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See Wydrzynski (1982)
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The Oxygen Evolving System of Photosynthesis

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ABSTRACT

The molecular mechanism of O₂ evolution by green plants is not yet known. A brief review of this topic is presented here. It is now established that (a) the ultimate source of O₂ is H₂O, not CO₂, (b) two pigment systems and two light reactions are required for the steady state O₂ evolution *in vivo*, but O₂ evolution is carried out by photochemical system II alone that also reduces plastoquinone, (c) four positive (oxidizing) equivalents must accumulate on the water side on a charge accumulator labelled "M" before O₂ can be evolved although water chemistry may begin earlier than the last step as some protons are released before this step; and (d) manganese and chloride are required for O₂ evolution with manganese undergoing dynamic changes during this process. M. Spector and D. Winget (1980) have isolated a manganese-protein that may indeed be the "oxygen evolving enzyme"; attempts are in progress for the isolation of this "oxygen evolving enzyme" in several laboratories.

Index Words : Bicarbonate|Emerson enhancement effect|manganese|nuclear magnetic resonance|Oxygen evolution|photosynthesis|photochemical system II|primary photochemical reaction|proton release.

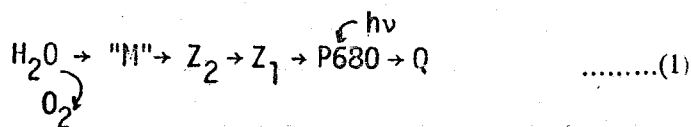
INTRODUCTION

In green plant photosynthesis, solar energy is converted into chemical energy on a large scale; this process provides us with food, fuel, fibre and oxygen for the sustenance of life on earth. An understanding of the molecular mechanism of this process may provide us information that could be later used for constructing efficient model systems to help solve the "energy crisis". By now, the steps involved in the fixation of CO₂ into carbohydrates [1], and the nature of electron carriers between

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the two photochemical systems (I and II) and beyond system I are fairly well understood [2, 3] although many important gaps need to be filled. There are two major areas that are in need of extensive further investigations: (1) mechanism of O_2 evolution [4]; and (2) mechanism of photophosphorylation [5]—the latter is being actively investigated in several laboratories, but only a few laboratories are engaged in the former investigation. This is mainly because no one has yet isolated the electron carriers involved in photochemical system II; only one laboratory* has isolated a manganese-protein that may be the "M" in the following scheme:



Here, "M" represents both the hypothetical oxygen evolving enzyme and the oxidizing equivalents' accumulator, Z_2 an intermediate electron carrier, Z_1 the electron donor to the reaction centre chlorophyll *a* P680 of the photochemical system II, and Q (a quinone) the stable electron acceptor of this system (see later for references). Except for the detection of Z_2^+ by Electron Spin Resonance (ESR) in the laboratory of K. Sauer, and our investigations on the water proton relaxation rates (PRR) for monitoring changes in the manganese (Mn)-containing oxygen (O_2) evolving machinery (M), no other intermediate between water and the reaction centre chlorophyll *a* (P680) have been monitored. The existence of Z_1 has been suggested from kinetic measurements on the absorbance change due to $P680^+$ to P680 conversion, and on the chlorophyll *a* fluorescence rise after short flashes of light.

A brief review of the present day concepts and of the literature follows.

SOURCE OF O_2

When chlorophyll *a*-containing plants are exposed to light, they produce O_2 . This capacity of plants was discovered by Priestly [6] and the role of light in it by Ingenhousz [7].

Wilstätter and Stoll [8] believed that CO_2 was the source of O_2 . This concept was abandoned due basically to three developments: (a) van Niel's comparative

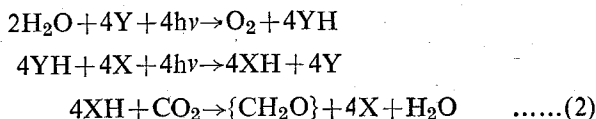
* M. Spector and D. Winget at the University of Cincinnati, Ohio (U.S.A.); their paper was *in press* in the *Proceedings of the National Academy of Sciences* (U.S.A.) at the time of the correction of this proof.

physiological arguments [9] : some photosynthetic bacteria deposited sulfur and used H_2S as the hydrogen source; photosynthesis was generalized as $\text{H}_2\text{A} + \text{CO}_2 + \text{light} \rightarrow \{\text{CH}_2\text{O}\} + \text{A}$; and in green plants A was O_2 and thus the source of O_2 was water, H_2O . (b) The discovery of the Hill reaction [10] : Hill found that isolated chloroplasts incapable of fixing CO_2 , could use artificial oxidants to evolve oxygen : $\text{H}_2\text{O} + \text{Fe}^{3+} \text{ oxalate} + \text{light} \rightarrow \text{O}_2 + \text{Fe}^{2+} \text{ oxalate}$. Since O_2 was evolved without CO_2 uptake, the latter could not have been a source of O_2 . (c) The ^{18}O experiments : Ruben *et al.* [11] showed that when cells of *Chlorella* (a green alga) were exposed to C^{18}O_2 and illuminated, the ratio $^{18}\text{O}_2/^{16}\text{O}_2$ in the evolved O_2 was different from that in CO_2 . However if $^{18}\text{O}_2$ was given as H_2^{18}O , then the ratio of $^{18}\text{O}_2/^{16}\text{O}_2$ in O_2 was similar to that in H_2O .

Warburg [12] and Metzner [13] have challenged the above arguments and, apparently, on proper grounds. There are obvious differences between bacterial and green plant photosynthesis and there is no reason why they could not perform reactions differently. Secondly, the Hill reaction requires CO_2 or bicarbonate (Warburg and Krippahl, [14]; Stemler and Govindjee [15]; also see reviews by Govindjee and R. Khanna, [16] and Govindjee and van Rensen, [17]. Thirdly, Ruben *et al.*'s experiments were done under conditions when there was plenty of time for exchange of $^{18}\text{O}_2$ between H_2O and CO_2 to take place—in fact, the intact algal cells used had enough carbonic anhydrase to hasten this equilibration reaction. It is now clearly established that a major role of CO_2 (or bicarbonate) in the Hill reaction is not on the oxygen evolution side but on the side that produces the reductant (see Wydrzynski and Govindjee [18]; Jursinic *et al.* [19]; Govindjee *et al.* [20]; Stemler [21]; Khanna *et al.* [22]; and Siggel *et al.* [23]. Finally, Stemler and Radmer [24] showed that in CO_2 -depleted chloroplasts (also depleted of carbonic anhydrase) injections of $\text{NaHC}^{18}\text{O}_3$ led to O_2 evolution, and all the evolved O_2 was regular $^{16}\text{O}_2$, not $^{18}\text{O}_2$. These experiments clearly establish that the ultimate source of O_2 is H_2O , but they cannot disprove that HCO_3^- may have a catalytic role in O_2 evolution. Kelley and Izawa [25] have, however, shown that HCO_3^- can replace chloride, but only to a very limited (5-10%) extent. Metzner [26] has suggested that bicarbonate (an anion) may be an intermediate on the water side. The energetics of water oxidation may take a new form if water oxidation would proceed in four steps [27]—it takes more energy to get the first electron out of water [26] and an anion (like chloride) may be useful in changing the energetics of the reaction. Thus it is considered likely that chloride (shown earlier [see ref. 25] to take part on the water side) may serve an important function. Whether it is an intermediate between H_2O and the oxygen evolving enzyme remains to be explored,

