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KINETICS AND INTERMEDIATES IN PHOTOSYNTHETIC OXYGEN EVOLUTION

Gerald Thomas Babcock

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KINETICS AND INTERMEDIATES IN PHOTOSYNTHETIC OXYGEN EVOLUTION

Gerald Thomas Babcock

Department of Chemistry and
Laboratory of Chemical Biodynamics
Lawrence Berkeley Laboratory
University of California
Berkeley, California

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ABSTRACT

Spinach chloroplasts and algae evolve little oxygen in response to the first two flashes in a series of 10 μsec flashes spaced one second apart, following a five minute incubation period in total darkness. All subsequent flashes stimulate oxygen evolution but the quantitative yield of oxygen per flash undergoes damped oscillations with period four, being maximal on the third, seventh, eleventh, etc., flashes. Kinetic analyses indicate that the period four oscillations are due to the linear accumulation of oxidized intermediates which are formed rapidly ($t_{1/2} \cong 400$ μsec) following each flash. Once formed, these intermediates are stable for seconds.

In contrast to this behavior, chloroplasts which have been inhibited on the water side of PSII oxidize the added donor systems, phenylenediamine or hydroquinone, in a manner which shows neither the induction period nor the oscillations with period four noted in normal oxygen evolution. Comparative rate measurements show that phenylenediamine gives results most clearly attributable to specific PSII oxidation in tris (0.8M, pH 8.0) or heat inhibited chloroplasts. When subjected to a series of saturating 10 μsec flashes, dark-adapted

tris-washed chloroplasts oxidize phenylenediamine on the first and all subsequent flashes. In the presence of DCMU only the first flash is effective, all following flashes give little or no signal. The onset kinetics for exogenous donor oxidation show half-times on the order of 200 μsec, comparable to those found with intact chloroplasts with water as the donor. These results are discussed in terms of a model in which donor oxidation occurs at a site close to the primary electron donor for PSII.

Bandwidth and hyperfine structure measurements of the EPR spectrum of Signal II, a free radical species which is observed only in oxygen evolving photosynthetic organisms, in spinach chloroplasts show that the signal reflects two alternative states. One state is characterized by a 16 G bandwidth and four partially resolved hyperfine components. The other state has 19 G bandwidth and five partially resolved hyperfine components. It is possible to interconvert these two states by changing the ionic strength of the chloroplast suspension.

In order to perform kinetic experiments on Signal II a method was developed which facilitates the isolation of fresh, untreated chloroplasts with low dark levels of Signal II. Under these conditions a single 10μsec flash is sufficient to generate greater than 80% of the possible light-induced increase in Signal II spin concentration. The risetime for this flash-induced increase in Signal II is approximately 1 sec. The close association of Signal II with PSII is confirmed by the observations that red light is more effective than is far red light in generating Signal II, and that DCMU does not inhibit the formation of the radical. Single flash saturation curves for the

flash-induced increase in Signal I and Signal II indicate that the quantum efficiency for Signal II formation is close to that for Signal I. While one or two flashes (spaced 10 msec apart) are quite efficient in generating Signal II, three or four flashes are much less effective. However, if this spacing is decreased to 100 μsec, three or four flashes become as efficient as one or two flashes. From observations of a deficiency of oxygen evolved during the initial flashes of dark-adapted chloroplasts, the conclusion is made that the species which gives rise to Signal II is able to compete with water for oxidizing equivalents generated by PSII. On the basis of these results a model is postulated in which Signal II arises from an oxidized radical which is produced by a slow electron transfer to the specific intermediates, S_2 and S_3, on the water side of PSII.

In chloroplasts at room temperature, Signal II dark decays to 50% of its total light-induced level in about 1 h. Single flashes increase the spin concentration in these aged chloroplasts but with decreased effectiveness compared with fresh, dark-adapted chloroplasts. CCCP decreases the decay time of Signal II from hours to seconds without appreciable altering the level of Signal II formed in saturating continuous light. However, both the formation time constant and the extent of Signal II increase stimulated by a single saturating flash are decreased in CCCP-treated chloroplasts. These results are interpreted in terms of the model, proposed above, in which Signal II is generated by oxidation-reduction reactions on the water side of PSII.

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To my parents

SECTION I

INTRODUCTION

INTRODUCTION

Photosynthesis forms one-half of the water/oxygen cycle in nature. Green plants and algae use water as a reductant in the light-driven process of carbon dioxide fixation. The oxygen released in this photooxidation of water is used in reactions, principally respiration linked to phosphorylation, which comprise the second half of the cycle. In these processes oxygen is used as an oxidant, being reduced to water.

The evolution of this cycle is both fascinating and instructive. In the most widely accepted hypothesis concerning the evolution of life, the earth solidified about five billion years ago (Berkner and Marshall, 1964) and initially was without an atmosphere (Latimer, 1950). A primitive atmosphere subsequently arose from secondary sources, most notably volcanic eruption, and was primarily composed of water, carbon dioxide, sulfur dioxide and nitrogen along with traces of a number of other gases. Early work on the composition of this primordial atmosphere has been summarized (Berkner and Marshall, 1964) and indicated the complete absence of oxygen in volcanic eruptions. More recent observations, particularly of the composition of gases emitted from volcanoes in Hawaii (Naughton et al., 1969; Muenow, 1973), indicate that small amounts of oxygen (about 0.1 ppm) are released from active volcanoes. However, the oxygen released from primitive volcanoes most likely reacted immediately with ferrous iron in lava oxidizing it to ferric iron (Dole, 1965), resulting in an early atmosphere that was essentially anoxygenic. The geochemical

evidence pointing to this conclusion has been summarized (Chamberlain, 1952; Rutten, 1962, 1971).

Early theories (see, for example, Gaffron, 1962) of the evolution of the atmosphere assumed that the principal source for the appearance of molecular oxygen was the photodissociation of water vapor. However, the work of Urey (1959) on the self-regulation of this process by oxygen shadowing and of Dole (1965) on the rates of hydrogen atom escape indicate that equilibrium for this photodissociation process was established at an oxygen concentration of 10^{-3} the present atmospheric level (PAL) of oxygen. Therefore the current theory for the prebiotic atmosphere postulates essentially a reducing atmosphere with only traces of oxygen. Under these conditions the ozone concentration at the earth's surface was only a tenth of present levels and extended to a height above the earth of one-fourth that at present (Berkner and Marshall, 1968). Since ozone constitutes the major component of the atmosphere shield against ultraviolet , the ultraviolet intensity incident upon the surface of the earth was much higher than it is today.

The now famous experiments of Miller and Urey (1959) have shown that under the prebiotic conditions described above, using energy inputs of ultraviolet radiation, heat, electric discharge and radioactivity, the simple organic compounds of the Oparin (1938) - Haldane (1954) organic soup are formed. Early attempts to detect porphyrins in similar experiments were unsuccessful (Gaffron, 1962); however, recently they have been shown to occur when the methane, ammonia, water vapor system is subjected to electrical discharge (Hodgson and

Ponnamperuma, 1968). A mechanism involving hydrogen cyanide has been proposed for this process and the catalytic effect of divalent cations has been noted (Calvin, 1969). Therefore the conclusion that the porphryin ring system, vital to both the photosynthetic and the respiratory systems, appeared early in the prebiotic organic milieu, is tenable.

The assembly of the simple monomeric organic compounds into polypeptides and polynucleotides, the role of abiotic catalysis and autocatalytic functions in this process and the origin of functional and informational macromolecules have been discussed by several authors (Good, 1973; Eigen, 1971; Pattee, 1971; Calvin, 1969). Since this area is one of active research with several hypothetical formulations I will not go into it in any detail.

In the prebiotic aqueous phase porphyrin may have played the role of a rogue molecule, reacting to form metalloporphyrins and then mediating both photochemical and catalytic electron transfers. Based on considerations of relative abundance, Krasnovsky (1971) has pointed out that the metalloporphyrins most likely to have occurred in the early environment are those formed either with magnesium or iron. Although the rates of the Fe (II) or Mg (II) reaction with porphyrins are very slow in water (Philips, 1963), once formed, both metalloporphyrins are thermodynamically quite stable in aqueous solution (Marks, 1969). In prebiotic bodies of water the pH is generally thought to have been between 7 and 9 (Rabinowitz _et al._, 1971), which would have stabilized both metalloporphyrins to the well known

dilute acid dissociation. The Fe metalloporphyrin is photochemically inactive but an excellent catalyst in electron transfer reactions which proceed with a net decrease in free energy. The Mg metalloporphyrin, on the other hand, is essentially a photocatalyst, mediating electron transfers in which free energy is stored in the products. Two characteristics of magnesium metalloporphyrins may have added considerably versatility to these photoreactions. The first is that chlorophyll (a magnesium metalloporphyrin with a hydrocarbon tail) is able to undergo photooxidation, photoreduction and reversible photosensitization reactions. Estigneev (1969) has demonstrated that in basic media in the presence of a fairly strong reductant and light the chlorophyll anion can be detected, while in acidic media with a suitable acceptor, the chlorophyll cation is observed. For example, Krasnovsky (1969) has observed the chlorophyll b photosensitized reduction of riboflavin by cysteine. The second characteristic is that the redox potential (reduction potential, i.e., for the reaction $M^+ + e^- \rightarrow M$) of chlorophyll is highly influenced by its environment. Similar effects have been noted for iron-porphyrins in which both the basicity of the porphyrin ring nitrogens and of the 5th and 6th ligands to the iron stabilize the ferric state with respect to ferrous, thus lowering the redox potential (Falks, 1964). Seely and Diehn (1969) have reported the potentials for the oxidation of chlorophyll a in a number of solvents and have found values for the reaction $Chl^+ + e \rightarrow Chl$ ranging from +0.75 in methanol to greater than +1.0 in t-butanol. They also report a

value of +0.60 for reduction of the chlorophyll cation to the triplet state. They conclude from these experiments that there must be some mechanism for nucleophilic stabilization of the Chl cation by solvent molecules. It is important to note that unstabilized Chl^+ has sufficient energy to oxidize water, although to date this has not been demonstrated _in vitro_ experimentally.

A second class of electron transfer catalysts also may have evolved prebiotically. These are the non-heme iron compounds of which ferredoxin is the best known example. They consist essentially of an iron-inorganic sulfur "rock" (generally Fe_2S_2 or Fe_4S_4) linked via cysteine thiol groups to a polypeptide chain (Carter _et al._, 1972). Granick (1965) has presented a model for their abiogenesis in which the first step involves FeS and FeS_2 cluster formation in monolayers on solid surfaces. The ferredoxins have very low redox potentials, generally in the range -0.2 to -0.5 volts, and are able to function as one (plant photosynthesis) or two (bacterial photosynthesis) electron carriers. Two excellent summaries of the properties and functions of known ferredoxins have appeared (Hall and Evans, 1969; Lippard, 1973).

The picture emerges, then, that in the prebiotic oceans considerable photochemical and catalytic electron transfer reactions occurred. These reactions, along with ultraviolet-mediated photoreactions and perhaps catalysis on clays, led to a diversification of the prebiotic hydrosphere. Had appreciable amounts of oxygen been present under these conditions it would have had severe inhibitory

effects by providing an oxidizing pathway resulting in an extremely stable final product, water. This argument has been cited as evidence for the anoxygenic character of the primordial atmosphere (Berkner and Marshall, 1964).

Electron transfer reactions, catalyzed by primitive enzymes similar to those described above, most likely provided the energy source for the first primitive cells. The method by which the energy in these reactions was conserved is uncertain; however, a number of considerations bear on this question. In today's biosphere, energy conservation occurs through the coupled phosphorylation of ADP to ATP catalyzed by a membrane bound coupling factor with ATPase activity [F_1 in mitochondria, CF_1 in chloroplasts (Beechey and Cattell, 1973)]. Whether this energy conservation is mediated by a specific high energy intermediate (Chance, 1972) or a transmembrane potential gradient (Mitchell, 1968) is an area of active research at present. However, the complexity involved in the membrane bound phosphorylation system indicates that perhaps a simpler system was originally used. A number of authors have suggested the orthophosphate (Pi) - inorganic pyrophosphate (PPi) system as a candidate for primordial energy conservation. This assignment is based both on the simplicity of the PPi phosphorous-oxygen-phosphorous linkage and the demonstration of the occurrence of PPi in prebiotic model systems (Schwartz, 1972; Miller and Parris, 1964). Support for this hypothesis has come from the "metabolic fossil" demonstrated in the photosynthetic bacterium, R. rubrum. H. Baltscheffsky (1971) and M. Baltscheffsky

(1969) have shown that under certain conditions, chromatophores (isolated membrane fragment vesicles obtained by sonication of the intact bacterial cell) produce PPi from Pi in a light-driven reaction and that the PPi formed can be coupled to energy requiring dark reactions via a pyrophosphatase.

The first organisms probably were not photosynthetic; rather they were able to couple primitive cell processes via the Pi - PPi system to the transfer of electrons from the highly reduced organic compounds in the environment to less reduced moelcular sinks. Broda (1970, 1971) has pointed out that these organisms may have resembled the hydrogen fermenters, typified by the clostridia found in today's biosphere. He bases this hypothesis on the fact that clostridia are obligate anaerobes and further on the fact that they are completely devoid of heme. Therefore the earliest organisms may have used only the non-heme iron and flavin compounds in an electron transport chain coupled to PPi formation. In light of the high potentials discussed above involved in magnesium porphyrin oxidation, this formulation is pleasing in that it allows life to appear without the complexity involved in first developing the controlled environment in which chlorophyll photooxidation occurs (Philipson, 1972) and then coupling an electron transfer chain with the photochemistry.

Within this hypothesis the rise of photosynthetic organisms would be triggered by the depletion of substrate suitable for fermentation. (It should be mentioned at this point that an alternative view, i.e., that photosynthesis was the first life process,

has been postulated (Granick, 1965; Gaffron, 1965). However, the arguments cited in the previous paragraph indicate that this was unlikely.) This evolutionary step may have occurred simply by replacing the exogenous electron donors to ferredoxin with the coupled photoreduction of ferredoxin and photooxidation of a Mg porphyrin. Iron heme compounds would have then rereduced the oxidized chlorophyll. In its earliest operation this type of photoactivity probably served only to provide energy for phosphorylation. The oxidized heme compounds would have served as excellent terminal acceptors in this light-driven "fermentation" reaction and thus a cycle developed: light-induced Chl oxidation and coupled non-heme iron reduction followed by successive electron transfer via flavin and quinone to the heme. Phosphorylation would have been coupled to the dark (non-light-driven) reactions; the energy conserved would have been used for the assimilation of exogenous organic compounds.

Considerable evidence supporting the role of a ferredoxin-like molecule as the primary acceptor (the primary acceptor and donor are the first stable chemical species observed following light-induced charge separation) in bacterial photosynthesis and in Photosystem I (see below) has accumulated. Using EPR techniques, Malkin and Bearden (1971) have shown the occurrence of an EPR signal (g = 1.94), characteristic of non-heme iron proteins, upon illuminating chloroplasts at 20°K. Similarly, Leigh and Dutton (1972) have observed a light-induced EPR signal (g = 1.82) in bacteria. Stoichiometric measurements indicate that these signals appear in a 1:1 ratio with the spin signal arising from the oxidized reaction center Chl (Bearden

and Malkin, 1972). Furthermore, chemical reduction of the g = 1.82 signal in bacteria prior to freezing leads to a loss in the light-induced oxidized reaction center signal. In these low temperature experiments it is assumed that only the primary donor and acceptor are produced and that secondary electron transfers do not occur.

The absorption characteristics of the magnesium porphyrin involved in this cycle is uncertain. The Chl in present day higher plants and algae has absorption bands in the blue (440 nm) and red (680 nm), while BChl absorbs at 370 nm, 590 nm and in the region around 850 nm. Olson (1970) has presented evidence, based on the biosynthesis of BChl, indicating that a form of Chl similar to that found in blue-green algae was first used in photoreactions. The control of the redox potential of the oxidized Chl in the photoactive centers (reaction centers) of these organisms may have been achieved in two related ways. Spectroscopic work (Sauer *et al.*, 1966; Katz *et al.*, 1963) has shown that Chl in suitable solvents (*e.g.*, CCl_4) can exist as dimers and the *in vivo* circular dichroism of reaction centers from bacteria and higher plants have indicated that the Chl molecules in these centers are coupled by exciton interaction (Philipson, 1972). Katz and Norris (1973) have presented arguments, based on NMR studies with Chl model systems, which indicate that Chl dimerized via water may exhibit spectral characteristics similar to one of the reaction centers in plants. Thus it appears that the nucleophilic stabilization of Chl^+ discussed above may occur in reaction centers by dimerization via a nucleophile.

The second mode of stabilization may be the delocalization of the positive charge over the two molecules in the dimer. Feher (1973), using ENDOR techniques, has shown that in the oxidized reaction center of R. spheroides the positive charge is delocalized over two BChl molecules. This charge delocalization would also tend to stabilize the oxidized reaction center. Further considerations on the stability of the reaction center will be discussed below.

Three mechanisms evolved to stabilize and funnel the photochemistry of the reaction center into energy conserving rather than photodestructive reactions. The first involved the build-up of a large quinone pool of intermediate redox potential (+0.04 V) (Reed et al., 1969) which functions to maintain the heme in the reduced state. This type of arrangement appears to be universal in photosynthetic bacteria and in Photosystem I of green plants, i.e., a c-type cytochrome is oxidized directly by the reaction center and the oxidized cytochrome is subsequently rereduced by electrons supplied by a large pool of quinone (generally 10-20 molecules per reaction center). This arrangement insures that a large concentration of oxidized, and therefore reactive, Chl does not occur. The second protective mechanism involves the development of kinetic balances such that the oxidation of the cytochrome occurs rapidly ($t_{1/2}$ = 1 μsec) (Parson, 1968). If the reaction center is not rereduced by the cytochrome, the reduced primary acceptor back reacts in a time (30 msec) (McElroy et al., 1969) long with respect to the oxidation of the cytochrome, but short enough to prevent accumulation of oxidized Chl. The third mechanism was the development

of carotenoids. Presumably these pigments first functioned in energy transfer (see below), but with rising oxygen concentrations, they assumed the function of Chl triplet state quenchers and served to prevent nonspecific Chl triplet state photooxidation. Clayton (1965) has pointed out that carotenoidless mutants of photosynthetic bacteria are able to grow anaerobically in the light, but in the presence of oxygen and light, massive photooxidation occurs.

The concept of the photosynthetic unit, first developed from the experiments of Emerson and Arnold (1932), indicates a further refinement of the emerging photosynthetic apparatus. The pigments in photosynthetic organisms have attained a kind of division of labor: The majority of the pigments do no photochemistry but serve to funnel light energy to the specialized reaction center Chl. This quite probably occurred early in the evolution of photosynthesis, perhaps in response to diminished light intensity due to the proliferation of light absorbing organisms. A second response to this competition may have been the development of a multiplicity of Chl forms with differing absorption spectra and eventually the incorporation of secondary pigments (e.g., carotenes and phycobilins) to assist in the collection of light. Rabinowitch and Govindjee (1969) discuss both the structure of these pigments and the modes of energy transfer. Also of interest is the recent work of Geacintov et al. (1972, 1973) on the orientation of Chl in vivo.

Many of the modifications and developments of the photosyntnetic apparatus described above may have occurred when the only light-driven

reaction was cyclic phosphorylation. However, as the supply of organic substrate suitable for photoassimilation was depleted, the exploitation of other carbon sources became necessary. This evolutionary stress appears to have been met in a number of ways. The major classes of photosynthetic bacteria are listed in Table 1 (p.32), together with a number of their characteristics. As shown under the column labeled "carbon source" the three classes of bacteria have retained the ability to photoassimilate organic compounds. Whether this is mediated by reversed electron flow via ATP hydrolysis or by light-induced non-cyclic generation of a reductant is uncertain at present (Gregory, 1971). However, all three classes have also developed methods of fixing carbon dioxide. This is accomplished in one of two ways: either the reductive pentose cycle (Calvin and Bassham, 1962) or the reductive citric acid cycle (Evans _et al._, 1966). The pentose pathway is the same as that found in algae and green plant carbon dioxide fixation and requires NADH and ATP, the reductive citric acid cycle is essentially a reversed Krebs' cycle and requires reduced ferredoxin along with NADH and ATP.

Before these two carbon fixing pathways could evolve, however, a hydrogen source is required for the light-driven NADH reduction. (The demonstration of a direct NAD non-cyclic reduction in bacterial systems is ticklish; in whole cells substrate photooxidation and NAD reduction can be observed (Amesz, 1963) whereas in chromatophore preparations NAD reduction appears to proceed by reversed (ATP dependent) electron flow (Vernon, 1968). Dutton and Leigh (1973) have recently reported that in purified reaction centers of _R. rubrum_

the midpoint potential of the g = 1.82 EPR signal, which they claim arises from the primary acceptor, is -40 mV and not pH dependent. However, in chromatophore preparations from the same organism, this midpoint potential is lower (-100 mV at pH 8) and pH dependent. It is quite possible then that in fractionating intact cells, to obtain chromatophores, similar alterations in the chemical environment of the non-cyclic NAD reduction mechanism may occur. For example, chromatophores exhibit ubiquinone behavior markedly different from that observed in whole cells (Vernon, 1964). Therefore the use of chromatophore preparations in studying electron transfer is fraught with artifacts.

In bacteria the hydrogen source for NAD reduction appears to have evolved in two distinct directions. The first, and perhaps the more primitive, utilizes a wide variety of organic compounds as substrate for photooxidation reactions. Van Niel (1941) has studied this reaction extensively and lists 12 organic acids (from two to nine carbon compounds) and a number of alcohols upon which bacteria may grow. The only products of these oxidations are carbon dioxide with the acids and acetone with the alcohols. This pathway is used extensively by the purple non-sulfur bacteria (e.g., Rhodopseudomonas spheroides, Rhodospirillum rubrum) and may have evolved by coupling exogenous organic reductants via flavoprotein dehydrogenases to the quinone pool. Horio and Kamen (1970) have noted that organic substrates reduce this pool in the dark in present day R. rubrum. This mechanism represents a simple extension of the protective role postulated above for the ubiquinone pool.

The second involves the photooxidation of inorganic sulfur compounds. Photosynthetic bacteria can be divided into two classes based on this sulfur metabolism. The simpler are the green sulfur bacteria (e.g., Chlorobium thiosulfatophilum) which are able to oxidize hydrogen sulfide or sodium sulfide to elemental sulfur. Van Niel (1941) has pointed out that these organisms are unable to oxidize the sulfur to higher oxidation states. In marked contrast to this behavior is that exhibited by the second class, the purple sulfur bacteria (e.g., Chromatium), which are able to oxidize hydrogen sulfide (S = -2) to sulfate (S = +6). This oxidation appears to proceed via two electron steps since the purple sulfur bacteria will also metabolize elemental sulfur (S = 0), thiosulfate (S = +2) and sulfite (S = +4) with sulfate (S = +6) as the end product. The characteristics of these processes will be dealt with in some detail.

The development of substrate oxidation by the purple non-sulfur followed by the green sulfur bacteria may have been the initiation of the evolutionary process leading to water oxidation in algae. The purple bacteria use BChl a as both the major antenna and reaction center pigment (P890 or P870) and consequently absorb maximally in the region 800-900 nm. (Reaction centers are usually designated as "P" followed by the wavelength of maximum bleaching upon the photooxidation of the reaction center.) The green bacteria, on the other hand, contain a form of Chl (Chlorobium Chl) which absorbs between 700 and 800 nm and subsequently transfers energy to the reaction center, P840, which is postulated to be a typical BChl a molecule (Olson, 1970).

Green bacteria show similarities to the oxygen evolving blue-green and green algae, especially in the organization of the membrane (Fowler et al., 1971), and may have been the more immediate precursor to oxygen evolving algae. In this formulation the purple sulfur bacteria would be an evolutionary refinement to the sulfur metabolism of the green bacteria and have presented a combination of the functions of the green and purple non-sulfur bacteria. Recent work (Kennel et al., 1972; Thornber and Olson, 1971) indicates that depending on the redox poise of the environment the purple sulfur bacteria, Chromatium, can oxidize either succinate, a reaction typical of purple non-sulfur bacteria, or hydrogen sulfide, a reaction characteristic of green bacteria. Thus the purple sulfur bacteria may have evolved from a purple non-sulfur bacteria by incorporating sulfur photooxidation. This is strengthened by the observation, mentioned above, that purple sulfur bacteria are able to use the waste, elemental sulfur, of green bacteria photosynthesis as substrate.

The cytochrome complement involved in these three types of bacterial photosynthesis offer insights into the mechanisms of the photooxidations. (Before proceeding into this topic, however, the reader should be warned that the subject of bacterial cytochromes is one that borders on chaos.) In photosynthetic bacteria there are both respiratory and photosynthetic cytochromes which must first be sorted through. In addition, I have encountered four types of classification for the photosynthetic cytochromes: (a) according to Soret band absorption maxima, (b) according to alpha band maxima, (c) according to midpoint redox potential, and (d) one that appears

to me to be completely random (e.g., cyt c_2, cyt cc', cyt B). For this subject a scorecard is a necessity. The purple non-sulfur bacteria contain both c and b type cytochromes. c-Type have alpha band maxima around 550 nm, and the heme is bound covalently via reaction of the heme vinyl groups with cysteines of the polypeptide. b-Type are usually of lower potential than c-type, have alpha band maxima around 560 nm and the heme is not bound to the protein covalently. Dutton and Jackson (1972) have studied the electron transfer reactions in R. rubrum and R. spheroides. Using the succinate/fumarate couple as substrate, ubiquinone is reduced via a flavoprotein and the ubiquinone is subsequently oxidized via cyt b and cyt c by the reaction center. Note that this reaction sequence is quite similar to mitochondrial electron transport in which the sequence is succinate, flavoprotein, ubiquinone, cyt b, cyt c, cytochrome oxidase and oxygen.

The sulfur metabolizers contain only c-type cytochromes. In Chromatium, cut c_{555} is a high potential (E_{m7} = +0.34) species (Thornber and Olson, 1971) and appears to function along with cyt cc' in cyclic and organic substrate photooxidation reactions (Gregory, 1971). These bacteria also contain a lower potential cyt c_{552} (E_{m7} = +0.04) which is functional in sulfur metabolism. The green sulfur bacterium, C. thiosulfatophilum, also contains a low potential cytochrome, cyt c_{553} (E_{m7} = +0.098) (Bartsch et al., 1967) which is similar to the low potential cyt c_{552} of Chromatium.

These two low potential cytochromes, cyt c_{552} of Chromatium and c_{553} of C. thiosulfatophilum have three quite interesting properties. They appear to be the site of sulfur interaction in the two bacteria,

they are oxidized directly by the reaction center (Hind and Olson, 1968) and they contain in addition to heme, a flavin molecule (Bartsch, 1968). Also important is the fact that similar heme-flavin-proteins have not been isolated from purple non-sulfur bacteria. Cyt c_{552} from Chromatium contains heme to flavin in a 2:1 ratio whereas cyt c_{553} contains only one heme and one flavin. This may be crucial to the ability of the purple bacteria to oxidize sulfide to sulfate, whereas the green bacteria are able to oxidize sulfide only to elemental sulfur. Unfortunately, little further work appears to have been done with these cytochromes. We may attribute, at least tentatively, the following characteristics to bacterial sulfur metabolism: (1) sulfur compounds are oxidized in two electron steps; (2) a flavo-heme-protein appears to be the interaction site; (3) although these cytochromes are of relatively low potential, there do not appear to be intermediate carriers between the cytochromes and the reaction center; (4) b-type cytochromes do not occur.

With the rise of sulfur metabolism and the accumulation of sulfate and partially oxidized intermediate in the, as yet, anoxygenic environment, the process of respiration became possible. Although some present day respirers are able to use fumarate as a terminal oxidant in respiration via a flavoprotein (Singer, 1971), this fumarate is usually of a non-photosynthetic source, e.g., glycolysis, and therefore may not have been present at high concentration in the early biosphere. Sulfate, however, was produced in high concentration by photosynthetic organisms and its reduction in respiration would have provided a sulfur cycle similar to the water/oxygen cycle in operation

today. Remnants from this earlier stage in evolution exist today and reduce sulfate via an electron transport chain consisting of flavoproteins, cytochrome and a terminal sulfate (or sulfite) reductase (Dolin, 1961). This terminal reductase and a similar one involved in nitrate-based respiration appear to be complex flavoproteins (up to eight flavin molecules per protein) which also contain non-heme iron (Mahler and Cordes, 1971). This sulfur cycle may have been long-lived, its development limited only by the scarcity of sulfur. Upon reaching this limit the stage was set for the development of oxygen evolution.

The reduction potential for oxygen is +0.82 V at pH 7 (Latimer, 1952). Therefore photosynthetic organisms capable of oxidizing water must develop a mechanism which generates an oxidant of higher potential than this. They have also retained the ability to generate a reductant with a potential lower than ferredoxin ($E_{m7} \cong -0.5$). The free energy available from single quanta of red light, which drives green plant and algal photosynthesis quite effectively (Sun, 1971), is insufficient to span this 1.3 V potential range (Ross and Calvin, 1967). The prototype algae which evolved from the bacteria overcame this thermodynamic difficulty by arranging two chlorophyll photo-oxidation-reduction reactions in series. One generates the high potential oxidant which oxidized water and simultaneously supplies a weak reductant to the other photoreaction which reduces ferredoxin. The reactions, both light-driven and dark steps, which generate oxygen, have been labeled Photosystem II (PSII) and the reactions which reduce ferredoxin, Photosystem I (PSI). Photosystem I

has many of the characteristics of bacterial photosynthesis. P_{700}, the reaction center in PSI, is reduced via a c-type cytochrome (unfortunately designated cyt f, the "f" coming from the Latin for leaf, frons) by a large quinone pool, there is a phosphorylation cycle around PSI, and NADP is reduced via a non-heme iron protein which appears to be the primary acceptor (Malkin and Bearden, 1971; Bearden and Malkin, 1972).

As shown in Table 1, the redox properties of P_{700} are very close to those for the bacterial reaction centers and consequently far short of the +0.82 necessary for PSII oxygen evolution. In order to gain an insight into the properties of the reaction center Chl for PSII, especially its high redox potential, consideration of the properties of these lower potential reaction centers is fruitful.

Three main lines of attack have been applied in attempting to understand the structure and functionality of these species. The first has been extensively developed by Sauer and coworkers (Sauer, 1973; Philipson, 1972) and involves the application of circular dichroism techniques to purified and partially purified reaction center preparations from green plants and bacteria. The technique is very sensitive to protein conformation and intermolecular interactions between the chlorophylls. Denoting the chlorophyll complement as P and the primary acceptor as A, the bleaching observed in photooxidizing the reaction center can be presented as

$$P\,A \xrightarrow{h\nu} P^{+}A^{-} \xrightarrow[\text{acceptors}]{\text{secondary}} P^{+}A$$

Chemical oxidation of the reaction center may be represented as

$$P\ A \xrightarrow[\text{oxidant}]{\text{chemical}} P^{+}A$$

To date the CD technique has been extensively applied in studying the Chl conformations in the PA complex. Upon oxidation, the absorptions due to the photooxidizable Chl are bleached and the interaction of the unbleached Chls in the reaction center, $P^{+}A$, can be studied. The results from these studies have shown extensive exciton interaction between the Chls in the reaction center, part of this interaction remains even after photooxidation. As yet the interactions of the oxidized Chl in the reaction center, $P^{+}A$, which absorbs at 1250, 1140, 980 and 420-450 nm in bacterial reaction centers (Clayton, 1962) and at 815 and 750 nm in PSI (Inoue et al., 1973), have not been examined with CD. Research into this area is currently underway.

The second technique, which employs EPR techniques, has been developed by Katz, Norris and coworkers (Katz and Norris, 1973; Norris et al., 1971) and by Feher and coworkers (McElroy et al., 1972; Feher, 1973). The method is essentially complementary to the CD measurements in that the unpaired electron of the oxidized Chl in the reaction center $P^{+}A$ is observed. This allows statements about the interactions of this oxidized Chl to be made. Norris et al. (1971) compared the EPR signal arising from oxidized P_{700} with the signal from a number of oxidized Chl model compounds and, basing their arguments on theoretical line width considerations, concluded that $P700^{+}$ best fit the assignment of $(Chl\text{-}H_2O\text{-}Chl)^{+}$. McElroy et al. (1972) concluded, on the basis of line narrowing in $P870^{+}$ ($\Delta H = 9.4 \pm 0.2$ G) compared to

monomer $BChl^+$ (ΔH = 13.0 ± 0.2 G) *in vitro*, that the *in vivo* signal probably arose from a dimer. More recently, Feher (1973) has reported that application of the ENDOR technique indicates that the unpaired electron in the *R. spheroides* oxidized reaction center is delocalized over two BChl molecules.

The final technique, also based on EPR, has been developed by Dutton and Leigh (1973; Dutton *et al.*, 1972) and involves chemical reduction of the primary acceptor and subsequent observation of light interaction with the reaction center PA^-. Under these conditions and at low temperature, EPR signals characteristic of the triplet state are observed following a laser pulse. The quantum yield for this triplet is high; Wraight *et al.* (1973) reported a value of 0.9.

The picture that emerges from these studies is that the Chls in the reaction center of bacteria and PSI interact extensively and are able to funnel energy efficiently via exciton transfer to the photo-oxidizable center. Charge separation may occur via the reaction (Chl^+-H_2O-Chl^-) (Katz and Norris, 1973). Following reduction of the primary acceptor the oxidized Chl may be stabilized by both the nucleophile, H_2O, and delocalization over the dimer as $(Chl\text{-}H_2O\text{-}Chl)^+$.

The reaction center of PSII most likely evolved from the structure postulated above. Olson (1970) has pointed out that two adjacent photosystems in an algal precursor may have been in communication via a shared quinone pool. Initially these two photosystems may have contained reaction center Chl arrangements similar to the structure described above and one of these may have gradually moved upward in redox potential by controlled destabilization of the Chl cation.

Olson (1970) has suggested that this destabilization occurred in small steps and that various nitrogen compounds served as electron donors to the emerging Photosystem II. The fact that organisms which use nitrate as terminal respiratory acceptor are functional today (Dollin, 1961) may be taken as support for this hypothesis. Opposing this view, however, is the failure to observe any photosynthetic organisms with nitrogen compounds as electron donors.

The mechanism of the controlled destabilization of the Chl cation to generate an oxidant capable of osidizing water is not at all understood. In fact, little is known about the architecture of the reaction center of Photosystem II (Clayton, 1972). However, the results of three sets of experiments allow at least speculative statements to be made about the sequence of events resulting in the reaction center of PSII. The first is that the Chl absorbance in the PSII reaction center is blue-shifted compared to P700. Action spectra show that the quantum efficiency for oxygen evolution is close to one electron transferred through PSII per two photons absorbed at 678 nm but that this efficiency drops rapidly in moving farther to the red. Photosystem I, on the other hand, shows a high quantum efficiency even in 700 nm light (Sun, 1971). In addition, a bleaching in the region 680-690 nm, first observed by Doring _et al._ (1967; 1969), has been tentatively attributed to the reaction center of PSII and designated P680 (Floyd _et al._, 1971). However, Butler (1972) has pointed out the dangers due to fluorescence artifacts in this measurement and notes that the concentration of P680 measured by Floyd _et al._ corresponds to 1:1000 Chl. Van Gorkom and Donze

(1973) have provided an explanation for the low P680 concentration in terms of a biphasic decay ($t_{1/2}$ = 35 μsec and 200 μsec) for the reduction of the oxidized reaction center.

