Light-Dependent Paramagnetic Centers in the Photosynthesis of Higher Plants

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I. INTRODUCTION

A. Structure of the Photosynthetic Apparatus of Green Plants

Photosynthetic systems are devices that, with high efficiency, convert the energy of the short-lived excited states of chlorophyll and some other pigments into the energy of stable chemical products. This is the basic process of bioenergetics. The highly efficient transformation of light energy over the rather large wavelength range in which natural photosynthesis takes place can be used as a model for artificial light-transformation devices that are now being developed.

The net chemical equation of green-plant photosynthesis can be written as follows:

$$CO_2 + H_2O \xrightarrow[\text{chlorophyll}]{\text{light}} (CH_2O) + O_2$$

This net process is really a long sequence of compartmented reactions, consisting of light and dark stages. In the light stage there is the chlorophyll-sensitized pho-

tooxidation of water producing molecular oxygen, O_2, the reduction of nicotinamide adenine dinucleotide phosphate (NADP), and the formation of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (P_i):

$$H_2O + NADP \xrightarrow{light} \tfrac{1}{2} O_2 + NADPH$$
$$ADP + P_i \rightarrow ATP + H_2O$$

Then the products of the light stage, NADPH and ATP, are used in a series of dark reactions of CO_2 reduction, which are not dependent on chlorophyll and light. We shall be interested only in the first, light stage, which occurs in specialized membrane structures—thylakoid membranes of chloroplasts and algae.

The minimal functional and structural unit capable of NADP photoreduction and of ATP synthesis seems to be a thylakoid, which is a more or less osmotically closed system, separated by a membrane composed of lipids, proteins, and pigments (Figure 1). The many components required for the realization of the net process mentioned above are fixed in the thylakoid membrane: pigments (about 5% of the weight of the membrane), proteins containing functional groups capable of redox transformations, and plastoquinones. In a mature chloroplast from a spinach leaf, one thylakoid disk contains about 10^5 molecules of chlorophyll and about 200 molecules of plastocyanin. Emerson and Arnold (1) showed that photosynthetic systems can use two consecutively acting light quanta, if the interval of time between them is not less than 20 ms. The O_2 yield is about 1 mole per 2400 moles of chlorophyll per flash. In bright sunlight every molecule of chlorophyll absorbs one quantum of light per 100 ms, and one quantum per 10 s at a moderate but still saturating light intensity. Thus the idea naturally arises that the excitation capture cross section is increased owing to the transfer of excitation energy from a large number of pigment molecules (not necessarily chlorophyll) to a small number of reaction centers (RC), where the primary chemical transformation takes place. Most of the pigment molecules thus form a light-harvesting matrix, and only a small fraction (less than 1%) undergo a photochemical reaction. The light-energy transfer from hundreds of pigment molecules to a reaction center is completed in 100-500 ps (2,3). This means that there is no time for the destructive oxidation processes of the chlorophyll matrix to take place, so the lifetime of a chlorophyll molecule *in vivo* is several days, in which time it absorbs 10^5-10^6 quanta. Thus, the quantum yield of the destructive processes is not more than 10^{-5}, whereas the quantum yield of primary photochemical reactions is almost unity. Not only does the transfer of energy to RCs have a high rate, but also the chemical

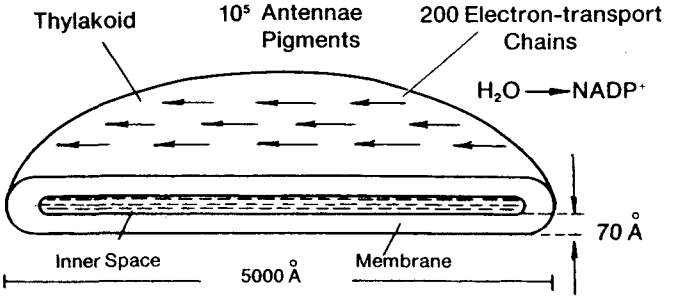


Figure 1. Diagram of a thylakoid. The membrane is built up of lipids, proteins, and pigments (chlorophylls and carotenoids).

transformations in the RCs themselves have rather short half-lives; from fluorescence measurements it has been found that these times are ca 10^{-10}s.

It has been definitely established that the transfer of excitation from antenna chlorophyll to RCs occurs in the form of singlet excitations. The most realistic model seems to be as follows: the transfer of excitation within the limits of small condensed associations of pigment molecules takes places through the exciton mechanism, and the transfer between these supramolecular aggregates (separated from each other by more than 10 Å is by Förster inductive resonance (4). The physical heterogeneity of chlorophyll and the chemical heterogeneity of the pigments results in the absorption of light by the light-harvesting matrix over a wide spectral range. The excitation transfer path is shortened owing to the predominant transfer of energy from short-wavelength to long-wavelength pigment species and finally to the RCs, which form the longest wavelength part of chlorophyll (5).

The high efficiency of the photochemical transformations in the RCs requires that the molecules taking part in this transformation form a tight complex so that the reactions among them are not limited by diffusion. The only processes that are truly photochemical are those in which chlorophyll *a* molecules take a direct part in the RCs of both photosystems. The other reactions are ordinary dark redox transformations, which take place without any light-excited states.

B. General Scheme of Electron Transport in the Photosynthesis of Higher Plants

The most important concept for understanding the mechanism of the light stage of photosynthesis is the concept of electron transport, i.e., the consecutive transfer of electrons from water, one at a time against the gradient of redox potential through a number of intermediate carriers. Two light-excited chlorophyll

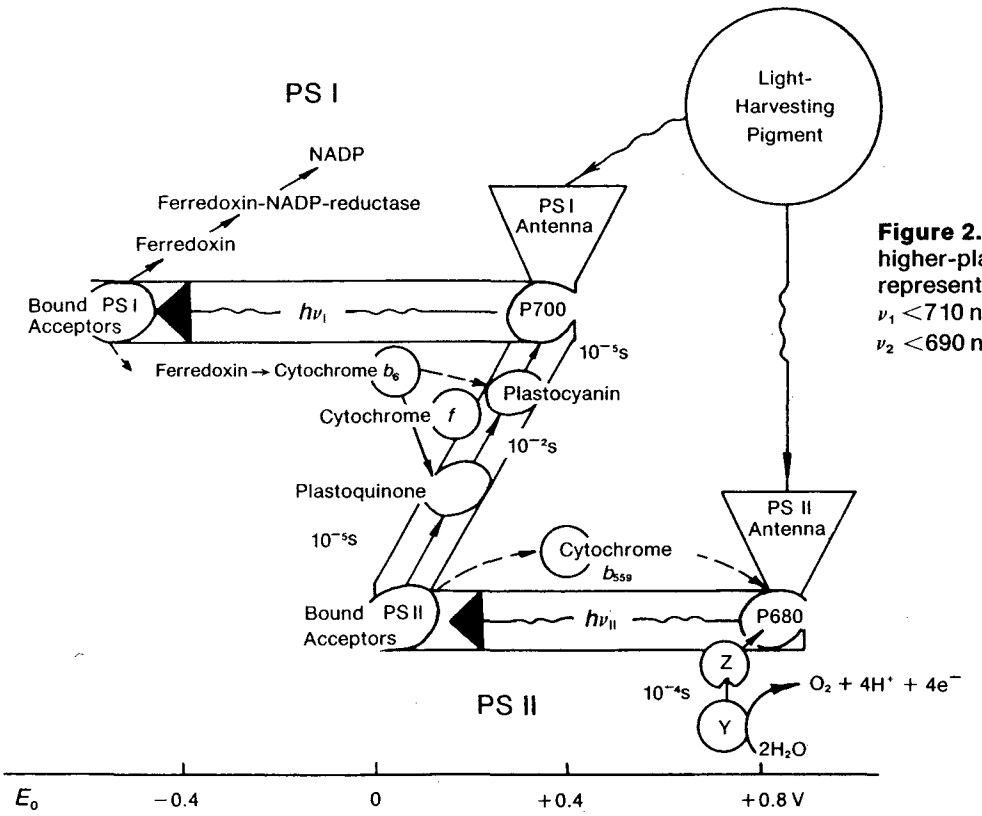


Figure 2. The Z-scheme for electron flow in higher-plant photosynthesis. The wavy arrows represent the two light reactions:
$\nu_1 < 710$ nm
$\nu_2 < 690$ nm, PS = photosystem.

molecules are involved in this electron transport. Chlorophyll in its excited state acquires redox properties very different from those exhibited in the ground state and provides the necessary energy for this unfavorable electron transfer. Chloroplast membranes can photoreduce viologen dyes (which replace the natural electron acceptor, NADP) with a standard redox potential of up to -0.6 V and oxidize water with the formation of at least 1 mole of ATP. If we remember that in order to oxidize water the electron acceptor must have a potential at least 0.1-0.2 V more positive than the potential of the H_2O/O_2 pair, i.e., about 1.0 V, we can estimate that the exciting light quantum should have an energy of eV. In view of the inevitable losses needed to stabilize the primary photochemical products, we must assume that this process requires at least two quanta of red light, each with an energy of about 1.8 eV. Independently of these energy considerations, many structural results (particularly the study of chloroplast fragments (6)) and the results of spectral and kinetic measurements (7) at least confirm the general validity of the concept of the consecutive transfer of electrons with the participation of the two photochemical systems (8).

Photosystem II includes a chlorophyll-protein complex with an action spectrum maximum in the range of 680-690 nm. It accepts electrons from water and donates them to plastoquinone. Photosystem I also contains a specific arrangement of chlorophyll and protein with an absorbance maximum at about 700 nm. It oxidizes plastohydroquinone and reduces NADP through a number of intermediate carriers. Figure 2 shows a widely accepted sequence of electron-transfer reactions. It also shows their redox potentials and the lifetimes (9) of the corresponding reactions. Not all the known facts support this popular concept (for a review see (10)). This "Z-scheme" should be regarded as the kinetically most accessible electron path in certain optimal conditions of the medium. Electron-path flexibility helps the system to adapt to the environment.

Both photochemical and some dark redox reactions occur between closely situated fixed carriers and do not depend on the diffusion of reagents either within the membrane or in the surrounding solution. The rates of some other reactions involving plastoquinone are limited by the mobility of plastoquinone in the membrane and/or by the rate at which ionic equilibria are established at the membrane interfaces. In the last few years a number of excellent review articles on the kinetics and mechanisms of photosynthetic electron transport have been published (4, 9-13).

C. The Main Types of ESR Signals Observed in Photosynthetic Systems

It is convenient to classify the problems relating to the mechanisms of photosynthesis into three groups. First,

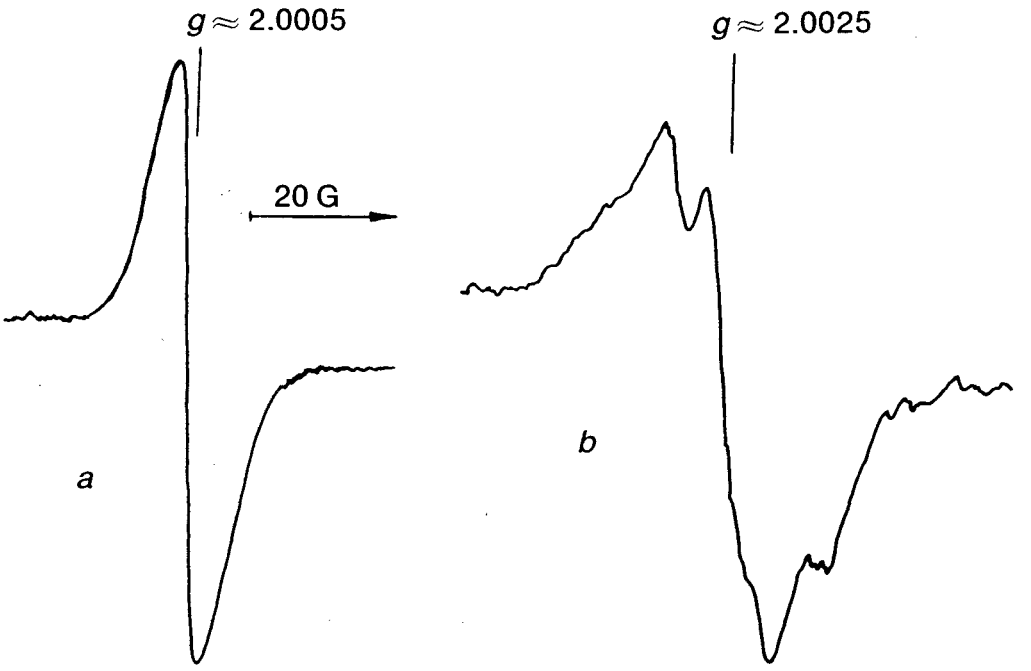


Figure 3. The two main signals from preparations containing photosystems I and II respectively. ESR signals I and II from mutant *Chlamydomonas reinhardi* cells deficient in photosystem II activity (a) and photosystem I activity (b).

there are the problems pertaining to the energy absorption and transfer of excitation from the matrix pigments to the RCs. They require purely physical research techniques and are characterized by extremely small time scales (from picoseconds to nanoseconds). Then there are the problems concerning the mechanisms of the primary chemical event, which belong to the field of biophotochemistry. The last group concerns the mechanisms of the dark electron transport among the carriers along the redox potential gradient, which is characterized by times from milliseconds to tens of milliseconds. In our review we shall limit ourselves to considering problems that can be solved by using the esr technique. It will be seen that these problems are fundamental and sufficiently wide-ranging to justify what at first sight may seem a purely technique-oriented approach.

Either all electron carriers in the photosynthetic chain are one-electron redox reagents or else they can take part in one-electron reactions, and so they can give esr signals in at least one of their redox states. However, 10-15 years ago the use of esr in photosynthetic studies was mainly limited to observations of two of the esr signals of the free-radical type (in the system *in situ*) (14): the signal from oxidized chlorophyll in the RCs of photosystem I (esr signal I) and that from the semiquinone center associated with photosystem II (15), which has not yet been identified unambiguously (esr signal II) (see Figure 3). At present it is possible to observe with certainty the signals of a much larger number of components. The possibilities of esr spectroscopy in this field have increased with the introduction of techniques in the temperature range of liquid helium. The obvious advantage of esr spectroscopy is its applicability to highly native preparations of arbitrary or variable optical density. Microwave radiation, unlike visible light in absorption and fluorescence spectroscopy, does not affect the state of the objects under study. The sensitivity of the technique is quite comparable to that of differential optical spectroscopy for strongly colored compounds (such as chlorophyll), and in many cases it is much higher (for compounds of low absorbance, such as iron-sulfur (Fe-S) centers). The study of Fe-S-centers *in situ* became possible only with the application of esr spectroscopy at liquid-helium temperatures (16).

Apart from the above-mentioned signals of chlorophyll radical cations and semiquinone radicals, it is still possible at room temperature to observe signals from Mn^{2+} and in some systems signals from flavosemiquinone radicals in certain flavoproteins. At temperatures less then 50 K, esr signals from the oxidized Cu-protein, plastocyanin, can be observed, and at still lower temperatures signals from reduced Fe-S centers bound in thylakoid membranes and from soluble Fe-S ferrodoxin protein are observable. In addition, the photosynthetic systems give a number of unidentified esr signals with both variable *g*-factors and line widths, which presumably indicate the presence of cluster structures formed by ferromagnetically coupled particles. Most of the signals mentioned above depend on light and thus appear to be directly connected with the functioning of the photosynthetic electron-transport chain.

D. The Main Problems Solvable by the ESR Technique

The application of esr in photosynthetic studies is aimed at solving problems of three types:

1. ESR is used as an analytical spectral technique that allows the identification of the separate components of a system *in situ*. A comparison is made with the esr spectra of the corresponding isolated components and/or compounds related to them.
2. ESR is used as a technique in structural studies, because the form of the esr spectrum, its symmetry, relaxation parameters, and so on can give valuable information about the structure of the paramagnetic centers, the type of environment, the ligand composition, and the nature of the interaction with the lattice.
3. The esr technique is used to determine the mechanisms and kinetics of electron transport in different parts of the electron-transport chain by observing the time dependences of the intensity of esr signals from certain components *in situ*, induced by light or other means.

We will consider below all these applications of esr in a number of cases. Studies of the esr spectra of exogenous paramagnetic additives (spin labels and probes) should also be mentioned as an approach to the structure and function of photosynthetic membranes. However, this aspect of esr application is not within the scope of this review, which is mainly concerned with the intrinsic paramagnetic centers of photosynthetic systems.

We will limit ourselves to a consideration of the photosynthesis of O_2-evolving species of higher plants and algae. Results from bacterial photosynthesis will be used only when it appears that the relevant processes of bacterial photosynthesis and photosynthesis in higher plants are of the same type and when there are no adequate experimental data available for higher-plant preparations.

The material of the review is systematized according to the actual sequence of events initiated on photon absorption by the reaction center in each of the two photosystems, i.e., from the charge separation to the formation of the final products, which are then used in CO_2 fixation.

II. PARAMAGNETIC CENTERS AT EARLY STAGES OF LIGHT-INDUCED CHARGE SEPARATION IN PHOTOSYSTEM I

A. Chlorophyll Functions in Photosynthesis

Organisms containing chlorophyll are the only ones capable of photosynthetic metabolism. This pigment has at least three functions: light absorption, excitation transfer to RCs, and photochemical transformation itself. The first function can also be performed by some auxiliary pigments such as carotenoids or phycobilins, but the other two can only be performed by chlorophyll. Light excitation may, in principle, result in three types of chlorophyll paramagnetic states: triplet chlorophyll, chlorophyll radical cation, and chlorophyll radical anion. There is no evidence for the formation of triplet chlorophyll or delocalized charge carriers (electrons or positive holes) in the light-harvesting pigment matrix. There are also no indications of triplet chlorophyll formation in the RCs of photochemical systems in normal photosynthesis, though some authors consider the possibility of primary photochemical product formation through the triplet chlorophyll in the RCs (17).