TWO LIGHT REACTIONS AND TWO PIGMENT SYSTEMS ARE REQUIRED FOR STEADY STATE O₂ EVOLUTION *IN VIVO*

Emerson and coworkers [28, 29] and Govindjee *et al.* [30] found that the minimum quantum requirement ($1/\phi$) of O₂ evolution is about 8-10 per O₂ molecule evolved. Franck and Herzfeld [31] and Rabinowitch [32] had suggested that $1/\phi$ of 8 could be easily explained if 2 quanta of light were used to transfer one electron from H₂O to CO₂ through two light reactions as 4 electrons must be removed from water before a molecule of O₂ could be evolved :



Here X and Y are electron acceptors of two separate light reactions.

It was in 1943 when Emerson and Lewis [28] discovered that the maximum quantum yield of O₂ evolution showed a drop at about 680 nm even when light was still being absorbed by chlorophyll *a*. This red drop phenomenon could not be quite understood at that time. Duysens [33] and French and Young [34] observed that light absorbed in phycobilins in red algae produced chlorophyll *a* fluorescence with high efficiency, but light absorbed in chlorophyll *a* itself produced chlorophyll *a* fluorescence with low efficiency. Duysens [23] interpreted this to mean that there existed two pools of chlorophyll *a*—one inactive and having low fluorescence yield and the other active and having high fluorescence yield. The latter pool must have been associated with phycobilins and the former must be comprised of a large portion of chlorophyll *a* present.

An important breakthrough was made by Emerson and coworkers [35, 36] when they recognized that the quantum yield of O₂ evolution in the far red region of the spectrum can be increased to the normal yield when the samples were supplemented by short wavelength light. This phenomenon, known as the Emerson enhancement effect, can be best rationalized as follows : Let $\text{RO}_2\lambda^2$, $\text{RO}_2\lambda^1$, $\text{RO}_2\lambda^1+\lambda_2$ be the rates of oxygen evolution in supplementary light, far red light and in the combined beams, and let us assume that (a) there are two pigment systems, (b) two light reactions are needed for O₂ evolution *in vivo*, and (c) the rate of overall reaction is governed by the rate of the slower of the two reactions. Following the present terminology (Duysens *et al.* [37]) light of λ_2 would be distributed in the two pigment systems with system II receiving a greater share of the energy (assume 60% in system

II and 40% in system I): thus $RO_2\lambda^2$ would be proportional to 40. On the other hand, light of λ_1 would be distributed unequally with system I receiving a greater share of the energy (at 710 nm, system I receives 90% and system II 10% of the energy); thus $RO_2\lambda^1$ would be proportional to 10. When the two beams are combined, system I reaction would be proportional to 130 and system II to 70; thus, the overall $RO_2\lambda^1+\lambda^2$ would be proportional to 70. However, if the two beams were given independently and separately, the sum of $RO_2\lambda^1+RO_2\lambda^2$ would have been only 50. Thus, there would be an enhancement equivalent to $70-50=20$ units. Emerson enhancement factor (E) would be :

$$\{RO_2\lambda^1+\lambda^2-RO_2\lambda^2\}/RO_2\lambda^1=70-40/10=3.$$

Emerson and Rabinowitch [36] presented the action spectra of this enhancement effect by varying λ_2 and by plotting E as a function of λ_2 . These spectra matched the fractional absorption spectra of the accessory pigments (chlorophyll *b* in green algae, fucoxanthol in diatoms, phycoerythrin in red algae and phycocyanin in cyanobacteria). Emerson argued that one pigment system contained accessory pigments and the other chlorophyll *a*. However, Duysens [33] had shown that energy absorbed by accessory pigments was always transferred to chlorophyll *a*. This problem was solved when Govindjee and Rabinowitch [38, 39] and independently French and coworkers [40] discovered a chlorophyll *a* band (Chl *a* peak at 670 nm) in the action spectrum of the Emerson effect when λ_2 was varied. Fork [41] clearly established the existence of chlorophyll *a* in system I in red algae when he plotted Emerson effect by varying λ_1 and keeping λ_2 (green light absorbed in phycoerythrin) constant; he observed both the red and the Soret bands of Chl *a*. (For a review on Emerson effect, see Myers [42]).

In 1960, several interesting observations were made : it was found by Govindjee *et al.* [43] that chlorophyll *a* fluorescence excited by red or blue light was quenched by light absorbed in the far red region of the spectrum; Kautsky *et al.* [44] explained the chlorophyll *a* fluorescence transient with the assumption of two light reactions and most importantly Hill and Bendall [45] proposed their two light reaction scheme of photosynthesis. This scheme was based on the redox potential of cytochromes; cytochrome (cyt) b_6 having a midpoint potential of ~ 0 eV and cytochrome *f* $+0.4$ eV. Hill suggested that one light reaction oxidizes water to O_2 and reduces Cyt b_6 , and the other light reaction oxidizes Cyt *f* and reduces $NADP^+$ (nicotinamide adenine dinucleotide phosphate). Oxidized cyt *f* is reduced by reduced cyt b_6 completing the chain leading also, as a bonus, to the formation of ATP as there is enough of a potential drop in this reaction. In 1961, several groups of

researchers provided firm data on such a scheme although the position of cyt b_6 , proposed by Hill, has been shown to be erroneous. Duysens *et al.* [37] coined the term pigment systems I and II responsible for the photooxidation of cyt f and H_2O respectively; and consequently, the light reactions performing these reactions are called light reactions I and II; these authors provided one of the best demonstrations of the series scheme through the antagonistic effect of light I and II on the redox level of cytochrome f . Kok and coworkers [46] provided data on the antagonistic effect of light I and II on the redox level of a chlorophyll a species labelled P700 that Kok [47] had discovered earlier and had suggested it to be the energy trap of photosynthesis. Supporting data on the Hill and Bendall scheme also came from the laboratory of Witt (Witt *et al.* [48]). For a present working model of this scheme, see Govindjee and van Rensen [17] and Figure 1. The legend of Figure 1 explains fully the details.