Malkin and Beraden (1973) have recently observed an EPR signal which they have suggested may be associated with the reaction center chlorophyll of PSII. The spectrum for the signal is very similar to that observed for the EPR spectrum of $P700^+$ in g value, linewidth, and absence of hyperfine structure. Preliminary quantitative measurements indicate that this spin signal is present in the concentration 1:330 Chl.

The similarities between the signal reported by Malkin and Bearden and P700 indicate that the unpaired electron of the oxidized Chl in the reaction centers of PSII and I exist in similar environments. This implies that in $P680^+$ the electron is delocalized over a dimer. The blue shift, however, may indicate that the water which was postulated above to be present in the P700 reaction center has been replaced in P680 by an alternative, and less nucleophilic, ligand. Katz and Norris (1973) have presented a number of Chl-ligand model compounds of the form -L-Chl_2-L- which show characteristic absorption in the region 680 to 690. Unfortunately, no work appears to have been done investigating the effect of the various ligands on the redox potential of the Chl dimers. Free radicals of these compounds were prepared and had linewidths from 1 to 3 gauss wider than the *in vivo* signal.

The third set of experiments involves the observation that the reaction center of PSII appears to function in a hydrophobic environment. In untreated chloroplasts only hydroxylamine (Cheniae and

Martin, 1971) and hydrogen peroxide (Inoue and Nishimura, 1971; Babcock, unpublished results) have been found to compete with water for oxidizing equivalents generated by PSII. Reagents, such as phenylenediamine, hydroquinone and ferrocyanide, appear to be excluded from the PSII reaction center in intact chloroplasts. An earlier report (Cheniae and Martin, 1970) that ascorbate is able to override oxygen evolution appears to be an artifact resulting from the ability of ascorbate to reduce superoxide (Allen and Hall, 1973). In contrast, P700 operates in a much more polar environment. Redox titrations of P700 have been performed using the ferri/ferrocyanide couple (Kok, 1961), and electron donors such as dichlorophenylindophenol and tetramethylphenylenediamine appear to have direct access to the reaction center. Therefore in the process of its evolution the reaction center of PSII appears to have moved into a more hydrophobic environment.

Krassner (1973) has studied the effect on the redox potential of iron-porphyrin compounds exerted by the polarity of the local environment. He concludes that the high redox potential of the heme in cytochromes compared to model heme complexes may be accounted for by the non-polar nature of the heme environment within the protein. Therefore a second mechanism involved in the destabilization of the oxidized reaction in PSII may be the hydrophobicity of the environment.

The evolution of water oxidation and concommitant oxygen release required the development of three interacting systems. We have considered the first, generation of a strong oxidant, in detail above. The second system is an organized mechanism which reacts quickly and

specificially with substrate water. This will be dealt with now and the third, protection of cell constituents from photooxidation, will be considered below.

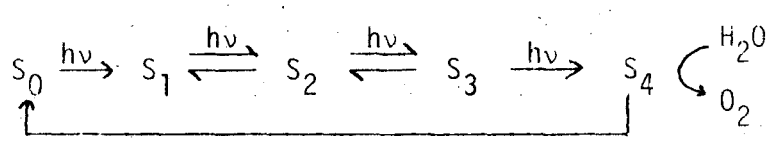
As postulated above, the oxidation of water occurs most probably in a hydrophobic environment. It has been known for some time that nonpolar moieties have a structuring effect on water which can be observed as an entropy decrease (Franks and Evans, 1945). Whether this effect has a direct stabilizing effect on proteins as suggested by Klotz (1965) or leads to the formation of hydrophobic bonds as suggested by Kauzmann (1959) and Scheraga (1964) has been recently discussed (Tait and Franks, 1971). A structure has been proposed (Richards, 1963) for the protein ribonuclease in which the depression in the surface of the molecule contains highly structured water molecules. Such a structure is envisioned for the site of water oxidation in PSII. The ordering of the water molecules by the hydrophobic site would facilitate, and may be necessary for, the formation of intermediates leading to the release of molecular oxygen. This model explains the observed inhibition of oxygen evolution in chloroplasts by chaotropic agents such as perchlorate anion, nitrate anion, urea and guanidine (Lozier et al., 1971). These agents are postulated to disrupt hydrogen bonds in water, subsequently making hydrophobic regions of proteins more accessible (Von Hippel and Schleich, 1969; Hatefi and Hanstein, 1969). These reagents, however, do not appear to disrupt photosynthetic reactions other than oxygen evolution since exogenous donors, hydroquinone and Mn^{+2}, are able to donate electrons to the reaction center with subsequent NADP reduction in this non-

oxygen evolving chloroplast preparation. Similarly, the oxygen evolving apparatus is disrupted by heat treatments (Yamashita and Butler, 1968) at times and temperatures (50°C, 3 min) which do not disrupt NADP reduction via PSII by exogenous donors. The model is also consistent with the H_2O^{18} experiments performed by Kutyurin (1971) from which he concludes that significant amounts of bound water exist in the oxygen evolving mechanism.

The oxidation of water to molecular oxygen is a four electron process. Within the postulated hydrophobic site for water oxidation the accumulation of these four oxidizing equivalents could occur in a number of ways. Three of the models will be described here. In the first, four PSII reaction centers, each storing one oxidizing equivalent per photon absorbed, would cooperate to provide the four oxidizing equivalents necessary to oxidize water. In the second model, cooperation is postulated between two PSII reaction centers, each of which is able to store two oxidizing equivalents. A model of this type has been proposed by Joliot et al. (1969) and involved the generation of atomic oxygen in a two electron oxidation process which occurred in each PSII center. Two oxygen atoms then reacted to form molecular oxygen. In the third model, each PSII center would act independently, accumulating oxidizing equivalents linearly with photons absorbed, until a total of four have been stored.

If single flashes of light, short enough so that only a single electron can be transferred through each PSII reaction center, are given, the measurements of the oxygen evolved in response to each flash should indicate which of the above mechanisms occurs. For

example, with four reaction centers cooperating, maximum oxygen should occur on the first flash. This experiment has been performed in a number of laboratories (Joliot *et al.*, 1969; Kok *et al.*, 1970; Weiss and Sauer, 1970; Weiss *et al.*, 1971; Babcock, this work, Section II.3) and the observation (see Fig. 3, this work) has been that little or no oxygen is evolved on the first two flashes, maximal amounts on the third and fourth and subsequent damped oscillations with period four. Kok *et al.* (1970) have presented a kinetic model for this process in which charge accumulation occurs independently within each reaction center. The independent action of reaction centers has also been shown in oxygen evolution experiments on partially inhibited chloroplasts (Kok *et al.*, 1970), on manganese deficient (see below) algae (Cheniae and Martin, 1973) and from fluorescence measurements (Joliot *et al.*, 1973). In order to account for the high yields of oxygen resulting from the third flash, Kok *et al.* (1970) postulated that the one electron oxidized state, as well as the completely reduced state, was stable. Denoting the oxygen evolving centers as S and its oxidation state by subscript, the Kok *et al.* model can be summarized as



S_0, S_1 -- Dark stable

Part of the work presented in this thesis deals with the kinetics of formation and decay of the intermediates presented in this scheme.

The electrons removed from the water side of PSII via P680 reduce the primary acceptor for PSII which is subsequently reoxidized by

oxidizing equivalents generated by PSI. Recent work (see Butler, 1973, for an excellent summary) has associated this primary acceptor with a spectral band shift around 550 nm, hence the designation of this acceptor as C550.

Using the flashing light technique it has become possible to study the behavior of PSII in all five of the S states. The state S_4 is quite labile and reacts to generate oxygen in less than 1 msec (Joliot et al., 1966). The states S_2 and S_3 have been studied in a number of ways, particularly by way of delayed emission which has the spectrum of Chl fluorescence and is thought to originate from a back reaction between the primary acceptor and donor (Lavorel, 1969). The halftime for the reaction S_n —— S_{n+1} following a flash is in the range 200-600 μsec (Kok et al., 1970; Babcock, Fig. 4, this work). Thus observation of delayed light emitted less than 200 μsec following a flash measures the state S_n, while the delayed light emitted after 1 msec measures the state S_{n+1}. Zankel (1971), measuring delayed light emitted in the range 45-160 μsec, observed maximum emission after the third flash, whereas Joliot and coworkers (Barbieri et al., 1970; Joliot et al., 1971) measured light emitted 30 msec after the flash and observed maximum emission following the second flash and oscillations with period four. Results similar to those reported by Joliot et al. have been described by Fleischman and Mayne (1973) and Hardt and Malkin (1973) for delayed light emission triggered by pH transitions, salt jumps and temperature jumps. These results imply that the states S_3 and to a certain extent S_2 are more reactive than S_0 and S_1. Results consistent with their interpretation have been

obtained for the light-induced generation of an EPR signal associated with Photosystem II (Babcock and Sauer, 1973a,b) and are described in this thesis. Tributsch (1971) and van Gorkom and Donze (1973) have interpreted this increased reactivity for the states S_2 and S_3 in terms of electric field effects existing within the reaction center. Vermeglio and Mathis (1973) have obtained evidence, from the low temperature photooxidation of a cytochrome (Cyt b_{559}) associated with PSII, indicating that conformation changes may accompany the formation of the states S_2 and S_3.

Table 1

Organism	Class	Carbon source	Absorption nm	RXN Center Chl & Absorption	RXN Center Potential	Hydrogen source	Cyto-chromes
R. rubrum	purple nonsulfur	CO_2 organic	800-900	BChl a, P870	+.44	organic	b,c
C. ethylica	green sulfur	CO_2 organic	700-800	BChl a, P840	+.24	some organic $S^=$	c
Chromatium	purple sulfur	CO_2 organic	800-900	BChl a, P885	+.49	$S^=$, S, $S_2O_3^=$, $SO_3^=$ organic	c
R. viridis	purple	CO_2 organic	970-1190	BChl b, P960	+.39 to +.45	organic	-
P. luridum	blue-green algae	CO_2 organic	550-700	Chl a, P700	+.38 to +.42	water, organic	c,b
Chlorella pyrenoidosa	green algae	CO_2 organic	600-700	Chl a, P700	+.43	water	c,b
Spinach	higher plant	CO_2	600-700	Chl a, P700	+.43	water	c,b

SECTION II

OXYGEN EVOLUTION IN FLASHING LIGHT

OXYGEN EVOLUTION IN FLASHING LIGHT

A. Introduction

The most striking recent development in the study of photosynthetic oxygen evolution has been the analysis of this process using sequences of short, saturating flashes and the subsequent realization that labile, photochemically produced intermediates are involved. Franck and Allen (1955) first noted that plants, incubated in the dark for several minutes, evolved no oxygen until the second flash in a series of brief (10^{-4} sec), strong flashes. This was confirmed by later investigators, and Joliot (1965) proposed a model explaining this observation as resulting from the activation of an oxygen precursor molecule in a one quantum process. However, experiments by Weiss and Sauer (1970), using 20-40 nanosecond pulses from a Q-switched ruby laser and 20 μsec flashes from a xenon flash lamp, demonstrated that at these flash times only a single electron is transferred through each PSII reaction center and showed that no appreciable amounts of oxygen were evolved until the third flash in a series. Similar experiments by both Joliot *et al.* (1969) and Kok *et al.* (1970), using refined polarographic techniques and 10 μsec flashes, have elucidated the entire pattern of oxygen evoked by a series of 20-30 flashes (Fig. 3). Both Joliot *et al.* (1969) and Kok *et al.* (1970) presented models to account for the pattern of oxygen evolution. The Joliot *et al.* (1969) model involved a complex switching mechanism and has been abandoned in favor of the Kok *et al.* (1970) model described in Section I.

In this section the polarographic technique for detecting oxygen evolved in response to single flashes is described and the pattern of oxygen evolution in response to 21 μsec flashes is presented. The activation and deactivation kinetics of the states S_2 and S_3 in the Kok et al. (1970) model for oxygen evolution have been studied using paired flashes with variable dark time between flashes.

B. Materials and Methods

(1) Light sources and optical arrangement

Short light flashes (2.1×10^{-5} sec) were produced from a xenon pulsed light system manufactured by ILC Co., Sunnyvale, Calif., as described by Weiss and Sauer (1970). Using a more efficient light-collecting optical arrangement we have lowered the lamp voltage necessary for saturating flashes and, consequently, it is now possible to flash once every second indefinitely. The flashes were focused by a lens system and filtered through Corning 1-69 infrared and 3-75 ultraviolet filters. Continuous light is obtained from a Bell and Howell 300-watt projector, filtered through 5 cm of water, and, if desired, appropriate Corning Glass color filters before being focused on the electrode by a pair of planoconvex lenses. Illumination was initiated using a mechanical shutter which had an opening time less than 20 msec.

(2) Electrodes and flow system

In the experiments described, both a teflon covered platinum and a bare platinum electrode were used. The teflon covered electrode is the modified Pickett apparatus described by Weiss and Sauer (1970). Because the teflon film (thickness = 6.4 microns) separates the oxygen evolving sample from the platinum electrode and consequently lengthens

the diffusion time necessary for the dissolved oxygen to reach the platinum surface, the response time of this apparatus is on the order of 300 msec.

In the bare electrode system, the teflon is removed and the algae or chloroplasts are in direct contact with the platinum electrode surface. In order to maintain electrical contact between the platinum and Ag/AgCl electrodes, and to avoid poisoning the photosynthetic mechanisms with Ag^+ ion, the reference (Ag/AgCl) electrode is placed in a cul-de-sac in the flow system downstream from the platinum electrode. The reference anode is approximately 1 cm from the main flow stream and 4 cm from the platinum cathode. The response time of this bare platinum electrode arrangement is 10 msec.

The sample compartment in both electrode systems is similar. The bottom is formed by the platinum electrode, either bare or teflon covered; the top is the same 1.6 mil (0.04 mm) dialysis membrane separating the algae from the flowing medium as described earlier (Weiss and Sauer, 1970). The sides of the compartment are defined by a spacer. During the course of this work a number of different spacers were tried. Initially a viton rubber coated fiberglass spacer was used. This was replaced by a 10 mil (0.25 mm) thick sheet of Mylar sandwiched between two 5 mil (0.13 mm) thick sheets of Dow Silastic #500-1 using General Electric silicone rubber adhesive sealant to hold the three sheets together. Finally, a bilayer spacer formed by black electrical tape and thin teflon sheeting [total spacer thickness = 5 mil (0.13 mm)] was used.

The flow system is essentially that described earlier (Weiss and Sauer, 1970) with three slight changes. The first is the use of an

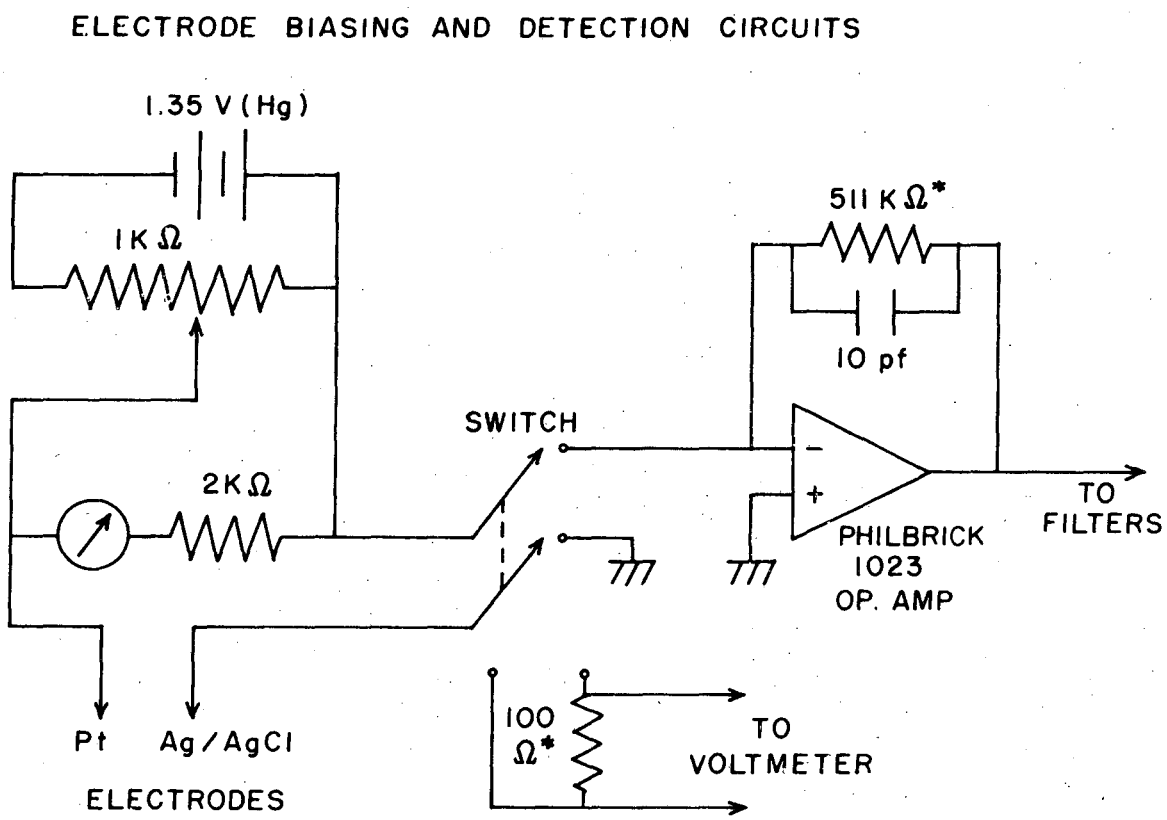


Figure 1. Electrode biasing and detection circuits. Details described in text. The voltmeter used was a Keithley 149 Milli-microvoltmeter. Resistors denoted by asterisks are precision (within 1% of the stated value) resistors, other resistors within 5% of the nominal value.

electrolyte solution (0.1 M KCl, 0.01 M phosphate buffer, pH = 7.6) instead of Myer's nutrient medium in experiments which involve the bare platinum electrode. The second is the elimination of the aerated, glass helices-packed column, which had been included to provide a more uniformly aerated flow medium. Experiments with and without this column under otherwise identical conditions show no differences in electrode response, current drift or noise. The reservoir, of course, is still aerated. Finally, the flow rate over the sample has been increased from 10 cc/min to 30 cc/min by raising the upper reservoir to a height of 4 feet above the electrode.

(3) Detection and recording circuits

The circuit used in providing a bias to the platinum electrode and in detecting oxygen transients is shown in Fig. 1. The double pole - double throw switch serves to connect either of two circuits with the electrode. The first, involving the 100 Ω resistor and a Keithley 149 millimicrovoltmeter, was adapted from Myers and Graham (1963) and is used in detecting slow (response time = 500 msec) phenomena. The second system involves the current-to-voltage transducing operational amplifier (Philbrick operational amplifier 1023). In this arrangement, both the Ag/AgCl reference electrode and the positive input terminal of the op amp are connected to system ground, and the negative input terminal, through the action of the operational amplifier, is a virtual ground. The voltage offset between positive and negative input terminals is biased with a 866 Ω resistor. This arrangement allows a current measurement to be made without introducing a resistor into the electrode circuit, thereby acting as a potentiostat

in supplying a well regulated EMF to the electrodes regardless of the current flowing in the circuit. Because of resistive and capacitive losses involved in the electrode arrangement, it is necessary to measure the potential between the platinum electrode and the electrolyte solution using a third electrode, either Ag/AgCl or calomel, and a null point potentiometer.

The current-to-voltage transducer is followed by a low pass (f_c = 5 kHz) third order Butterworth active filter (Operational Amplifier Devices 45 J) which attenuates high frequency noise components. The dark current ($\sim$ 3 μamp) due to the high concentration of oxygen dissolved in the flowing electrolyte is eliminated using either a high pass (f_c = 0.5 Hz) active filter (Philbrick operational amplifier SQ 10a) or a biased unity gain amplifier (Philbrick SQ 10a). The output of this system can be fed into either an oscilloscope (Tektronics RM 31, Type D Preamplifier) or into a Sanborn Single Channel Recorder (Sanborn Model 151-100A, Driver Amplifier and Power Supply Model 150-400; rise time = 5 msec).

(4) Algae and chloroplasts

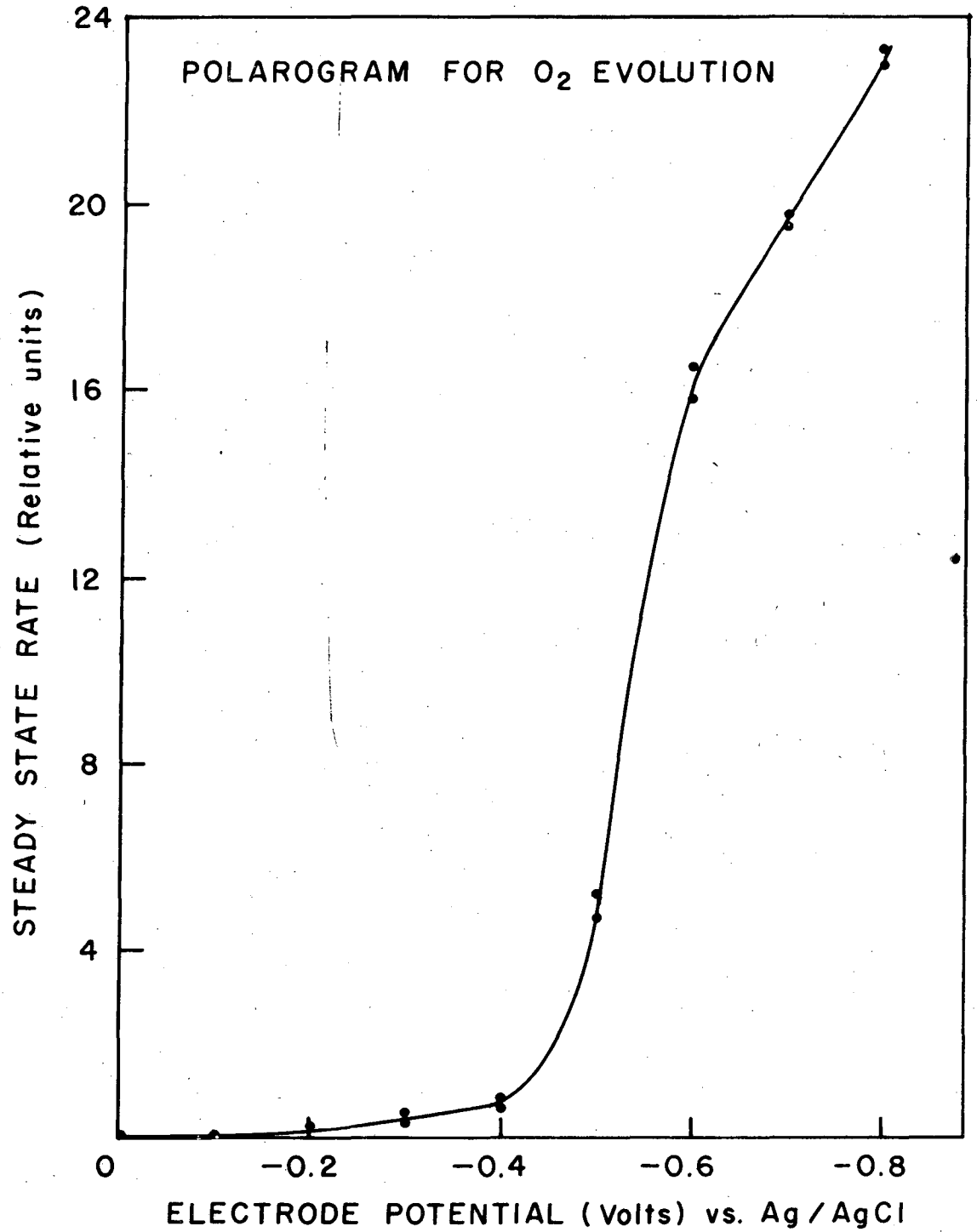
The algae used in these experiments were *Chlorella pyrenoidosa* grown under a variety of conditions, generally in batch culture. No obvious differences were noted with any of the various samples. Chloroplasts come from six to eight week old spinach plants grown from seed in a growth chamber. The isolation procedures are either those described by Kelly (1968), or those used by Butler and Yamashita (1968b) to obtain "intact chloroplasts". Estimations of chlorophyll content were made according to the method outlined by Sun (1971).

In our experiments we used chloroplasts which contained between 0.3 mg/ml of chlorophyll and 0.9 mg/ml. The reaction mixtures used in each experiment will be described in the discussion of that experiment.

C. Results

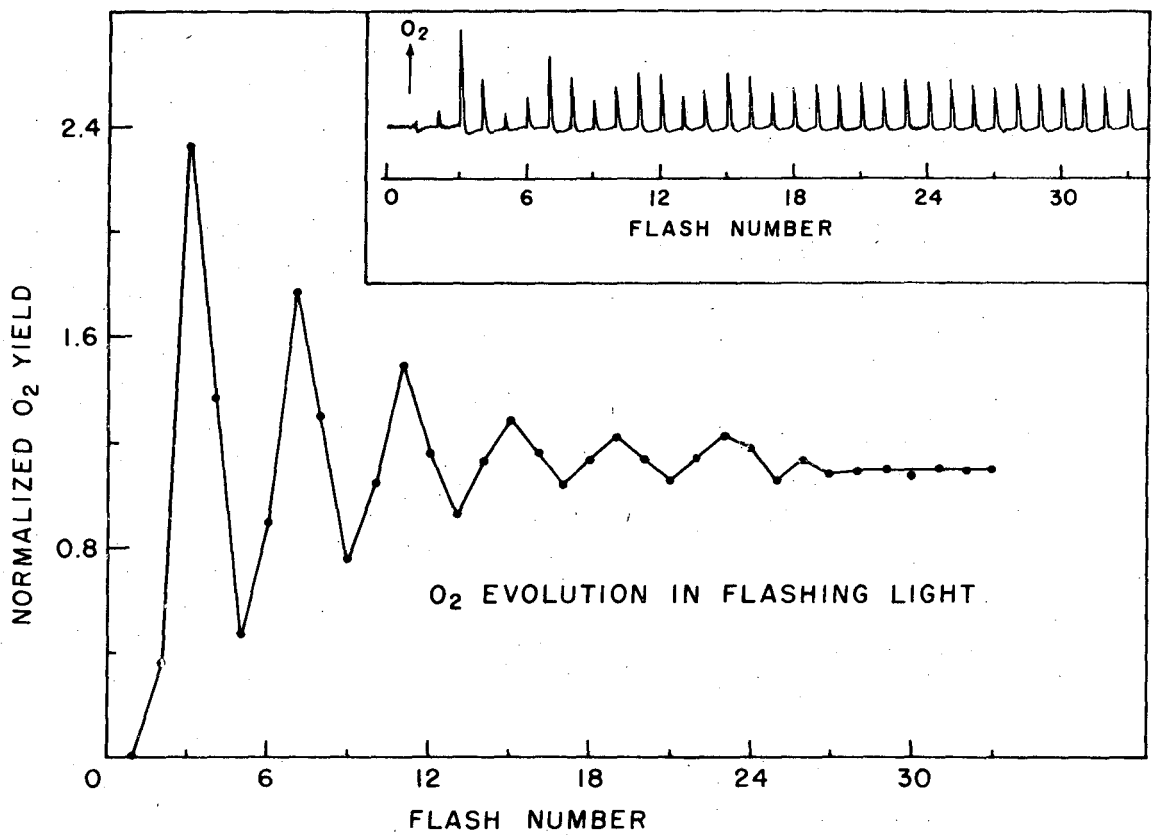
(1) Bare electrode oxygen polarogram

Fig. 2 shows the dependence of electrode response upon the applied potential in monitoring photosynthetically generated oxygen. In these experiments chloroplasts were prepared according to Yamashita and Butler (1968b) and were 0.4 mg/ml in chlorophyll; the reaction mixture contained 0.0025 M NADP, 41 μg Fd/ml, 0.012 M tris (pH = 7.6), 0.008 M $MgCl_2$, and 0.016 M NaCl. A new sample was injected into the electrode for each different voltage setting. Because this type of experiment precludes the usual normalization of results, it was necessary to treat each sample as uniformly as possible in order to obtain repeatability. Therefore, for all samples the following protocol was observed as closely as possible: 7-1/2 min after applying the voltage the chloroplasts were injected and allowed to settle for 10 min in the dark; 40 xenon flashes were given, followed by 5 min dark, and another 40 flashes followed by another 5 min dark period. The sample was then illuminated for 4.0 sec with weak continuous red light followed by 5 min dark and another 4 sec illumination with red light. The signal after the second 4 sec illumination is plotted as the ordinate on the polarogram. The absence of a well defined plateau is characteristic of polarograms obtained with biological samples (Myers and Graham, 1963), and necessitates the careful regulation of



XBL736-4843

Figure 2. Polarogram for oxygen evolution. Reaction mixtures and experimental protocol described in text. Weak continuous red light was obtained using Corning 2-64 Glass Color Filter.



XBL 736-4842

Figure 3. Oxygen evolution in flashing light. Inset: experimental trace from Sanborn recorder, chloroplasts and reaction mixtures as in Fig. 2, saturating 21 microsecond flashes spaced 1 sec apart were used to stimulate oxygen evolution. Main: average of eight experiments performed exactly as described in the inset.

the applied voltage mentioned previously. In contrast, for oxygen polarograms obtained without chloroplasts, a diffusion limited current plateau exists in the region of applied potential between -.55 and -.80 volts.

(2) Pattern of oxygen yield in flashing light

The chloroplasts and reaction mixtures for these experiments were prepared in the same manner as those used in obtaining the polarogram. The inset in Fig. 3 shows a typical trace obtained with saturating 21 μsec flashes spaced 1 sec apart. The sample had been kept in the dark for 5 min preceding this series of flashes. In this experiment a small oxygen uptake is observed on the first flash, which is probably attributable to Photosystem I effects (Mehler, 1951). This phenomenon appeared rather randomly in our experiments, its appearance and magnitude varying from experiment to experiment. In all of our experiments, however, we observed a small amount of oxygen on the second flash; its magnitude relative to the steady state is typical. The large third spike as well as the oscillations with a period of four are evident in this trace. In this experiment the high pass filter was used to eliminate the d.c. dark current. This arrangement distorts the decay of the oxygen spikes. However, using the unity gain bias amplifier, it can be shown (Ley and Babcock, unpublished) that the decay of the signal is exponential and therefore that the height of the spike is proportional to area. In all quantitative experiments the spike height was used as an index to oxygen evolved.

Fig. 3 presents data averaged from eight experiments carried out under the same conditions as described above. Flash number is plotted as the abscissa; the average normalized flash yield (flash yield

divided by steady-state yield) is plotted on the ordinate. As shown, the oxygen pattern oscillates through six or seven cycles before reaching a steady state.

(3) Kinetics of activation and deactivation of oxygen producing intermediates

Abundant evidence exists which indicates that the unique pattern of oxygen evolution shown in Fig. 3 results from the linear accumulation of charge on the oxidizing side of PSII (Kok *et al.*, 1970; Bouges-Bocquet, 1973; Section III). The first two non-oxygen evolving flashes serve to build a pool of oxidized intermediates, the S states in the Kok *et al.* (1970) model, which cooperate on the third flash to produce oxygen. In the series of experiments described here, the kinetics of the principal intermediate formed after the first flash, S_2, and after the second flash, S_3, have been determined. Since the state S_2 requires two flashes to reach the oxygen evolving state S_4 by varying the time between flashes 1 and 2 and monitoring the oxygen yield resulting from the third flash (Y_3), the kinetics of S_2 can be determined. Similarly the S_3 state kinetics may be observed by varying the time between flashes 2 and 3 and monitoring Y_3. The half time for the formation of these intermediates may be obtained by gradually decreasing the time between the two flashes until the yield of oxygen on the third flash indicates that the two flashes are so closely spaced that the organism behaves as if they were one flash. A plot of the time, either Δt_{12} for S_2 or Δt_{23} for S_3, between these flashes vs. yield on the third flash gives the half time for the formation of the intermediate. Fig. 4 is a plot of the results from such an experiment for S_3 using

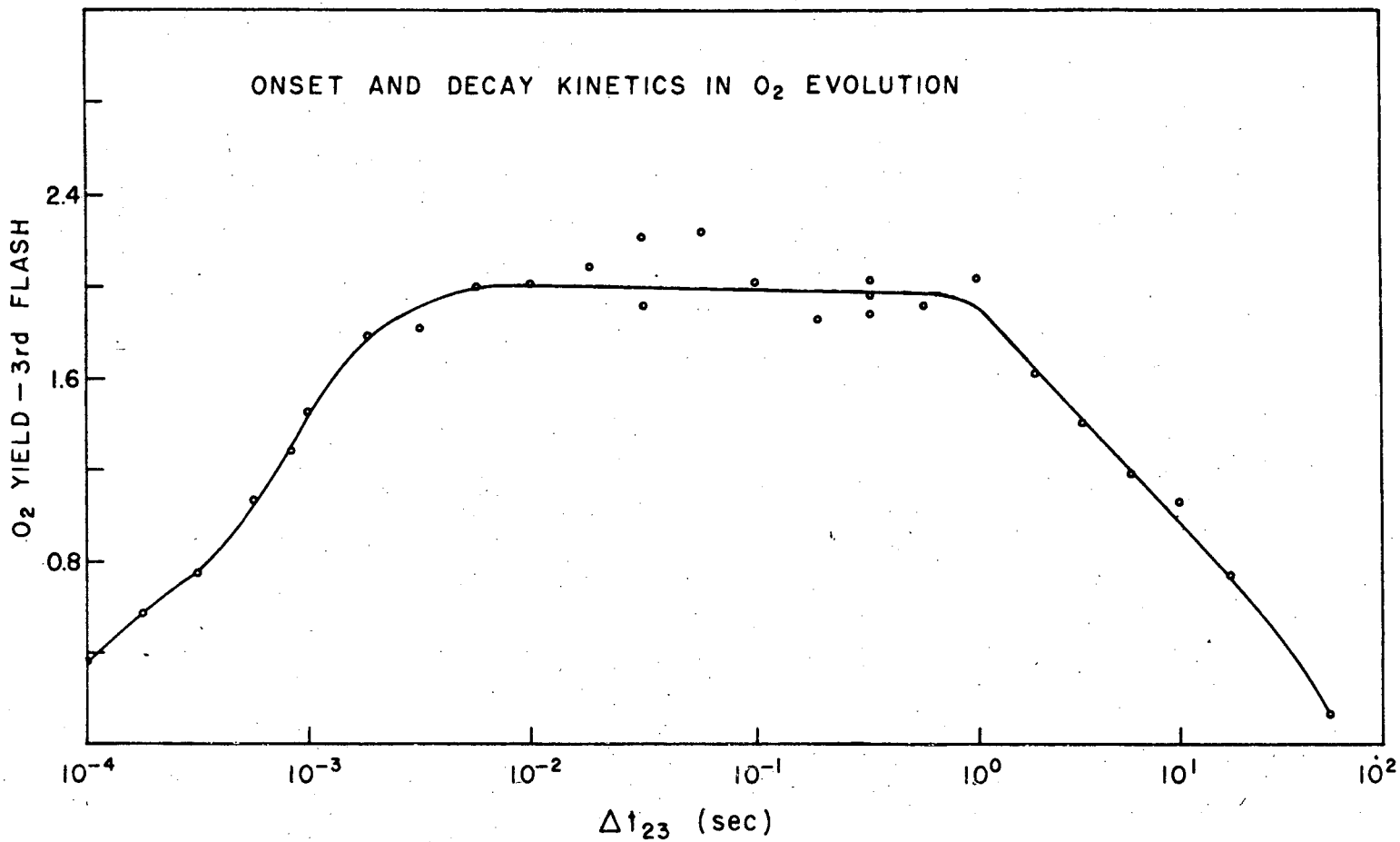


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Figure 4. Onset and decay kinetics for the state S_3. The yield of oxygen on the third flash, Y_3, is plotted as a function of the time between the second and third flash (Δt_{23}). 1 sec between other flashes, Y_3 normalized with respect to the steady state yield. Other conditions described in the text.

the teflon covered electrode and the Keithley voltmeter as the amplifier. Chloroplasts prepared according to Kelly (1968) and containing 0.33 μg/ml chlorophyll were suspended in a reaction mixture which was 0.045 M tricine (pH = 7.5), 0.0045 M $MgCl_2$, 5.3 x 10^{-5} M DPIP, 50 μg/ml in ferrodoxin and 83 μg/ml in plastocyanin. $t_{1/2}$ is approximately 750 μsec for the rise of S_3. Analogous experiments for S_2 indicate that this state is formed slightly more rapidly, $t_{1/2}$ = 500 μsec. In the plot of Δt_{12}, or as shown in Fig. 4 Δt_{23}, vs. Y_3 there is a plateau extending from Δt = 10 msec to Δt = 1 sec in which Y_3 does not vary and indicates that both S_2 and S_3 are stable in this time range. At Δt greater than 1 sec deactivation begins. Fig. 4 shows the deactivation kinetics; the half time for the decay of S_3 is seen to be approximately 10 sec. A similar value was obtained for S_2 in chloroplasts.

