change and variable-yield fluorescence. The reactions were measured in spinach chloroplasts and isolated chloroplasts or whole cells of green and blue-green algae.

Experiments showing that all system II requiring reactions, as well as photoreduction and cyclic photophosphorylation (which require only system I) were equally inhibited suggested that the site of the UV inhibition was at some point in the electron transport chain near system II. Ascorbate-DCIP mediated reduction of NADP was not affected.

A peak in the action spectrum for UV inhibition at 250 nm and an apparent decrease in the plastoquinone (PQ) content of
chloroplasts after irradiation suggested that PQ was the locus of the inhibition. However, experiments in which pure, unirradiated PQ was added to irradiated chloroplasts did not restore photochemical activity. In addition, examination of whole cell systems, using various algae, revealed that when photosynthetic activity had been totally abolished, only about 40% of the endogenous PQ had been destroyed. Experiments comparing the effects of petroleum ether extraction of the PQ from lyophilized chloroplasts versus UV irradiation of chloroplasts revealed striking differences. From this type of data it was concluded that destruction of PQ was not the major cause of the UV inhibition.

The effects of UV irradiation on the 520 nm absorbance change were also examined. It was found that the system II (DCMU sensitive) portion of the absorbance change was inhibited at a rate parallel to the inhibition of photosynthesis. The system I (DCMU insensitive) portion was also inhibited, but required two to three times the amount of irradiation. Experiments to compare the rate of inhibition of the system I portion of the 520 nm absorbance change with PQ destruction did not reveal any apparent positive correlation. Periods of irradiation sufficient to abolish totally the system I portion of the absorbance change had no effect on the ability of chloroplasts isolated from this material to reduce NADP with the ascorbate-DCIP couple. Extraction of the PQ from chloroplasts abolished both
portions of the 520 nm absorbance change, indicating that PQ is required for the change but is not the site of the UV inhibition.

Carotenoid, lipid and protein content and complement in irradiated and unirradiated chloroplasts and cells were also compared. In no case was any major alteration noted.

Measurements were made of the effects of UV irradiation on the variable-yield fluorescence of isolated chloroplasts and whole cells. It was expected that as photosynthetic activity decreased the variable-yield fluorescence would increase until the maximum attainable yield was reached when photosynthetic activity had been totally abolished. Surprisingly, it was found that the variable-yield fluorescence decreased after UV irradiation and, more important, the maximum attainable yield also decreased. This was interpreted as indicating that the fluorescence emitter, the system II trapping center, was destroyed.

Considering all of the results discussed above, it seems likely that UV irradiation disrupts a physical entity consisting of the system II trapping center, the initial electron acceptor from system II, and at least one component beyond the site of DCMU inhibition. This interpretation further suggests that system II consists of a physical unit containing several components of the electron transport chain.
The Effects of Ultraviolet Irradiation on the Photosynthetic Apparatus

by

Kenneth Edward Mantai

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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>CCCP</td>
<td>m-chlorocyanocarbonylphenyl hydrazone</td>
</tr>
<tr>
<td>CMU</td>
<td>p-chlorophenyl-1,1-dimethyl urea</td>
</tr>
<tr>
<td>DCIP</td>
<td>2,6-dichlorophenolindophenol</td>
</tr>
<tr>
<td>DCMU</td>
<td>3-(3,4-dichlorophenyl)-1,1-dimethyl urea</td>
</tr>
<tr>
<td>DNP</td>
<td>2,4-dinitrophenol</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide dinucleotide phosphate</td>
</tr>
<tr>
<td>nm</td>
<td>$10^{-9}$ meters</td>
</tr>
<tr>
<td>PCV</td>
<td>packed cell volume</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PMS</td>
<td>phenazine methosulfate</td>
</tr>
<tr>
<td>PPNR</td>
<td>photosynthetic pyridine nucleotide reductase</td>
</tr>
<tr>
<td>PQ</td>
<td>plastoquinone</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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THE EFFECTS OF ULTRAVIOLET IRRADIATION ON THE PHOTOSYNTHETIC APPARATUS

INTRODUCTION

General Definition and Description

If a single process may be termed the key for the continued existence of life on this planet, that process is photosynthesis. Not only does photosynthesis provide reduced substrates for the host organisms which are capable of performing the process, but indirectly for all of the earth's heterotrophs as well. During early stages of evolution, photosynthesis was responsible for the shift of the earth's atmosphere from a reducing to an oxidizing one, thereby greatly increasing the amount of free energy readily available to the metabolic pathways of existing organisms and allowing life to become more than a marginal phenomenon. Photosynthesis serves to link the seemingly limitless energy supply provided by the sun with the limited energy supply of the earth, thus keeping the earth at a near steady state.

Man's comprehension of the process did not really begin until the mid-eighteenth century, but by the beginning of the nineteenth century the work of Priestley, Ingen-Housz, Senebier and de Saussure had clearly established the basic aspects of photosynthesis. During this period knowledge of the photosynthetic process could be
summarized by the following reaction:

$$\text{CO}_2 + \text{H}_2\text{O} \rightarrow (\text{CH}_2\text{O}) + \text{O}_2.$$  

The stoichiometry of the above equation led to the belief that the oxygen evolved came from carbon dioxide (Gray, 1868), and it was not until the brilliant work of van Niel (1931) on the photometabolism of photosynthetic bacteria that the modern concept of photosynthesis began to materialize. Van Niel summarized his many findings into a simplified representation for the general mechanism of photosynthesis according to the following equation:

$$\text{CO}_2 + 2\text{H}_2\text{A} \rightarrow (\text{CH}_2\text{O}) + 2\text{A} + \text{H}_2\text{O}$$

where the $\text{H}_2\text{A}$ could be any of a number of reduced substrates, from water in the case of green plant photosynthesis to various reduced sulfur or organic compounds in the photosynthetic bacteria. Moreover, this overall equation itself could be broken down into a series of three reactions, one requiring light, and the other two being dark reactions. His formulation, as applied to higher plant photosynthesis, can be written as follows:

1. light reaction: $\text{H}_2\text{O} \xrightarrow{\text{hv}} \text{chlorophyll} [\text{H}^+] + [\text{OH}^-]$

2. dark reaction: $4[\text{H}^+] + \text{CO}_2 \xrightarrow{\text{enzymatic}} \text{CH}_2\text{O} + \text{H}_2\text{O}$

3. dark reaction: $4[\text{OH}^-] \xrightarrow{\text{enzymatic}} \text{O}_2 + 2\text{H}_2\text{O}$
With the further discoveries during the 1930's that carbon dioxide fixation was not a process unique to photosynthesis, it was evident that the photosynthetic apparatus could be separated into two parts: (1) the photochemistry of the chlorophyll molecules and production of reducing power and (2) the enzymatic reaction of carbon dioxide itself. This thesis will be concerned only with those reactions leading to the production of reducing power (and ATP).

**The Hill Reaction**

The concept that photosynthesis could be separated into a series of light and dark events was further substantiated by subsequent discovery of the "Hill reaction": a process wherein the evolution of oxygen occurs without the concomitant reduction of CO₂.

The ability of isolated chloroplasts or dried leaf powders to liberate small amounts of oxygen when exposed to light was first observed in the late nineteenth century (Rabinowitch, 1945). A large number of experiments were later performed by Inman (1935; 1938a; 1938b), who showed that proteolytic enzymes destroyed the ability of leaf powders to evolve oxygen and that evolution was limited to the pH range 5-7. He also stressed that the similarities between the effects of temperature, pH and trypsin action on oxygen evolution and protein denaturation indicated that enzymatic reactions were involved. However, the rates of oxygen evolution that he observed were very
low and usually lasted for not more than an hour. This suggested that the oxygen was actually coming from a peroxide present in the preparations. It was thought that peroxide formation by leaf fragments occurred either by normal plant metabolism or as an artifact of the isolation procedure (Rabinowitch, 1945).

It remained for Robert Hill and coworkers, using oxygen detection techniques gained from the study of hemoglobin, to discover that various iron compounds could act as electron acceptors and give stoichiometric yields of oxygen (Hill, 1939). Since most oxidants used for "Hill reaction" measurements have redox potentials greater than zero, to confirm that the Hill reaction was actually a true representation of part of photosynthesis, it was necessary to show that compounds with strongly negative redox potentials could also be reduced. (preferably NADP, the in vivo acceptor in photosynthesis.) Davenport, Hill and Whatley (1952) isolated a soluble factor from chloroplasts which catalyzed the reduction of methemoglobin and cytochrome c; they called this material "methemoglobin reducing factor." Unfortunately they did not test the ability of this factor to catalyze the reduction of NADP, but a few years later San Pietro and Lang (1958) isolated "photosynthetic pyridine nucleotide reductase," a flavoprotein containing non-heme iron, which would catalyze the reduction of NADP. All evidence indicated that this factor is identical to that described by Davenport, Hill and Whatley.
Quantum Yield and Action Spectra

In any photochemical process such as photosynthesis, it is important to determine both the pigments involved in the reactions and the efficiencies with which these pigments operate. In a biochemical system as complex as a cell, it is not always obvious which pigments are actually operating in the photochemical system. Action spectra, determined by measuring the rate of the reaction as a function of wavelength (performed by irradiating with equal intensities or number of quanta of each wavelength and measuring the rate of the reaction), will normally produce a curve roughly analogous to the absorption spectrum of the pigment(s) involved. It should be stressed, however, that an action spectrum does not give a quantitative measure of the efficiency of utilization of the light.

The first action spectrum for photosynthesis was published by Engelmann (1883), using green filamentous algae and employing motile bacteria to measure oxygen production. He showed that in green algae the action spectrum paralleled the absorption spectrum of chlorophyll, while in the brown, blue-green and red algae the action spectrum followed the absorption spectrum of the respective accessory pigments. Since that time, action spectra for many of the partial reactions of photosynthesis have been recorded and confirmed.
Once it has been determined which pigments are acting in a
photochemical system, it is of great interest to know the efficiencies
with which each pigment performs. To determine this, not only the
rate of the reaction (i.e., oxygen evolved or carbon dioxide reduced),
but also the amount of light absorbed by the system at each wavelength
must be carefully measured.

Emerson and Lewis (1942 and 1943) were the first workers to
make precise quantum yield measurements throughout the visible
spectrum. Their spectra revealed two wavelengths at which major
decreases in the quantum yield occur. The first occurred at about
480 nm, the absorption maximum of the carotenoids, and the second
a drastic decrease in yield at wavelengths beyond 685 nm. This was
particularly surprising in that chlorophyll still absorbs quite strongly
at this wavelength. Theoretically, the quantum yield of any photo-
chemical reaction should remain constant throughout the absorption
range. In the case of chlorophyll, any quantum absorbed in the region
of 650-710 nm will excite a π electron into the first excited singlet
state. The slightly different energy content of quanta on either side
of the actual peak will excite the electron into different vibrational
energy levels of the first excited singlet state, with the difference in
energy between these vibrational levels being very small compared
to the energy difference between the ground state and the first ex-
cited singlet state. Therefore, any quantum which can excite the
molecule into the first excited singlet state should allow the photochemical reaction to proceed. It is apparent, then, that if quanta absorbed on the short wavelength side of the red peak of chlorophyll can initiate the photochemical reaction, those absorbed on the long wavelength side of the peak should also be equally efficient. This perplexing problem was resolved with the development of the two photosystem hypothesis for photosynthesis.

**Chromatic Transients, Enhancement and Two Light Reactions**

The first evidence for more than one light reaction came from the work of Blinks (1957), who found that by illuminating the red alga *Porphyra perforata* with alternating wavelengths of light, one quite efficient for photosynthesis (absorbed by the accessory pigments) and the other relatively inefficient (absorbed by chlorophyll a), the rate of oxygen evolution did not remain constant. He termed these effects "chromatic transients" and attributed them, at least in part, to changes in respiratory oxygen uptake caused by one of the wavelengths. This assumption proved to be at least partially correct.

In the same year, independent investigation by Emerson (Emerson, Chalmers and Cedarstrand, 1957) of the decrease in quantum yield at long wavelengths ("red drop") revealed that the decline in quantum efficiency occurred only (principally) on the long
wavelength side of the red absorption band of chlorophyll in vivo but that the efficiency could be increased by adding a weak background light of 644 nm. Continued study in many laboratories has confirmed that the efficiency of a combination of near-red (e.g., 650 nm) and far-red (e.g., 700 nm) monochromatic light is greater than the sum of the efficiencies of either wavelength alone. This phenomenon is usually termed the "Emerson enhancement" effect.

Myers and French (1960a) determined action spectra for the short wavelength effect with both enhancement and chromatic transients and concluded that chlorophyll b played a predominant role in the short wavelength effect in higher plants. Moreover, because the action spectra for both processes were identical, they concluded that both must be related to the same biochemical or photochemical sequence of reactions. Their data definitely suggested that two distinct light reactions were operating in photosynthesis.

In a second paper, Myers and French (1960b) reported that the two wavelengths did not have to be applied simultaneously to obtain enhancement. A dark time of as much as 1.2 seconds between illuminations with either beam was possible without substantially reducing the enhancement effect. This important finding suggested that the reaction sequence includes classical biochemical reactions as well as photochemical steps and that at least some of the products must be stable.
Hill and Bendall (1960) summarized the growing volume of data on the existence of two light reactions into the now familiar "Z" scheme. This scheme is a series formulation, that is, one in which the product of one light reaction is utilized by the other. There have been attempts to visualize a parallel sequence where the products of each photoact react with each other in dark enzymatic steps, but the series formulation appears to offer the best working hypothesis at the present time and will be utilized in this thesis. A currently acceptable form of this scheme is depicted in Figure 1.