PHOTOSYNTHETIC UNIT AND REACTION CENTRES

In 1932, Emerson and Arnold [49] discovered that *Chlorella* cells evolve a maximum of 1 molecule of O_2 per 2400 chlorophyll molecules present when brief light flashes, separated by optimum dark periods, are given. Brief saturating flashes were used so that only the light reaction would occur in all the centres and the optimum dark time between flashes was used to ensure that none of the photoproducts is wasted. The high number of chlorophyll molecules needed for one O_2 molecule was rather surprising because photosynthesis at low light intensities is known to be rather efficient. This was the beginning of the concept of photosynthetic unit, several hundred chlorophyll molecules cooperating to produce O_2 . In 1936, Gaffron and Wohl [50] presented arguments that in such a unit, energy of several quanta (4 at that time) must be collected through excitation energy migration at some special sites and used for O_2 evolution. The concept of an assembly of pigment molecules (bulk or antenna) associated with an energy trap or reaction centre molecule is now widely accepted.

There are two pigment systems and two light reactions : (cf. Fig. 1) thus one electron transfer from H_2O to $NADP^+$ requires 2 light reactions and since 4 electrons must be removed from water to get one O_2 molecule, there is a total of 8 primary events. The photosynthetic unit size of 2400 chlorophyll per O_2 can be divided into 1200 per pigment system, or 600 per electron transferred, or 300 per light reaction. Thus if there is one reaction centre molecule for pigment system I (PSI) and one for pigment system II (PSII), we expect their concentrations to be 1/600 total

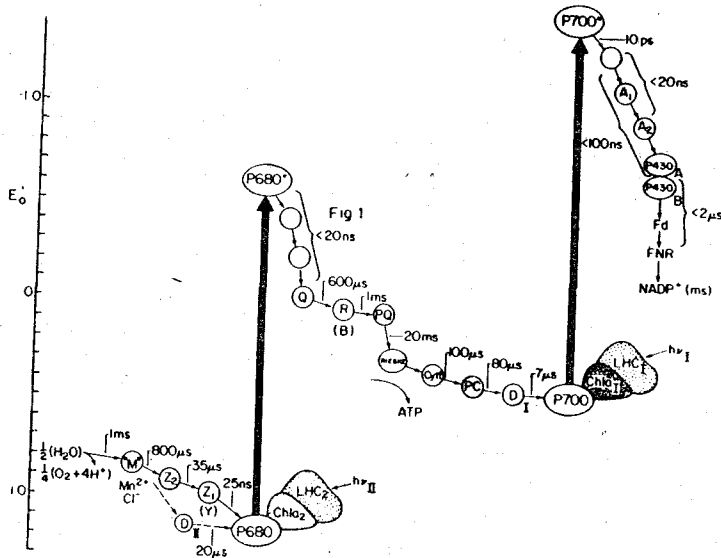
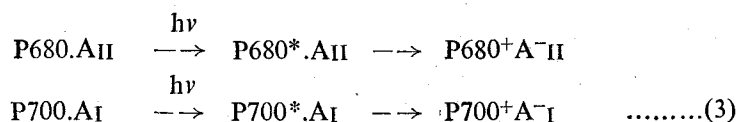


Fig. 1. A Working Model for Non-Cyclic Electron Flow from Water (H_2O) to Nicotinamide Adenine Dinucleotide Phosphate ($NADP^+$). This model includes approximate half-times of some recently measured reactions, and of some well known reactions; still others are estimates based on indirect measurements or by analogy to other systems. Most of the electron carriers suggested to be in the chain are included even though proof for some of these are not yet available. Cytochrome *b*₆ and cytochrome *b*-559, that may serve on cyclic pathways, are not included in the chain. Empty circles indicate species that may exist as it appears from some recent data, but their nature is not at all known. The left side of the diagram represents standard redox potentials in volts. Redox potentials refer to the pair of oxidized and reduced carriers-- but for simplicity in the diagram, only one form is shown. This becomes particularly important for reaction centre chlorophylls of photochemical system II (P680) and of photochemical system I (P700). [Contrary to convention, possible potentials for the excited reaction centres are also shown only to indicate higher reducing power of the excited species than the ground state species] The symbols from left to right are: "M": charge accumulator-Mn-containing hypothetical oxygen evolving enzyme; Z_2 : electron donor--speculated to contain tightly bound manganese, quinone and measurable as ESR signal II_v when oxidized; Z_1 : electron donor to P680; this is referred to as Y by scientists in Europe and "M" is referred to as Z by these scientists; D: endogenous donor alternate to Z_1 ; Q: quinone--first stable acceptor; R: also a quinone--a 2-electron carrier; it is referred to as "B" by the French group; PQ: plastoquinone pool; Rieske: iron centre (recently suggested to be there, equivalent to "M" of Levine); cyt f: cytochrome f; ATP: adenosine triphosphate; PC: plastocyanin; D₁: bound plastocyanin, electron donor to P700; A₁, A₂: observed through kinetic components of P700 and through ESR signals; the order of B and A should be reversed; P430: some type of bound ferredoxin; Fd--Ferredoxin; FNR--Ferredoxin-NADP-Reductase. The pigment systems I and II are composed of antenna complexes I and II along with their respective reaction centre chlorophyll molecules. Antenna complexes are denoted as core complexes (Chl a₁ and Chl a₂) and light-harvesting complexes (LHC₁ and LHC₂).

chlorophylls or 1/300 chlorophylls of either of the two systems. The reaction centre chlorophyll of PSI is P700 and was discovered by B. Kok as noted earlier.

The reaction centre chlorophyll of PSII is P680 and was discovered by Döring *et al.* [51] (also see Rabinowitch and Govindjee [52]; Govindjee *et al.* [53]).