D. Discussion

The pattern of oxygen evolution in flashing light shown in Fig. 3 has been most adequately explained by the four-step, linear charge accumulation model postulated by Kok et al. (1970). This model has been discussed in Section I, so only a few additional comments will be presented here.

The most unusual aspect of the oxygen evolution pattern is the high yield of oxygen observed on the third flash. The stability of the state S_1, postulated to account for this result, has been well documented (Kok et al., 1970; Joliot et al., 1971). However, whether this stable state is located on the oxidizing side of PSII or on the reducing side is uncertain. If it were on the reducing side, the

transfer of two electrons on one of the first three flashes would be necessary to account for the large Y_3. Indirect evidence has accumulated recently supporting the reducing side hypothesis. Light saturation curves indicate that higher intensities by approximately 50% are required to saturate the oxygen yield of the third flash compared to that for the steady state (Babcock, unpublished). A two-electron transfer on one of the first three flashes would explain this requirement for higher light energy. Bouges-Bocquet (1973) demonstrated that the equilibrium between S_0 and S_1 has a potential close to the zero volt potential of the plastoquinone pool. Intermediates on the water side of PSII would be expected to have potentials much closer to the +.8 volt potential of water. Kok and Dubcek (1972) have studied the reduction of the fluorescence quencher, Q, by single flashes in DCMU-treated chloroplasts and conclude that this pool is heterogenous and may require more than a single electron for complete reduction. Joliot and Joliot (1971) have reached similar conclusions studying dithionite effects on fluorescence quenching. Butler and Cramer (1969) performed redox titrations on fluorescence quenching and observed two components, one with a potential of -.035 volts and a second with a potential around -.270 volts. Finally, the results described in Section III indicate that in tris-treated chloroplasts two electrons may be transferred through PSII on the first flash in dark-adapted samples. Unfortunately, clearly definitive experiments addressed to this question are lacking.

The values for the formation half times for the states S_2 and S_3 are in agreement with values obtained in similar experiments by

other authors. Kok et al. (1970) observed 200 μsec for S_3 and 400 μsec for S_3, and Bouges-Bocquet (1972, 1973) reports values between 200 and 600 μsec for these two states. Kok and Forbush (1968), measuring fluorescence quenching in flashing light, determined that the recovery time for the fluorescence quencher following a single flash is 600 μsec. Since this quencher is generally assumed to be the primary acceptor of PSII (Duysens and Sweers, 1963), the reoxidation of this acceptor may be the rate-limiting step for reactions occurring on the oxidizing side of PSII. Recently, however, experiments by Joliot and Joliot (1971b), Delosme (1971), and Butler (1973) have indicated that attributing all fluorescence quenching to reactions on the reducing side of PSII may be simplistic. These authors suggest that the quenching process may involve the cooperation between entities involved on both sides of PSII. In this case the formation kinetics presented in this Section and the fluorescence experiments of Kok and Forbush (1968) would be measurements of the same process, i.e., the rate limiting step in the reaction center of PSII.

The deactivation kinetics presented in Fig. 4 for the state S_3, and similar experiments for S_2, show that both states are stable for seconds in chloroplasts isolated from spinach. For the green alga, Chlorella pyrenoidosa, Joliot et al. (1971) showed that the deactivation for S_2 and S_3 in this organism is about five times faster than that for chloroplasts. Ley and Babcock (unpublished) found results similar to Joliot et al. (1971) for Chlorella, but for the blue-green alga, Anacystis nidulans, found that the deactivation rate in this organism was even more rapid than Chlorella. Thus it appears that

in the course of its evolution from the less complex blue-green algae through green algae to green plants the oxygen apparatus has developed in a way which prolongs the lifetime of intermediate oxidation states.

SECTION III

OXIDATION OF EXOGENOUS DONORS BY SYSTEM II

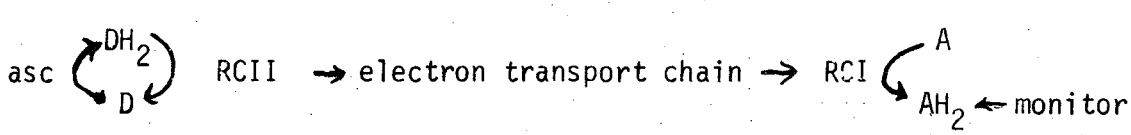
OXIDATION OF EXOGENOUS DONORS BY SYSTEM II

A. Introduction

The results presented in Section II and the Kok *et al.* (1970) model invoked to explain them provide insight into the behavior of the oxygen evolution machinery. However, this model is essentially a formal kinetic model, and the S state formulation provides little insight into the molecular identity of the intermediate species, or the organization of the enzymatic processes necessary for oxygen evolution. A much better approach to this facet of the problem has been developed in recent years through the use of treatments which inhibit oxygen evolution followed by the use of electron donors which are subsequently able to restore the flow of electrons through the reaction center of Photosystem II. This type of approach has been used extensively in studying Photosystem I, particularly using DCMU to inhibit electron flow from water followed by the addition of the ascorbate/DPIP couple to restore electron flow through System I exclusively (Sun and Sauer, 1971).

To be useful in probing the mechanism of Photosystem II the treatment employed must be able to inhibit on the oxidizing side of System II, *i.e.*, it must be able to simplify the system by separating the reaction center Chl (RC II) from some or all of the oxygen evolving machinery. Several such treatments have been introduced recently: Extraction with tris buffer at high pH (Yamashita and Butler, 1968b), incubation with hydroxylamine (Cheniae and Martin, 1970), moderate heat treatment (Katoh and San Pietro, 1967), chloride ion deficiency (Izawa *et al.*, 1969), treatment with chaotropic agents

(Lozier et al., 1971), incubation with phenyl mercuric acetate (Honeycutt and Krogmann, 1972), trypsin digestion (Selman and Bannister, 1971), and isolation of mutants with lesions on the water side of PSII (Epel and Levine, 1971). In addition, the preparation of System II particles from chloroplasts usually results in a loss in oxygen evolving capacity (see, for example, Vernon and Shaw, 1969; Wessels et al., 1973). Under these treatments the chloroplasts or particles are no longer able to reduce NADP with water as an electron donor. However, if an exogenous donor is supplied, NADP is again reduced in light-driven electron transfer reactions that generally proceed via both photosystems. A number of exogenous donors have been used, among them hydroquinone, manganous ion, and phenylenediamine (for a more complete listing and references, see Haveman et al., 1972). Generally, ascorbate is added to the system in order to maintain the donor in its reduced state and electron flow is monitored by recording changes in concentration of an added oxidant. Schematically, this process is represented as

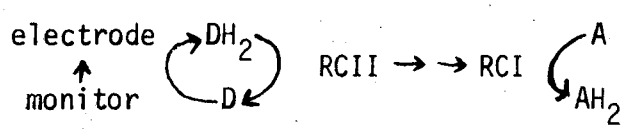


where DH_2 and D are the reduced and oxidized forms of the added donor, AH_2 and A are the reduced and oxidized forms of the added electron acceptor, asc is ascorbate, and RCI and RCII are the reaction centers for Photosystems I and II.

Unfortunately, this method has a number of disadvantages: (1) Most Hill oxidants, such as ferricyanide or DPIP, react directly with

the added donors. Thus far, only NADP and methylviologen, both Photosystem I acceptors, have been used as oxidants. Therefore the process of interest, the oxidation of the donor, is fairly far removed from the process which is actually monitored. (2) The detection of oxidant is relatively insensitive, e.g., single flash experiments are impractical, and consequently it is difficult to obtain information concerning the kinetics of donor oxidation. (3) Not all donors are specific to the oxidizing side of Photosystem II. Some, for example, couple both between the two photosystems and on the water side of System II (Cheniae and Martin, 1970; Haveman et al., 1972).

In this Section a system is described which overcomes the difficulties mentioned above by combining the use of inhibitory treatments and added electron donors with the increased sensitivity afforded by polarographic detection. Essentially the method consists of replacing ascorbate, which has been used by previous workers to maintain the donor in its reduced state, with a polarized platinum electrode. The oxidized donor, which has been produced in the light, then diffuses to the electrode surface where it is re-reduced and the resulting signal is amplified and recorded. Schematically,



The advantages of this method are immediately obvious. We are using chloroplasts which have been treated in a way that simplifies the fairly complicated four-electron process which yields molecular oxygen. Therefore, we are able to observe reactions much closer to the actual reaction center chlorophyll. The increased sensitivity

and fast time response of the polarized electrode detection system allows the use of short single flashes in studying reaction kinetics for these donors. We have also eliminated the cumbersome and often inaccurate (Mantai and Hind, 1971; Allen and Hall, 1973) detection systems used when changes in Hill oxidant concentrations are monitored as an indication of electron flow through Photosystem II. In this Section, results obtained by applying this polarographic method, using primarily tris-washed chloroplasts and phenylenediamine as donor, are described.

B. Materials and Methods

(1) Light sources, optical arrangement, electrodes and flow systems

Short light flashes were produced from a xenon pulsed light system manufactured by ILC Company (Sunnyvale, Calif.) and focused on the sample as described in Section II. A pulse forming network (PFN-6) has been obtained from ILC which produces 10 μsec flashes rather than the 21 μsec flashes described earlier. Continuous light is obtained from a Bell and Howell 300 watt projector and, when desired, filtered through Corning Glass Color Filters. Monochromatic light and intensity measurements were obtained as described by Sauer and Biggins (1965). The electrode system is the bare electrode which has been described in Section II. In order to obtain a reproducible platinum surface before each series of experiments, the platinum is rubbed gently with mild abrasive, finely powdered sapphire (Trade name, Saffron), and washed with a 1% solution of Triton X-100. It is then rinsed for about 15 min with distilled water and assembled. The 5 mil bilayer spacer was used in all experiments. The flow system is identical to that described

(Section II) except the upper reservoir is now aerated with 100% air rather than the 96% air, 4% CO_2 used previously. This change had no effect on the response of the electrode.

(2) Chloroplasts and reaction mixtures

"Intact" and "Tris-washed" spinach chloroplasts were prepared according to the methods of Yamashita and Butler (1968b). Stock suspensions of these chloroplasts, containing between 2 and 5 mg Chl/ml, were diluted 9 to 1 with a standard reaction mixture containing 0.02 M NaCl, 0.01 M $MgCl_2$, 0.015 M tris (pH = 7.6) buffer. NADP, DCMU, ferredoxin and other reagents were added according to the experiment and are noted in the figure captions. Chl analyses were carried out as described in Section II. Final Chl concentrations in all experiments were between 0.2 and 0.5 mg Chl/ml.

(3) Reagents

All reagents except those noted below were used as obtained from the manufacturer. DCMU was obtained from duPont and recrystallized from methanol before use. Hydroquinone and phenylenediamine were obtained from J. T. Baker Company and sublimed at 40 microns pressure and 80°C. Ferredoxin was purified from spinach as described by Sun and Sauer (1971).

C. Results

(1) Donor systems

Lozier *et al.* (1971) observed the photooxidation of Mn^{2+} in chloroplasts treated with chaotropic agents and Ben Hayyim and Avron (1970) reported the photooxidation of Mn^{+2} to Mn^{+3} in untreated chloroplasts. In the latter experiments the unstable Mn^{+3} is trapped as the

manganipyrophosphate and assayed by quantitative oxidation of potassium ferrocyanide. Kolthoff and Lingane (1952) reported that manganipyrophosphate is reduced at a dropping mercury electrode with a half-wave potential of +0.1 volts vs. S.C.E. However, all attempts to observe Mn^{+2} photooxidation, with or without added pyrophosphate, using the bare platinum electrode were unsuccessful.

Similarly, attempts to monitor the oxidation of ascorbate polarographically proved unsuccessful. At high concentrations (0.01 M to 0.05 M) in intact or tris-washed chloroplasts, with or without DCMU, a large negative going signal was observed at -0.6 volts. This type of signal corresponds to a decrease in the dark current which results from a consumption of oxygen, most probably a reduction of O_2 to H_2O_2 via a Mehler type reaction (Mehler, 1951). The pronounced insensitivity to DCMU, a well known System II inhibitor, indicates that ascorbate is involved in oxidation processes other than those mediated by this photosystem (Allen and Hall, 1973).

Hydroxylamine has been used extensively as an electron donor to System II. Bennoun and Joliot (1969) observed a signal from the oxidized derivative of hydroxylamine at a modulated platinum electrode when chloroplasts which had been supplied with this donor were illuminated. We have repeated these experiments and observed the signal. However, there are a number of disadvantages in using this compound:
(1) Relatively high concentrations are necessary (about 0.01 M) to override competing signals from O_2 produced photosynthetically.
(2) Hydroxylamine has at least three effects in chloroplasts--inhibitor of oxygen evolution, electron donor and phosphorylation uncoupler

(Cheniae and Martin, 1971). (3) The chemistry of hydroxylamine is complex so that it may be possible that interfering reactions complicate its oxidation. For these reasons these experiments were not pursued in any more detail.

The electron donors hydroquinone (HQ) and phenylenediamine (PD) have been used by Yamashita and Butler (1968b) to restore electron flow through System II in tris-washed chloroplasts. Kolthoff and Lingane (1950, 1952) have shown that these compounds are suitable for polarographic study at stationary platinum electrodes, the half-wave potentials being between 0.0 and -0.4 volts. Using tris-washed chloroplasts under aerobic conditions in the absence of ascorbate and electrode potentials from 0.0 to -0.6, signals corresponding to both oxidized hydroquinone and oxidized phenylenediamine have been observed. Polarograms for the detection of oxidized PD and HQ are shown in Fig. 5. The experiments were performed by illuminating the tris-washed chloroplasts with a series of 20 flashes, spaced 1 sec apart, and averaging the magnitude of the signals from the last 10 flashes for each voltage setting. The polarogram for each species shows a plateau in the region between -0.3 and -0.6 volts. The half-wave potentials for HQ and PD, -0.2 volts, are sufficiently separated from the more negative half-wave potential of oxygen (Fig. 2), that a clean separation between the two signals is possible. Since most of the inhibitory treatments discussed in the introduction to this Section do not completely inhibit oxygen evolution, this feature is useful in eliminating oxygen signals from the functional water oxidizing centers.

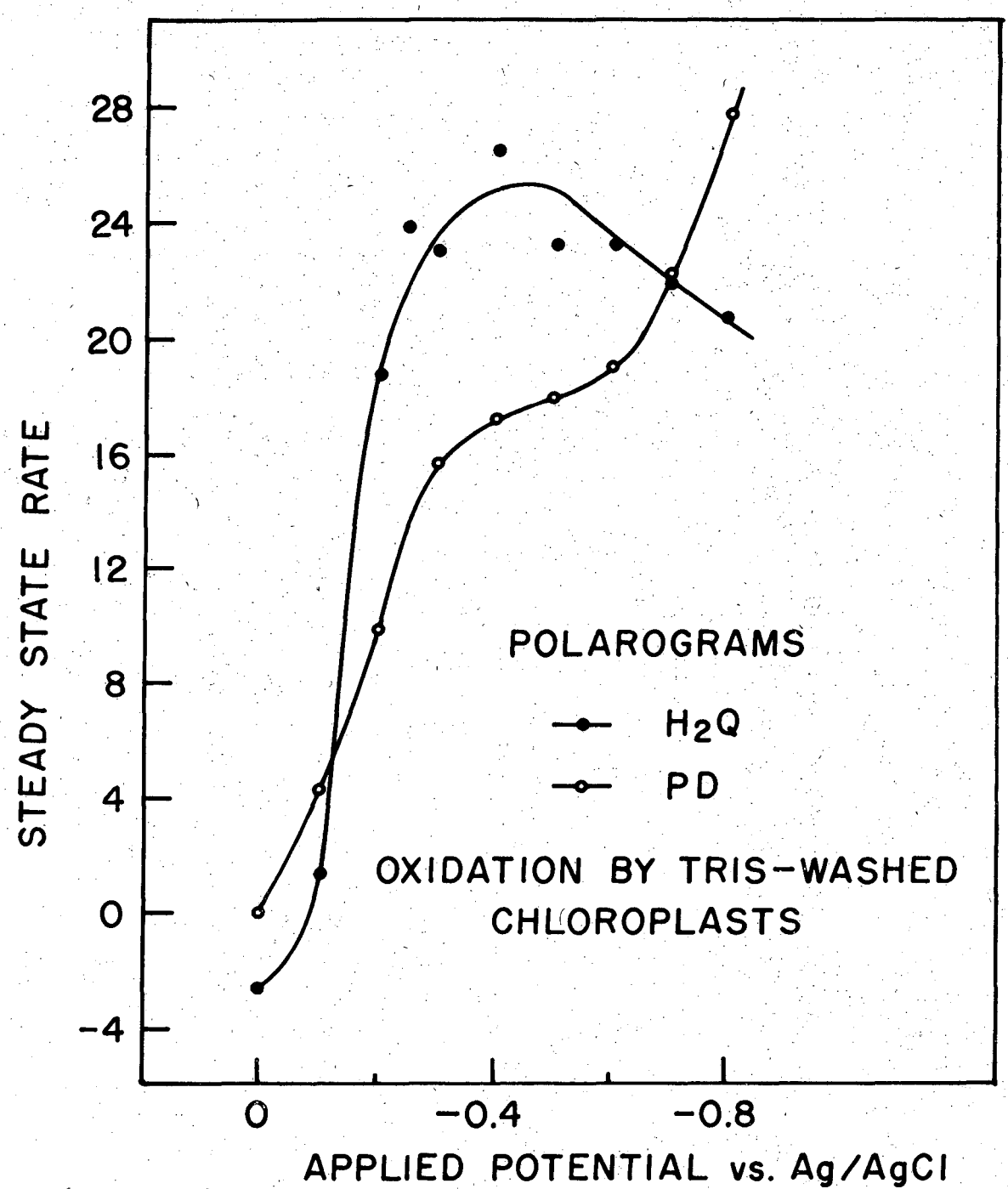


Figure 5. Polarograms for HQ and PD oxidation in tris-washed chloroplasts. Tris-washed chloroplasts prepared as described in Materials and Methods as are reaction mixtures used; donor concentration 5 X 10^{-4} M. Experimental protocol described in the text.

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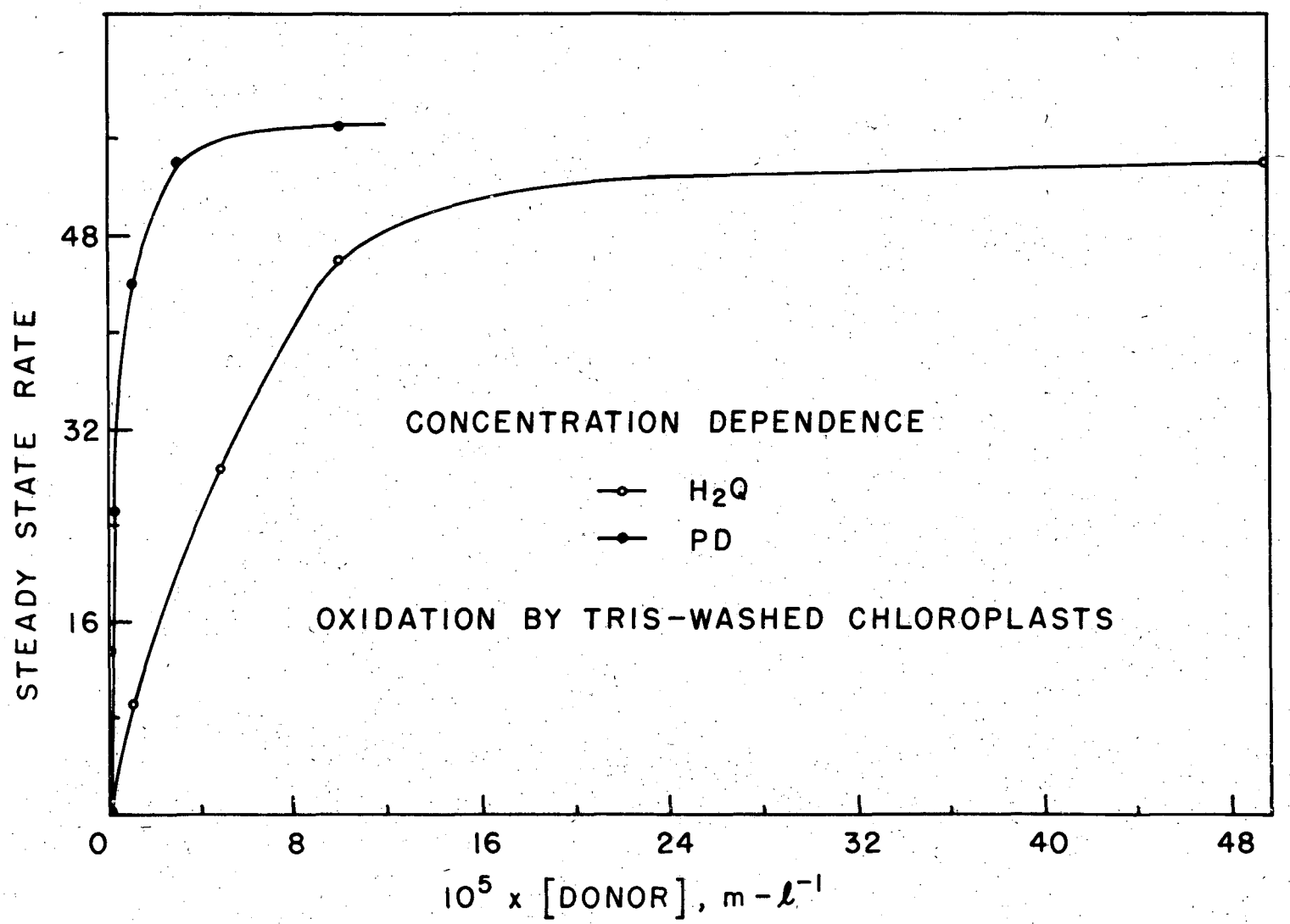

Figure 6. Concentration dependence for PD and HQ oxidation in tris-washed chloroplasts. XBL 736-4845

Reaction mixtures and chloroplasts as described in Materials and Methods. Illumination by 10 flashes.

The decrease in signal magnitude for HQ in going to more negative applied voltages probably indicates a reaction between HQ and oxygen producing quinone and hydrogen peroxide. A similar reaction has been reported by Trebst et al. (1963). Mantai and Hind (1971) have shown an analogous reaction between hydrazine and oxygen which is mediated by the combined action of light, chloroplasts and Mn^{+2}. In the bare electrode system the reaction of oxygen would be observed only at voltages more negative than -0.5 (see oxygen polarogram, Fig. 2). This would produce a negative-going signal at these voltages which would subtract from the positive-going oxidized HQ signal. Ferredoxin appears to catalyze this reaction between HQ and oxygen since the addition of the protein to tris-washed chloroplasts increases the magnitude of HQ oxidation, detected polarographically at -0.4 volts, by a factor of 4 (Table III).

This hypothesis is supported by concentration studies for PD and HQ, Fig. 6, which indicate that while PD oxidation in tris-washed chloroplasts half saturates at a concentration of 5×10^{-6} M, the photooxidation of HQ, in the presence of ferredoxin, requires an 8-fold increase in concentration for half saturation at 4×10^{-5} M. Therefore it appears that there is more than a single site for HQ oxidation.

Polarographic detection of significant oxidation of the PD or HQ donor systems in intact chloroplasts has not been observed. It appears that the mechanism for water oxidation must be disrupted by one of the treatments described in the introduction to this Section before these exogenous donor systems have access to the reactions

occurring on the water side of PSII.

(2) Spectroscopic detection of acceptor reduction compared with polarographic detection of donor oxidation

If PD is donating electrons only to the oxidizing side of PSII, a comparison between the rates of NADP reduction and PD oxidation in tris-washed chloroplasts should give the same value. The results of experiments in which the reduction of NADP was monitored are shown in Table II. In intact chloroplasts water serves as the electron donor; with tris-washed chloroplasts, either PD or HQ as indicated in the table. These results can be summarized as follows: (1) tris-washing inhibits the reduction of NADP with water as electron donor, as reported by Yamashita and Butler (1968b); (2) the donor system, PD/asc, restores about 60% of the electron flow to NADP, while the system, HQ/asc, restores about 40%; (3) the restoration of electron flow by either donor system is DCMU sensitive indicating that System II is necessary for NADP reduction by these donors.

The results of analogous experiments in which the oxidation of the electron donor is monitored polarographically are shown in Table III. In these experiments the chloroplast reaction mixture suspension was injected into the electrode and a series of 10 μsec saturating flashes spaced 1 sec apart was given. The steady-state yield of oxidized product [which is proportional to the rate of the reaction (Kok et al., 1970)] was obtained as the average of 10 flashes. For the Pd → NADP reaction these results are in good agreement with those obtained from the spectroscopically obtained results, i.e., with or without Fd in the system the degree of restoration of electron flow

Table II

Spectroscopic Detection - NADP Reduction

Chloro-plasts	Reaction Mixture*	Donor System	Rate μmoles NADP (mg Chl hr)	Percent of Intact
Intact	NADP, Fd	H_2O	10.2	100
Tris	NADP, Fd	H_2O	<1.0	<10
Tris	NADP, Fd, HQ, asc	HQ/asc	4.0	39
Tris	NADP, Fd, HQ, asc DCMU	HQ/asc	<1.0	<10
Tris	NADP, Fd, PD, asc	PD/asc	6.4	63
Tris	NADP, Fd, PD, asc DCMU	PD/asc	<1.0	<10

*Concentrations: [NADP] = 2.5×10^{-4} M; [HQ] = 2.5×10^{-5} M; [asc] = 2.5×10^{-4} M; [PD] = 2.5×10^{-5} M; [DCMU] = 1×10^{-5} M; [Fd] = 4 μg/ml; [Chl] = 13 μg/ml.

Continuous actinic light at 678 nm; incident intensity 3 nanoeinsteins-cm^{-2}-sec^{-1}.

Table III

Polarographic Detection - Donor Oxidation

Chloro-plasts	Reaction Mixture*	Donor System	Rate relative units (mg Chl)	Percent of Intact
Intact	NADP, Fd	H_2O	20.9	100
Tris	NADP, Fd	H_2O	2.5	12
Tris	NADP, Fd, HQ	HQ	58.5	280
Tris	NADP, HQ	HQ	13.7	66
Tris	NADP, Fd, PD	PD	15.6	75
Tris	NADP, PD	PD	14.7	70

*Concentrations: [NADP] = 2.5×10^{-3} M; [HQ] = 5×10^{-4} M; [PD] = 5×10^{-4} M; [Fd] = 41 μg/ml; [Chl] = 200 μg/ml.

Actinic flashes (10 μsec) of white light.

is about 70%. With HQ as donor in tris-washed chloroplasts, the effect of ferredoxin discussed above is evident. Without added ferredoxin, HQ oxidation is about 65% that observed in intact chloroplasts with water as electron donor (compared with 40% restoration of NADP reduction by HQ/asc shown in Table II). However, added ferredoxin increases the rate of HQ oxidation to almost three times that observed with intact chloroplasts for water oxidation. The results from these two sets of experiments and those discussed above indicate that PD is less ambiguous as an electron donor than HQ.

(3) PD oxidation by tris-washed chloroplasts in flashing light

The pattern of PD oxidation in flashing light (10 μsec flashes, 1 sec apart) in preilluminated tris-washed chloroplasts is shown in Fig. 7a. The yield from each flash is about the same and the situation is similar to steady-state oxygen evolution in intact chloroplasts (see inset, Fig. 3). If DCMU is added to these tris-washed chloroplasts prior to preillumination, the photooxidation of PD in flashing light is eliminated on all flashes. This result indicates that the photooxidation of PD is mediated by PSII and confirms the results shown in Table II in which DCMU eliminated the PD/asc supported reduction of NADP in tris-washed chloroplasts.

The mode of action of DCMU is generally thought to involve the inhibition of the reoxidation of the primary System II acceptor, Q, following its reduction by light (Kok and Forbush, 1968; Bennoun and Joliot, 1969; Bennoun and Li, 1973; but see also Rosenberg et al., 1972). Since Q is largely in the oxidized state in the dark, DCMU-treated, tris-washed chloroplasts which have been maintained in the dark should produce oxidized PD only on the first flash, which serves to reduce Q. Since the reoxidation of Q^- is then blocked by DCMU, subsequent flashes should be ineffectual in oxidizing PD. This experiment is shown in Fig. 7d and the predicted result is observed, i.e., in dark-adapted, DCMU-treated chloroplasts oxidized PD is generated only on the first flash. Similar experiments with dark-adapted, DCMU-treated chloroplasts, monitoring oxygen evolution, show no oxygen evolved on any flash. However, since in these chloroplasts

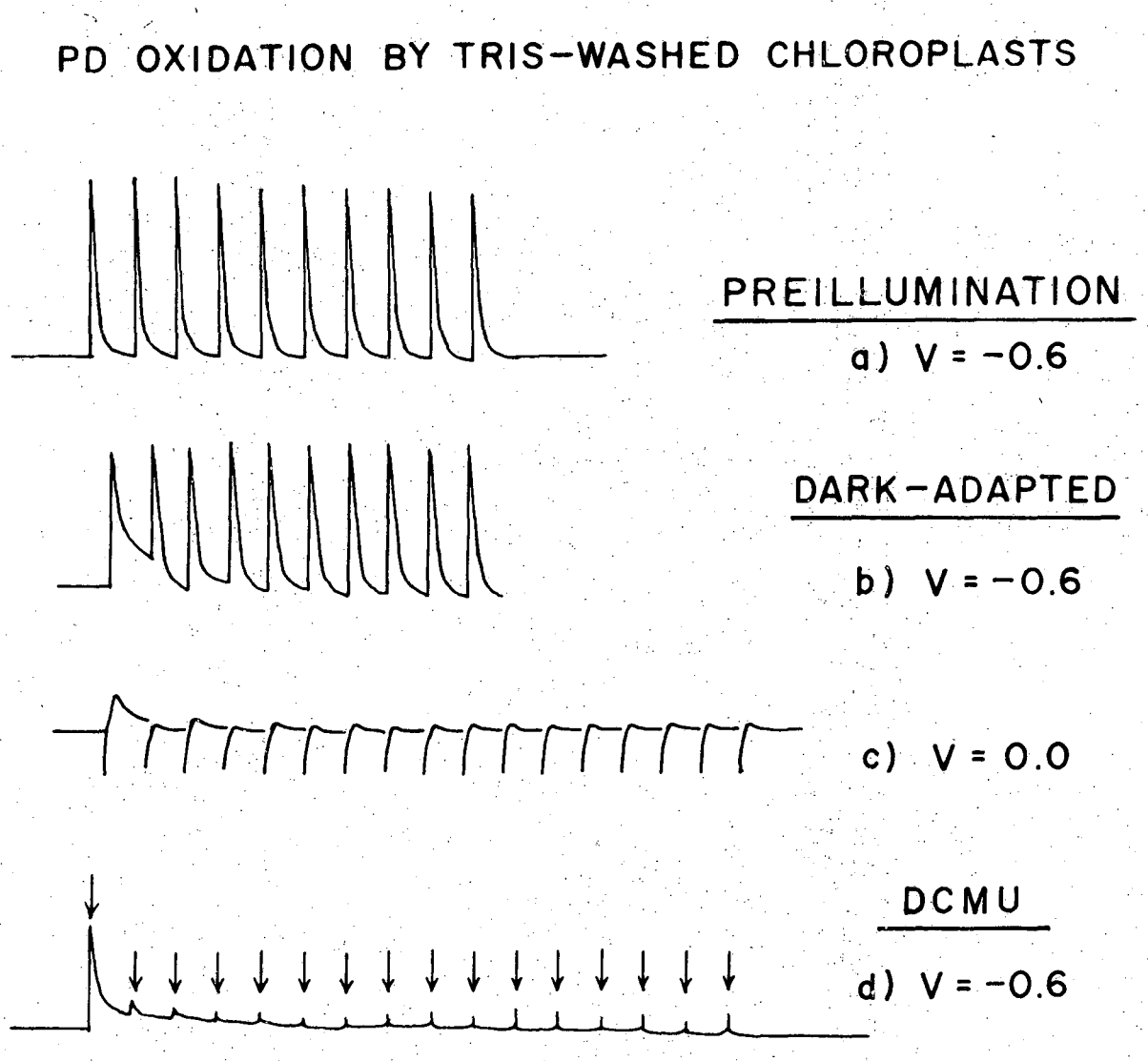

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Figure 7. Experimental traces for PD oxidation in tris-washed chloroplasts. Reaction mixtures as described in Materials and Methods; PD concentration in (a), (b) and (c) was 5 X 10^{-4} M, in (d) 1X 10^{-4} M. Dark adapted chloroplasts were allowed 5 min dark before initiation of flash sequence. Preillumination was with 10 flashes spaced 1 sec apart and ending 1 sec before initiation of sequence.

little oxygen is evolved until the third flash (Fig. 3), this result is expected.