Neither steady-state nor short-lived signals of triplet chlorophyll have so far been observed in green-plant preparations. However, these signals appear in bacterial photosynthesis (18) if normal electron transport is interrupted in some way. We shall return later to the mechanism that gives rise to these signals.

In 1947, Krasnovsky (19, 20) showed that chlorophyll in solution is capable of undergoing reversible redox transformations in the presence of suitable electron donors and acceptors. It cannot be said *a priori* which process, the oxidation or reduction of singlet excited chlorophyll, is the primary photochemical event of photosynthesis *in situ*. Optical data show only photobleaching of the absorption maximum of ground-state chlorophyll (21), but it has been shown recently that at the same time a wide band in the far-red spectral range appears, which corresponds to a product of chlorophyll oxidation (22). Both possible reactions, oxidation and reduction, must lead to the formation of paramagnetic free-radical species: singly oxidized or singly reduced chlorophyll.

B. The Primary Electron Donor in Photosystem I: Pigment P700

The esr signal that appears in green plants as a result of the primary photochemical reaction was first observed by Commoner et al in 1956 (23). This singlet signal has a Gaussian shape of 7.5 G line width with $g \simeq 2.0025$, but does not reveal any hyperfine structure and hence gives no information about the nature of the corresponding paramagnetic center. The origin of the signal and the functional meaning of the corresponding center *in situ* were established mainly by a comparison with optical spectroscopic data. Kok (21) observed light-induced photobleaching *in vivo* with a maximum at 702-705 nm. This was interpreted as a result of the oxidation of a long-wave form of chlorophyll *a* in the RC.

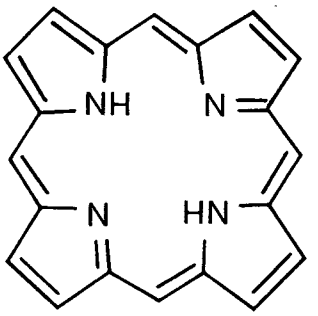
This center was called pigment P700. Later it became clear that this effect was caused by the primary reaction in the centers of photosystem I. That the process is one of oxidation follows from the similar absorption changes and esr signals, that are obtained by dark oxidation using different oxidants. Further experiments (24, 25) clearly showed that the esr signal I ($P700^+$) at room temperature coincides with optical photobleaching in the range 702-705 nm in its dependence on the redox potential of the medium. The kinetics of the optical effect and the esr signal in dark-light-dark transitions also coincide.

1. The Nature of the Light-Induced Free-Radical Center

Information about the structure of the free-radical center *in vivo* has been obtained by comparing the transformation products of the photochemical and dark transformations of chlorophyll *in vitro,* from data on the effect of isotopic H-D substitution *in vivo* and *in vitro,* and also from electron nuclear double resonance (ENDOR) data (26-30). It is usually assumed, by analogy with *in vitro* systems, that the oxidized pigment in the RCs of photosystem I is a radical cation. In fact it is only known that this oxidation product is in a doublet state (the esr triplet chlorophyll spectrum is completely different). Thus, the + sign in $P700^+$ indicates more the state of oxidation than the presence of a charge (31). However, direct electrophoretic measurements for chlorophyll solutions *in vitro* have shown that the species formed by oxidation are positively charged (28).

The esr signal that appears when photosynthetic systems are illuminated is, in all parameters except line width, close to the signal arising due to light-induced or dark chemical oxidation of chlorophyll *a* in solution. (The structure of chlorophyll *a* is shown in Figure 4.) Chlorophyll is practically never encountered in its pure form, except in some mass-spectral experiments (32). In all cases chlorophyll *in vivo* and *in vitro* produces a species with one or two ligands, which may be either a solvent molecule or a functional group of another chlorophyll molecule. The tendency of chlorophyll to solvation and aggregation is due to the unsaturated coordination of the Mg atom bound to the four pyrrole N atoms of the macrocycle (33). The Mg atom in chlorophyll is a strong electrophilic agent, capable of coordinating different molecules that contain electron-donor atoms of oxygen or sulfur with lone electron pairs. In weakly nucleophilic solvents (such as acetone and diethyl ether) chlorophyll binds one molecule of the solvent, thus forming a complex Chl *a* · L_1 (L denotes the ligand). In strongly nucleophilic solvents of the pyridine type, complexes are formed with saturated ligand shells, Chl *a* · L_2. In solution, monomeric chlorophyll complexes are strongly fluorescent and have an absorption band near 665 nm.

The amperometric technique shows that chlorophyll and the related porphyrins and metalloporphyrins, i.e., species containing macrocycles of the following type



in solution can undergo two consecutive one-electron oxidations (28, 34). Among the synthetic analogs the most completely studied reactions are those of metaloctaethylporphyrins (MeOEP) and metaltetraphenylporphyrins (MeTPP). The oxidation of Mg and Zn porphyrins is achieved either electrochemically, chemically with iodine or bromine in certain solvents, or photochemically (35-39). In the *in vitro* oxidation of chlorophyll *a,* a singlet esr signal of Gaussian shape of 9 G line width is observed, which makes it impossible to obtain the spin-density distribution in the oxidation product directly. The narrowing of the line upon deuteration indicates that at least partially, the line width is determined by unresolved proton hyperfine structure (HFS). However for singly oxidized ZnTPP, MgTPP, and MgOEP, esr signals with partially or completely resolved HFS are obtained (Figure 5). They clearly indicate that in each case the radical is formed by the removal of an

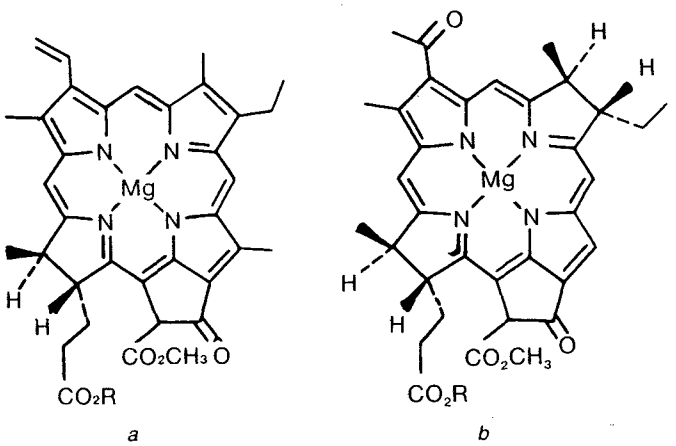


Figure 4. Structure of the chlorophylls *a* and *b.* R is phytyl:

$-CH_2-CH=C(CH_3)-(CH_2)_3-CH(CH_3)-(CH_2)_3-CH(CH_3)-(CH_2)_3-CH(CH_3)-CH_3$

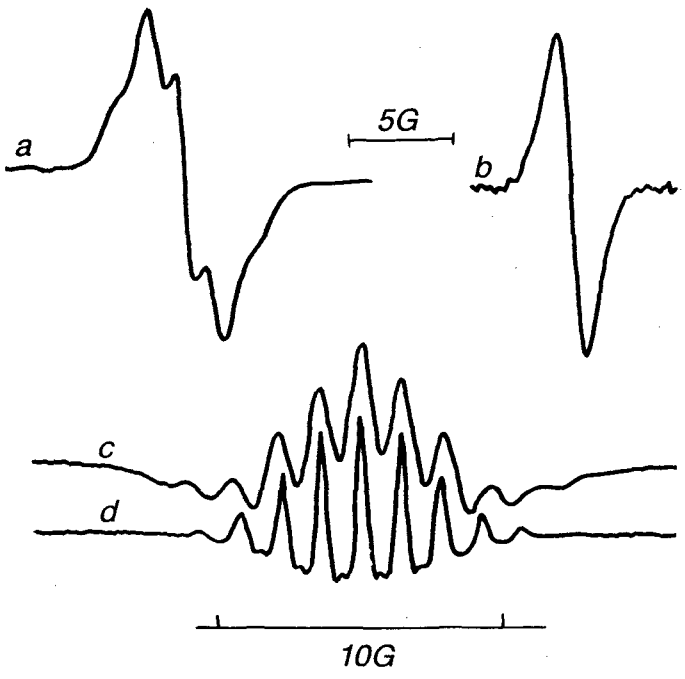


Figure 5. First derivative esr spectra of (a) $MgOEP^{+\cdot}$ ClO_4^- and (b) $(MgOEP\text{-}meso\text{-}d_4)^{+\cdot}$ ClO_4^- in methanol at $-50°C$. Second derivative esr spectra of (c) $MgTPP^{+\cdot}$ ClO_4^- and (d) $(MgTPP\text{-}d_{20})^{+\cdot}$ ClO_4^- (perdeuterated phenyl groups) in chloroform. From ref. (28) with permission.

electron from the porphyrin molecular orbital. However, esr spectra of the cation radical of MgOEP and MgTPP differ greatly in their HFS patterns. For MgTPP, the HFS from the four equivalent N atoms and from phenyl protons was observed, whereas for MgOEP a signal consisting of five hyperfine lines from four equivalent *meso* protons is observed, while the splitting on the nitrogen nuclei is absent. This assignment agrees with the data obtained from H-D substitution. From these results it follows that the radicals have π-electron structure and that two types of distribution of spin density in radical cations of metalloporphyrins are possible.

The optical spectra of these two oxidized porphyrins are quite different (28). The molecular orbital (MO) calculations show that for oxidized chlorins two almost degenerate states separated by an energy gap corresponding to 2000 cm^{-1} are possible (40). The two calculated spin densities on structurally distinct atoms in metalloporphyrin radical cations agree with the experimentally obtained hyperfine constants from esr spectra of $MgOEP^+$ and $MgTPP^+$. A comparison of the data for metalloporphyrins producing esr signals with resolved HFS with the data for oxidized chlorophyll *in vitro* producing an unsplit singlet signal, has shown that the electronic structures of all these oxidized forms are very similar. All these compounds behave in a similar way under electrochemical oxidation and have similar redox potentials. Metal coordination has a rather weak effect on the chemical, electrochemical, and spectral properties of the compounds.

The esr spectra obtained for synthetic metalloporphyrins made it possible to conclude that one-electron oxidation leads to the formation of π-radical cations. It follows that in the case of chlorophylls *a* and *b* π-radical cations are also formed. This conclusion is confirmed by ENDOR data (30, 40, 41). Figure 6 shows the ENDOR spectrum of radical cations of chlorophyll *a* at 108 K. Hyperfine interaction constants determined by this technique are in agreement with the prediction that the radical cation of chlorophyll *a* is in the state $^2A_2$ (symmetry group C_{2v}), as are $ZnTPP^+$ and $MgTPP^+$. The hyperfine splitting on β-protons in radical cations of chlorophyll *a* decreases as the temperature increases. This also happens in the case of $ZnTPC^+$ (40). Thus, the distribution of spin density depends on the external conditions (temperature and possibly solvent). It is therefore possible that a radical cation can exist as a mixture of electron tautomers whose equilibrium is determined by the parameters of the medium.

2. Metalloporphyrin Radical Anions

As will be seen below, apart from radical cations of oxidized chlorophyll, radical anions of reduced chlor-

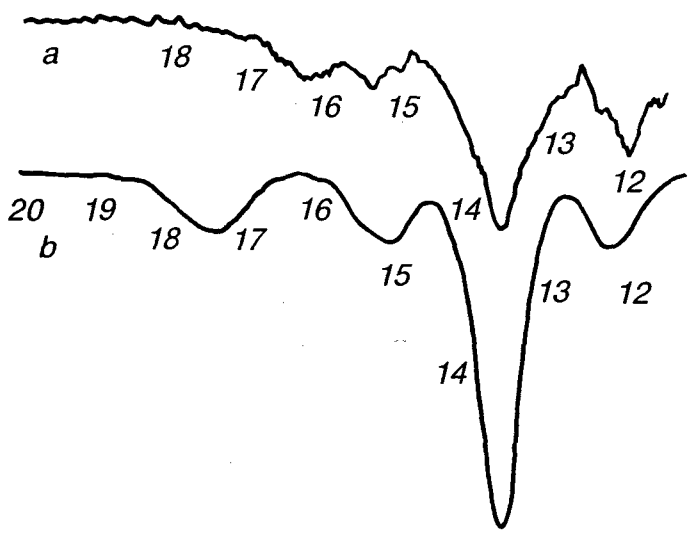


Figure 6. The ENDOR spectra of the blue-green alga *Synechococcus lividus* oxidized by ferricyanide at 108 K (A) is compared with that of *in vitro* chlorophyll a^+ in $CDCl_3$-CD_3OD (4:1) oxidized by I_2 (B). Numerical values of the peaks are in megahertz (2.8 MHz ≡ 1 G). Only the high-frequency half of each spectrum is shown. The hyperfine coupling constant is twice the difference between the frequencies of the peak and the center of the spectrum. From ref. (30) with permission.

ophyll *a* and pheophytin *a* may be formed in photosynthetic systems. They also have been obtained electrochemically as well as photolytically and radiolytically *in vitro* (42-44). The properties of these reduced forms indicate that these particles are also π-radicals. The esr spectrum of Chl *a* radical anions at low temperatures is a singlet with a *g*-factor that is practically the same as the *g*-factor of radical cations. Its line width is about 13 G, i.e., much greater than the line width of a singlet line from radical cations (9 G). The unpaired electron density distribution in the porphyrin ring is confirmed by a broadening of the signal when the isotopic substitution $^{12}C \rightarrow {}^{13}C$ is made. Fujita et al (44) have recently shown that radical anions of pheophytin *a* and of Chl *a* can be obtained by electrolysis in highly purified anhydrous solvents under strictly anaerobic conditions and are stable for several months. The reaction is reversible. A comparison of esr and ENDOR spectra of these species in dimethylformamide and dimethylformamide-d_7 has shown that there is no proton exchange between the solvent and radical cations even on prolonged standing. Structureless esr signals of Chl $a^{-\cdot}$ and Pheo $a^{-\cdot}$ become highly saturated as the microwave power increases and the saturation effect increases with decreasing temperature, which makes observation of these signals *in situ* more difficult.

MO calculations (44-47) for metalloporphyrin and metalloporphyrin radical anions have led to predictions on the distribution of spin density, in accordance with ENDOR spectra of radical anions (40, 44) of natural isotopic composition as well as of deuterated derivatives. The calculations predict a considerable splitting of the signal on N nuclei of pyrrole rings II and IV and a remarkable spin density on the Mg atom. Hyperfine interaction with Mg can in principle be demonstrated experimentally by observing the esr or ENDOR spectra of oxidized pigments containing ^{25}Mg ($I = 5/2$). This would make it possible to differentiate the signals of Chl $a^{-\cdot}$ and Pheo $a^{-\cdot}$ *in vivo* and *in vitro*. This approach has already been used for radical cations of bacterial chlorophyll and for some synthetic metalloporphyrin radical cations for which the spin density on the central metal atom was much smaller than that assumed for radical anions (48).

3. Chlorophyll Dimers in Reaction Centers

The esr signal I of oxidized $P700^+$ centers in many respects coincides with the esr signal of radical cations of chlorophyll *a in vitro,* but differs from it in one significant parameter, the line width, which is 9-10 G for monomeric chlorophyll *in vitro* and 7.5 G *in vivo* (23, 49). H-D substitution narrows the lines (50), but the ratio of the line widths *in vivo* and *in vitro* remains the same. The difference in esr line widths corresponds to the shift of the absorption band from 665 nm for monomeric chlorophyll to 704 nm for chlorophyll in the RCs of photosystem I (21). These differences have proved to be quite useful for determining the nature of the photoreaction centers of photosystem I. Fifty years ago the long-wave shift in the absorption spectra was thought to be due to the influence of the environment of the chlorophyll *in vivo* (51). Later it was regarded as a confirmation of the chlorophyll-aggregated state in the photosynthetic mechanism (5, 52). The narrowing of the esr line of oxidized chlorophyll *in situ* as compared to monomeric chlorophyll in solution was explained by the unpaired electron density, which is uniformly distributed in two chlorophyll molecules forming a dimeric structure. Much research has been devoted to the theoretical analysis of possible dimeric and oligomeric structures and to obtaining and investigating the properties of model associates that simulate the properties of the pigment in the reaction center of photosystem I (30). These models are constructed on the basis of the above-mentioned unsaturated coordination of the Mg atom in the chlorophyll molecule and the presence of electron-donor ester and keto groups in chlorophyll.

Chlorophyll oligomers can be divided into two groups. In oligomers of $(\text{Chl } a)_n$ composition ($n = 2$-20), the side groups of other chlorophyll molecules are directly bound to the Mg atom. The other group of model aggregates is made up of oligomers in which the bond between any two chlorophyll molecules is formed by a bifunctional ligand molecule such as water. In the presence of equimolar quantities of Chl *a* and pyrazine in CCl_4, for example, these associations can reach colloidal dimensions (30, 33). The addition of water to solutions of chlorophyll *a* in aliphatic hydrocarbons leads to a change of color (the absorption maximum shifts from 670-680 nm to 740 nm) and also to some changes in the infrared spectrum, which indicates C=O···Mg bond fission. If Chl *a* is replaced by ethylchlorophyllide, in which the phytyl chain is substituted for ethyl, the condensed hydrate can be obtained in a crystal form. Its structure is determined by X-ray diffraction (53, 54). This structure is formed by parallel layers of the same geometry as the monolayer chlorophyll at the solution-air interface. These chlorophyll films also absorb at 735 nm (55). Under photochemical oxidation of any of these systems, a product with singlet esr signals of Gaussian form of different line widths is obtained (56). Light-induced signals seemed to be observed only for chlorophyll-water adducts and were unstable in the dark. The 740 nm adduct esr signal is unusually narrow (of about 1 G line width). This is very unusual for such a large molecule as chlorophyll, with many structurally different H and N atoms. The narrowing of the signal is

due to the delocalization of the spin density among many molecules in the adduct. Since the electron delocalization in this case is equivalent to spin delocalization, the average spin density at each magnetic nucleus (under conditions when the delocalization frequency is higher than the hyperfine interaction frequency) decreases as the number of structurally identical nuclei increases. For an association of n identical molecules the line width H_n of the oligomer is related to the line width H_m of the monomer in the following way: $H_n = H_m / \sqrt{n}$ (57). For Chl $a^{+\cdot}$ the line width is 10 G. If the adduct contains, for example, 100 chlorophyll molecules, the line width of the corresponding radical cation must be about 1 G. The P740 associations contain up to 200 molecules, and the esr line width for the largest associates decreases to 0.5 G.