The Emerson enhancement effect can be explained in terms of this formulation by considering that system II absorbs light in the near-red region of the spectrum extending to about 680 nm. System I, on the other hand, absorbs to a wavelength of about 710 nm, as well as the shorter wavelengths; hence short wavelength light will excite both systems to some extent and produce a high quantum yield. However, at wavelengths beyond about 680 nm, system II no longer absorbs and consequently the quantum yield drops.

The assignment of system II to the process of oxygen evolution is well substantiated (Blinks, 1957 and 1959; Blinks and van Niel, 1963; Gaffron and Bishop, 1963). The two systems appear to have distinct pigment complements, with system II containing the accessory pigments (chlorophyll b, phycobilins, chlorophyll c and d depending on the organism) and a special form of chlorophyll a.
Figure 1. Two light reaction hypothesis of photosynthesis.
System I contains a longer wavelength absorbing form of chlorophyll as a bulk energy gathering component, a pigment absorbing at about 705 nm (probably also a chlorophyll) and P700, the "trapping center".

**Photophosphorylation**

As early as 1943, Ruben (1943) had pointed out that the reduction of carbon dioxide could not be carried out efficiently with NADPH alone, but would require an additional source of energy, probably ATP. The first reports of light-mediated production of ATP from ADP were by Arnon, Allen and Whatley (1954) in chloroplasts and by Frenkel (1954) in bacterial chromatophores.

From studies on a number of cofactors, selective inhibitors and various other experimental conditions, it has become apparent that photophosphorylation can be separated into three types. The first is cyclic phosphorylation, requiring only system I, in which there is no stoichiometry between electron flow and ATP formation, i.e., the electrons are cycled through the system by an added catalyst such as PMS, menadione or DCIP. The second, non-cyclic phosphorylation, is coupled to reduction of a Hill oxidant (with resultant oxygen evolution) and a stoichiometric relation exists between substrate reduced and ATP formed. The maximal P/2e ratio appears to be one, although values as high as four have been reported (Lynn and Brown, 1967). Pseudocyclic phosphorylation, the third
type, is essentially the same as non-cyclic phosphorylation except that the electron acceptor used is immediately reoxidized by molecular oxygen, giving an apparent cyclic flow. Most experiments measuring photophosphorylation to date have utilized a cyclic system with PMS as the catalyst.

An understanding of the mechanism of phosphorylation has been an elusive problem for many years. Various high energy intermediates analogous to those proposed for oxidative phosphorylation have been suggested (Shen and Shen, 1962; Hind and Jagendorf, 1963). Recently, Mitchell (1966; Mitchell and Moyle, 1965) proposed a chemi-osmotic mechanism for phosphorylation where the driving force comes from a pH gradient across a membrane. It has since been shown that a simple acid to base transition in chloroplast fragments will support production of ATP (Hind and Jagendorf, 1965; Jagendorf and Uribe, 1966). A review of the evidence for this hypothesis has been published by Jagendorf (1967).

**Difference Spectroscopy**

The measurement of changes in absorption of ultraviolet and visible light at specific wavelengths to determine a change in the level of oxidation or reduction of various compounds has played an important role in the development of current concepts concerning electron transport in mitochondria and, more recently, in photosynthetic systems. Measurements of both the amount of absorbance change and the kinetics of the change have provided considerable insight into the components involved in electron transport and also the sequential order of their participation.
In photosynthetic systems there are a number of absorbance changes in the region between 400 nm and 700 nm which appear to be directly related to photosynthetic activity. Several of these absorbance changes provide convenient in vivo measures of oxidation-reduction states of the systems involved. For the purposes of this thesis only one of these absorbance changes is of importance; it involves an increase in absorption at 520 nm when the sample is exposed to light. Duysens (1954), who made extensive studies on the light-dark difference spectra throughout the visible spectrum, was the first to report this change. Chance and Strehler (1957) concluded that the 520 nm absorbance change was a photooxidation reaction which possessed complex kinetics. They showed that under aerobic conditions the absorbance change was biphasic but that anaerobic conditions produced only a uniphasic signal.

H. T. Witt and his group have been the major source of information dealing with the 520 nm absorbance change. They suggest that there are two absorbance changes near 520 nm, one due to the formation of a special chlorophyll a derivative and the other caused by a reduction of chlorophyll b by system II (Witt et al., 1965). Of major concern for this thesis is the suggestion that this absorbance change at 520 nm is a quantitative indicator for PQ reduction (Weikard, Müller and Witt, 1963). Measurement of this absorbance change, then, could provide a rapid, sensitive and in vivo....
method of measuring PQ in the visible region of the spectrum. PQ reduction can also be seen by a decrease in absorption at 260 nm (Klingenberg et al., 1962), but measurement in the UV requires special optical equipment (e.g., quartz optics, UV sensitive photomultiplier, etc.).

Fluorescence

There are three possible ways for the energy of excited chlorophyll molecules to be dissipated. One of these, radiationless dissipation as heat, is unmeasurable and assumed to be constant. The other two, fluorescence and photochemistry, are competing reactions; if the efficiency of the photochemical reaction is increased, the amount of energy released as fluorescence will decrease. Measurement of either fluorescence or the photochemical reaction should provide information as to the photochemical efficiency.

There is, at present, a vast and somewhat controversial volume of literature on the fluorescence properties of photosynthetic systems. The following discussion will be brief and concerned only with those aspects pertinent to this thesis (for a comprehensive discussion of many aspects of fluorescence, see Butler, 1966).

Fluorescence emission spectra from higher plants are characterized by several peaks in the red-far red region of the spectrum (at room temperature the main peaks are at 685 nm and 730 nm).
The peak of particular interest to this thesis lies at about 685 nm and appears to consist of two components. The first, a constant-yield fluorescence, which is probably emitted from the bulk absorbing chlorophyll, is not affected by changes in the efficiency of the photochemical reactions. The second component, having a variable-yield fluorescence, is directly related to the photosynthetic capacity of the system, presumably emanating from the system II trapping center. The trapping center (probably a special chlorophyll molecule) can be visualized as the couple between the energy-gathering system and the biochemical sequence of reactions. The energy levels of the trapping center molecules are probably lower than the bulk absorbing pigment molecules, insuring that once the quanta reach this point they cannot reenter the bulk pigment complex.

The two components of the fluorescence at 685 nm can be interpreted as follows. The bulk absorbing chlorophyll and the component to which it transfers quanta are present in large excess, and at any given time only a small portion of these molecules will be in the excited state. This means that the absorbed quanta always have a "place" to go and the fluorescence will be due solely to the probability that the quanta will be reemitted before they can be transferred. Therefore the yield will be constant for any given exciting intensity. When the quanta reach the trapping center, however, the situation is quite different. In this case large numbers of
quanta arrive at a site with limited turnover capacity; the probability is high that an incoming quantum will find the trapping center already converted by a previous quantum. The yield of this fluorescence will depend, in large part, on the ability of the system to unload the trapping center and allow additional quanta to be accepted. If the system is inefficient in removing this energy, fluorescence will be high and any treatment increasing electron flow will tend to suppress this fluorescence yield.

Kautsky, Appel and Amann (1960) attempted to relate the variable fluorescence yield to electron flow and they interpreted their results in terms of a two light-reaction system, apparently independently from the work of Emerson. The first demonstration of the two photosystem effect on fluorescence was made by Govindjee et al. (1960), who compared the fluorescence yield when light which activated both photosystems was used versus the sum of the yields of two beams used separately, with each beam activating only one of the photosystems. The former case had a lower yield, consistent with its higher quantum yield for photosynthesis.

Duysens (1963; Duysens and Sweers, 1963) proposed a mechanism of fluorescence based on an oxidation and reduction of an electron transport chain between the two photosystems. In this scheme, the fluorescence of chlorophyll $a_2$ is quenched by a hypothetical compound $Q$ when $Q$ is in the oxidized state, but not when reduced.
(QH). QH is then reoxidized by P 700, either directly or through a series of intermediates. This scheme is remarkably similar to that of Kautsky, who earlier proposed a substance A which acted in a manner similar to that of Duysens' Q. Several other theories explaining fluorescence have been proposed (Franck and Rosenberg, 1964; Arnold and Davidson, 1963; Clayton, 1965a and 1965b), but Duysens' hypothesis is most generally accepted today.

Several inhibitors of oxygen evolution in photosynthesis have been shown to increase the variable-yield fluorescence (Zweig, Tamas and Greenberg, 1963). In a similar manner, various mutants of Scenedesmus have shown that genetic alterations which prevent the reoxidation of QH show a maximal variable-yield fluorescence (Butler and Bishop, 1963). It would appear, then, that fluorescence measurements (of the variable-yield component) can provide information as to the efficiency of the mechanism of the electron transport chain.

**Quinones of the Photosynthetic Electron Transport System**

Studies of the molecular components of the electron transport chain in both mitochondria and chloroplasts have revealed the presence of several compounds which apparently take part in the electron transfer reactions. In photosynthetic organisms these include cytochromes b₆ and f, plastocyanin and quinones. Various roles have
been proposed for these quinones in both chloroplasts and mitochondria, but only their role in chloroplasts will be considered here. Their ability to undergo oxidation and reduction and their localization in particles capable of electron transport have suggested a crucial role for the quinones in the electron transfer reactions.

There are three major groups of quinones which have been isolated from photosynthetically active plant material. They include a series of substituted benzoquinones, napthaquinones and tocopherolquinones. At present there is very little evidence for a specific role for the napthaquinones and tocopherolquinones; these two groups will not be considered further here.

Köfler isolated the first substituted benzoquinone (2,3-dimethyl-5-solanesyl-benzoquinone) from plant material in 1946 (Arnon and Horton, 1963). This compound was given the trivial name plastoquinone (PQ) by Crane (1959) and was shown to be localized in the chloroplasts. A possible role for PQ was suggested by Bishop (1959), who found that extraction of lyophilized chloroplasts by petroleum ether removed their ability to reduce Hill oxidants. This photoreductive ability could be recovered either by readdition of the extract or authentic PQ. The β-carotene present in the extract was shown to be completely inert in restoring Hill reaction activity. These observations were subsequently confirmed, and it was shown that cyclic photophosphorylation with PMS as cofactor was similarly affected
(Krogmann, 1961; Krogmann and Olivero, 1962). It has also been suggested that the reduced form of PQ may be required for at least some reactions (Wood and Crane, 1965).

Various naturally occurring analogs of PQ have been described, all possessing the same spectral properties (absorption maxima at 255 nm and 262 nm). These have been named PQ A, PQ B, PQ C and PQ D (Kegel, Henninger and Crane, 1962; Henninger and Crane, 1963). The structures vary only in the side chain (Das et al., 1965; Das et al., 1967; Griffiths, 1966). PQ B appears to be an ester of PQ C and PQ D an isomer of PQ C. Recently it has been suggested that the designation PQ D be dropped in favor of a subscript of PQ C (Barr, Henninger and Crane, 1967). To further complicate the situation, each of the various fractions (A, B, C, and D) can be separated into at least six subfractions (Griffiths, Wallwork and Pennock, 1966). This is probably to be expected since there are nine double bonds in the side chain which are available for substitution.

Any classification of the various PQ analogs is trivial unless some variation in the specificity can be shown. Many experiments attempting to show this relationship have been performed, but all have suffered from irreproducibility. The very nature of the procedures employed probably precludes any conclusive data. Virtually all of these experiments have been carried out with lyophilized chloroplasts; the lyophilization procedure itself is harsh. Extraction with
various organic solvents removes many compounds other than PQ and almost certainly disrupts the structural integrity of the chloroplasts. The readdition of the various quinones is done simply by evaporating a quinone solution onto the chloroplasts, immediately raising the question as to whether the quinone is returning to its original site. Moreover, some of the material is deposited on the walls of the container, making a quantitative description of the re-added quinone impossible. In spite of the above difficulties, Crane's group, in particular, has published data showing the supposed specificity of the various quinones in a number of chloroplast systems (e.g., Wood, Bhagavan and Crane, 1966; Henninger and Crane, 1966). The only apparent and consistent conclusion which can be drawn at this time is that PQ A is generally required to restore activity to extracted chloroplasts.

Another technique which has lent support to the hypothesis that PQ is an integral part of the electron transport chain is direct spectrophotometric measurement of the oxidation and reduction of PQ in vivo, as mentioned above. These measurements were made at 260 nm (Klingenberg et al., 1962). Based on the rapidity of the absorbance change near 260 nm, the temperature independence of its magnitude at all light intensities and an observed oxidation by 700 nm light and reduction by 650 nm light, Witt and his group concluded that PQ is situated between the two light systems but very
close to the light reactions of system II, probably functioning as the initial electron acceptor for photosystem II (Witt, Müller and Rumberg, 1961).

Ultraviolet Inhibition of Photosynthesis

The use of specific inhibitors has played a major role in the elucidation of both mitochondrial and chloroplast electron transport mechanisms. Ultraviolet irradiation has been shown to be a potent and specific inhibitor of photosynthesis and appears to offer unique possibilities as a tool to study photosynthetic reactions.