The present day concepts are that P700 molecules are dimers of chlorophyll *a* connected with water-like molecules (see Norris *et al.* [54]; and Shipman *et al.* [55]). On the other hand, Jack Fajer and coworkers have recently suggested that P680 is a monomer chlorophyll, but it may be associated with a protein. The primary reactions of the two light reactions may be written as :

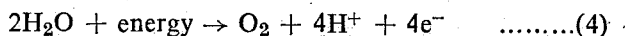


where, AII and AI are primary electron acceptors and P680 and P700 are primary electron donors of PSII and PSI, respectively. The primacy of a reaction is often judged by three criteria (one of them being not enough) :

(a) it should have a high quantum yield of the reaction (close to 1.0) so that it be the major reaction; (b) it should be the first chemical reaction (in photosystem I, it has been shown to take ~ 10 ps, see *e.g.* Fenton *et al.* [56] and (c) it should occur at low temperatures as it is a photochemical reaction in a complex. The first reaction in Eq. 3 is connected to the oxygen evolving system (see scheme (1)).

THE OXYGEN EVOLUTION REACTION

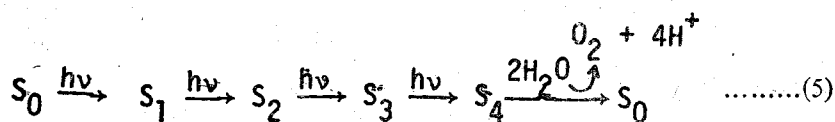
The average redox potential of $\text{H}_2\text{O}/\text{O}_2$ system is +0.8 eV, but the redox potential of one of the reactions could be much larger. It is very difficult to extract the first electron from H_2O molecule (see *e.g.* Renger, 27, 57). The evolution of O_2 from water requires the following reaction :



This reaction requires a lot of energy (indirectly supplied by light energy here). There could be two opposite models of O_2 evolution : (a) cooperating chains and (b) independent chains. In cooperating chains, either (1) positive charge stored on P680 can migrate to another chain and once four such charges are stored, O_2 can be evolved from reaction with water, or (2) chemical species equivalent to "O" atoms

may be formed before O_2 is evolved. In the independent chain concept, four successive reactions on one and the same centre are required. Thus, in the independent chain hypothesis, no O_2 should be evolved unless four oxidizing equivalents are stored on the same reaction chain, and, this could be accomplished with a series of four brief saturating light flashes separated by appropriate dark time between the flashes, necessary for the recovery of the reaction centres. Experimental results support the independent chain hypothesis [58]. The earliest results of Allen and Franck [59] showed that in anaerobic cells no O_2 was evolved in the first flash. The first important experimental result was that by Joliot *et al.* [60] who showed that in aerobic suspension of chloroplasts or algae exposure to a series of short (μs range, shorter than the recovery time of the reaction centre complex) and bright (so that each centre received one photon) flashes led to an oscillatory pattern in a plot of O_2 /flash as a function of flash number (Fig. 2). This experiment showed a periodicity of four in the pattern, a damping of oscillations with flash number, and most interestingly a peak on the 3rd instead of the 4th flash. Kok and coworkers [61] were able to confirm these results and provide a simple theory for these results (also see Mar and Govindjee, 62, for a discussion that a fit of data by a certain model does not make that model uniquely correct).

Kok's O_2 Clock. In Kok's O_2 model, the oxygen evolving system exists in five states labelled S_0, S_1, S_2, S_3 and S_4 with the subscripts indicating the number of positive charges. As a consequence of each light reaction ($h\nu$) the S states advance to the next higher S states, and the O_2 is evolved when S_4 reacts with H_2O :



This model predicts the periodicity of four in plots of O_2 /flash as a function of flash number. In order to explain the O_2 in the 3rd flash, and other characteristics, Forbush *et al.* [63] suggested that the oxygen evolving system exists in dark-adapted chloroplasts in two states S_0 and S_1 , in a ratio of $\sim 1:3$. That is, S_1 is a stable species in darkness. Thus, the third flash will give the O_2 in the first series of flashes.

The damping in the flash pattern with increasing flash number was explained to be due to the mixing of S states with time and also due to a certain probability of inefficiency of the process called misses. Whenever there was O_2 in the 2nd flash,

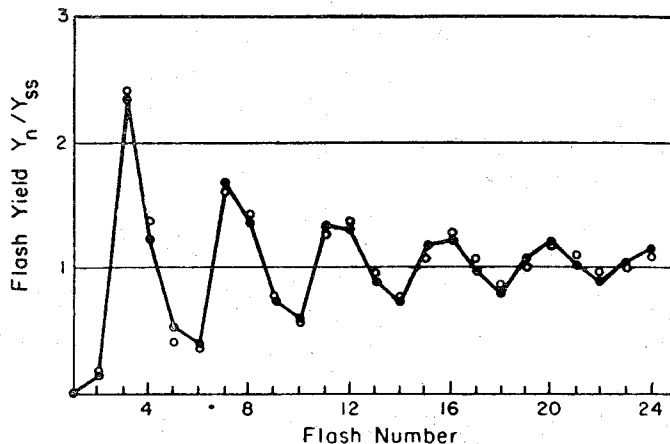


Fig. 2. Yield of Oxygen per Light Flash as a Function of Flash Number from Isolated Broken Spinach Chloroplasts. Y_n —Yield of O_2 in the n th flash, Y_{ss} —Yield of O_2 in steady state. Suspensions were dark-adapted for 40 min before a series of short ($\sim 10\mu s$) saturating flashes, spaced ~ 1 s apart, were given. O_2 was measured with a platinum-Ag, AgCl rate electrode. Experimental data are shown with solid points. Open circles are theoretical points based on Kok's model with the following assumptions: misses (α), 10%, double-hits (β), 5%, concentrations (relative) of S_0 and S_1 are 25% and 75%, respectively, in dark-adapted chloroplasts (data of Forbush *et al.* [63]; reproduced from ref. 2, page 392).

it was explained to be due to the flashes being too long causing "double hits" (i.e., S states advanced twice); this also affected the flash pattern (see Weiss *et al.*, 64; Mar and Govindjee, 62; Joliot *et al.*, 65).