It is clear that the dimensions of an aggregate are not the only factor controlling the line narrowing. It is also necessary that the structure of an aggregate (the mutual orientation of the monomeric units) should allow for the effective delocalization of the spin density. In the case of dehydrated Chl a_n aggregates, chemical oxidation leads to the appearance of esr signals of 10 G line width, which means that in the absence of water the aggregation due to C=O···Mg bonds does not allow spin-density delocalization. The stoichiometry of chlorophyll-water bonding in a hydrophobic medium seems to be different. According to Fong et al (58), when the chlorophyll hydrate is illuminated in a mixture of pentane and cyclohexane at 10°C in strictly anaerobic conditions, a short-lived slightly asymmetric esr signal of about 7 G line width appears at $g \simeq 2.003$. At -140°C the signal becomes irreversible in darkness, its asymmetry increases, and its line width decreases.

After a long exposure with an excess of water, a polymer (Chl $a \cdot 2H_2O)_n$ appears with an absorption maximum at 740 nm. This polymer produces a short-lived singlet esr signal of 1.3 G line width. Fong et al (58) claim that the signal, reversible in darkness, belongs to dimeric chlorophyll dihydrate (Chl $a \cdot 2H_2O)_2^{+\cdot}$. Another, more stable signal they assume to be due to dimeric monohydrate chlorophyll (Chl $a \cdot H_2O)_2^{+\cdot}$. Both signals can appear as the result of excited chlorophyll-water interaction, water being an electron acceptor. They explain the dark decay of the signal by the interaction of (Chl $a \cdot 2H_2O)_2^{+\cdot}$ radical cations with water as an electron donor. However, the existence of such reactions is yet to be confirmed by direct measurements, and in any case their quantum yield is rather small.

The 40% line narrowing *in vivo,* as compared with monomeric chlorophyll *in vitro,* closely corresponds to the decrease of the original line width by a factor of $\sqrt{2}$, i.e., it indicates a water-linked chlorophyll dimer as the source of the signal. This conclusion is confirmed by ENDOR data for systems *in situ.* Chlorophyll a^+ ENDOR spectra *in vivo* and *in vitro* are compared in Figure 6 (30, 40). The ENDOR results agree with the twofold decrease of the hyperfine interaction constants, which indicates that the spin density is distributed between two equivalent centers in the dimeric molecule. The hyperfine interaction constant for the ith proton in the aggregate is $a_{id} = a_{im}/2$, where a_{im} is the hyperfine interaction constant for a single proton of the monomer.

A number of structures have been suggested for the modeling of a specific pair of chlorophyll molecules in the RCs of photosystem I. It has been assumed in all cases that the porphyrin macrocycles are oriented parallel to each other and are more or less rigidly fixed by covalent or hydrogen bonds. An acceptable model for "special pair" chlorophyll should explain why it is more readily oxidizable than monomeric chlorophyll. The dimer will be more oxidizable when the highest occupied molecular orbitals of the two monomeric subunits can interact to generate two "supermolecular" highest occupied orbitals, from the upper of which it will be easier to remove an electron, since this orbital lies at a higher energy than does the highest occupied orbital of the monomer. In addition, the arrangement of the two chlorophylls must provide for satisfactory overlap of the π-systems and equality of corresponding sites in the two chlorophyll subunits, to facilitate a near equal distribution of unpaired-electron density in the special pair, predicted by esr and ENDOR spectra.

A few models, which to some extent meet all the above-mentioned criteria, are shown in Figure 7. In the first model (59, 60) the two chlorophyll molecules are held together by a water molecule bound to the Mg atom of one chlorophyll molecule while at the same time linking, by hydrogen bonds, a ketonic carbonyl group to a carbomethoxy-carbonyl group of ring V in the other chlorophyll molecule. This model meets the requirement of parallel macrocycles, and theoretical calculations show it is in agreement with the observed infrared spectra. However, because of the presence of a carbomethoxy group between the two macrocycles, the latter cannot have their π orbitals overlapping, which contradicts the effective delocalization of spin density on oxidation. Besides, the chlorophyll molecules here are asymmetric, though it is not clear whether this asymmetry is high enough to be incompatible with esr or ENDOR data.

A modification of this model in which the distance between the two macrocycles is reduced (30) is shown in Figure 7b. This model is based on X-ray diffraction data for ethylchlorophyllide $a \cdot 2H_2O$ (54). Here the dimer is just a fragment of the chlorophyll-water adduct oligomer structure and differs from the above model by the absence of the hydrogen at the methyl ester group.

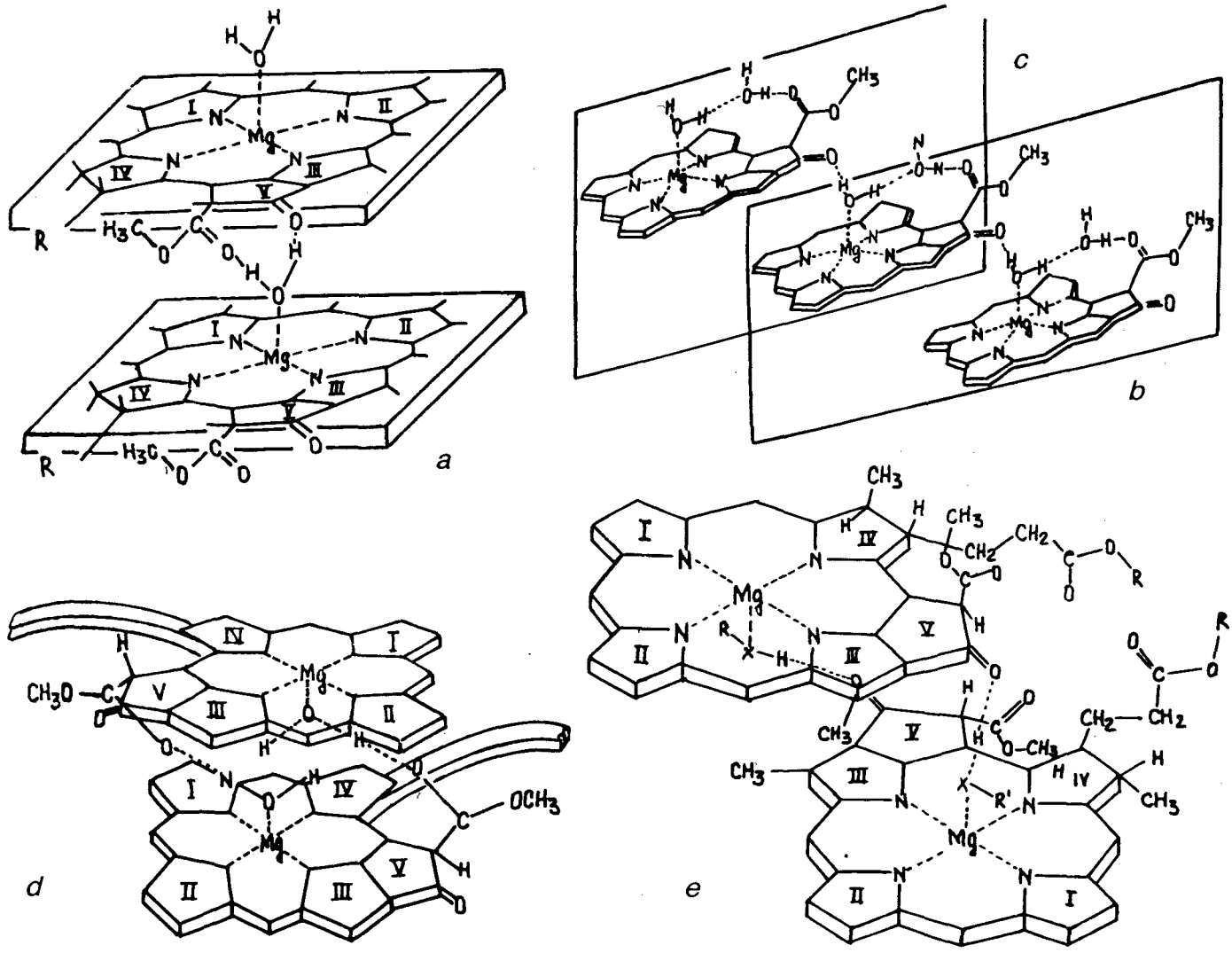


Figure 7. Several models for "chlorophyll special pair": a) a molecule of water is coordinated to the Mg atom of one chlorophyll and simultaneously hydrogen-bonded to the ketonic C=O and carbomethoxy C=O functions of the second chlorophyll (59). The model is asymmetric. The two chlorophylls are not equivalent, as the lower Chl *a* is acting only as acceptor. b) An asymmetric model based on the structure of crystalline ethylchlorophyllide *a*·$2H_2O$ (30, 54). c) Another version of the same model (61); the two Chl *a* molecules in the special pair are equivalent and are related by translational symmetry. d) Model of Fong (62). In this model the two Chl *a* molecules are crosslinked by interaction with the carbomethoxy C=O functions. The arrangement has C_2 symmetry, but the Chl *a* macrocycles are rotated at a 60° angle and are 6 Å apart. e) Special pair model with C_2 symmetry as proposed by Shipman et al (63). In this version the ketonic C=O functions are hydrogen-bonded. Various nucleophiles R'XH, where X = O, N, or S and R' = H or alkyl can act as crosslinking agents.

Exciton calculations (61) have shown that for this kind of dimer there must be a shift of the red absorption maximum up to 693 nm, which agrees with the data for P700 *in vivo*. The chlorophyll molecules are not quite the same here either. The macrocycles are separated by a distance of 3.6 Å, which is smaller than the sum of their van der Waals radii, i.e., the required overlap of the π systems is ensured. Another pair of molecules may be distinguished in the same structure, as is shown in Figure 7c. They form a dimer consisting of completely identical, but not quite symmetrical subunits. In both cases the third molecule disturbs the structure of the acceptor chlorophyll molecule, so that the other two molecules, regarded as a dimer, become more similar. Fong (62) suggested a completely symmetrical C_2 structure, shown in Figure 7d. The two chlorophyll molecules are held together by two water molecules. Each water molecule is at the same time bound to the Mg atom and a ring V methyl ester group of the other chlorophyll. Ketonic groups do not take part in the dimer formation.

However, the construction of molecular models has shown that the distance between the macrocycles in this case is 5.7 Å, i.e., greater than the sum of the van der Waals radii. Calculations of the optical properties of this structure do not give the experimentally observed long-wave shift (30, 63). The ketonic groups, which have stronger electron-donor properties than does the methyl ester group, are not used at all. Experimentally obtained dimers (64), which it was suggested correspond to this model, were constructed in such a way that the carbomethoxy functions (according to infrared spectra) did not in fact take part in the dimerization (65).

Shipman et al (66) and Boxer and Closs (67) have suggested a model which seems to overcome the difficulties of Fong's model, but which meets the requirement of maximal symmetry of the monomers (Figure 7e). This last model is attractive also because it allows coordination not only with water, but with other nucleophiles. *In situ* these could be nucleophilic protein side groups. It opens up the possibility of binding RC dimeric chlorophyll to the proteins that are isolated with dimeric chlorophyll from plant material during fragmentation and purification of reaction center preparations. The dimer structure is maintained by hydrogen bonds between keto functions and water (or some other nucleophilic group $-SH, -NH_2, -OH$); π-system overlap is also achieved. An experimental realization of the model (66) seems to be a system containing 0.1 *M* chlorophyll *a* in toluene in the presence of 1.5 *M* excess ethanol. As the solution cools, the initially observed absorption maximum at 668 nm disappears and is replaced by a maximum at 702 nm. According to spectral data, neither the keto nor ester carbonyls take part in coordination at room temperature. However, in the cooling process a keto group becomes bound by a hydrogen bond, and the ester group remains free.

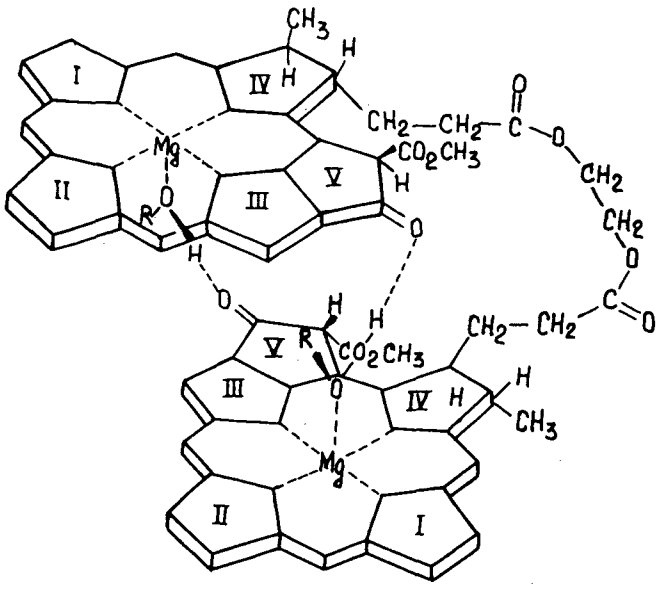


Figure 8. Covalently linked chlorophyll molecules in the folded configuration. Dotted lines indicate H bonds.

A comparatively new direction in the modeling of the *in vivo* chlorophyll dimer is the synthesis of compounds in which two porphyrin rings are linked by one or two covalent bridge structures (67-71). Between the optical properties of Zn-porphyrin dimers with peptide bridges and corresponding monomers, there are no differences that are comparable to the differences between the monomeric chlorophyll and dimeric chlorophyll in photosystem I. The problem of photosynthetic dimer modeling has stimulated much research into synthesis, which has been highly successful. For example, bis(pyrochlorophyllide *a*) ethylene glycol diester was obtained (67). Its absorption maximum in benzene or carbon tetrachloride in the presence of excess water or primary alcohols was observed at 694 nm. Wasielewski et al (72) have developed synthetic methods that have resulted in the preparation of covalently bound dimeric species of chlorophyll *a* (Figure 8). The ethylene glycol bridges are flexible enough not to interfere with the formation of hydrated symmetrical structures containing their macrocycles at the closest possible position. At the same time they assist in stacking by constantly keeping the two monomeric subunits close together. In benzene in the presence of water, this compound had a spectrum with an absorption band at 694 nm and a minor peak at 677 nm. This spectrum is close to the chlorophyll spectrum *in vivo*. The esr spectrum of the corresponding oxidation product does not appear to have been investigated in detail, but there is little doubt that the spectrum would be similar to that of radical cations of $P700^+$ dimeric chlorophyll.

C. The System of Bound Electron Acceptors of Photosystem I

It has been firmly established that chlorophyll *a* is a primary photoexcited electron donor in photosystem I and forms a dimer of a specific structure. Thus, the problem of determining the primary charge-separation mechanism is essentially reduced to that of establishing the nature of the earliest electron acceptor(s). After excitation by a laser pulse, chlorophyll a_1 (P700) is oxidized in less than 20 ns (41). However, it is known that quantum stabilization occurs much more quickly, i.e., in less than 30 ps (2, 3). Thus, the early electron transfer occurs at a very high rate, incompatible with any processes limited by diffusion. Evidently, the participants in this reaction are constantly forming a complex, and indeed the electron transfer in this complex

occurs independently of the temperature, even at the temperature of liquid helium.

In some early publications it was suggested that a chlorophyll molecule is an early electron acceptor (73). For a long time there was no experimental support for this hypothesis. ESR data showed that the early acceptor is an Fe-S center with a more negative potential than that of free ferredoxin. This problem is still being intensively investigated; however, the latest experiments again lead to the conclusion, though on quite different experimental grounds, that the primary acceptor must be a pigment molecule. This conclusion is equally valid for all types of reaction centers, for the RCs of photosystems I and II and those of bacterial photosynthesis as well.

For bacterial photosynthesis it was established from optical and esr data that the earliest acceptor is not ubiquinone, as was thought earlier, but bacteriopheophytin, a Mg-free analogue of bacteriochlorophyll. When reduced, this acceptor forms a radical anion, which is identified by its optical and esr spectra (74). Later Klevanik et al (75, 76) established that in photosystem II pheophytin takes part as an intermediate carrier in the electron transfer between the pigment P680 in the reaction center and plastoquinone, which for a long time was regarded as the primary electron acceptor of photosystem II. Finally it was also shown for photosystem I by optical spectroscopy and esr that there exists at least one more carrier between the Fe-S center and pigment P700. Some indirect data indicate that chlorophyll *a* molecules might be this carrier. Energy considerations make this assumption quite probable.