The first account of UV irradiation affecting photosynthesis was reported by Arnold (1933), using whole cells of Chlorella pyrenoidosa. He showed that the inhibition was apparently first order and that the chlorophyll molecules were not chemically altered. Endogenous respiration was insensitive to the irradiation and he concluded that the hypothetical substance destroyed was proportional to, and a very small fraction of, the total chlorophyll content. Eighteen years later Arnold's group reported a series of experiments expanding those of the earlier paper (Holt, Brooks and Arnold, 1951). They observed that not only photosynthesis, but also the Hill reaction and photoreduction (in adapted algae), are inhibited to the same degree by UV irradiation. The inhibition of photoreduction, a reaction requiring only system I, is of particular interest (Bishop, 1962 and
1967). These experiments were performed before the development of the two photosystem hypothesis, hence the importance of this observation was not realized. The implications involved will be discussed later. They also examined the activity of several enzyme systems present in their chloroplast preparation (catalase, polyphenol oxidase and cytochrome oxidase) and found them to be unaffected by the irradiation.

Redford and Myers (1951) compared the effects of UV irradiation on many cellular processes of Chlorella and obtained results essentially similar to those of Holt, Brooks and Arnold. They reported a logarithmic inhibition of photosynthesis which appeared to be a function not only of dosage of UV irradiation but also of intensity, higher intensities being more effective. They felt that two types of effects were required to explain the inhibition of photosynthesis, but that the effects could not be identified with the techniques available to them.

A possible link between UV inhibition and PQ was suggested by Bishop (1961) when he found an apparent parallelism between PQ content and loss of photosynthetic activity following UV irradiation of spinach chloroplasts. At that time, no attempt was made to reactivate irradiated chloroplasts by readdition of unirradiated PQ. Experiments of this type have been done, but with inconclusive results (Shavit and Avron, 1963). These workers were able to show a
stimulation of Hill activity in irradiated Swiss chard chloroplasts by adding PQ, but a similar stimulation also occurred when PQ was added to unirradiated chloroplasts. A decrease in PQ content, (as measured by a decrease in absorption at 255 nm) with increasing UV irradiation, as reported by Bishop, was noted. They also showed that cyclic photophosphorylation (PMS) was considerably less sensitive than Hill activity to UV irradiation. Thus the earlier report by Arnold’s group of inhibition of photoreduction is of particular interest. These two results would localize the site of UV inhibition in the electron transport chain itself, not in either of the "light systems".

There have also been some studies on the effects of UV irradiation on whole leaves. Anderson and Spikes (1954) observed that rhubarb chard (Beta vulgaris) leaves treated with UV irradiation showed a rapid decolorization of the anthocyanin pigments in the petiole and midribs. By two weeks after irradiation the treated portions of the leaves became necrotic and atrophied. They also noted a loss of green color after 24 to 36 hours. This loss of chlorophyll could be prevented by placing the plants in the dark, although this did not prevent the treated leaves from dying. There did not appear to be any new synthesis of chlorophyll in the treated leaves. Of more importance to this thesis was their observations that both a photochemical and an enzymatic portion of the Hill
reaction (in chloroplasts which were isolated from these leaves) were affected by treatment with UV irradiation. This finding will be considered in detail in the Results and Discussion section.
STATEMENT OF PURPOSE

One of the most useful tools in studies on the mechanism of photosynthesis has been the use of selective enzymatic inhibitors. Various observations on the effects of UV irradiation on photosynthesis have shown that UV irradiation exhibits a pronounced specificity in its inhibition. The action spectrum for this inhibition has a peak of activity at about 250 nm, suggesting that destruction or alteration of a compound, or compounds, absorbing at this wavelength is responsible for the inhibition. Since plastoquinone (PQ) is an essential reactant in the photosynthetic electron transport system and has an absorption maximum at 255 nm, the possibility has been raised that alteration of PQ might be the principal cause of UV inhibition.

One purpose of this study will be to examine the interrelationships between UV inhibition of photosynthesis and PQ destruction by the irradiation. Several partial reactions of photosynthesis in isolated chloroplasts and whole cells will be examined, and the effects compared to those on photosynthesis, in an attempt to define the site of the inhibition in relation to the currently accepted scheme for electron transport. In particular, the effect of UV irradiation on photosystem I and photosystem II reactions and their relation to PQ and the electron transport chain will be discussed. Some of the partial reactions which will be utilized are the Hill reaction (system
II), photoreduction in adapted green algae, cyclic photophosphorylation, ascorbate-DCIP mediated reduction of NADP (all system I reactions) and also the 520 nm absorbance change and variable-yield fluorescence. It is anticipated that information obtained in this study will help elucidate the nature of the 520 nm absorbance change as well as its relation to UV inhibition.

Several classes of compounds found in photosynthetic systems will also be examined with respect to their potential role in the UV inhibition of photosynthesis. Some of these compounds have been suggested to play a structural role in the photosynthetic apparatus and the possibility that the inhibition may be due to a structural breakdown will be explored.
METHODS AND MATERIALS

Algal Culture

Various types of algae were employed in these studies. Ana-cystic nidulans was grown on medium C of Myers (Kratz and Myers, 1955). Another blue-green type alga, Cyanidium caldarum, was grown on a medium developed by Ascione, Southwick and Fresco (1966). Autotrophic cultures of Chlorella pyrenoidosa and Scenedesmus obliquus were grown on nitrate medium (Kessler, Arthur and Brugger, 1957). All of the above organisms were cultured in glass culture tubes (length 42 cm, diameter 2.7 cm) into which a mixture of 4% CO₂ in air was introduced from the bottom. Light was provided by a combination of warm-white and gro-lux fluorescent lamps. The tubes were maintained in a growth chamber at 25°C.

For some experiments, heterotrophic cultures of Scenedesmus were employed. The medium used consisted of nitrate medium supplemented with 0.5% glucose and 0.25% yeast extract. The cultures were maintained in the dark at 25°C on a rotating shaker.

Cultures were normally five to seven days old when used.

Chlorophyll Concentration Determination

Measurement of chlorophyll concentration was done by the procedure of Arnon (1949). This method is based on the extinction
coefficients of chlorophyll \( a \) and \( b \) at 645 nm and 663 nm in 80% acetone. The total chlorophyll concentration can be calculated from the following equation:

\[
\text{total chlorophyll conc. (mg/1)} = 20.2 A_{645} + 8.02 A_{663}. 
\]

When both blue-green and green algae were used in the same experiment, comparisons were made on a PCV basis only.

**Chloroplast Preparation**

Chloroplasts were normally prepared from spinach (in some cases Swiss chard) purchased from a local market. The leaves were deveined, deribbed and kept in the cold until ready for use. About 200 g of washed leaves were ground in 250 ml of 0.5 M sucrose-0.03 M KCl solution in a Waring Blender (Variac set at 70 V) for about one minute. The resulting homogenate was filtered through four layers of cheesecloth to remove larger pieces of debris, transferred to centrifuge tubes and centrifuged at 500 \( \times \) g for five minutes. The pellet was discarded and the supernatant centrifuged for 10 minutes at 5-10,000 \( \times \) g, depending on whether whole chloroplasts or chloroplast fragments were desired. The resulting supernatant was discarded and the pellet resuspended in enough sucrose-KCl solution to give the desired chlorophyll concentration. The resulting chloroplast suspension was divided into convenient aliquots and frozen.
(-35°C) until used.

Lyophilized chloroplasts were prepared as indicated above except that the final chloroplast pellet was washed twice with cold distilled water to remove the sucrose and resuspended in a minimum amount of cold distilled water. The chloroplast-water suspension was immediately placed in lyophilization jars and frozen by immersing the vessels in a mixture of acetone and dry ice. The vessels were then placed on a VirTis Mobile Unitrap freeze-dry apparatus (Model 10-100) until dry (usually about three hours). The lyophilized chloroplasts were stored in small glass weighing bottles, containing desiccant, in a freezer until used.

For photophosphorylation experiments the following chloroplast isolation procedure was used (adapted from Avron, 1960). Washed, deveined and deribbed spinach leaves were ground by hand in an ice cold mortar and pestle. Glass beads (0.35 mm) were added to facilitate grinding. A few milliliters of homogenizing medium (0.4 M sucrose, 0.05 M Tris, 0.01 M NaCl, pH 7.8) were added as the grinding proceeded. During the grinding 0.02 M Na-ascorbate was also included in the homogenizing medium. The slurry obtained was squeezed through two layers of cheesecloth and centrifuged at 500 X g for 10 minutes. The pellet was discarded and the supernatant centrifuged at 10,000 X g for 10 minutes. The resulting pellet was washed once in homogenizing medium to give a chlorophyll concentration of
about 0.3 mg/ml and the chloroplasts used immediately. The entire procedure was carried out at 0-4°C.

Chloroplast fragments from whole cells of the green alga *Scenedesmus* were prepared by the method of Pratt (1968). About 1 cc of cells were suspended in sufficient "breaking" solution (0.02 M KPO₄, pH 7.0) to give a total volume of 20 ml and placed in a 50 ml stainless-steel cup which was then filled with 0.35 mm glass beads. The filled cup was vibrated at full power on a Vibrogen-Zellmühle (Edmund Bühler, Tübingen, Germany) for five minutes. The cup was kept at 0-4°C by circulating ice water around it. After vibrating, the glass beads were removed from the suspension by filtering through a fritted-glass filter. The beads were then washed with buffer to remove any remaining cells or chloroplasts. The filtrate was centrifuged for 10 minutes at 1000 X g followed by centrifugation of the supernatant for 15 minutes at 15,000 X g. The chloroplast pellet was then suspended in another buffer solution (0.4 M sucrose, 0.01 M NaCl, 0.05 M Tris, pH 7.8) to give a final chlorophyll concentration of about 0.2 mg/ml and frozen until used.

**Chloroplastin Preparation**

Washed and deveined spinach leaves (about 200 g) were ground with 200 ml ice cold 0.35 M NaCl for four 30 second periods in a Waring Blender. The homogenate was strained through cheesecloth
and centrifuged at $500 \times g$ for five minutes. The supernatant was then centrifuged at $2,500 \times g$ for 10 minutes and the pellet washed twice with about 100 ml of 0.35 M NaCl.

The washed chloroplasts were incubated for 20 minutes at room temperature with a 1% digitonin solution (0.05 ml/g fresh leaf). The suspension was then centrifuged at $25,000 \times g$ for 30 minutes, the green supernatant carefully collected and the pellet discarded. Cold (-15°C) ethanol (0.3 ml/ml extract) was added to the supernatant slowly with stirring. After standing for 15 minutes, the suspension was centrifuged at $25,000 \times g$ for 20 minutes. The pellet was suspended in a minimum amount of 1% digitonin solution and lyophilized as described above.

**Isolation of Chloroplast Quinones**

A variety of methods were utilized for isolation of the various quinones, depending upon the type of material to be extracted and the degree of purity desired. For extraction of quinones from whole algal cells (either green or blue-green algae), the cells were extracted with warm methanol, centrifuged at $1500 \times g$ for two or three minutes and the extraction repeated until the pellet was white. The extracts were combined and evaporated to dryness under vacuum. The residue was then redissolved in a small amount of an appropriate solvent and stored in a freezer until needed.
Isolated chloroplasts were extracted with 80% acetone, centrifuged (1500 \times g) and the pellet reextracted until no green color remained. The 80% acetone extract was in turn partitioned with an equal volume of redistilled petroleum ether (boiling range 36-40^\circ C) in a separatory funnel. After phase separation the petroleum ether fraction was removed and the 80% acetone reextracted until clear. The petroleum ether fractions were combined and washed twice with 50 ml aliquots of water to remove traces of acetone. A small amount of anhydrous sodium sulfate was added to remove any remaining water. The extract was then evaporated to dryness under vacuum and treated as described above.

Lyophilized chloroplasts or chloroplastin were normally extracted by adding 3.0 ml of petroleum ether (heptane, hexane or cold petroleum ether were sometimes used) to 60 mg of the material in a ten Broeck homogenizer. The plunger was worked a set number of times and the resulting suspension centrifuged at 1500 \times g. The resulting extract was then treated as described above.

All of the above extracts contained large quantities of carotenoids and chlorophyll as well as quinones (extracts from lyophilized material contained only quinones and carotenoids). Prior to quantitation of the quinones present, further purification was necessary.

In early experiments this purification was accomplished by column chromatography employing silicic acid as adsorbent. The
extract was dissolved in a small volume of 25% chloroform-75% iso-octane and applied to a column previously washed with 25% chloroform-75% iso-octane. Addition of 25% chloroform-75% iso-octane rapidly eluted the β-carotene off the column, while the quinones, carotenoids, and chlorophylls remained near the top of the column. After the carotene band had passed through the column, 75% chloroform-25% iso-octane was added; this eluted the quinone fraction off the column but left the remaining pigments behind. The yellow quinone band was collected, evaporated to dryness under vacuum and finally redissolved in absolute ethanol. The concentration of PQ present was estimated by measuring the absorbance change (ΔA) at 255 nm upon reduction with sodium borohydride. The concentration in mg/l is equal to the ΔA/19.6. It should be pointed out that the silicic acid column gives a mixture of PQ's; at least PQ A and PQ B, as well as vitamin K₁ and vitamin E.

In order to separate further the various quinones, thin-layer chromatography of the quinone fraction was employed. By streaking the quinone fraction on thin-layer plates of silica gel G and developing 100% chloroform reasonable separation of PQ A, vitamin K₁, and vitamin E was obtained, although PQ A and vitamin K often overlapped. The bands were then removed from the plates, eluted with ethanol and the PQ concentration determined as described above.