The pattern of PD oxidation in dark-adapted, tris-washed chloroplasts is shown in Fig. 7b. In contrast to oxygen evolution in intact chloroplasts, this experiment shows that oxidized PD is produced on each flash, including the first two. This pattern of PD oxidation on the first 10 flashes can be observed with or without added NADP. For later flashes without the added acceptor the yield of oxidized PD decreases, indicating that the plastoquinone pool located between the two photosystems has become reduced. The yield of the first flash, and to a lesser extent the third flash, is anomalous in that the total area beneath the spike is greater than on other flashes. The shape of the spike also indicates that the signal is composed of two components, one which is detected rapidly and a second which appears more slowly in time. Fig. 7c is a repeat of the experiment in 7b except that the voltage applied to the electrode has been raised to 0 volts. At this potential only the slower component is detected as a positive going signal, indicating that this component is chemically distinct from the more rapidly detected species. Parker and Adams (1956) have shown that under certain conditions of anodic polarography, the polarogram for PD oxidation splits into two waves, indicating that PD is able to undergo one or two electron oxidation. The difference between the two half-wave potentials is on the order of 0.15 to 0.20 volts. In addition, Piette et al. (1962) have shown that the PD^{+2} ion is more easily reduced than the PD^{+1} ion. In the data presented in Figs. 7b and 7c the faster component is more difficult to reduce than the

slower component and is assigned to the PD^{+1} ion. The slower component, whose half-wave potential is about 0.2 volts more positive than the fast component, can then be assigned to the PD^{+2} ion.

As shown in Fig. 7a and for later flashes in 7b, the appearance of the slower ocmponent requires a dark interval before a series of flashes is able to evoke it. Fig. 8 presents the kinetics for this induction period. Tris-washed chloroplasts with added PD were incubated in the dark for various times and the magnitude of the slow component on the first flash after this period is plotted as a function of the dark time. The half time is on the order of 40 sec and and about 3 min dark is required for the complete regeneration of the slow component. This time is comparable to times found for the full induction of the oscillatory behavior of oxygen evolution in flashing light (Joliot et al., 1969).

Similar experiments with HQ as donor in dark-adapted, tris-washed chloroplasts show analogous behavior, in that the yield of oxidized HQ on the first flash is about twice that of subsequent flashes. However, with antimycin A-treated or heated chloroplasts (51°C, 3 min) the yield of oxidized PD on the first flash shows only the fast component.

(4) Activation kinetics for PD oxidation in tris-washed chloroplasts

As shown above, PD oxidation by tris-washed chloroplasts exhibits a pattern in flashing light quite distinct from that for water oxidation in intact chloroplasts. In order to gain information on the site of PD oxidation, activation studies, similar to those shown in Fig. 4 for oxygen evolution, were carried out. Fig. 9 presents the results

of these experiments. Two closely spaced flashes were given to pre-illuminated tris-washed chloroplasts and the yield of oxidized donor produced on the second flash is plotted as a function of the time between the two flashes. As this dark time decreases toward the value for the half time of PD donation the yield of the second flash decreases; this corresponds to the falling portion of the curve in the region between 10^{-3} and 10^{-4} sec. The half time for PD oxidation, 200-300 μsec, is in the same range as that reported in Section II and by other authors (Kok et al., 1970; Bouges-Bocquet, 1973) for water oxidation in intact chloroplasts.

D. Discussion

Of the donor systems studied, PD gives results most clearly attributable to specific oxidation by Photosystem II. This conclusion is based on three lines of evidence. The first is the good agreement between the rate of PD oxidation detected polarographically in tris-washed chloroplasts and the rate of NADP reduction detected spectroscopically in tris-washed chloroplasts supplied with the PD/asc couple (Tables II and III). The second is the sensitivity of PD oxidation to the PSII inhibitor, DCMU, shown in Fig. 7d. The third line of evidence is the observation, discussed in connection with Fig. 7b, that PD oxidation in dark-adapted, tris-washed chloroplasts occurs for the first several flashes with or without the addition of NADP. This behavior is similar to that observed in oxygen evolution experiments and indicates that the plastoquinone pool located between the two photosystems (Amesz, 1973) is acting as a sink for electrons coming from PSII, either from water in intact chloroplasts or PD in tris-washed chloroplasts.

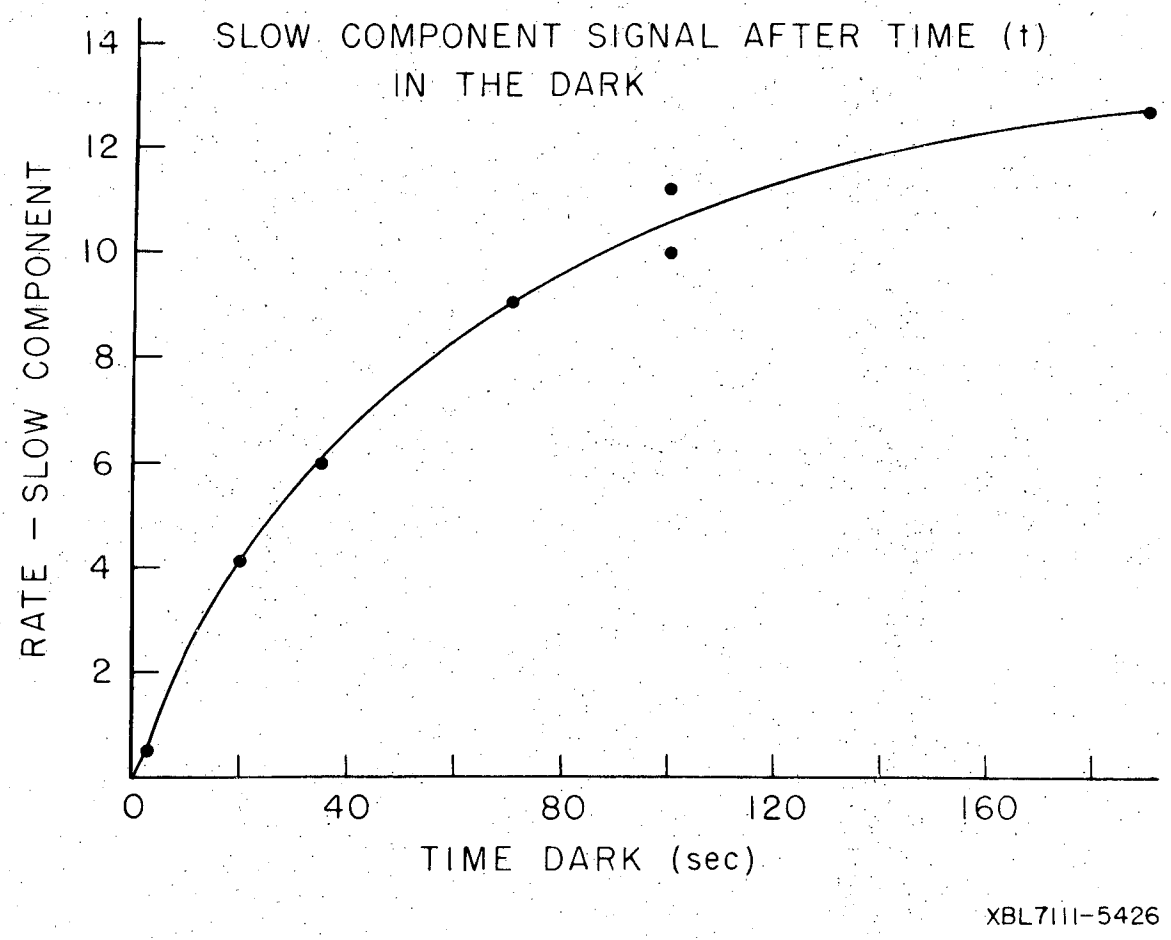

Figure 8. Recovery kinetics for slow component. Reaction mixture as in Materials and Methods; PD concentration was 3×10^{-4} M. Results from each experiment were normalized with respect to the steady yield after 10 flashes.

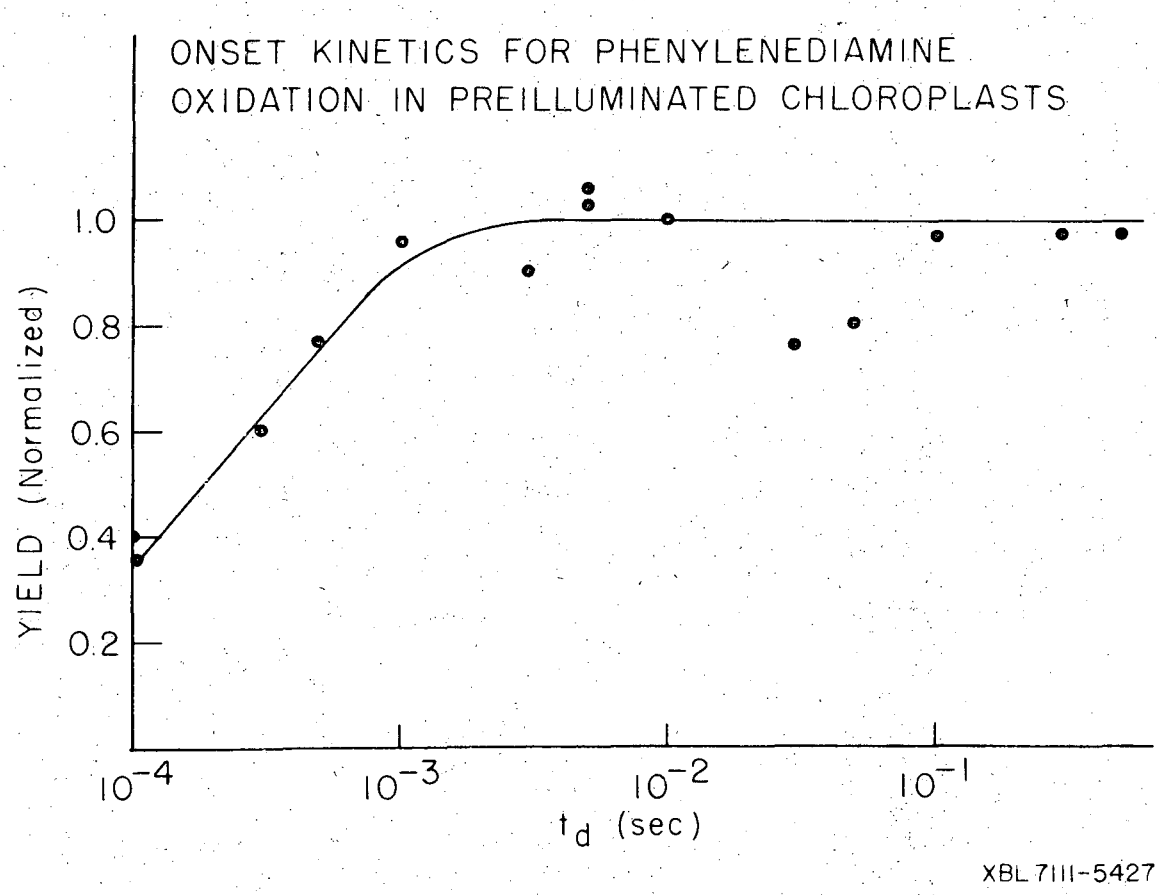

Figure 9. Onset kinetics for PD oxidation in tris-washed chloroplasts. Reaction mixture as described in Materials and Methods; PD concentration was 1×10^{-3} M. Experimental procedure as described in the text.

The principal results of the flashing light studies on PD oxidation in tris-washed chloroplasts shown in Figs. 7, 8, and 9 can be summarized as follows: (1) PD oxidation occurs on the first and all subsequent flashes in dark-adapted or preilluminated chloroplasts. These results provide direct evidence that charge separation in PSII occurs on all flashes and that the two-flash induction period observed in oxygen evolution experiments serves to accumulate oxidizing equivalents for the oxidation of water. (2) Onset kinetics (Fib. 9) show that PD is one electron oxidized at a site which has a reset time similar to that observed for oxygen evolution intermediates. (3) In dark-adapted chloroplasts both PD^{+1} and PD^{+2} are generated. There are no oscillations with flash number in the yield of PD^{+1} indicating that the site for PD oxidation is close to the primary donor or the PSII reaction center Chl itself. However, PD^{+2} production is maximal on the first flash and oscillates with period two, but with diminishing yield, on subsequent flashes. (4) Following illumination a dark time is required before PD^{+2} can again be evoked by flashes. The time course for this dark process (Fig. 8) is similar to that observed for the recovery of the characteristic period four oscillations in oxygen evolution. (5) DCMU inhibits the oxidation of PD on all flashes in preilluminated DCMU-treated chloroplasts; for dark-adapted chloroplasts the yield of oxidized PD on the first flash is not affected by DCMU; however, subsequent flashes produce no oxidized PD.

The following model accounts for the observations on the generation of PD^{+1}. The reaction center and water oxidizing site in PSII is designated as Q-P680-Z-S_n where Q is the primary acceptor, P680

is the reaction center Chl, Z is the primary donor, and S_n is the pool in which charge accumulation occurs (with n = 0 to 4 indicating extent of oxidation). The absorption of a photon by dark-adapted chloroplasts would then result in the following reactions:

$$Q\text{-}P680\text{-}Z\text{-}S_0 \xrightarrow{h\nu} Q^-\text{-}P680^+\text{-}Z\text{-}S_0 \qquad \text{III-1}$$

$$Q^-\text{-}P680^+\text{-}Z\text{-}S_0 \xrightarrow{\text{dark}} Q^-\text{-}P680\text{-}Z^+\text{-}S_0 \qquad \text{III-2}$$

$$Q^-\text{-}P680\text{-}Z^+\text{-}S_0 \xrightarrow{\text{dark}} Q^-\text{-}P680\text{-}Z\text{-}S_1 \qquad \text{III-3}$$

The fast component of PD oxidation, leading to the one-electron oxidized PD^{+1}, observed on the first and subsequent flashes, would result from the reduction by PD of one of the species $P680^{+1}$, Z^+, or S_1. As mentioned above, the onset kinetics (Fig. 9) show that PD oxidation occurs at a site which has a reset time similar to that observed for oxygen evolution intermediates. In oxygen experiments this reset time is usually associated with the reaction shown in equation III-3, but may also correspond to the reoxidation of Q^- (see Discussion, Section II) or with the reaction in equation III-2.

In this model DCMU inhibits by blocking the reoxidation of Q^-. In preilluminated chloroplasts, Q has been reduced during the preceding illumination period and subsequent flashes evoke no further electron transfer. In dark-adapted chloroplasts, Q is oxidized before the flash series but the first flash transfers sufficient electrons to reduce Q completely, and no further flash-induced electron transfer occurs. Yamashita and Butler (1968b) have reached a similar conclusion on the basis of fluorescence studies for the mode of action of DCMU in tris-washed chloroplasts.

The slower component, corresponding to PD^{+2}, which occurs on the first, third and fifth flash is more difficult to explain. The oscillation with period two and the recovery kinetics of this component shown in Fig. 8 suggest that it may originate from a more oxidized S state, either S_2 or S_3. In this model the recovery kinetics would correspond to the kinetics for reestablishing equilibrium between S_0 and S_1. Following the first flash S_0 would be advanced to S_1 and produce PD^{+1}, S_1 would be advanced to S_2 and produce PD^{+2}. However, additional assumptions, perhaps involving alternate primary acceptors as suggested by Joliot and Joliot (1971) to account for fluorescence data, would be necessary to explain the period two oscillations in the slow component.

A second model would implicate electron carriers which lie off the linear pathway from P680 to S_n shown in equation III-1 as the source for the slower component. One of these carriers is cyt b_{559} (Butler, 1973; Vermeglio and Mathis, 1973) and another is a free radical species which has been designated Signal II (see Section IV). In tris-washed chloroplasts both of these species have been shown to undergo PSII mediated photooxidation (Knaff and Arnon, 1969; Lozier and Butler, 1973). The addition of an electron donor appears to quench this oxidation, but unfortunately this process has not been studied in detail. The reduction of one or both of these electron carriers may account for the generation of the PD^{+2} observed on the first flash shown in Figs. 7b and 7c. The fact that the PD^{+2} signal is absent in heat-treated or antimycin A-treated, tris-washed chloroplasts indicates that this may be the case because these treatments

alter the potential of cyt b_{559} (Wada and Arnon, 1971; Cramer and Bohme, 1972) so that it is no longer reducible by PD. Flashing light studies of cyt b_{559} behavior in tris-washed chloroplasts would be useful.

Finally, our experiments do not definitely associate the slower component exclusively with reactions occurring on the water side of PSII. Vermeglio and Mathis (1973b) have noticed oscillations with period two in the oxidation of cyt f, presumably a PSI reaction. That the recovery kinetics of the slow component are similar to those for intermediates involved in oxygen evolution, and that the slow component is not observed in intact chloroplasts argue against this PSI assignment. An action spectrum for the generation of the slow component would resolve this question.

SECTION IV

DECAY KINETICS AND SPECTRAL PROPERTIES OF EPR SIGNAL II

DECAY KINETICS AND SPECTRAL PROPERTIES OF EPR SIGNAL II

A. Introduction

At room temperature oxygen-evolving photosynthetic materials generate two free radical species which are detectable using EPR spectroscopy (Commoner et al., 1956; Sogo et al., 1957) (Figure 10). The first, which has been termed Signal I, has rapid rise and decay kinetics and has been established as arising from the oxidized reaction center of Photosystem I, $P700^+$ (Beinert and Kok, 1964; Kohl, 1972). The second, Signal II, has been less well characterized. It has been reported to have a g value of 2.0046, a line width of about 20 gauss, hyperfine structure resulting from interaction with protons and decay kinetics on the order of hours (Heise and Treharne, 1964). The studies of Weaver and Bishop have shown Signal II to be absent in photosynthetic bacteria, in algal mutants lacking the ability to evolve oxygen and in algae grown on a manganese deficient medium (Weaver, 1962; Weaver and Bishop, 1963). Chloroplast preparations which have lost oxygen evolving capacity through heating or sonication also lack the spin signal (Treharne et al., 1963). Chloroplast particles enriched in Photosystem II activity show an increased Signal II magnitude, whereas Photosystem I particles are deficient in this feature (Kohl, 1972). On the basis of these findings Signal II has been associated with the oxygen evolving Photosystem II in algae and green plants (Weaver, 1968).

Kohl and coworkers, using deuteration, extraction and readdition procedures and in vitro studies on model compounds, have presented

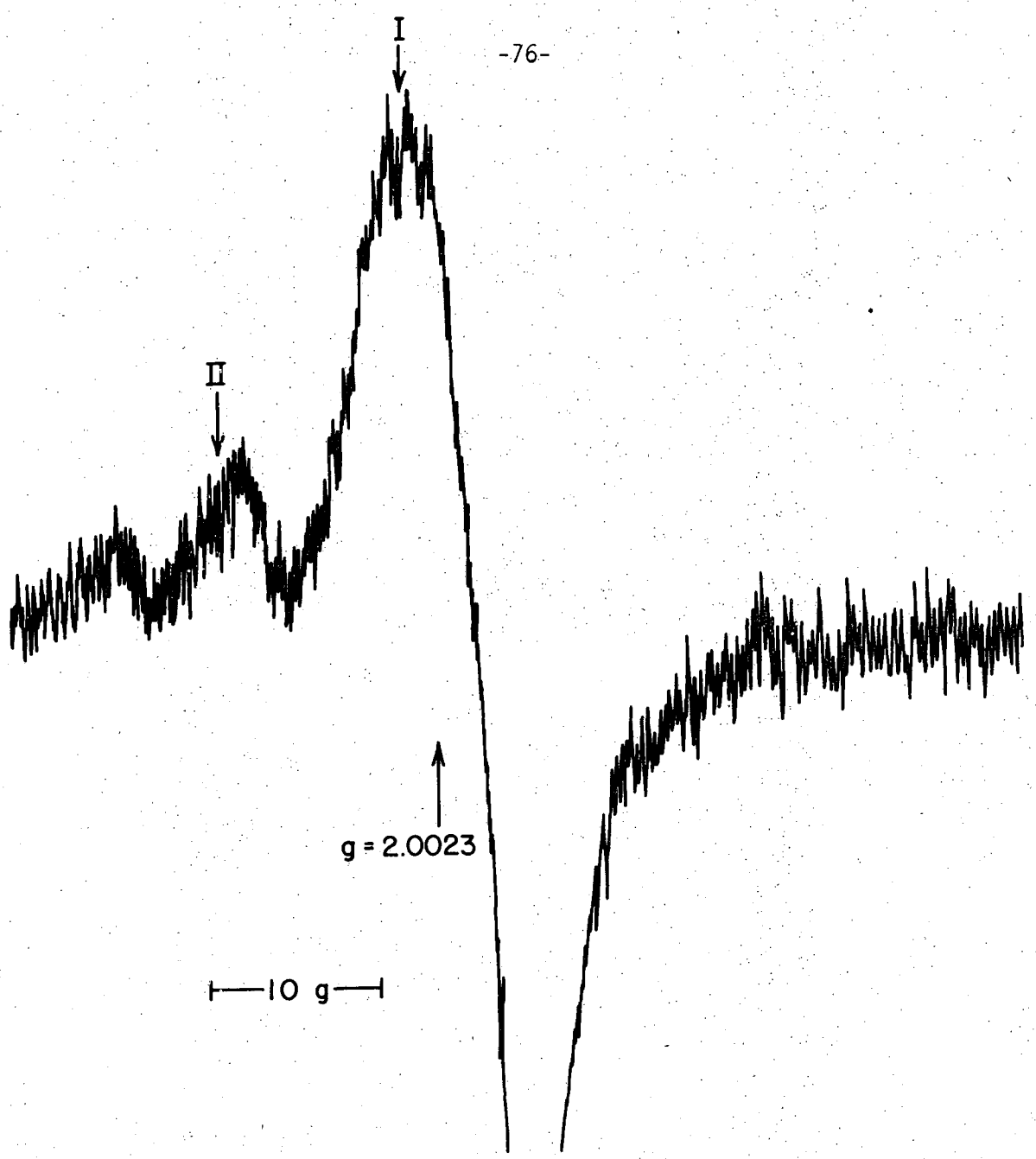

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Figure 10. EPR spectrum (1st derivative) of spinach chloroplasts illuminated with broad band continuous light. NADP concentration was 1 X 10^{-3} M and Fd was 41 ug/ml. The microwave power was 50 mW, instrument time constant was 0.3 sec, scan rate was 12.5 G/min. The low field maximum of Signal I is labelled with "I", one of the low field hyperfine components of Signal II is labelled with "II".

evidence suggesting that the molecular species giving rise to Signal II may be plastoquinone or a species closely related to it (Kohl et al., 1965; Kohl and Wood, 1969; Kohl et al., 1969). Kinetic evidence linking this observation with the functional pool of plastoquinone located between the two photosystems is lacking (Esser, 1972; Amesz, 1973). There are, however, several different pools of quinone present in the chloroplast, so this observation does not invalidate the assignment of Signal II to a plastoquinone derivative (Lichtenthaler, 1969). A review article on the properties of both Signals I and II has recently appeared (Kohl, 1972).

Recently, speculations on the functional location of Signal II have centered on the water side of Photosystem II, where long-lived intermediates involved in the water oxidation process have been demonstrated (Kok et al., 1970). The basis for this assignment comes from both the long decay time of the radical and its behavior to reagents such as hydroxylamine, anilinothiophene and CCCP, which speed the decay of both Signal II (Okayama et al., 1971; Lozier and Butler, 1973) and oxidized precursors involved in oxygen evolution (Renger, 1971).

Kinetic analysis of Signal II has been greatly hampered by its slow decay, which is on the order of hours at room temperature. Kinetic measurements after such long times are difficult to interpret because of severe aging effects (Section VI). In the experiments presented in this section we have established conditions under which fresh chloroplasts with low dark levels of Signal II can be obtained.

In addition, we have found that the species giving rise to Signal II exists in two alternative states. The EPR spectrum of one of these states has 5 hyperfine components and is similar to that reported by other workers (Kohl, 1972). The EPR properties of the second state include a narrower bandwidth and only 4 hyperfine components. We have carried out preliminary experiments exploring the factors which control the interconversion of these two states.

B. Materials and Methods

(1) Chloroplast preparation

Spinach (*Spinacia oleracea* var. early hybrid No. 7) was grown in growth chambers under conditions as outlined by Sun and Sauer (1971). Chloroplasts were isolated by grinding for 10 sec in a Waring blender using an isolation solution consisting of 0.4 M sucrose, 0.1 M tricine (pH = 7.6), 0.01 M NaCl (unwashed sucrose chloroplasts) or 0.35 M NaCl, 0.02 M tris (pH 8.0) (unwashed salt chloroplasts). The chloroplasts were then filtered through 8 layers of cheesecloth, spun for 1 min at 3000 x g in a Sorval RC2B centrifuge, and the pelleted chloroplasts were resuspended in the isolation solution. All operations were carried out at 4°C. Washed chloroplasts were prepared by resuspending either of these types of chloroplasts in fresh isolation medium and subsequently centrifuging at 1000 x g for 10 min. Chloroplasts referred to in the text as "dark-adapted chloroplasts" were prepared in the same manner except that the spinach leaves were picked after they had been in the dark for for at least 8 hr, and all subsequent isolation steps were carried

out in the dark. Chlorophyll concentrations were between 2 and 4 mg/ml. EDTA (10^{-4} M) (final concentration) was present in all experiments to eliminate the Mn^{+2} signal which otherwise would distort the baseline. In control experiments the same results were obtained with or without added EDTA.

(2) Light sources

Broad band, continuous white light was obtained from a microscope illuminator and was passed through a water filter and a Corning 1-69, 3-74 filter combination. The intensity at the sample for this continuous light was 45 milliwatts per cm^2. Red (650 nm) or far red (700 nm) continuous light was provided by a tungsten lamp, a Bausch and Lomb monochromator (Model 33-86-03, entrance slit = exit slit = 2 mm; dispersion, 6.5 nm/mm), and appropriate Optical Industries interference filters to eliminate higher order diffractions. Light intensity was adjusted using appropriate Balzers neutral density filters and measured with a Hewlett-Packard Radiant Flux Detector (Model 8334A). Illumination was initiated using an electromechanical shutter which has an opening time less than 10 msec.

(3) EPR measurements

A Varian E-3 (X band, 9.5 GHz) EPR spectrometer fitted with a slotted cavity to permit *in situ* illumination was used in recording spectra and kinetic changes in chloroplast suspensions contained in a quartz EPR flat cell (nominal optical path length = 0.2 mm). The cavity was continuously flushed with dry, room-temperature nitrogen gas. The modulation amplitude in recording spectra was 5.0 gauss. Spectra were recorded by sweeping from low field to high field with

the spectrometer time constant, microwave power and scan rate as noted in the figure legends.

Signal averaging was performed using a 1024 channel Enhancetron signal averager. The output of the Varian E-3 spectrometer, with a time constant as noted in the text, was fed into the averager. Appropriate timing circuits synchronized the initiation of the averager sweep and the field sweep for recording spectra. Low temperature EPR experiments were performed using 3 mm cylindrical (I.D.) quartz sample tubes. A Varian low temperature accessory (Model #E-4557-9) was used to maintain the temperature within ±5°C of the nominal value.

C. Results

(1) Effect of dark adaptation on Signal II decay

Figure 11 shows EPR spectra of chloroplasts in the light and in the dark after illumination. In the light, both Signal II and Signal I are observed, although the magnitude of Signal I is low because no electron acceptor system, such as ferredoxin/$NADP^+$, has been included in the chloroplast suspension. Upon darkening, Signal I decays quickly whereas the extent of Signal II decay is slight. In his recent review article, Kohl (1972) mentions that in the dark Signal II has hyperfine structure in the region labeled "I" in Fig. 11, such that the ratio of the peak in this region to the peak at the position labeled "II" in Fig. 11 is 3/4. We have found that this ratio varies considerably and is dependent on the method of chloroplast isolation. In the following subsection we consider the sources for this variation

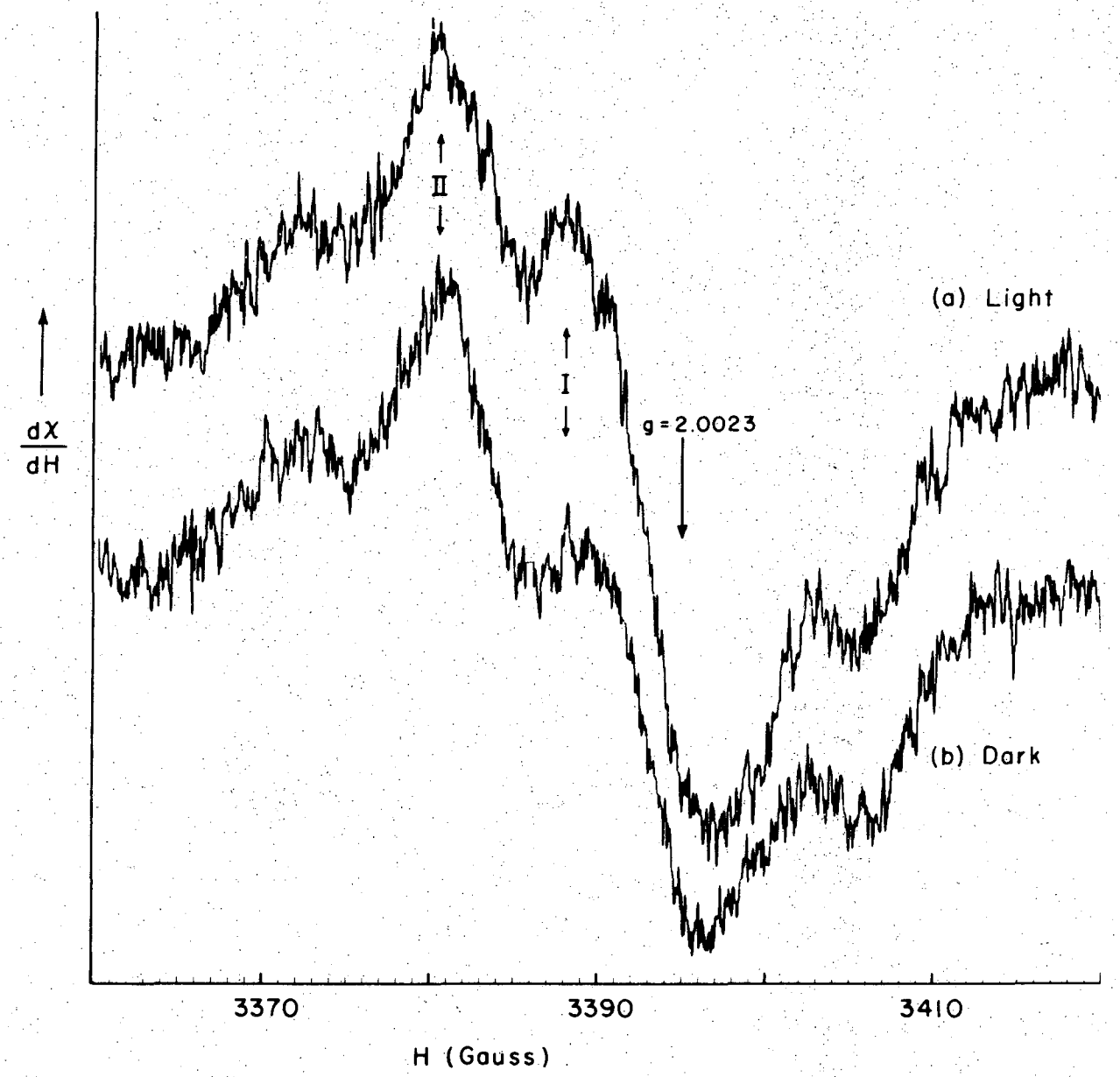

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Figure 11. EPR spectra of chloroplasts resuspended in isolation solution in the light (a) and in the dark immediately after illumination (b). Broad band continuous light was used to illuminate the sample. The instrument time constant was 0.3 sec, the scan rate was 25 G/min and the microwave power was 16 mW. Low field maximum for Signal II labelled as "II", for signal I as "I".

in the structure of Signal II in detail.

Cycles of red and far red light have been shown to have no effect on the decay of Signal II (Esser, 1972), and exogenous redox systems appear to be excluded from the site of Signal II formation. Fresh chloroplasts prepared from spinach picked during the growth chamber light cycle show high dark levels of Signal II and exhibit little additional light-induced increase. We have found that incubation of these chloroplasts for 2-4 h at 0°C in a darkened ice bucket leads to a 20-30% decrease in the signal, which is regained upon illumination. It appears that a soluble endogenous factor facilitates this decay, since washed chloroplasts show very little (less than 10%) Signal II decrease even after 5 or 6 h of dark incubation.

We have found, however, that if spinach leaves are picked toward the end of the dark period of their growth cycle and the chloroplast isolation procedure is carried out in the dark, the magnitude of Signal II prior to illumination is reduced. Fig. 12 shows EPR spectra of such dark-adapted chloroplasts before and after illumination. The chloroplasts in this experiment, prepared from leaves which had been in the dark approximately 8 hr, show a 45% increase in Signal II upon illumination.

The effect of the microwave power level on the Signal II amplitude and on the ratio of Signal II before and after illumination in dark-adapted chloroplasts is shown in Fig. 13. Curve (a) indicates that Signal II saturates at fairly low power in agreement with Kohl (1972), and decreases slightly at higher powers. However, the ratio of Signal II in dark-adapted chloroplasts to Signal II in these same

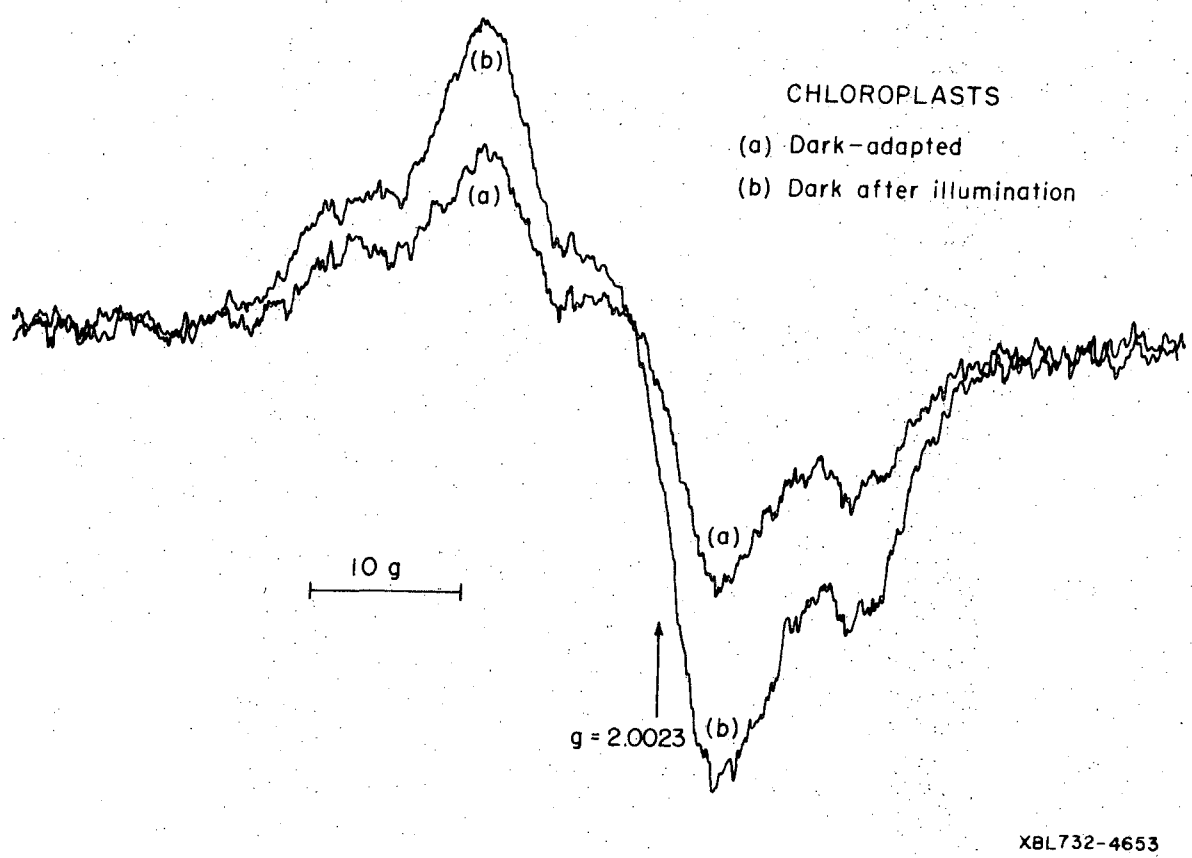

Figure 12. EPR spectra of dark-adapted chloroplasts in the dark before (a) and immediately after (b) illumination. The sample was illuminated with broad band white light for 2 min before spectrum (b) was recorded. The instrument time constant was 1.0 sec, scan rate was 25 G/min, microwave power was 50 mW.