Redox titration data of the $P700^+$ esr signal and the signals of the bound Fe-S centers and the photosystem II early acceptor have shown that the P700 redox potential is about +0.4 V (22, 77), whereas the potential of the pigment in the photosystem II reaction center, P680, cannot be less than +0.8 V (since photosystem II brings about the decomposition of water into O_2, then E_{H_2O/O_2} = 0.8 V). The half-wave potentials in electrochemical oxidation of chlorophyll *a* and pheophytin *a* (in dimethylformamide) are −0.88 and −0.64 V (44). In photosystem I the reaction

$$P700 + \text{Chl } a \xrightarrow{\text{light}} P700^{+\cdot} + \text{Chl } a^{-}$$

must be accompanied by a free-energy change ΔE = 1.3 eV (−0.9 to +0.4V), whereas for the hypothetical reaction

$$P700 + \text{Pheo } a \xrightarrow{\text{light}} P700^{+\cdot} + \text{Pheo } a^{-\cdot}$$

the change is ΔE = 1 eV (− 0.6 to + 0.4 V).

In photosystem II the change in free energy for the hypothetical reaction

$$P680 + \text{Chl } a \xrightarrow{\text{light}} P680^{+\cdot} + \text{Chl } a^{-\cdot}$$

would be ΔE = 1.7 eV, while for the reaction

$$P680 + \text{Pheo } a \xrightarrow{\text{light}} P680^{+\cdot} + \text{Pheo } a^{-\cdot}$$

the change is ΔE = 1.4 eV. Since 700 nm and 680 nm photons absorbed by photosystems I and II have energies of 1.77 and 1.82 eV, respectively, then taking into account the losses necessary for the stabilization of the separated charges, it follows that the energy of a quantum is more completely used in the transfer of an electron to pheophytin in photosystem II or to chlorophyll in photosystem I. The pheophytin redox potential (−0.64 V) and the chorophyll redox potential (−0.88 V) are quite sufficient for reduction of the plastoquinone (E = −0.2 V) and the bound Fe-S chloroplast center (E = −0.6 V), respectively. Independent of these energy considerations, experiments have shown that in the reaction center of photosystem I there are several components among which, at any temperature, electron transfer is possible in the forward direction (i.e., from the light-excited chlorophyll dimer) and among some of which back transfer is possible (charge recombination). In investigations of early acceptor properties, one trend is clearly seen. As techniques of measuring short-lived and weak changes in the optical absorption and esr spectra become better, the measured redox potentials of the earliest acceptors become greater, thus approaching the calculated redox potential of excited chlorophyll. At the same time the rate of charge recombination of the dimeric chlorophyll radical cation (the oxidized primary donor) with the component considered to be a primary acceptor also increases over a wide temperature range. The main results are obtained by studying chloroplasts and their fragments by the application of esr and optical spectroscopy at liquid-nitrogen and liquid-helium temperatures (4-77 K) (77-91).

1. The Composition of the Acceptor Complex of Photosystem I

Malkin and Bearden (78) observed esr signals of Fe-S centers (Figure 9) from illuminated chloroplasts at 10 K. These centers produced signals at g-values of 2.05, 1.94, and 1.86—close to those of reduced soluble ferredoxin. Illumination at 715 nm was as effective as at 615 nm. From this it was concluded (and confirmed by later experiments) that these centers are related to photosystem I. A quantitative comparison of the $P700^+$ content and the Fe-S centers, as well as a comparison of

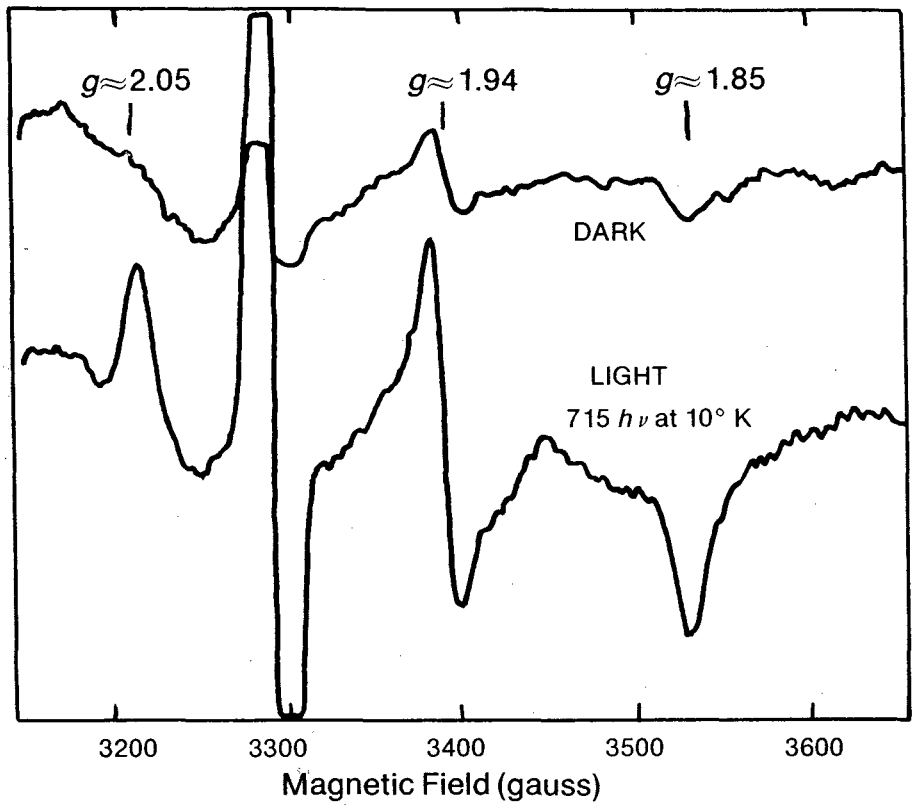


Figure 9. Photoreduction of a bound Fe-S center in intact spinach chloroplasts after illumination at 10 K. From ref. (16), with permission.

their kinetic characteristics in the interval of 10-100 K, shows that there is a good correlation between P700 oxidation and Fe-S center reduction in chloroplasts and in the particles enriched in photosystem I (79-81).

In addition, in frozen chloroplasts exposed to light a signal with *g*-factors of 2.05, 1.93, and 1.89 appears; this signal does not change in the dark at low temperatures. This signal can also be obtained by dark reduction by dithionite. Estimates of the redox potential of this center vary between −530 and −580 mV (81-83). Independent optical data on the bound acceptor were obtained by Ke et al (82), who found short-lived photobleaching at 430 nm (P430) and established a correlation between P430 and low-temperature signals with *g*-factors of 2.05, 1.95, and 1.87. This component was regarded as possibly being the primary acceptor in photosystem I. However, data were subsequently obtained that indicated an intermediate electron carrier (or carriers) between P700 and the Fe-S centers with these *g*-factors.

McIntosh and Bolton (84) and Evans et al (89) observed at about 10 K a new esr signal with *g* values of 2.07, 1.86, and 1.78 (Figure 10); this signal appears reversibly either under an intense low-temperature illumination of photosystem I fragments in the reducing medium (simultaneously with the esr I signal) or under strong dark reduction in the presence of certain redox mediators (the corresponding component was called the "X center"). The redox potential of this center is about −730 mV. Later, Demeter and Ke (86), studying reversible and irreversible absorption changes induced by light in a strongly reducing medium, also concluded that there was an intermediate carrier between P700 and P430. The charge separation between these components is reversible even at 20 K, and the degree of reversibility is determined by the fraction of reduced P430 present and reaches 100% when the potential of the medium is about −670 mV. This potential exceeds the potentials of viologen dyes, which still can be photoreduced by chloroplasts. It seems that an electron is transferred onto the intermediate acceptor and then onto P430 if the latter has not been previously reduced. The charges of both $P700^+$ and this intermediate acceptor also recombine quickly even at liquid-helium temperature, if further transfer of electrons onto more distant acceptors is impossible.

However, an esr signal from the reduced X center (the A_2 center (87)) can also be observed, with $g \approx 1.78$, 1.88, and 2.08, without pretreatment by strong reducing agents. In this case a stable steady-state signal of the same type is observed when the sample is gradually frozen in strong light. The signal remains stable in darkness and is not accompanied by an esr signal I from the $P700^+$ centers. Evidently, this result can be explained by the fact that in the process of cooling, the electron transfers on the oxidizing side of photosystem I are stopped at a higher temperature than that of X

reduction. Thus, the states with reduced P700, oxidized secondary donor (plastocyanin), and reduced acceptor X^- become fixed. The combination of charges [Pc^+ P700 X^-] is stable at cryogenic temperatures.

Recently Mathis et al (92) studied photosystem I chlorophyll-protein complexes obtained by dodecylsulfate treatment, in which no photochemical activity had been observed before (93) or in which charge separation was very ineffective (94). In these preparations they detected P700 oxidation induced by a short light pulse; after this, $P700^+$ reduction by charge recombination with an electron acceptor occurred, in 0.5 ms at 5 K and in 10 ms at 294 K. Later a similar result was obtained for chloroplasts incubated in the presence of a reducing agent. Thus, the reversibility of low-temperature P700 oxidation (and acceptor reduction) can be obtained both by the reduction of the majority of the bound acceptors or by their removal or denaturation.

Using very sensitive, high-speed equipment, Sauer et al (95) studied laser-induced reactions of photosystem I in subchloroplast fragments at room temperature and have shown that the electron capacity of the acceptor pool is four electrons for one P700 center:

$$P700 \longrightarrow A_I \longrightarrow A_2 \longrightarrow P430 \begin{bmatrix} A \\ B \end{bmatrix}$$

If all the components of this pool are oxidized, two flashes are required for complete P430 reduction. This center is identified with the bound Fe-S center found earlier, whose redox potential is about −500 mV (P430). If all the P430 are initially reduced, the flash will reduce the A_2 component, which corresponds to the center with a more negative redox potential (about −700 mV). The esr signal from this center was determined by McIntosh and Bolton (84) and by Evans et al (84, 89) at a temperature close to that of liquid helium. If this acceptor is also previously reduced, the charge separation is limited by two components, P700 and A_1. The times for charge recombination between P700 and each of the bound acceptors at room temperature are: 30 ms for $P430^-$, 250 μs for A_2^- and 3μs for A_1^-. These components are all capable of taking part in photochemical charge separation at liquid-nitrogen and liquid-helium temperatures, and the charges recombine between $P700^+$ and A_1^- or A_2^- at a very high rate. At 5 K the reoxidation of A_1^- due to its reaction with $P700^+$ takes about 3 ms, though the reaction of $P700^+$ with $P430^-$ is practically inhibited. According to data that Ke presented in a report delivered at an international seminar in Moscow in September 1978, the absorption spectra of the earliest intermediate products of the photosystem I reaction contain features characteristic of both $P700^+$ and the chlorophyll *a* radical anion. It is suggested that the esr signal observed when chloroplasts in a strongly reducing medium

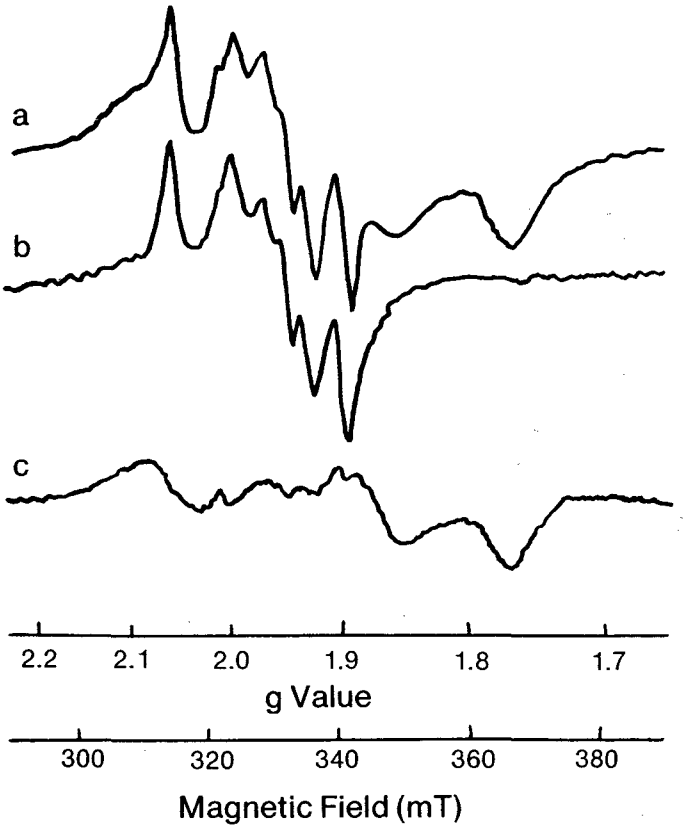


Figure 10. ESR spectrum of component X (A_2). Photosystem I particles frozen with Fe-S centers A and B reduced (a), or centers A and B and component X reduced (b), and their difference corresponding to the spectrum of X (c). From ref. (91), with permission.

are illuminated in the temperature range of 4-77 K, contains a contribution from radical anions of the chlorophyll acceptor. This assumption is confirmed by the asymmetry of the esr signal that has been observed at these reducing potentials. The signal is distorted by the superposition of the signal from the radical anion primary acceptor. Thus, the A_1 component appears to be chlorophyll *a*.*

2. Radical Pairs as an Early Product of the Photochemical Reaction

Experiments using the chemically induced dynamic electron spin polarization (CIDEP) technique provide another source of information about the early reactions

*Recently the observation of an esr signal of 14*g* line width has been reported in photosystem I particles illuminated at room temperature and frozen in the light in the presence of a strong reducing agent. This may correspond to the electron acceptor A_2. [P. Heathcote, K.N. Timofeev, and M. C. W. Evans, *FEBS Lett.* **101**, 105 (1979)].

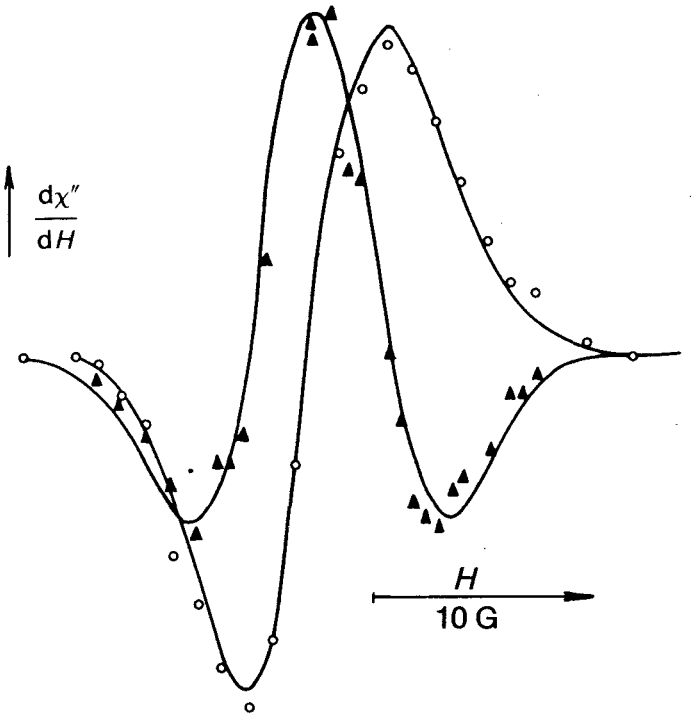


Figure 11. Calculated (solid lines) and experimental esr spectra for the oriented and unoriented polarized signal from spinach chloroplasts. Solid triangles are experimental intensities for flow-oriented chloroplasts. Open circles are experimental intensities for unoriented chloroplasts. From ref. (99), with permission.

in photosystem I centers (96-99). The study of signals from triplet chlorophyll *in situ* provides a third source. The CIDEP in photosynthetic systems was discovered by Blankenship et al (100) and by McIntosh and Bolton (101) and was investigated in detail in Sauer's laboratory (98, 99).

ESR signals observed from various photosystem I preparations in the first few microseconds of pulsed excitation display spin polarization. The pulse induces an esr signal with a g-value of 2.0025, which coincides with the g-value of the esr signal I from $P700^{+\cdot}$ centers, but with an anomalous intensity distribution along the magnetic field (Figure 11). Microwave radiation emission prevails in the low-field region, whereas in the high-field region enhanced absorption occurs. This means that the free-radical center with this esr signal has a nonequilibrium spin distribution. The shape of this signal changes with the orientation of chloroplasts in the flow. These effects were analyzed (99) according to the spin polarization theory developed earlier by Adrian (102) for radical pairs in solution. The spin-state population changes because of coherent mixing of the triplet and the singlet states of the weakly coupled partners in a radical pair. This mixing, caused by the presence of local magnetic fields and by spin exchange, takes place more quickly than the incoherent spin-lattice relaxation. The sign of the effect (emission or enhanced absorption of microwave radiation) can give information about the state preceding the radical pair. CIDEP data give information about both partners of a radical pair, though sometimes only the esr signal from one of them can be observed directly.

In addition, the dependence of the spectrum with spin polarization on the orientation of the membrane in the magnetic field is useful in studying the anisotropy of the magnetic interactions within the radical pair and the orientation of the free-radical species relative to one another and to the membrane. Since the spin polarization occurs before the establishment of equilibrium with the lattice, fast kinetic techniques must be used in experiments. In practice a photosynthetic system, such as a chloroplast suspension, is illuminated by short light pulses in the esr cavity, and then the kinetics of the changes in the amplitude of the first derivative of the esr absorption are observed at various fixed field intensities near the absorption maximum. Then the esr spectra, corresponding to various times after the pulse, are constructed on the basis of the kinetic data. It is possible in practice to reduce the esr spectrometer dead time to 2 μs. Hence, of course, the earliest processes are lost since the primary photochemical reaction has a much shorter lifetime. Nevertheless, two different esr signal components are observable, one of which has a comparatively long lifetime of 30 ms and the usual shape of an esr I signal, while its amplitude corresponds to the contents of P700 in the specimen. The other component, with a significantly larger amplitude and shorter lifetime, has an anomalous shape, which indicates the occurrence of CIDEP. The shapes of the long-lived component and of the steady-state esr I signal do not depend on chloroplast orientation in the magnetic field. However, the shape of the anomalous signal does depend to a great extent on the chloroplast orientation in the magnetic field (Figure 11). Also the shape of the anomalously polarized signal depends on the resolution time of the spectrometer. Thus, it was concluded that the relaxation rate is different for different hyperfine lines of the free-radical center.

