MECHANISM OF OXYGEN EVOLUTION IN PHOTOSYNTHESIS

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INTRODUCTION

The evolution of oxygen results from a combination of photochemical and enzymatic reactions in the chloroplast of green cells. Concerning the photochemistry of the primary photochemical act, Livingston has said, "Physiologists and biochemists appear to believe that this question was answered long ago by physicists, while physicists find the problem distressingly complicated and therefore uninteresting" (ref. 1, p. 832). I would like to take this occasion to plead equal ignorance on behalf of the biochemists regarding the oxygen evolution system.

Although a broad outline of the problem can be given, and one can talk about the mechanism in general terms, many details of this problem still remain to be supplied. It is paradoxical that the first aspect of photosynthesis to be recognized was oxygen evolution, but this phase of the photosynthetic process is more poorly defined today than the other major related processes in the chloroplast. We know the oxygen most probably comes from water, that manganese ion is required, and that the reaction is driven by the light-activated pigment system containing chlorophyll and other light-absorbing molecules. The nature of the enzymes involved, and the intermediates produced are a mystery. The current interest in the oxygen-evolution problem, however, coupled with the techniques currently available promise that many of the unknown details concerning this process will soon become known.

To the English minister, Joseph Priestly, goes the credit for the discovery of oxygen evolution by green plants. His bioassay for oxygen has not found favor with workers in the field today, but it was appropriate and adequate for the task then at hand. The ability of a mouse to live in a closed system with air made "putrid" or "obnoxious" by burning candles or exhalations of animals was shown to be dependent upon the presence of plants. He also discovered bubbles of "dephlogisticated" air (oxygen) which were formed

by algae which had contaminated his water containers.

The requirement for light was clearly documented by Ingen-Housz, who correlated the ability of plants to purify bad air with the periods of sunlight. The subsequent work of Senebier on "fixed air", the designation of "fixed air" as carbonic acid, the inclusion of water as one of the reactants due to the quantitative study of de Saussure, and the fundamental concept advanced by Mayer that light energy was changed into chemical energy allowed the formulation of the photosynthetic process in the following terms:

$$(1)\quad CO_2 + H_2O + \text{light} \longrightarrow \text{organic matter} + O_2 + \text{chemical energy}$$

For a more detailed presentation of the historical development, the following references may be consulted (ref. 2, 3). In a general sense, equation 1 is still considered valid, since it does describe the gross changes occurring during photosynthesis. Subsequent workers in the field have been engaged in filling in the innumerable details associated with the conversions

$$(2a)\quad CO_2 \longrightarrow \text{organic matter}$$

$$(2b)\quad H_2O \longrightarrow O_2$$

$$(2c)\quad \text{light} \longrightarrow \text{chemical energy}$$

I shall consider the conversion of water into oxygen, both as a unique physiological process and as one of the many coordinated biochemical processes which are driven by the photochemical apparatus in the chloroplast.

LIST OF ABBREVIATIONS

DPN, DPNH	Oxidized and reduced diphosphopyridine nucleotide
TPN, TPNH	Oxidized and reduced triphosphopyridine nucleotide
ADP, ATP	Adenosine diphosphate and adenosine triphosphate
DPIP, $DPIPH_2$	Oxidized and reduced 2, 6-dichlorophenolindophenol
TPIP, $TPIPH_2$	Oxidized and reduced 2, 3, 6-trichlorophenolindophenol
FMN	Flavin Mononucleotide
CMU	p-chlorophenyl-1, 1-dimethylurea
DCMU	3-(3, 4-dichlorophenyl)-1, 1-dimethylurea
ESR	Electron spin resonance
PPNR	Photosynthetic pyridine nucleotide reductase

PLANT PHOTOSYNTHESIS: GENERAL ASPECTS

Oxygen Evolution as an Expression of the Photochemically Generated Oxidizing Potential of Chloroplasts

Two oxygen-containing compounds enter into the photosynthetic process, carbon dioxide and water, and either could be considered as a possible source for the molecular oxygen produced. To the early investigators, it appeared obvious that oxygen was produced from carbon dioxide, while water was either neglected or considered only as a general nutrient. In 1864, Berthelot suggested that water was decomposed into oxygen and hydrogen, with the hydrogen then reducing carbon monoxide which had been formed from carbon dioxide. On the strength of the stoichiometry observed between oxygen evolution and carbon dioxide uptake, Willstätter and Stoll (ref. 4) in 1918 rejected the idea that water was the source of the oxygen, and retained the old idea of a decomposition of carbon dioxide as a source of oxygen.

With the development of the concept that biological oxidations take place via hydrogen transfer between compounds, the idea that carbon dioxide was the source of oxygen became less acceptable. In 1931 the first formulation of photosynthesis as a definite hydrogen transfer reaction involving water as the primary compound was published by van Niel (ref. 5). Drawing upon his wide experience with the metabolism of sulfur bacteria which live from the energy gained by transferring hydrogen from hydrogen sulfide to oxygen, van Niel postulated the same sort of hydrogen transfer from water in plant photosynthesis. The two situations can be represented by the following equations:

$$(3)\quad H_2S \longrightarrow 2H+S$$

$$(4)\quad H_2O \longrightarrow 2H+1/2O_2$$

The really unique feature of photosynthesis, then, would be the way in which water serves as a hydrogen donor.

In 1937 it was shown by Hill (ref. 6) that isolated chloroplasts produced oxygen when illuminated, resulting in a coupled reaction in which no carbon dioxide, but added electron acceptors such as ferric oxalate were reduced. This made untenable the idea that oxygen came from carbon dioxide.

Evidence that water was the source of photosynthetic oxygen was obtained through the use of water enriched with the O^{18} isotope. The experiments of Ruben et al. (ref. 7) and later Vinogradov and Teis (ref. 8) demonstrated that oxygen evolved during photosynthesis in such enriched water had an isotope ratio similar to the water in the medium. The original

investigations were open to the criticism that they failed to consider the possibility of exchange reactions inside the cell involving the oxygen of water and carbon dioxide. Subsequent experiments performed with isotopic components in equilibrium proportions yielded the same results, however. Brown and Frenkel (ref. 9) have critically evaluated this aspect in their review, and they concluded that absolutely definitive experiments have not been accomplished, since the published results all indicate a considerable portion of the evolved oxygen could derive from carbon dioxide. They point out, however, that they "do not wish to suggest an alternate concept but want only to emphasize that the experimental evidence from tracer oxygen experiments in several different laboratories not only shows wide disagreement but, on several counts, it is simply inadequate to support or deny the widely accepted theory of water as the sole source of photosynthetic oxygen."

In terms of the overall stoichiometry of the photosynthesis reaction, it is logical that the molecular oxygen must ultimately come from water. However, a number of oxygen-containing compounds could well be intermediates in the reaction, and one such compound is the phosphate ion. Roux et al. performed experiments with tobacco leaves in a medium containing O^{18}-labeled phosphate and measured the isotope abundance in the oxygen produced and in the residual "leaf water" (ref. 10). The evolved oxygen had a higher ratio of O^{18} to O^{16} than did the "leaf water", which led them to advance the idea that phosphate was involved in some fashion, and was functioning closer to the terminal oxygen evolution step than was water.

Opposite results have been obtained by Engelsma, who performed experiments with both Chlorella cells and spinach chloroplasts (ref. 11). In neither case was the O^{18} concentration in the evolved gas higher than that found in ordinary oxygen or in air, indicating phosphate was not involved in oxygen evolution. However, information concerning a possible exchange of oxygen between the phosphate and water (thus diluting out the isotope) was not available, so these data should not be taken as conclusive.

Oxygen Evolution by Isolated Chloroplasts: The Hill Reaction

Once oxygen had been identified with photosynthesis, efforts were made to see if the "chlorophyll-body", or chloroplast could perform this function outisde the living cell. In 1881 Engelmann succeeded in isolating chloroplasts, and looked for oxygen evolution from these bodies utilizing an extremely sensitive microscopic method based upon the chemotactic response of motile bacteria to oxygen under anaerobic conditions (ref. 12). Haberlandt in 1887 (ref. 13) and Ewart in 1896 (ref. 14) also demonstrated an evolution of oxygen from isolated chloroplasts, thus leading to acceptance of the idea that the chloroplast was the locus of the photosynthetic process. This idea has held to the present time, as far as the oxygen evolving system is concerned. Due to difficulties in experimental technique, the idea that the chloroplast was also the site of carbon dioxide fixation was subsequently rejected, only to be revived in recent years through the work of Arnon and co-workers (ref. 3).

The early measurements of oxygen evolution by chloroplasts required very sensitive measuring methods. Beijerinck (ref 15) in 1901 used luminous bacteria as an oxygen detecting device, and measured oxygen evolved from a cell-free extract of clover leaves. Molish (ref. 16) used the same method in an extended investigation and concluded that photosynthesis, like fermentation, could proceed outside the living cell.

The real cornerstone for the cell-free study of oxygen evolution was laid by Hill in 1937, who employed his knowledge of the hemoglobin-oxyhemoglobin system to devise a spectrophotometric method for oxygen detection based upon the formation of oxyhemoglobin (ref. 6). Using this procedure he was able to repeat the previous experiments on oxygen evolution by chloroplasts, and made the most significant observation that this oxygen evolution could be coupled to the reduction of some added electron acceptor (he used ferric potassium oxalate and showed a reduction of the iron). This showed that oxygen evolution could be divorced from carbon dioxide fixation and demonstrated that the latter was a secondary effect, caused by some reducing power generated in the light by the chloroplast. This allowed the oxygen evolution system to be routinely studied with the chloroplast, and opened up the modern era wherein the oxidation-reduction reactions catalyzed by chloroplasts can be studied experimentally. Hill suggested (ref. 17) and Spikes later demonstrated (ref. 18) that with potassium ferricyanide as the oxidant, the stoichiometry of the reaction was as follows:

$$(5)\quad 4K_3Fe(CN)_6 + 4K^+ + 2H_2O \longrightarrow 4K_4Fe(CN)_6 + 4H^+ + O_2$$

The Hill reaction has been demonstrated with many different oxidants. The reduction of these Hill reagents is coupled in each case to oxygen evolution, and they all act as electron acceptors for the electron (hydrogen) removed from water by the photochemical act. Accordingly, the use of different Hill oxidants is merely a variation upon the same general theme, and more attention cannot be given to them in this discussion. For additional information the articles by Holt and French (ref. 19) and Clendenning (ref. 20) may be consulted. The following have been shown to function as Hill oxidants: ferricyanide (ref. 21, 18), p-benzoquinone (ref. 22), other quinones (ref. 23), 2, 6-dichlorophenolindophenol (ref. 24), chromate (ref. 19), nitrite (ref. 22), indigo carmine (ref. 26) and other dyes. In addition, triphosphopyridine nucleotide (TPN) can be reduced in a similar system if a specific chloroplast enzyme, photosynthetic pyridine nucleotide reductase (PPNR), is added to the system (ref. 27).

Requirement for Structured Chloroplast

Considerable effort has been expended trying to correlate the photochemical properties of chlorophyll with the role it must play in catalyzing oxidation-reduction reactions in the chloroplast. A large body of information

has accumulated on the photochemistry of the chlorophylls (ref. 1, 2, 28), and it is well known that they have the ability to catalyze oxidation-reduction reactions when dissolved in organic solvents, when present as colloidal dispersions or in conjunction with detergents or lipoidal proteins in aqueous solution (ref. 28). Using ascorbate or phenylhydrazine as an electron donor, the photoreduction of dyes, flavins and pyridine nucleotides by chlorophyll (and other porphyrins also) can be accomplished (ref. 1, 28, 29). Depending upon the conditions, chlorophyll solutions can catalyze either photooxidations (in the presence of oxygen) or photoreductions (in the presence of ascorbate, phenylhydrazine, etc.) Aqueous, colloidal suspensions of either chlorophyll a, chlorophyll b, or the corresponding pheophytins (as well as hematoporphyrin) photoreduce pyridine nucleotides when ascorbate is present as electron donor under anaerobic conditions and when the enzyme PPNR is present to catalyze the reaction (ref. 29). This reaction demonstrates that chlorophyll can catalyze a photoreduction of pyridine nucleotides (required for carbon dioxide fixation in the whole cell) and is a case where chlorophyll catalyzes a photochemical oxidation-reduction reaction with a storage of energy. It could represent one of the two postulated photochemical reactions which are discussed below, but would in no wise be a model for the photoreaction leading to oxygen evolution.

All attempts to produce an artificial chlorophyll system for the photoreduction of carbon dioxide and simultaneous oxygen production have failed, and Livingston has stated (ref. 1, p. 830), "It is most improbable that a homogeneous solution will ever be devised which will be capable, with sensible efficiency, of using visible light to bring about the reduction of carbon dioxide by water, eliminating oxygen. For such a process to succeed, either the energy of four or more photons must be accumulated by a sensitizer molecule or else a series of fairly stable reaction intermediates must be formed..... Natural photosynthesis undoubtedly involves the formation and reactions of a series of intermediates, but occurs in a spatially organized system containing a number of enzymes and carriers."

The chlorophyll in plant cells is contained in the chloroplast in a definitely organized state (ref. 30). The pigment is restricted to lamellae, which in general extend throughout the chloroplasts of algae, but in higher plants the pigments are usually confined to circular regions in the stroma lamellae. A collection of these regions, located one on top of another, is known as a granum. Such organization could facilitate the photosynthetic process by allowing the chlorophyll and other pigment molecules to form a condensed system with physical properties different from those of the individual molecules and essential to the initial photochemical act. One possible property acquired by such systems is the presence of conduction bands in the condensed system, allowing electron flow between pigment molecules (ref. 31). Another benefit resulting from such organization could be a spatial separation of the oxidizing and reducing sites of chloroplasts, thus preventing back reaction between the oxidized and reduced products.

The requirement for an organized enzyme system to accomplish oxygen evolution should also be considered. Looking at equations 6 - 9

given below, which assume a liberation of oxygen from some oxidized product of water at the oxidation level of a peroxide, and assuming one hydrogen transfer per quantum in the initial photochemical act, it is incumbent upon the chloroplast system to channel the action of at least four light quanta into the evolution of one molecule of oxygen. This requires the formation of stable chemical intermediates, which is accomplished by the enzymes (as yet unknown) in the organized chloroplast.

One becomes accustomed to thinking of photosynthesis, and particularly oxygen evolution, as taking place only in the organized chloroplast. However, Wolken has reported that digitonin extracts of Euglena chloroplasts retained three of the activities associated with photosynthesis, reduction of a dye, liberation of oxygen and fixation of inorganic into organic phosphate (ref. 32). Further work on this very interesting system, which Wolken calls "chloroplastin", is required to determine how far the original structured elements of the chloroplast are broken down by this treatment.

A Scheme for Plant Photosynthesis

Evidence is accumulating that there are two separate photochemical acts involved in plant photosynthesis, involving different pigment systems and different but coupled photoreactions. This will be discussed below, but is mentioned here only to emphasize that the photochemistry of chlorophyll a may not be involved in the oxygen-producing system at all. Regardless of one or two photochemical acts, the following general statements may be made concerning the function of the light-absorbing pigment system in photosynthesis. (1) Absorption of a light quantum by a pigment molecule produces an excited molecule containing the additional energy of the quantum. Such molecules can either give up electrons or accept electrons, and thus may catalyze either oxidations or reductions. (2) Regardless of which reaction takes place first (it is most likely the former), the net result of the formation of the excited pigment molecule is a transfer of electrons from a donor molecule through the pigment molecule (or molecules) to an acceptor molecule which has a lower oxidation potential compared with the donor. Thus the energy contained in the quantum is converted into chemical energy. (3) The initial donor and acceptor molecules must be insulated chemically or physically from each other to prevent immediate back reaction, in order that the system may realize a storage of chemical energy. (4) Although it may or may not be the direct acceptor molecule, TPN eventually accepts the electron (hydrogen), thus becoming reduced and capable of functioning in carbon dioxide reduction. (5) Although water may or may not be the direct electron donor, it is the ultimate donor molecule, and some oxidized product of water produces the oxygen evolved in photosynthesis. (6) In principle this could be accomplished by one photochemical act (one pigment system with associated donors and acceptor molecules) or by two separate photochemical acts. If two such acts are involved, they must be coupled by some common intermediate(s) to explain the strict stoichiometry observed between carbon dioxide fixation and oxygen evolution.

(7) Since the immediate biochemical expression of photosynthesis is electron transfer between the donor and acceptor molecules, the possibility exists of accompanying these electron transfers with ATP formation. Any electron transfer reaction proceeding in the dark and liberating sufficient energy could couple to ATP formation if the necessary enzymes are present. One logical sequence of electron transfer reactions producing ATP is the back-flow of the electrons from the initially reduced molecule to the initially oxidized molecule.

Drawing diagrams to represent the photosynthetic process is currently popular, and Fig. 1 can be added to the pile. Box 1 is intended to represent the light-absorbing unit which is composed of pigment molecules (chlorophylls and other pigments) and serves to funnel light excitation energy to reaction sites X and Y. The dashed lines represent electrons being transferred by the pigment system as a result of excitation. In this representation only one light-absorbing system is considered. There is ample evidence to show that this is an oversimplification, since plant photosynthesis involves two separate but coupled light reactions, which will be discussed below. In terms of overall mechanism, however, the one-light concept is sufficient to present the significant facts.

Box 2 represents the initial biochemical oxidizing and reducing moieties, YOH and XH, respectively. The nature of Y and X is not known. X could be a chlorophyll molecule itself, and Y has been represented as a cytochrome by Arnon (ref. 33) in his earlier work. However, it is difficult to visualize a cytochrome functioning in a normal manner operating at this point in the scheme. One reason for including cytochromes in the plant system, as Arnon has done, is the fact that plant cytochromes become oxidized when light is turned on. However, when one considers the evidence pointing toward two separate light reactions in plants, the cytochrome function is more logically placed on the light reaction not involved in oxygen evolution. This will be discussed in more detail below. The XH and YOH generated in the manner depicted can then be utilized in CO_2 fixation or oxygen evolution respectively, or can back react in a controlled fashion to produce ATP via the process of cyclic photophosphorylation. Box 3 represents the reactions taking place during the Hill reaction, where A represents any of the Hill reagents listed above. In this reaction the CO_2 fixing system is inoperative.

In the literature on photosynthesis the oxidizing and reducing equivalents produced photochemically have been represented using the photolysis of water as the primary photochemical event:

$$(6)\quad 4X + 4Y + 4H_2O + \text{Light} \longrightarrow 4XH + 4YOH$$

$$(7)\quad 4XH + CO_2 \longrightarrow 4X + CH_2O + H_2O$$

$$(8)\quad 4XH + A \longrightarrow 4X + 2AH_2 \quad \text{(Hill Reaction)}$$

$$(9)\quad 4OH \longrightarrow O_2 + 2H_2O$$

The YOH and XH in these equations are those represented in Fig. 1. The main advantage of a diagram such as Fig. 1 is the emphasis placed upon the primary photochemical act, which initiates all the subsequent dark reactions by driving electrons against a chemical energy gradient to form stable intermediates which can subsequently react in a series of biochemical oxidation-reduction reactions to produce the reactions commonly associated with photosynthesis, carbon dioxide fixation and oxygen evolution.

It should be pointed out that the two terms, "hydrogen transfer" and "electron transfer" are both used in biochemical literature to describe biological oxidation and reduction reactions. Even though the elements of a hydrogen atom may be transferred in a reaction, this could still take place as separate transfers of the electron and proton. In the present discussion the term "electron transfer" will be used predominantly. When this applies to chemical reactions resulting from the initial photochemical act, the electron transfer will be accompanied by a net (not direct) proton (hydrogen ion) transfer to form the final reduced products such as TPNH, etc.