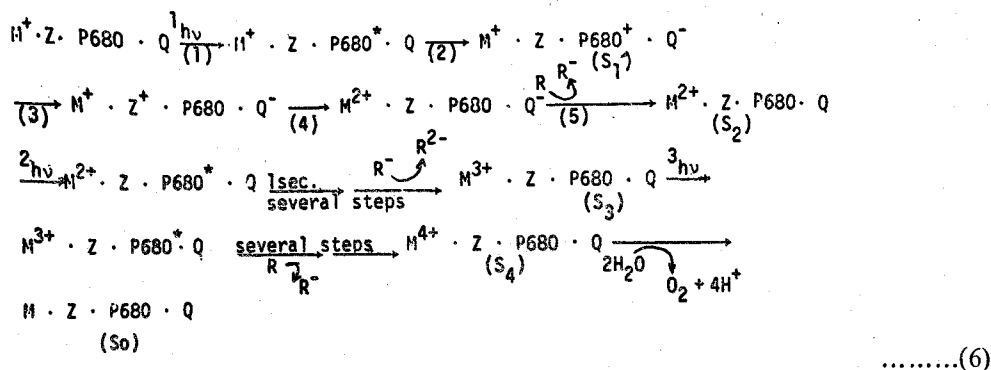
Mar and Govindjee [62] have already emphasized that a number of models can fit the existing data, but Kok's picture is preferred as it is the simplest of all.

The above model assumes that the charges produced on one centre are independent of all the other centres. This was confirmed when (see Joliot and Kok, 58) it was found that the pattern of O_2 /flash as a function of flash number remained unaffected when most of the centres were closed by the addition of the herbicide diuron. If there was a cooperation among the various centres, closed centres would have transferred their energy to open centres causing O_2 evolution even in the first or second flash.

Kinetics of Different Steps in the O_2 Clock. Joliot *et al.* [66] and later Sinclair *et al.* [67] have been able to measure the time of O_2 evolution from the last

step : $S_4 + 2H_2O \rightarrow O_2 + S_0$ by the delay in phase of O_2 evolution as follows. A sinusoidal wave of light was used to expose the samples and from a delay in the phase when O_2 was evolved, and after correcting for diffusion of O_2 (to the platinum electrode), the time of the last step was measured to be ~ 1 ms.

The time for the relaxation of the "S" states between each flash was measured as follows. Kok *et al.* [61] and Bouges-Bocquet [68] varied the dark time between flash 1 (that leads to formation of S_2) and flash 2 (that leads to formation of S_3) to measure the relaxation of S_1 to S_2 (the states formed directly after illumination were called the prime states). O_2 was measured in the 3rd flash, the latter being given 1s after the 2nd flash. A plot of Y_3 (O_2 in the 3rd flash) versus dark time between flash 1 and 2 showed a rise in O_2 yield with a half-time of $\sim 600 \mu s$. This was the time of the relaxation of S_1^- to S_2 . In this fashion, relaxation times for other steps were also measured. (Stemler *et al.* [69] note that this relaxation step is slowed down to about 10 ms when chloroplasts are depleted of CO_2 .) This relaxation step has been interpreted to be due to the step labelled (5) in the following scheme ;

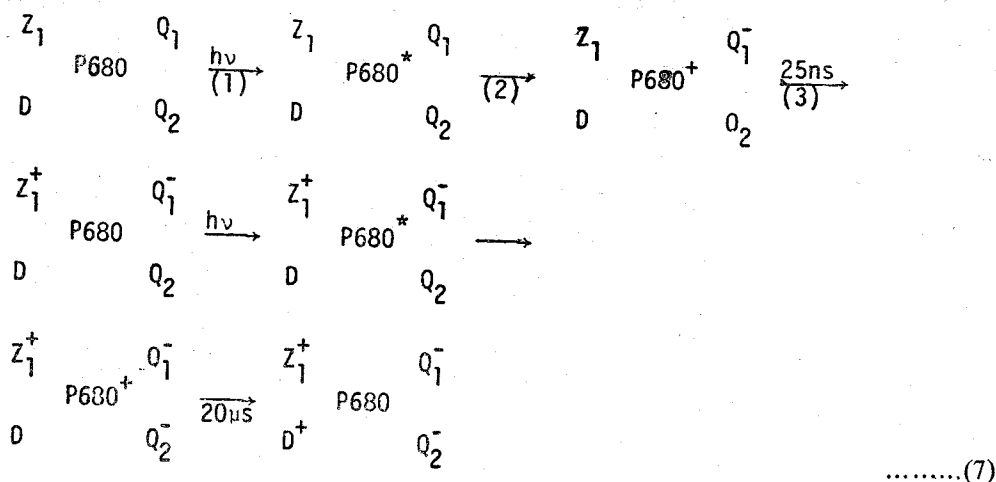


where M is the charge accumulator, Z is the electron donor to the reaction centre chlorophyll P680, Q is the electron acceptor, R is the 2 electron acceptor, and superscript on $h\nu$ (light flash) represents the number of the flash. Thus, the relaxation step is taken to be the discharge of the electron from Q^- (the reduced form of the electron acceptor) on to R (a secondary electron acceptor [20, 70, 71] that can accumulate up to 2 electrons and is like Q [72, 73], a quinone molecule [74]. R^{2-} transfers the pair of electrons on to the plastoquinone pool (PQ), see Fig. 1.

The states S_2 and S_3 deactivate in darkness to S_1 , S_0 and S_1 are stable. The deactivation of the S states has been studied by the same technique as described above but here the dark time between flashes had to be varied in the second and

minute range (see reviews by Joliot and Kok, 58, Diner and Joliot, 75; and Radmer and Chéniaie, 3, for further details on the oxygen evolution mechanism).

The Double Hits in System II. From the above it should be clear that double hits on the S' states should be very small if the light flashes are short ($\sim 1\mu\text{s}$). This is indeed so. However, Kok and coworkers [76] have shown that the addition of ferricyanide causes an anomalous amount of double hit in the first flash as O_2 in the second flash was found to be anomalously high under these conditions. In addition to double hits seen through O_2 evolution, a new concept of double hits at the reaction centre itself is emerging. It is now considered likely [77, 78] that there are two electron acceptors (Q_1 and Q_2) and donors (Z_1 and D) with Q_1 and Z_1 being involved in the fast and efficient reaction and Q_2 and D in a slower reaction as follows :

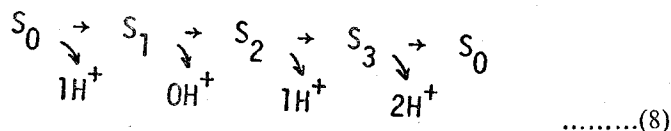


Van Best and Mathis [79] have indeed found that, in dark-adapted chloroplasts, after the first flash, P680^+ recovers to P680 within 25 ns (step (3) in scheme 7). In repetitive flash experiments (G. Renger, personal communication), or in steady state conditions, this recovery may be as slow as $0.4\mu\text{s}$ (L.N.M. Duysem and coworkers). Whether the above scheme is correct or whether there are heterogeneous reaction centres (slow and fast) connected to their respective electron carriers is yet to be established.