In later experiments it was found that the silicic acid column
step could be eliminated and the various quinones separated by thin-layer chromatography alone. The crude extracts were applied to silica gel G plates and developed in benzene-heptane (60:40). This procedure separated the quinones into three groups of two: PQ A and vitamin K₁ were recovered from a band at Rf = 0.4, PQ B and vitamin E travel at an Rf of about 0.2 and PQ C and PQ D at an Rf of 0.05. The exact location of the various bands was determined by spraying a very small portion of the plate with a 0.5% solution of Rhodamine B in ethanol. The plates fluoresce orange-red under UV irradiation with the quinones appearing as dark purplish spots. The remainder of the band was then removed, eluted with an appropriate solvent (usually acetone) and rechromatographed to separate the individual quinones, if desired.

Separation of PQ C from PQ D and PQ B from vitamin E was accomplished by rechromatographing with dichloroethane as the solvent but satisfactory separation of PQ A and vitamin K required the use of reversed-phase chromatography. Plates were prepared by developing the usual silica gel G plates in 5% silicone (Dow-Corning 550 Fluid) in chloroform (v/v). The plates were then dried and stored in a desiccator until used. Samples were spotted on these plates and developed in 80% n-propanol-20% water. Vitamin K₁ moved with an Rf of about 0.60 while PQ A had an Rf of only 0.30.

Ordinarily the purity of each quinone was checked by determining
its spectrum with a Beckman DK 2A recording spectrophotometer. Generally the purity obtained by the above procedure was excellent.

For readdition experiments with petroleum ether extracted chloroplasts, the quinones to be used were dissolved in a small volume of petroleum ether, added to the lyophilized material in a small homogenizer and mixed. The suspension was then evaporated to dryness under vacuum and the material scraped out for use. Due to the hygroscopic nature of the lyophilized material, the above procedure was carried out as rapidly as possible.

Carotenoid Isolation and Purification

Isolation and separation of carotenoids was done essentially by the method of Dersch (1960). The chloroplast samples were first extracted with 80% acetone followed by partitioning into petroleum ether. The petroleum ether extract was washed, dried and evaporated as described in the quinone isolation procedure. The residue was taken up in 30 ml of 10% KOH in methanol and incubated for 10 minutes at 40°C. At the end of the incubation period the extract was cooled, 70 ml of diethyl ether were added and the mixture gently shaken. Glass-distilled water was added until phase separation occurred and the lower phase (water-methanol) was drawn off and discarded. The ether extract was washed two or three times with water, dried with sodium sulfate and finally evaporated to dryness
under vacuum. The residue was then taken up in a small volume of a suitable solvent for streaking on thin-layer plates. The gel on the plates consisted of 30 g silica gel G and 18 mg calcium hydroxide suspended in 66 ml of $5 \times 10^{-3}$ M Na-ascorbate. The plates were developed in a mixture of petroleum ether, isopropanol and water (100:10:0.25). The carotenoids were examined visually on the plate with no further attempt made to quantitate the amounts of each present (see Results and Discussion).

**Lipid Isolation and Purification**

Chloroplast samples containing about 23 mg chlorophyll were centrifuged for 10 minutes at 39,000 $\times$ g to remove them from the suspending medium (sucrose-KCl). The pellet was extracted with 25 ml of hot methanol and the resulting suspension centrifuged at 1500 $\times$ g for two or three minutes. The supernatant was carefully removed and the pellet extracted with 20 ml hot methanol-chloroform (1:3). This suspension was again centrifuged as above, the supernatant removed and the pellet reextracted with hot methanol-chloroform (1:1). The centrifugation was repeated and the pellet extracted with hot chloroform. This suspension was filtered and the residue finally reextracted with hot methanol. All of the extracts were pooled and evaporated to dryness under vacuum. The residue was taken up in a small volume of chloroform and 0.5 ml spotted on one corner of
a standard 15 x 15 cm plate (silica gel G). The plate was developed in one dimension in chloroform-methanol-water (65:25:1), followed by development in the other dimension in n-butanol-acetic acid-water (40:25:5). Spots were detected either by treating with iodine vapor (lipids appear as brown spots) or by spraying with a 0.5% Rhodamine B solution in ethanol. Spots were examined visually under UV with no further attempt to quantitate the various lipids present (see Results and Discussion).

Separation of Water Soluble Proteins of Algae

Scenedesmus cells were grown heterotrophically for six days and removed from the growth medium by centrifugation. The cells were suspended in 0.15 M Tris at pH 7.5 and broken in a French press. A small amount of DNAse was added to prevent increased viscosity caused by coagulation of DNA.

The resulting suspension of broken cells was brought to 90% acetone concentration at 0°C and the precipitate collected by centrifugation. The precipitate was dried under vacuum and dissolved in 0.15 M Tris, pH 7.5. An ammonium sulfate precipitation was performed, with the fraction at 35-90% saturation at 0°C collected. This precipitate was collected by centrifugation, redissolved in a small amount of the Tris buffer (pH 7.5) and the total protein measured by determining the optical density at 260 and 280 nm.
Samples of the protein were then separated by gel electrophoresis (EC 470 Vertical Gel Electrophoresis Cell, E-C Apparatus Corporation).

**Whole Cell Reactions**

Measurement of photosynthesis with whole cells was done either on a Gilson Respirometer (Gilson Medical Electronics) or an Oxygraph (Gilson Medical Electronics). When the respirometer was used, 50 µl of cells were suspended in 3.0 ml of Warburg No. 9 carbonate buffer. The Oxygraph required 25 µl suspended in 2.7 ml of nutrient medium.

Photoreduction was measured with a conventional Warburg apparatus because of the permeability of the Tygon tubing used on the respirometer to H₂. Fifty microliters of cells were suspended in 3.0 ml of 0.05 M phosphate buffer at pH 7.0 and placed in Warburg vessels. The vessels were gassed with 96% H₂-4% CO₂, under continuous shaking, for 10 minutes after which time the cells were allowed to adapt in 96% H₂-4% CO₂ in the dark overnight. The reactions were run in the presence of 5 × 10⁻⁶ M DCMU. White light with an intensity of about 10⁶ ergs cm⁻² sec⁻¹ was used for actinic radiation.
Chloroplast Photoreactions

The Hill reaction, with ferricyanide or benzoquinone as the oxidant, was measured manometrically on a Gilson Respirometer. The reaction mixture consisted of 24 µmoles $K_3Fe(CN)_6$ or 4.6 µmoles of p-benzoquinone, 100 µmoles phosphate buffer, pH 7.0, chloroplasts containing about 0.35 mg chlorophyll and water to 3.0 ml. The reactions were run at 20°C in a gas phase of air. White light, with an intensity of about $10^6$ ergs cm$^{-2}$ sec$^{-1}$, was used as actinic radiation.

DCIP reduction was measured spectrophotometrically on a Zeiss PM QII spectrophotometer and the changes in absorbance plotted with a Photovolt Varicord recorder, Model 43. The reaction mixture contained 0.1 µmoles DCIP and chloroplasts containing about 35 µg chlorophyll in 3.0 ml of sucrose-KCl solution. Routinely no buffer was included in this reaction mixture. Experiments performed with phosphate buffer, pH 7.0, did not reveal any alteration of activity. Actinic light was provided by a 1000 watt projection bulb. This light was first passed through a Corning glass cutoff filter (2-61)(transmits wavelengths greater than 600 nm) focused onto the top of a standard 1 cm cuvette through a hole in the top of the sample compartment. The change in absorbance at 610 nm is related to µmoles substrate reduced by the following equation (Pratt,
µmoles DCIP reduced/ml = 0.0563 ΔA_{610 nm}

Cytochrome c and NADP reduction were also measured spectrophotometrically at 550 nm and 340 nm respectively. The reaction mixture contained 250 µmoles Tris, pH 7.8, 2 mg cytochrome c or 0.8 µmoles NADP, saturating PPNR (prepared according to San Pietro and Lang, 1958), chloroplasts containing 35 µg chlorophyll and water to a volume of 3.0 ml. The coefficients for conversion of absorbance changes in µmoles substrate reduced are:

µmoles cytochrome c reduced/ml = 0.0510 A_{550 nm}
(Miller and Evans, 1956)

µmoles NADP reduced/ml = 0.1613 A_{340 nm}
(Bergmeyer, 1963, p. 1030)

Ascorbate-DCIP coupled reduction of NADP was performed as indicated above except that the reaction mixture also contained 20 µmoles Na-ascorbate, 0.1 µmoles DCIP and was run in the presence of 5 × 10^{-6} M DCMU.

Oxidation of reduced cytochrome c by chloroplastin was performed in a manner similar to the above assays. The reaction mixture contained 2 mg reduced cytochrome c (prepared by adding a small amount of sodium dithionite to 50 mg of cytochrome c
dissolved in 5 ml of 0.01 phosphate buffer, pH 7.0, and dialyzing against water for several hours), 0.1 µ moles benzyl viologen, 0.2 µ moles KCN, saturating plastocyanin, 115 µ moles phosphate buffer, pH 6.5, all in a final volume of 3.0 ml of water.

Photophosphorylation

Photophosphorylation assays were carried out in 50 ml Erlenmeyer flasks on a Gilson Respirometer adapted to hold the flasks. The reaction mixture (adapted from Avron, 1960) contained 45 µ moles Tris, pH 7.8, 60 µ moles NaCl, 12 µ moles MgCl₂, 40 µ moles ascorbate, 12 µ moles Pi, 50 µ curies ³²Pi, 12 µ moles ADP, 0.09 µ moles PMS, chloroplasts containing 50 µg chlorophyll and water to a volume of 3.0 ml. The reactions were run with continuous shaking in a water bath at a temperature of 25°C for 10 minutes. Actinic light was supplied from beneath and was passed through red plastic passing wavelengths greater than 600 nm. Actinic intensity was approximately \(8.5 \times 10^4\) ergs cm\(^{-2}\) sec\(^{-1}\). The reactions were terminated by adding 0.3 ml of 20% TCA.

The assay procedure for determining ³²P incorporation follows that of Avron (1960). This method consists of complexing the unesterified Pi present as a phosphate-molybdate complex, extracting it with organic solvents and counting the remaining aqueous phase for ³²P incorporated into organic compounds, principally ATP.
Aliquots of the aqueous phase were placed on planchets, allowed to dry and counted on a Nuclear Chicago gas flow detector (Model D 47). Two planchets were made for each sample and each planchet was counted twice. Counting was performed by recording the time required to obtain 10,000 counts. Dark controls were run for each treatment as well as a zero time control.

In order to calculate the µmoles ATP produced from the raw counting data, the counting efficiency had to be determined. This was done by placing a known amount of highly diluted 32P, including the normal reaction mixture, on a planchet, drying and determining the count rate. The counting efficiency was calculated by dividing the count rate by the DPM introduced. For these experiments the counting efficiency was 46%.

Photophosphorylation rates are usually expressed as µmoles ATP produced/mg chlorophyll/hour. The corrected count rates were converted to rates of ATP synthesis by the following equation:

$$\text{rate} = \frac{(\text{sample cpm} - \text{control cpm})}{\text{added cpm}} \times \frac{(6) (21.9) (13) (1000)}{X}$$

where X is the number of µg of chlorophyll added, (13) is the number of µmoles of Pi added, (21.9) is the dilution factor in the preparation of the planchets and (6) converts the rate/10 minutes to rate/hour.
Ultraviolet Irradiation

Ultraviolet irradiation of samples was carried out in the device shown in Figure 2 (Anderson, 1956). The outer chamber was used to cool the sample; when chloroplasts were being irradiated, ice water was circulated through the chamber, but when algal suspensions were being irradiated, tapwater at about 20°C was used. The inner chamber contained the sample being irradiated, which was in direct contact with the wall of the UV source (G.E. 15 watt germicidal lamp). This lamp emits approximately 85% of its energy at 253.7 nm with the total intensity emitted being about $3.8 \times 10^4$ ergs cm$^{-2}$ sec$^{-1}$. When ice water was circulated through the outer chamber the temperature of the chloroplast suspension remained between 0 and 4°C.

The main disadvantage of the device was its rather limited capacity. The largest sample size which could be used was 27 ml. To keep the irradiation time reasonable (about one hour for total inhibition of photosynthesis), chloroplast suspensions had to have no more than about 0.5 mg chlorophyll/ml and algal suspensions no more than 25 µl of cells/ml. In order to avoid settling and to insure uniform irradiation of all cells, particularly with algae, the device was continuously shaken.
Figure 2. Schematic diagram of irradiation vessel. Cooling jacket consisted of cylinder around entire sample compartment through which cooling water was circulated. Apparatus was connected to a shaking device to eliminate settling of contents and insure equal irradiation of all cells. See Methods and Materials for details of operation.
Single-Beam Spectrophotometry

Measurements of the 520 nm light-dark absorbance change were made on a single-beam spectrophotometer capable of making measurements in light scattering samples. The basic design is similar to that of de Kouchkovsky and Fork (1964) with some modifications (Pratt, 1968). A schematic diagram of the instrument is presented in Figure 3. Although not shown in the diagram, a simple lens system was required for focusing both the measuring and actinic beams. The measuring light was obtained from a quartz-iodide lamp operated from two 12 V lead storage batteries connected in series. For later experiments a 6 V, 18 amp microscope lamp was substituted. This lamp was operated from a Sorensen QSB6-30 regulated power supply. The proper wavelength for the measuring beam was provided by using a 525 nm Schott interference filter (all interference filters used had a half-band width of about 10 nm). Another 525 nm Schott interference filter, a blue Corning glass filter (4-71) and an infrared-absorbing filter were placed over the photomultiplier tube (RCA 6217). The sample holder was then placed directly over these filters. Actinic light was obtained from either a prefocused 500 watt tungsten filament projector lamp or a PEK 150 W xenon lamp (Model 911). To isolate the actinic from the measuring light, three red cutoff filters (2 Corning 2-58 and 1 2-59), an infrared-absorbing filter and
Figure 3. Schematic representation of the single-beam spectrophotometer. (Pratt, 1968)
a 5 cm water bath were used. The measuring light intensity was approximately 2500 ergs cm\(^{-2}\) sec\(^{-1}\) and the actinic intensity about 60,000 ergs cm\(^{-2}\) sec\(^{-1}\) with the tungsten filament and 90,000 ergs cm\(^{-2}\) sec\(^{-1}\) with the xenon source. The photomultiplier high voltage was provided by a Keithley 240 regulated high voltage supply, and was operated at 900 volts.