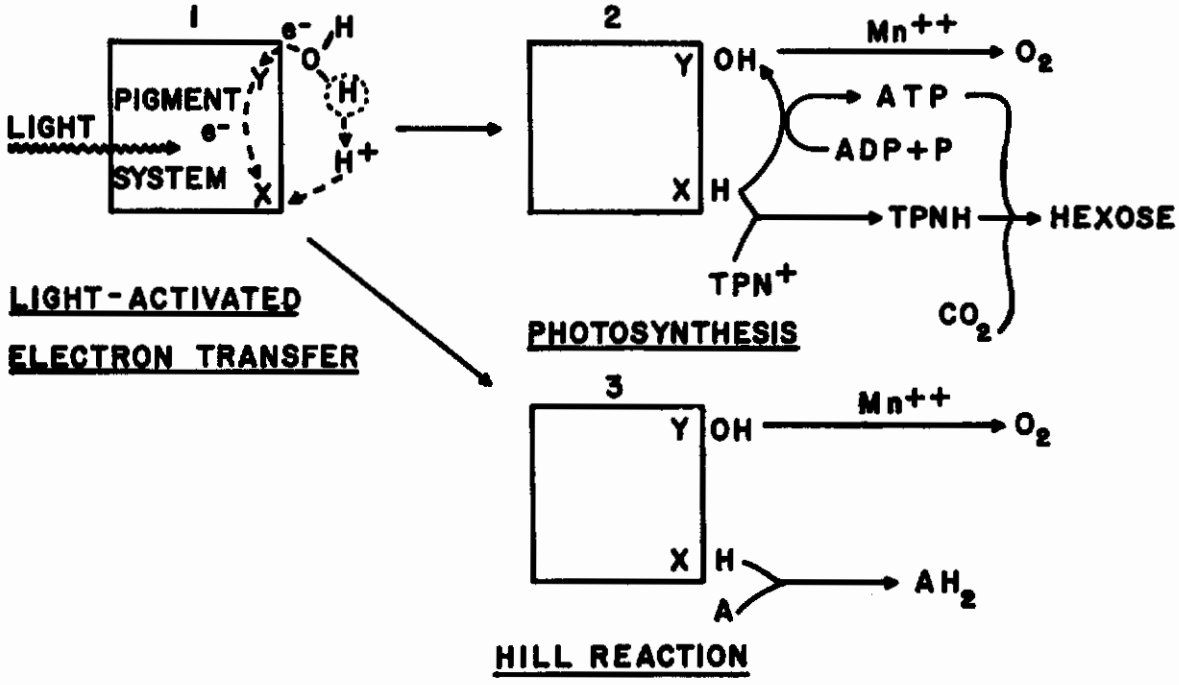


FIG.1. PHOTOSYNTHETIC PROCESS OF GREEN PLANTS

OTHER EXPRESSIONS OF THE PHOTOCHEMICALLY GENERATED OXIDIZING POTENTIAL

The previous section considered oxygen evolution as the biochemical result of a photochemically generated oxidizing potential by the pigment system of the chloroplasts. There are other biological expressions of this oxidizing power, and a discussion of them is worthwhile since there may be some common features of these processes and oxygen evolution.

Bacterial Photosynthesis

In 1931 van Niel erected a milestone in the history of photosynthesis by advancing the idea that plant and bacterial photosynthesis are functionally the same and differ only in the nature of the donor molecule supplying the hydrogens involved in carbon dioxide reduction (ref. 5). Since water serves this function in plants, the oxidized product is seen as molecular oxygen. In the sulfur photosynthetic bacteria, hydrogen sulfide (as well as other sulfur compounds) serve this function, and the more oxidized forms of sulfur accumulate in the medium. This concept readily explains why bacteria do not evolve oxygen, and elevated the concept of water-splitting in plant photosynthesis to the important place it holds today. It is not appropriate to extensively discuss the photosynthetic bacteria at this time, and the discussion will be limited primarily to the expression of the photochemically generated oxidizing potential in bacteria. For a more detailed discussion of this subject, the reader is referred to the publications of van Niel (ref. 34, 35), Stanier (ref. 36) and Gest and Kamen (ref. 37).

The photosynthetic bacteria can assimilate carbon dioxide photosynthetically. The purple sulfur bacteria (Thiorhodaceae) and the green sulfur bacteria (Chlorobium) will do so in the presence of hydrogen sulfide or other sulfur compounds, while the purple non-sulfur bacteria (Athiorhodaceae) need an organic acid such as butyrate for this process. The fixation process continues so long as the sulfur compounds or aliphatic acids are present, but ceases when they become exhausted. Examination of the growth medium of the sulfur bacteria reveals that an oxidation of the sulfur atoms has taken place during the process, with sulfate accumulating. Thus it becomes apparent that an oxidizable substrate is required for photosynthesis (carbon dioxide fixation) and the substrate molecule becomes oxidized during the process. This same general reaction can be demonstrated with all photosynthetic bacteria.

The pigment system is contained in a small body called the chromatophore, and consists of either bacteriochlorophyll or chlorobium chlorophyll plus a characteristic array of carotenoids and xanthophylls. In considering the wide variety of substrate molecules which can serve as the ultimate hydrogen donor for the photochemical act, it appears very unlikely that the photochemical system can accommodate each of these molecules directly, but more likely they donate their hydrogens (electrons) indirectly through

some common intermediate. There is considerable evidence that cytochromes play this role. All species of photosynthetic bacteria investigated to date have been shown to contain cytochromes of the c type, even though some are strictly anaerobic bacteria which have no oxidative metabolism in the dark involving oxygen (ref. 38-40). The cytochromes are natural candidates for this role, and several investigations have shown that one of the earliest and most pronounced effects of illuminating the bacterial chromatophore system is an oxidation of the characteristic cytochrome c in the chromatophore. Smith et al. have shown an oxidation of cytochrome c_2 in R. rubrum upon illumination (ref. 41). Similar reactions have been shown with Chromatium by Olsen and Chance (ref. 42) and have served as the basis for quantum yield measurements for bacterial photosynthesis (ref. 43). The close proximity of the cytochromes and chlorophyll and the physical nature of the act was shown by the fact that with Chromatium, the oxidation of cytochrome c proceeded even at -78° C with no significant decrease in rate (ref. 44).

Further evidence for the photooxidative power generated by bacterial chromatophores is furnished by the experiments of Frenkel, in which reduced FMN was oxidized upon illumination under anaerobic conditions, and DPN was concomitantly reduced (ref. 45). Likewise, the experiments of Vernon (ref. 46) and Vernon and Ash (ref. 47) showed that chromatophores of R. rubrum catalyzed a photooxidation of reduced cytochrome c and oxidation reduction dyes under anaerobic conditions. These photooxidations were coupled to the photoreduction of DPN. Thus there is ample evidence for the photochemically generated oxidizing potential of bacterial chromatophores, and it has been demonstrated in a variety of ways.

Fig. 2 presents a general scheme of bacterial photosynthesis as it is known today. The inclusion of a photosynthetic phosphorylation pathway reflects the information gained from the experiments of Frenkel (ref. 48), Geller and Lipmann (ref. 49), Vernon and Ash (ref. 47) and Arnon et al. (ref. 33).

One last piece of evidence pointing toward a common photochemical mechanism for the photosynthetic bacteria is the constancy of the quantum requirement for carbon dioxide fixation using a variety of hydrogen donors (ref. 50).

The next section treats the special case of plant photosynthesis called photoreduction, in which algae can be forced to perform a bacterial-type photosynthesis not involving oxygen evolution. Furthermore, under selected conditions chromatophores of R. rubrum and spinach chloroplasts will catalyze the same photoreaction (a photoreduction of DPN and photooxidation of ascorbate (ref. 26)).

Photoreduction in Algae

For a review of this experimentally important mode of plant photosynthesis, the reader should consult the works of Rabinowitch (ref. 2), Kessler (ref. 51), and the review by Gaffron (ref. 52). The pioneer work of

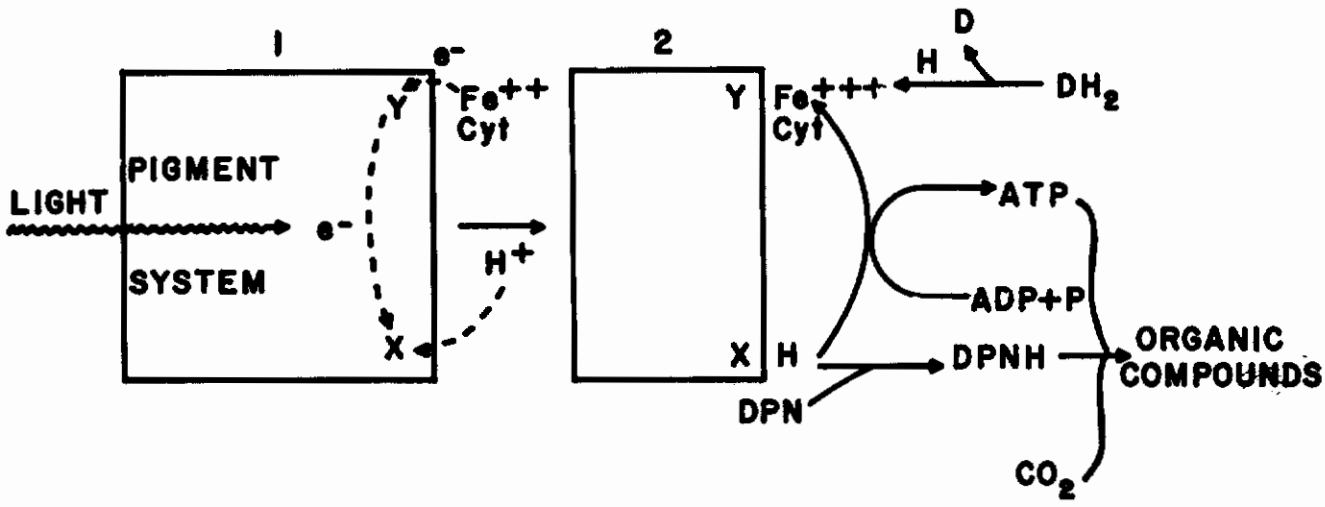


FIG.2. THE PHOTOSYNTHETIC PROCESS OF BACTERIA

Nakamura with Oscillatoria (a blue alga) and of Gaffron with a green alga, Scenedesmus, showed that a bacterial type of photosynthesis could be forced upon plant cells capable initially of the usual plant photosynthesis. When exposed to hydrogen gas under anaerobic conditions, the cells formed a hydrogenase which then allowed the cell to consume hydrogen while reducing carbon dioxide in light of low intensity. This consumption continued if the light intensity was kept below a certain threshold of low intensity. When this intensity was surpassed, the cells quickly reverted to the normal photosynthetic process involving oxygen production, indicating a destruction of the hydrogenase either by oxygen or its precursors formed photosynthetically. Fig. 3, which is taken from one of Gaffron's early papers (ref. 53), shows the initial photoreduction process (gas uptake) followed by a reversion to normal photosynthesis. The reversion was prevented by hydroxylamine. Since the initial observations of Gaffron on Scenedesmus, many species of algae have been adapted to perform the photoreduction process, as tabulated by Kessler (ref. 51).

As pointed out by Gaffron (ref. 52), this phenomenon may be explained in one of two ways. Either the adaptation process interferes by allowing the hydrogen to compete with the oxygen evolving system for YOH, or oxygen is evolved as usual but is reduced by the hydrogen via the hydrogenase formed adaptively. The former explanation is the correct one as shown by the experiments of Horwitz and Allen (ref. 54), who investigated the photoreduction process under a stream of either nitrogen or hydrogen gas. Fig. 4 is taken from their paper, and shows that a normal photosynthetic evolution of oxygen took place in atmospheres of either gas. The streams of either hydrogen or nitrogen removed the oxygen as it was produced, thus preventing its build-up and subsequent inactivation of the hydrogenase system at high light intensity. When returned to light of low intensity, photoreduction was again the preferred reaction. The rapid inactivation of the photoreduction process and the reversion to normal photosynthesis observed at high light intensities in static atmospheres apparently results from a photooxidation of the hydrogenase by the photosynthetically evolved oxygen.

Photoreduction involves then, a competition for the photochemically formed YOH. If YOH reacts with the adaptively formed hydrogenase, hydrogen gas is taken up and photoreduction occurs. If it reacts with the oxygen evolving system, normal photosynthesis results. This is expressed by Horwitz and Allen (ref. 54), "..... although adapted algae can perform a true photoreduction, they do not necessarily lose the capacity to evolve oxygen. True photoreduction with hydrogen and photosynthesis are not mutually exclusive processes. Both processes can exist side by side in adapted algae. Either photoreduction or photosynthesis can predominate depending upon conditions."

The process of photoreduction is a very valuable tool in studies on the mechanism of oxygen production, since it provides a means of investigating the oxygen evolution system apart from the reducing system produced by the chloroplasts. During normal photosynthesis, or during the Hill reaction, one cannot be certain if a particular inhibitor or activator

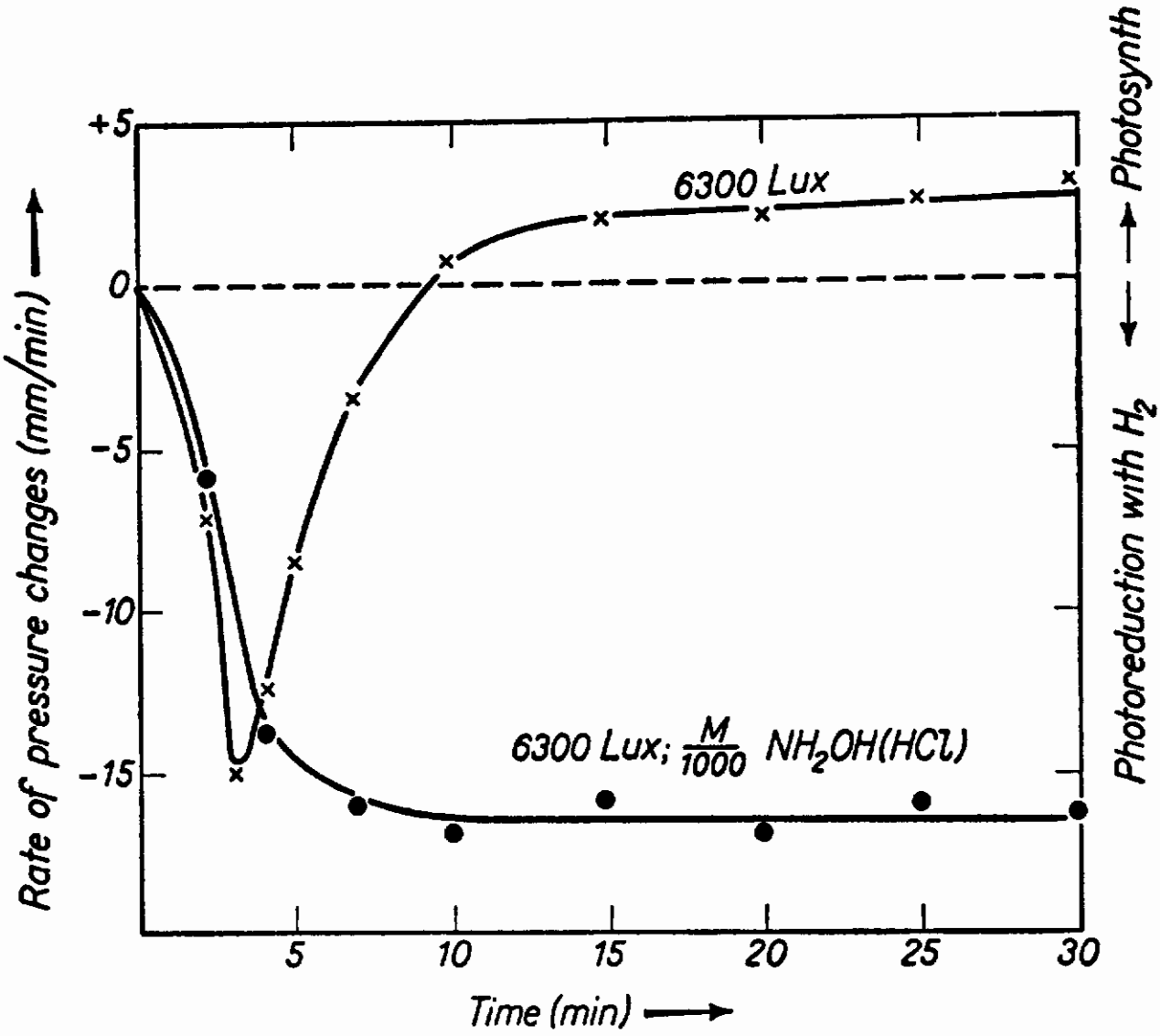


Fig. 3. Deadaptation at high light intensity of Scenedesmus which had been adapted for photoreduction, and the prevention of the deadaptation by hydroxylamine. Taken from Gaffron (53).

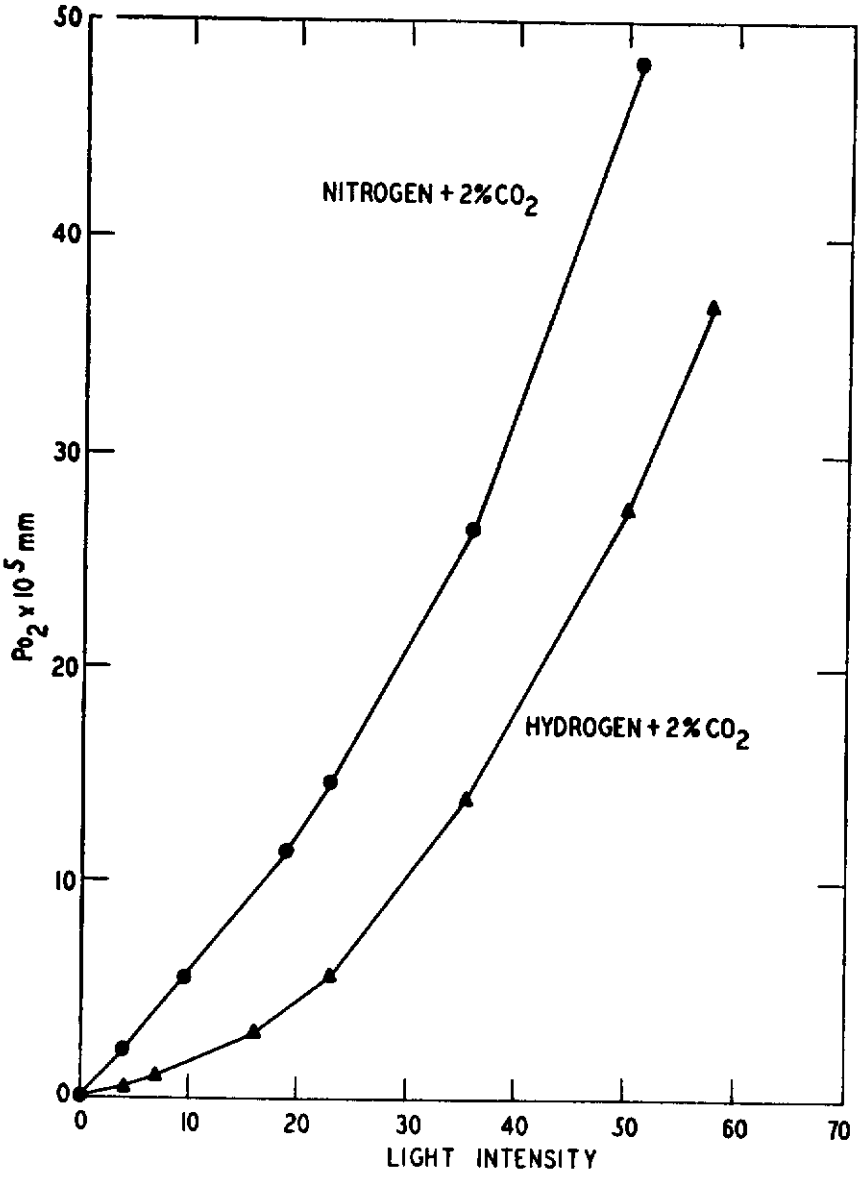


Fig. 4. Evolution of oxygen by adapted Scenedesmus with simultaneous flushing by nitrogen or hydrogen gas to produce very low partial pressures of oxygen. The removal of oxygen allows the photoreduction process to proceed simultaneously with the normal oxygen evolution process. Taken from Horwitz and Allen (54).

acts upon the oxygen evolving system or on the reducing system, since the inhibition of either step would stop the entire system. In the case of photoreduction, however, both systems are functioning separately, and the ability of the algal cell to revert to normal photosynthesis at high light intensity in a static atmosphere provides a very sensitive tool for studying the oxygen system. By means of this technique, a number of potent inhibitors of photosynthesis have been shown to act on the oxygen evolving system. Gaffron in 1942 showed that the inhibitor of photosynthesis, hydroxylamine, was acting on the oxygen evolving system, since adapted algae were protected from reversion by the presence of .001 M hydroxylamine (Fig. 3). A more potent inhibitor of the oxygen evolving system is the herbicide DCMU, (3-(3, 4-dichlorophenyl)-1, 1-dimethylurea), which completely inhibits photosynthesis at a concentration below that of the chlorophyll. Fig. 5, taken from the work of Bishop (ref. 55), shows that at 3×10^{-6} M DCMU, algae adapted for photoreduction do not revert at a light intensity of up to 8 Kilolux.