McIntosh and Bolton (96, 97) noticed the possibility of esr spectra distortions in CIDEP experiments because at high modulation frequencies (100-1000 kHz) conditions for slow passage through the resonance are not fulfilled: even at room temperatures the spin-lattice relaxation time of $P700^{+}$ centers may be of the order of 1 μs. These distortions may be similar to the anomalous polarization of the esr signal. Because of this they observed only the direct esr absorption signal, without high-frequency modulation. This made it possible to reduce the dead

time of the instrument to 0.4 μs. The results showed that the anomalous intensity distribution in the esr spectrum that appears immediately after excitation of the system by a short pulse is really caused by the CIDEP. The spectrum at 100 K contained both absorption and emission components, situated asymmetrically relative to the center of the esr signal I.

One of the main peculiarities of the signal was a shift in the direction of the weak field, as compared to signals observed at room temperature. McIntosh et al (97) suggest that the existence of short-lived signals with a range of *g*-values of 2.045-2.065 indicates the participation of an alternative electron acceptor in charge separation, apart from the chlorophyll *a* suggested by Dismuckes et al (98), the lifetime of which in a reduced state increases as the temperature decreases. It is possible that these two intermediate acceptors, functioning between P700 and A_2 (component X in the terminology of McIntosh and Bolton (84)), are situated in different photosystems I, i.e., the reaction centers of photosystems I are in fact heterogeneous. However, this problem requires further research.

According to redox titrations the amplitudes of the short-lived emission and enhanced absorption signals decrease with an increase in the redox potential of the medium in the interval 400-600 mV. The amplitude of the steady-state esr signal I simultaneously increases. The spin polarization was also observed in subchloroplast fragments of photosystem I, though the orientation effects did not occur in these preparations (the particles are approximately spherical and so do not orient in the flow). The steady-state esr signal I and the polarized signal have the same *g*-value. There is no doubt now that both signals belong to oxidized chlorophyll *a*, the primary electron donor of photosystem I. The observed polarization and orientation effects can be explained by supposing that electron transfer from dimeric chlorophyll P700 to the primary acceptor causes the formation of radical pairs in which both radicals are close enough to make possible a partial overlapping of their orbitals and an effective spin exchange. The initial spin configuration is the same as that of the initial excited P700 from which electron transfer occurs. This means that the configuration is a singlet and that no polarization can take place in the initial state. However, in a weakly coupled radical pair there is a coherent mixing of the singlet and the triplet states due to local magnetic fields (hyperfine and spin orbital), different for each radical. In solutions and, it seems, in membrane structures, this mixing leads to spin polarization. Since the $P700^+$ has an isotropic *g*-tensor (the corresponding steady-state signal is isotropic and does not depend on the orientation) while the anomalously polarized signal depends appreciably on the orientation, it must be assumed that this anisotropy is determined by the acceptor component of the radical pair. The anisotropy of the signal disappears since the electron transport separates the oxidized primary donor and the sequentially reduced acceptors more rapidly than the spin relaxation takes place (98, 99).

The previously suggested triplet mechanism for the formation of the anomalous polarized signal (100,101) does not explain the signs of the polarization for different hyperfine lines of the $P700^+$ esr signal (i.e., the dependence of the amplitude on the field). A detailed theory of the effect has been developed in the work of Friesner et al (99), who examined a model of the radical pairs that considers the influence of the anisotropy of the *g*-tensor of the primary and secondary electron acceptors, as well as the fixed orientation of all three participants in this reaction in the membrane. The results obtained can be described, both qualitatively and quantitatively, on the basis of the electron-transfer scheme:

$$P700 \longrightarrow A_1 \text{ (Chlorophyll } a\text{)} \longrightarrow A_2 \text{ (Fe-S center X)} \longrightarrow P430 \text{ (binuclear Fe-S center } \left[\begin{smallmatrix} A \\ B \end{smallmatrix}\right]\text{)}$$

The first of these acceptors is, apparently, an organic molecule with a practically isotropic *g*-tensor very close to those of $P700^+$. This acceptor is most probably the chlorophyll *a* molecule. The secondary acceptor A_2 must have a highly anisotropic *g*-tensor and be rather rigidly oriented relative to the plane of the membrane (which is confirmed by direct measurements of esr signals from this center in oriented chloroplasts; see below). Neither the model that takes into account only the spin exchange between $P700^+$ and acceptor A (a small organic molecule with an isotropic *g*-tensor) nor the model that considers only the interaction between $P700^+$ and acceptor A_2 (which has an anisotropic *g*-tensor) provides an explanation of the experimental results. Only a two-site model is adequate for a quantitative interpretation of both the intensity of the polarized signals and their orientational dependence. Such a model accounts for the consecutive electron migrations from P700 to A_1 and further to A_2, and both acceptors must be in a state of spin exchange with $P700^+$ (with appreciably different values for the exchange integral, the exchange being more effective for A_1^- than for A_2^-). Some details are still

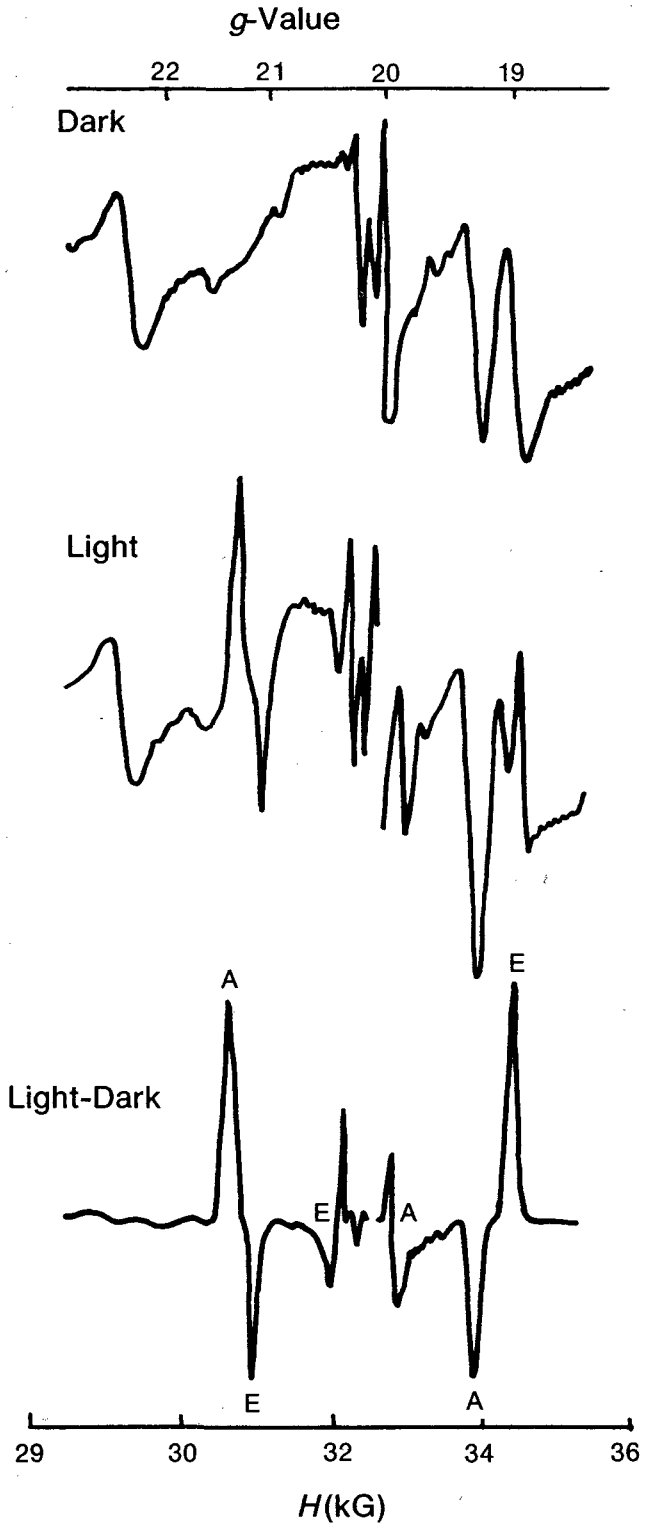


Figure 12. ESR spectra at liquid-helium temperature of chromatophores from chromatium D. The redox potential was −260 mV. The lower trace is the difference between light and dark signals. Peaks A and E result from microwave absorption and emission respectively. This spectrum is similar to the triplet bacteriochlorophyll in solution. From ref. (103), with permission.

unclear. For example, the polarized signal in non-oriented samples is narrower than the signal from $P700^+$ (5.6 G instead of 7.5 G), but the mechanism is still to be explained. New experiments and theoretical studies will be necessary for the lifetimes of the reduced intermediate acceptors to be estimated by the CIDEP approach and for the nature of A_1 and A_2 to be finally established.

The theory also has given a satisfactory explanation of the esr triplet signals, which are observed in certain photosynthetic systems when electron transport is blocked in the vicinity of the reaction centers (Figure 12). So far no one has been able to observe triplet chlorophyll esr signals in preparations from higher plants or algae. These signals were observed only in preparations of reaction centers from photosynthesizing bacteria in conditions of vigorous reduction. They displayed properties rather unusual for triplet states (103,104).

As is well known, the energy levels of a triplet molecule are determined by dipole interaction between two unpaired electrons and in esr experiments by the interaction with the external magnetic field. In this case the triplet substates are denoted by $|T_{+1}\rangle$, $|T_0\rangle$, and $|T_{-1}\rangle$, and for each orientation of the triplet molecule there is a system consisting of three levels between which two transitions are possible $\Delta m_s = \pm 1$. Evaluations of the zero-field-splitting parameters, D and E, which characterize the coupling between two unpaired electrons as well as the symmetry of the system, have been obtained from esr spectra of triplet chlorophyll both *in vivo* and *in vitro* (Table I) (103-111). The triplet signal of bacteriochlorophyll *in situ* differs from the signals of various chlorophylls *in vitro* in two respects. First, the D and E parameters are smaller *in situ* than *in vitro*. This corresponds to a spin-density distribution outside a single molecule different from that for chlorophyll triplets in dilute solution.

The other important characteristic of esr triplet spectra is the kinetics of the population and depopulation of triplet sublevels. In this respect, chlorophylls *in situ* and *in vitro* also differ. Generally speaking, all the spectra of triplet chlorophylls display spin polarization due to non-Boltzmann populations of the levels. This feature can be explained by the polarization of singlet-triplet transitions (109,110) and is related to the fact that triplet chlorophyll appears as a transformation product of an excited singlet molecule. However, *in situ* the central sublevel T_0 is more populated for all molecular orientations than the other two sublevels. Katz et al (30,111) have suggested an interpretation of the spectra of triplet chlorophyll *in situ*. This interpretation is also based on radical pairs that are considered as precursors of triplet chlorophyll in the reaction center. As in CIDEP theory, it is suggested that during light-induced charge separa-

tion an ion-radical pair is initially formed in the singlet state, similar to the initial singlet state of excited chlorophyll. This pair then turns into normal products if other, more distant electron acceptors are present. If these are initially reduced, the charges recombine within the radical pair.

In principle, the radical pair that is initially formed in the singlet state also has a triplet state with three spin sublevels, T_+ (RP), T_0 (RP), and T_- (RP). As the distance between two unpaired electrons in the radical pair is quite large, the states T_0 (RP) and S_1 (RP) are quite close to each other in energy, whereas the levels T_- (RP) and T_+ (RP) lie respectively higher and lower than T_0 (RP), and the degree of mixing due to local field inhomogeneity is higher for the states S_1 (RP) with T_0 (RP) than for the same singlet S_1 (RP) with the two other triplet substates. The T_+ (RP) and T_- (RP) levels can be populated by nonresonance mixing with other levels. However, reverse electron transport, i.e., transition from the state T_0 (RP) to the state T_0 (D) (triplet of the donor molecule) proceeds faster than does nonresonance relaxation. This leads to a more preferable population of the T_0 (D) state, whereas the T_+ (D) and T_- (D) levels remain unpopulated.

The nature of this triplet is not clear at the present stage of research. It may be a complex with the change transfer between two pigment molecules in the dimer $P^{+\cdot}-P^{-\cdot}$, i.e., essentially a biradical ion pair, or else it is a complex with supermolecular orbitals with two parallel spins, as in the case of triplet monomeric chlorophyll (30). It is also not clear what kinetic peculiarities of the chemical transformations (i.e., electron transfer) and/or spin relaxation are preventing observation of triplet states in the photochemical reaction centers of higher plants, unlike the case for bacterial photosynthesis.

D. Bound Fe-S Centers on the Reducing Side of Photosystem I

The first Fe-S protein, ferredoxin, was isolated from spinach leaves by Tagawa and Arnon in 1962 (112). At first it was supposed that it was the only protein of this type that took part in photosynthetic electron transport. Later, however, it became clear that ferredoxin is easily washed out from photosynthetic membranes and that after its removal the chloroplasts still contain about 30 nmole of acid-labile sulfur and nonheme iron per 1 mg of chlorophyll, or 15 moles of Fe-S centers per 1 mole of photochemical reaction centers, i.e., more than the heme iron (5-10 nmole per 1 mg of chlorophyll). Techniques exist for a direct chemical determination of nonheme iron and acid-labile sulfur, but they do not allow the determination of the composition of the corresponding centers. Optical methods are not sensitive enough for experiments *in situ* since proteins of the ferrodoxin type have weak absorption bands in the 350-450 nm region ($\epsilon \approx 10\ mM^{-1}cm^{-1}$) that are masked by strong absorption of chlorophyll ($100\ mM^{-1}cm^{-1}$), which is present in great excess. The investigation of the bound Fe-S centers (i.e., centers not removable by washing) in photosynthetic membranes became possible only due to the application of esr. The reduced Fe-S centers at a temperature lower than 80 K produce characteristic esr signals sensitive to light and providing definite information about the structure of paramagnetic complexes. These signals are caused by the antiferromagnetic interaction between high-spin Fe (III) and high-spin Fe (II) (with electron spins 5/2 and 2 respectively), which results in the formation of a paramagnetic center with a total effective spin of 1/2 and a g-value of about 2.0. The g-values of the strongest corresponding esr lines are usually within the range 1.89-1.96, the commonest being 1.94, hence this signal is usually referred to as "the signal 1.94." Reduced Fe-S centers give spectra of rhombic ($g_x \neq g_y \neq g_z$) or axial symmetry ($g_\parallel \neq g_\perp$). Signals from different Fe-S centers often overlap. They can be separated by redox titration, i.e., by recording signals from frozen suspensions of photosynthetic membranes in light or in dark, in the presence of oxidants or reductants that determine the redox potential of the medium and mediators that equilibrate the Fe-S centers with the medium.

Table 1. Zero-Field-Splitting Parameters for *in vitro* and *in vivo* Triplet States.

Species	$D(10^4\ cm^{-1})$	$E(10^4\ cm^{-1})$
Chl *a*	270-320*	40
B Chl *a*	224	53
Rhodospirillum rubrum cells	185	33
Rhodopseudomonas sphaeroides cells	182	35

*Dependent on solvent and concentration

The reduction of the membrane-bound Fe-S center in light gives rise to an esr signal (Figure 9) close in g-value to the signal from the reduced soluble ferredoxin. However, the signals from bound Fe-S centers differed greatly from the esr signal of soluble ferredoxin in their line widths (15 G and 50 G, respectively). A similar signal was observed later from illuminated photosystem I subchloroplast particles (77, 83, 92, 94, 113, 114) as well as from many other photosynthetic systems: green algae (115), blue-green algae (115,116), etc. In subchloroplast preparations enriched in photosystem II, these signals have not been observed (117).

A stoichiometric ratio of 1 : 1 (118, 119) was established between the number of $P700^+$ paramagnetic centers and the number of reduced Fe-S centers appearing under the illumination of photosynthetic preparations at low temperatures. The reaction induced by light at 77 K is irreversible. At temperatures above 80 K it becomes more or less reversible (120,121). The 40% decrease in the $P700^+$ esr signal at 120 K is followed by a corresponding decrease in the Fe-S-center signal. In photosynthetic preparations obtained by treatment with sodium dodecyl sulfate, no stable charge separation at low temperature was observed, though at 300 K in these preparations the photooxidation of P700 took place, producing a corresponding esr signal (94,122). The photooxidation of P700 was inhibited by the treatment of chloroplasts with concentrated urea solutions in the presence of ferricyanide (123). Here labile sulfur is oxidized to free sulfur. The P700 pigment itself is apparently not changed by this treatment, since its chemical oxidation can still be observed in darkness and is accompanied by the same esr signal as is the photooxidation of untreated preparations. Dithiothreitol, which restores the structure of the active Fe-S center, also restores the P700 photooxidation.

After dark reduction of the Fe-S centers at redox potentials of the medium below −540 mV, stable light-induced charge separation at cryogenic temperatures becomes impossible. At more negative potentials of the medium (below −590 mV), the observed spectrum changes: its g-values become 1.89, 1.92, 1.94, and 2.05. These four signals cannot belong to a single Fe-S center with a total spin of 1/2, because one center can produce no more than three signals. Therefore it may be conjectured that in this case there is a superposition of at least two partially overlapping signals. The comparison of various data leads to the conclusion that on the acceptor side of photosystem I there are two types of Fe-S centers: A centers, with g values of 1.86. 1.94, and 2.05 and a redox potential of −540 mV, that are capable of accepting electrons from P700 at cryogenic temperatures, and B centers with g-values of 1.89, 1.92, and 2.05 and a redox potential of −590 mV. At cryogenic temperatures B centers are less able to accept electrons from P700 than A centers. According to Malkin and Bearden (16), in photosystem I particles the number of B centers photoreduced at 10 K is 10-25% of the number of photoreduced A centers. In *Dunaliella parva* these authors observed about equal photoreduction of A and B centers. Both centers can be photoreduced at 300 K in the presence of electron donors to photosystem I in the medium (e.g., reduced dichlorophenol indophenol). In the dark these donors do not reduce A and B.