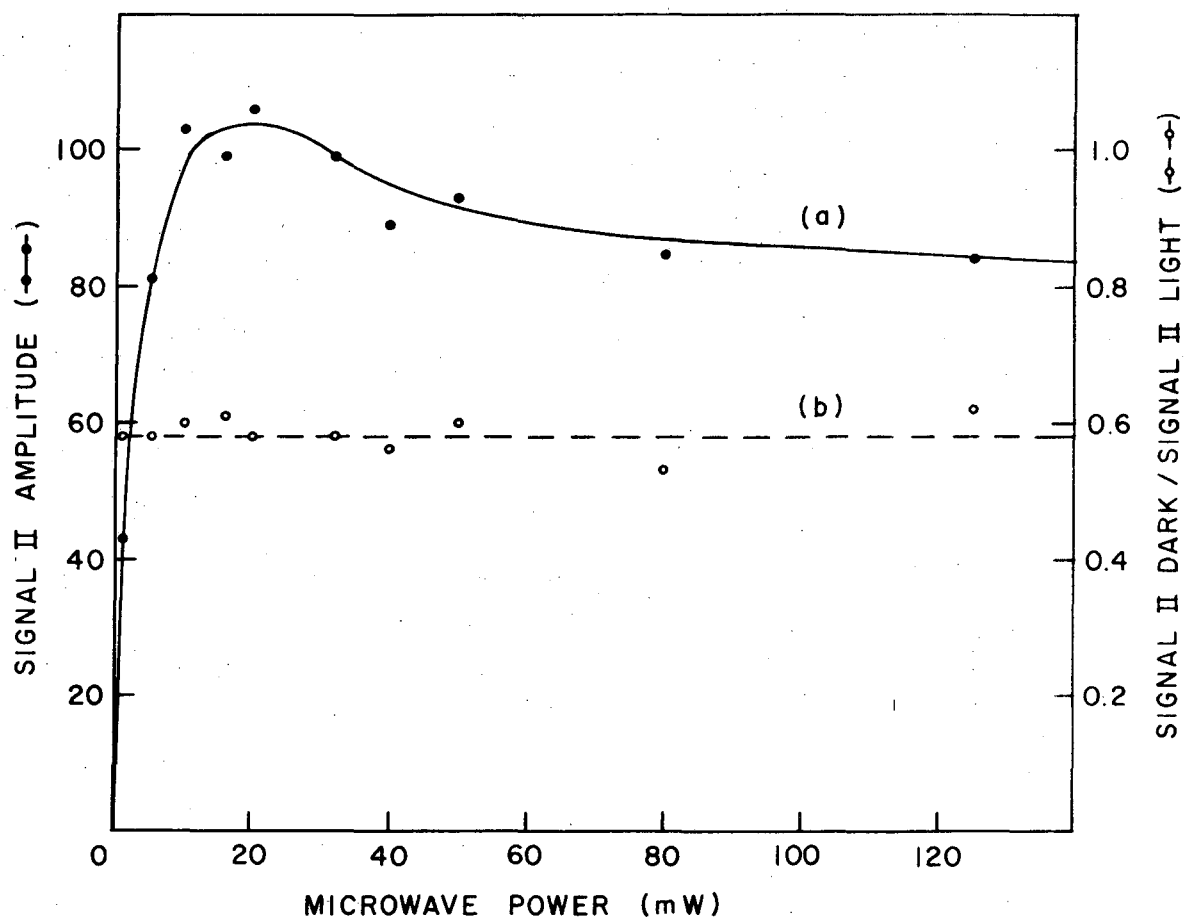

Figure 13. EPR Signal II amplitude (curve a, —●—●—) XBL734-4774 and the ratio of Signal II before and after illumination (curve b, - -o- -o- -) as a function of microwave power. A fresh sample of untreated, dark-adapted chloroplasts was used for each experimental point. The instrument time constant and scan rate were as described in Fig. 12.

chloroplasts following illumination is not influenced by the microwave power as shown in curve (b) in Fig. 13. This ratio remains constant at about 0.60 in this set of experiments for microwave powers between 1.0 and 125 mW.

Fig. 14 summarizes a series of experiments in which chloroplasts were prepared from leaves picked at various times after initiation of the dark period. Within the first 2 hr after darkening there is a decrease of about 25% in the signal, which may correspond to the 20-30% decrease (see above) that has been found to be associated with dark incubated chloroplasts at 0°C. Following this initial decrease there is a slower decrease to about 50-60% of the light-induced signal after 12 hr in the dark. We have consistently found that Signal II appears to decay only to this 50% level in the dark *in vivo*. Lozier and Butler (1973) have reported a similar 50% plateau in the decay of Signal II following illumination in isolated spinach chloroplasts at room temperature. The possible significance of this effect will be discussed in detail below.

(2) EPR spectra of Signal II - Alternative forms

Fig. 15 presents EPR spectra of spinach chloroplasts measured in the dark under various conditions. Magnetic field positions corresponding to partially-resolved hyperfine peaks and troughs in the spectra have been labeled with the letters A-F. Fig. 15a shows the EPR spectrum for Signal II which we observe routinely from unwashed sucrose chloroplasts. There are four partially resolved components and the bandwidth, measured as the magnetic field difference between the low field maximum at B and the high field minimum at D, is 16 G.

Figure 14. The decay of Signal II *in vivo*. Spinach plants in the growth chamber entered the dark period of their growth cycle at time zero. At various times following this, chloroplasts were isolated under rigorously dark conditions. Spectra were recorded before and after illumination with broad band white light, using the time constant and scan rate described in Fig. 12. The magnitude of Signal II was measured as the difference between the low field maximum at 3380 G and the high field minimum at 3396 G in Fig. 11. The ratio of Signal II before illumination to Signal II following illumination is plotted as a function of the time the spinach leaves were picked.

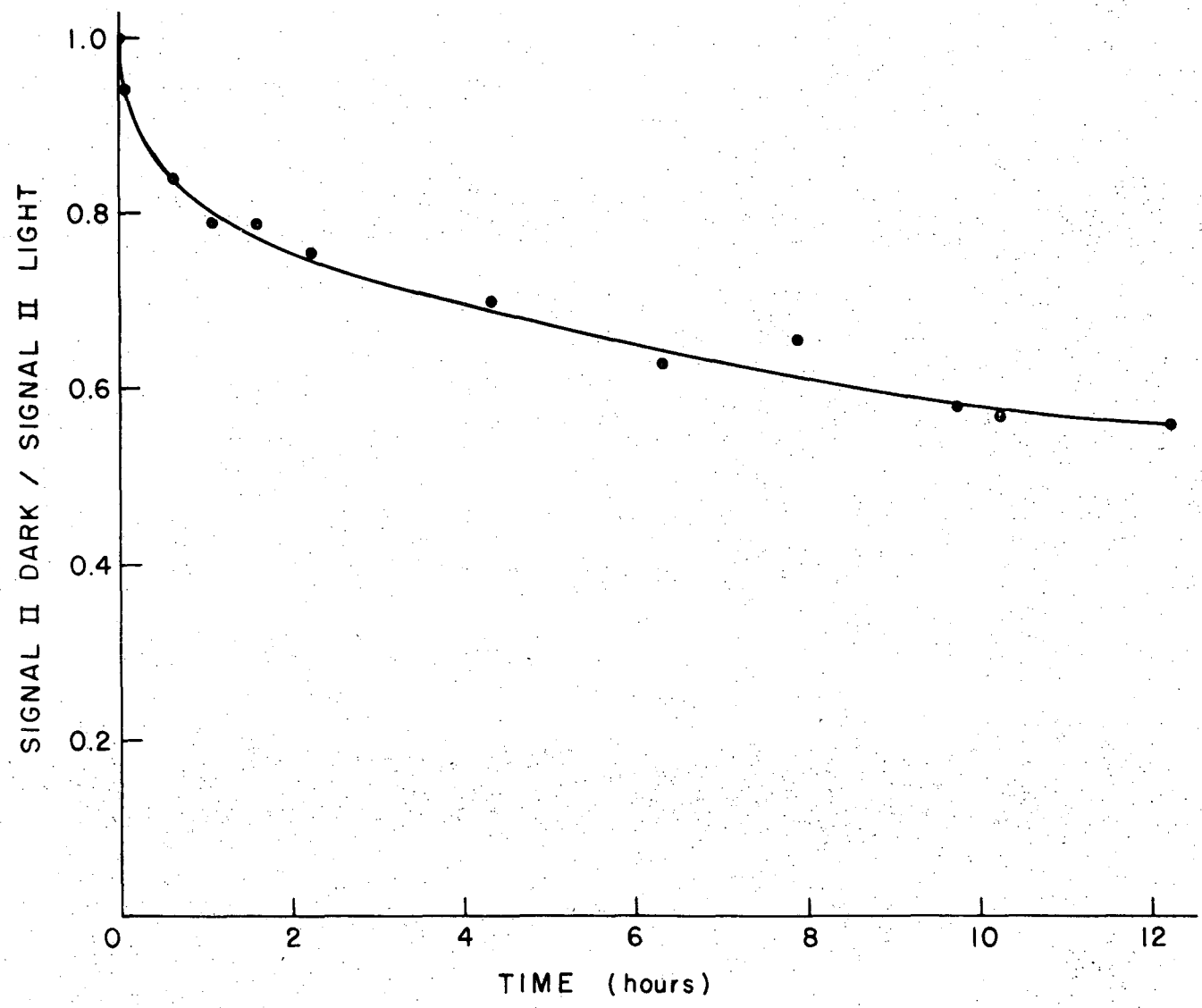

Figure 14.

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Under these conditions the shoulder at A is prominent, the amplitude of the peak C is small compared to that at B, and there is no obvious shoulder or peak at field position E.

If these unwashed sucrose chloroplasts are subsequently washed with the sucrose isolation medium, the spectrum shown in Fig. 15b results. This spectrum is now similar to that reported by other workers (Kohl, 1972). The low field shoulder at A is less well resolved; the peak at C is much larger; there is a shoulder at D and a high field minimum at E. The apparent bandwidth, measured between B and E, is 19 G. For the remainder of this work we shall refer to the 16 G spectrum of Signal II in Fig. 15a as Signal II(16) and to the 19 G spectrum in Fig. 15b as Signal II(19).

Signal II(19) in washed sucrose chloroplasts can be converted to Signal II(16) by increasing the ionic strength. For example, we have found that the addition of 30 mM $MgSO_4$, 40 mM K_2SO_4, 30 mM $MgCl_2$, 30 mM Na_2MoO_4 or 10 mM $K_4\ Fe^{II}(CN)_6$ to washed sucrose chloroplasts converts Signal II(19) to Signal II(16). Fig. 15c shows the EPR spectrum of Signal II in washed sucrose chloroplasts in which the ionic strength has been increased by the addition of 10 mM potassium ferrocyanide. This trace shows structure very similar to that recorded for Signal II(16) in unwashed sucrose chloroplasts (Fig. 15a). The shoulder at A is again more apparent, the amplitude at C is reduced, and the high field minimum has shifted from E to D, resulting in a bandwidth of 16 G.

In these experiments the ionic strength of the added salt was the critical factor. For example, 10 mM $K_4\ Fe^{II}(CN)_6$ (μ = 0.1 M) was sufficient, whereas 10 mM $MgCl_2$ (μ = 0.03 M) was without appreciable

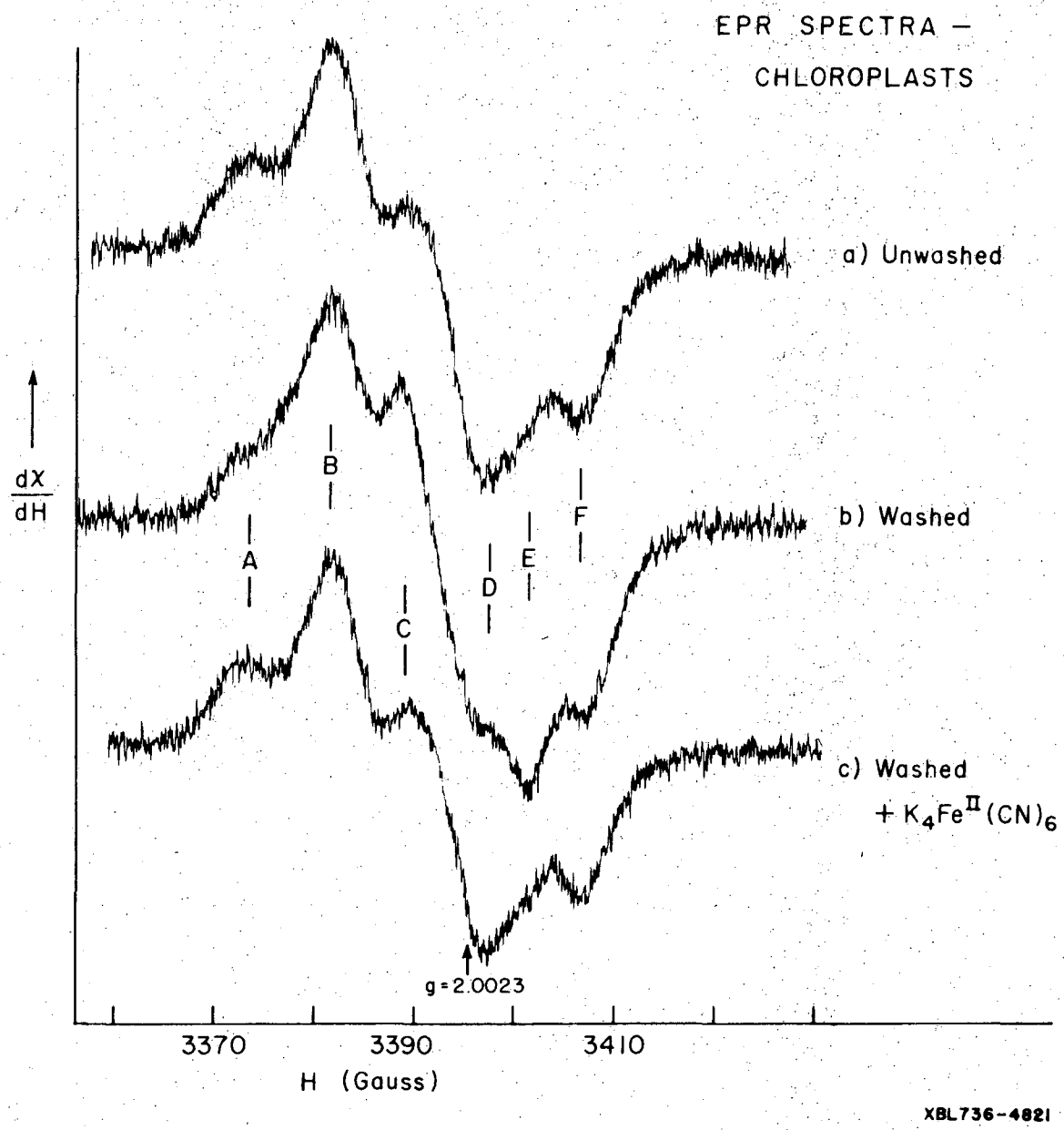

Figure 15. EPR spectra of spinach chloroplasts, isolated in sucrose isolation medium, a) resuspended in isolation medium, b) washed once in sucrose isolation medium and resuspended in isolation medium, c) as in b) with 10 mM $K_4Fe^{II}(CN)_6$ added following resuspension. All samples were illuminated with continuous, broad band light for 30 sec before the spectra were recorded. The microwave power was 16mW, instrument time constant was 0.3sec, and a scan rate of 12.5 G/min.

effect on the conversion process. However, if 30 mM $MgCl_2$ (μ = 0.09 M) was added to washed sucrose chloroplasts, Signal II(16) was observed. In these experiments we found, in general, that the ionic strength of the added salt had to be greater than μ = 0.08 M to convert Signal II(19) to II(16).

This conversion of Signal II(19) to Signal II(16) is not facilitated by 30 mM sodium ascorbate, indicating that the mechanism for this conversion does not involve reduction. Further evidence supporting the conclusion that oxidation-reduction reactions are not involved comes from the observation that 10 mM potassium ferricyanide added to washed sucrose chloroplasts converts Signal II(19) to Signal II(16) as effectively as does 10 mM potassium ferrocyanide. At this concentration ferricyanide is a strong enough oxidant to cause appreciable oxidation of P700 and hence the presence of Signal I obscures Signal II in the region around field positions C and D. However, the shoulder at A in ferricyanide-treated chloroplasts is as well resolved as it is in Fig. 15a or 15c, indicating that Signal II(16) is present. This experiment also demonstrates that Signal II(19) does not result from an addition of Signal I and Signal II(16), in agreement with the interpretation of Kohl (1972).

In a second set of experiments designed to test the effect of ionic strength on the structure of Signal II, we prepared unwashed and washed salt chloroplasts. The final NaCl concentration in both types of chloroplasts was 0.35 M (μ = 0.35 M) and in each case Signal II(16) was observed. These experiments indicate that Signal II(19) results in washed sucrose chloroplasts from an ionic strength

decrease caused by the washing procedure and not, for example, by the removal of soluble factors contributing directly to Signal II(16). This suggests that the interconversion of the two states of Signal II is mediated by an alteration in the structure of the radical species giving rise to the signal.

In order to determine the relative number of spins in Signal II(19) and Signal II(16), we performed double integration for Signal II(19) in samples of washed sucrose chloroplasts. Using chloroplasts from the same preparation, we then converted Signal II(19) to Signal II(16) and carried out double integration for Signal II(16). Comparing these two values we found that the ratio of spins in Signal II(19) to spins in II(16) was 1.0 ± 0.05 for three different preparations of washed sucrose chloroplasts. We conclude from these experiments that the species giving rise to Signal II can exist in two alternative states and that converting from one state to the other by ionic strength changes does not alter the number of spins detected.

Preliminary results suggest that the interconversion between the two states of Signal II is temperature sensitive. Unwashed sucrose chloroplasts which exhibit Signal II(16) at room temperature show Signal II(19) when frozen to -60°C or to -196°C. However, as discussed in greater detail below, illumination does not play a role in the interconversion of these two states of Signal II.

We observed both Signal II(16) and II(19) in chloroplasts prepared from market and greenhouse spinach and in pea chloroplasts. In each of these samples it is possible to convert Signal II(19) to II(16) by ionic strength increase. In whole leaves of growth chamber

spinach we observed a 19G bandwidth for Signal II, indicating that the predominant *in vivo* state is that giving rise to Signal II(19). We also investigated the structure of Signal II in the algae *Chlorella pyrenoidosa* and *Chlamydomonas reinhardi*. In agreement with published spectra by Weaver (1962), Kohl (1972) and others we observe essentially Signal II(19). The ratio of peak intensities at field point C to that at field point B in these algae varies between 0.50 and 0.80, compared to the C/B ratio in Signal II(16) which is less than 0.25. The bandwidth for Signal II in these algae is 19 G.

(3) Light-induced increases in Signals II(16) and II(19)

We presented data above on the light-induced increase in Signal II in dark-adapted chloroplasts. The experiments were done with spinach chloroplasts which exhibited Signal II(16). We have repeated these experiments with chloroplasts which show Signal II(19) and with chloroplasts in which Signal II(19) has been converted to Signal II(16) by ionic strength increase.

The left side of Fig. 16 shows spectra of Signal II recorded for dark-adapted chloroplasts before (curves 1) and in the dark after (curves 2) illumination. On the right in the figure are the spectra of the light-induced increase in Signal II, which is obtained by subtracting curve 1 from curve 2 for each of the three experiments. Fig. 16a was done with unwashed sucrose chloroplasts, Fig. 16b with washed sucrose chloroplasts, and Fig. 16c with washed sucrose chloroplasts to which 10 mM $K_4Fe^{II}(CN)_6$ was added. The data show that in unwashed chloroplasts and in washed chloroplasts plus ferrocyanide the spectrum of the light-induced increase is that of Signal II(16).

Washed sucrose chloroplasts (Fig. 16b) however, exhibit Signal II(19) before and after illumination and, as expected, the difference spectrum is also that of Signal II(19). The results indicate that Signals II(16) and II(19) undergo light-induced increases without change in spectral characteristics.

The chloroplasts used in the experiments in Fig. 16a were prepared from spinach plants which had been in the dark about 4 hr. The ratio of the amplitude of Signal II before illumination to Signal II following illumination is 0.7, in agreement with data presented in Fig. 14 on the in vivo decay of Signal II. In Figs. 16b and 16c a second preparation of chloroplasts, isolated from spinach plants which had been in the dark for 10 hr, was used. In both Figs. 16b and 16c the amplitude of Signal II before illumination accounts for about 55% of the amplitude of Signal II following illumination. This result agrees with the in vivo decay of Signal II in spinach plants which we measured earlier using unwashed sucrose chloroplasts and indicates that the state in which the Signal II precursor exists has little effect on the decay or light-induced increase in Signal II.

D. Discussion

Previous work on Signal II has concentrated on its molecular identity and its general location with respect to the two photosynthetic light reactions. The main conclusions from this earlier work has been the identification of Signal II with either plastiquinone or a plastoquinone derivative and the general association with the oxygen evolving photosystem. Very few kinetic experiments have been

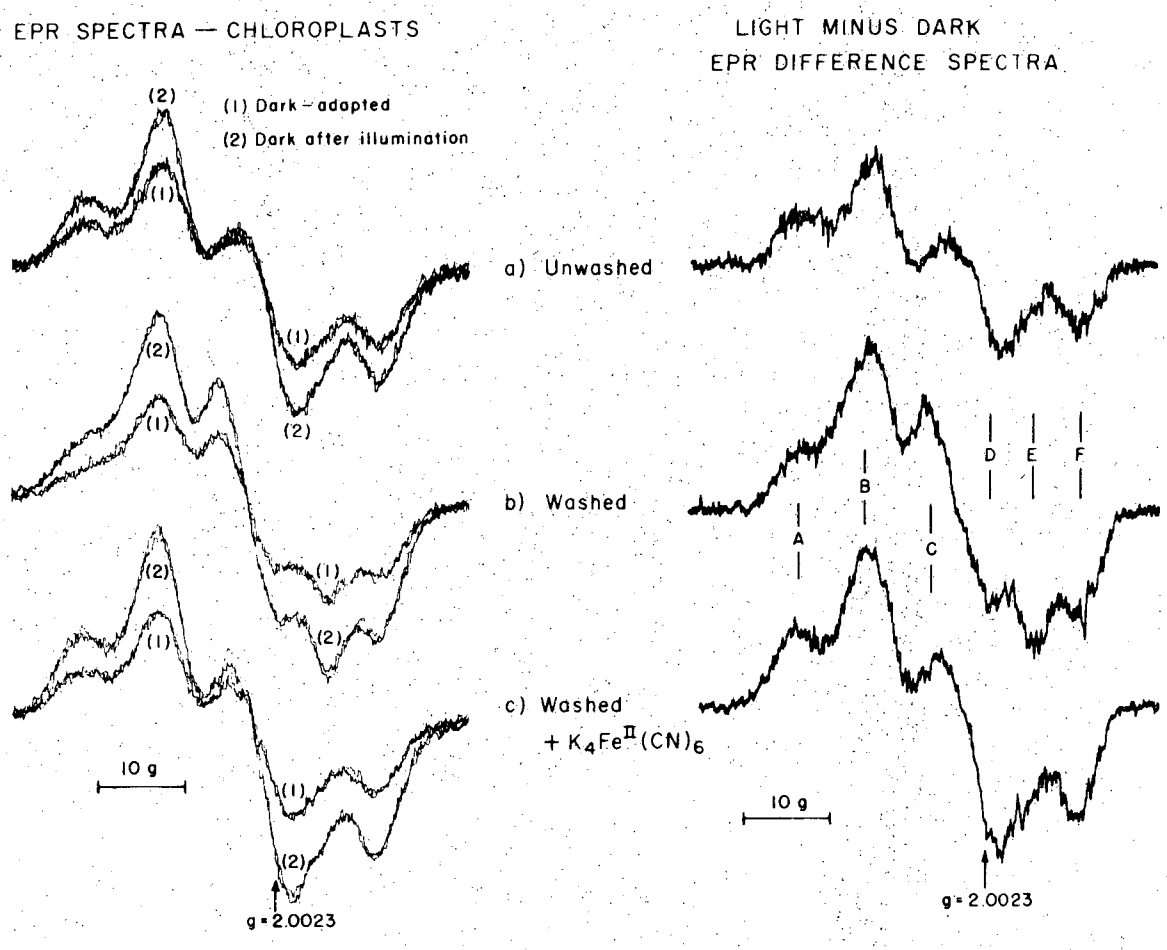

Figure 16. Left: EPR spectra of dark-adapted chloroplasts, isolated in sucrose medium, in the dark (1) before and (2) after illumination; two scans are shown for both (1) and (2). Right: light minus dark EPR difference spectra obtained by subtracting (1) from (2) for each of the samples on the left. a) Unwashed, b) washed, and c) washed + $K_4Fe^{II}(CN)$ sucrose chloroplasts. Broad band light was used for illumination. The subtractions were performed electronically with the signal averager. The microwave power was 5 mW, instrument time constant was 0.3 sec, and the scan rate was 25 G/min.

reported, primarily because of the difficulty associated with the long decay of the radical; consequently, more specific information regarding its location and mode of generation has been lacking.

We have developed a procedure that allows us to obtain fresh untreated chloroplasts with low dark levels of Signal II radicals. In the following section this procedure will be used in order to perform kinetic experiments on the light-induced generation of Signal II. However, in developing this procedure several characteristics of Signal II have come to light.

The *in vivo* decay of Signal II shown in Fig. 14 indicates that this radical is unusually stable. By contrast, the decay of Signal I following cessation of illumination is on the order of tens of msec (Kohl, 1972). In addition, only about half of the radical has decayed even after 12 hr dark, and from the shape of the decay curve it appears that a plateau is reached at the level of half decay. Lozier and Butler (1973) and experiments reported below (Fig. 32) indicate that the dark decay of Signal II in isolated chloroplasts at room temperature also reaches a plateau at about 50% of the light-induced level. Thus it appears that Signal II is heterogeneous, at least with respect to decay kinetics, in that about 50% of the radicals decay more rapidly than the other half. This behavior is similar to that displayed by another enigmatic component which has been associated with Photosystem II, cyt b_{559}. Epel *et al.* (1972) have shown that there are two distinct pools of this cytochrome in oxygen-evolving organisms and that these two pools may be distinguished on the basis of redox behavior. In addition, both cyt b_{559} and Signal II

do not appear to be involved in the main electron transfer reactions in normally functioning chloroplasts. Cyt b_{559} is largely reduced in these chloroplasts and Signal II is present as a free radical. Illumination does not appear to alter these states. The purpose which these pools of stabilized components serve is unclear at present. However, as discussed in the Introduction to this Section for Signal II and by Epel et al. (1972) for cyt b_{559}, their integrity appears necessary for oxygen evolution.

As shown in Fig. 15, we have demonstrated that the species which gives rise to Signal II can exist in either of two states. The EPR properties of Signal II in these two states differ both in hyperfine structure and bandwidth. The bandwidth of Signal II(16) is 16 G, and we observe four partially-resolved hyperfine components; the bandwidth of Signal II(19) is 19 G with five partially-resolved hyperfine components. We have shown that reversible interconversion between these two states can be achieved by changing the ionic strength of the chloroplast suspension. At this time we do not understand the mechanism of the ionic strength induced changes in Signal II structure.

The existence of two different states for the species which gives rise to Signal II is similar to the situation for flavins and flavoproteins. Palmer et al. (1971) have presented the bandwidth of the EPR spectra for 13 flavoprotein free radicals. These flavoproteins fall into two categories: one class has a bandwidth of about 15 G, the other has a bandwidth of 19 G. In some cases (e.g., glucose oxidase) it is possible to convert from one bandwidth to the other by a

pH change. The optical properties of these two classes of flavoprotein free radicals also show characteristic behavior: the 19 G species is usually blue, the 15 G species is usually red. On the basis of optical, magnetic resonance and model system studies, the blue, 19 G species has been associated with the neutral flavin semiquinone radical of the flavoprotein, while the red 15 G species is the anionic flavoprotein radical. In addition, the cationic flavoprotein free radical appears to have EPR properties similar to the neutral radical species (Beinert, 1972), while the metal chelate formed from the neutral flavin semiquinone has an EPR spectrum similar to that of the anionic flavoprotein radical (Muller et al., 1967). Therefore, the difference in both optical and EPR characteristics of the flavoproteins can be attributed to the proton at the N(5) position in the flavin (Beinert, 1972). This proton is present in the blue, 19 G neutral or cationic flavins and absent in the red, 15 G anionic or metal chelated species.

Model compound studies of flavins indicate that the hyperfine splitting pattern of the neutral flavin semiquinone radical is more complex than that of the anionic flavin radical (Beinert, 1972). This is also the case in the benzoquinone model system. The anionic p-benzoquinone radical has five hyperfine lines caused by interaction of the unpaired spin with the four equivalent ring protons (Venkataraman and Fraenkel, 1955; Wertz and Vivo, 1955). The monoprotonated neutral p-benzosemiquinone radical has eighteen hyperfine lines, indicating that the hydroxylic proton and two sets of two identical ring protons interact with the spin (Gough, 1966). Thus the protonation of atoms

which share unpaired electron density results in the observation of a more complex hyperfine splitting pattern. This protonation phenomenon may be the basis for the observation of four partially-resolved hyperfine components in Signal II(16) and five partially-resolved hyperfine components in Signal II(19). This interpretation is strengthened by the deuteration experiments of Kohl et al. (1965) which showed that the hyperfine components of Signal II were due to the interaction of the unpaired electron with protons.

The work of Kohl et al. (Kohl et al., 1969; Kohl and Wood, 1969) and Weaver (1968) have implicated plastoquinone or a plastoquinone derivative as the source of Signal II. The similarities which we find between flavin free radicals and Signal II behavior indicate that this species is also an attractive candidate. The ionic strength-induced changes in the EPR spectrum of Signal II which we report, however, can be accommodated by either molecule. At low ionic strength the Signal II species, which almost certainly is located in the thylakoid membrane, may be the protonated 19 G neutral semiquinone of either a flavin or a plastoquinone derivative. As the ionic strength is increased, conformational changes in the membrane may result in loss of a proton to form the 16 G anion or the metal chelate. Results of kinetic experiments which support this hypothesis will be presented in the next Section.

SECTION V

KINETIC STUDIES ON THE FORMATION OF EPR SIGNAL II

KINETIC STUDIES ON THE FORMATION OF EPR SIGNAL II

A. Introduction

Kinetic measurements on Signal II have been greatly hampered by the long decay time exhibited by the spin signal (Figs. 14 and 32). Thus most of the information regarding the functional location of Signal II has been derived from experiments involving measurements other than those designed to obtain kinetic information. The rationale and conclusions from these experiments have been summarized in the Introduction to Section IV.

Use of the isolation procedure described in the preceding Section, however, has allowed us to make kinetic analyses of the increase in Signal II magnitude induced by either 10 μsec flashes or continuous, monochromatic light in fresh, untreated chloroplasts. We have found results which support a model in which Signal II arises via electron transfer to oxidized intermediates between the Photosystem II reaction center chlorophyll and the site of water oxidation.

B. Materials and Methods

(1) Chloroplasts, light sources and EPR measurements

Unwashed sucrose chloroplasts, prepared as described in Section IV, were used in all experiments except those described in Table IV. For those experiments washed sucrose chloroplasts were used. The chlorophyll concentration in all EPR experiments was between 2 and 4 mg Chl/ml. EDTA (10^{-4} M) was added to eliminate the baseline distortion due to Mn^{+2}. Chlorophyll concentration in monitoring oxygen evolution was 0.2 mg Chl/ml.

Continuous, monochromatic light was obtained as described in Section IV. Xenon flashes were obtained from a flash system similar to that described by Weiss and Sauer (1970) and in Section II, except that a capacitor bank was used which gave flashes of 10 μsec duration (measured at half height) instead of the 28 μsec flashes which they used. The light was filtered through a Corning 1-69 heat filter and a Corning 3-74 ultraviolet filter and was focused on the slotted microwave cavity in EPR experiments or on the platinum electrode in oxygen experiments using appropriate lens systems.

EPR measurements were performed as described in Section IV. The microwave power in all experiments was 50 mW; modulation amplitude in recording spectra was 5.0 gauss; this was increased to 6.3 gauss in kinetic experiments to increase the signal-to-noise ratio. Spectra were recorded by sweeping from low field to high field with the spectrometer time constant and scan rate as noted in figure legends. In kinetic experiments in which signal averaging techniques were applied, the output of the E-3 spectrometer was fed into a 1024 channel Enhancetron signal averager. Timing circuits provided pulses which triggered the averager and, after a preset delay time, initiated the flash lamp pulse. All experiments were carried out at room temperature.

(2) Oxygen measurements

Oxygen evolution from chloroplasts in response to individual flashes was measured using an arrangement similar to that described by Weiss and Sauer (1970) and in Section II. We have modified the teflon covered electrode described in their work so that it is possible

to perform experiments without the teflon membrane, thus decreasing the response time of the electrode to approx. 10 msec. The Ag/AgCl reference electrode is located 4 cm downstream from the platinum electrode in a reservoir of electrolyte. The current increase resulting from chloroplast oxygen evolution is represented as the voltage output from a current-to-voltage transducing operational amplifier. This voltage is subsequently amplified and recorded using a Sanborn recorder (risetime = 5 msec). In the experiments described in this work flash lamp pulses were spaced 1 sec apart and were of saturating intensity. All experiments were carried out at room temperature.