Other inhibitors which have been shown to function primarily on the oxygen evolving system are vitamin K, phthiocol, and o-phenanthroline (ref. 52). Recently Bishop has examined a new class of herbicides, the amino trazines, and has found them to also function by inhibiting the oxygen producing system, although they are less potent than DCMU (ref. 56).

It has been known for some time that manganese is an essential element for algal growth. Pirson reported in 1937 that manganese deficient cells showed a decreased rate of photosynthesis and an increased rate of respiration (ref. 57). Furthermore, addition of manganese restores photosynthesis within 30 minutes. The extensive investigation at our laboratory over the past several years has further demonstrated the requirement for manganese ion for both growth and Hill reaction activity for Chlorella cells (ref. 58).

The use of normal and manganese-deficient algae adapted for photoreduction allowed a definitive placement for the manganese ion in the oxygen evolving system. Fig. 6 shows the decreased rate of photosynthesis and the increased rate of photoreduction (at low intensities) with the deficient cells. Furthermore, the deficient cells were protected against reversion at high light intensities. The only explanation for these data is that manganese functions on the oxygen evolution system. The cells reverted to normal photosynthesis at higher light intensities because some manganese remained in the cells (it is required for the dark respiratory reactions of the cells, at about 1/1000 the concentration needed for photosynthesis). It is impossible to completely inhibit photosynthesis in the living cell by means of manganese deficiency and keep the cells alive.

Without repeating all the scheme shown in Fig. 1, it is possible to relate photoreduction to photosynthesis by considering only the fate of the YOH produced photochemically. The competition between the oxygen evolving system and the adaptively formed hydrogenase for the YOH is shown below:

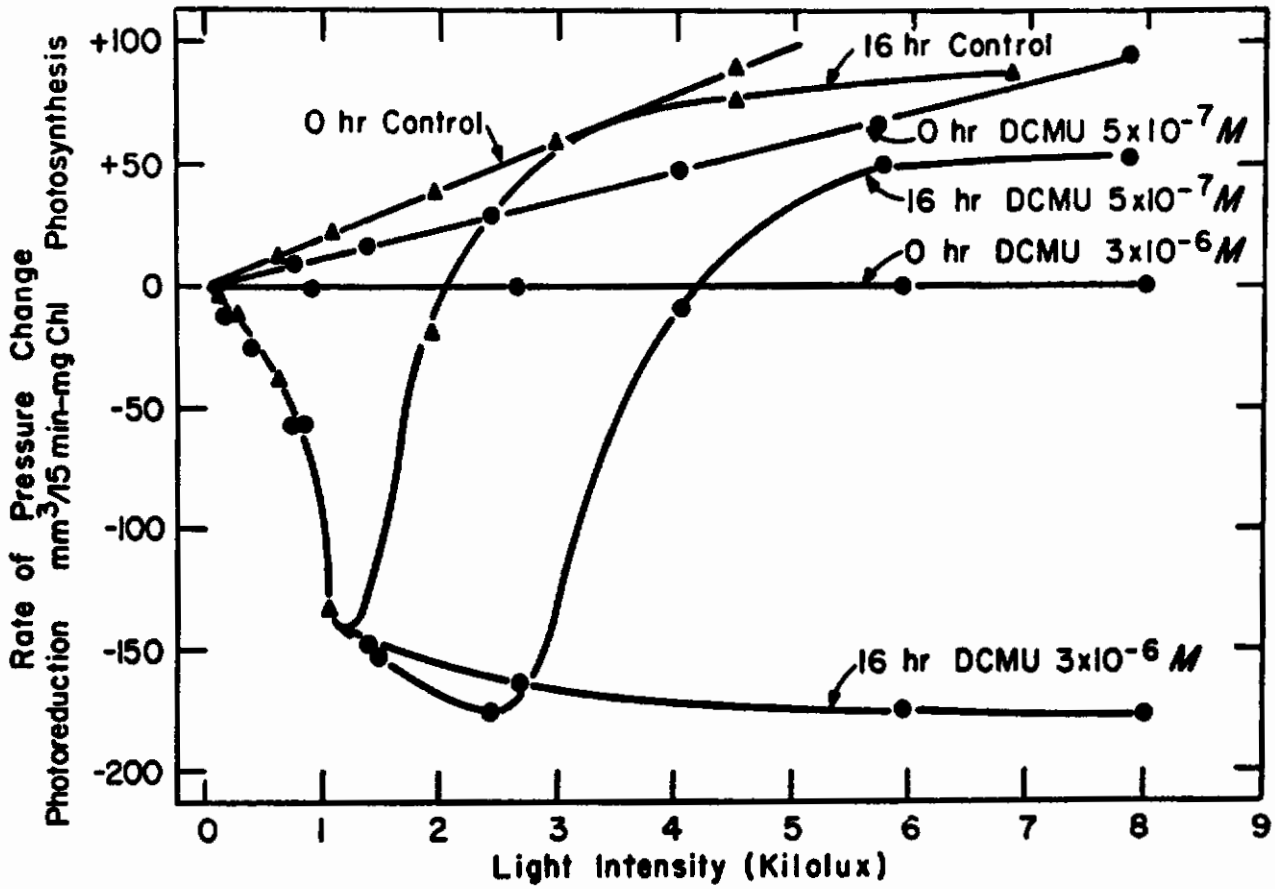


Fig. 5. Prevention of deadaptation of Scenedesmus by the herbicide, DCMU. The presence of this herbicide stabilizes the photoreduction process by preventing oxygen evolution, which would result in destruction of the hydrogenase. Taken from Bishop (55).

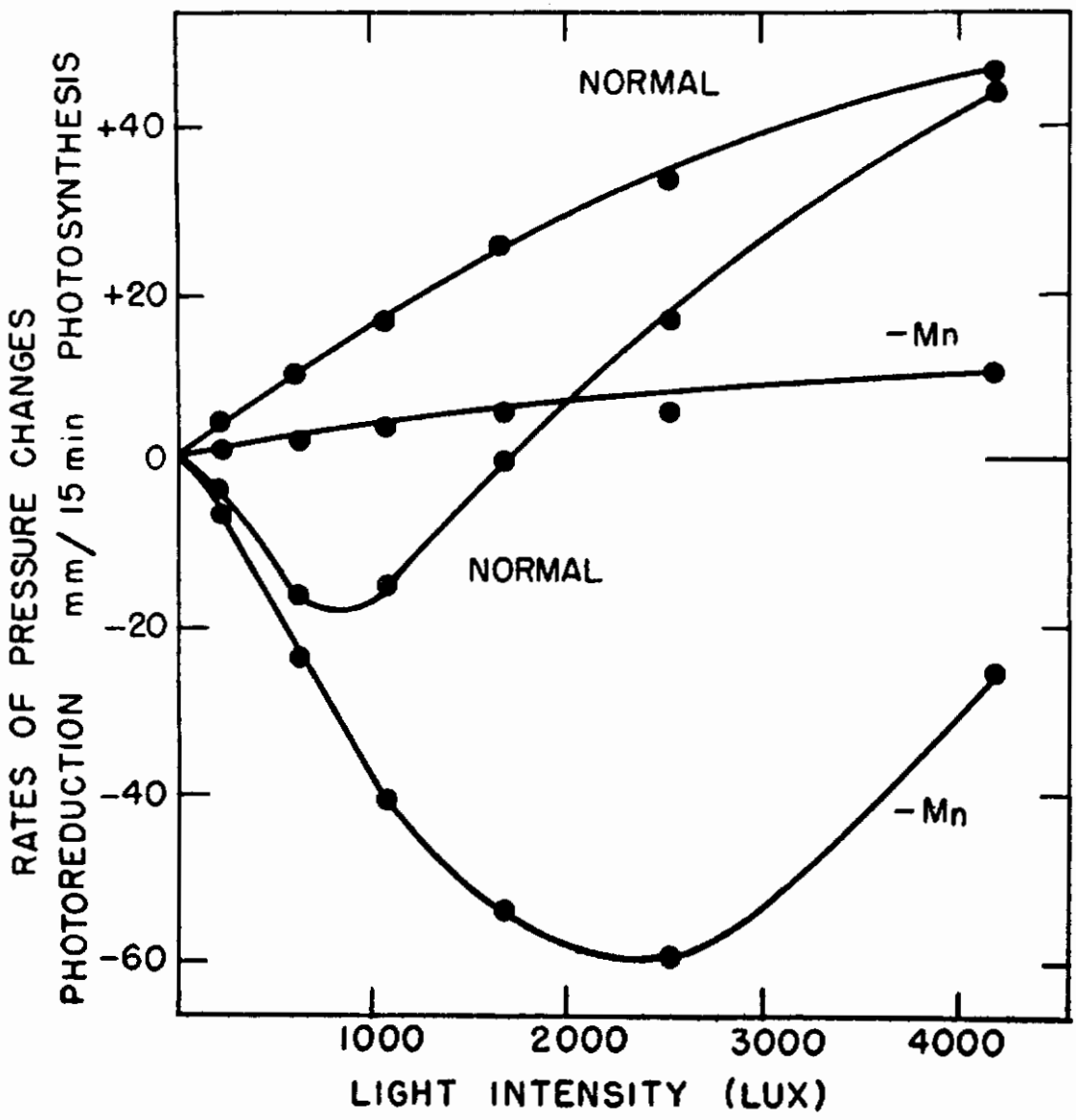
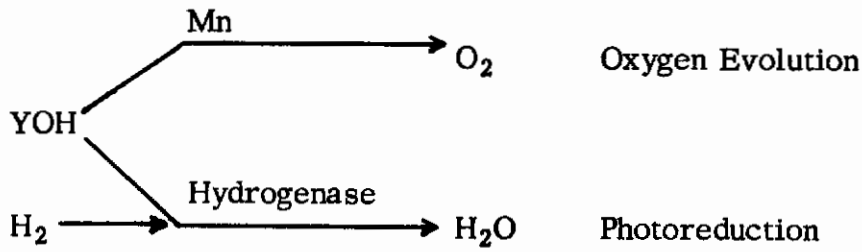


Fig. 6. Photosynthesis and photoreduction by normal and manganese-deficient Ankistrodesmus braunii cells. A manganese-deficiency inhibits photosynthesis (the curves showing initial and steady increases in pressure), but it partially stabilizes the photoreduction process of adapted cells (the curves showing initial decrease in gas pressure). Taken from Kessler (59).



Photooxidation of Ascorbate in the Presence of 2, 6-dichlorophenolindophenol

In a very significant series of investigations, Mehler discovered that chloroplasts were capable of consuming oxygen in the presence of ascorbate, particularly following a preliminary reduction of quinone in a regular Hill reaction (ref. 60, 61). Following this clue, Vernon and Kamen examined the photooxidation of ascorbate in the presence of 2, 6-dichlorophenol-indophenol (DPIP) by both spinach chloroplasts and chromatophores of R. rubrum (ref. 62) and concluded that the photooxidation was accomplished by the photochemical oxidizing system of the chloroplast (YOH), while the oxygen uptake was due to a simultaneous reduction of molecular oxygen by the photoreducing component, XH.

The original proposal of Vernon and Kamen was subsequently shown to be correct by allowing chloroplasts poisoned with inhibitors of the oxygen evolving system to photooxidize ascorbate (with DPIP) under strictly anaerobic conditions (ref. 26, 63). Under these conditions TPN could be reduced photochemically in a coupled reaction, with ascorbate being oxidized. Ascorbate oxidation substituted for the water oxidation (and oxygen production) observed normally. Table 1 shows that chloroplasts which have been poisoned with DCMU will no longer reduce either DPN, TPN, FMN, vitamin K_3 or the dye indigo carmine unless the ascorbate-DPIP couple is added. Ascorbate serves as an alternate electron (or hydrogen) donor, and the dye DPIP serves as an intermediate electron carrier between ascorbate and the photochemical system.

The experiments of Jagendorf and Margulies also show that the ascorbate-DPIP couple can serve as an electron-donating system for YOH, thus leaving XH available to carry out photoreductions. The inhibition of TPN photoreduction by p-chlorophenyl dimethyl urea CMU (ref. 64), by storing bean leaves in the cold (ref. 65) and by aging chloroplasts (ref. 26), is relieved by the addition of ascorbate and DPIP to the system. The couple ascorbate-PMS also allowed photoreduction of TPN in the presence of inhibitors of the oxygen evolving system.

Marre et al. (ref. 66) have shown that chloroplast fragments have an enzyme which catalyzes the reduction of dehydroascorbate by TPNH in the dark. Chloroplasts which have lost this activity carry out a photooxidation of ascorbate in the air, but not under nitrogen gas. However, if TPN and a TPNH trapping system are added, the photooxidation of ascorbate proceeds under nitrogen, which indicates that TPN is serving as the electron acceptor

Table 1
Ability of DPIP-ascorbate couple to overcome inhibition caused by DCMU, ammonium ion and hydroxylamine. The basic system contained fresh spinach chloroplasts, Tris buffer and PPNR in addition to the oxidant listed. The experiments involving FMN were performed in red light, and PPNR was added only to those systems involving TPN and DPN. Taken from Vernon and Zaugg, 26.

Conditions	Extent of Photoreduction				
	TPN redn.	DPN redn.	Indigo carmine redn.	FMN redn.	Vitamin K_3 redn.
	umoles/ml	umoles/ml	umoles/ml	umoles/ml	umoles/ml
Control	0.193	0.168	0.102	0.013	0.071
+DPIP, ascorbate	0.164	0.137	0.064	0.022	0.034
+DCMU	0.003	0.003	0.001	0.005	0.009
+DCMU, ascorbate	0.042	0.035	0.009	0.010	0.015
+DCMU, DPIP, ascorbate	0.179	0.146	0.060	0.019	0.024
(Boiled chloroplasts)	0.001	0	0	0.005	0.001
Control	0.154	0.168	0.126	0.013	0.068
+DPIP, ascorbate	0.098	0.137	0.100	0.021	0.038
+Hydroxylamine	0.020	0.014	0.002	0.004	0.010
+Hydroxylamine, ascorbate	0.035	0.024	0.041	0.018	0.015
+Hydroxylamine, DPIP, ascorbate	0.121	0.098	0.094	0.058	0.023
Control	0.188	0.130	0.072	0.036	0.074
+DPIP, ascorbate	0.153	0.056	0.059	0.027	0.056
$+NH_4^+$	0.010	0.009	0.001	0.001	0.004
$+NH_4^+$, ascorbate	0.030	0.018	0.026	0.007	0.010
$+NH_4^+$, DPIP, ascorbate	0.122	0.054	0.054	0.021	0.037

for the electron donated by ascorbate and transferred via the photochemical system.

With ascorbate (in the presence of either DPIP or PMS) as the electron donor for the photosynthetic reactions, water is replaced as the hydrogen donor and no oxygen evolution is observed. A chloroplast functioning in this manner is in many ways analagous to the bacterial chromatophore; both catalyze a photoreduction of pyridine nucleotide and a simultaneous, coupled photooxidation of a hydrogen donor molecule other than water. Data supporting this concept are shown in Table 2, which shows that both photosynthetic systems will catalyze a photooxidation of ascorbate and a linked photoreduction of DPN. Although TPN is the pyridine nucleotide reduced preferentially by spinach chloroplasts in the presence of photosynthetic pyridine nucleotide reductase (PPNR), use of a partially purified enzyme preparation containing a transhydrogenase allowed DPN reduction to proceed. These data emphasize the similarity of the bacterial system and the chloroplast system when the oxygen evolution mechanism is not operating. The system responsible for water oxidation and oxygen evolution in plants functions only as a source for the hydrogens transferred to pyridine nucleotide. The reduced pyridine nucleotide and ATP are then used in carbon dioxide fixation, and this is the main benefit to the plant from the photosynthetic process.

Table 2

Similarity of aged spinach chloroplasts and Rhodospirillum rubrum chromatophores in catalyzing linked photooxidation of ascorbate and photoreduction of DPN. DPIP was present to couple with the photooxidizing system, and pyruvate and lactic dehydrogenase were present to trap the DPNH formed in the reaction. Taken from Vernon and Zaugg, ref. 26.

Experiment	Ascorbate oxidized	Lactate produced
	μmoles/ml	μmoles/ml
Aged chloroplasts.....	0.42	0.39
Bacterial chromatophores.....	0.57	0.30

TWO LIGHT REACTIONS

The chemical reactions of photosynthesis are driven by photochemical reactions resulting from absorption of light quanta by the pigment systems of the plant and bacteria. All plants contain chlorophyll a, and it must be regarded as the indispensable pigment as far as plant photosynthesis is concerned. Chlorophyll b is also found in all higher plants, but is absent from the blue-green, brown and red algae, from diatoms and from the green algae of the genus Vaucheria (ref. 67). Also present in the cells are carotenes (mostly B-carotene) and oxygenated derivatives of carotene, the carotenols. The carotenes function primarily as agents to protect the chlorophylls against photooxidative destruction (ref. 68), and the carotenols function as light absorbers capable of transferring their energy to the chlorophyll system (ref. 69). Also present in some algae are the phycobilins, which are open-chain tetrapyroles. As shown below, these seem to function in a separate photoreaction from that involving chlorophyll a, but also are capable of transferring their energy to chlorophyll a.

With the perfecting of absorption spectrophotometry, more than one form of chlorophyll a has been identified in plants. French has been primarily responsible for perfecting these techniques, and has shown that there are forms of chlorophyll a which show absorption maxima at 673, 683, 695 and 705 mu (ref. 70, 71). It is apparent that to bring about the evolution of oxygen and photoreduction of pyridine nucleotide, the plant chloroplast brings into play a wide variety of light absorbing pigments and chemically reacting components, and the great problem facing us is to determine just how these different systems interact in such a beautifully coordinated fashion to bring about the final reactions we observe.

Using monochromatic light of different wave lengths to drive the photosynthetic process, it can be shown readily that light absorbed by pigments other than the chlorophylls is active in photosynthesis. The light absorbed by the accessory pigments could be active in photosynthesis in two ways: either the pigments catalyze the same reaction as the chlorophylls, or they transfer their energy to the chlorophyll a molecules which then couple directly with the chemical system. Since the accessory pigments can transfer their energy to chlorophyll a and cause its fluorescence in the intact chloroplasts (ref. 69), it seemed logical that their function was merely that of a light absorbing system which allowed a greater collection of light energy for coupling to the chemical system via chlorophyll a. However, the early experiments of Emerson and Lewis with Chlorella (ref. 72) revealed that light in the region of 460 to 500 mμ had a poor efficiency for photosynthesis, and furthermore a striking decline in the quantum yield was found on the far red side of the spectrum, where light was absorbed primarily by chlorophyll a. A similar decline has been seen in higher plants (ref. 73). These data were difficult to explain on the basis of a single photochemical function in the chloroplast.

When the absorption spectrum for red algae was compared with the

action spectrum for photosynthetic oxygen evolution, a surprising feature was apparent; light absorbed by the chlorophylls was less active for photosynthesis than was light absorbed by the accessory pigment phycoerythrin (ref. 74). Fig. 7 shows the data obtained by Haxo and Blinks for Schizymenia pacifica, and shows the relative inactivity of monochromatic light absorbed by the chlorophylls. These experiments led to the idea that there were both "active" and "inactive" chlorophyll molecules in the red algae, although the nature of the inactive chlorophyll was not known.