Many aspects concerning the composition and functional role of these two Fe-S centers are not yet clear. Ke et al (82) and Evans et al (83) obtained contradictory data on the number of electrons accepted during the reduction of A and B. The data of the latter, according to which this reduction is one-electron, seem more plausible on the basis of the known properties of isolated Fe-S proteins. Further, it is not clear why B-center reduction is accompanied by the inhibition of the signal with the g-value of 1.86. A comparison of the intensities of all the esr signals of the reduced A centers with the total intensities of reduced A and B signals shows that the disappearance of the line at $g \approx 1.86$ may be related to the strengthening of the line $g \approx 1.89$, to which it is shifted. Evans et al (81) suggested that the disappearance of the 1.86 signal is caused by the total change of shape of the signal of the reduced A center, due to spin interactions with the paramagnetic B center. For this effect to be possible, these centers must be situated quite close to each other, presumably in the same protein. In fact, nobody has succeeded in obtaining photochemically active preparations that contain either A centers only or B centers only. Attempts to isolate such a protein have failed, too. It is possible that these two Fe-S clusters, initially identical and bound with the same protein, are quite similar in their properties, but the reduction of one of the centers slightly changes the properties (the redox potential, kinetic availability, electron structure) of the other center. There is no information about the possibility of the consecutive electron transfer from A to B or vice versa.

A number of speculations have been made about the functions of A and B centers. Bolton (124) suggested that the B center takes part in the cyclic electron flow and that the A center takes part in the reduction of NADP. This would mean that the A and B centers are contained in different proteins and, probably, even on different sides of the membrane. Arnon et al (116), on the basis of esr data on blue-green algae fragments, suggested that only B centers take part in the electron transfer to NADP. This conclusion was based on the change in the steady-state intensity of the line with $g \approx 1.89$. However, they did not take into account the contribution of the A center through the shift of the signal with $g \approx 1.86$. Finally, according to the data of Dismukes and Sauer (87), the peak with $g \approx 1.86$ belongs only to the A center, and its increase upon reduction of the B center is caused by the interaction between these centers. The signal with $g \approx 1.96$ is here considered as one of the components of the esr signal from the B centers.

The chemical nature of membrane Fe-S centers can be determined by various techniques, mainly by comparison of the esr spectra of photosynthetic preparations with the esr spectra of synthetic cluster structures containing Fe and S and soluble Fe-S proteins of known

structure (125,126). In isolated proteins the active centers can contain either two or four Fe atoms. Cammack and Evans (127) compared the esr spectra of photosystem I fragments reduced after 80% dimethyl sulfoxide treatment and similarly treated soluble Fe-S proteins in which the structure of the active center was known, namely chloroplast ferredoxin containing two Fe atoms per center, and soluble bacterial ferredoxins with two centers, each containing four atoms of Fe. The data obtained lead to the conclusion that the properties of the bound Fe-S centers in the chloroplast are close to those of bacterial ferredoxins and that one center also contains four Fe atoms. This interpretation is complicated by the fact that dimethyl sulfoxide facilitates dimer-tetramer transitions in some synthetic Fe-S clusters, modeling the active center of ferredoxin (128). This analogy to the structure of the Fe-S centers of bacterial ferredoxins was taken as the basis for the present concept of the structure of the bound Fe-S centers in photosystem I. The spatial structures of the 4Fe-4S centers in *Chromatium* ferredoxin (four Fe atoms) and in *Peptococcus aerogenes* and *Micrococcus aerogenes* ferredoxins were established by X-ray diffraction (129, 130). In all cases the structures shown in Figure 13 proved to be the same. Each Fe atom is situated in the tetrahedral environment of sulfur ligands. Fe is coordinated with three bridge atoms of inorganic sulfur and one S atom of a cysteine residue. In *P. aerogenes* protein there are two clusters that are situated at a distance of 11.5 Å from each other.

The compounds $(Fe_4S_4(SR)_4)^{4-}$, where R = $CH_2C_6H_5$ or C_6H_5 (131,132), have been synthesized as models of 4Fe–4S active centers. X-ray diffraction has confirmed the similarity between their structures and the structures of the active centers, soluble Fe-S proteins with four Fe atoms. Thus it is quite probable that membrane-bound Fe-S centers have the same structure. This is important as these centers cannot be investigated by direct structural methods. The esr and Mössbauer spectra of synthetic analogues and soluble Fe-S proteins are also quite similar. Low-molecular-weight analogues were also obtained directly from proteins by substitution of cysteine residues for benzyl thiol ligands (128).

The other intermediate electron carrier, with esr lines at $g_x = 1.78$, $g_y = 1.88$, and $g_z = 2.08$ (the X center, A_2), could not be identified with the same certainty. Its content in chloroplasts is equal to that of P700 (91). The redox potential of this center, which is about −700 mV, is appreciably more negative than the potentials of the A and B centers. Such a high negative potential is not typical for Fe-S proteins. Usually the redox potentials of 2Fe–2S–, 4Fe–4S–, and 8Fe–8S– proteins are from −400 to −550 mV. However, there are some exceptions to this rule. The "superreduced" form 4Fe-4S-

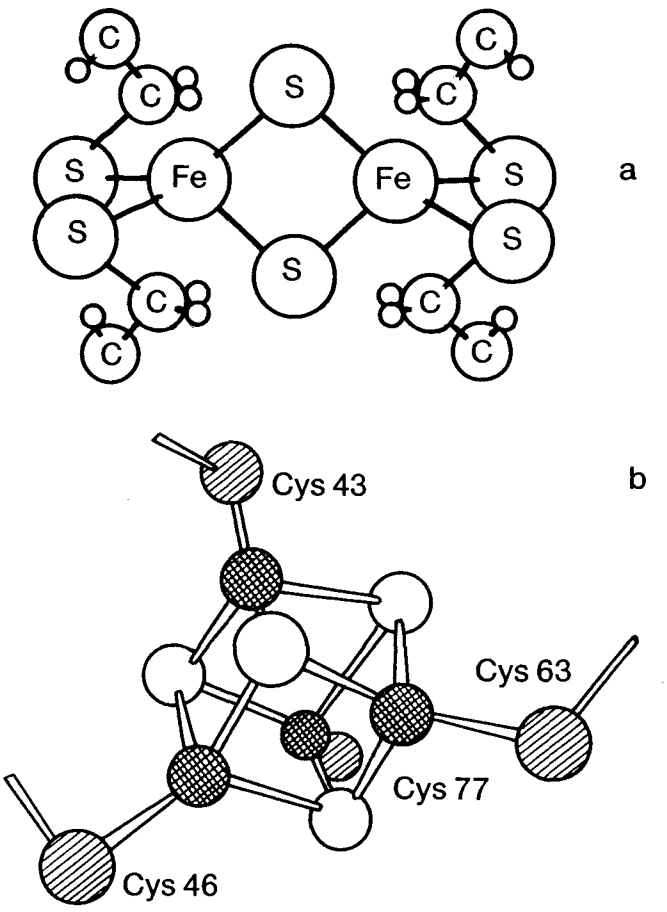


Figure 13. The three-dimensional structures of soluble 2Fe–2S plant ferredoxin (a) and of the 4Fe–4S center (b). The black cirlces are Fe atoms, the open circles are inorganic S atoms.

ferredoxin from the photosynthesizing bacteria *Chromatium* has $E_m = -640$ mV in the presence of 70% dimethyl sulfoxide (133). This center is paramagnetic in the oxidized state and diamagnetic in the reduced state. Superoxidized ferredoxins are also known (134). For example, the 4Fe-4S protein from *Chromatium* has a redox potential of +350 mV.

Carter et al (129) have suggested that 4Fe-4S proteins can be found in three redox states. The first is an oxidized paramagnetic state C^+ with total spin $S = 1/2$, which is observed for the high-potential *Chromatium* protein. The second is a diamagnetic state C ($S = O$) which is equivalent to the oxidized state of all the other Fe-S proteins. The third and most highly reduced paramagnetic state is typical for the majority of the reduced Fe-S proteins containing 4Fe-4S centers. These proteins have an esr signal close to $g \approx 1.94$. These reduced states have been observed for the Fe-S centers of various *Clostridia* (134), for hydrogenases and soluble ferredoxins of various bacteria (135), and for model

low-molecular-weight complexes of similar structure (136). However, the redox potentials of model complexes are much lower than those of proteins. This shows the significant role of the protein environment in the redox properties of Fe-S centers. In particular the effect of the hydrogen bonds between cysteine sulfur and the proton-donor protein groups, and also the effect of a hydrophobic environment on the redox properties of Fe-S centers have been considered (130,133).

The states of the Fe atoms in four- and eight-nuclear Fe-S centers have not been established. In the Mössbauer spectra of bacterial Fe-S proteins with four or eight Fe atoms, signals characteristic of Fe(II) and Fe(III) have not been separated (137). Only an increase in chemical shift upon reduction has been found. The formal valence state of the three forms of Fe-S proteins can be written as follows: C^+ (3 Fe(III) + 1 Fe(II)); C (2 Fe(III) + 2 Fe(II)); C^- (1 Fe(III) + 3 Fe(II)). However, the effective antiferromagnetic coupling among the Fe atoms to a great extent averages the states of Fe. It has been suggested that the center with $g \approx 1.78$, 1.83, and 2.08 in higher-plant photosynthesis belongs to the "super-reduced" Fe-S clusters mentioned above. The absence of a signal from its initially oxidized form is probably caused by relaxation broadening. The alternative suggestion has been made by Bolton (124) who assumed that this center is a nonheme-iron complex with the quinone of the same type as the Fe-ubiquinone complex in the reaction centers of photosynthesizing bacteria.

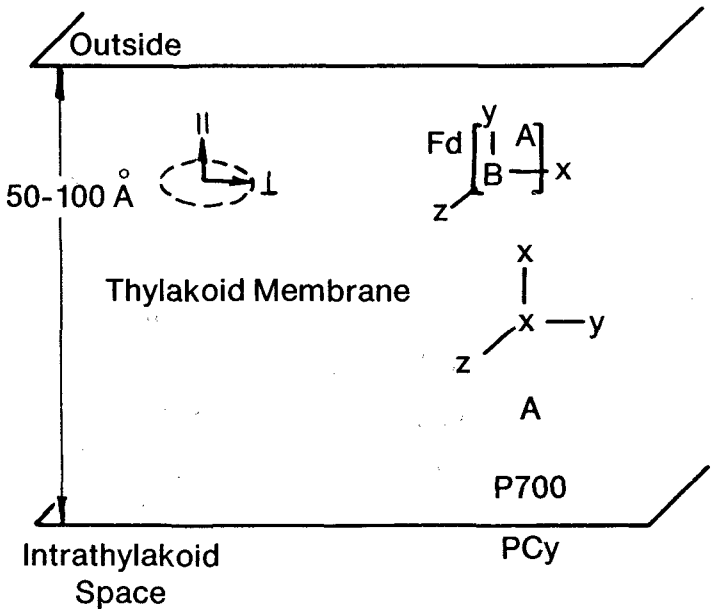


Figure 14. The orientation of principal g-axes of component X and of the bound Fe-S center B, Fd(B), relative to the thylakoid membrane and a possible arrangement of these and other photosystem I components. PCy is plastocyanin. From ref. (87), with permission.

This suggestion agrees with the fact that photosystem I preparations always contain a certain amount of plastoquinone, but there are no data indicating the participation of this plastoquinone in the early reactions of photosystem I.

Additional information on the consumption and relative orientation of photosystem I acceptor centers has been recently obtained by Dismukes and Sauer (87) from the angular variation effects of the corresponding esr signals in chloroplasts. This work opens a new area of research that seems to be very promising. The experiments are based on the orienting effect of a strong magnetic field (10-20 kG) on chloroplasts, discovered by Ceacintov et al (138). An analysis of optical absorption changes and chlorophyll fluorescence polarization in oriented chloroplasts leads to the conclusion that the planes of the thylakoid membranes of the chloroplasts (which have the shape of prolate ellipsoids) are mostly oriented normal to the magnetic field vector. If the suspension of chloroplasts oriented by the magnetic field is frozen, then the orientation induced by the magnetic field remains even after the field is removed. For the Fe-S center A, orientation dependence has not been found, but the lines corresponding to center B were significantly changed when the sample, frozen in a magnetic field of 9 to 20 kG, was rotated by 90° relative to the initial orientation. In this case the line at $g_x = 1.89$ reaches its maximum when the membrane is oriented at right angles to the magnetic field. From this it is concluded that the x-axis of the g-tensor lies in the membrane plane, and the y-axis is oriented normal to the membrane plane.

The questions arise: How can the highly symmetric cubic structure of the 4Fe–4S cluster form a paramagnetic center with the esr spectrum, which depends on the orientation in the magnetic field? And, why do the two apparently structurally similar centers A and B behave differently? If we assume that the electron in the reduced center A can reside on any two Fe atoms forming the diagonal of the cube, there can be no angular dependence of the esr signal because on the average in half of the centers A, the g-tensor will be oriented with respect to the membrane (and the field) in one way, and in the other half, in the other way. However, the center B can be reduced only after reduction of the center A, and this possibly creates the nonequivalence of the two pairs of Fe atoms, which causes the anisotropy of the orientation relative to the membrane.

The intensities of all three components of the esr signal A_2 (or X) of the carrier were found to be dependent on the orientation, no matter how the signal is obtained. It was shown that for this center the minor axis of the g-tensor ($g_x = 1.78$) is oriented normal to the membrane. Upon rotation of the sample practically no line

shifts were observed, only some changes in the relative intensity of the lines. The strong orientational dependence of esr signals from center X is in agreement with the fact that this component is closely associated with the donor P700 center and that it determines the orientational dependence of CIDEP signals from radical pairs.

Since the ordering of the thylakoid membranes themselves is far from ideal (the ordering cannot be perfect in closed membrane vesicles because of edge effects) the degree of ordering of the Fe-S centers relative to the membrane must be very high. The orientation of the *g*-tensors of some photosystem I components, obtained from angular dependence data of esr signals is shown in Figure 14.

III. OTHER ELECTRON DONORS AND ACCEPTORS OF PHOTOSYSTEM I

In contrast to the very complicated system of bound electron acceptors of photosystem I, further electron transfer takes place with the participation of only two soluble and distinct components, ferredoxin and the flavoprotein enzyme ferredoxin-NADP reductase.

A. Soluble Ferredoxin

The physiological properties of soluble ferredoxins were studied by Arnon and his colleagues (reviewed in (10)), who note its many functions as an electron donor acting in various metabolic pathways (in the reduction of NADP, nitrite, and sulfite, as a cyclic electron transport mediator, and as a participant in other reactions). This protein, with a molecular weight of 12,000 and a redox potential of −420mV, has been isolated in crystalline form (139). The structure of its active center has been examined in detail. It has been shown that plant ferredoxin molecules contain two Fe atoms and two S atoms (140). A model of the spatial structure of this center is shown in Figure 13. The binuclear center of plant ferredoxin contains two atoms of labile sulfur and four cysteine residues in the ligand environment of each iron atom. Synthetic low-molecular-weight compounds of $(FeS(SR)_2)_2^{2-}$ composition are suggested as models. The structure of one of these compounds, $(FeS(SCH_2)_2C_6H_4)_2^{2-}$ was established by X-ray diffraction (141). The optical, nmr, and Mossbauer spectra of this compound are similar to the corresponding ferredoxin spectra. At low temperatures oxidized ferredoxins with two Fe atoms are diamagnetic, due to the zero total electron spin caused by the antiferromagnetic interaction between the two Fe(III) atoms with spin $S = 5/2$. When the temperature increases, they exhibit paramagnetism. This is caused by admixing excited paramagnetic states with total spins of 1, 2, . . . , to the initial state of the Fe-S complex having zero total spin. The reduced ferredoxin contains one atom of Fe(III) ($S = 5/2$) and one atom of Fe(II) ($S = 2$), so the total spin is 1/2. The esr spectrum observed at temperatures below 50 K has a rhombic symmetry with $g_x = 1.89$, $g_y = 1.95$, and $g_z = 2.05$. A comparison of the esr spectra of reduced soluble ferredoxins of natural isotopic composition with the spectra of ferredoxins labeled with ^{57}Fe and ^{33}S has shown that the unpaired electron interacts by the hyperfine interaction mechanism with iron, with acid-labile sulfur, and with one or more S atoms in cysteine (142, 143).

The ENDOR technique has given important information about the structure of the active center (142). It has revealed that the two Fe atoms of reduced ferredoxin, in spite of the effective antiferromagnetic interaction between them, are not equivalent. ENDOR measurements of spinach ferredoxin containing ^{57}Fe have given the following values of the hyperfine interaction constants with iron nuclei:

Fe(III): $A_x = 51$ G, $A_y = 50$ G, $A_z = 42$ G

Fe(II): $A_z = 35.5$ G.

Additional information on the electron structure of the iron environment has been obtained from the esr and ENDOR spectra of *Synechococcus lividus* cultivated with D_2O (144). It has been shown that the protein contains several classes of protons strongly interacting with the active center and nonexchangeable with the medium. There are also four types of protons that are exchangeable with the medium. These results correlate with ^1H nmr data, according to which there also exist eight types of protons, nonexchangeable with the medium, whose magnetic relaxation is significantly accelerated upon ferredoxin reduction due to the appearance of a paramagnetic center near these protons. The corresponding ^1H nmr signals are identified as arising from four methylene groups of cysteine residues in the ligand environment of the iron. Their chemical shifts do not completely coincide with one another and depend on the temperature in different ways. From this it also follows that an unpaired electron is unequally distributed between two iron atoms (145). Electron exchange between Fe atoms, according to these data, takes place at a low rate, which does not cause either a broadening or a coalescence of nmr signals.