C. Results

(1) Effect of single flashes on Signal II induction in dark-adapted chloroplasts

By setting the magnetic field of the spectrometer at the low field position labeled "II" in Fig. 11 we are able to monitor the kinetics of light-induced changes in Signal II. The effect of a series of 10 μsec flashes on radical concentration in dark-adapted chloroplasts is shown in Fig. 17. In this experiment Signal II before illumination was about 50% of the signal found after the flashes. As can be seen in Fig. 17, a single flash is sufficient to induce about 80% of the increase. Subsequent flashes increase the signal only slightly and, significantly, there are no oscillations with these later flashes such as those observed in experiments monitoring oxygen evolution as a function of flash number (Kok *et al.*, 1970). The characteristic slow

decay of Signal II is apparent in this experiment.

During a single 10 μsec flash, at most a single electron can be transferred through each of the photosystems (Weiss and Sauer, 1970), yet under these conditions we find that 80% of the light-induced increase in Signal II occurs. This observation implies that the species which gives rise to Signal II is present in relatively small concentrations compared to the total amount of chlorophyll in the chloroplast. We have confirmed this hypothesis by determining the ratio of spins in Signal II to the number of spins in Signal I in saturating light. We used the method of double integration as outlined by Chang and Johnson (1967) and found a value for this ratio which is close to unity in fresh chloroplasts.

(2) Quantum efficiency for Signal II formation in flashing light

We have determined single flash saturation curves for both Signal I and Signal II. These results are plotted in Fig. 18 as the fraction of Signal I or II formed as a function of the light intensity of a single flash. In these experiments dark-adapted chloroplasts, to which the acceptor system ferredoxin/NADP had been added, were used. The extent of Signal II formation resulting from a single flash of intensity J was divided by the extent of Signal II formation after 10 saturating flashes to obtain the fraction of Signal II formed at intensity J. Then, by changing the magnetic field from position II to the position labeled "I" in Fig. 11 without changing either lamp or sample placement, we determined the saturation behavior of Signal I. Since Signal I decays rapidly, the average of 36 flashes was used in these experiments. The extent of Signal I formation for a flash of

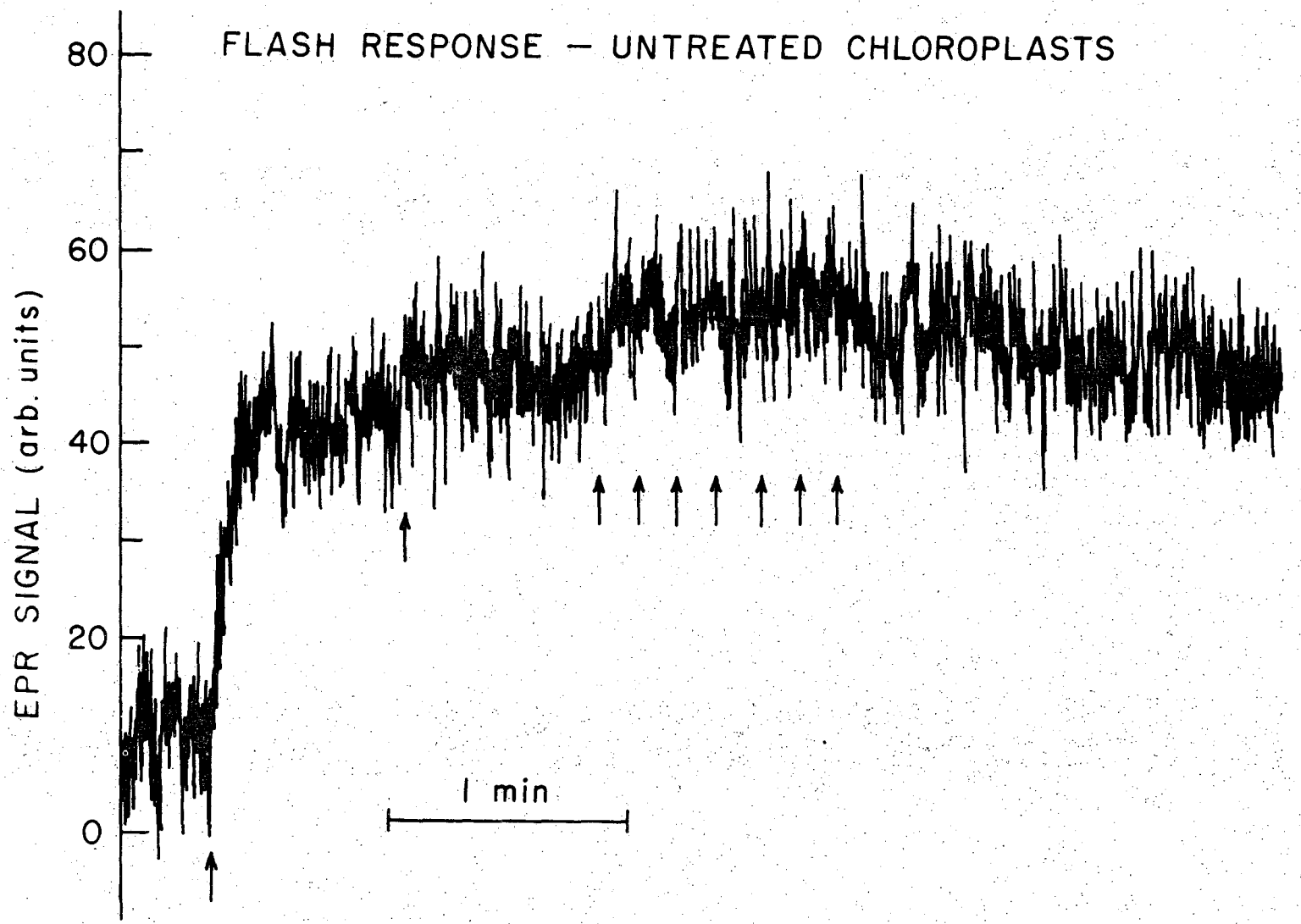

Figure 17. Response of Signal II in dark-adapted, untreated chloroplasts to light flashes (10 μsec). A single saturating flash was given at each of the arrows. Magnetic field set at peak labelled "II" in Fig. 11. Instrument time constant was 0.3 sec.

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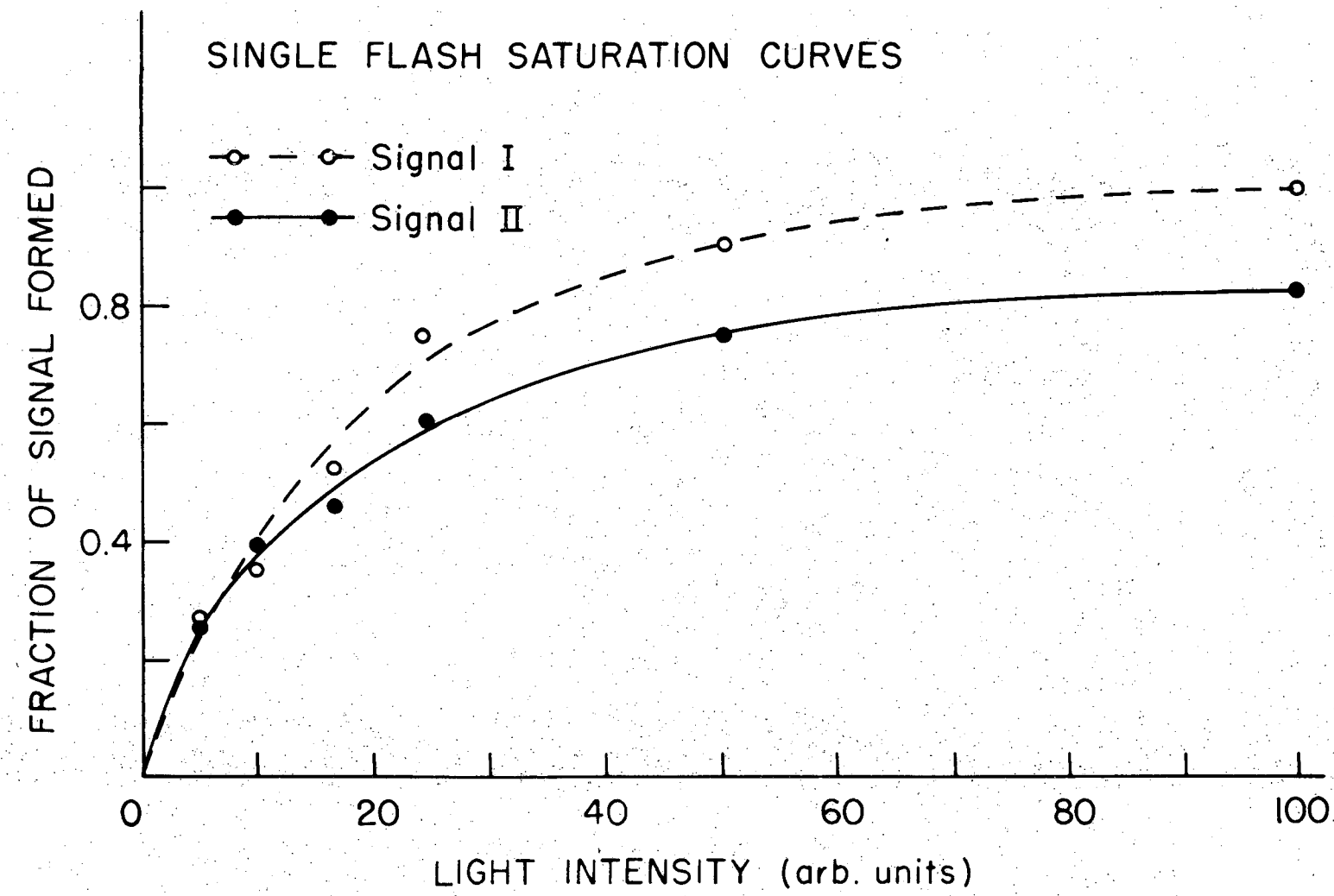

Figure 18. Single flash saturation curves for Signal I (--o--o--) and Signal II (—●—●—) in untreated chloroplasts. Instrument time constant: 0.3 sec for Signal II; 10 msec for Signal I.

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intensity J was divided by the extent of Signal I formation for a saturating flash to obtain the fraction of Signal I formed at intensity J.

Half saturation for both Signals I and II occurs at the same light intensity, which together with the results described above indicates that the quantum efficiency for Signal II formation in dark-adapted chloroplasts approaches that for Signal I formation. These results appear to be at variance with data reported by Treharne and Vernon (1962) which indicated that Signal II saturated at an intensity at least an order of magnitude lower than Signal I in whole Chlorella cells. However, from their experimental description it appears that their work was done under steady-state conditions which, because of the long decay time for Signal II, would yield a saturation intensity significantly lower than initial rate or single flash saturation values. In experiments which we have performed with Chlorella we find that a single flash is less effective in generating Signal II than in spinach chloroplasts.

(3) Effect of DCMU on Signal II formation in dark-adapted chloroplasts

Lozier and Butler (1973) and Weaver and Weaver (1963) have reported that DCMU does not inhibit the light response of Signal II. We repeated these experiments using dark-adapted chloroplasts and, as shown in Fig. 19, confirmed the finding that DCMU does not inhibit the formation of Signal II in continuous light. The DCMU concentration in this experiment was 2×10^{-4} M with a molar ratio of DCMU to Chl of 0.1, which is sufficient to inhibit oxygen evolution in chloroplasts

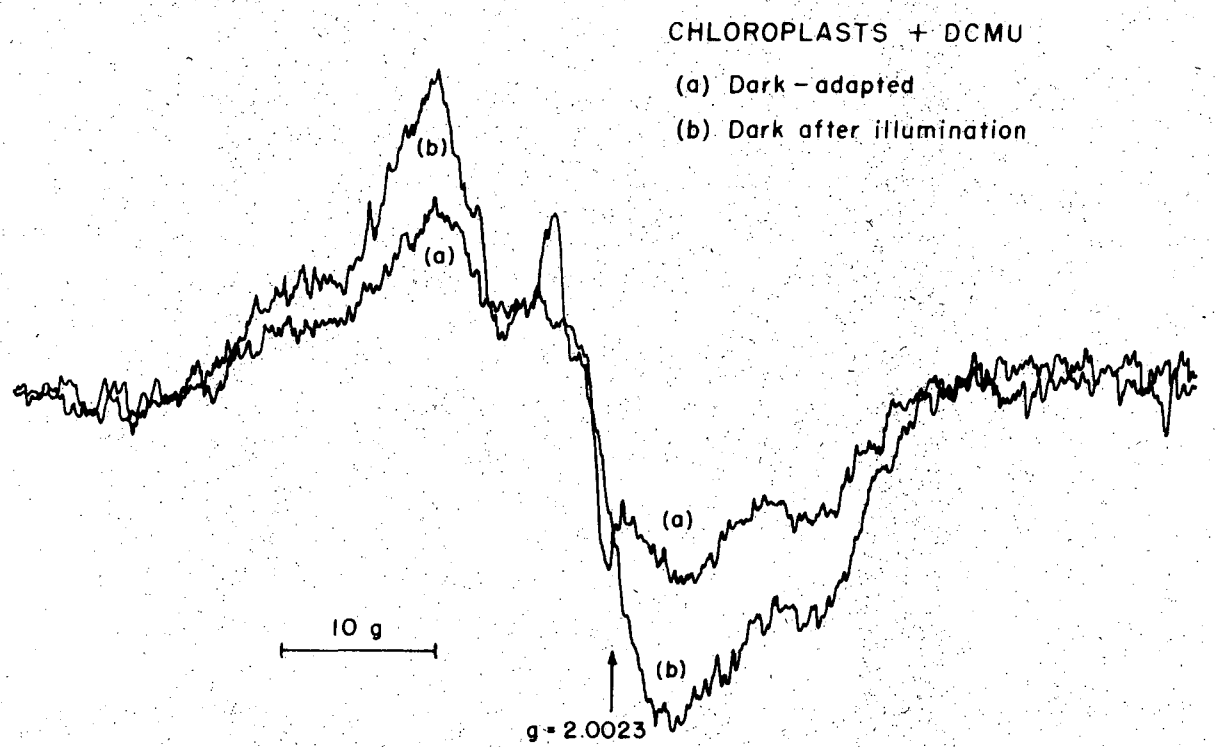

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Figure 19. EPR spectra of dark-adapted, DCMU-treated chloroplasts before (a) and immediately after (b) illumination with broad band white light. Chl concentration; 2.2mg/ml, DCMU concentration; 2 X 10^{-4} M. The instrument time constant and scan rate were as described in Fig. 12. The narrow signal in the center of the spectra is due to ascorbate free radical which is present in variable concentrations in spinach leaves (Walker, 1971).

completely. However, when these dark-adapted, DCMU-treated chloroplasts are subjected to a series of 10 μsec saturating flashes we find that the first flash evokes only one-third of the maximal light-induced response, and approximately 10 flashes are needed to induce Signal II fully. This is to be contrasted with untreated chloroplasts (Fig. 17) in which a single flash produces more than 80% of the light-induced signal and no further increase is observed following the third flash. Thus the effect of DCMU is to lower the quantum efficiency of Signal II formation without inhibiting the maximal extent of its response.

(4) Effect of red vs. far red illumination on Signal II formation

The insensitivity of the extent of Signal II formation to DCMU indicates two possible sites for its location. One places Signal II on the PSI side of the DCMU block, in which case far red light should be more effective than red light in stimulating its formation; the other possibility would locate Signal II on the PSII side of the block with red light more stimulatory than far red. In order to test these two possibilities we have done studies of the rate of Signal II formation in 650 nm and 700 nm continuous light. The experiments were done at low incident light intensities, since rates of formation yield more precise information than steady-state Signal II levels, for reasons mentioned above. At the high optical densities used in this study ($O.D._{650\ nm} = 4$, $O.D._{700\ nm} = 0.8$) essentially all of the light is absorbed at either wavelength, so that no corrections involving the extinction coefficients at 650 and 700 nm are applied. The results of the experiments are shown in Fig. 20. They indicate that for approximately equal incident photon fluxes, the initial rate of

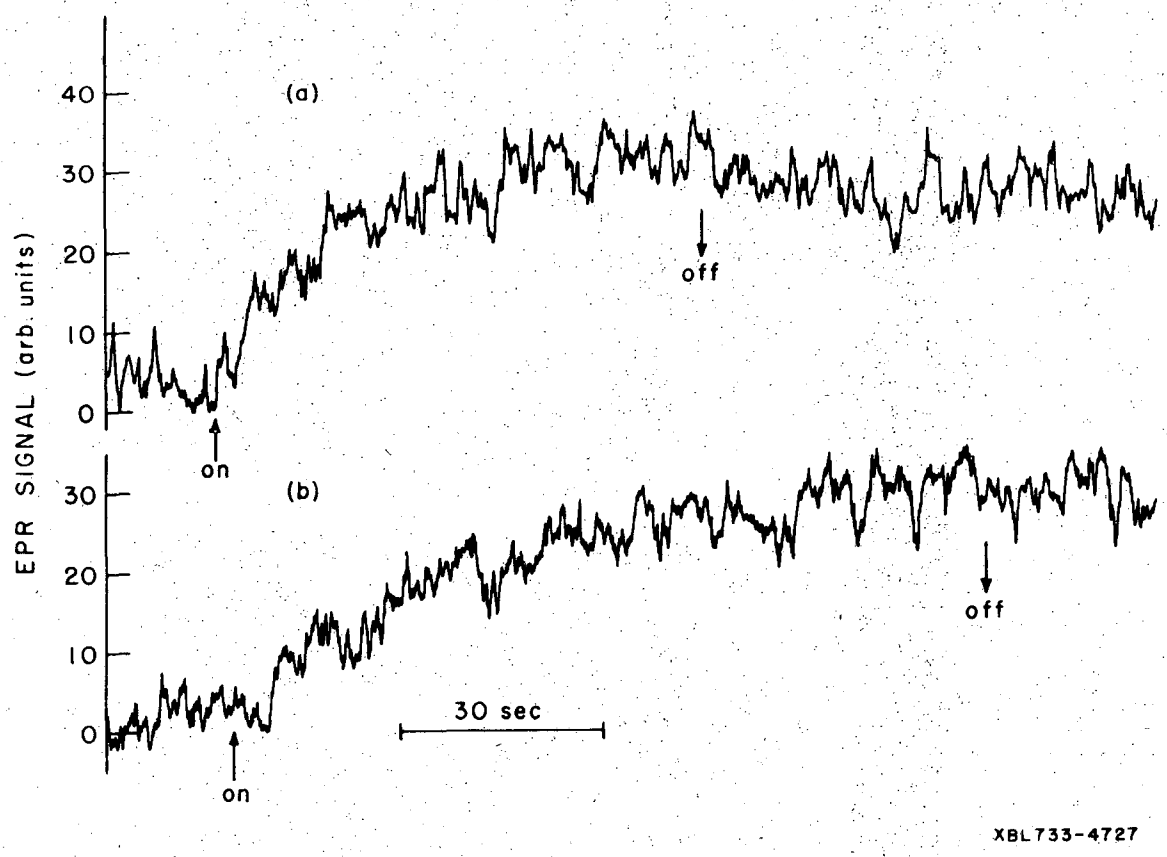

Figure 20. Time course of Signal II generation in dark-adapted, untreated chloroplasts in 650 nm (a) and 700 nm (b) light. Light on and off as indicated. The instrument time constant was 0.3 sec, light intensity at the sample was 65 μwatts/cm^2 for the 650 nm light, 70 μwatts/cm^2 for the 700 nm light.

Signal II formation in 650 nm light is more than twice the initial rate in 700 nm light. This is in agreement with the results of Allen et al. (1962) which indicate that Signal II is preferentially excited by shorter wavelengths of light, while the Signal I action spectrum persists to longer wavelengths. It is also consistent with the evidence cited in the Introduction associating Signal II with Photosystem II.

(5) Oxygen evolution in flashing light in dark-adapted and preilluminated chloroplasts

The results described above suggest that Signal II arises from a species located on the PSII side of the DCMU block. DCMU is known to act very close to the primary PSII photochemistry by blocking electron transfer from the primary acceptor to secondary acceptors in the chain between PSII and PSI. However, fluorescence induction studies of Joliot and Joliot (1971) indicate that the primary acceptor pool may be inhomogeneous. Therefore, there exists the possibility that Signal II arises from either the oxidizing side or reducing side of PSII. Since we have shown in Fig. 17 that Signal II arises via an electron transfer step that occurs largely on the first flash, we have carried out a series of experiments in which we monitored oxygen evolution in response to individual flashes in order to investigate these possibilities in more detail. Briefly (see Discussion), we expect dark-adapted (low Signal II) chloroplasts to show equal or higher yields of oxygen on the third flash compared to preilluminated (high Signal II) chloroplasts if the species giving rise to Signal II were a potential electron acceptor supplementing the primary acceptor on the reducing side of PSII. The opposite effect would be expected if Signal II arose as a result of

electron dontation to species on the water side of PSII. The results of the experiments are shown in Fig. 21. In the thoroughly dark-adapted chloroplasts the level of Signal II was 55% of the signal after the train of pulses. Preilluminated chloroplasts were obtained by exposing a portion of the same chloroplast sample to room light for 2 min, which served to induce Signal II fully, prior to injection into the electrode. Each sample was allowed 10 min dark time on the oxygen electrode before the flash sequence was initiated. A comparison of the two curves indicates that in fully dark-adapted chloroplasts the oxygen yield resulting from the third flash is lower and the yield of the fourth flash higher than in preilluminated chloroplasts. Dividing the oxygen yield of the third flash, Y_3, by that for the fourth flash, Y_4, we find values for the Y_3/Y_4 ratio of 1.2 for the dark-adapted chloroplasts and 1.9 for the preilluminated sample. According to the analysis described above and in the Discussion, these results favor a model in which Signal II originates as a consequence of electron donation on the water side of Photosystem II.

(6) Risetime of Signal II in response to a single flash

In constructing models locating Signal II on the water side of PSII we have found two which adequately explain the data thus far. The first of these places Signal II as an intermediate between the site of water oxidation and the reaction center chlorophyll P680. This model associates Signal II directly with the oxidized intermediates, which the experiments of Joliot *et al.* (1969), Kok *et al.* (1970), and Weiss and Sauer (1970) have demonstrated in the water oxidation process. These oxidized intermediates correspond to the S states in the Kok

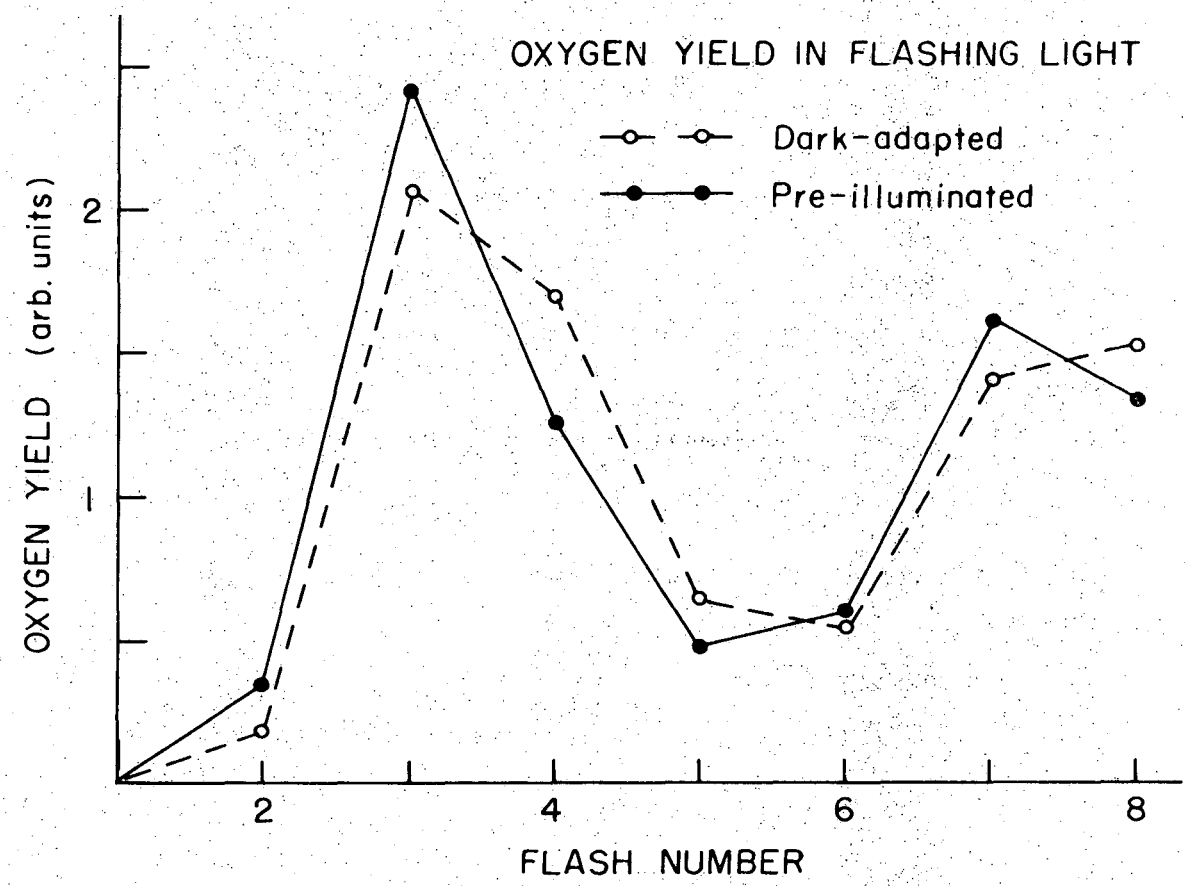

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Figure 21. Oxygen evolution in response to a series of saturating 10 μsec flashes from dark-adapted and preilluminated chloroplasts. Individual flashes in each series were space 1 sec apart. Oxygen yield in response to each flash was normalized with respect to a steady state value of 1, which is reached after 25 flashes.

et al. (1970) model for oxygen evolution. The second model places the species giving rise to Signal II off this electron transport pathway, but its formation would occur through interaction with intermediates in the chain between water and PSII.

The experiment described above (Fig. 17) in which we monitored the response of Signal II to single flashes argues against the assignment of Signal II to one of the S states in the electron transport chain between P680 and the water splitting site. This experiment shows that the concentration of the radical does not vary with flash number, whereas there should be marked oscillations in the concentrations of the oxidized intermediates involved in water splitting (Kok et al., 1970). We have obtained further evidence against the identification of Signal II with an S state directly on the pathway from the water oxidation site to P680 by determining the risetime of Signal II in response to a single flash. The results of this experiment, shown in Fig. 22, indicate that Signal II is formed rather slowly after a flash. The halftime for its rise is approximately 1 sec, which is three orders of magnitude greater than the values found for the risetimes of the intermediates involved in the water splitting process in experiments measuring oxygen evolution in Section II. Therefore both its response to a series of flashes and its risetime in response to a single flash argue against the direct assignment of Signal II to one of the S states involved in water oxidation.

(7) Multiple flash studies of Signal II formation

If Signal II arises indirectly via interaction with oxidized intermediates on the pathway from the water oxidation site to P680,

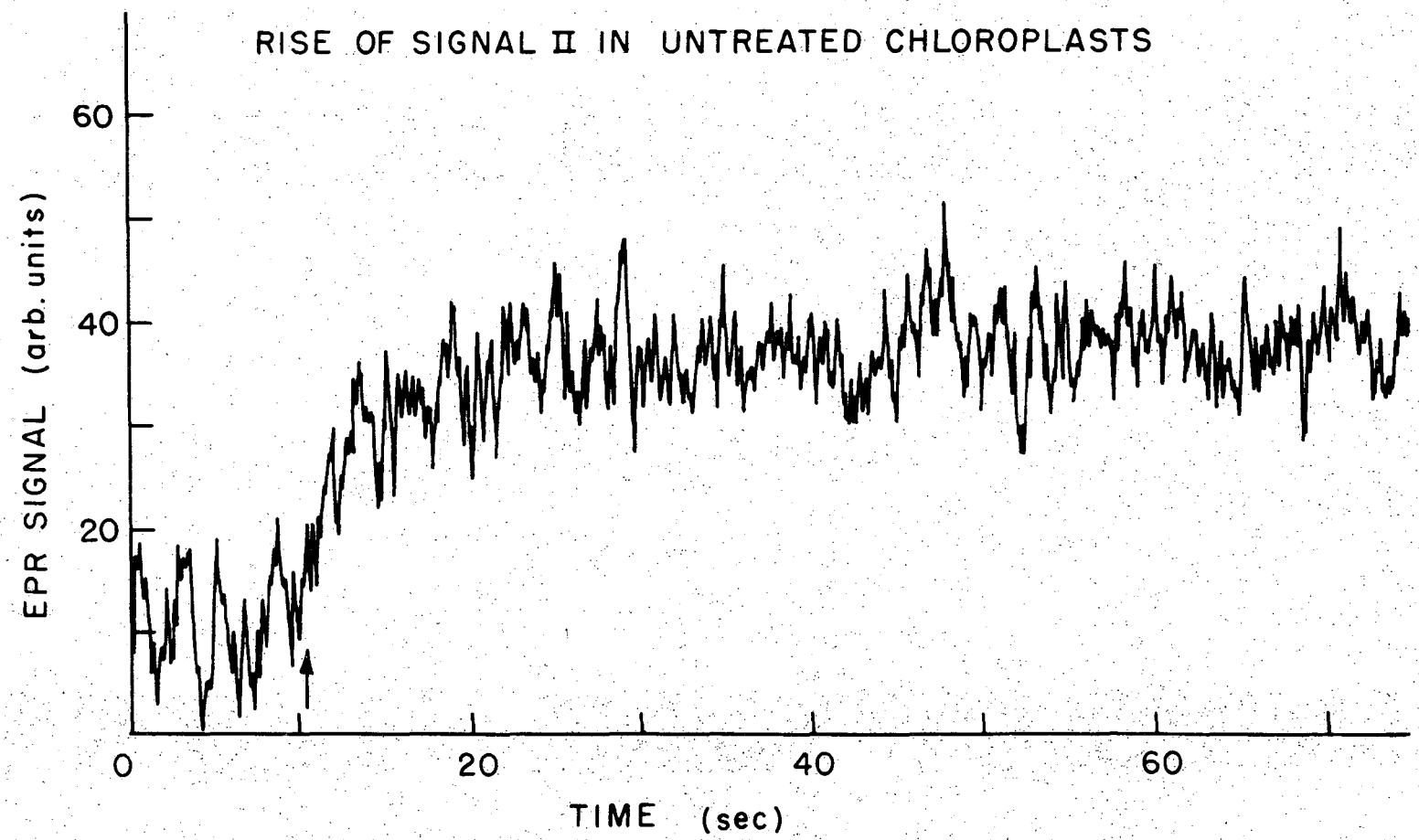

Figure 22. Time course of the response of Signal II in dark-adapted, untreated chloroplasts to a single saturating flash. Lamp discharged at arrow, time constant was 0.3 sec.

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we expect its extent of formation to be related to the concentrations of one or more of these oxidized species formed on a flash or in a series of flashes. As mentioned above, a number of workers (Kok et al., 1970; Joliot et al., 1969; Section II) have shown that following a flash the risetime for concentration changes in these oxidized species is less than 10 msec. On the other hand, Fig. 22 indicates that the rate of formation of Signal II is much slower following a flash. We have taken advantage of this disparity in rate constants to test the second model mentioned in the previous section. In a series of closely spaced flashes, with the dark time between flashes short compared to the risetime for Signal II, the species which generates the radical should be sensitive to the concentration of oxidized intermediates present at the conclusion of the flash series. The pattern of oxygen evolution shown in Fig. 21 has been most successfully explained by postulating a build-up of oxidized intermediates on the first and second flashes which are subsequently discharged in the water splitting process on the third and fourth flashes. Thus, after two flashes we expect a large concentration of highly oxidized intermediates, and after four flashes a much lower concentration.

The effects of these two flash patterns on the extent of Signal II formation are shown in Fig. 23. The spectra of dark-adapted chloroplasts obtained (Curves 1). Then, either two (Fig. 23a) or four (Fig. (23b) saturating flashes were given and the second spectra (Curves 2) were taken. At the conclusion of this scan ten saturating flashes were given and the third spectra (Curves 3) were recorded. Different samples from the same chloroplast preparation were used for the two

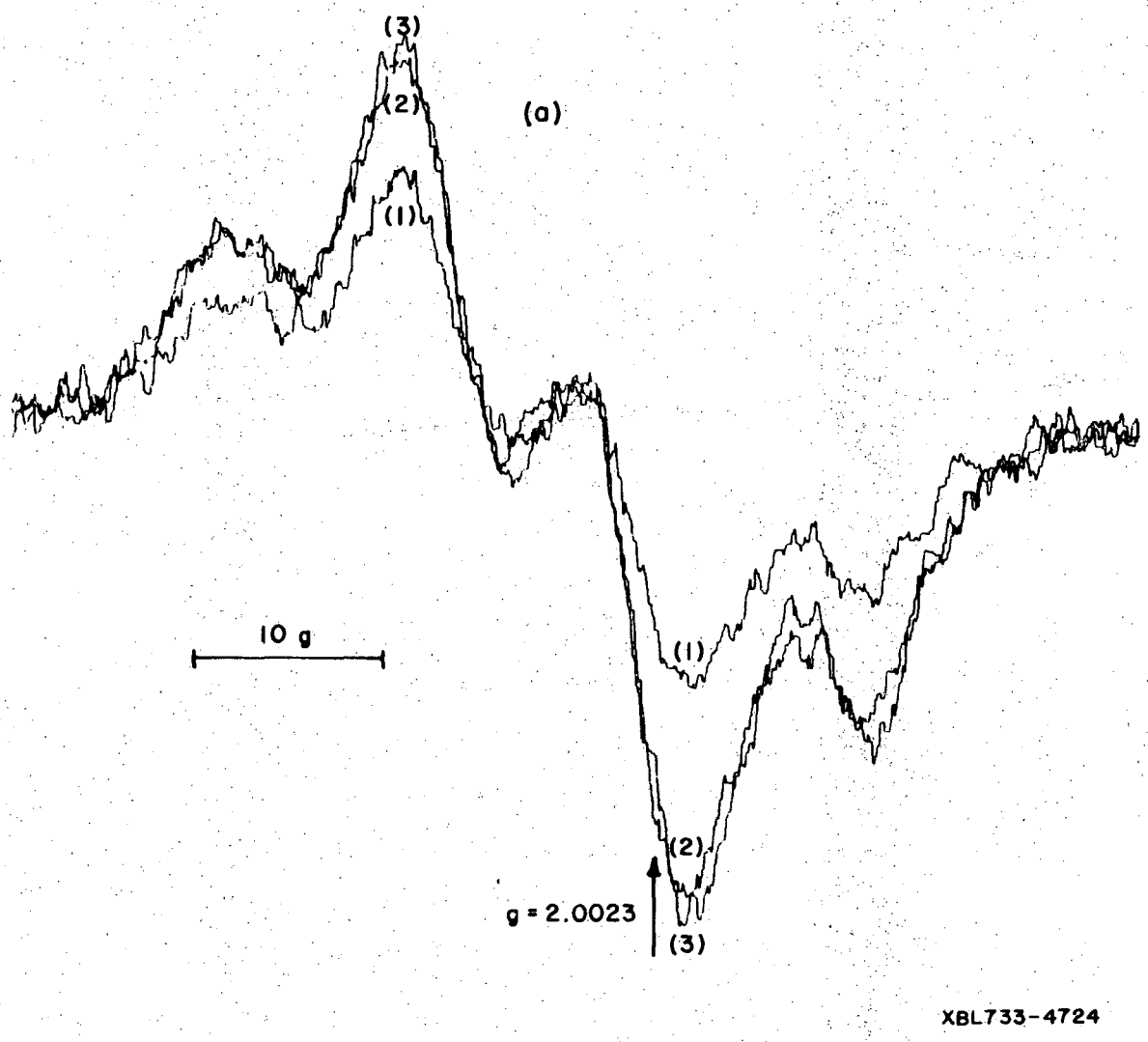

Figure 23. Response of Signal II in dark-adapted, untreated chloroplasts to 10 μsec saturating flashes: (a) two flashes separated by 10 msec, (b) four flashes separated by 10 msec. Each experiment was performed with a fresh sample of dark-adapted chloroplasts as described in the text. Instrument time constant and scan rate as described in Fig. 12. Total time between initiation of Curve 1 and completion of Curve 3 was 10 min.