The output from the photomultiplier was displayed either on an oscilloscope (Hewlett-Packard 140A with Polaroid camera) or a high-speed strip recorder (BLH Meterite, BSA 250 with PR 301 B amplifier).

**Fluorescence**

Fluorescence measurements were made using two procedures. The first method used the same instrument as described above except that the measuring beam wavelength was provided by a broad band cutoff filter (Kodak #70) passing wavelengths between 660 nm and 720 nm. A 697 nm or a 686 nm interference filter (Schott or Baird-Atomic) and a broad band filter passing wavelengths from 650 nm to 750 nm (Balzer K-7) were used over the photomultiplier tube. Actinic light was provided by the xenon source at a wavelength of 430 nm (430 nm Baird-Atomic interference filter, infrared-absorbing filter, Kodak Wratten filter #47 and a 5 cm CuSO\(_4\) solution).

The second method eliminated the need for a measuring beam
and allowed the measurement of the effects of one wavelength of actinic light on the fluorescence elicited by another wavelength. In this instrument the fluorescence activating beam was chopped by a mechanical light chopper (Princeton Applied Research, Model BZ-1) at 500 cps. The output from the photomultiplier was passed through a tuned amplifier (Princeton Applied Research, Model JR-4) which only accepts signals of a predetermined frequency. By setting this frequency to match that of the chopper, and with proper synchronization, only signals arising from the chopped beam will pass to the display. This instrument also eliminated the need for a large biasing voltage in the electronic circuit. In the first method described, the measuring beam produced a rather large base current and the signal was measured as a change in this base current. Since the instrument measured a small signal on a large base current, any noise in the base current tended to swamp the signal. With the second method, the chopper produces a light-dark square wave (light when the beam is passed and dark when the chopper blade blocks the beam) which is converted to a DC output by the tuned amplifier. Therefore, when the activating beam is off, the tuned amplifier sees no AC signal and the DC output is zero. This instrument is shown in block diagram in Figure 4. The time constant used was <10 msec for the experiments with chloroplasts but up to 1 sec for experiments with whole cell systems. These long time constants preclude any kinetic analysis
Figure 4. Schematic diagram of fluorescence apparatus.
of the results.

In a typical experiment, 430 nm light was used to activate fluorescence (chopped beam) and 650 nm light (Baird-Atomic 650 nm interference filter, infrared-absorbing filter and a 5 cm water bath) from a 1000 watt AC projection lamp used to "drive" the photo system (see Results and Discussion). The intensity of the 430 nm activating beam was between 800 and 900 ergs cm\(^{-2}\) sec\(^{-1}\) and the intensity of the 650 nm beam about 3000 ergs cm\(^{-2}\) sec\(^{-1}\). The sample holder used had a cross sectional area of 10 cm\(^2\). For experiments using algal cells, 10 or 20 µl of cells suspended in 1.0 ml of growth medium were used and any additions, such as DCMU, were made to give a total volume of 1.0 ml. When isolated chloroplasts were used an amount equal to 70 µg chlorophyll was suspended in 1.0 ml of sucrose-KCl. Again any additions were made to give a total volume of 1.0 ml.
RESULTS AND DISCUSSION

Effects of UV Irradiation on Various Partial Reactions of Photosynthesis

Early studies on the killing of small organisms by ionizing radiations led workers to test the effects of various types of radiation on specific cellular reactions. Arnold (1933) made the first studies on the effects of UV irradiation on photosynthesis using manometric techniques to measure the photosynthetic rates. The 253.7 nm mercury line was used; a recent action spectrum of UV inhibition indicates a peak at about 250 nm (Jones and Kok, 1966a). Later studies (Holt, Brooks and Arnold, 1951; Redford and Myers, 1951) extended these experiments to include photoreduction in adapted algae and the Hill reaction in both isolated chloroplasts and whole cells. Holt, Brooks and Arnold showed that photosynthesis and p-benzoquinone photoreduction in whole cells of Chlorella were equally inhibited as were ferricyanide and phenolindophenol reduction by isolated chloroplasts. At the time these experiments were performed, the two photosystem hypothesis had not yet been proposed; hence Holt, Brooks and Arnold could make only a general interpretation of their results. It is assumed that all of these artificial electron acceptors require only system II activity, although Kok (Kok and Cheniae, 1966) proposes that almost all Hill reaction oxidants are reduced
by system I.

The results mentioned above strongly suggest that the site of the UV inhibition is before the earliest point where electrons can be withdrawn from the electron transport chain, probably near system II. It is difficult, however, to compare results from whole cell systems with those from isolated chloroplasts. Although it definitely appeared that system II was inhibited, possible effects on system I cannot be determined from the above data.

With the above facts in mind, it was thought desirable to perform a series of experiments similar to those of Holt, Brooks and Arnold but using isolated chloroplasts only and measuring various photoreactions requiring system II only, system I only or both systems. The reduction of several Hill oxidants was used as a system requiring only photosystem II and NADP reduction as a reaction requiring both photosystems (Hoch and Martin, 1963; Sauer and Biggins, 1965). System II can be bypassed by adding DCMU to chloroplasts and using ascorbate as electron donor with catalytic amounts of DCIP as a coupling agent (Vernon and Zaugg, 1960). This reaction requires only system I. Figure 5 shows the effects of UV irradiation on these several reactions. The results are similar to what might be predicted from the experiments of Holt, Brooks and Arnold; all reactions requiring system II activity are equally inhibited, whether system I is also required or not, and reactions utilizing only system
Figure 5. Effects of UV irradiation on various Hill reactions and ascorbate-DCIP mediated reduction of NADP. Control rates (expressed as μholes substrate reduced/mg chlorophyll/hr) are as follows: DCIP = 64.5, NADP = 129.7, Fe(CN)$_6^{3-}$ = 40, p-benzoquinone = 37.6, ascorbate-DCIP reduction of NADP = 54. For experimental conditions see Methods and Materials.
I activity (ascorbate-DCIP coupled reduction of NADP) are not affected. Trebst and Pistorius (1965) independently found similar results.

While the above experiments indicate that system I is not inhibited by UV irradiation, they do not demonstrate whether the block lies on the reducing or oxidizing side of system II. The experiment by Holt, Brooks and Arnold on the effects of UV irradiation on in vivo photoreduction is particularly important in answering this question. Photoreduction requires only system I activity (Bishop, 1962 and 1967). If this reaction is inhibited, the UV block must be on the reducing side of system II. Figure 6 shows the results of such an experiment confirming that photoreduction is indeed inhibited at a rate equal to photosynthesis. It might be concluded from the above results that the site of the UV inhibition of photosynthesis lies in the electron transport chain between the two light systems, or at a phosphorylation site.

Effects of UV Irradiation on Photophosphorylation

Photophosphorylation is intimately coupled to electron transport and any consideration of the effect of UV irradiation on "photosynthetic capability" must include this process. Non-cyclic photophosphorylation, coupled with oxidation of water and reduction of NADP, was shown to be inhibited to the same degree as the Hill
Figure 6. Comparison of effects of UV irradiation on photoreduction and photosynthesis in *Scenedesmus*. Photosynthesis was measured manometrically with 50 µl of cells suspended in 3.0 ml of carbonate buffer. Control rate = 436.3 µl O₂ evolved/50 µl cells/hr. Photoreduction was measured manometrically with 50 µl of cells suspended in 3.0 ml of phosphate buffer, pH 7.0. Control rate = 25.9 µl H₂ uptake/50 µl cells/hr. See Methods and Materials for details.
reaction (Jones and Kok, 1966b), but the inhibition of cyclic photophosphorylation appears to be more complex. Shavit and Avron (1963) reported that in their system cyclic photophosphorylation with PMS as catalyst was inhibited only 30% when Hill activity (DCIP reduction) was totally abolished. However, Trebst and Pistorius (1965) found that PMS-mediated cyclic photophosphorylation was inhibited about 65% when Hill activity was essentially zero. A partial inhibition seemed difficult to explain assuming that the UV inhibition of electron transport was due to inactivation of a single site. For this reason these experiments were repeated. The results of such an experiment are plotted in Figure 7. It is apparent that in this system inhibition of PMS-mediated photophosphorylation closely parallels loss of Hill reaction activity. Examination of the experimental conditions used in the three laboratories reveals a possible explanation. PMS-mediated photophosphorylation does not show a typical light saturation curve characteristic of photosynthesis. In spinach chloroplasts, Hill activity and FMN-mediated photophosphorylation are saturated at about 2,000 foot candles but PMS-mediated photophosphorylation requires at least 4,500 foot candles (Jagendorf and Avron, 1958). For Swiss chard chloroplasts, over 14,000 foot candles are required (Avron, 1960). When the results obtained with the various light intensities used are compared, there appears to be a correlation between the amount of inhibition of PMS-mediated
A Cyclic Photophosphorylation

Figure 7. Comparison of effects of UV irradiation on Hill reaction and cyclic photophosphorylation. Control rates are as follows: DCIP = 168.9 µmoles reduced/mg chlorophyll/hr, ATP = 33.3 µmoles esterified/mg chlorophyll/hr. See Methods and Materials for experimental conditions.
photophosphorylation and the light intensity employed. Shavit and Avron, who observed the least inhibition, used over 11,000 foot candles (in Swiss chard chloroplasts). Trebst and Pistorius used about 3,300 foot candles (spinach chloroplasts) and the results presented in this thesis were obtained with less than 1,000 foot candles (also spinach chloroplasts). This intensity is very near saturating for the Hill reaction. These observations suggest that at high light intensities PMS can partially by-pass the UV-sensitive site of electron entry.

Of possible importance is the observation by Jones and Kok (1966b) that, although NADP reduction mediated by ascorbate-DCIP is not inhibited by UV irradiation, phosphorylation associated with this mode of electron transport is. They propose two possible explanations: (1) Phosphorylation occurs at a site in the electron transport chain which can be bypassed by DCIP without loss in the rate of electron transport, although the phosphorylating pathway is preferred, or (2) UV irradiation acts as an uncoupler of phosphorylation with this system without affecting electron transport. At present there is insufficient data to favor either of these alternatives, or to suggest others.
The Role of Quinones in UV Inhibition

As mentioned previously, 253.7 nm ultraviolet light was used in all of the early studies on UV inhibition of photosynthesis. Based on the absorption maxima of PQ at 255 nm (Crane and Lester, 1958) and its requirement in electron transport (Bishop, 1959), Bishop (1961) suggested that PQ might be the locus of the inhibition. He reported a decrease in PQ content corresponding to loss of photochemical activity after UV irradiation. However, he did not attempt to reactivate the chloroplasts by adding unirradiated PQ. Several attempts to accomplish this have been unsuccessful (Shavit and Avron, 1963; Trebst and Pistorius, 1965; Jones and Kok, 1966b). Although their readdition experiments were unsuccessful, Trebst and Pistorius concluded that PQ was indeed the site of the inhibition.

All of the above experiments were carried out with isolated chloroplasts. Chloroplasts, in spite of the fact that they will perform essentially all of the reactions of photosynthesis, must be considered an in vitro system. For this reason, a series of experiments were performed using whole cells of various algae. Whole cells were irradiated until photosynthesis (actually the 520 nm absorbance change, see below) was totally inhibited and the PQ content examined. The results of several experiments with Chlorella are listed in Table 1. Whole cells did not show a correlation between
PQ content and rate of inhibition of photosynthesis. The reason for the widely differing rates of apparent PO destruction compared to inhibition of photosynthesis or the Hill reaction is not known, although Shavit and Avron (1963) showed that various compounds, particularly ascorbate, had a protective effect on the UV inhibition in chloroplasts. This protective effect was more than just absorption of the UV by the ascorbate. They did not, however, measure the decrease in PQ content in the 'protected' chloroplasts.

Table 1. Comparison of the effects of UV irradiation on the destruction of PQ and the inhibition of photosynthesis in Chlorella. See text for details.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>% PQ destroyed</th>
<th>△A at 520 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>1</td>
<td>41</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>31</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>49</td>
<td>$5 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

Experiments were performed measuring the effects of UV irradiation on pure PQ in vitro. Figure 8 illustrates that PQ (dissolved in ethanol) is destroyed when treated with UV irradiation. Initially an apparent photoreduction of the PQ occurs (indicated by the increase in absorption at 290 nm and the decrease at 255 nm), followed by rapid and total disruption of the spectrum. The reaction
Figure 8. Effect of UV irradiation on the absorption spectrum of PQ in ethanol solution. Curves show various times of irradiation.
probably consists of the formation of dimers or higher molecular weight products of PQ (Eck and Trebst, 1963; Trebst and Pistorius, 1965). This interpretation is partially supported by the fact that chromatography of irradiated pure PQ indicates that several new compounds are formed, coinciding with the disappearance of the original PQ spot. No attempt was made in these studies to isolate and analyze the photolytic products.