PROTON RELEASE DURING O_2 EVOLUTION

In the early model of Kok (scheme 5), it was assumed for simplicity that all four protons are released in the S_4 to S_0 reaction. Recently this has been modified

independently by three groups of researchers Junge *et al.* [81]; Fowler [80]; and Saphon and Crofts [82]. One scheme [82] is that protons are released as follows :



W. Junge (personal communication), however, believes that the H^+ release pattern is in between 0, 1, 1, 2 and 1, 1, 1, 1. This suggests that water may indeed have begun to undergo chemistry prior to the last step contrary to the earlier belief. The concept that protons could be released in several steps has also come independently from our analysis of Nuclear Magnetic Resonance (NMR) measurements on thylakoids exposed to a series of light flashes (see Govindjee *et al.* [83]; Govindjee, [84]). The details remain to be understood.

BIOCHEMISTRY OF O_2 EVOLVING SYSTEM

The nature of the "S" states, particularly that of the intermediates "M", " Z_2 " and " Z_1 " remains unknown. However, some inroads have recently been made in this area.

The Z Complex. Sauer and coworkers [85-87] have identified an ESR signal labelled IIvf (II very fast) that is, in all likelihood, due to Z_2^+ ; its decay measures the electron flow from H_2O to Z_2^+ with a half time of ~ 800 to $900\mu s$; its rate of formation has been established to be approximately $10\mu s$. This signal could indeed be from some species having the character of a quinone as suggested by its band shape [88]. Furthermore, there are indications, from biochemical experiments, that quinones are involved on the water side [89, 90]. Schmid *et al.* [91] have prepared an antibody against a 10,000 molecular weight polypeptide that inhibits electron flow on the water side (between "M" and P680). Thus, Z_2 or Z_1 may be proteinaceous or attached to a proteinaceous moiety. The role of tightly bound manganese is not yet known. Perhaps, Z_2 and Z_1 are complexes of quinone-manganese and protein. On the other hand, R. Khanna (Ph. D. thesis, University of Illinois, 1979) has found that most of the tightly bound manganese in thylakoids are present in the light-harvesting pigment-protein complex. It is, however, likely that contamination with manganese-containing superoxide dismutase may account for some of this manganese.

It is quite possible to stop the electron transfer from the charge accumulator (M) to P680 by various methods (Fig. 3) : alkaline Tris-washing (0.8M) [92]; and heating chloroplasts for 50° for 5 min. [93]. These treatments seem to affect the charge accumulator, but, electron flow through PS II and PS I can go on at full

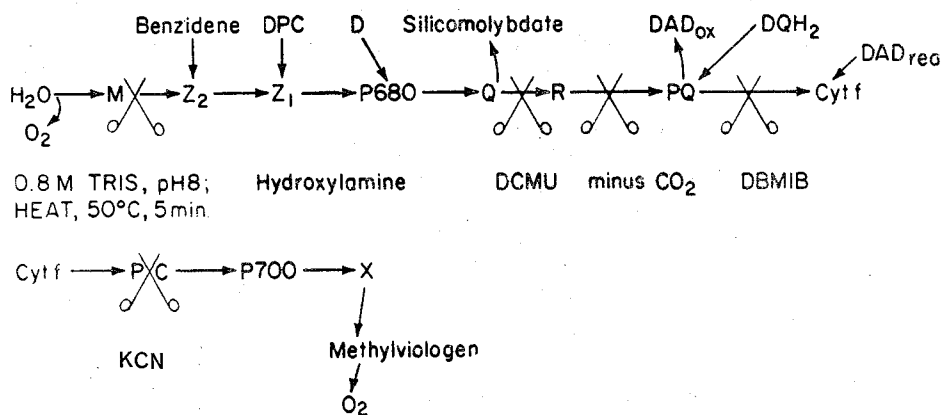


Fig. 3. Electron Flow from Water to "X", the Primary Electron Acceptor of Photochemical System I, Electron Donors, Acceptors and Inhibitors. This diagram, like Fig. 1, is based on the work of several investigators (see e.g. Trebst and Avron, ref. 151, pp. 253-282). D, endogenous electron donor to P680, the reaction centre chlorophyll *a* of photochemical system II; DAD, diaminodurene or 2, 3, 5, 6-tetramethyl-*p*-phenylenediamine; DBMIB, dibromothymoquinone or 2, 5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone, DCMU, 3-(3', 4'-Dichlorophenyl)-1, 1-dimethylurea; DPC, diphenyl-carbazide; DQH₂, reduced duroquinone donates electrons not at PQ, as indicated, but just beyond it; KCN, potassium cyanide; OX, oxidized, red, reduced. Scissors indicate where the inhibitions of electron flow take place. Arrows pointing into the main chain indicate the donation sites, and arrows pointing away from the main chain indicate the acceptance sites. For all other symbols, see the legend of Fig. 1.

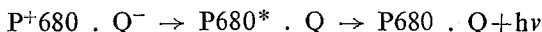
speed if certain exogenous electron donors are supplied. Among these electron donors are : diphenylcarbazide, DPC [94]; hydroxylamine [95]; benzidine [96]; iodide [97]; hydroquinone [96]; tetraphenylboron [98]; MnCl₂ [99]; and H₂O₂ [100]. It appears, from a survey of the literature, that some of these donors like DPC and MnCl₂ donate electrons closer to the reaction centre P680 (perhaps, to Z₁) whereas others like benzidine, etc. donate farther from it (perhaps, to Z₂) [101]. None of these experiments have, as yet, permitted a knowledge of the nature of Z₁ and Z₂.

The "M" Complex. Since O₂ evolution/flash as a function of flash number oscillates with a periodicity of 4, it is considered likely that any component that will

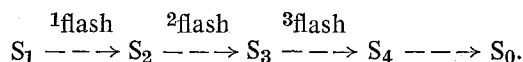
oscillate with that period will be related to this system directly or indirectly. Several such phenomena have been observed.