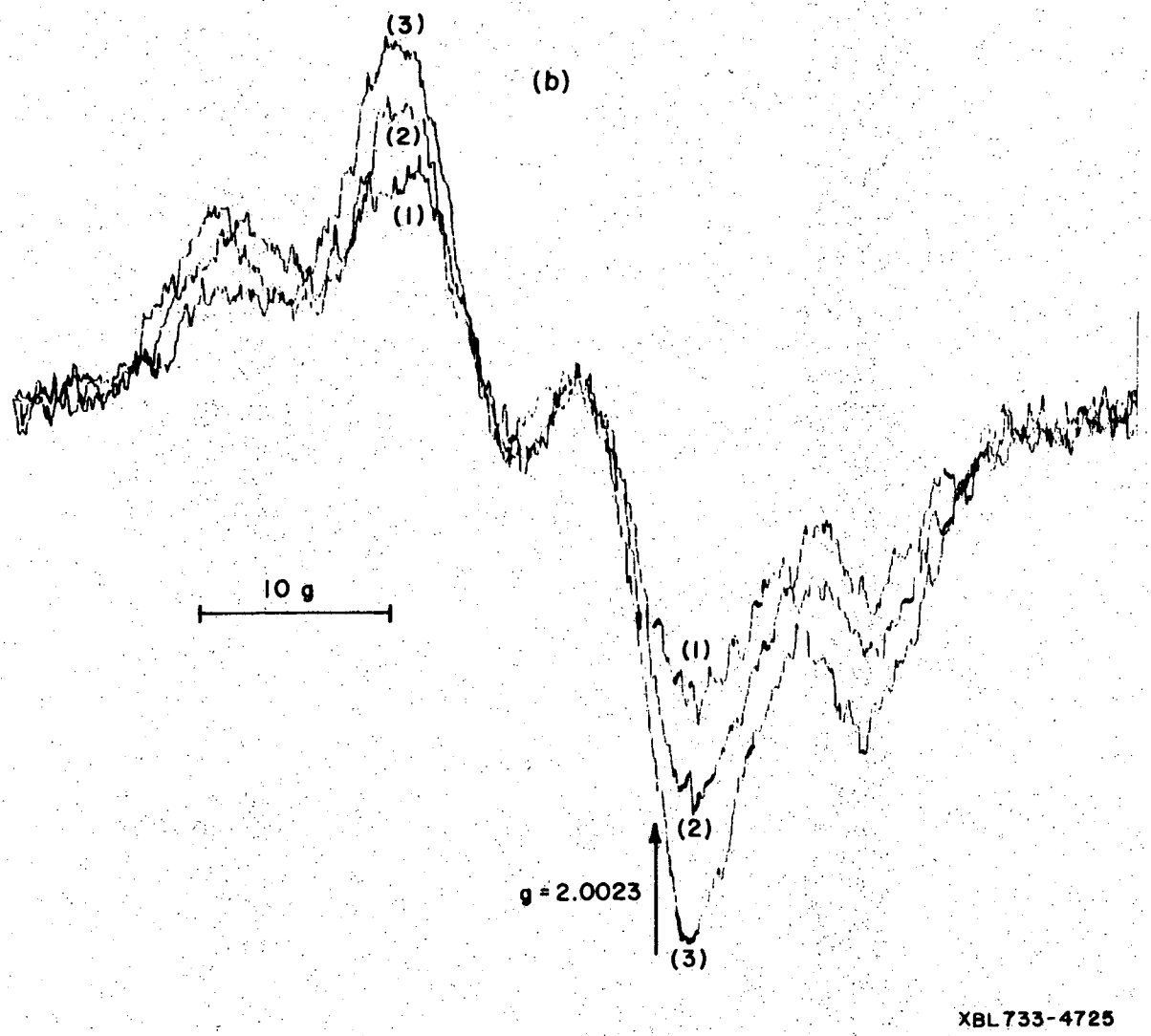

Figure 23. Continued.

experiments because of the long decay of Signal II. In each experiment the dark signal (Curve 1) was about 58% of the fully induced signal (Curve 3). However, two flashes, 10 msec apart, generated 90% of the light-induced signal whereas four flashes 10 msec apart produced only 40% of the maximum light-inducible signal. Ten msec was chosen as the dark time between flashes because four flashes spaced 10 msec apart yield maximal amounts of oxygen per flash; i.e., the intermediates in the water splitting process are fully advanced within 10 msec after a flash. Three flashes, 10 msec apart, behave in a manner similar to four flashes, whereas a single flash has effects similar to two flashes. These results are shown in Fig. 24a, in which we summarize the data from the four experiments. The results are presented in histogram form to emphasize that each experiment was performed with a different sample and that the effects we see are not oscillations; for example, two flashes followed by a 1 sec dark period and then two flashes 10 msec apart does not decrease the level of Signal II. In all experimental approaches we have explored we have found no method involving light which decreases the concentration of Signal II spins.

After four flashes the oxygen evolving system has been largely discharged and, to a first approximation, is similar, with respect to the concentration of oxidized intermediates, to the situation before the first flash. The fifth and sixth flashes yield little oxygen but serve to restore a pool of oxidized species which are discharged on the seventh and eighth flashes. Therefore Signal II should react to five flashes 10 msec apart as it did to a single flash and to six flashes as it did to two flashes. Because of limitations in

Figure 24. Response of Signal II in dark-adapted, untreated chloroplasts to various numbers of 10 μsec saturating flashes spaced (a) 10 msec or (b) 370 msec apart. Each bar corresponds to an experiment on a fresh sample of dark-adapted chloroplasts in which the sample was given the designated number of flashes followed by single flashes to complete the induction of Signal II. The ratio of the increase in Signal II stimulated by this set of flashes to the total light-induced increase in Signal II is plotted as a function of the number of flashes in each set. Response to the flashes was measured at the low field peak of Signal II with an instrument time constant of 1.0 sec.

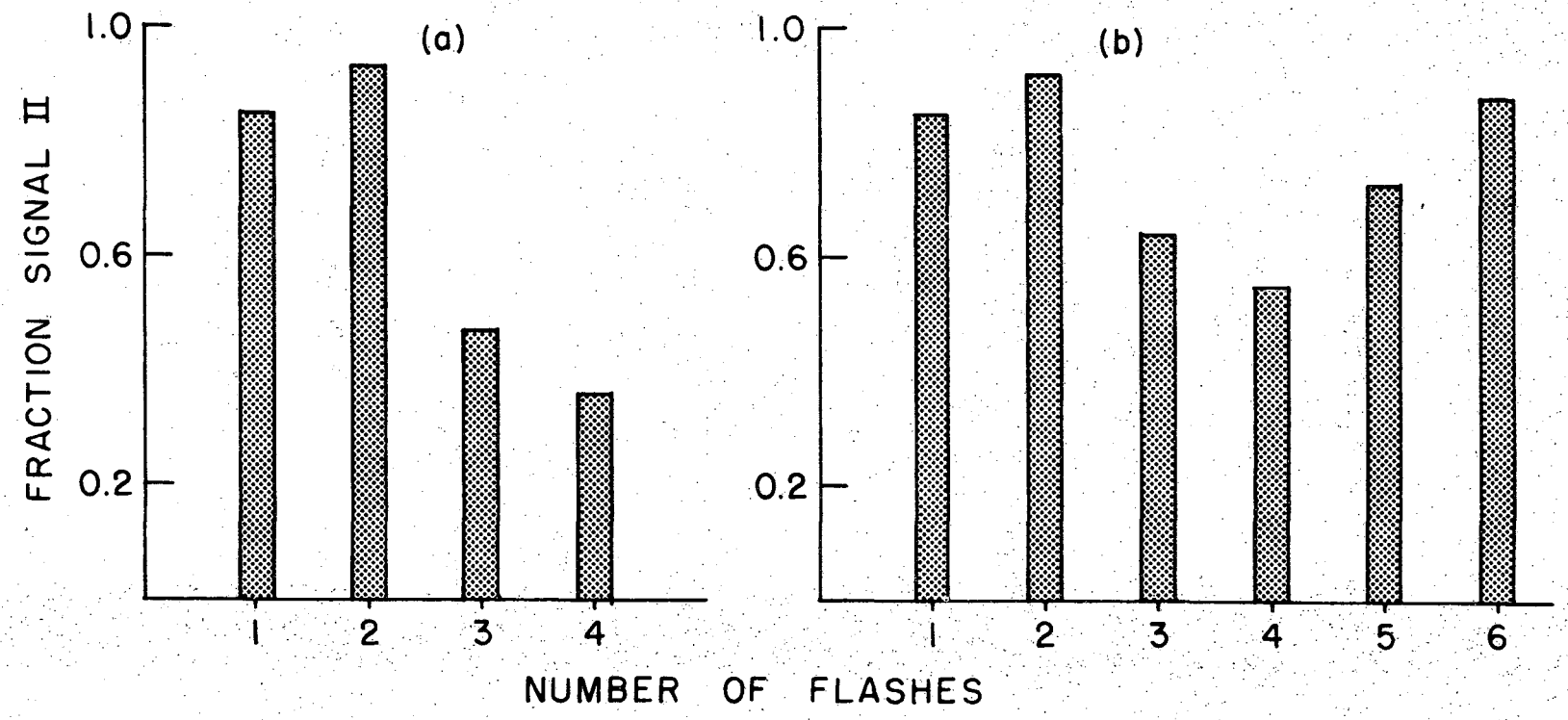

Figure 24.

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the flash apparatus, it was necessary to increase the time between flashes to 370 msec to give five or six flashes in a sequence. The results of these experiments are shown in Fig. 24b. Again, one or two flashes yield greater than 80% of the light-induced increase in Signal II. For this longer dark time between flashes, the distinction between the effects of one or two flashes and three or four flashes is somewhat less pronounced. With five flashes the fraction of Signal II formed is increased, and with six spaced 370 msec apart this increase is even more substantial, in accord with the model.

Three or four flashes spaced 100 μsec apart yield only small quantities of oxygen compared to the case in which the spacing is 10 msec (Section II). This observation has been taken as evidence that the relaxation time for concentration changes in the water splitting process is somewhat longer than 100 μsec but shorter than 10 msec. We have performed experiments of this nature, similar to those described in Fig. 4 for oxygen evolution, for Signal II formation in response to two, three and four flashes in which we varied the time between flashes from 100 μsec to 10 sec. The results of these experiments are shown in Fig. 25. Again, each point represents an experiment with a fresh sample. In order to increase signal-to-noise we performed the experiments kinetically by monitoring the signal level as shown in Fig. 17, except that the instrument time constant was increased to 1.0 sec. In these experiments the dark signal was 55 to 60% of the fully induced Signal II. Fig. 26 shows typical data; this experiment was done with four flashes spaced 3.7 msec apart followed by single flashes to complete the induction of Signal II. The fraction of

Figure 25. Response of Signal II in dark-adapted untreated chloroplasts to set of 2 (—o—o—), 3 (—x—x—), or 4 (—●—●—) 10 μsec saturating flashes in which the time dark (t_d) between flashes in the set was varied. Each point corresponds to an experiment on a fresh sample of dark-adapted chloroplasts. Normalization of the response of Signal II was carried out as described in Fig. 24.

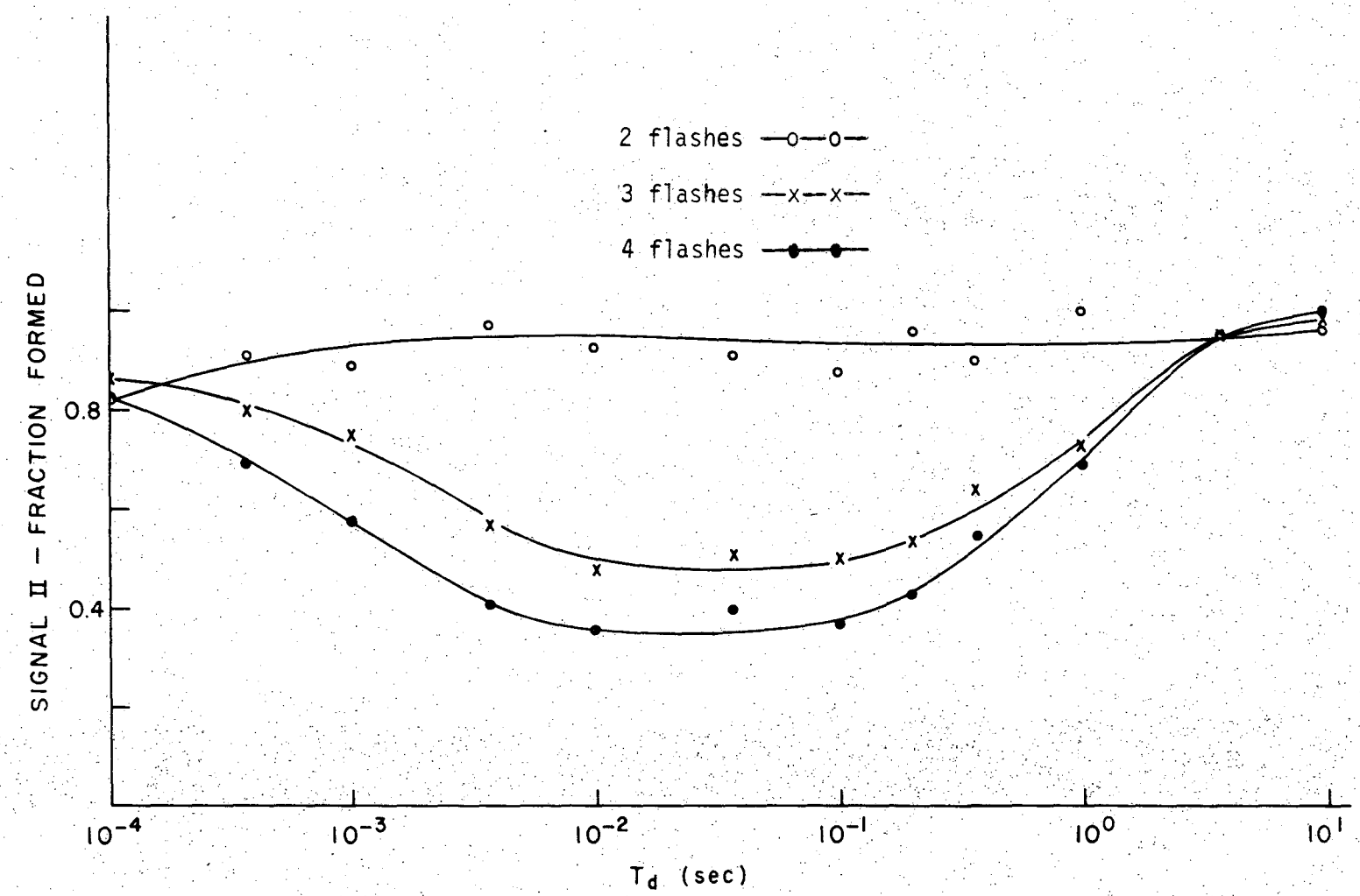

Figure 25.

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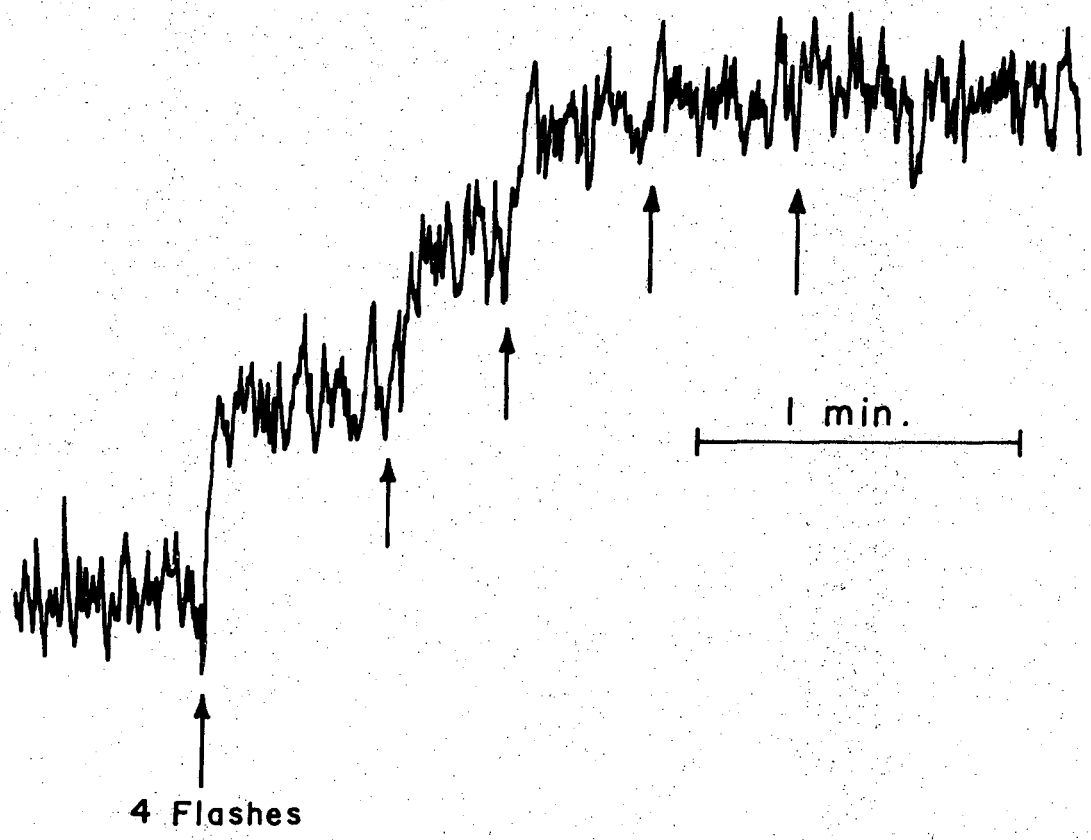

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Figure 26. Response of Signal II to 4 saturating 10 μsec flashes spaced 3.7 msec apart, followed by single flashes to complete the photoconversion. At the first arrow the four flashes were given; at the subsequent arrows only a single saturating flash was given. The instrument time constant was 1.0 sec.

Signal II formed is then calculated by dividing the extent of Signal II formation resulting from the initial set of flashes by the fully generated light-induced signal. Referring to Fig. 25, two flashes, regardless of the dark time between the two, always generate greater than 80% of the light signal. Between 100 μsec and 1 msec there is a slight increase in the effectiveness of the two flashes, which probably indicates that at the shorter time the chloroplasts are able to process only the first flash whereas at the longer time both flashes are effective in producing oxidized intermediates. The effect of three or four flashes is remarkably different. Between 100 μsec and 10 msec the fraction of Signal II generated by the flashes decreases reflecting the increasing effectiveness of the set of three or four flashes in discharging the pools of oxidized intermediates formed during the flashes. Between 10 and 100 msec is a plateau region for both three and four flashes followed by a region from 100 msec to about 4 sec in which the fraction of Signal II increases. This rising section of the curve reflects the observed rise of Signal II, which we showed (Fig. 22) to have a halftime on the order of 1 sec. As the time dark between flashes approaches this halftime, proportionately more of the Signal II precursor reacts with the intermediate(s) formed after each flash and not, as with the shorter intervals, only with the intermediates present following the final flash. At times greater than 4 sec, Signal II is fully generated after the second flash and additional flashes have no further effect. At all times less than 4 sec three flashes are slightly more effective in generating Signal II than four, indicating that four flashes more

completely discharge the pool of oxidized intermediates formed during the sequence.

(8) Flashing light studies on Signal II(19) formation

All of the experiments thus far reported in this Section were performed on unwashed sucrose chloroplasts which exhibit Signal II(16). However, in the preceding Section we showed that in washed sucrose chloroplasts, Signal II exists in an alternative state, Signal II(19), with EPR properties distinct from Signal II(16). There the hypothesis was made that the state of the Signal II precursor, leading either to Signal II(16) or Signal II(19), has little effect on the kinetics of Signal II induction. We have explored this hypothesis in more detail by studying the light induced increase in Signal II(19) in flashing light. The experiments are analogous to those performed for Signal II(16) shown in Fig. 24, and the results are shown in Table IV. One or two flashes spaced 10 msec apart are quite effective in increasing Signal II(19). Three or four flashes 10 msec apart are much less effective; however, if the spacing between four flashes is decreased to 100 μsec, more than 80% of the maximum light-induced increase occurs. These results are the same as we report above for the flash-induced increase in Signal II(16) in unwashed spinach chloroplasts. We performed analogous flashing light experiments in washed sucrose chloroplasts in which Signal II(19) has been converted to Signal II(16) by ionic strength increase and find parallel results. In both sets of experiments we observed a 1 sec halftime for the rise of Signal II following a flash, which is the same as reported above. These data strengthen

Table IV

FLASH-INDUCED INCREASE IN SIGNAL II(19)

A fresh sample of dark-adapted, washed sucrose chloroplasts was used for each experiment. The flash-induced increase in Signal II(19) was monitored at the low field peak B in Fig. 15 with an instrument time constant of 1 sec and microwave power of 5 mW. The dark-adapted sample was given the designated number of flashes with a spacing, t_d, between flashes. Following this initial set of flashes, single flashes were given to complete the induction of Signal II(19). The increase in Signal II(19) resulting from the initial set of flashes divided by the total Signal II(19) increase is tabulated in the right column.

Number of flashes	t_d	$\left[\frac{\text{Flash-induced Signal II(19) increase}}{\text{Total Signal II(19) increase}}\right]$
1	10 msec	0.77
2	10 msec	0.88
3	10 msec	0.51
4	10 msec	0.40
4	100 μsec	0.84

the hypothesis proposed in the preceding Section that both states of the Signal II precursor exhibit the same light-induced kinetic behavior.

D. Discussion

The results from the experiments described in this Section can be explained in terms of the model shown in Fig. 27. C550 represents the primary acceptor as described by Erixon and Butler (1971). P680 is the reaction center chlorophyll (Floyd et al., 1971), and S_0 through S_4 represent successively more oxidized intermediates involved in the water splitting process. These states have been described in detail by Kok et al. (1970). Briefly, S_0 and S_1 are stable states persisting in the dark. S_4 is a strong enough oxidant to oxidize water and, once formed, does so in less than 1 msec (Joliot et al., 1966). S_2 and S_3 are oxidized states formed rapidly after a flash and are stable for 10-20 sec in the dark after formation. Signal II arises from a radical which is formed via electron transfer to the intermediates, S_2 and S_3. The rate constants, k_2 or k_3, for this reaction are approximately 1 sec^{-1}, which is low compared to the rates of advance of the S states following a flash.

The evidence from our experiments supporting this model has been briefly discussed in the Results section. Thus, the model explains the greater stimulation of Signal II by red than by far red light observed both by us and by Allen et al. (1962), and the failure of DCMU to inhibit the formation of the radical. The fact that in DCMU-treated chloroplasts more than a single flash is required to saturate the signal probably reflects a competing back reaction between the reduced primary acceptor and an oxidized intermediate on the water side of PSII which is stimulated by DCMU. The stimulation of this back reaction has been postulated to account for the increased

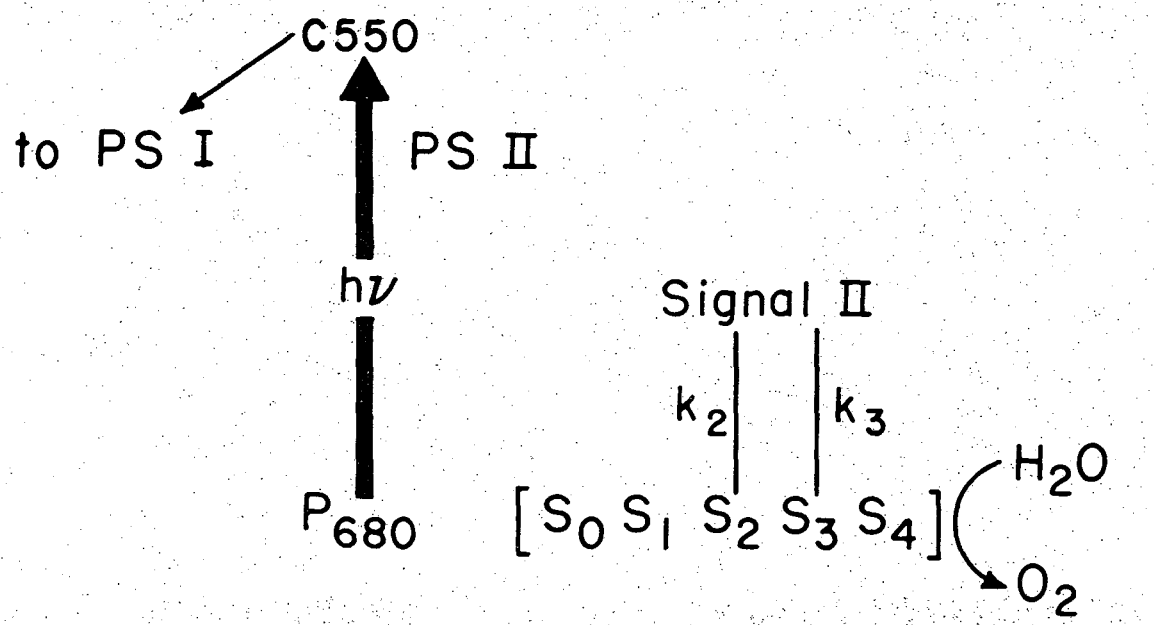

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Figure 27. Model for Signal II generation. Details described in text.

delayed fluorescence observed in chloroplasts treated with DCMU (Jursinic and Govindjee, 1972). Bennoun (1970) has carried out a detailed study of the kinetics of the back reaction in DCMU-treated chloroplasts and found that the time constant for this process is comparable to the 1 sec halftime we have observed for Signal II formation. Since S_2 and S_3 have lifetimes on the order of 10-20 sec in untreated chloroplasts, it appears that DCMU decreases the effectiveness of a single flash in generating Signal II by decreasing the lifetimes of the intermediates which give rise to the radical. However, because the reaction center is regenerated by the back reaction and is therefore able to be reexcited, subsequent flashes eventually fully induce Signal II. Bennoun (1970) has shown that hydroxylamine inhibits the back reaction in DCMU-treated chloroplasts by a rapid rereduction of the oxidized intermediates on the water side of PSII and Lozier and Butler (1973) have shown that under these conditions the light response of Signal II is completely inhibited. Similarly CCCP, which stimulates the rereduction of S_2 and S_3, has been shown to inhibit the light response of Signal II in DCMU-treated chloroplasts (Lozier and Butler, 1973). Therefore the formation of Signal II is quite sensitive to the lifetimes of oxidized intermediates, and treatments which destabilize the S states serve to reduce the efficiency of Signal II generation (Section VI).

The high quantum efficiency for Signal II formation following a single flash (Fig. 18) is a consequence of the relative stability of the S states in untreated chloroplasts. The experiments of Kok _et al._ (1970) and Joliot _et al._ (1969) have shown that the S states are

advanced with high quantum efficiency by a single flash and that following the flash their decay time is on the order of 10-20 sec. This time is long compared to the 1 sec onset time for Signal II generation, and it allows for efficient formation of the radical. A corollary to this argument is that with four flashes spaced 10 msec apart the quantum efficiency for Signal II formation is decreased since the lifetime for the S_4 state is only on the order of a millisecond. This analysis also explains the lowered quantum efficiency we observe for Signal II formation in Chlorella since Joliot et al. (1971) have shown that the lifetimes for S_2 and S_3 are about five times shorter in this alga than in spinach chloroplasts. Similarly we have found with CCCP-treated chloroplasts that the quantum efficiency for Signal II formation is decreased (Section VI), which is a consequence of the action of CCCP in decreasing the lifetime of the oxidized intermediates following a flash.

In the model proposed in Fig. 27, Signal II arises by an interaction with S_2 or S_3, in which the species giving rise to Signal II is oxidized by an S state. This interaction may be represented as

$$S_n \xrightarrow[P680]{h\nu} S_{n+1} \qquad (1)$$

$$S_{n+1} + F \xrightarrow[k_{n+1}]{} S_n + F^{+\cdot} \qquad (2)$$

where S_n is an S state with n=1 or 2, S_{n+1} is one equivalent more oxidized than S_n and F represents the species which, when oxidized to the radical $F^{+\cdot}$, gives rise to Signal II. The behavior of the state S_n in this scheme is such that at the conclusion of the process its final

oxidation state is the same as its initial state even though a photon has been absorbed by Photosystem II. This situation in which an S state is left unchanged by a flash has been termed a "miss" in the Kok et al. (1970) model for oxygen evolution in flashing light (Forbush et al., 1971). Thus, the process of Signal II formation viewed via oxygen evolution results in an increased number of misses on the first three or four flashes. This increased number of misses accounts for the lowered yield of oxygen on the third flash and increased oxygen yield on the fourth flash shown in Fig. 21. Computer programs to fit oxygen evolution curves such as those generated by the preilluminated chloroplasts in Fig. 21 usually contain a "miss parameter" to account for S states which are not advanced by a flash. Our results indicate that this parameter may be larger for the first few flashes than it is for later flashes (Ley and Babcock, unpublished results).

We postulate that the Signal II precursor, F, can be oxidized by either the state S_2 or S_3 on the basis of the results presented in Fig. 24. Following a single flash roughly 75% of the System II centers are in the state S_2 and 25% in the state S_1. Under these conditions we observe 80% of the light-induced increase in Signal II. Following two flashes 10 msec apart 75% of the centers are in the S_3 state and 25% in the state S_2. With this flash pattern we observe greater than 95% photoproduction of Signal II. Therefore the concentration of $[S_2 + S_3]$ following a flash sequence parallels the extent of the light-induced generation of Signal II.

The results of Fig. 25 present the strongest evidence in support of our proposed model. The concentration of $[S_2 + S_3]$ is always high

after two flashes regardless of the time dark between the two and correspondingly the fraction of Signal II formed under these conditions is high. After three or four flashes [S_2 + S_3] are formed to an appreciable extent only if the flashes are spaced less than about 5 msec apart (Section II). At times longer than this the oxygen system is able to process each of the flashes individually and the high concentrations of S_2 and S_3 produced by the first two flashes are discharged on the third and fourth. Similarly, the fraction of Signal II formed in response to three or four flashes is high at very short flash intervals and declines as the time between flashes is increased, until a plateau at about 0.4 in the fraction of Signal II formed is reached from 10 to 100 msec for four flashes and at about 0.5 through this time range for three flashes. This plateau region is non-zero due to the fact that not all of the S states have been completely cycled during the four flashes; the concentrations of S_2 and S_3 following the fourth flash are non-zero as indicated by the finite oxygen yields of the fifth and sixth flashes.

The similarities in the flash-induced changes exhibited by Signal II(16) and Signal II(19) shown in Fig. 24 and Table IV, and in the continuous light changes reported in Fig. 16 of the preceding Section, preclude the possibility of gross structural changes occurring in the species giving rise to Signal II in converting between the two states. Rather, they indicate that both states of F exist in similar environments and react with similar rates with the same reaction partners, the states S_2 and S_3 on the water side of System II. Only a

slight structural perturbation is indicated in the conversion between the two Signal II states.

This conclusion is supported by the inhibition studies that we report in Section VI. The data indicate that the kinetic behavior of the Signal II precursor is quite labile, since the reasonably mild treatments which we have used, particularly aging at room temperature for 1 hr, drastically alter the induction of Signal II in flashing light. Thus, if major structural changes were involved in the conversion between Signal II(16) and II(19), we might expect this to be reflected in the induction kinetics.

The kinetic behavior of Signal II identifies it as the first endogenous species besides water (or reduced primary acceptor, in a back reaction) that is able to interact directly with the oxygen evolving complex in photosynthesis at physiological temperatures. The work of Knaff and Arnon (1969), Erixon and Butler (1971a,b), and others has shown that at low temperatures Cyt b_{559} is able to donate electrons to an intermediate on the oxidizing side of PSII, probably P680, but the effect is lost upon increasing the temperature above -100°C (Butler et al., 1973). We have also shown that the Signal II precursor is able to interact with the specific intermediates S_2 and S_3, but not with S_0 and S_1. Bennoun and Joliot (1969) have shown that hydroxylamine is able to override oxygen evolution, but this most likely occurs by a direct interaction with the reaction center chlorophyll or its primary donor. Similarly, we have studied the oxidation of phenylenediamine and hydroquinone in tris-washed chloroplasts in flashing light (Section III) and have found neither

the oscillations nor the two flash induction period found in oxygen evolution, indicating that in this system these reductants interact by a mechanism similar to that exhibited by hydroxylamine.

In this study we have focused on the kinetics and location of the Signal II species. Consequently our experiments yield no new information as to the molecular identity of the radical. Kohl and coworkers (Kohl and Wood, 1969; Kohl et al., 1969) have presented evidence implicating a derivative of plastoquinone as the source for the Signal II spin, mainly on the basis of extraction, deuteration and readdition experiments with chloroplasts and model compound studies in vitro. If this assignment proves correct, the experiments reported here provide the first evidence for a known species other than chlorophyll located on the oxidizing side of Photosystem II. Our model postulates that Signal II characterizes an oxidized radical; however, in experiments where we have treated chloroplasts with hydroquinone and ascorbate we note only a slight increase in its rate of decay; treatment with an oxidant, ferricyanide, has the same effect. Recently Lozier and Butler (1973) reported that in tris-washed chloroplasts the decay of Signal II is greatly enhanced by ascorbate and we have found similar effects for ascorbate on the decay of the radical in System II particles prepared as described by Malkin (1971). Signal II appears to share with the oxygen evolving system the characteristic of being normally unavailable to exogenous redox couples, and only under fairly extreme conditions do they become accessible. Since Photosystem II generates very strong oxidants it operates much more efficiently when the access of potential reductants other than water is limited.