Vitamin K\textsubscript{1} is also rapidly destroyed by UV irradiation in \textit{vitro} (Figure 9). However, experiments with spinach chloroplasts have shown that substantial amounts of vitamin K\textsubscript{1} remain after Hill activity has been totally abolished.

Readdition experiments of the type mentioned above were also attempted using both PQ and vitamin K\textsubscript{1}. Results of such an experiment are shown in Table 2. PQ did partially reactivate irradiated chloroplasts, but a similar enhancement of activity was also noted in the control (see also Shavit and Avron, 1963). Addition of vitamin K\textsubscript{1} to irradiated chloroplasts had no measurable effect although a slight stimulation of the control was noted. The amounts of either PQ or vitamin K\textsubscript{1} added were calculated to approximate the amount of each quinone normally found in chloroplasts. The control in these experiments consisted of lyophilized chloroplasts to which 3.0 ml of petroleum ether was added and mixed. This petroleum ether was then evaporated from the chloroplast suspension to account for
Figure 9. Effect of UV irradiation on the absorption spectrum of vitamin $K_1$ in ethanol solution. Curves show various times of irradiation.
effects due to the extraction procedure. This caused a considerable decrease in activity compared to chloroplasts which had not been so treated. The crudeness of this procedure makes a definite interpretation of the results extremely difficult.

Table 2. The effects on Hill reaction (cytochrome c reduction) of adding PQ or vitamin K₁ to irradiated or unirradiated spinach chloroplasts. See Methods and Materials for details on experimental conditions. Values are expressed as μmoles cytochrome c reduced/mg chlorophyll/hr.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Hill reaction activity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Irradiated</td>
<td>Unirradiated</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>32.0</td>
</tr>
<tr>
<td>vitamin K₁</td>
<td>0</td>
<td>34.5</td>
</tr>
<tr>
<td>PQ</td>
<td>6.12</td>
<td>46.8</td>
</tr>
</tbody>
</table>

Although the above results would tend to eliminate both PQ and vitamin K₁ as the site of UV inhibition, there are a number of possible complications which makes a straight-forward interpretation impossible. Since PQ exists in chloroplasts in very high concentrations (0.1 mole/mole chlorophyll), it is possible that the major portion of the pool is inactive and possibly less susceptible to UV inactivation. The crude techniques employed could certainly account for the inability to reactivate irradiated chloroplasts by adding PQ.
To help clarify this situation, a number of experiments were performed comparing the effects of extraction versus UV irradiation. Table 3 shows the effect of PQ extraction on various Hill reactions and NADP reduction mediated by the ascorbate-DCIP couple. Some striking differences are evident when these results are compared with those in Figure 5. UV inhibition affects all photoreductions requiring system II equally, while PQ removal (the extraction technique used affected all of the PQ analogs to the same degree; the ratio of PQ A:B:C+D remained essentially constant in the chloroplasts) shows a varying effect depending on the Hill oxidant used in the reaction. Inhibition of cytochrome c reduction (a Hill reaction analogous to NADP reduction but more convenient to measure) is directly proportional to the amount of PQ removed. However, in the case of DCIP reduction, total removal of the PQ results in only a 50% reduction in activity. This suggests that DCIP can accept electrons at more than one site, a hypothesis supported by the kinetic data of Joliot (1965). Surprisingly, NADP reduction with the ascorbate-DCIP couple was also partially inhibited by removal of PQ.

Henninger and Crane (1966) have recently reported similar results with this system and attributed their observations to a requirement for PQ C, but not PQ A in that system. The results of Tables 3 and 4 show that readdition of the total quinone fraction gives considerable stimulation, but readdition of individual PQ analogs gives only a
Table 3. The effects of petroleum ether extraction on various partial reactions of photosynthesis in lyophilized spinach chloroplasts. See Methods and Materials for reaction mixtures and experimental conditions. Control values are as follows (μmoles substrate reduced/mg chlorophyll/hr): DCIP = 43.2, cytochrome c = 28.4 and NADP reduction with the ascorbate-DCIP couple = 7.4.

<table>
<thead>
<tr>
<th>No. extractions</th>
<th>% PQ remaining*</th>
<th>Readditions</th>
<th>% Control activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CYT C</td>
<td>DCIP</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>none</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>31</td>
<td>none</td>
<td>21</td>
</tr>
<tr>
<td>4</td>
<td>&lt;10</td>
<td>none</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>31</td>
<td>total ext.</td>
<td>63</td>
</tr>
<tr>
<td>1</td>
<td>31</td>
<td>PQ</td>
<td>78.5</td>
</tr>
<tr>
<td>4</td>
<td>&lt;10</td>
<td>total ext.</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>&lt;10</td>
<td>PQ</td>
<td>56</td>
</tr>
</tbody>
</table>

*Ratio of PQ A:B:C+D remained constant.

Table 4. The effect on Hill reaction activity of adding individual PQ analogs to extracted spinach chloroplasts. See Methods and Materials for addition procedure and reaction mixtures. Values are expressed as μmoles substrate reduced/mg chlorophyll/hr.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DCIP</td>
</tr>
<tr>
<td>Non-extracted</td>
<td>83.8</td>
</tr>
<tr>
<td>None</td>
<td>24.4</td>
</tr>
<tr>
<td>PQ A</td>
<td>31.6</td>
</tr>
<tr>
<td>PQ B</td>
<td>30.3</td>
</tr>
<tr>
<td>PQ C+D</td>
<td>26.0</td>
</tr>
</tbody>
</table>
slight stimulation, supporting the results of Henninger and Crane. The above observations provide further evidence that PQ (any analog or total) is not the site of UV inhibition of the Hill reaction.

The results also suggest that the UV block occurs before the active site of PQ in the electron transport chain. This follows by considering that total extraction of PQ completely blocks electron flow to NADP or cytochrome c but not to DCIP (Table 3). Therefore, DCIP must be able to accept electrons, although with reduced efficiency, from some component before PQ. UV irradiation totally blocks DCIP reduction, meaning that the UV inhibition must be at or before either point where DCIP can accept electrons in the electron transport chain.

Effects of UV Irradiation on the 520 nm Light-Dark Absorbance Change

Measurement of absorbance changes in vivo as an indication of photosynthetic competence offers many advantages over the more usual chemical or biochemical methods. It is very rapid, can be repeated a large number of times in a short period of time and the system is not changed by the measurements. Also, once the technical problems have been overcome, the method is extremely sensitive; it is possible to measure absorbance changes of 0.001 absorbance units. Another advantage is the short time constant of the
electronic circuits. For the instrument used the time constant was less than ten milliseconds.

There are several reasons why the absorbance change at 520 nm was chosen for study. It is relatively large, which lessens technical problems, and it also occurs in a region of the spectrum where chlorophyll does not absorb strongly, thus simplifying the problem of isolating the measuring and actinic beams. However, the major reason, in terms of this study, is that it has been suggested that this spectral change is due to a PQ-chlorophyll b interaction (Witt et al., 1965). It was thought that this would provide another method to explore the relationship, if any, between PQ and UV inhibition.

Figure 10a shows the typical 520 nm absorbance change in aerobic whole cells, in this case Scenedesmus. In this and all succeeding figures on the 520 change a vertical line beneath the trace will indicate when the actinic beam was turned on and a vertical line above the trace when the actinic beam was turned off. The change is biphasic, consisting of a portion with rapid on-kinetics and slow off-kinetics and a second portion with slower on-kinetics and rapid off-kinetics. This biphasic change appears to be common to all plants capable of photosynthesis, excluding the photosynthetic bacteria. The relative size of each portion and to some extent the kinetics vary among organisms but the general appearance is always the same. Addition of DCMU totally inhibits
Figure 10. Effect of UV irradiation on the 520 change in *Scenedesmus*. Cells were grown autotrophically. 100 μl of cells were suspended in 3.0 ml of nutrient medium for 520 change measurements. See Methods and Materials for details.
the second phase of the change without affecting the first. For convenience, the first (DCMU insensitive) phase of the change will be referred to as the system I portion and the second (DCMU sensitive) phase as the system II portion. This distinction is probably not biochemically valid (see Pratt, 1968, for a more complete discussion of the characteristics of the 520 nm absorbance change).

The system I portion of the 520 change is absent in isolated chloroplasts from either algae or higher plants, although a DCMU insensitive signal can be obtained with the ascorbate-DCIP couple. No added oxidant is required. A typical 520 nm absorbance change of isolated chloroplasts is seen in Figure 12a.

The effect of UV irradiation on the 520 change in Scenedesmus is shown in Figures 10 and 11. As is apparent from Figure 10, the system II portion of the change is considerably more sensitive than the system I portion. Although not evident in Figures 10 or 11, often there is an increase in the size of the system I portion of the change as the system II portion is inhibited. This is probably due to some type of competition between the two phases for the chromophore(s). Much longer periods of irradiation are required to inhibit the system I portion; usually two to three times as much time is required. The differences in the time scales in Figures 10 and 11 are due to different cell concentrations in the sample being irradiated; denser suspensions required longer periods of irradiation.
Figure 11. Long term effect of UV irradiation on the 520 change in *Scenedesmus*. Conditions as in Figure 10.
Table 12. Effect of UV irradiation on the 520 nm change in spinach chloroplasts. Chloroplasts containing 500 μg chlorophyll were suspended in 3.0 ml of sucrose-KCl solution for measurement of 520 nm change. See Methods and Materials for details.
A comparison of the rates of inhibition of the 520 nm absorbance change and photosynthesis indicates that the loss of photosynthetic activity parallels the loss of the system II portion of the signal in *Scenedesmus* (Figure 13). Figure 14 depicts the effect of UV irradiation on the 520 nm absorbance change in *Chlorella*. In this organism both portions of the signal are inhibited at about the same rate, which in turn parallels the loss of photosynthetic activity. The 520 change (system II portion in *Scenedesmus*) thus appears to offer a rapid and quantitative assay for photosynthetic activity after UV irradiation.

As mentioned above, isolated chloroplasts do not show a system I absorbance change. However, the effect of UV irradiation on the system II portion follows the same pattern as in whole cells (Figure 12). Again there is a parallel in the loss of the signal and photosynthetic activity (Hill reaction). After the endogenous absorbance change (system II) has been totally inhibited, a large DCMU insensitive signal can be detected in the presence of the ascorbate-DCIP couple. This appears to correspond directly to the system I portion of the change in whole cells.

Although the experiments described above suggest that UV destruction of PQ cannot be correlated with the inhibition of photosynthesis (hence the system II portion of the 520 change), on the basis of the periods of irradiation required there was some reason to
Figure 13. Comparison of effects of UV irradiation on photosynthesis and the system II 520 change in Scenedesmus. Photosynthesis was measured manometrically with 80 μl of cells suspended in 3.0 ml of Warburg's buffer #9. Control rate was 106 μl O₂ evolved/50 μl cells/hr. The 520 change was measured with 64 μl of cells in 2.0 ml of nutrient medium. See Methods and Materials for details.
Figure 14. Effect of UV irradiation on the 520 change in Chlorella. For 520 change measurements 100 µl of cells were suspended in 3.0 ml of nutrient medium. See Methods and Materials for details.
believe that a correlation might exist between PQ destruction and the inhibition of the system I portion of the signal. For this reason a number of experiments were performed where whole cells were irradiated until the system I portion of the 520 nm change was totally inhibited, and their PQ content examined. Table 5 shows the results of experiments with the green alga Scenedesmus and the blue-green algae Cyanidium and Anacystis. Although considerable variation exists in the PQ determinations, in no case was the degree of destruction of PQ as great as the inhibition of the system I portion of the 520 change. As previously stated for the Hill reaction, it seems reasonable to conclude that destruction of PQ is not the cause of the UV inhibition in either the system I or the system II portion of the 520 nm absorbance change.

In regard to the experiments with the blue-green algae, there is some controversy whether the blue-greens actually possess a true 520 nm absorbance change or not. Although Inselberg and Rosenberg (1964) have reported observing a 520 change in the blue-green alga Porphyridium, Fork (personal communication) feels that the change observed in the neighborhood of 520 nm is not the "true" 520 nm absorbance change. This hypothesis is based on the findings that the blue-green algae do not possess the negative absorbance change at 475 nm. It has been suggested that the absorbance changes at 520 nm and 475 nm are due to the same component, chlorophyll b
<table>
<thead>
<tr>
<th>Organism</th>
<th>% Decrease in PQ</th>
<th>% Decrease in Sys. I 520 change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanidium</td>
<td>58</td>
<td>&gt;95</td>
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<tr>
<td></td>
<td>49</td>
<td>&gt;95</td>
</tr>
<tr>
<td>Scenedesmus</td>
<td>75</td>
<td>93</td>
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<tr>
<td></td>
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<td>93</td>
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<td></td>
<td>72</td>
<td>&gt;90</td>
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<tr>
<td>Anacystis</td>
<td>81</td>
<td>90</td>
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(Rumberg, 1964). Since the blue-green algae do not possess chlorophyll b, it has been concluded that they lack a true 520 change. Although the absence of the negative change at 475 nm in the blue-green algae was not confirmed in this study, the signal observed at 520 nm shows characteristics very similar to those observed in Scenedesmus. These characteristics include (1) biphasic nature, (2) similar kinetics, (3) sensitivity of second portion to DCMU, (4) parallel in the inhibition of the second portion of the change and inhibition of photosynthesis during UV irradiation and (5) the relative insensitivity of the first portion to UV irradiation. Therefore, for the purposes of this thesis it will be assumed that the blue-green algae do indeed possess a "true" 520 nm absorbance change.