Warburg, et al. showed in 1955 that addition of weak blue light to a Chlorella suspension photosynthesizing in red light caused an enhancement in the rate which was more than the additive effect of the two light beams (ref. 75). Emerson, Chalmers, and Cederstrand (ref. 76) investigated the problem in detail, and showed that a mixture of red and shorter wave length light gave a higher rate of oxygen evolution than the sum of the rates obtained by single illumination in separate systems. Thus, the absorption of light of one wave length allowed the chloroplast to utilize more efficiently the light absorbed at another wave length, or it "enhanced" the other system. Using red light absorbed primarily by chlorophyll a, the enhancing effect of other wave lengths of light showed maximal effects at 480 and 655 m u (ref. 77), which implicates chlorophyll b in the system enhancing chlorophyll a action for Chlorella. In Navicula the action spectrum for enhancement (where light at 690 m μ was supplied to give the basal photosynthetic rate) corresponded to fucoxanthol absorption, in Anacystis (a blue-green alga) to phycocyanin, and in Porphyridium (a red alga) to phycoerythrin. Subsequent investigations also showed that in general two systems could be detected, one involving a long wave length form of chlorophyll a and the other either a short wave length form of chlorophyll a, chlorophyll b or the phycobilin pigments (ref. 70, 71, 78, 79). The concept of two separate, but coupled light reactions being required for the photosynthetic process in green plants explained the earlier observations of Haxo and Blinks (ref. 74) that light absorbed by the accessory pigments of alga was more efficient on the quantum basis than was light absorbed by the chlorophyll system.

Another phenomenon relating to the differential effect of light quality upon photosynthesis was reported by Blinks (ref. 80, 81). By following the rate of oxygen evolution from Ulva by means of a sensitive and fast oxygen electrode, he was able to show that after attaining a steady state rate of photosynthesis at 490 m μ, changing to light of 540 m μ (of an intensity which would give the same steady state rate) produced fluctuations in the rate of oxygen evolution. Similar results were obtained with alternating light of 640 and 688 m u, suggesting that different pathways were activated by the different light beams. Myers and French determined the action spectrum for production of the upward spike when changing from light of 700 m μ with Chlorella. This was shown to correspond with the action spectrum for enhancement of oxygen production when light of 700 m u was used for the basal rate. These data are shown in Fig. 8, and show conclusively that the same processes are involved in both the enhancement and chromatic transient phenomena, and show further that the two systems involved in Chlorella are

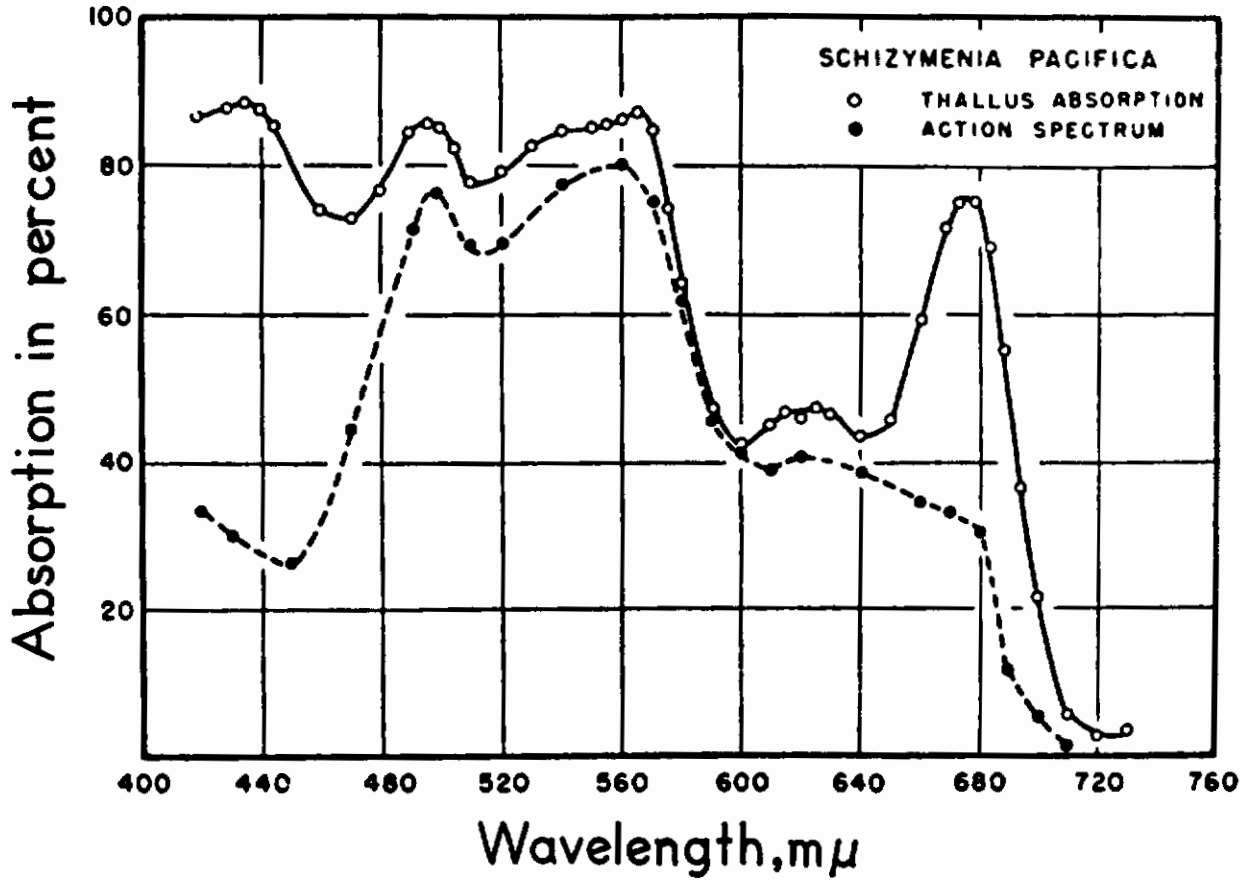


Fig. 7. The action and absorption spectra of a red alga, showing that light absorbed by the chlorophyll a system is less active than light absorbed by the phycoerythrin for oxygen production. Taken from Haxo and Blinks (74).

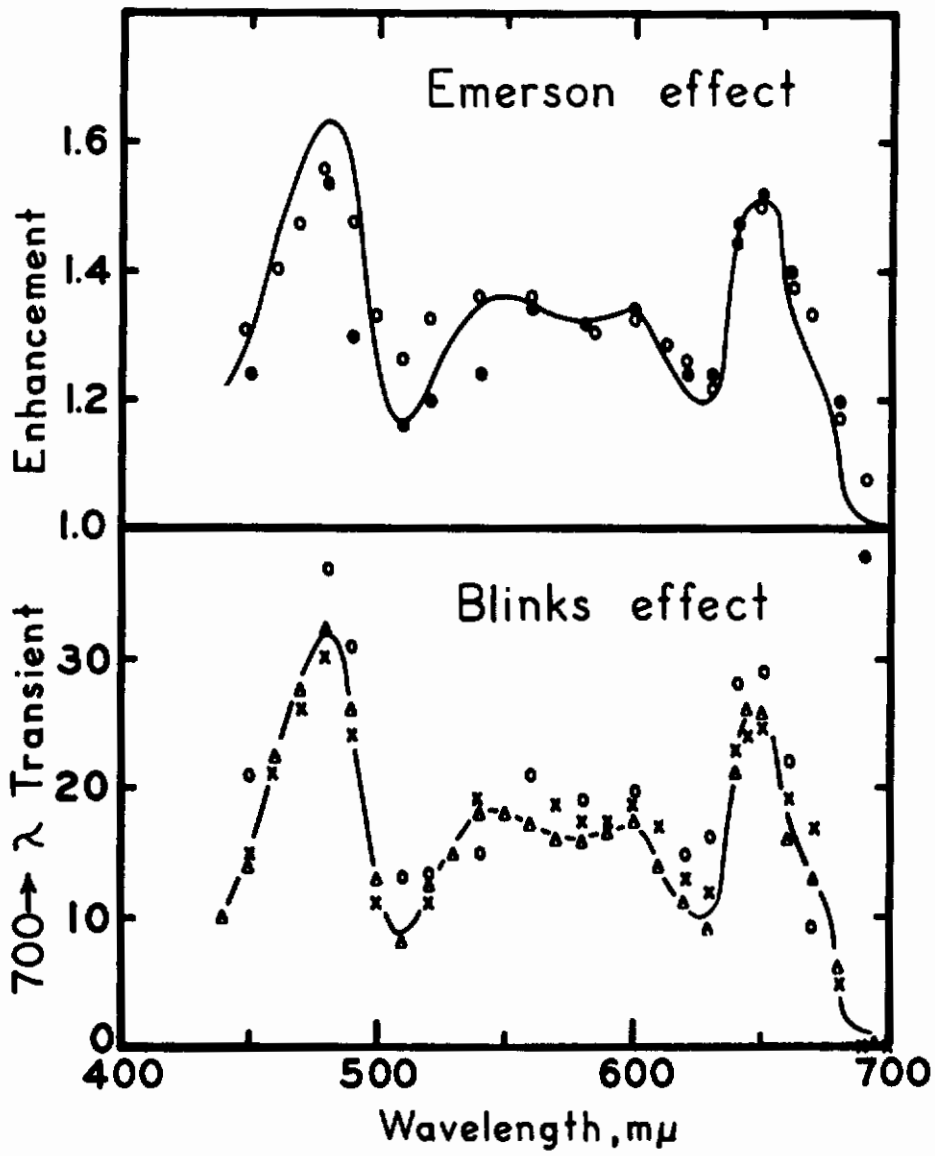


Fig. 8. The action spectra for chromatic transients (Blinks effect) and for enhancement (Emerson effect) of oxygen production by Chlorella. Taken from Myers and Rench (78).

chlorophyll a (which absorbed the light at 700 mμ) and chlorophyll b (whose absorption spectrum corresponds to the action spectra shown in Fig. 8).

By doing the reverse experiment, it was possible to determine which form of chlorophyll enhanced the photosynthetic evolution of oxygen caused by light absorbed by the chlorophyll b system in Chlorella (ref. 71, 78). With light of 650 mμ providing the basal rate, the action spectrum for enhancement showed a peak corresponding to the absorption spectrum of chlorophyll a 695. Furthermore, the very significant observation was made that the enhancement occurred if the light was given in flashes separated by several seconds of darkness, indicating that the coupling of the two light reactions is chemical in nature (able to couple during the dark period) and not electronic (photochemical).

French and Fork have also shown that with Porphyridium cruentum, green light of 570 mμ caused a sharp increase in the rate of oxygen evolution as measured by the oxygen electrode (ref. 70). Light in the red, at 695 mμ, caused a photostimulation of respiration, or oxygen uptake. The action spectrum for photosynthesis (positive spike corresponding to oxygen production) corresponded to the absorption spectrum of the phycoerythrin, while the action spectrum of the photostimulation of respiration (negative spike corresponding to oxygen uptake) corresponded to the absorption spectrum of chlorophyll a in this organism. Again the two pigment systems were clearly and distinctly separated in terms of their transient effects upon oxygen production.

The evidence for two pigment systems operating in photosynthesis is great. The fact that the enhancement can be observed when the light flashes are separated by several seconds of darkness speaks against two light quanta being combined in the photochemical reactions of photosynthesis as has been proposed by Franck (ref. 82, 83). Accepting that the synergistic effect of light of different wave lengths arises from coupled chemical reactions tied to two primary photochemical events, there are still two ways one could conceive of this happening. One possibility is that the two light reactions are coupled in series to cover the electron transfer span from water (oxygen production with E_0' of .8) to TPN (E_0' of -.32 v) in two steps. An alternate mechanism would have the two quanta joined in some non-series fashion, so that the energy could be concentrated at one reactive site (perhaps the oxygen evolving site) and still transfer the electron to the TPN system in one step. From the evidence available at the present time, it appears that the former, or series coupling of the two systems is to be preferred. Accordingly, this mechanism will be used in considering the various aspects of the coupled light reactions discussed below.

From the experiments described above, it is apparent that the pigment complement of chloroplasts can be functionally divided into two systems. One absorbs light at shorter wave lengths, consists of the phycobilins and/or chlorophyll b and/or the shorter wave length forms of chlorophyll a and is more directly involved with the oxygen evolving mechanism. Some long wave length form of chlorophyll a, absorbing at wave lengths of 683, 695, 700 or 705 (ref. 70, 84), drives the other half of the couple. From the experiments

of French and Fork which associate the chlorophyll a with photostimulation of respiration, the chlorophyll a system is shown to function more directly in the reduction of TPN.

A representation of the two separate, series-linked light reactions is shown in Fig. 9. The two systems have been designated according to the convention proposed by Duysens, et al. (ref. 85). System 2 represents the light absorbing system and accompanying enzymatic components involved in the oxidation of water and the accompanying transfer of the electrons from water through Y to an electron acceptor of intermediate oxidation potential designated by the letter A. The reduced form of A would then transfer the electron to B by means of a coupled, light-independent enzymatic reaction. System 1 represents the other pigment system and accompanying enzymes which absorb light and transfer electrons from the reduced form of B to X, resulting in the formation of XH which can then reduce TPN. In this scheme the use of YOH and XH found in the photosynthesis literature is retained. Y represents the molecule initially oxidized under the influence of light absorbed by system 2, and YOH represents the oxidized product of water formed by interaction of Y and water. A represents the electron acceptor for electrons transferred via system 2, B represents the electron donor for system 1, and X represents the electron acceptor for system 1 which couples with TPN to produce TPNH.

Similar schemes have been advanced previously. Hill and Bendall (ref. 86) proposed a scheme in 1960 depicting two light reactions, and they suggested that A could be cytochrome b_6 and B was probably cytochrome f. Duysens, et al., (ref. 85) proposed a scheme in 1961 involving two light reactions, in which systems 1 and 2 coupled through only one compound, viz. A and B were identical. Witt, et al., (ref. 87, 88) have published schemes based upon their very elegant study of the rapid absorbancy changes taking place upon illumination of the chloroplast system, and their experimental evidence favors such a two system process. They identify B as being cytochrome f, and from observations with chloroplasts extracted with petroleum ether (which extracts plastoquinone) they suggest that A is either plastoquinone or is very closely linked to plastoquinone.

French and Fork (ref. 70) have also described the photosynthetic process in terms of two light reactions, based upon their experiments on oxygen production as a function of different wave lengths of incident light. Kok and Hoch also have obtained experimental evidence for two light steps in photosynthesis, centering around a very exciting pigment called P_{700}, of which he has spoken in this symposium (ref. 84). This compound absorbs light at 700 mμ, and appears to be a special form of chlorophyll a. It functions in system 1, or the one leading to reduction of pyridine nucleotide. Long and short wave length lights affect this compound in opposite ways, and it appears to be intimately associated with the cytochromes and other compounds involved in photosynthesis.

The occurrence of two separate but coupled light reactions to drive the photosynthetic machinery is now commonly accepted, and it then becomes necessary to identify both the light absorbing pigment systems and also the components working in the dark reactions.

INTERACTIONS IN THE TWO-LIGHT SYSTEM

Oxygen evolution is a terminal reaction of the coupled, two-cycle scheme presented in Fig. 9. Since photosynthesis in the plant cell involves oxygen evolution definitely coupled to TPN reduction, the natural system involves both cycles. Therefore, it is germane to this presentation to consider a few aspects of the two-cycle system which relate to electron transfer through these systems.

Location of Photosynthetic Phosphorylation Step

There is sufficient evidence available now to show that one phosphorylation (the conversion of ADP and inorganic phosphate into ATP) takes place during the reactions comprising the transfer of electrons from water to such acceptors as TPN, ferricyanide, and FMN in chloroplasts. The dependence of phosphorylation upon the reduction of these compounds by the chloroplast in the light has been shown by many investigators who report one ATP formed per two electrons transferred to the individual compounds (ref. 89-95). This suggests a similar coupled phosphorylation site for all three reactions.

In theory there are three places where the phosphorylation site could be placed on the coupled two cycle system shown in Fig. 9. One possibility is on the sequence of reactions leading to oxygen evolution. Since indophenol dyes are reduced in a regular Hill reaction in which oxygen is evolved, and this process does not result in ATP formation (ref. 94), this possibility is ruled out.

Another possible location for ATP formation is the reaction in which XH reacts with TPN (or ferricyanide or FMN) to produce the reduced form of the acceptor. This would require that the potential of XH be sufficiently below that of TPN to furnish sufficient energy for the phosphorylation during the electron transfer reaction. This is energetically feasible, since XH can be coupled to the photoproduction of molecular hydrogen in bacterial chromatophores and chloroplasts (ref. 3, 95). However, several lines of evidence seem to rule out this area as the phosphorylation site.

Bishop has shown that chloroplasts contain a substituted benzoquinone which is necessary for the Hill reaction, using either ferricyanide or DPIP as the electron acceptor (ref. 96). In comparing these two Hill oxidants, it was shown by Avron, et al. (ref. 94) that ferricyanide reduction results in a phosphorylation while DPIP photoreduction does not. Since the reduction of such widely differing electron acceptors as TPN, FMN and ferricyanide results in an accompanying phosphorylation with a similar yield of ATP per two electrons involved, it indicates that DPIP is being reduced at another site which must be A.

The common requirement for plastoquinone in all these Hill reactions could be explained by its functioning either in the oxygen evolution mechanism, or at site A shown in Fig. 9. There is no biochemical precendent for a

TWO LIGHT REACTIONS IN SERIES

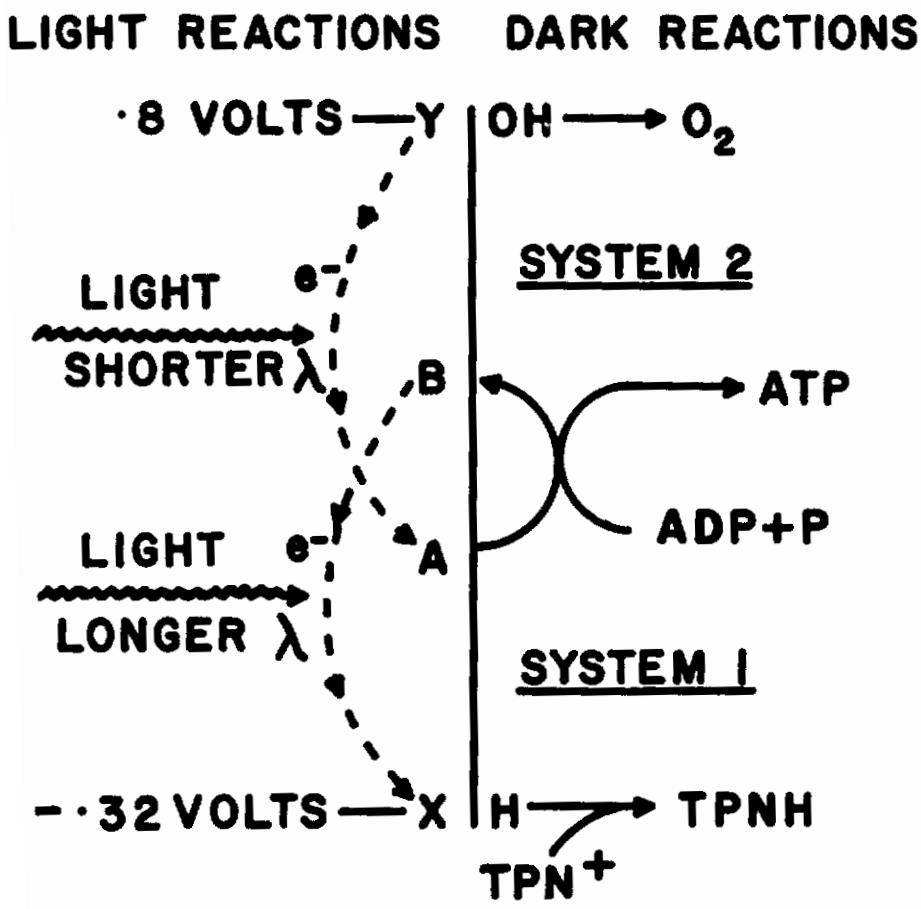


FIG. 9.

quinone functioning at such a high potential as that required for direct participation in the oxygen evolution system. The potential of site A has been estimated by Witt, et al. (called X in their scheme) as being zero (ref. 87), and they conclude that X is plastoquinone. Since the potential of site A is appropriate for plastoquinone, it is logical to assume that plastoquinone does act at site A.

TPN can be photoreduced by chloroplasts utilizing ascorbate and DPIP as the electron donating system in lieu of water (ref. 26). Arnon has shown that there is a phosphorylation associated with this reaction (ref. 97), and in a recent report has discussed the plastoquinone requirement for this reaction (ref. 98). Whereas plastoquinone is not needed for the transfer of electrons from ascorbate to TPN, it is required for the associated phosphorylation. If the phosphorylating step associated with this plastoquinone requirement was located between XH and TPN, this would require the quinone to function at a potential which must be close to -.32 volts, the potential of the TPN system. When these facts are considered together with the fact that the potential of the closely related ubiquinone is .098 volts (ref. 99), it is logical to place both the quinone and phosphorylation step at A as shown in Fig. 9.