The Mössbauer spectrum of oxidized plant ferredoxin containing ^{57}Fe consists of two overlapping doublets, determined by quadrupole splitting. The splitting constants and chemical shifts show that both atoms of high-spin iron in oxidized ferredoxin are situated in similar environments. Upon reduction the Fe-S signal remains practically unchanged; however, the entire Mössbauer spectrum becomes more complicated

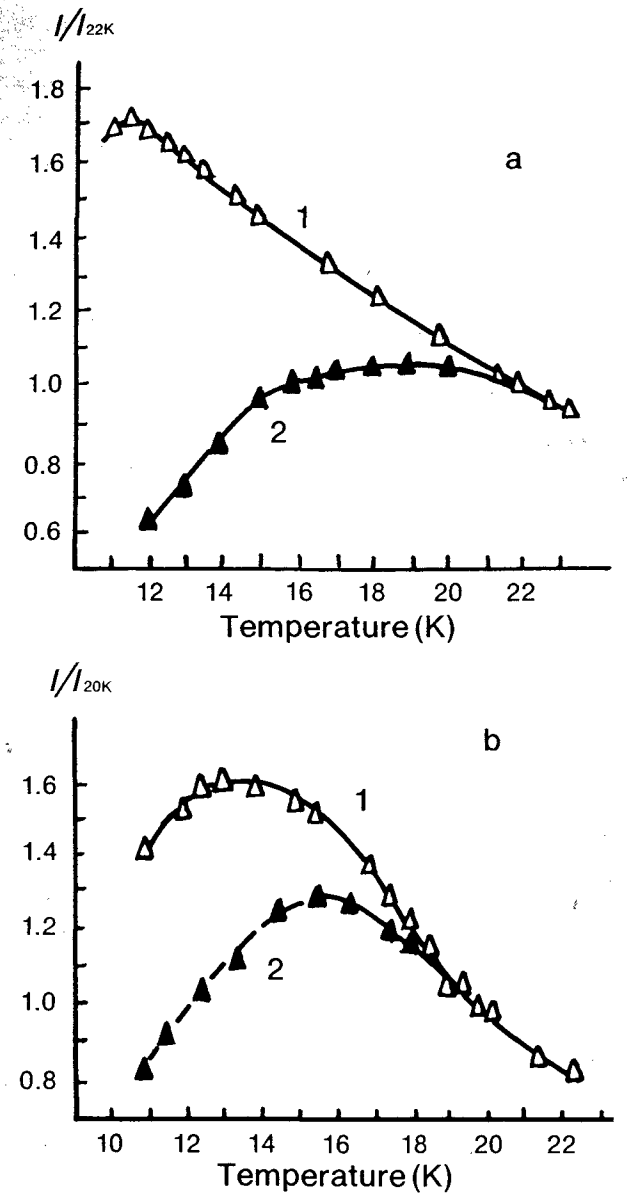


Figure 15. The intensity of the line at $g_x \approx 1.89$ in esr spectra of soluble ferredoxin (a) of bean chloroplasts (b) as a function of the temperature: *1*) nonequilibrium states; *2*) after relaxation to equilibrium. From ref. (150), with permission.

because of the appearance of a new doublet which belongs to an Fe(II) atom, also in a high-spin state.

The values of the exchange integrals, *I*, characterizing the interaction between the iron atoms in spinach ferredoxin are -183 cm^{-1} for oxidized protein and -100 cm^{-1} for reduced protein (146). The Fe-S ferredoxin centers are situated inside the protein globule and are inaccessible to solvent molecules. This conclusion is based on the fact that ferredoxin does not accelerate the magnetic relaxation of water protons as measured by ^{1}H nmr, either in the oxidized or in the reduced state (147). Ferredoxin *in vivo* acts as a one-electron carrier, but *in vitro* a different, two-electron reduction is possible (148, 149).

For soluble ferredoxin and bound Fe-S centers in *Vicia faba* bean chloroplasts, some differences in the state of the active center and its close environment have been found that are caused by the structural nonequilibrium of the system (150). The esr spectra of ferredoxin in solution and Fe-S centers in chloroplasts were observed in the temperature range 10-30 K. The reduction of Fe-S centers was achieved by two methods: 1) by dithionite treatment in the presence of methylviologen; 2) by solvated electrons generated by radiolysis at 77 K. The observed esr spectra of Fe-S proteins reduced by these two methods did not differ from one another in shape. However, the temperature dependence of the intensity of the resonance signal with a *g*-value of 1.89 is quite different (Figure 15). The exchange integral is very sensitive to small distortions of the geometry of the paramagnetic center and its close environment. The decrease of the exchange integral when the structure of the center departs from equilibrium may appreciably enhance the spin-lattice interaction. At fixed temperature the esr signal will be saturated at higher microwave power, and at fixed power the saturation will be achieved at a lower temperature.

It has been suggested that during intense electron flow *in vivo* the rate at which electrons leave the excited P700 centers will be so great that the environment of the Fe-S center will not be able to relax after each reduction and reoxidation, and that in the steady state the protein conformation near the Fe-S center is nonequilibrium. The situation is somewhat similar for Fe-S center reduction in low-temperature radiolysis. The nonequilibrium chemical properties of proteins may be quite different from their equilibrium properties. This has been shown for many proteins *in vitro* (151) and is of importance for biological applications, particularly those involving the biochemistry of electron transfer.

B. Flavodoxin

The functions of soluble ferredoxin, at least in some photophysiological reactions, can be performed by flavodoxin, a protein that contains flavin mononucleotide (FMN) as a cofactor. Some algae use this protein as a photosystem I electron acceptor when there is an Fe deficiency in the medium (152). The reduction of this protein is accompanied by the appearance of an esr singlet signal with a *g*-value of 2.002 from flavosemiquinone radicals. The esr signal from ferredoxin is observed only at quite low temperatures, but that from flavodoxin semiquinone radicals is observed at room temperature. This opens new possibilities, not yet fully exploited, of using the esr technique to study photoinduced

processes *in vivo* without fixing the redox state by freezing.

C. Ferredoxin-NADP Reductase

The next component of the chain accepting electrons from ferredoxin is ferredoxin-NADP reductase, which contains flavin adenine nucleotide (FAD). This protein has been isolated in crystalline form and studied in detail by spectral and biochemical techniques (153, 154). Although flavosemiquinone radicals are one of the classical objects of esr study, radiospectroscopy has not yet made any noticeable contribution to the study of its functions *in situ*. This can be partially explained by the high rate of dismutation of ferredoxin-NADP-reductase flavosemiquinone radicals and by the impossibility of generating these radicals at low temperatures, as in this case electron transfer on flavin does not occur. Ferredoxin-NADP-reductase flavosemiquinone radicals are formed (at room temperature) in photosystem I by a pulse lasting less than 1 μs. The subsequent dismutation of semiquinone radicals with the formation of diamagnetic particles occurs with a half-life of about 300 μs whereas the reoxidation of flavin by NADP requires about 500 μs (155). Evidently at the level of this component various electron-transfer chains effectively exchange electrons, though the mechanism of such an exchange is still unknown.

With that, we complete our survey of reactions occurring in the light stage of photosynthesis on the reducing side of photosystem I. Further processes occur among the soluble components in chloroplast stroma. Though there is little doubt that at least in some of these reactions paramagnetic states appear as intermediate products, these states have not been found by esr *in situ*, and their study is beyond the scope of this review.

D. The Reactions on the Oxidizing Side of Photosystem I

The reactions on the oxidizing side of photosystem I consist of the reduction of $P700^+$ centers due to the interaction with secondary electron donors, also fixed in the thylakoid membranes of the chloroplast. Two membrane-bound proteins, cytochrome *f* and plastocyanin, were considered to be possible immediate electron donors to $P700^+$. The problem of the relative localization of these carriers in the chain was the subject of some controversy, which was reflected in the literature (156). The sequence of electron transfer now appears to be as follows (157, 158):

$$\text{plastohydroquinone} \xrightarrow{20\ \mu s} \text{cytochrome } f \xrightarrow{200\ \mu s} \text{plastocyanin} \xrightarrow{20\ \mu s} \text{P700}$$

though direct electron transfer from plastohydroquinone to plastocyanin, is also possible (155,156). Several milliseconds after short-pulse illumination of *Chlorella* cells, the contact between cytochrome *f* and plastocyanin breaks and is reestablished only after 0.5 s (159). Electron pathway variability at the cytochrome *f* level is considered in connection with the differences in the relative contents of these proteins in the thylakoid membranes, which form grana, and in the agranal regions (159). This is consistent with the assumption that cytochrome *f* participates in cyclic electron flow as the electron donor for plastocyanin and in noncyclic electron transport, plastohydroquinone acts as a direct electron donor for plastocyanin. For some lower plants variability in donor composition (plastocyanin and cytochromes) for P700 was found, dependent on Fe and Cu content of the medium (160).

All the cytochromes in chloroplasts (cytochromes *f*, b_{559}, b_6) are low-spin complexes and so do not give esr signals. The recent results of Beinert et al (161, 162) for mitochondrial cytochromes show, however, that during electron transfer, high-spin complexes can also be formed. The corresponding esr signals at 10 K have very high *g*-values.

Plastocyanin, a copper protein discovered by Katoh (163,164), is easily separated from the thylakoid membranes by sonication or detergent treatment, but unlike soluble ferredoxin it cannot be washed out from chloroplasts by isotonic solutions. This is explained by the fact that plastocyanin is located mainly near the inner surface of the thylakoid membranes, while ferredoxin is located on the outer surface. X-ray diffraction shows that the copper complex in plastocyanin forms a distorted tetrahedral structure in which the cysteine SH group, the methionine thioester group, and the two imidazole N atoms of histidine are copper ligands (165). It is difficult to follow the redox reactions of plastocyanin *in situ* by its absorption spectra (a wide band centered at 597 nm) because of its low extinction coefficient and overlapping with some other more intense bands of other components of the system. Hence the esr technique is valuable for studying plastocyanin function in photosynthesis.

The esr spectrum of oxidized plastocyanin is characterized by axial symmetry with $g_\perp = 2.05$, $g_\parallel = 2.2$, and a small hyperfine splitting, which is unusual for copper complexes. Only the signal corresponding to $g_\parallel$ is distinctly split into four lines. This is due to hyperfine interaction with the copper nucleus ($I = 3/2$) (Figure 16).

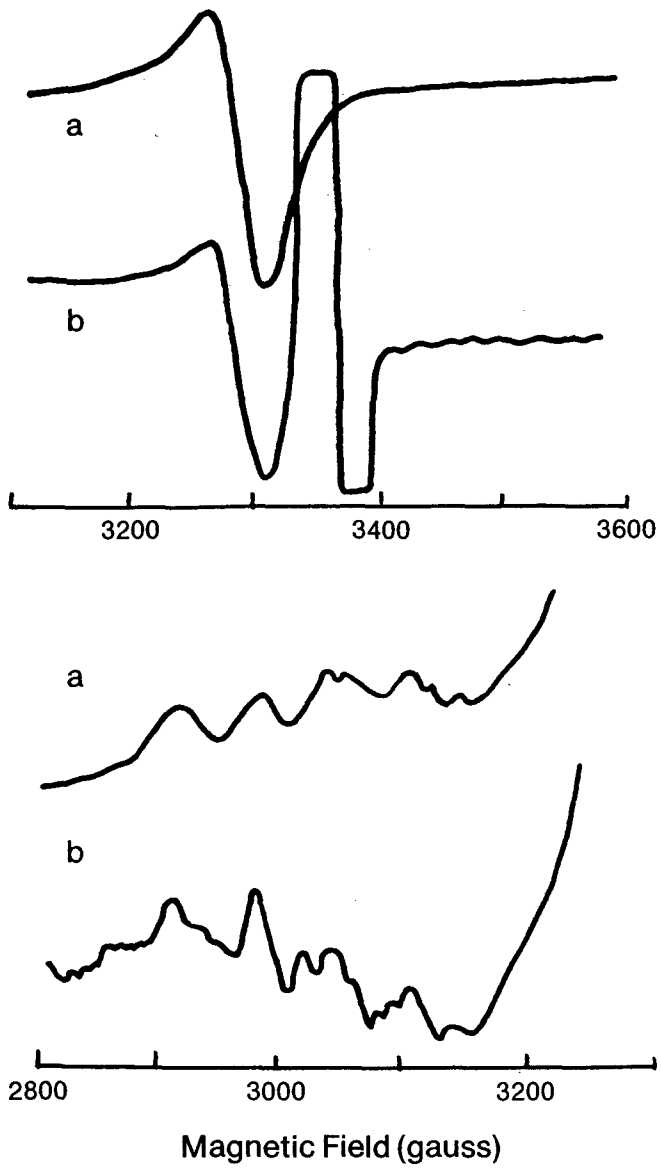


Figure 16. ESR spectrum of oxidized isolated plastocyanin (a) and of membrane-bound plastocyanin in spinach chloroplasts (b) at 25 K. Bottom: the low-field portion of the spectra of these samples. From ref. (166) with permission.

The location of plastocyanin between the two photosystems agrees with the redox potential of this protein (340 mV) and the dependence of its redox state *in situ* on the wavelength of the light. The oxidized plastocyanin esr signal appears on illumination of chloroplasts by far-red light, which mainly excites photosystem I (166,167), and disappears with weak red light, which excites both photosystems (168). For the esr signal of plastocyanin in chloroplasts, no orientation dependence has been observed (87).

Plastocyanin oxidation in chloroplasts in light is observed only at room temperature, but not at 77 K, which shows that plastocyanin reactions with photochemical centers are not primary, but secondary events.

Malkin and Bearden, who first observed the esr signal from oxidized plastocyanin in chloroplasts *in situ* (168), showed that the phosphorylation uncoupler NH_4Cl, which accelerates the electron flow, also stimulates plastocyanin reduction, as do ADP and P_i. Thus, it was shown that the coupling site of electron transport and phosphorylation is situated at a point of the chain preceding plastocyanin. Diuron, which blocks electron transport at the level of the bound acceptor of photosystem II, promotes plastocyanin oxidation.

At present it is not clear whether plastocyanin is the only copper protein in chloroplasts. According to Katoh et al (169), plastocyanin contains only about half of the Cu in chloroplasts. Malkin and Bearden did not find any other esr signals from Cu proteins in chloroplasts. However, this does not exclude the possibility that other Cu complexes are present but are not revealed in the esr spectra. In spite of the ease of the quantitative removal of plastocyanin from chloroplasts, subchloroplast fragments containing photosystem I and photosystem II always contain a certain amount of Cu. This can be observed in the characteristic esr spectra of the Cu complexes, which appear when plant material is treated with xanthate ($ROCS_2^-$) or fructose after acid degradation (170). In heavy subchloroplast particles isolated by treatment with digitonin, triton X-100, and NaCl solutions and thoroughly purified of P700 contamination, a signal from a Cu complex was observed. The temperature dependence of the esr signal from oxidized Cu protein in chloroplasts indicates the heterogeneity of this complex: a part of its esr signal is inhibited as the temperature increases from 13 to 50 K, and another part was observed at an even higher temperature, up to 77 K (171).

IV. PRIMARY REACTIONS IN PHOTOSYSTEM II

A. The Scheme of Electron-Transport Reactions in Photosystem II

In photosystem II electron transfer takes place from water to plastoquinone. Many experiments done on this fragment of the electron-transport chain have made it possible to describe in detail some phenomenological properties of reactions both on the oxidizing and on the reducing sides of photosystem II. However, the immediate participants in the primary photochemical event are far from being as completely characterized as their counterparts in photosystem I. It is now firmly established (mainly by application of high-speed difference spectroscopy and fluorescence techniques) that the

primary event, as in photosystem I, is the photoexcitation of a specific chlorophyll *a* form in the chlorophyll-protein complex. This chlorophyll molecule in the excited state is an electron donor for the primary acceptor and forms a tight complex with it. The principal difference between photosystem II reaction centers and those of photosystem I is that not only the primary acceptor but also the secondary donor form part of this complex so that the oxidized form of the photoexcited pigment is very short-lived. This makes the observation of the corresponding esr signal difficult.

Döring et al (172) found light-induced transient changes of the optical absorption in chloroplasts, with a maximum of about 680 nm. These were ascribed to the oxidation of chlorophyll in the photosystem II reaction center (P680). In native systems the reduction of $P680^+$ takes place in less than 10 μs (173). Hence it is not surprising that the photooxidation product could not be identified by the esr technique in the same way as in photosystem I. From various optical measurements, one can give the following scheme for the photosystem II electron-transport reactions:

$$YDPa_1a_2a_3 \xrightarrow{\sim 10ns} YDP^+a_1^-a_2a_3 \xrightarrow{<10\mu s} YD^+Pa_1a_2^-a_3 \xrightarrow{\sim 200\mu s} Y^+DPa_1a_2^-a_3 \xrightarrow{\sim 1\ ms} Y^+DPa_1a_2a_3^-$$

$$\uparrow \quad 30\text{-}150\mu s \quad \downarrow$$

In this scheme P is the photoexcited pigment P680; D is the secondary electron donor, whose nature is unknown; Y is the enzyme system of water decomposition; and a_i is the ith electron acceptor. The acceptors have been identified as pheophytin *a* (a_1), bound plastoquinone (a_2), and free plastoquinone in the plastoquinone pool between the two photosystems (a_3). All half-lives are given for room temperature, and for completely native samples the reduction time of $P680^+$ by the secondary donor D, according to Mathis's data (174), is about 30 ns, and in chloroplasts carefully treated with tris buffer it is about 10 μs. The reactions among P, D, a_1, and a_2 in both forward and backward directions take place at a high rate and also at low temperatures. The remaining components do not take part in electron transfer in frozen samples. The total electron-transport rate in this chain is limited by the rate of a_3 (plastoquinone) reduction. However, there are steps of charge recombination of P^+ with a_1^- and a_2^-, which occur at a high rate. Charge recombination in a normally functioning system does not occur because it is inhibited by the fast oxidation of D. Thus, this secondary reaction ensures the stabilization of separated charges in photosystem II. Such a donor does not exist in photosystem I. Separated charge stabilization in photosystem I is provided by fast electron transfer to a number of secondary acceptors, which recombine with $P700^+$ only at a very slow rate.