Experiments (similar to those summarized in Table 3) comparing the effects of extraction of PQ and UV irradiation on the 520 change in chloroplasts should provide evidence for the possible site of UV inhibition. The results of some experiments of this type are shown in Figures 15 and 16. In these experiments the results are compared to the effects of DCMU because the DCMU-treated system, although inhibited, is still "intact," and the ascorbate-DCIP couple should give a maximal response. From the data in Figure 15 it is apparent that DCMU and UV irradiation have identical effects on the system II part of the 520 nm absorbance change. This is to be expected from the Hill reaction data previously discussed. UV
Figure 15. Comparison of effects of DCMU and UV irradiation on the 520 change in spinach chloroplasts. Experimental conditions as in Figure 12. Additions were as follows: DCMU = 37.5 µmoles, ascorbate-DCIP = 20 µmoles ascorbate and 200 µmoles DCIP.
Figure 16. Comparison of effects of DCMU and PQ extraction on the 520 change in lyophilized spinach chloroplasts. Conditions as in Figure 12.
irradiation does not affect ascorbate-DCIP coupled reduction of NADP and only inhibits the system I portion of the 520 change after long periods of irradiation in whole cells. The effects of extraction of PQ, however, are quite different. Extraction of PQ totally abolishes the system II portion of the change (Figure 16d), in agreement with Witt (Weikard, Müller and Witt, 1963), even though there is only a partial inhibition of the DCIP Hill reaction. The DCMU insensitive, ascorbate-DCIP-mediated absorbance change is also completely inhibited. In Figure 16f the small absorbance change with ascorbate-DCIP is due to incomplete extraction of the PQ. Those chloroplasts also showed a small amount of cytochrome c reduction activity. Readdition of the petroleum ether extract gave a partial restoration of the 520 change, with the degree of restoration similar to the amount of Hill reaction activity recovered. The data suggests that, although both portions of the 520 change require the presence of PQ, the cause of the UV inhibition of the change is not exclusively due to PQ destruction.

Another important question must be raised in regard to the system I portion of the 520 change. Is the absorbance change related to the photoreductive (or oxidative) capacity of photosystem I? Witt's group feels that this change is due to a chlorophyll a reaction (Moraw and Witt, 1961).

In an attempt to answer this question, a suspension of whole
cells of *Scenedesmus* was irradiated until the system I portion of the 520 change had been totally inhibited. Then the cells were broken, the chloroplasts isolated and a measurement made of their ability to reduce NADP in the presence of the ascorbate-DCIP couple. Figure 17 shows the results of such an experiment. The initial increase in the system I portion of the absorbance change has been discussed above. It is apparent that even though this portion of the absorbance change has been abolished, the chloroplasts still retain system I photochemical activity. Thus this part of the 520 change does not appear to be related to electron flow and may be the result of some side pathway. From other lines of evidence Pratt (1968) came to a similar conclusion. It is not known whether the actual chromophore(s) responsible for either or both portions of the absorbance change is located in the system I physical complex. The system II portion of the signal does appear to be directly related to electron flow and can be used as a measure of this flow.

**Effects of UV Irradiation on Other Chloroplast Constituents**

There are many compounds present in chloroplasts other than PQ which absorb at about 250 nm and hence might be involved in the UV inhibition of photosynthesis. Jones and Kok (1966b) reported that irradiation of a heptane extract of lyophilized chloroplasts
Figure 17. Comparison of effects of UV irradiation on the system I S20 change and ascorbate-DCIP mediated reduction of NADP in Scenedesmus. Control value for NADP reduction = 18.3 µmoles/mg chlorophyll/hr. See Methods and Materials for details on reaction mixtures and chloroplast isolation.
caused a large decrease in absorption from 400 to 500 nm which they attributed to destruction of the carotenoids. However, this decrease has not been observed in in vivo studies (Shavit and Avron, 1963). This does not eliminate the possibility that some specific carotenoid, essential for photosynthetic activity and present in low concentration, is destroyed by UV irradiation.

To test this possibility, a sample of isolated spinach chloroplasts was irradiated until no Hill reaction activity remained and its carotenoid content and complement compared to an unirradiated sample. The results of this type of experiment are shown in Figure 18. The thin-layer plates were only examined visually on the assumption that if a specific carotenoid was responsible for the inhibition it would be almost totally destroyed when Hill reaction activity was completely inhibited. No readily observed change was noted in either the content or complement of carotenoids in spinach chloroplasts (Figure 18). Experiments with Swiss chard chloroplasts yielded similar results.

Various other compounds in the lipid class absorb around 250-260 nm. These compounds, if present in PQ samples, can completely obscure the characteristic 255 nm peak of PQ. It has been reported that treatment of chloroplasts with lipase abolishes Hill reaction activity without affecting system I reactions (Okayama, 1964). This effect is very similar to that observed after UV
Figure 18. Comparison of carotenoids from control and irradiated spinach chloroplasts. Plates consisted of silica gel G, Ca(OH)$_2$ and ascorbate. Solvent consisted of petroleum ether: isopropanol: water (100:10:0.25). See Methods and Materials for details of extraction and preparation of plates. Bands were identified as follows: (1) beta carotene, (2) alpha carotene, (3) alpha carotene 5,6-epoxide, (4) hydroxyechinenone, (5) lutein, (6) unidentified, (7) violaxanthin and (8) neoxanthin.
irradiation. For this reason experiments similar to those with the carotenoids were carried out with the lipid fraction from spinach chloroplasts. Results of one such experiment are shown in Figure 19. Again, no apparent decrease in content nor change in complement of the chloroplast lipids can be seen. No attempt was made to identify each spot, but those near the top of the plate consist mostly of galactolipids and those nearer the origin are the phospholipids. The hatched spots represent chlorophyll and various chlorophyll breakdown products.

Chloroplast proteins (particularly those rich in aromatic amino acids) absorb ultraviolet light. Many of the known electron transport chain components are protein in nature (e.g., cytochromes f and b, and plastocyanin), and their destruction or denaturation would certainly be expected to lead to a decrease in photosynthetic activity. The water-soluble proteins of chloroplasts were examined electro- phoretically, and again no changes were observed between irradiated and unirradiated samples.

**Effects of UV Irradiation on Fluorescence**

Fluorescence yield can be used as a measure of the efficiency of electron flow. The other methods employed to measure photosynthetic activity all measure electron flow directly. Fluorescence provides a method where electron flow can be measured indirectly.
Figure 19. Comparison of lipids from control and irradiated spinach chloroplasts. Silica gel G plates were used. Solvent 1 consisted of n-butanol: acetic acid : water (40:25:5) and solvent 2 of chloroform : methanol : water (65:25:1). See Methods and Materials for details on extraction procedure. Spots were detected by spraying with a 0.5% solution of Rhodamine B in ethanol and observing under UV. No attempt was made to identify individual lipids. Hatched spots represent chlorophyll or chlorophyll breakdown products.
Since variable-yield fluorescence competes with electron flow for energy, any treatment which blocks electron flow should increase the fluorescence yield.

Experiments were performed to compare the effects of UV irradiation on photosynthesis and fluorescence in whole cells and on the Hill reaction and fluorescence in isolated chloroplasts. Before describing the results of these experiments a brief description of the characteristics of the fluorescence measurements is appropriate. Figure 20a shows a typical fluorescence pattern in unirradiated spinach chloroplasts. In this and succeeding figures on fluorescence measurements, a vertical line beneath the trace indicates when the 430 nm beam (activating beam) was turned on and when the 430 nm beam was turned off indicated by a vertical line above the trace. An upward pointing arrow above the trace indicates when the 650 nm beam (reducing beam) was turned on and a downward pointing arrow when it was turned off. The time constants of the tuned amplifier are indicated for each Figure.

Several reactions take place upon illumination which are evident in the fluorescence measurements. Light at 430 nm causes an instantaneous increase in the fluorescence level followed by a second slower increase. The initial, rapid part of the fluorescence represents the constant-yield fluorescence emanating from the bulk chlorophyll. This fluorescence
Figure 20. Fluorescence characteristics of spinach chloroplasts. Chloroplasts containing 150 µg of chlorophyll were suspended in 1.0 ml of sucrose-KCl solution. All additions were made to give a total of 1.0 ml. Additions were as follows: DCMU= 1.5 µmoles, CCCP= 2.0 µmoles, Fe(CN)$_6^{3-}$=4 µmoles. See Methods and Materials for details. Time constant = < 10 msec.
remains unchanged by any treatment affecting electron flow. The second slow increase in yield is a portion of the variable-yield fluorescence and indicates the efficiency of the chloroplasts in transferring electrons in photosynthetic reactions. In very active chloroplasts this portion of the variable-yield fluorescence is very small, indicating that the reoxidation of the pools is carried out efficiently in the electron transport chain. The rate limiting step is probably enzymatic rather than photochemical.

Addition of 650 nm light produces another increase with rapid on-kinetics (Figure 20a). It should be remembered that this increase is a change in the 430 nm-activated fluorescence yield induced by the 650 nm beam. The 650 nm beam will also produce fluorescence, but it will not be detected because it does not have the proper frequency. The rationale of this increase in the 430 nm-induced fluorescence is that the 650 nm beam activates system II much more strongly than system I and will, therefore, tend to reduce all of the pools between the two light systems. This reduction means that more of the 430 nm quanta arriving at the trapping center will find it reduced and will be reemitted as fluorescence. When the 650 nm light is turned off, a biphasic decay in the 430 nm fluorescence yield ensues. The first phase is instantaneous but the second is much slower ($t_{1/2}=0.95$ sec). The cause of this biphasic decay is not known.

Addition of DCMU (Figure 20b) blocks electron flow and
provides a measure of the maximum variable fluorescence. Figure 20b illustrates that this is indeed the case. This level should be a relative assessment of the concentration of the emitter. The induction period is greatly reduced, confirming that the DCMU block is near the system II trapping center, and furthermore, that the pools preceding it are small and rapidly reduced. A small 650 nm stimulation occurs, due to incomplete inhibition by DCMU. The decay of this small stimulation does not appear to have the slow portion of the decay. If true, the pool producing this decay must be beyond the site of the DCMU inhibition.

The reasoning that the initial induction as well as the 650 nm stimulation are due to redox states of various pools is substantiated by several other observations. Addition of CCCP, an uncoupler of phosphorylation, which thereby stimulates electron flow, has several effects (Figure 20c). First, it lowers the level of the initial variable-yield fluorescence. This would be expected if it indeed increases electron flow, as it will allow more rapid reoxidation of the pools, thus reducing the probability that a 430 nm quanta will find a trapping center already reduced. A second effect is a great decrease in the rate at which the new equilibrium is reached upon the addition of 650 nm light (Figure 20c). Again this is consistent with the proposed increased electron flow. One interesting point is that the decay kinetics are not affected. It would appear that the decay should also
be increased if there has been an increase in electron flow. It may be that the pool causing this slow decay is not directly involved in electron transport but rather may be a side reaction which is induced by the saturating red light. There are other observations which suggest that this may be an oversimplification, but these are not pertinent to this thesis.

The addition of a Hill reaction oxidant has effects similar to that of CCCP (Figure 20d). The initial induction is greatly reduced, consistent with the idea that an oxidant will increase electron flow over that present with only endogenous oxidants (mainly $O_2$). The magnitude of the 650 nm stimulation is also reduced and the phase of this stimulation with slow decay kinetics appears to be completely abolished. This is apparent with either ferricyanide or DNP as oxidant (the DNP was originally used as a phosphorylation uncoupler, but its ability to act as a Hill oxidant completely overrode any uncoupling effects). A reasonable interpretation is that the oxidant accepts electrons at some point before the pool producing the slow kinetics, thereby bypassing the reduction of this pool.

At no time were any multiple induction effects of the type reported by Malkin and Kok (1966) observed in isolated chloroplasts.

Whole cells of *Scenedesmus* gave results similar to those with chloroplasts (Figure 21). It was found that the condition of the culture had profound effects on the various treatments. If the culture was
Figure 21. Fluorescence characteristics of heterotrophic *Scenedesmus* cells. Measurements were made with 12.5 µl of cells in 1.0 ml of nutrient medium. Additions were made to give a total volume of 1.0 ml. Additions were as follows: DCMU = 2.5 µmoles, CCCP = 2.0 µmoles. See Methods and Materials for details. Time constant = <10 msec.
quite young the cells were very active and induction effects were at
a minimum. In these cells the intensity of the 650 nm beam produced
effects very similar to those observed in chloroplasts. In older cul-
tures the photosynthetic capacity had decreased, as indicated by the
increased induction level, and the 650 nm beam reduced the system
to the extent that the variable-yield fluorescence level reached that
obtained with the addition of DCMU. Qualitatively, however, there
did not appear to be any major differences.

On the basis of the observations discussed above, it was as-
sumed that treatment of chloroplasts or whole cells with UV irradi-
ation would produce an increase in the variable-yield fluorescence.
It was also expected that this increase would parallel the decrease
in Hill reaction or photosynthetic activity: as electron flow became
inhibited, the fluorescence yield should increase. However, rather
than increase, the variable-yield fluorescence decreased and at a
rate equal to that of the decrease in Hill reaction activity in chloro-
plasts or photosynthesis in whole cells (Figure 22). The base level
fluorescence was also decreased, particularly in whole cells, but
to a much smaller degree. The decay in both Hill reaction activity
and variable fluorescence in chloroplasts appears to be largely first
order.