The action spectra for the photoreduction of TPN and accompanying phosphorylation have been determined by Black, et al., with chloroplast fragments (ref. 100). The spectra showed only minor differences, with the red peak for TPN reduction occurring at 675 mμ and the corresponding peak for phosphorylation occurring at 680 mμ. The average quantum requirement was determined as 15 per ATP formed, and 9 for TPN reduction. This would correspond to 18 per oxygen molecule evolved, or about half the quanta required for intact cells. These features of the so called non-cyclic phosphorylation relating to TPN reduction are in sharp contrast to the cyclic photosynthetic phosphorylation catalyzed by phenazine methosulfate, PMS, which is discussed below. In the present case, there is a very close relationship between the terminal electron transfer reactions responsible for oxygen evolution and TPN reduction and the concomitant phosphorylation observed.

Site of Reduction of Ferricyanide

The precise stoichiometry of four ferric ions reduced per oxygen molecule evolved in the ferricyanide Hill reaction has been shown by Spikes (ref. 18). This transfer of electrons from water to ferricyanide is accompanied by the formation of one ATP molecule per two electrons if ADP and inorganic phosphate are also present (ref. 89, 94). Accepting the need for two light reactions and the location of the phosphorylation site at or near plastoquinone, it follows that the phosphorylation accompanying a ferricyanide Hill reaction is as shown in Fig. 10. Bishop has shown that plastoquinone is required for ferricyanide reduction by chloroplasts (ref. 96).

The reduction of ferricyanide by chloroplasts does not require PPNR, but otherwise resembles the reduction of TPN. In both cases the

FERRICYANIDE HILL REACTION

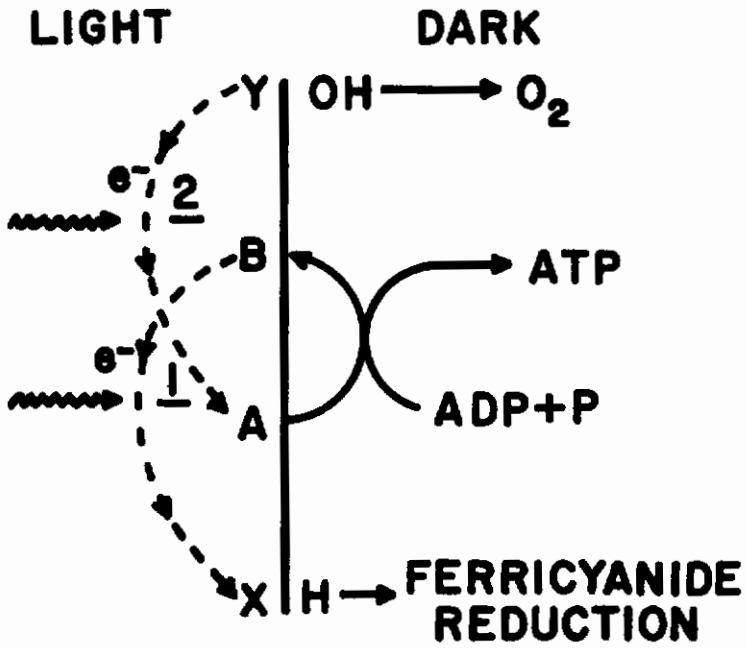


FIG. 10.

phosphorylation is coupled to the reduction of the added oxidant, the rate of reduction of both compounds is increased 3-4 fold by the addition of ADP and P (although the enzyme must be in excess in the case of TPN, plastoquinone is needed for both reactions and the action spectra are of the type relating to the complete photosynthetic process (ref. 84, 100). Although the two compounds differ quite widely in oxidation potential, their similarity of action in this case is not surprising. Minakami, et al. have recently investigated the sites of interaction of ferricyanide in the electron transport chain of mitochondrial preparations, and find the fastest reduction of ferricyanide occurs at the level of the flavoprotein associated with DPNH dehydrogenation (ref. 101).

Site of Reduction of Quinone and DPIP

During the reduction of indophenol dyes by illuminated chloroplasts, there is no accompanying formation of ATP, as shown by the experiments of Krogmann and Jagendorf with TPIP (2, 3, 6-trichlorophenol indophenol) (ref. 91). Likewise, there is no accompanying phosphorylation (ref. 89, 102) when benzoquinone is reduced under anaerobic conditions. In the presence of air, however, ATP is produced in a quinone Hill reaction (ref. 103). Also, TPIP will catalyze a phosphorylation reaction with chloroplasts in the presence of oxygen. Krogmann has proposed that under aerobic conditions the initial reduction of the dye in a Hill reaction is followed by a reoxidation of the reduced dye with YOH. The corresponding production of XH in the coupled system produces the ATP (ref. 104). Since the initial reduction of these compounds in a Hill reaction does not result in ATP formation, they should be regarded as reacting at A, as shown in Fig. 11.

The Hill reaction with indophenol dyes requires the presence of plastoquinone in the chloroplast and no ATP is formed in the reaction. Plastoquinone is also required for the phosphorylation associated with photoreduction of TPN by ascorbate-DPIP (ref. 98). At first glance, these data would seem to be incompatible. However, it has been shown by Ziegler and Doeg that the reduced form of the closely related benzoquinone coenzyme Q (ubiquinone) found in mammalian mitochondria reacts very rapidly in a non-enzymatic reaction with DPIP (ref. 105). A similar reaction would be logical for plastoquinone in the chloroplast and would explain (1) the lack of ATP formation even though plastoquinone is involved in the electron transfer reaction and (2) the very rapid rate of indophenol reduction in the Hill reaction (ref. 91). A similar rapid, non-enzymatic reaction between reduced ubiquinone and methylene blue has been reported by Redfearn (ref. 106).

The very interesting experiments of Levine and Smillie with mutant strains of Chlamydomonas reinhardi give strong evidence that DPIP is reduced via system 2 and does not involve both systems (ref. 107). One of their mutants (ac-21) will not reduce TPN in a regular Hill reaction yet will conduct an ascorbate-DPIP linked reduction of TPN, showing that the block in this mutant is not in the TPN reducing system. This mutant will, however, reduce DPIP in a Hill reaction showing the block is not in the oxygen system.

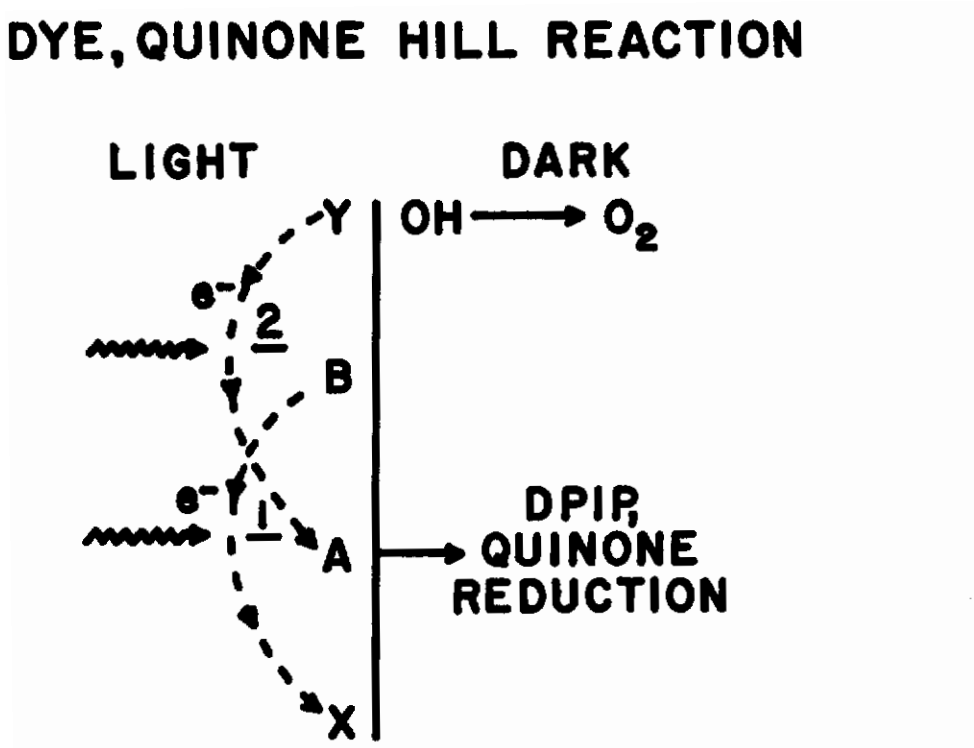


FIG. II.

The logical interpretation is that this mutant has a metabolic block somewhere between A and B which still allows DPIP reduction at site A. Indeed, the scheme of photosynthesis they advanced to explain these data is almost identical to the one proposed here.

The primary reason for locating both DPIP and quinone reduction at site A is that phosphorylation does not accompany their reduction under anaerobic conditions (ref. 89, 91, 102). This indicates that both cycles are not operating in a coupled manner. There are several data which should be considered, however, since they appear to be contradictory to this concept. These will be discussed below.

The action spectrum for DPIP reduction in a Hill reaction by chloroplasts was shown by Chen to follow closely the absorption spectrum of the chloroplasts (ref. 108), although there was a decline in activity at wave lengths longer than the main absorption band. Govindjee and Rabinowitch (ref. 79) have reported the quantum yield at different wave lengths for the quinone Hill reaction with whole Chlorella cells, showing that they are very similar to those of photosynthesis and show the characteristic decline above 680 mμ. San Pietro, et al. (ref. 109) have shown that the action spectrum for TPN photoreduction is also quite similar to the absorption spectrum of the chloroplast suspension used, with the usual drop in activity in the red. One might consider these similarities of absorption and action spectra as evidence that all three reductions used the same mechanism, and involved both light systems. However, one point should be kept in mind. In their examination of the action spectrum of enhancement in Chlorella, French (ref. 71) and Myers and French (ref. 78) showed that the pigment active in the long wave length system 1 (showed enhancement of chlorophyll b) was chlorophyll a 695. The active pigments in the short wave length system 2 (showed enhancement of chlorophyll a 695) were chlorophyll b, chlorophyll a 673 and perhaps chlorophyll a 684. This indicates that light of wave lengths up to 684 mμ is active in system 2, and this is already on the long wave length side of the absorption maximum of chloroplasts or or Chlorella. Likewise, the decline in quantum yield of photosynthesis with green algae does not commence until about 680 mμ (ref. 76), indicating that light of this wavelength is equally effective to both systems. Thus the bulk of the light absorbed by the chlorophyll system can be effective in system 2, and the similarity of the absorption spectrum of chloroplasts and the action spectrum of DPIP or quinone reduction in the Hill reaction does not preclude these reactions proceeding primarily via system 2.

Govindjee, et al. have also reported that the quinone Hill reaction in Chlorella showed the usual enhancement effect of photosynthesis, indicating that both light systems would be functional. Attempts to confirm this result have been unsuccessful, however, (ref. 110) and further information is needed on this most important point.

Ehrmantraut and Rabinowitch have extensively studied the kinetics of the quinone Hill reaction with chloroplasts and Chlorella cells, and report quantum requirements of 11-13 quanta per oxygen evolved (ref. 111). These values agree well with the values for photosynthesis and a ferricyanide

Hill reaction, and again would indicate that the quinone Hill reaction requires both system 1 and system 2 operating in the chloroplast. However, it is well known that quinone acts as a self-poison, and as the concentration of quinone is increased both the rate and final yield of oxygen decrease (ref. 111). Also, the photosynthesis and quinone Hill reaction vary widely in their response to light intensity (ref. 111). It seems entirely possible, therefore, that the reported quantum requirement for the quinone Hill reaction does not represent the chloroplast functioning as efficiently as it does in photosynthesis. In experiments of this type, failure to observe a lower quantum requirement with quinone does not rigorously exclude the possibility that only system 2 is required for the reaction, since it could be functioning in a less efficient manner. This applies also to the indophenol system, with which Chen reported a minimum quantum requirement of 8 quanta per dye molecule reduced, which would be 16 quanta per oxygen evolved (ref. 108).

The experiments of Witt and Muller (ref. 112) also indicate that DPIP is reduced at site A. In following the absorbancy changes at 515 mμ as a function of periods of illumination and darkness, they demonstrated that the 515 mμ component (related to plastoquinone and having an oxidation potential estimated to be about zero) was reduced in the light and that addition of DPIP caused an oxidation of the reduced compound. This clearly shows that DPIP can interact at this point in the photosynthetic system.

It is to be understood that as additional data become available, the concept of benzoquinone and DPIP reduction at site A may have to be changed. However, with the information presently at hand, the formulation shown in Fig. 11 agrees best with the experimental observations.

Oxidation of $DPIPH_2$

As discussed in a previous section, chloroplasts have the ability to either oxidize water in the regular photosynthetic process (produce oxygen) or to oxidize ascorbate in the presence of DPIP (cause a net oxidation of ascorbate). Since ascorbate reduces DPIP non-enzymatically, the addition of these compounds results in reduced indophenol dye, $DPIPH_2$ and excess ascorbate. The $DPIPH_2$ is then the compound which interacts with the oxidizing equivalents produced photochemically, and the excess ascorbate serves as a reservoir of reducing power to rereduce any $DPIPH_2$ oxidized.

The oxygen evolving system can be made non-functional by poisoning with DCMU, hydroxylamine (ref. 55) or CMU (ref. 64), by aging (ref. 26), by storing bean leaves in the cold (ref. 65), by exposure to high concentrations of Tris buffer (ref. 113) and by a chloride deficiency (ref. 33). With the oxygen evolving system non-functional, a photoreduction of oxidants such as TPN and DPN can still be accomplished by adding ascorbate and DPIP. The question becomes then, where is the $DPIPH_2$ oxidized?

Considering the scheme given in Fig. 9, there are three sites where $DPIPH_2$ could react to allow the chloroplast to carry out photoreductions via XH. These three sites are Y, A and B. Reaction of $DPIPH_2$ at sites Y and A

would still allow photosynthetic phosphorylation to proceed, while reaction at site B would not. Arnon has reported that the reduction of TPN by chloroplasts using the ascorbate-DPIP system as the electron donor does result in ATP formation (ref. 97), with a ratio of ATP to TPNH close to 1. This would exclude B and indicate that either Y or A is the site of $DPIPH_2$ oxidation, since reaction at either of these would allow the phosphorylation step to proceed.

Arnon has determined a partial action spectrum for TPNH formation accompanying ascorbate-DPIP oxidation by chloroplasts in the light (ref. 3, 97). Oxygen evolution in a regular Hill reaction showed a peak around 644 mu in these experiments, while the TPNH reduction accompanying ascorbate-DPIP oxidation showed a minimum at about 661 mμ and then increased at longer wave lengths up to 699 mμ. The latter reaction did not show a maximum in the spectral region investigated, but the distinction between the two systems is apparent. Hoch and Kok (ref. 114) have also determined the action spectrum for this reaction, and report a peak at about 700 mμ. These data show that the ascorbate-DPIP reduction of TPN by illuminated chloroplasts proceeds by the longer wave length system, or system 1 in Fig. 9. All these data exclude the operation of system 2 and point to site A as the locus of $DPIPH_2$ oxidation by illuminated chloroplasts. This is shown in Fig. 12.

One other line of evidence points toward site A as the locus of $DPIPH_2$ oxidation. Witt, et al. (ref. 87, 88, 112) have investigated the characteristic changes in absorbancy of chloroplasts taking place upon illumination. Those occurring at 515 mμ are ascribed to plastoquinone by these workers and are related to site A in Fig. 9 of this paper. The compound represented by the changes at 515 mu can be reduced by light of shorter wave length (system 2) and is oxidized in the dark by DPIP. Addition of $DPIPH_2$ to the system causes a three-fold increase in the changes at 515 mμ upon illumination, which is evidence that it can reduce this component. All these data are in agreement with the idea that $DPIPH_2$ is oxidized at site A.

The evidence available indicates that site A is probably plastoquinone. In the preceding section it was pointed out that DPIP, when functioning as a Hill oxidant in the oxidized form, is most likely reduced at site A. This is logical since benzoquinones do react very rapidly with DPIP in a non-enzymatic reaction (ref. 105). Since the potential of the DPIP system (E_0' = .217 volts at pH 7) is not far from that of ubiquinone (E_0' = .098 volts), which is closely related to plastoquinone, it would be possible for this couple to work in both directions in an efficient manner. It may also be that the oxidation potential of the plastoquinone is closer to that of DPIP when the quinone is contained in the ordered chloroplast. When the chloroplast is presented with the oxidized form of the dye, DPIP, it would be reduced at site A, oxygen would be given off at Y upon illumination, and a Hill reaction would take place via system 2. When the chloroplast is presented with the reduced dye, $DPIPH_2$ and some compound such as TPN which can react at X, then $DPIPH_2$ would couple to site A and transfer electrons through B to X and result in TPN reduction in the light. Jagendorf and Marguiles (ref. 64)

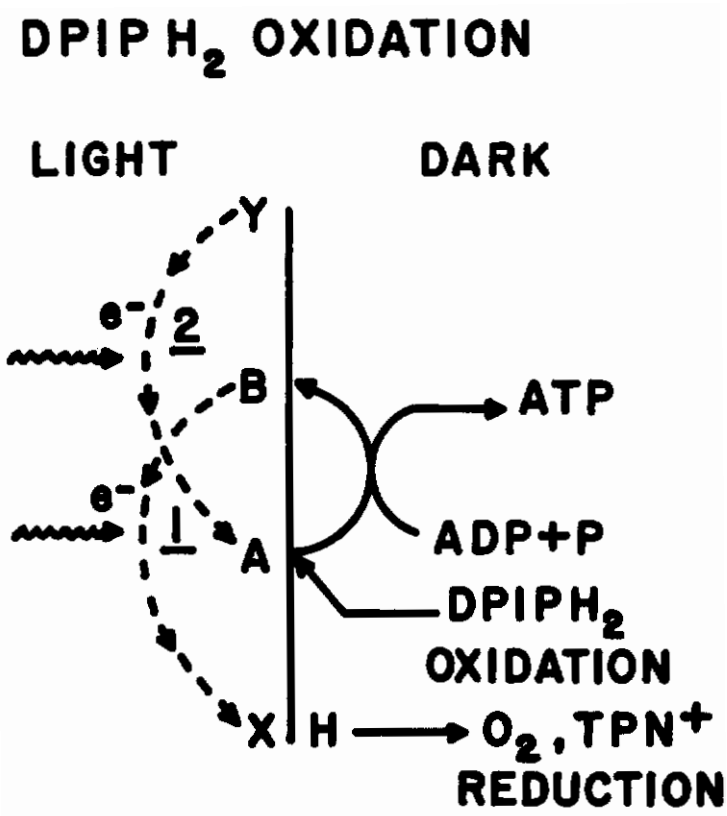


FIG. 12.

showed that a couple consisting of ascorbate and PMS acted like the ascorbate-DPIP couple in chloroplast reactions. As shown below, it is probable that the reduced form of PMS also reacts at A, which is in agreement with the $DPIPH_2$ interaction.

The very interesting yet somewhat complicated experiments of Krogmann and co-workers must be considered in light of the proposal that $DPIPH_2$ is oxidized at site A. When $TPIPH_2$ was provided in the reduced form (with ascorbate or glutathione) a coupled uptake of oxygen and formation of ATP was observed (ref. 115, 116). This process was termed oxidative photophosphorylation, since it proceeded only in the presence of oxygen and oxygen was taken up in the process. The phosphorylation resulting from oxidation of $TPIPH_2$ (with ascorbate of glutathione) must involve the passage of electrons from A to B subsequent to the interaction of the reduced dye at site A. Oxygen would be required to accept the hydrogen from XH, thus allowing the process to proceed.

The TPIP Stimulated Phosphorylation Reaction

Addition of TPIP to a chloroplast system stimulates ATP formation (after the initial reduction of the dye (ref. 115, 116). By the use of oxygen isotopes it has been shown that the presence of TPIP also results in a higher rate of oxygen uptake and oxygen evolution by the same chloroplasts (ref. 104). Furthermore, the formation of ATP was inhibited by CMU to the same extent that a Hill reaction would be inhibited under similar conditions (ref. 64). This indicates that ATP formation resulting from the presence of TPIP is linked to the oxygen evolution system and must be a function of the normal formation of oxygen from YOH, the transfer of electrons from Y through A and B to X, where they form XH. The XH then reacts with molecular oxygen, completing the electron transfer sequence and allowing a phosphorylation reaction to take place during electron transfer from A to B.