Delosme [102] observed that chlorophyll *a* fluorescence ($<1\mu\text{s}$ after the beginning of the light flash) shows peaks at the 1st and the 5th flashes. This is explained as follows. If we accept the hypothesis [103, 104] that P^+680 is a quencher of chlorophyll *a* fluorescence, then we could say that, after the first flash, P^+680 goes to P680 rapidly by electron transfer from Z_1 (S_1 to S_2 transition occurs in the first flash). Furthermore, if this donation time is dependent upon the "S" state, fluorescence yield would oscillate accordingly. For example, it would have to be suggested that this donation time is fast in the S_0 and S_1 states but slower in the S_2 and S_3 states as $[\text{S}_2] + [\text{S}_3]$ peak at the 3rd and 7th flashes where the fluorescence yield has minima in its oscillatory pattern. This phenomenon is thus indirectly related to the S state.

Another phenomenon that shows an oscillatory pattern is that of the delayed light emission, DLE [105 to 107]. Delayed light is generally assumed to be due to the back reaction of PSII primary photochemistry [108] :



However, the creation of P^+680 in dark could depend upon the S states and of Q^- on the reducing side of PSII. Zankel [105], using brief flashes, observed that DLE $10\mu\text{s}$ after the flash peaked at the 3rd and 7th flashes whereas Barbieri *et al.* [106] observed that DLE 300ms after the flash peaked at 2nd and 6th etc. flashes. This is explained as follows : The following major transitions occur as the number of flashes, in a series of flashes, is increased :



If DLE efficiency or the production of P^+680 is greater with S_4 followed by S_3 , S_2 etc [109], then DLE would be maximum after 3 flashes provided it is measured before S_4 goes to S_0 . This was possible in Zankel's experiment since DLE was measured $10\mu\text{s}$ after flash. However, in Barbieri *et al.*'s experiment, DLE was measured 320 ms after the flash, and thus, after the 3rd flash, S_4 had already been converted to S_0 . Therefore, the maxima were after 2nd flash (S_3), 6th flash, etc. By following DLE after individual flashes, one could relate DLE to the deactivation of S states. However, this phenomenon of DLE is indirectly related to the "M complex" and can only be an indicator of it; it provides no information on the biochemistry of the "M" complex.

A third phenomenon, thermoluminescence [110] *i.e.*, production of glow peaks as an illuminated sample is slowly warmed, also shows oscillations when the

intensity of these peaks is plotted as a function of flash number during illumination [111, 112]. This, again, can be used as an indicator of the S states. Perhaps, their physical nature as regards their temperature dependence can be inferred from these data, but, not their chemical nature.

There are two observations that have the possibility of providing information on the chemical nature of the "M" complex. Pulles [113] has observed oscillations in an absorbance change (ΔA) in the 290-320 nm region with a periodicity of four when ΔA is plotted as a function of flash number. This ΔA as a function of wavelength is flat in the 290 to 320 nm region. It remains to be seen where this change peaks when these observations are extended into the shorter wave region of the spectrum. One could speculate that these changes arise from the oxygen evolving enzyme itself, but, this should be stated with great reservations and caution.

There is a good deal of evidence that manganese is necessary for oxygen evolution (see refs. 114 to 116). No evidence exists that *proves* that manganese undergoes functional changes during oxygen evolution. ESR technique has been rather unsuccessful because bound manganese does not show the usual 6 line signal, and 6-line signal is generally found to be absent in healthy chloroplasts. These, however, could be obtained by releasing manganese by various means. In spite of these negative results, Siderer *et al.* [117] claim to have observed bound manganese in their chloroplasts. This will not be discussed any further.

At Urbana, we initiated measurements on Nuclear Magnetic Resonance (NMR) of thylakoid membranes with the hope of studying the role of manganese in them. Paramagnetic ions (particularly Mn^{2+}) are known to speed up the relaxation of water protons (nuclei, 'H; not to be confused with released protons, H^+). Wydrzynski *et al.* [118 to 120] and R. Khanna (Ph.D. thesis, University of Illinois, 1979) have established that conditions that (a) release manganese from thylakoid membranes lead to decreases in water proton relaxation rates (PRR); (b) directly or indirectly reduce the "S" states increase the PRR; and (c) directly or indirectly oxidize the "S" states decrease the PRR. These results are taken to mean that in dark-adapted chloroplasts manganese exists in a mixture of oxidation states. From an analysis of PRR as a function of manganese content of the thylakoid membrane and its O_2 evolving capacity, it was concluded that the "loosely" bound manganese—related to O_2 evolution—is being monitored by PRR [120]. A further analysis [119, 120] of the dependence of PRR as a function of the frequency of rf pulse suggested that the major species being monitored is Mn^{2+} since Cu^{2+} , another species with similar characteristics, present in plastocyanin, is not accessible to water protons. However, other Cu^{2+} containing membrane-bound components have not yet been ruled out.

Since PRR is not a highly specific way of monitoring only manganese functional in O_2 evolution, it also monitors the very loosely bound form of manganese that is totally unrelated to O_2 evolution (R. Khanna, 1979). A clear relationship of PRR data to the "M" complex (the charge accumulator) was, however, established when it was observed that PRR after a series of flashes show oscillations when plotted as a function of flash number [83, 121 to 124] (Fig. 4). Analysis of such

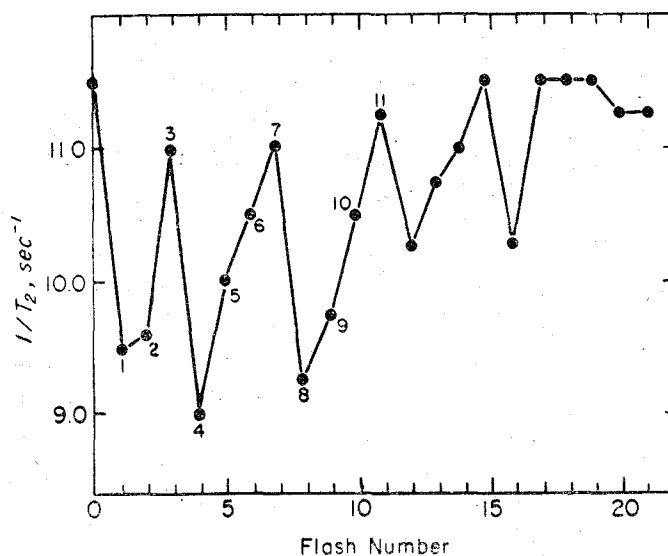


Fig. 4 Water Proton Transverse Relaxation Rate ($1/T_2$) as a Function of Light Flash Number in Isolated Broken Chloroplasts from Spinach (Data of Wydrzynski *et al.*, ref. 121.) Proton relaxation rates (PRR) are affected strongly by changes in the concentration of Mn (II) (see refs. 120, and 83) and by changes in the number of ligands bound to water molecules. *Note* : PRR pattern, shown here, is similar to the pattern of O_2 flash shown in Fig. 2 in one respect : maxima occur in the 3rd, 7th, and 11th flashes. This periodicity of 4 suggests that during O_2 evolution, there is also a cycling of changes in manganese. Difference in the two patterns are explained by additional assumptions. For a more complete interpretation, see refs. 83, 84, 123 and 124.