Not only did the initial variable-yield fluorescence decrease
with increasing time of UV irradiation, but the total variable-yield
Figure 22. Effect of UV irradiation on fluorescence characteristics of spinach chloroplasts. Measurements made with chloroplasts containing 170 µg chlorophyll in 1.0 ml of sucrose-KCl solution. See Methods and Materials for details.
fluorescence attainable (in the presence of DCMU) also decreased at the same rate as Hill reaction activity (Figure 23). Kok et al. (1967) reported that at 77°K the fluorescence yield of UV treated chloroplasts did not rise above the initial value.

A similar decrease in fluorescence after treatment of chloroplasts with lipase has been reported (Okayama, 1964). Okayama measured fluorescence at 685 nm and observed that the fluorescence level dropped to about 50% of the initial value after digestion with the enzyme. Hill reaction activity was completely abolished after such treatment. However, system I reactions (cytochrome c oxidation or reduction of NADP with the ascorbate-DCIP couple) were not appreciably affected. These results are remarkably similar to those obtained after UV irradiation. Okayama concluded that lipase digestion converts a highly fluorescent form of chlorophyll a, which participates in oxygen evolution, into an inactive, non- or weakly fluorescent form, thereby destroying the oxygen evolving system.

Experiments with Scenedesmus were not as conclusive. Many fluorescence experiments were performed with the single beam spectrophotometer or the phased fluorescence apparatus. In some experiments a definite biphasic decrease in the total variable-yield fluorescence was noted, but the reproducibility was not good. In the best experiments the two phases appeared to be correlated with photosynthetic activity and the system I 520 nm absorbance change.
Figure 23. Effect of UV irradiation on maximal variable fluorescence of spinach chloroplasts. Control value of DCIP reduction = 28.6 µmoles/mg chlorophyll/hr. See Methods and Materials for experimental conditions.
These relationships are shown in Figures 24 and 25. Lack of reproducibility was a constant problem with whole cell systems. Several factors were probably involved. Whole cells are much more heterogeneous in size and shape than isolated chloroplasts, meaning that scattering characteristics will vary with the age of the culture and with time in a given sample due to settling of the cells. Filtering the cell suspension to remove the largest cells and to make the suspension somewhat more homogeneous helped considerably, as did keeping the volume of suspending liquid as small as possible.

All of the above experiments were performed with the assumption that the variable-yield fluorescence at 686 nm is due solely to system II. To test this assumption measurements were made of the fluorescence characteristics of chloroplastin (see Methods and Materials). Chloroplastin shows no system II photochemical activity but reduces NADP with the ascorbate-DCIP couple. As expected, only a base level fluorescence was evident. There was no stimulation either by 650 nm light or with DCMU.

The most logical interpretation of this fluorescence data is that the emitter itself is either destroyed or completely uncoupled from the system. Another possibility is that, even though photosynthetic electron transport is blocked, UV irradiation increases the efficiency of quenching of the fluorescence. The first hypothesis would appear to be the most reasonable on the basis of simplicity; it is easier to
Figure 24. Effect of UV irradiation on variable fluorescence of *Scenedesmus*. Cells were grown heterotrophically. For fluorescence measurements 12 µl of cells were suspended in 1.0 ml of growth medium, and fluorescence measured at 686 nm. Photosynthesis was measured on an Oxygraph with 25 µl of cells suspended in 1.5 ml of Warburg's buffer #9. See Methods and Materials for details. Closed circles indicate biphasic decay. Open circles represent only the initial phase of the decay.
Figure 25. Comparison of effects of UV irradiation on the system I 520 change and the second phase of variable fluorescence in Scenedesmus. For 520 change measurements 75 µl of cells were suspended in 3.0 ml of nutrient medium. For fluorescence measurements 25 µl were suspended in 1.5 ml of nutrient medium. Cells were grown heterotrophically.
consider that one process is accounting for all of the observed results rather than two or more essentially unrelated processes proceeding at the same rate.

Assuming that the reaction involved is a destruction of the fluorescence emitter, several qualifying statements are also required to explain all of the data. The fluorescence emitter must consist of a physical complex which includes several members of the electron transport chain. This must be the case to account for the observed inhibition of both photoreduction in adapted algae and cyclic photophosphorylation. Neither of these reactions should require the trapping center of system II. Moreover, the site where electrons enter the chain in these two reactions must be at least one component removed from the trapping center. This follows from a consideration of DCMU inhibition. Fluorescence yield in the presence of DCMU shows a definite, though small, lag before reaching a maximum. If DCMU acted between the trapping center and the first electron acceptor, the fluorescence rise curve should be instantaneous, because any quenching would be blocked. The existence of a lag indicates that there is a pool of oxidized quencher which can accept electrons from the trapping center until the pool becomes completely reduced. The length of the lag period should be an indication of the concentration of this oxidized quencher(s). DCMU, however, has no effect on either photoreduction or cyclic photophosphorylation,
indicating that the point where electrons enter the chain in these processes is beyond the site of DCMU inhibition. UV inhibition of these two reactions shows that the component which accepts their electrons must also be in the system II "unit". Therefore, these observations require that at least three components make up the system II unit; the trapping center (fluorescence emitter), the initial electron acceptor (Q) and at least the first acceptor after the site of DCMU inhibition. There may also be several other entities on the oxidizing (O₂ evolving) side of system II which should be included.

With the exception of a small decrease in the PQ level, there was no indication of destruction of any specific compound. This, plus the findings that a relatively non-specific lipase destroyed system II activity, lends support to the notion that system II is a strict physical unit and UV irradiation acts by disrupting the structure of this unit. This hypothesis is shown in schematic form in Figure 26. The physical size and nature of this system II unit could not be determined from these experiments.

Preliminary experiments examining the lamellar structure of Scenedesmus cells before and after UV irradiation with the electron microscope show a possible breakdown in the lamellar integrity. Much more extensive work is required before this structural breakdown, if real, can be correlated with loss of photosynthetic activity.
Figure 26. Scheme showing system II complex which is destroyed by UV irradiation. Solid line indicates probable extent of unit. Dashed line indicates unknown component(s) which may also be an integral part of the complex.
CONCLUSION

This study of the effects of UV irradiation on the photosynthetic apparatus has provided information which allows certain conclusions to be reached with respect to the following subjects: (1) the role of PQ in UV inhibition of photosynthesis, (2) the nature and cofactor requirements of the 520 nm absorbance change and (3) the extent and composition of a system II complex.

The Role of PQ in UV Inhibition of Photosynthesis

Several reports have indicated that the PQ content of isolated chloroplasts declines at a rate parallel to the decrease in photosynthetic activity (Bishop, 1961; Shavit and Avron, 1963; Trebst and Pistorius, 1965). In the studies reported here, it was shown that although an apparent parallelism does occur in isolated chloroplasts, in whole cell systems of various algae the PQ content decreases much more slowly than photosynthetic activity. Moreover, experiments comparing the effects of PQ extraction versus UV irradiation in chloroplasts demonstrated that removal of PQ produces results quite different from UV irradiation. The data indicated that the site of UV inhibition must be before the site of PQ in the electron transport chain. This conclusion is based, in part, on the observation that UV irradiation totally blocks the reduction of DCIP with
water as electron donor, but extraction of the PQ only partially abolishes this reduction. Therefore, DCIP must be capable of accepting electrons, although at reduced efficiency, from some component before PQ in the electron transport chain and that the UV block must be before this site. It has been suggested that PQ is the initial electron acceptor for system II (Amesz, 1964; Witt, Müller and Rumberg, 1963) but the above results indicate that there are probably one or more components before PQ in the electron transport chain. It can be concluded from these observations that PQ is not the site of UV inhibition of photosynthesis and it is probably not the initial electron acceptor for system II.

The 520 nm Light-Dark Absorbance Change

There is a great deal of controversy concerning the nature and components involved in the 520 nm absorbance change. The studies reported in this thesis allow certain conclusions to be drawn regarding the components involved and to some extent the nature of the change. Reports from Witt's laboratory have suggested that at least part of the signal is due to interaction of PQ and chlorophyll b (Witt et al., 1965). Experimental evidence presented here tends to confirm this hypothesis, insofar as the requirements for PQ is concerned. It was found that extraction of the PQ abolishes both portions of the signal and that readdition of PQ restores activity. No
firm conclusion can be drawn concerning the requirement of the system II portion of the 520 change for PQ. Pratt (1968) has suggested that this portion requires system II activity only as a source of electrons. Extraction experiments indicated that PQ removal blocks electron transport to system I. It cannot be determined from these experiments whether the loss of the system II signal is due to a specific requirement for PQ or whether it is due to blockage of electron flow. The system I change is somewhat more clear cut. Extraction of PQ partially restricts electron flow from the ascorbate-DCIP couple through photosystem I but such extraction totally abolishes the system I portion of the absorbance change. This suggests that the system I signal requires the presence of PQ, even though the PQ is not required for electron flow through system I. Experiments comparing the rate of inhibition of the system I change with the decrease in PQ content during UV irradiation show that the inhibition of the signal does not parallel the rate of PQ destruction. Thus, although the system I 520 change requires PQ, and the PQ is partially destroyed by UV irradiation, the inhibition of the signal is not completely due to the loss of PQ.

The results also indicate that the system I 520 change is not directly coupled to electron flow through photosystem I. The type of data obtained does not allow speculation as to whether the change is a photooxidation or a photoreduction, but in isolated chloroplasts the
ascorbate-DCIP couple is required for the system I change. This is difficult to interpret because there is no apparent system I change when normal electron flow from system II is available. In this case only the system II portion is evident. Addition of the ascorbate-DCIP couple greatly increases the size of the endogenous signal without producing the typical biphasic change characteristic of whole cells. It is not clear why the couple is needed to elicit this signal in chloroplasts.

**The System II Complex**

The results of many types of experiments on the effects of UV irradiation on various partial reactions of photosynthesis all suggested that the inhibition was a specific one, probably affecting a single component of the electron transport chain. With the exception of PQ, which was discussed earlier, no decrease in any compound could be detected. Lipids, carotenoids, chlorophyll and chloroplast proteins were examined, with negative results. However, simply because a component is not chemically destroyed by the UV treatment does not eliminate it as a possible site for the inhibition. The functional photosynthetic apparatus might well be classified as a solid-state system with the physical integrity of the components in relation to one another being of crucial importance for efficient operation of the reaction sequence. Relatively minor changes in this structure could have
profound effects on photochemical activity.

Examination of the fluorescence characteristics of chloroplasts and whole cells revealed that the probable cause of the UV inhibition was a physical breakdown of some structural unit associated with system II. The variable-yield fluorescence competes with the photochemistry for energy arriving at the trapping center and any treatment which inhibits electron flow should increase the variable fluorescence. This is indeed the case when inhibitors such as DCMU are added to chloroplast or whole cell systems. In the presence of DCMU electron flow is completely blocked and the variable-yield fluorescence approaches a maximum. At any given actinic light intensity, this maximum fluorescence should be a relative measure of the concentration of the system II trapping center.

UV irradiation of chloroplasts or whole cells was expected to produce an increase in the variable-yield fluorescence which would parallel the decrease in Hill reaction activity. Experiments showed, however, that rather than increase, the fluorescence yield decreased, as also did the maximum attainable yield. Apparently, irradiation causes a decline in the concentration of the system II trapping center. It is assumed that this trapping center involves some chlorophyll molecules and, furthermore, it is known that UV does not affect the structure of the chlorophyll (Arnold, 1933). A reasonable conclusion is that the trapping center is a physical complex which includes a
specially-bound chlorophyll and (presumably) lipoprotein and that UV irradiation disrupts this complex, blocking both electron flow and fluorescence. Consideration of other data requires the inclusion of additional components in this complex.

The complex must consist of the trapping center and at least two other electron transport components. This follows because numerous reactions which do not require system II photochemical activity (photoreduction and cyclic photophosphorylation) are inhibited to the same degree. Since DCMU does not inhibit these reactions, the site of its action must be before the component where electrons from photoreduction and cyclic photophosphorylation can enter the pathway. However, because the fluorescence yield, in the presence of DCMU, does not reach a maximum instantaneously, a pool of oxidized acceptor must exist between the DCMU site and the trapping center. The lag time before the maximum level of fluorescence is reached is small, indicating that the pool, or pools, are small and become reduced rapidly.

Since the inactivation of the many partial reactions proceeds at nearly identical rates, they all must be dependent upon the same complex. In the simplest case, the system II complex must consist of the trapping center, one electron transport component which accepts electrons from the trapping center and a second component which can accept electrons from either hydrogen gas in photoreduction.
or from PMS in cyclic photophosphorylation. DCMU blocks between these two components. This scheme is illustrated in Figure 26.

There also may be several other factors involved but the data are not sufficient to distinguish any more than the three components listed above. For example, the number and type of components on the oxidizing side of the trapping center is completely obscure.

It has been postulated that system I consists of several electron transport components also bound in a strict unit (Pratt, 1968). This hypothesis was based on experiments done with mutations of the green alga, Scenedesmus. From the data in this thesis it is concluded that system II is also a strict physical unit which is specifically disrupted by UV irradiation. It is not known why the system I unit is not similarly affected. Certainly the reactions catalyzed by this system do not appear to be so strongly structurally dependent as the system II type reactions.
BIBLIOGRAPHY