SECTION VI

THE EFFECT OF INHIBITORY TREATMENTS ON THE KINETICS OF SIGNAL II IN FLASHING LIGHT

A. Introduction

Lozier and Butler (1973) showed that a number of reagents, including CCCP and hydroxylamine, decrease the decay time of Signal II from hours in untreated chloroplasts to seconds at high concentrations of these compounds. The effects of these reagents on a number of other reactions involved in photosynthesis have been studied in detail. Vredenberg (1969) showed that CCCP and nigericin increase the rate of decay of variable fluorescence following illumination, while Kimimura et al. (1971) showed that the level of variable fluorescence during illumination decreased with increasing CCCP concentration. These same authors also showed that the inhibition of the Hill reaction which they observe with CCCP is caused by a decreased quantum efficiency for the light reaction and not by an inhibition of a dark step. Renger (1969, 1972a) studied the action of CCCP and related compounds on intermediates on the water side of PSII (the S states in the Kok et al. (1970) model for oxygen evolution) and found a decrease in the lifetime of these intermediates with increasing CCCP concentration. While one effect of hydroxylamine is likewise to decrease the lifetimes of these intermediates, it appears that the modes of action of hydroxylamine and CCCP in achieving this are different. Bennoun and Joliot (1969) showed that hydroxylamine reduces the S states chemically, while Renger (1972b) ruled out this possibility for CCCP on the basis of stoichiometric arguments. Instead, Renger et al. (1973) proposed a model for the action of CCCP and similar reagents involving a more efficient re-reduction of the S states by reduced components of the electron transport chain on the acceptor side of PSII. This model is

consistent not only with their results on lifetimes of oxidized equivalents in the water splitting process but also with the fluorescence and Hill reaction inhibition data.

In the preceding Section we presented a kinetic model for the light-induced generation of Signal II in which the Signal II precursor, F, is one electron oxidized to the free radical by either of the states S_2 or S_3 on the water side of PSII. Since the risetime of Signal II is about 1 sec (Fig. 22), the model would predict that any treatment which decreases the lifetimes of S_2 and S_3 to less than a second would decrease the extent of Signal II formation following a flash. In the experiments described in this Section we studied the effect on the kinetics of Signal II caused by CCCP and similar reagents which increase the decay of these oxidized intermediates. We also investigated the effect on the decay and flash-induced generation of Signal II caused by aging, heating, and similar treatments which are known to disrupt the water splitting process (Cheniae, 1970).

B. Materials and Methods

(1) Chloroplast preparation and reagents

Unwashed sucrose chloroplasts were prepared as described in Section IV. Chlorophyll concentration in all experiments was between 2 and 4 mg Chl/ml; EDTA (10^{-4} M) was added to all samples to eliminate the Mn^{+2} baseline distortion.

CCCP, valinomycin, and sodium ascorbate were obtained from Calbiochem; tris and tricine from Sigma. ANT was prepared according to methods outlined by Büchel and Shäfer (1970). Stock solutions of

CCCP, ANT, and valinomycin were prepared in 95% ethanol. The final ethanol concentration in all chloroplast samples was less than 1%. Aging and heating were carried out as described in the text.

(2) Light sources and EPR measurements

Ten μsec white light flashes and continuous white light were obtained from sources as described in the preceding Section. EPR experiments were carried out under conditions described previously or in the text.

Signal averaging was performed using a 1024 channel Enhancetron signal averager. The output of the Varian E-3 spectrometer, with a time constant as noted in the text, was fed into the averager. Appropriate time circuits synchronized the initiation of the averager sweep and the lamp discharge. Experiments involving a flowing sample suspension were carried out using an EPR flat cell connected in a closed loop via tygon tubing to a peristaltic pump. The flow rate was adjusted so that a new sample was pumped into the flat cell in the interval between scans. Total volume of chloroplasts necessary to fill the system was 7 ml; each 7 ml sample was used for 20 min and discarded.

C. Results

(1) Effects of CCCP on Signal II decay and induction

Lozier and Butler (1973) have shown that CCCP dramatically increases the rate of decay of Signal II. Fig. 28 presents the concentration curve for the effect of CCCP on both the decay time and light-induced response of Signal II. The decay of Signal II in untreated chloroplasts

in the EPR cavity at room temperature is on the order of an hour. Fig. 28a shows that with increasing concentrations of CCCP this decay time decreases to seconds. In contrast, however, the magnitude of Signal II formed in saturating light (Fig. 28b) is only slightly decreased even at high CCCP concentration. For example, at 3×10^{-4} M CCCP the magnitude of Signal II, measured at field point B in Fig. 15, is only 20% less than in untreated chloroplasts. The chlorophyll concentrations used in these EPR experiments are much higher than those used in experiments monitoring oxygen evolution or fluorescence changes. Calculating the ratio of CCCP to chlorophyll for these systems, however, we find that CCCP exhibits its effects on Signal II in the same [CCCP]/[Chl] range as observed in experiments on oxygen evolution or fluorescence changes.

We have taken advantage of the rapid decay of Signal II in CCCP-treated chloroplasts to study the flash-induced increase in radical concentration. Previously we showed that in rigorously dark-adapted chloroplasts Signal II is present at about 50% of its fully induced magnitude. Using CCCP, however, it is possible to obtain chloroplasts with no Signal II spins in the dark.

A typical experiment showing the rise of Signal II in CCCP-treated chloroplasts stimulated by 10 μsec flashes is shown in Fig. 29. The CCCP concentration is 3×10^{-5} M and the interval between successive flashes is 2 sec. The flash-induced increase in Signal II is measured with the magnetic field set at the low field maximum B in Fig. 15. Signal averaging techniques were used in this experiment; the data shown are the average of 90 scans. At this CCCP concentration the

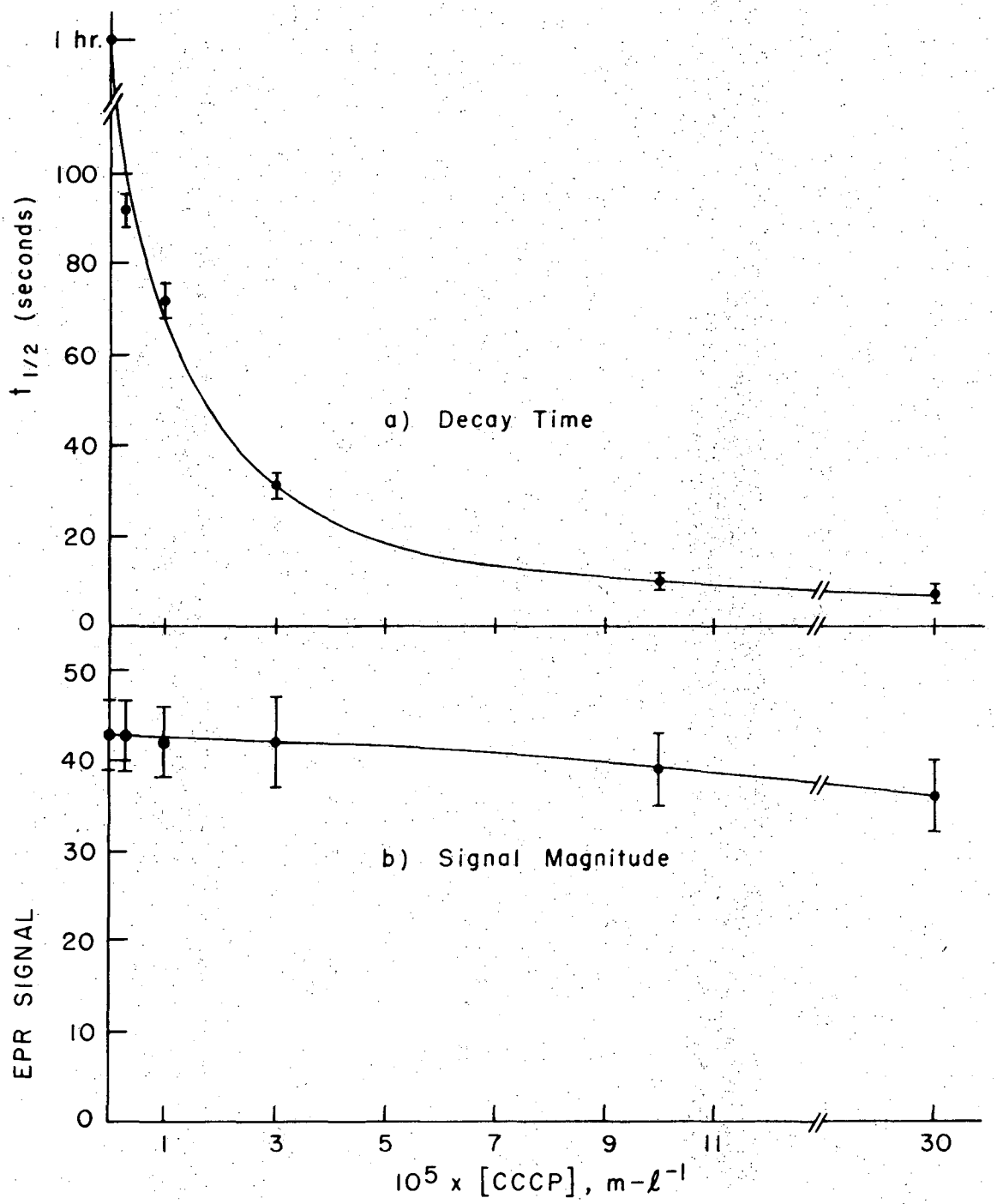

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Figure 28. EPR Signal II decay time (a) and signal magnitude (b) as a function of CCCP concentration. $t_{1/2}$ was measured as the time between cessation of illumination and the half decay for Signal II. Signal magnitude measured at peak B in Fig. 15 in saturating light. Microwave power was 20 mW, time constant was 1.0 sec.

first flash induces only about 25% of the total light-induced increase in Signal II. Increasing the intensity of this flash does not lead to the generation of more spins. This result is markedly different from that of our previous experiments (Section V) with untreated dark-adapted chloroplasts, where a single saturating flash generated about 80% of the light-induced increase in Signal II. Thus, it appears that CCCP lowers the effectiveness of a single flash in increasing the Signal II spin concentration. A second feature of this experiment is the much faster rise of Signal II following the flash compared to the 1-sec halftime in untreated chloroplasts (Fig. 22).

Fig. 30 shows a concentration study for the CCCP-induced decrease in the effectiveness of a single flash in generating Signal II. The experiments were carried out in a manner similar to those shown in Fig. 29. The increase in Signal II resulting from the first flash in a series divided by the total increase generated by 20 flashes is plotted as a function of CCCP concentration. With increasing CCCP, the first flash becomes progressively less effective, indicating that CCCP decreases the fraction of Photosystem II reaction centers capable of generating Signal II on a single flash.

Renger (1972b) has performed extensive experiments on a class of compounds including CCCP and ANT which decrease the lifetimes of the S states in the Kok et al. (1970) model for oxygen evolution. Recently Renger et al. (1973) have shown that these compounds exert their effect by increasing the reaction rate between these oxidized intermediates and an unidentified reduced component of the photosynthetic electron transport chain on the acceptor side of PSII. His model provides an

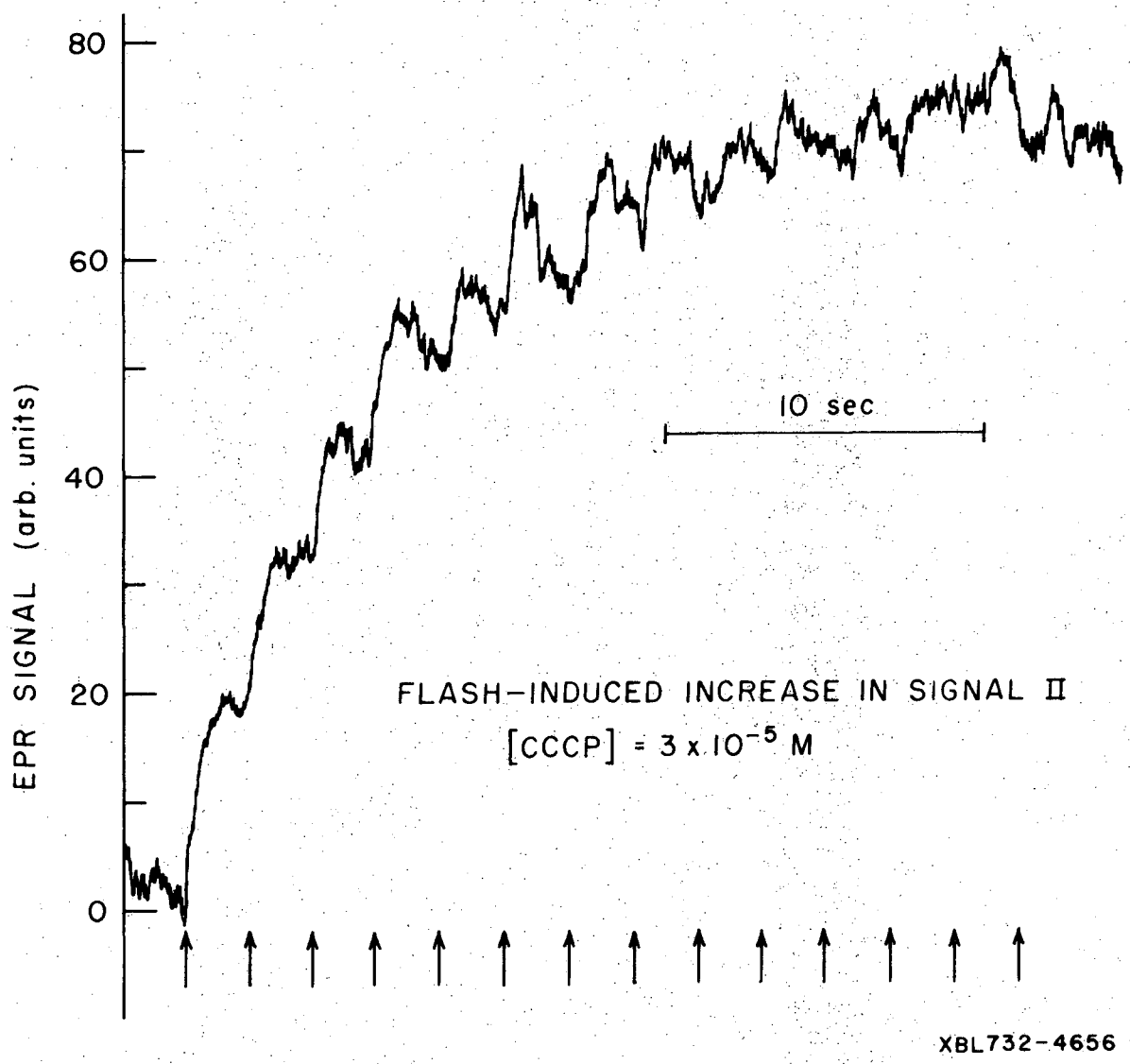

Figure 29. Response of Signal II in chloroplasts treated with 3×10^{-5} M CCCP to flashes. A single saturating flash was given at each arrow. Signal II increase measured as in Fig. 28. Microwave power was 20 mW, time constant was 0.3 sec.

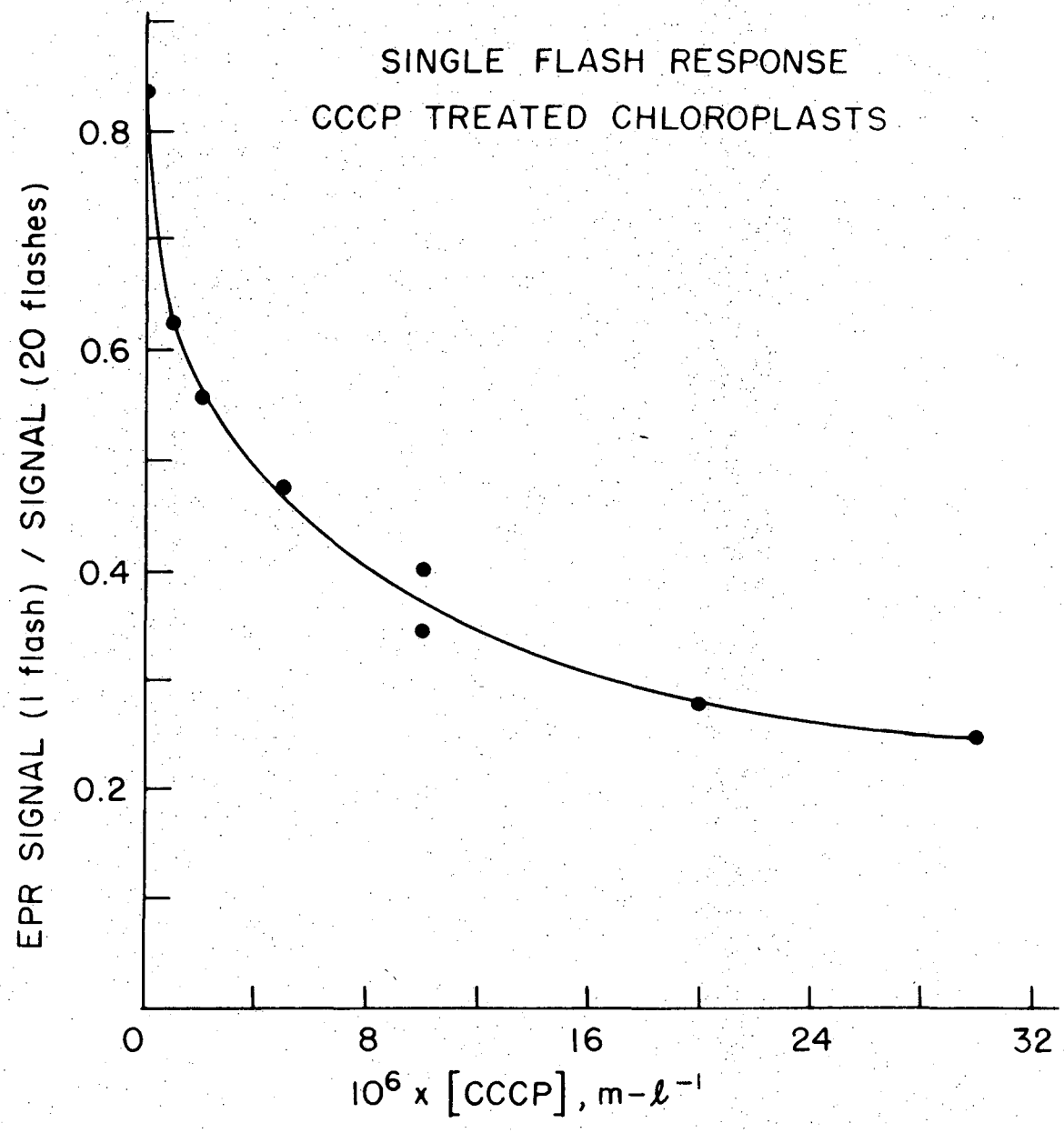

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Figure 30. Response of Signal II to flashes in chloroplasts as a function of CCCP concentration. The extent of Signal II formation stimulated by the first flash divided by the total increase in Signal II evoked by 20 flashes spaced 2 sec apart is plotted on the ordinate. Conditions as in Fig. 29.

explanation for the effect of CCCP on Signal II generation, since such a back flow of electrons would decrease the effectiveness of a single flash in any reaction dependent upon long-lived oxidized intermediates. We have proposed that Signal II arises via an oxidation-reduction reaction between the states S_2 and S_3 on the water side of PSII and F (Section V). Furthermore, we have shown that following a flash this oxidation proceeds fairly slowly; the halftime for its rise is about 1 sec. Therefore, any factor which appreciably decreases the lifetime of the oxidized intermediates S_2 and S_3 would be expected to decrease the extent of Signal II formation evoked by a single flash. We have already shown that this is the case in DCMU-treated chloroplasts (Section V).

In untreated chloroplasts the lifetimes of the states S_2 and S_3 are on the order of 10-20 sec (Joliot et al., 1971; Fig. 4, Section II). Since these lifetimes are long compared with the 1-sec halftime for the rise of Signal II, this 1-sec time constant reflects the intrinsic rate of reaction between the Signal II precursor and the states S_2 and S_3. Our model predicts, however, that the CCCP-induced decrease in the lifetimes of the S states to less than 1 sec would result in a decrease in the time constant for Signal II formation. This decrease in time constant would accompany the CCCP-induced decline in the extent of Signal II generated in response to a flash. The experiment shown in Fig. 29 implies that this is the case. There we noted that the time constant for Signal II formation had decreased significantly compared to its 1-sec halftime in untreated chloroplasts. We present further evidence to support this conclusion in Fig. 31. In this experiment we

have monitored the rise of Signal II following a single 10 μsec flash in chloroplasts treated with 1×10^{-4} M CCCP. Signal averaging techniques and the flow system were used in this experiment. A single saturating flash was given once per second; the trace in Fig. 31 is the average of 6000 scans. Clearly the extent of Signal II formation is significantly lowered compared to chloroplasts in the absence of CCCP. However, the flash-induced increase in spin concentration in Fig. 31 occurs in a time limited by the 5 msec instrument time constant, indicating that the rise of Signal II under these conditions is much more rapid than in untreated chloroplasts. This experiment also demonstrates that, with 5 msec time resoltuion, we observe no fast decaying transients in Signal II induction in CCCP-treated chloroplasts.

We have carried out experiments with a number of other compounds, including NH_4Cl, valinomycin, and ANT, to study their effects on Signal II. Neither NH_4Cl nor valinomycin influenced the decay of Signal II; the radical behaved as it does in untreated chloroplasts. The effects of ANT are similar to those reported for CCCP in that it increased the rate of decay of Signal II and decreased the effectiveness of a single flash in increasing Signal II spin concentration. Renger (1972b) has shown that ANT is similar to CCCP in that both accelerate the deactivation of intermediates on the water side of PSII, whereas NH_4Cl and valinomycin have been shown not to have such an effect.

(2) Aging effects on Signal II

The oxygen-evolving system in chloroplasts is fragile; mild heating or aging for short periods at room temperature leads to an

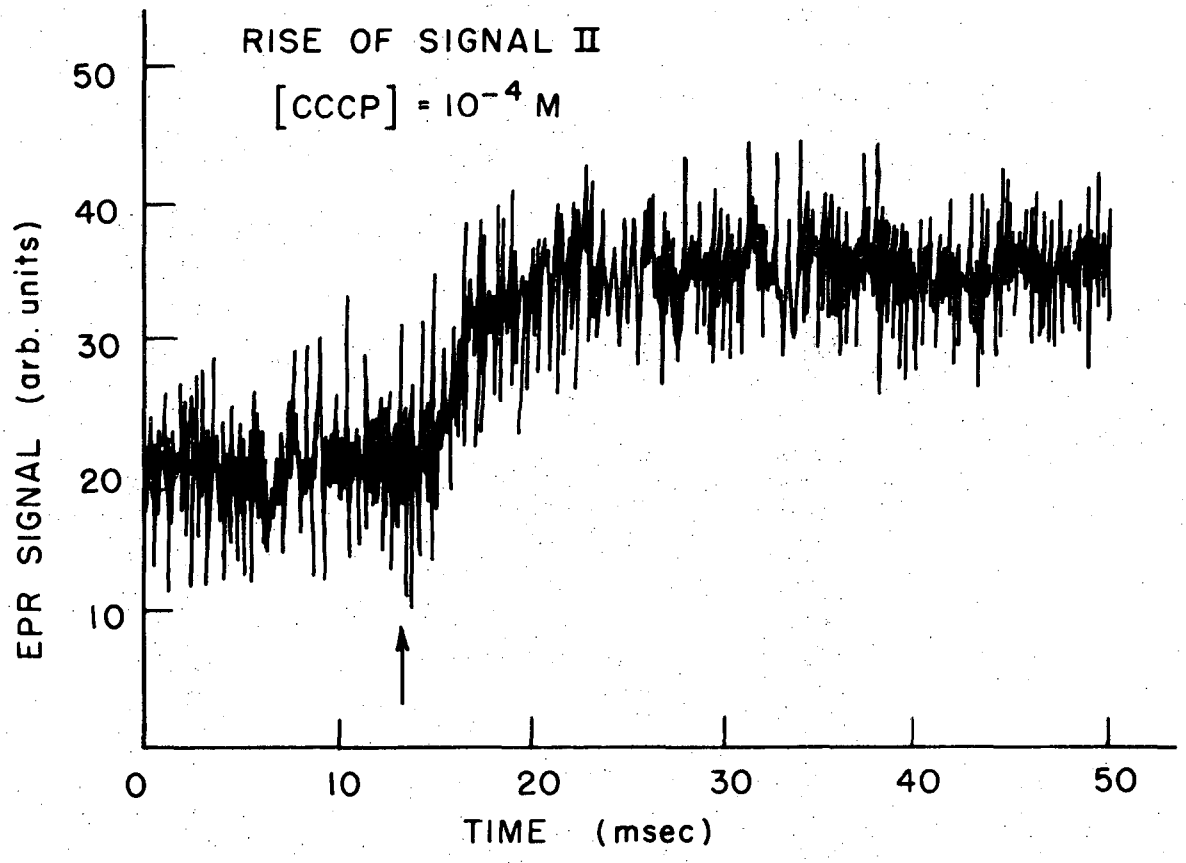

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Figure 31. Time course of the response of Signal II to a single saturating flash in chloroplasts treated with 1×10^{-4} M CCCP. The arrow designates the time at which the lamp was discharged. The flash-induced increase measured at peak B in Fig. 15 with an instrument time constant of 5 msec. The flow system was used in the experiment, the data are the average of 6000 scans. The microwave power was 50 mW.

inactivation of the system (Cheniae, 1970). We investigated the effects of these treatments on the decay of Signal II and its generation in response to flashes.

Previously (Fig. 14, Section V) we demonstrated that the decay of Signal II in vivo in spinach plants is very slow, reaching a level of about 50% of the fully generated signal only after 12 hr in complete darkness. Recent experiments by Lozier and Butler (1973) in which they monitored the decay of Signal II in isolated chloroplasts at room temperature indicated that under these circumstances the spin concentration decays much more rapidly. Their data show that Signal II decays to a level half that of the fully generated signal after only 1 hr in the dark.

We have extended this experiment by applying saturating flashes to stimulate the regeneration of Signal II following the dark decay. The results of this experiment are shown in Fig. 32. A sample of chloroplasts in the flat cell was illuminated for 1 min with continuous white light, at time zero this light was extinguished. The magnitude of Signal II, measured as the peak to trough amplitude between B and D in Fig. 15, is plotted as a function of the time dark. As shown in Fig. 32, Signal II declines to about 50% of its light-induced level after 1 hr, which agrees quite well with the data of Lozier and Butler (1973). Having allowed the signal to decay to this level, we then applied a single saturating flash and recorded the spectrum immediately following. As shown in Fig. 32, this single flash generated only 25% of the maximum light-inducible signal in these aged chloroplasts. Subsequently, sets of flashes with the number of flashes and time between

Figure 32. The effect of aging in the dark at room temperature on the decay and flash-induced response of Signal II. A sample of fresh, untreated chloroplasts was illuminated for 30 sec with broad band light and at zero time the lamp was switched off. Spectra were recorded in the dark and the magnitude of Signal II, measured as the difference between the low field peak at B and the high field trough at D in Fig. 15, is plotted as a function of dark time. When Signal II had decayed to about 50%, saturating flashes were given. Each arrow corresponds to the flash regime described in the legend, t_d denotes the dark time between flashes in a given set. The filled squares indicate the peak to trough magnitude of Signal II recorded immediately following a flash sequence. Microwave power was 50 mW, instrument time constant was 1 sec, and scan rate was 25G/min.

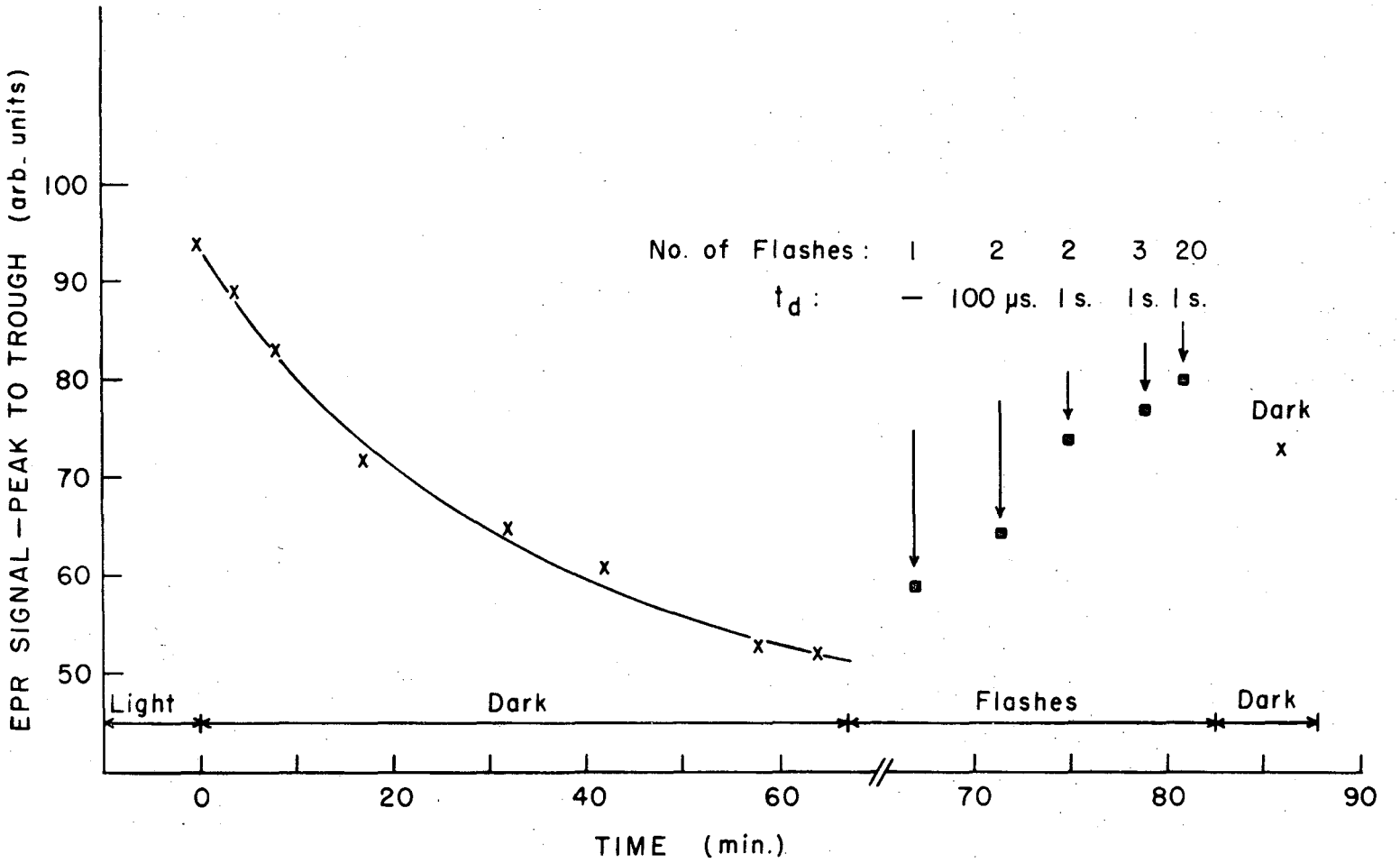

Figure 32.

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flashes indicated in the plot were given and Signal II recorded after each set. It is apparent from the figure that the behavior of Signal II has been altered by the room temperature aging. In untreated chloroplasts, freshly prepared from dark-adapted leaves, a single flash generates greater than 80% of the light-induced increase in Signal II, whereas with these aged chloroplasts only 25% is regenerated by one flash. In addition, the fully induced signal after 20 flashes 1 sec apart is about 20% less than the magnitude of Signal II at the initiation of the aging process.

We have performed similar experiments with heated (51°C for 2 min) and tris-washed chloroplasts and with PSII particles prepared as described by Malkin (1971). Under these treatments the behavior of Signal II is similar to that observed with the aged chloroplasts. The decay of Signal II is much more rapid even than with aged chloroplasts, and a single flash is less effective in generating Signal II than is the case for untreated chloroplasts. These systems all share with aged chloroplasts the characteristics of having an impaired oxygen-evolving system.

D. Discussion

The action of CCCP in chloroplasts is complex (Vredenberg, 1969; Kimimura *et al.*, 1971; Cramer *et al.*, 1971; Shavit *et al.*, 1970). It increases the proton permeability of the chloroplast membrane and, at moderate concentrations (10 μM), uncouples phosphorylation Karlish *et al.*, 1969). However, the effects of CCCP which we report are not due simply to its uncoupling action since we have shown that neither NH_4^+ nor valinomycin affect Signal II.

Renger (1972b) shown that CCCP belongs to a class of reagents which accelerate the decay of the S states. This acceleration is mediated by a reaction between these oxidized intermediates on the water side and reduced components on the acceptor side of PS II. In the model that we have proposed, Signal II is generated via a reaction involving the oxidized intermediates, specifically the two intermediate states, S_2 and S_3. Our model predicts that any treatment which decreases the lifetime of these S states to a time comparable to or less than the time constant for Signal II generation would decrease the extent of formation of Signal II following a single flash. Fig. 30 shows that this is indeed the case. With increasing CCCP concentration the effectiveness of a single flash in generating Signal II decreases. A second prediction which our model makes is that, as the lifetimes of S_2 and S_3 are decreaseed, the time constant for Signal II generation should decrease; i.e., as the lifetimes for S_2 and S_3 decrease, only those Signal II precursors that react during this shortened time will produce spins. The data of Figs. 29 and 31 show that prediction is also consistent with experiments.

Lozier and Butler (1973) reported that neither DCMU nor CCCP alone inhibited the light-induced formation of Signal II. They showed, however, that if chloroplasts were treated simultaneously with these two reagents, the light-induced Signal II response was abolished. Homann (1971) has shown that in chloroplasts treated with DCMU, CCCP strongly inhibits the reoxidation of Q^- following illumination. Renger et al. (1973) have interpreted this result as indicating that CCCP prevents the back reaction between Q^- and oxidized intermediates

on the water side of PSII by inducing reduction of the oxidized intermediates. Thus Q^-, S_2 amd S_3 remain reduced and further electron flow through PSII is inhibited. Since our model postulates an oxidation of the Signal II precursor by S_2 or S_3, we predict the result observed under these conditions, i.e., an inhibition of the light response of Signal II.

The other treatments that we have used--aging, heating, tris-washing, and preparation of System II particles--are less well characterized than the action of CCCP, but all are known to inactivate the oxygen-evolving system. These effects are of at least three types: a loss of oxygen-evolving capacity, a shift toward lower values of the midpoint potential of cytochrome b_{559} (Wada and Arnon, 1971; Erixon et al., 1972), and an alteration of the kinetics of Signal II. We have proposed that the oxygen-evolving system and the species giving rise to Signal II are located in a hydrophobic environment (Section V). Under normal circumstances of membrane integrity, aqueous reagents are quite efficiently excluded from this site. The treatments which we have described may denature this hydrophobic region allowing access by normally excluded reagents, including exogenous reductants and reduced intermediates on the acceptor side of PSII. The experiments of Lozier and Butler (1973) in which they showed that added ascorbate greatly increases the decay of Signal II in tris-washed chloroplasts support this idea. The function of the CCCP-like compounds may be to accelerate this denaturation reaction.

The inhibition experiments which we have reported here support the model, proposed both by us (Section V) and by Butler and co-workers

(Lozier and Butler, 1973; Okayama et al., 1971), in which Signal II is generated by reactions occurring on the water side of Photosystem II.

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TECHNICAL INFORMATION DIVISION
LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720