It remains a question, therefore, why this reaction does not proceed readily in the absence of TPIP or why TPIP should stimulate a reaction that could take place anyway. One explanation for this phenomenon is related to the extent of reduction of A. Following the reduction of TPIP in a Hill reaction, one would expect A to be predominantly in the reduced form, since it would be in equilibrium with the reduced dye and would also be subject to reduction from the photochemical reactions of system 1. This should also be reflected in a more reduced state for B and would make system 2 more efficient in transferring electrons to X to form XH. This would result in a more rapid oxygen uptake, which would be coupled to oxygen evolution from YOH and ATP formation between A and B. It is probable that quinone reacts in a similar fashion as the indophenol dyes. The reduction of quinone under nitrogen is not accompanied by ATP formation (reaction at site A), but its subsequent reoxidation in the air results in one ATP per hydroquinone reoxidized (via the sequence A to B to X) (ref. 103).

When chloroplasts are illuminated in the presence of ethanol and

catalase, oxygen is consumed and acetaldehyde is produced (ref. 60, 61). In this case oxygen is still being produced by the oxygen evolving system, but XH reacts with oxygen to produce hydrogen peroxide, which is reacted with ethanol via catalase to produce acetaldehyde (ref. 117). Addition of ascorbate and DPIP to such a system results in a decrease in the rate of oxygen evolution and an increase in the rate of oxygen uptake (ref. 118). This is consistent with the concept of $DPIPH_2$ being oxidized by the chloroplast in the light, resulting in a transfer of electrons to XH.

The ascorbate-DPIP couple has been an important tool in investigation of the electron transfer reactions of chloroplasts. It is significant that the same couple can function in both the chloroplast system of plants and the chromatophore system of bacteria. In Table 2 it was shown that in the presence of ascorbate and DPIP, both chloroplasts and chromatophores could catalyze the same reaction, e. g. a photooxidation of ascorbate (via DPIP) and a photoreduction of DPN (a crude preparation of PPNR contains a transhydrogenase which allows DPN to be photoreduced). The photosynthetic apparatus in bacteria is in many respects similar to system 1 of plants. Indeed it seems that the difference between plants and bacteria is that plants have the additional photosynthetic machinery associated with system 2, thus allowing the plant to use water as the hydrogen donor for the photosynthetic process.

Arnon has confirmed the earlier report that chromatophores of Rhodospirillum rubrum can catalyze a photoreduction of DPN coupled to the photooxidation of ascorbate-DPIP, and has extended the work to show that this reaction was coupled with ATP formation. Fig. 12 shows the proposed mechanism of $DPIPH_2$ interaction with the Chloroplast system. If system 2 were removed from this figure, the remaining scheme would apply just as well to the bacterial system as to the chloroplast. It should be mentioned also, that the chromatophores of R. rubrum have been shown to catalyze a photooxidation of $DPIPH_2$, other reduced dyes and ferrocytochrome c (ref. 46, 47) in the absence of excess ascorbate. This is a direct demonstration of the photooxidations which have been discussed in this section, but which were not seen directly since excess ascorbate was present to rereduce the dye once it was oxidized.

The above discussion on the site of action of $DPIPH_2$ has been based upon information gained from a number of investigators in different laboratories. The selection of A for the site of action, as shown in Fig. 12, appears to be the most logical one. Accepting the phosphorylation site as shown in Fig. 9 between the two systems, it was possible to discard site B as a possibility for interaction of $DPIPH_2$, since the photoreduction of TPN by ascorbate-DPIP results in ATP formation. The choice of A over Y as the site of interaction was made primarily from the action spectra of TPN reduction associated with $DPIPH_2$ oxidation (ref. 97, 114). Obviously, more information is needed concerning the action spectra for the various partial reactions now possible using the DPIP system. A more complete set of data is required for the ascorbate-DPIP coupled reduction of TPN, and especially the action spectrum for ATP formation accompanying this reaction is needed

In addition, the action spectrum of TPIP-stimulated ATP formation and oxygen exchange would show if the mechanisms proposed above are correct.

Stimulation of ATP Formation by Cofactors Other Than PMS

There are a number of compounds which stimulate the formation of ATP by chloroplasts in the light. FMN and vitamin K_3 have been extensively used in this capacity, and most information is available on their mode of action. Following the initial discovery that these compounds stimulated photosynthetic phosphorylation (ref. 119, 120), it was assumed that they catalyzed a cyclic flow of electrons in the chloroplast which resulted in ATP formation, since no change in gas pressure was observed, and they were active under apparently anaerobic conditions. It was subsequently shown that this was not correct, and that these compounds catalyzed the ATP formation process by accepting the electrons from XH and then reacting in their reduced forms with oxygen. The photoreduction of FMN has been shown by Good and Hill to be accompanied by oxygen evolution (ref. 121). It was also shown that at low concentrations these compounds did require oxygen in order to function as catalysts of phosphorylation in the light (ref. 122 - 124). At higher concentrations their activity was independent of oxygen concentration. This probably resulted from their avid consumption of the photosynthetically produced oxygen, since inclusion of catalase and ethanol to remove hydrogen peroxide produced by reoxidation of the $FMNH_2$, and thus to further remove oxygen, again restored the oxygen dependency (ref. 125).

Further proof that the stimulation of ATP formation by vitamin K_3 and FMN resulted from a stimulation of oxygen exchange (more evolution via the normal process and more uptake by reoxidation of the reduced cofactor) came from isotope exchange experiments (ref. 92, 125, 126). Both FMN and menadione catalyzed an oxygen exchange reaction of sufficient speed to account for their effect on ATP formation. Final convincing evidence for this mechanism of stimulation comes from the experiments in which oxygen evolution was inhibited by DCMU, CMU or other inhibitors. These inhibitors inhibited ATP formation catalyzed by these cofactors to the same extent they affected oxygen evolution in the Hill reaction (ref. 127).

The data given above show that these cofactors of photosynthetic phosphorylation are active by virtue of their ability to act in an oxygen exchange reaction involving the whole electron transfer system of the chloroplast, as shown in Fig. 13. Arnon has stated that in addition to this method of catalysis, these compounds can also catalyze a cyclic photosynthetic phosphorylation, in which oxygen is not evolved nor is there any net reduction by XH (ref. 95, 97, 128). The basis for this concept lies in the fact that when FMN and vitamin K_3 are utilized as cofactors for ATP formation in the presence of air, the reaction requires oxygen evolution. Thus chloride deficiency, the presence of CMU or o-phenanthroline (all inhibitors of oxygen evolution) cause inhibition of ATP formation. However, in the absence of air (under nitrogen) these conditions have much less capacity to

STIMULATION OF PHOTOSYNTHETIC PHOSPHORYLATION BY COFACTORS

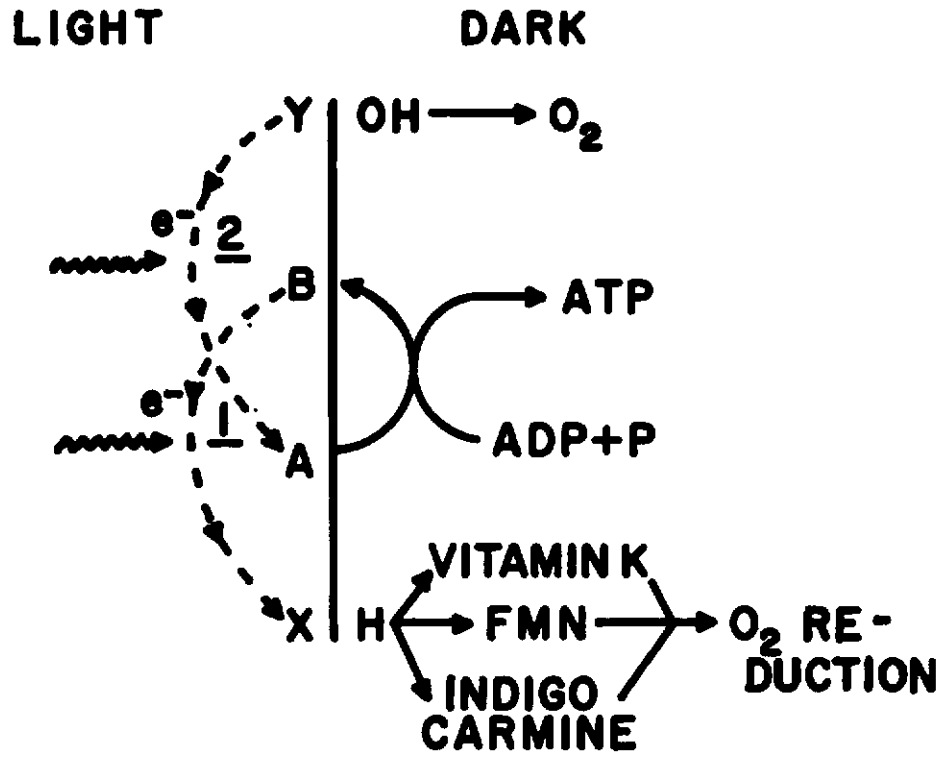


FIG. 13.

inhibit ATP formation. The logical explanation advanced by Arnon is that in the presence of oxygen, any FMN or vitamin K_3 reduced by XH and re-oxidized by oxygen cannot enter into the reaction again unless it is re-reduced. This rereduction of FMN or menadione requires a functioning oxygen evolution system in order for water to reduce these cofactors. Thus, inhibitors of oxygen evolution inhibit the photophosphorylation. Under nitrogen there is no reoxidation of the cofactors by oxygen (or only partial reoxidation by the oxygen produce photosynthetically) and thus the cofactors are not removed from action. Of the two factors, vitamin K_3 appears to function better in a cyclic fashion. Other laboratories have not been able to obtain similar results, however (ref. 64, 65, 124).

In a series of experiments involving FMN and vitamin K_3 catalysis of phosphorylation in the absence and presence of catalase and ethanol, it was shown that vitamin K_3 was more resistant to the effect of catalase and ethanol (ref. 125). If these two compounds were functional only through the oxygen exchange mechanism, the removal of extra oxygen (and the photosynthetically produced oxygen) via the catalase and ethanol coupled to the peroxide produced when the reduced factor is reoxidized would stop both reactions. The partial resistance of vitamin K_3 to catalase and ethanol indicated some cyclization of electrons with this compound. Accordingly, one may possibly assign to vitamin K_3 the dual role of functioning in a non-cyclic fashion via oxygen exchange, and also in a cyclic fashion.

In considering all the information available, it would seem that the scheme shown in Fig. 13 explains the primary mode of action of these activators. Although less is known about indigo carmine, it probably works in the same manner. Under aerobic conditions where these compounds catalyze an oxygen exchange, both systems 1 and 2 would be operative, and this would be a photosynthetic phosphorylation of the non-cyclic type with oxygen as the oxidant for XH. Under anaerobic conditions only system 1 would be required to function, and the phosphorylation with vitamin K_3 would be of the cyclic type (ref. 95, 125). Again action spectra of these two types of reactions would be extremely useful in determining the accuracy of the suggested mechanism of action of these compounds. Kok has reported that the FMN and vitamin K_3 stimulated phosphorylation reactions show an action spectrum that does not peak at 700-710 mμ as does the PMS stimulated system (ref. 84). This would indicate these compounds were functioning primarily in the non-cyclic mechanism involving oxygen exchange. However, the conditions of these reactions (degree of anaerobicity) were not reported.

Fork reports that vitamin K_3 phosphorylation by chloroplasts under aerobic conditions show a maximum at 668 mμ (ref. 129), so, in this case, the chloroplasts are using both systems 1 and 2, and the vitamin K_3 is functioning in a non-cyclic manner in an oxygen exchange reaction.

Stimulation of ATP Formation by PMS

Jagendorf lists three criteria for determining if catalysis of

photosynthetic phosphorylation by a given compound is by means of oxygen exchange or by a cyclic electron transfer process (ref. 127). In all these respects PMS differs from FMN and menadione: (a) It does not catalyze an oxygen exchange reaction (ref. 92), (b) the presence of inhibitors of oxygen evolution does not affect its activity (ref. 130), and (c) it is independent of oxygen concentration except at the lowest concentrations (ref. 122). The oxidized form of PMS, pyocyanin, is as effective, or more so, than is PMS, and, in the presence of oxygen and light, PMS is converted to pyocyanin. Hill and Walker have shown that PMS has little or no activity when oxygen is excluded (ref. 131), so the small concentrations of oxygen required for PMS activity may be related to this conversion. The cyclic mechanism of PMS activation of photosynthetic phosphorylation is shown in Fig. 14.

Although PMS can catalyze a purely cyclic photophosphorylation, it is also capable of catalyzing a non-cyclic type associated with oxygen exchange. The reduced dye is readily oxidized by molecular oxygen, and once this happens, it must be reduced again by means of XH formation coupled to oxygen evolution. Thus, in the presence of air, inhibitors of oxygen evolution such as CMU will inhibit the PMS stimulated ATP formation. This inhibition becomes less as the system is flushed with nitrogen gas (ref. 132).

The coupling of PMS in the anaerobic photosynthetic phosphorylation system is shown between XH and A in Fig. 14. The subsequent transfer of electrons from A to B in the dark would result in ATP formation, and the entire process could be maintained by system 1. The action spectrum for PMS catalyzed photophosphorylation under anaerobic conditions has been shown by Kok and Hoch to peak in the area of 710 mμ, which definitely places this reaction in the realm of the long wave length pigment system associated with system 1 (ref. 84). Under aerobic conditions, the PMS reaction shows a maximum in the red at a shorter wave length, with Jagendorf, et al. reporting a value of about 675 mμ (ref. 133) and Black, et al. reporting a value of 680 mμ (ref. 100). This is in agreement with the proposed scheme, since with no oxygen present the cyclic type of phosphorylation catalyzed entirely by system 1 would take place. With air present, some of the reduced PMS could react with it, and to compensate for this oxidative removal of the reduced PMS, both systems 1 and 2 would have to function in order to rereduce the PMS.

Petrack has reported that chloroplast particles from blue-green algae readily lose their phycocyanin to the suspending medium, and that such particles are unable to evolve oxygen in the light (ref. 134). However, such particles still catalyze the PMS photosynthetic phosphorylation. This is again an example of the PMS phosphorylation reaction taking place when the oxygen evolving system is non-functional and again points to system 1 as the site for PMS phosphorylation. In addition, PMS stimulates the phosphorylation process in the photosynthetic bacteria while FMN and menadione do not (ref. 47, 49), and the similarity between the bacterial system and system 1 of chloroplasts has been pointed out above.

PMS STIMULATION OF PHOTOSYNTHETIC PHOSPHORYLATION

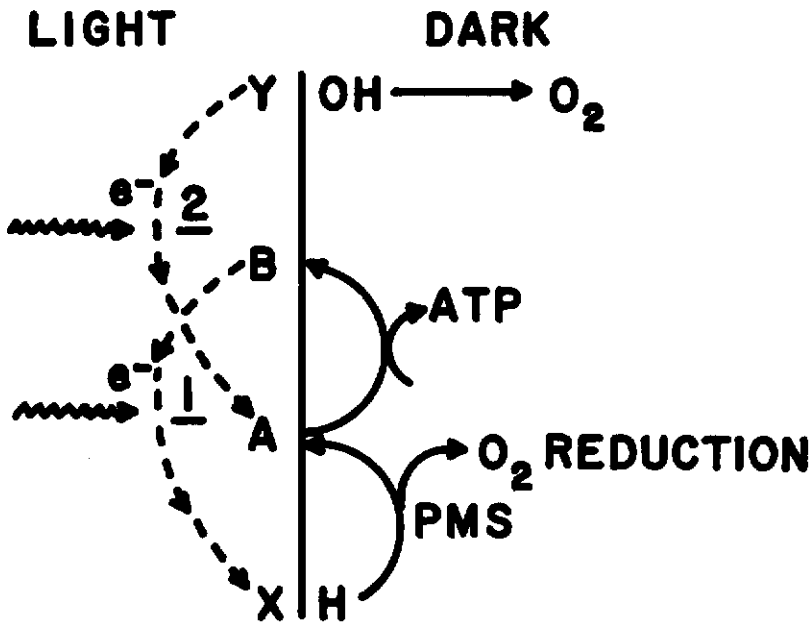


FIG.14.

Another distinguishing characteristic of PMS in the photophosphorylation reaction is the response it shows to light intensity. Whereas both FMN and vitamin K_3 are more active than PMS at low light intensities, their activity saturates at about 20,000 lux while the PMS system does not saturate even at 50,000 lux. At this light intensity the PMS system is more active than either of the other systems (ref. 95). This again is consistent with the scheme shown in Fig. 14 since the PMS reaction involves only system 1, and apparently this system does not saturate at these high light intensities. The FMN and menadione systems require the operation of both systems 1 and 2.

Black, et al. have reported that an average of 180 quanta were required per ATP formed in the PMS system, whereas the TPN related phosphorylation (which would be similar to the FMN and vitamin K_3 systems) required an average of 15 quanta (ref. 100). Thus, the PMS system is very inefficient in utilizing the light energy. This helps to explain the response of the PMS system to varying light intensities.

CHLOROPLAST COMPONENTS NOT DIRECTLY RELATED TO OXYGEN EVOLUTION

The functional components of the chloroplast will be discussed in light of the proposal represented in Fig. 9, in which two separate photochemical systems are coupled via dark, enzymatic reactions to effect the removal of hydrogen from water and transfer it to TPN. This section will deal with the components listed as A, B, and X in Fig. 9.

Nature of A

The scheme presented in Fig. 9 is generally similar to those advanced by a number of investigators. As proposed, sites A and B would consist of the terminal ends of an electron transport chain. This span would involve one site for ATP formation when electrons were passed from A to B. As shown by Hill and co-workers, chloroplasts are known to contain two cytochromes, cytochrome b_6 and cytochrome f, which are characteristic of the green parts of the cell and thus are very likely involved in the photosynthetic apparatus (ref. 135, 136). Hill and Bendall (ref. 86) have proposed a scheme similar to that given in Fig. 9 and have suggested that cytochrome b_6 corresponds to A in Fig. 9. They present this merely as a suggestion. However, there have been some investigations on the absorbancy changes occurring at 560 mμ upon illumination, which could be interpreted as a reduction of cytochrome b_6 under the influence of light (ref. 137-139).

Bishop has examined the function of the very important compound plastoquinone in chloroplasts (ref. 96). Earlier experiments by Lynch and French (ref. 140) had shown that chloroplasts extracted with petroleum ether were inactive in the Hill reaction, and partial restoration was effected by adding B-carotene back to the chloroplasts. Bishop, however, showed that it was not the B-carotene which was responsible, but rather vitamin K contained in the preparation (ref. 96). Although vitamin K derivatives did restore this activity, Bishop showed that the normal chloroplast contained not vitamin K, but a benzoquinone very similar in structure to ubiquinone, which is an essential component of the electron transport chain in mammalian mitochondria. Plastoquinone had previously been isolated and studied by Crane (ref. 141). Fig. 15 is taken from the paper by Bishop and shows the plastoquinone requirement for a ferricyanide Hill reaction with petroleum-ether-extracted chloroplasts (ref. 96). The same paper shows that the quinone is also needed for the reduction of DPIP in a Hill reaction.