data [83, 84, 122, 123] suggest, but do not prove, that manganese may be undergoing redox changes during the operation of the "S" cycle if it is assumed that PRR monitors mainly changes in Mn^{2+} . At this time, an effect through changes in the conformation of the system or through changes in the number and the strength of

the ligand bound cannot be excluded. Our working model [84], although highly speculative, suggests that manganese is not being sequentially oxidized with light flashes: reduction at intermediate steps may imply that water chemistry does not have to wait until the last step, and, thus, protons may be released at steps earlier than the last step. It seems that both this "analysis" and the H^+ release data suggest that the water chemistry is not a one step reaction as casually implied by Kok's earlier model; Kok's model would be ultimately modified to include the present concepts.

An anion chloride has been shown to be involved in the oxygen evolution machinery of photosynthesis [2⁵, 125, 126]. Exogenous iodide [97] has been shown to be a good donor to PS II, but, not chloride. It is considered likely that it either plays a role in stabilizing Mn complexes or that it is an actual intermediate in the chain. Its role remains to be established.

Attempts to isolate the oxygen evolving enzyme had not been successful in the past. [4, 127]. Some Mn-containing complexes had been isolated [128, 129] but their relationship to the oxygen evolving system is not at all clear.

A most promising isolate is that by M. Spector and D. Winget at the University of Cincinnati (mentioned earlier); this preparation has an approximate molecular weight of 65,000; it restores O_2 evolution when it is added to the cholate-treated thylakoid membrane incorporated into an artificial lipid vesicle. This preparation contains 2 Mn atoms per protein. I consider this work as a breakthrough in this field. A goal of several research groups is to isolate this enzyme complex and study its biochemistry. Combination of genetics and improved methods of isolation of membrane-bound enzymes may be necessary for achieving this goal.

Bicarbonate anion has been implicated to function in the O_2 evolving mechanism [13] but it has been clearly shown that the major function of bicarbonate anion is on the reducing site of PS II [16, 17].

The only biochemically characterized component in PS II is cytochrome b 559 [130] and curiously it does not play any direct role in O_2 evolution. At 77°K, it does become an efficient donor to P^+680 [131]. It may, however, play some regulatory role in PS II chemistry.

Role of Manganese. We have hinted several times that manganese plays a role in the intermediate ("M" complex) accumulating charge. As noted earlier, manganese is known to be essential for oxygen evolution and can take on a number of relatively stable oxidation states, making it a likely candidate for the "charge accumulator" (see below). There has been no direct experimental evidence yet to

prove that chloroplast manganese undergoes changes in oxidation state during photosynthesis. Our experimental results on proton relaxation rates (cited earlier) are, however, the first demonstration of dynamic changes in manganese related to the "M" complex; and, the simplest interpretation of these results is in terms of redox changes between Mn^{2+} and Mn^{3+} . In order to further resolve the function of manganese, it is necessary to combine the use of NMR with ESR and other techniques (e.g. EXAFS). As noted earlier, our present results (obtained in collaboration with the research group of H.S. Gutowsky of our Chemistry Department) suggest that the water proton relaxation rates monitor, although indirectly, membrane-bound manganese as well as the individual charge accumulating states in the oxygen evolution mechanism. In our future research, we propose to identify (a) the nature of manganese in dark adapted chloroplasts; and (b) the relationship of the dynamic role of manganese, as involved in charge accumulation, with oxygen evolution. Finally, we hope to obtain more exact information about the biochemistry of oxygen evolving machinery. A brief review on the role of manganese follows.

Manganese is required for O_2 evolution [4, 132]. Manganese-deficient plants show reduced capacity for O_2 evolution, but Mn-sufficient plants have normal rates of O_2 evolution (see e.g., Pirson [133], Kessler [134], Eyster *et al.* [135] and Spencer and Possingham [136]). There are apparently three pools of manganese: very-loosely bound, loosely-bound and tightly-bound. The very-loosely-bound pool seems to be unrelated to O_2 evolution (R. Khanna, S. Rajan, unpublished), the loosely-bound pool ($\sim 4-6$ atoms/reaction centre II) is related to O_2 evolution (see Cheniae [132], and Cheniae and Martin [137]). The function of tightly bound pool is unknown; it may be involved in the Z complex as suggested above. The loosely-bound manganese is removed from its native place by various treatments [92, 93, 137, 138]. The appearance of free manganese by such treatments is monitored by the appearance of the 6-line hexa-aqua manganese ESR signal [138, 139]. Alkaline TRIS-washing has been shown to give variable results [140, 141, 142, 143, 144]. Blankenship and Sauer [139] have shown that manganese is released, by TRIS-washing, to the inside of the thylakoid vesicles; this suggests that the manganese containing component is located on the inner side of the thylakoid membrane.

Since (1) a large portion of the chloroplast manganese is associated with the photochemical system II-enriched particles [145], (2) Mn-deficient chloroplasts can perform normal electron flow from artificial donors (see Fig. 3) like DPC, benzidine, etc.) to $NADP^+$, and (3) photochemical system I reactions are unaffected by manganese depletion, the site of manganese requirement is clearly on the O_2 evolving mechanism.

Manganese is certainly a candidate for the charge accumulating species "M" as it can take on a number of stable (and unstable) oxidation states. Several manganese models for O₂ evolution exist; these include those by Olson [146], Renger [147], Earley [148] and Govindjee [84]. In addition, exogenous Mn (II) can be photo-oxidized, by chloroplasts to Mn (III) (see McKenna and Bishop [149] and Homann [150]). However, at present there is no *direct* evidence for changes in the oxidation state of bound manganese during O₂ evolution.

In conclusion, biochemistry of the oxygen evolution mechanism remains an important problem for future investigators; its knowledge is very poor and its biochemistry has been rather difficult. Direct proof for the involvement of redox changes in manganese is still lacking. This inner sanctum of photosynthesis requires exploration by combined efforts of geneticists physiologists, chemists and physicists.

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