As discussed below, site B is most likely cytochrome f. With this as the electron donating compound for the photochemistry of system 2, the assignment of cytochrome b_6 and plastoquinone at site A gives the biochemically pleasing situation of an electron transport chain similar to that observed in mitochondria and covering a potential span which is small enough

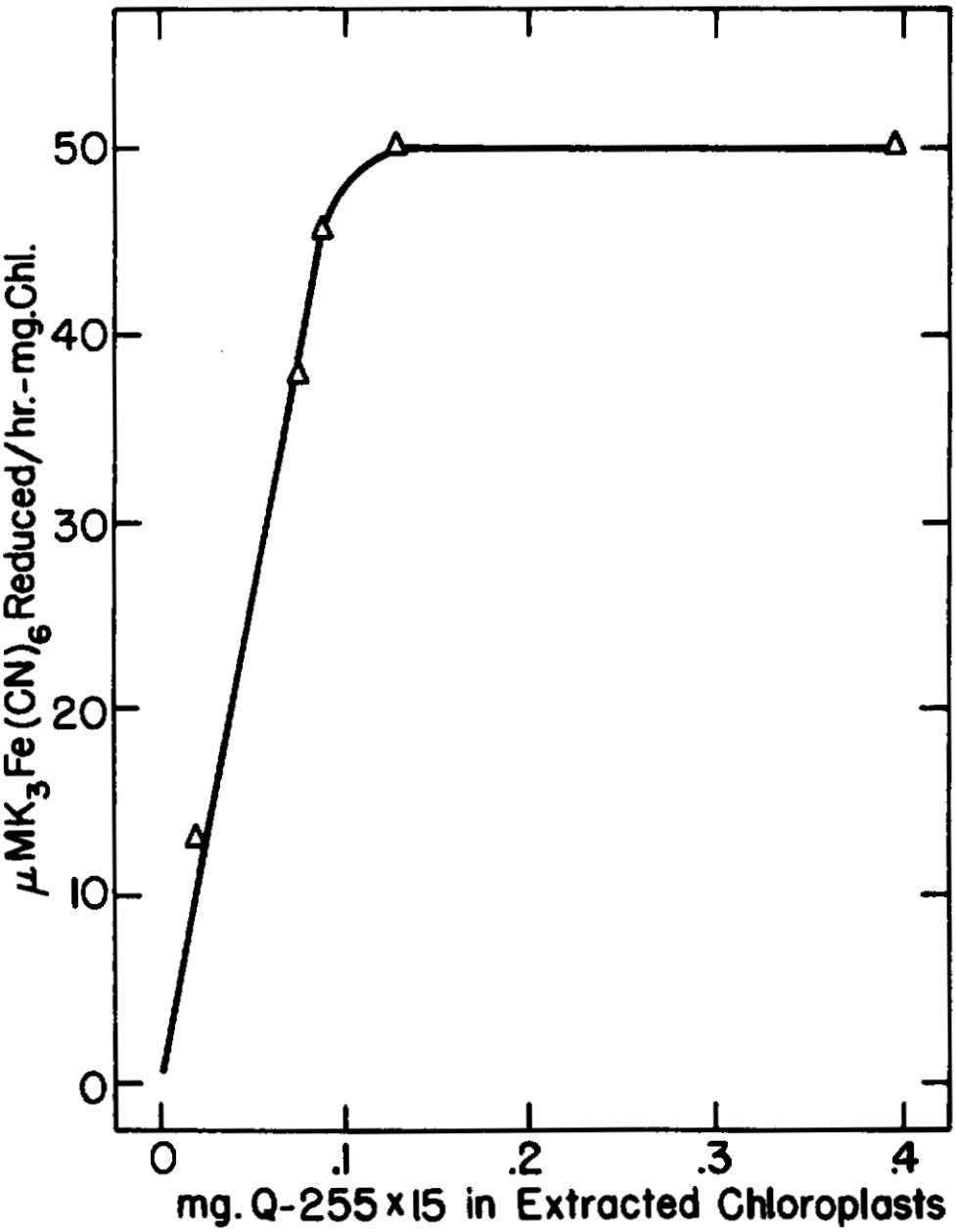


Fig. 15. Plastoquinone requirement for Hill reaction by sugar beet chloroplasts. The rate of ferricyanide reduction is shown to be a function of the plastoquinone remaining in chloroplasts extracted in varying degrees to remove plastoquinone. Taken from Bishop (96).

to be rapidly interacting yet large enough to provide the energy for the production of one ATP. Such a system working in a coupled manner would explain the observed relationship between TPN or ferricyanide reduction and ATP formation. A direct reduction of DPIP at A would explain the lack of phosphorylation associated with this dye and would also account for the distinctive nature of the Hill reaction when it is reducing DPIP. Also, the other related phenomena are consistent with this formulation.

Witt, et al. (ref. 87, 88) have reported on a series of investigations into the absorbancy changes associated with illumination of algae and chloroplasts. It is impossible to go into much detail concerning this very significant work, but a few points should be mentioned. By following absorbancy changes produced in very short periods after turning on and off the illunina-ting source and by determining the effect of temperature, redox reagents and various treatments, they have been able to related certain changes with the primary photochemical acts and other changes with the dark enzymatic reactions. The absorption changes designated as type 2b are temperature dependent, are associated with a system of oxidation potential of approximately zero volts, are lost when the chloroplasts are extracted with petroleum ether, and they are restored when the extracted material is recondensed on the chloroplasts. These absorption changes are caused by a reduction process, and excitation by light of 710 mμ (system 1) decreases the observed changes. All these characteristics fit with the scheme shown in Fig. 9, with A representing either plastoquinone or plastoquinone in redox coupling with some other agent. It seems plausible, then, that site A in Fig. 9 involves at least plastoquinone and may also include cytochrome b_6.

Nature of B

There seems to be a general agreement that the compound represented as B in Fig. 9 is cytochrome f. This cytochrome was discovered and characterized by Hill and Scarisbrick (ref. 135), is found only in chloroplasts, and becomes oxidized under the influence of light, as shown by a decrease in absorption at 555 mμ (ref. 137, 142 - 144). A very distinct demonstration of the oxidation of cytochrome f by chloroplasts was shown by Witt, et al. (ref. 87), in which Chlorella cells were illuminated in 710 mμ light, corresponding to system 1 in Fig. 9. This oxidation was temperature insensitive, taking place at -150° C. In algae, cytochrome f is replaced by another high potential cytochrome (ref. 145). In both cases the potential of the cytochromes agrees with the potential of . 37 volts calculated for B by Witt, et al. (ref. 87, 88).

As mentioned above, system 1 of plant photosynthesis is very similar to the photosynthetic apparatus of the photosynthetic bacteria. When only system 1 operates in plants, a cytochrome is oxidized as one terminal reaction, and TPN is reduced at the other terminal point. This reaction is catalyzed by a chlorophyll a, and the oxidation of the cytochrome is not temperature dependent. With bacterial systems the same reactions take place. Chance and Nishimura have shown that in the case of Chromatium,

light causes a temperature-insensitive oxidation of cytochromes (ref. 44). Smith, et al. have shown a similar type of reaction in Rhodospirillum rubrum (ref. 41). Using chromatophores of R. rubrum, Vernon has demonstrated a photooxidation of added ferrocytochrome c (and redox dyes in the reduced form, including $DPIPH_2$) under strictly anaerobic conditions, as shown in Fig. 16 (ref. 46). This oxidation was dependent upon the presence of an electron acceptor such as fumarate or DPN and has all the essential components of system 1 as shown in Fig. 9. The similarity of the bacterial system with the partial plant photosynthetic reactions catalyzed by plant chloroplasts when only system 1 is operative, has been shown and discussed by other workers (ref. 52, 33, 97, 127).

When only one light reaction was considered, the experimentally shown oxidation of cytochrome f by chloroplast systems in the light was taken to mean that it functioned close to Y and was oxidized by Y (ref. 33). This presented an untenable situation in terms of current knowledge of the cytochromes, since the proposed cytochrome would have to function at a potential of .8 volts to couple with the oxygen evolving system. With the knowledge that two light reactions were involved, however, this problem was obviated, and it allows the known experimental facts of cytochrome oxidation in the light to be correlated with a cytochrome function at a lower potential.

The Nature of X

In the various schemes representing photosynthesis, X has been used to represent the compounds which, through a photochemical act involving the chlorophyll system of the chloroplasts, serves as the hydrogen donor for carbon dioxide fixation. Through the extensive investigations of San Pietro's group, it was shown that the first readily demonstrable reaction of XH was the coupled reduction of TPN via the enzyme PPNR (ref. 27, 146). In addition to TPN, XH is capable of reducing those Hill reagents which are coupled to ATP formation. The rate of reduction of one of these, ferricyanide, is stimulated by the addition of ADP and inorganic phosphate to the same extent that TPN reduction is (when PPNR is present in excess).

It is very possible that the X shown in Fig. 9 may be the P700 compound which has been investigated by Kok and his group. This P700 pigment is a chlorophyll a bound to some other compound. Kok has suggested a lipoprotein as the binding agent (ref. 147). The bleaching of the 700 band responds to many of the reagents which effect the photosynthetic process. Many of these changes are consistent with the idea that under the influence of light P700 donates an electron to some acceptor (TPN, cytochrome c, ferricyanide) thus becoming oxidized. Subsequently, the oxidized P700 can accept an electron from B (cytochrome f) to resume its initial form. Since B (cytochrome f) is coupled to system 1 and is reduced by it, this would explain the reverse effects of long and short wave lengths upon the compound. The experiments of Witt, et al. (ref. 88) correlate the oxidation of the P700

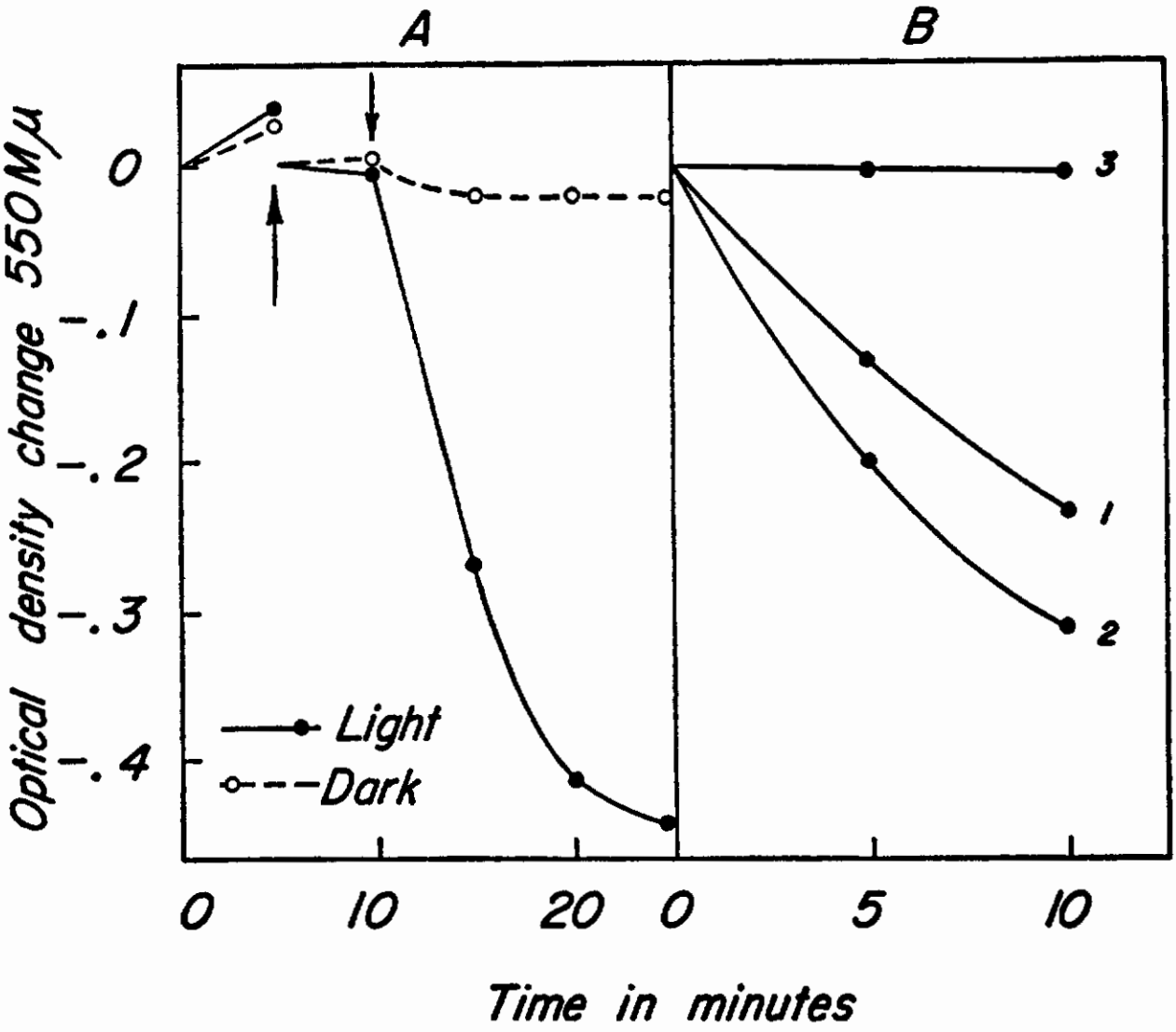


Fig. 16. The photooxidation of mammalian cytochrome c by chromatophores of Rhodospirillum rubrum under anaerobic conditions. This photooxidation reaction, represented by the solid line in A, was dependent upon the presence of an electron acceptor which could couple with it via the photochemical and enzymatic components of the chromatophore, as shown by the reaction following addition of fumarate at the last arrow in A. Taken from Vernon (46).

with a subsequent oxidation of the cytochrome component of chloroplasts.

Relating to the above discussion is the fact that isolated chlorophylls have the ability to catalyze a photoreduction of TPN in aqueous media when ascorbate is the electron donor (ref. 29). Fig. 17 shows the photoreductions effected by this system. It should be pointed out, however, that this reaction showed no specificity for chlorophyll type, nor did it even require the special features of the chlorophyll molecule, since pheophytins and other porphyrins were also active. It does point out, however, that chlorophylls can couple to the TPN system, thus, allowing its reduction in the light.

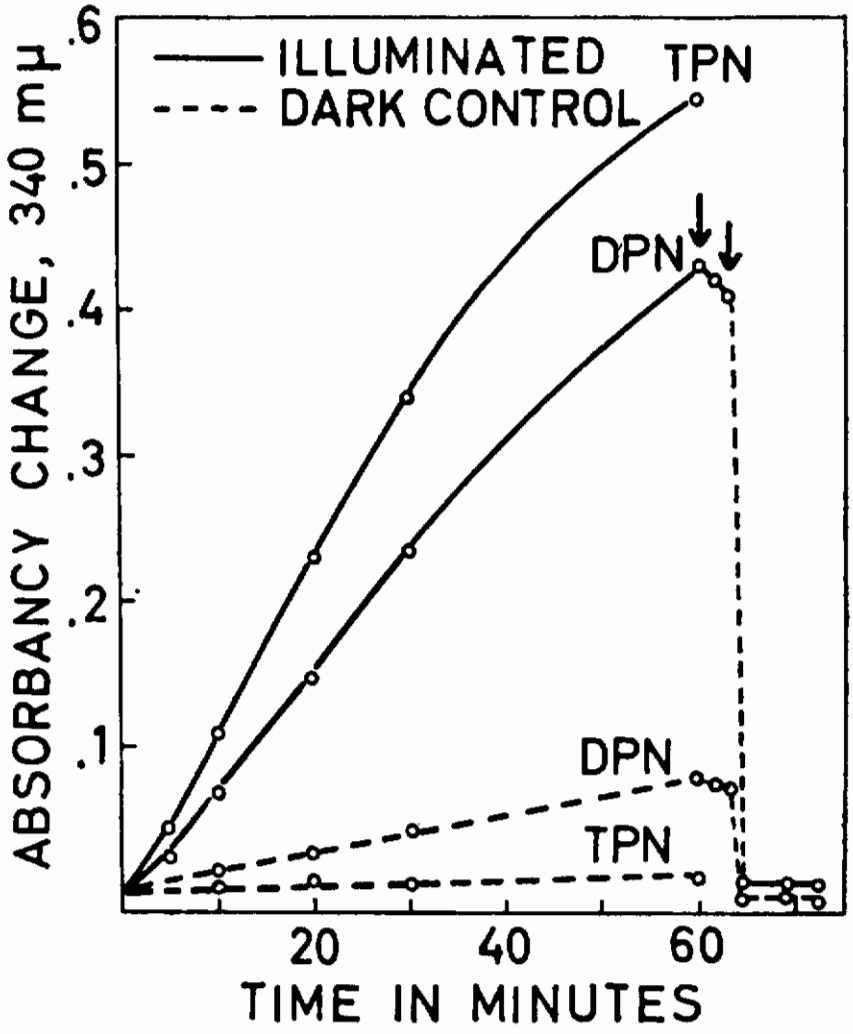


Fig. 17. Photoreduction of DPN and TPN by a crude chlorophyll preparation prepared by acetone extraction of spinach. These experiments were performed in aqueous media, and the photoreductions required the presence of PPNR. At the last arrow, lactic dehydrogenase and pyruvate were added to the DPN system. Taken from Vernon (29).

THE OXYGEN EVOLVING SYSTEM

A Form of Chlorophyll as Y

Franck has advanced the idea that the splitting of water to produce oxygen takes place while the water molecule is bound to the chlorophyll molecule (ref. 82, 83). The effect of light would be to remove the hydrogen from water to form OH, which would be at the oxidation level of a peroxide. The OH would then be transferred to some enzyme system which would accomplish the dismutation necessary to produce oxygen from this oxidized fragment of water. Franck retains the idea that the acceptor molecule is phosphoglyceric acid (PGA), although evidence points toward the fact that PGA reduction is a secondary reaction catalyzed by TPNH which is formed from XH. The site of water attachment to the chlorophyll was suggested as carbons 9 and 10 in ring V, which are highly active.

Vishniac and Rose reported that illumination of chloroplasts in tritiated water resulted in the incorporation of tritium into the chlorophyll (ref. 148). This would fit with Franck's scheme of water participation in the reaction, but these experiments have not been repeated in other laboratories.

There is no direct evidence for the hypothesis that water is oxidized while it is bound to a chlorophyll molecule. However, there is ample evidence to show that water is bound to chlorophyll and that this profoundly affects its physical properties. Livingston has shown that whereas chlorophyll dissolved in absolutely dry benzene shows no fluorescence, the admission of traces of water vapor produces a fluorescent state of the chlorophyll molecule (ref. 149, 150). In studying the ESR signals of crystalline chlorophyll upon illumination, Holmogorov and Terenin observed that the presence of water vapor was necessary for the production of the light-dependent signals (ref. 151). Anderson and Calvin, have shown that the ESR signals produced by extracts of Chlorella are strongly influenced by the presence of water (ref. 152). If, as postulated, the chlorophyll does oxidize water directly, it must be coupled to an enzymatic system to allow the oxidized water moity to be converted into oxygen. Concerning the enzymatic reactions little or nothing is known, and knowledge in this area would allow more definitive experiments on the role of chlorophyll in water splitting.

Plastocyanin

A blue pigment has been isolated by Katoh and Takamiya from photosynthetic tissue and probably has a function in photosynthesis (ref. 153, 154). This pigment is a copper protein and is only present in photosynthetic tissues, with a ratio of about 1 plastocyanin to 300 chlorophyll molecules. The compound can be reduced in a Hill reaction and stimulates the reduction of DPIP and cytochrome c in a Hill reaction system. The stimulation

of the DPIP Hill reaction would indicate that it is functioning in system 2. The reduced form is not oxidized by oxygen. Since copper proteins are intimately involved in enzymatic reactions involving oxygen, with the enzyme serving as an oxidase, it is unusual that this copper protein is not oxidized by molecular oxygen. Further investigation of this very interesting and potentially significant compound is urgently needed to see if it is involved in oxygen production.

Percarbonic acid

Although it does not fit into the generally accepted idea that oxygen arises from an oxidative splitting of water as the primary reaction, the hypothesis of Warburg and Krippahl should be mentioned (ref. 155). In line with their active interest in the stimulating effect of carbon dioxide on the Hill reaction, they proposed that carbon dioxide bound to the chlorophyll was converted to percarbonic acid (OHCOOOH) which was then split to form oxygen. Although internally consistent, there is little experimental evidence to support this idea, and it neglects the data which show that photosynthetic oxygen comes from the water.

Carotenoids

The effect of light on the various carotenoids and xanthophylls in photosynthetic tissues has been investigated. Dourough and Calvin first tested the idea that xanthophylls are involved in the oxygen evolution system by examining the oxygen content of xanthophylls from algae grown on oxygen-18 water in the light (ref. 156). The oxygenated derivatives of carotenes are present in photosynthetic tissues in high concentrations, and it is logical to consider them as possible intermediates in the oxygen evolution mechanism. Yamamoto, et al. (ref. 157, 158) have investigated the biosynthetic source of the oxygens in the leaf zanthophylls luetin, violaxanthin and neoxanthin, and found that the hydroxyl groups derive their oxygen from molecular oxygen, while the epoxide oxygen comes from water. Studies by Sapozhnikov (ref. 159) and Krinsky (ref. 160) show that light affects a number of carotenoid systems, causing a decrease in the epoxy form and an increase in the hydroxylated forms. Furthermore, this conversion was inhibited by the specific inhibitor of photosynthesis, hydroxylamine (ref. 159). These data suggest a role of the epoxy xanthophylls in the oxygen evolving system. However, the most recent experiments of Yamamoto, et al. with isolated chloroplasts performing a Hill reaction show no incorporation of the oxygen from water into the oxygenated carotenoids violaxanthin and neoxanthin (ref. 161). Thus the carotenoids are not involved in the immediate reactions leading to oxygen evolution. The influence of light upon these compounds in the intact cell must be of a secondary nature.

Chloride

Chloride ion is required for a functional oxygen evolution system (ref. 33, 97, 162). This ion is required for the Hill reaction, for ATP formation associated with oxygen evolution (non-cyclic type), and for the FMN catalyzed phosphorylation related to oxygen exchange. Chloride is not required, however, for ATP formation associated with cyclic electron flow. Likewise, it is not required for the phosphorylations associated with electron transfer in bacterial chromatophores. Thus, it is clearly relegated to a position of cofactor in the oxygen evolution system. The mechanism of action, however, is not known.

Manganese

As discussed in the section on photoreduction in algae, manganese is an essential element for the photosynthetic process (ref. 57 - 59). Investigations to date have shown that a functional oxygen evolving system requires manganese, but in any partial photosynthetic system not involving oxygen evolution, manganese is not required. Table 3 contains a list of those photosynthetic processes which are either sensitive or insensitive to the presence of manganese. Some discussion of these is in order.

Table 3
Evidence for the Involvement of Manganese
in the Oxygen Evolution System of Photosynthesis

Processes Not Affected by Manganese Deficiency
1. Photoreduction. Kessler (ref. 59).
2. Photoreduction of TPN with DPIP-ascorbate. Eyster (ref. 163).
3. Photosynthetic phosphorylation catalyzed by pyocyanin. Spencer and Possingham (ref. 164).
4. Temperature-insensitive photoreactions. Kessler, et al. (ref. 165).
Effects of Manganese Deficiency
1. Inhibition of photosynthetic phosphorylation with FMN. Spencer and Possingham (ref. 164).
2. Increase in fluorescence. Kessler, et al. (ref. 166).
3. Decrease in delayed light emission. Kessler, et al. (ref. 166).
4. Loss of light-dependent ESR signal. Treharne, et al. (ref. 167).
5. Decrease in temperature-dependent absorbancy changes. Kessler, et al. (ref. 165).

Processes Not Affected by Manganese Deficiency

1. Photoreduction by adapted algae. As mentioned above, this process is catalyzed by algae which have adaptively formed the enzyme hydrogenase. Such algae can then utilize hydrogen gas for the reductant to react with the

photochemically generated oxidizing power (either at Y or at B in Fig. 9). Such cells do continue to evolve oxygen at a reduced rate, and it appears that they can carry out two reactions simultaneously: (1) photooxidation of water to produce oxygen and (2) photooxidation of hydrogen gas via hydrogenase (ref. 52). The photoreduction process is not appreciably affected by a lack of manganese (ref. 59) and does not involve oxygen production.

2. Photoreduction of TPN with DPIP-ascorbate as the electron donating system. This reaction is performed by chloroplasts which are deficient in manganese to the degree that they do not perform a quinone Hill reaction (ref. 163). With the substitute reductant they need not produce oxygen, and this insensitivity to manganese is expected.

3. Photosynthetic phosphorylation catalyzed by pyocyanin. This reaction is catalyzed by cyclic electron flow not involving oxygen evolution (ref. 164). This process is insensitive to inhibitors of the oxygen producing system, as are also items 1 and 2 above.

4. Temperature-insensitive photoreactions. The temperature insensitive absorbancy changes studied by Witt, et al. are related to the primary photochemistry of the chlorophyll system (ref. 87, 88) and occur prior to the oxygen evolution step. Such absorbancy changes are not appreciably affected by a state of manganese deficiency (ref. 165).

Effects of Manganese Deficiency

1. Inhibition of photosynthetic phosphorylation with FMN as cofactor (ref. 164). As shown above, this process is a phosphorylation associated with an oxygen exchange reaction and requires a functional oxygen evolution system.

2. Increase in fluorescence and decrease in delayed light emission (ref. 166). The increase in fluorescence would correlate with the disruption of the oxygen evolution system, since this would allow more of the activated chlorophyll to return to the ground state by emitting light in the process of fluorescence. The decrease in the amount of delayed light emission also correlates well, since a non-functional oxygen evolution system would result in fewer chemical intermediates of the photosynthetic process, and thus decrease the amount of delayed light emission.

3. Decrease in temperature-dependent absorbancy changes (ref. 166). These changes are correlated with the chemical reactions which follow the initial photochemical processes. With the oxygen evolving system impaired, fewer chemical changes would be possible, and the related absorption changes would be depressed.

4. Loss of light dependent ESR signal (ref. 167). Normal cells display ESR signals corresponding to free electrons or free radicals when illuminated. These signals disappear when Chlorella cells are made deficient in manganese. The correlation of these signals with particular areas of the photosynthetic mechanism is not definite, but there are two lines of reasoning that place the responsible moieties somewhere in system 1

of Fig. 9. In the first place, light-dependent ESR signals are displayed by bacterial photosynthetic systems, which in all probability have only one light reaction corresponding to system 1 of plants. Furthermore, Hoch and Kok have reported that preparations of their P700 pigment show the characteristic light-dependent ESR signal shown by whole plant cells, and that the number of unpaired spins is roughly equivalent to the number of P700 molecules present (ref. 168). Since P700 functions in system 1 of the plant system, the unpaired spins reflected in the ESR signal are most likely located in this system.

In addition to the effects of manganese listed above, there are a few other known manganese phenomena which may be related to the photosynthetic process. One interesting characteristic of manganese is its specific function in peroxidase systems. Akazawa and Conn demonstrated that peroxidase will catalyze an oxidation of DPNH with molecular oxygen with manganese as a specific cofactor (ref. 169). Klebanoff investigated the DPNH oxidase system with regard to the manganese effect (ref. 170), and also has shown that ergothioneine (a sulfhydryl derivative of histidine) oxidation by hydrogen peroxide in the presence of peroxidase is stimulated markedly and in a specific manner by manganese (ref. 171). Habermann and Gaffron have reported an unusual photooxidation reaction catalyzed by FMN in the presence of peroxidase. In this reaction ascorbic acid is photooxidized in a sequential fashion, and manganese is specifically required (ref. 172).

The experiments relating catalase and peroxidase function to manganese are interesting in that the systems may be functionally similar to the oxygen evolving system. I emphasize that they may be similar, since there is no evidence that either catalase or peroxidase is involved in the oxygen evolving system. However, the reactions do involve manganese, a hemeprotein and either peroxide or oxygen. All these compounds are present in the plant system and could serve in the oxygen evolving system.

There has been extensive speculation that catalase or a peroxidase may be the enzyme involved in the oxygen evolving system, converting the YOH into molecular oxygen (ref. 2, 52), but to date there has been no evidence for a function of these peroxide enzymes in the system. Indeed, the experiments of Mehler (ref. 60, 61) have shown that the photosynthetic process does not produce hydrogen peroxide which would be available to added catalase. To date, the only hydrogen peroxide produced in photosynthetic reactions results from the reoxidation of reduced cofactors such as FMN by molecular oxygen, and this hydrogen peroxide was detected in the experiments of Mehler. Even though there has been no evidence for a catalase or peroxidase involvement in oxygen evolution to date, it would seem that the peroxidase system involving manganese merits much more study from the photosynthetic viewpoint.

Another interesting series of experiments has been conducted in Calvin's laboratory by Markham (ref. 173) and Yamamoto (ref. 174) on manganese phthalocyanine. Phthalocyanine is a porphyrin related to chlorophyll, but is much more stable. This compound shows many of the photo-

chemical and electronic properties predicted of the active pigment in photosynthesis (ref. 31). When complexed with manganese, the resultant compound will combine reversibly with molecular oxygen in pyridine solution. The compound resulting from oxygen addition can go through a number of modifications involving changes in the oxidation state of the manganese. Oxygen can be liberated again from one of these by boiling the solution. Although of a preliminary nature, these very interesting experiments may lead to a model system which can lead to oxygen evolution from water and thus represent the function of manganese in the intact chloroplast.

Finally, the experiments of Kenton and Mann pertain to this discussion. With illuminated chloroplasts they were able to oxidize manganese to the trivalent state (ref. 175). Although the reaction was very slow and was not examined in detail, it does show the possibility of effecting changes in the valency of manganese by the illuminated chloroplast.

INDUCTION EFFECTS

Induction Phenomena

There is a great body of information concerning the induction periods of photosynthesis relating to dark and light periods, aerobic and anaerobic conditions, etc. (ref. 2, 52). The majority of these effects relate to the slower enzymatic reactions involving carbon dioxide fixation and the formation of ATP (ref. 52). Olson, et al. utilized an oxygen electrode to follow fast changes in the oxygen tension and demonstrated that following a period of darkness, Chlorella cells gave a very short burst of oxygen upon illumination (ref. 176). Fig. 18 is taken from this paper and shows the initial burst of oxygen gas, followed by a period of uptake of oxygen and then a subsequent evolution of oxygen which took place in two steps. The period of the short initial burst was no more than 3 seconds. The extent of the rapid initial burst was affected by the oxygen concentration, but was only slightly affected by changes in temperature, being far less sensitive to low temperatures than was the steady state rate of oxygen production.

The oxygen burst observed upon illumination of Chlorella cells correlates with the chromatic transients observed by Blinks (ref. 80, 81) and by French and Fork (ref. 70). When this is considered in light of the two light reactions, it seems logical that the oxygen burst is a reflection of system 2 being immediately ready to function upon illumination. Once the components of system 2 (Y and A) become completely oxidized and reduced respectively, the reaction must stop until system 1 can couple and catch up to system 2. The subsequent transition to steady state oxygen production would reflect this coupling of the two systems. The transitory step observed following the uptake could be the saturation of system 1. The final rate would then reflect the completely operating system including CO_2 fixation. The increase in the initial burst observed at higher oxygen concentrations could be due to a higher initial state of oxidation of A prior to illumination.

The concept that the oxygen evolving system coupled to system 1 is poised and ready to operate immediately upon illumination is supported by the fact that generally the Hill reaction does not show the general induction phenomena observed with whole cells where the carbon dioxide fixing system must be included. Allen and Franck (ref. 177) have utilized the extremely sensitive phosphorescence quenching method for measuring oxygen and have shown that with Scenedesmus obliquus cells, one single, intense half-millisecond flash of light will not support oxygen evolution from the cell when the whole photosynthetic apparatus is involved. However, the single flash does produce oxygen when the cells are performing a quinone Hill reaction. In passing, it should be mentioned that Allen, using the sensitive method for oxygen detection has shown that when the atmosphere was maintained at very low partial pressure of oxygen, Chlorella cells still were capable of supporting oxygen evolution at a rate that was as great or higher than that observed under air (ref. 178). This renders untenable the idea that oxygen is necessary for photosynthesis.

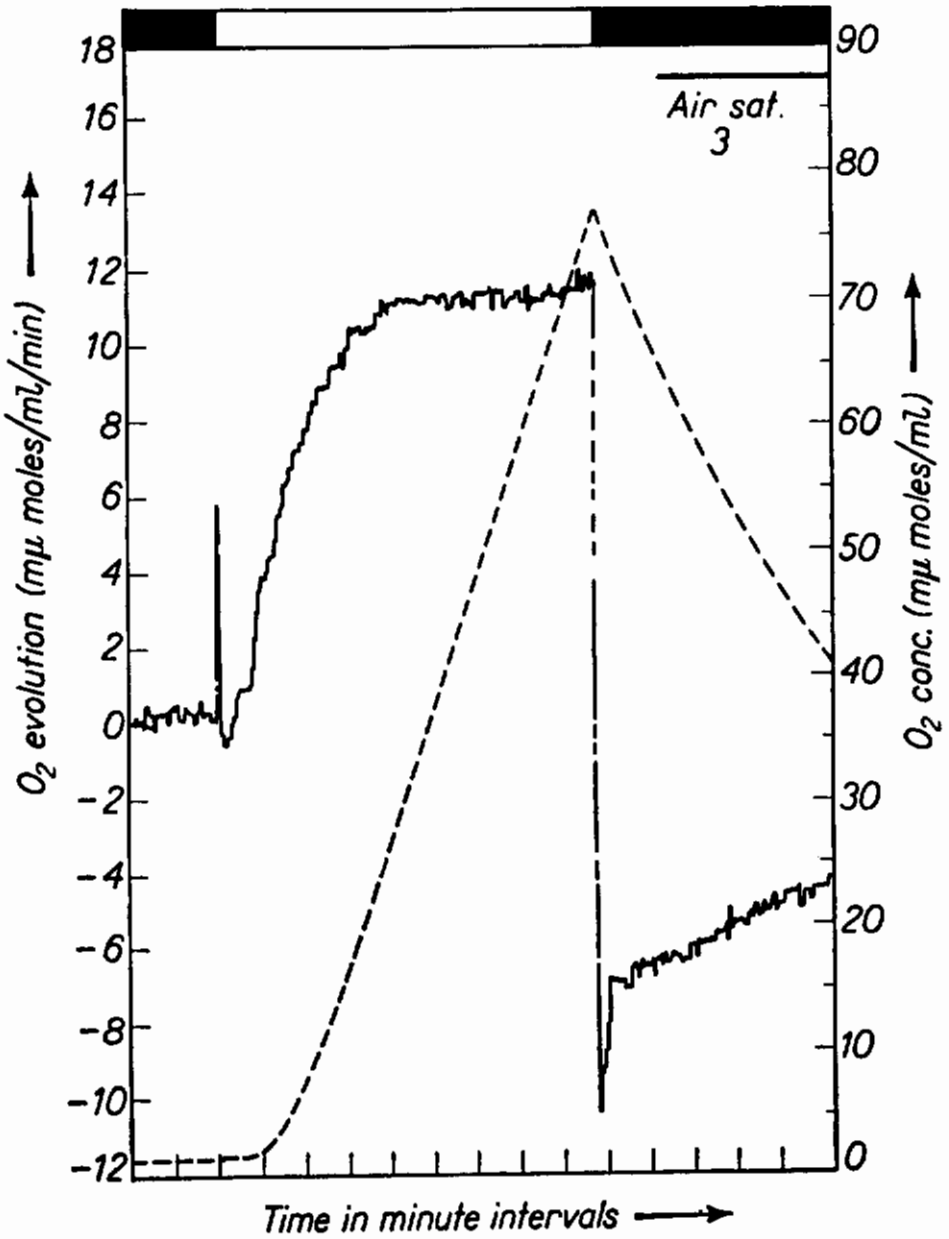


Fig. 18. Oxygen evolution from Chlorella cells upon illumination following a dark period. Use of an oxygen electrode allowed the detection of a short burst of gas immediately upon illumination, which lasted less than three seconds. The sharp uptake of oxygen upon cessation of illumination is shown. Taken from Olson, et al. (175).

INHIBITORS OF OXYGEN EVOLUTION

Jagendorf has summarized very nicely the information currently available on inhibitors of oxygen evolution. Table 4 is taken from his publication (ref. 127). An inhibitor is known to function on the oxygen evolving system if it inhibits a photoreaction which is dependent upon oxygen evolution, but does not inhibit a photoreaction which is independent of oxygen evolution. The three cases are: (1) inhibits photosynthesis but not photoreduction, (2) inhibits Hill reactions or the reactions involving oxygen exchange (particularly with FMN), but does not inhibit cyclic electron flow, and (3) relief of an inhibition by ascorbate-DPIP or ascorbate-PMS, which systems serve as alternate electron donors.

Table 4
Inhibitors of Oxygen Evolution. Taken from Jagendorf (ref. 127)

Compound or treatment	Criterion: Inhibits photosynthesis but not photoreduction	Criterion: Inhibits Hill reactions or O_2 exchange but not cyclic electron flow	Criterion: Inhibition relieved by $DPIPH_2$-ascorbate or PMS-ascorbate
NH_2OH	5a	-	16
Phthiocol	6	-	-
o-Phenanthroline	6	8, 17	-
CMU	-	8, 9, 17	9
DCMU	3	13, 15	11, 16
2-Nonyl-4-hydroxy-quinoline-N-oxide	-	2	-
Mn deficiency	4, 10, 14	-	-
Cl deficiency	-	1	-
"Aging"	-	-	16
Brief heating	-	-	7
Bean leaf cold storage	-	12	12
Exposure to high Tris	-	13, 15	15

a References:

1. Arnon (33)
2. Avron (179)
3. Bishop (55)
4. Eyster, et al. (58)
5. Gaffron (53)
6. Gaffron (180)
7. Hinkson and Vernon (112)
8. Jagendorf and Avron (130)
9. Jagendorf and Margulies (64)
10. Kessler (59)
11. Krogmann (115)
12. Margulies and Jagendorf (65)
13. Nakamoto, et al. (122)
14. Pirson, et al. (181)
15. Stern (113)
16. Vernon and Zaugg (26)
17. Whatley, et al. (182)

SUMMARY

The evolution of oxygen by green plant cells during photosynthesis has been discussed in terms of its being a result of water serving as the electron donor for a photochemically generated oxidation site, Y. Transfer of an electron from water to Y would produce YOH, which, through a light-independent enzymatic reaction produces oxygen. The nature of Y and the subsequent enzymatic steps are not known, although a number of inhibitors of photosynthesis have been shown to be active because of their ability to inhibit the oxygen evolution step. Table 4 gives a list of these. There appears to be no common chemical feature among the various compounds, although a number of the compounds are substituted amines.

Oxygen evolution requires a structured system in the chloroplast. The entire photosynthetic process involves two systems which respond differently to monochromatic light of appropriate wave lengths. Using the nomenclature of Duysens, system 2 is activated by light of shorter wave length (absorbed by chlorophyll b in plants and Chlorella, and by phycobilins in other algae), catalyzes the oxidation of water (leading to oxygen production) and reduction of a compound, A, of intermediate potential. A may be either cytochrome b_6, plastoquinone, or a composite of both. Transfer of the electrons from A to another compound B is enzymatic in nature and the reactions lead to the production of ATP. The reduced form of B then gives up its electrons to the other photochemical system, system 1, which transfers the electron to a low potential compound which can reduce TPN. System 1 is activated by light of longer wave length which is absorbed by some form of chlorophyll a.

A number of alternate oxidation reactions can be catalyzed by chloroplasts, and these may replace the water oxidation process (thus allowing no oxygen production). Algae can be adapted to produce an enzyme, hydrogenase, which then allows the cell to oxidize hydrogen in lieu of water. This allows the algal cell to practice a photosynthesis that is very similar to the bacterial process, since the photosynthetic bacteria differ from plants in that they utilize some molecule other than water as the hydrogen donor for the photochemical system. 2, 6-dichlorophenolindophenol (DPIP) and phenazine methosulfate (PMS) maintained in the reduced form by excess ascorbate can also serve very effectively as the electron donor for the photochemical act. This again substitutes for oxygen evolution. When the two separate light reactions are considered, there are two possible sites of interaction of these electron donors. From action spectra for the reactions involving these electron donors, it is concluded that they are oxidized primarily by system 1, leaving the oxygen-evolving system idle.

With the oxygen-evolving system 2 inoperative, the plant chloroplast resembles the chromatophore of photosynthetic bacteria. Photochemical oxidation and reduction reactions are catalyzed, and the accompanying phosphorylation reactions are located on this system. System 2, then, can be considered as the accessory required by the plant cell to allow it to utilize

water as the hydrogen donor for photosynthesis. Induction phenomena (instantaneous burst of oxygen upon illumination), the specific action of inhibitors, and the requirement of manganese allows experimental separation of the two systems.

Previously advanced schemes have shown cytochromes functioning in the oxygen evolution system. The present evidence is that they do not function there, but serve as the electron donor for system 1 (e.g. are compound B in Fig. 9). The P700 pigment studied by Kok and co-workers functions in system 1 and may well be the long wave length sink for excitation energy which under the influence of light transfers electrons to TPN and becomes oxidized. It subsequently would be reduced by B, which is most likely cytochrome f (or the analagous cytochrome in algae).

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