B. ESR Signals from Oxidized Chlorophyll in Photosystem II

It is clear from the primary reaction scheme that the steady-state concentration of $P680^+$ cannot be high if all reactions proceed at their normal rates.

In experiments at cryogenic temperatures it was possible to slow down some of these reactions, thus making some of their intermediate products accessible for esr observations. Malkin and Bearden (175) used short laser pulses to illuminate a suspension of frozen (at 15 K) chloroplasts and subchloroplast particles, enriched in photosystem II. They discovered a singlet signal, reversible in darkness, with a lifetime of about 30 ms (Figure 17). At oxidative potentials of the medium, an esr signal of similar shape, irreversible in darkness, appeared together with this short-lived signal. It was assumed to belong to one of the alternative secondary donors. It is possible that this donor is active only when other donors are previously oxidized and is yet another chlorophyll molecule in the P680 environment. The appearance of the light-induced, dark-reversible signal from oxidized chlorophyll in photosystem II in the temperature range 108-180 K is described in (176).

At room temperature the following two conditions must be fulfilled for the observation of $P680^+$: 1) the reactions with the secondary donor D must be inhibited, or the donor D must be oxidized; 2) the recombination reaction with acceptor a_2 must also be inhibited. The first condition is easy to fulfill. It is enough to slow down or completely stop the oxidation of water (through enzyme system Y), for D to be oxidized under steady-state illumination. The second condition is not so easily achieved. The solution is to replace the reaction with the normal acceptor a_3, which limits the rate of consecutive electron transfer, by a more effective process that does not use a_3. For this purpose silicomolybdate was used as an electron acceptor; this accepts electrons directly from acceptor a_2 (bound plastoquinone) in a reaction insensitive to diuron (177,178). In the presence of silicomolybdate, illuminated subchloroplast particles (which contain practically no $P700^+$ contamination as judged by optical or esr criteria) emit a singlet esr signal with $g \approx 2.0025$ of 9G line width. This signal is fast reversible in the dark (Figure 18, (179)). The difference in

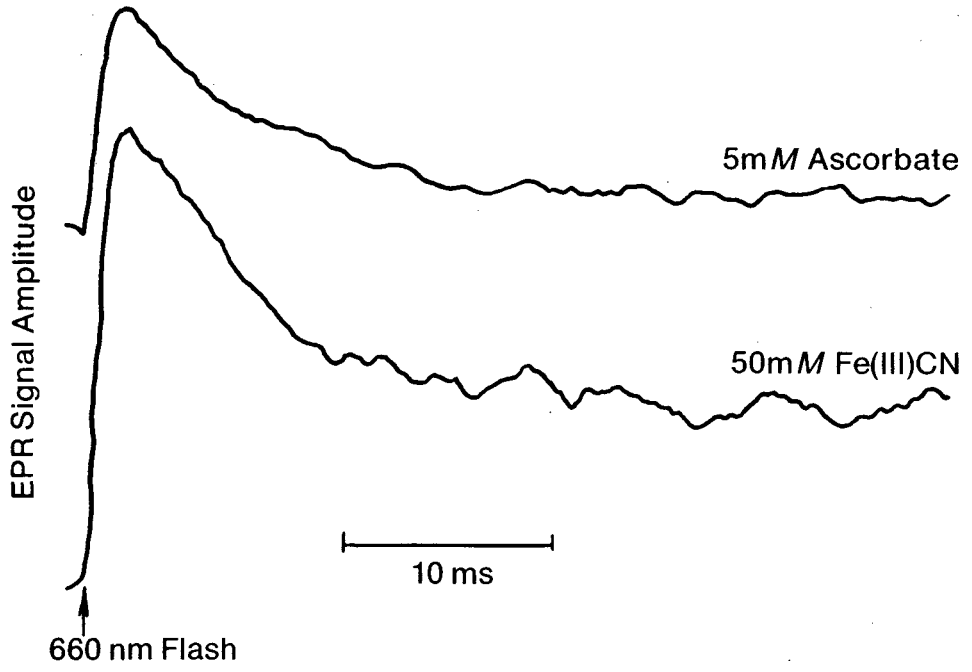


Figure 17. The kinetics of flash-induced change of the esr signal at $g \approx 2.0025$ at 35 K. Chloroplast fragments were incubated with ferricyanide or ascorbate prior to flash activation at 660 nm. The reversible part of the signal has been attributed to $P680^+$. The dark decay corresponds to the back reaction between $P680^+$ and the reduced primary acceptor of photosystem II. From ref. (175), with permission.

line widths between the light-induced signal of photosystem II (9G) and the esr signal I ($P700^+$, 7.5 G) was not related to the electron acceptor's nature: photosystem I particles in light with silicomolybdate gave a normal esr I signal. With other acceptors, such as ferricyanide, photosystem II particles did not give rise to a signal in light that was reversible in darkness. The esr signal of photosystem II was appreciably different from the esr I signal from chloroplasts in its dependence on the light intensity (Figure 19). Its dark decay at 0° C can be approximated by a bi-exponential function with characteristic times 13.5 s and 2.1 s. Initially the preparation contained approximately equal amounts of these two kinetically distinct centers. The steady-state intensity of the light-induced signal from photosystem II was determined by the rate of its dark decay both when the temperature varied and when electron donors of photosystem II (hydroxylamine, Mn^{2+} ions) were introduced. This is consistent with the assumption that the signal appears as a result of photooxidation, and not as the result of secondary dark oxidation of any component acting as an electron donor for $P680^+$ centers. Even if a secondary electron donor is the source of the signal, it must be very close, in both its structure and in its functional properties, to the primary photooxidizable electron donor of photosystem II.

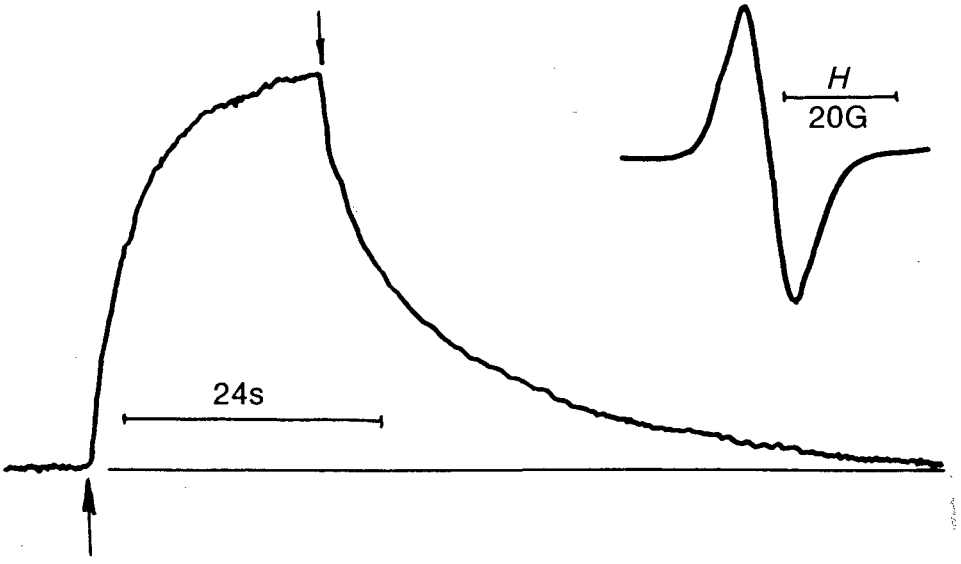


Figure 18. ESR signal at $g \approx 2.0025$ in photosystem II fragments in the light and the kinetics of its rise under saturating light (12 W/m²) and dark decay at 0°C in the presence of 1m*M* silicomolybdate. [From ref. (182)]

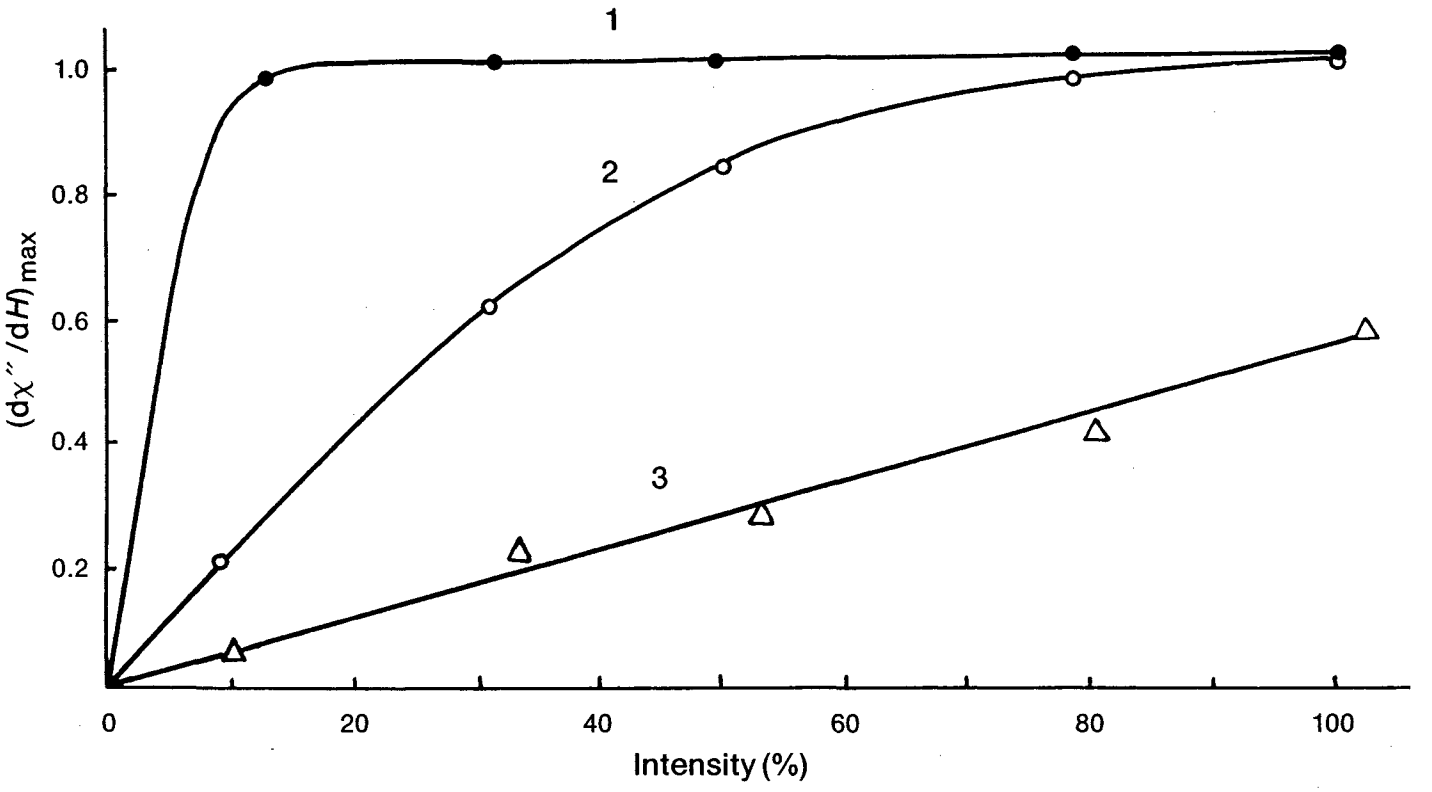


Figure 19. The light saturation curves of esr signals: *1*) signal I from diuron-treated chloroplasts; *2,3*) silicomolybdate-dependent signal from photosystem II particles; *1,3*) at 22°C; *2*) at 2°C; 100% of light corresponds to 12 W/m². Intensity is in percent, and $(d\chi''/dH)_{max}$ is the amplitude of the first derivative of esr absorption.

The accessibility of the bound electron acceptor Q (or A_2) for external electron donors and acceptors increases as the *p*H of the medium decreases (180). At *p*H 4 this led to the appearance in light of a singlet signal from oxidized chlorophyll, which quickly decays in the dark (181). This chlorophyll may either be the photooxidized primary donor or one of the alternative secondary donors of photosystem II.

Silicomolybdate does not lead to the appearance of an esr singlet signal under illumination of photosystem II preparations at 77 K. Evidently, this is related to the fact that at 77 K silicomolybdate does not oxidize the donor which is accessible for the $P680^+$ centers. The properties of the silicomolybdate-dependent esr signal can be studied at low temperatures, if this signal is "frozen in" by cooling the samples in light. At room temperature the photosystem II signal does not differ from the $P700^+$ signal in its relaxation properties. However, at 77 K the microwave-saturation curves of these two signals are quite different (182). The esr signals were compared with the optical effects induced by light in photosystem II preparations in the presence of silicomolybdate, and it was shown (183) that the appearance of the signal correlates with photobleaching, with maxima at 685 nm and 435 nm. This spectrum coincides with that obtained by Döring et al (172), who attributed it to the photooxidized pigment in the photosystem II reaction center. Optical changes were compared with the intensity of the photoinduced singlet signal calibrated by the esr signal of nitroxyl radical, and the molar extinction of the corresponding center was evaluated (0.66×10^5 $M^{-1}cm^{-1}$ (184), coinciding with the molar extinction of P700 pigment, from the data of Hijama and Ke (185)).

When studying reactions induced by light or oxidants, it is always necessary to consider the possibility of pigment oxidation in the light-harvesting matrix. This process does in fact occur at room temperature in the presence of certain combinations of oxidants. For example, if a mixture of silicomolybdate and potassium chloroiridate is present in photosynthetic preparations, including those that contain no P700, an esr signal appears in light and is stable for tens of minutes in darkness (182). On the background of this dark signal, a dark-reversible signal appears from photosystem II fragments in light; the latter is identical with the one described above. A mixture of chlorophylls *a* and *b* in an aqueous-alcohol medium in the presence of this combination of oxidants gives the same dark-stable signal. A similar signal was obtained from a suspension of photosystem II particles that were pretreated at 80°C

for 10 min. The esr signal reversible in darkness was not observed in this case.

Pigment oxidation in the light-harvesting matrix was also observed in photosystem I preparations at low-temperature illumination, in the presence of a large excess of oxidants (77). The esr signal differs in its relaxation parameters from the esr signal I from the P700 centers. Its appearance in light at 15 K is not accompanied by the reduction of acceptor Fe-S centers. This fact can be used to differentiate the $P700^+$ signal and the signal from oxidized antenna chlorophyll (85). In photosystem II preparations at cryogenic temperatures in the presence of excess ferricyanide, an esr signal also appears in light (186), which must come from oxidized matrix chlorophyll or from a secondary donor of chlorophyll nature. All these signals are stable in darkness.

C. Bound Acceptors in Photosystem II

The role of plastoquinone as one of the participants in the earliest photosystem II reactions induced by light absorption at very low temperatures was shown using esr techniques by Knaff et al (187). After vigorous extraction of plastoquinone from photosystem II subchloroplast particles, the reversible component of the photoinduced esr signal from oxidized chlorophyll (which had previously been observed for oxidizing potentials of the medium at 15 K) was lost. This component reappears upon reconstruction with plastoquinone. The charge recombination time of $P680^+$ and the bound plastosemiquinone radical indicated by these data is about 3-5 ms at 15 K. The formation of plastosemiquinone radicals by photosystem II photoexcitation was shown earlier by differential absorption spectroscopy (188). These results, however, do not exclude the existence of other carriers between the P680 center and the bound plastoquinone, which recombine with P680 at rates too high for esr observations.

According to Klevanik et al (75, 76), the photosystem II primary acceptor is pheophytin *a*, which forms a tight complex with P680. Its reduction product, the pheophytin radical anion, is identified by its differential optical spectrum. The accumulation of pheophytin radical anions upon steady-state illumination of photosystem II subchloroplast fragments in the presence of diuron, which blocks the oxidation of bound plastosemiquinone at negative redox potentials of the medium (-200 mV), occurs through the formation of a complex in the state [P680 pheo $a^{-\cdot}$ $Pq^{-\cdot}$]. The recombination of $P680^+$ and pheo *a* is inhibited by a not very effective but ever operative process, the reduction of $P680^+$ by an exogenous electron donor present in excess. However, attempts to observe the esr signal from the pheophytin radical anion in these conditions have so far been unsuccessful as have attempts to observe the esr signal from the plastosemiquinone radical anion of the reduced acceptor Q^-. The reason for this is probably the coupling among the various paramagnetic centers (for example, between the pheophytin radical anion and the plastosemiquinone radical anion) or the formation of a complex with a transition metal ion, such as iron. A similar problem was solved earlier in connection with the charge-separation mechanism in bacterial photosynthesis (74). A signal from the ubisemiquinone radical anion in bacteria grown on a medium deficient in Fe in the presence of oxidants was observed at room temperature (24). The esr signal from the bacteriopheophytin radical anion was observed at temperatures below 17 K (189). It had a *g*-factor of 2.003 and was split into components separated by intervals of 63 G.

It now seems probable that iron components not identical with the components related to photosystem I are present near the reaction centers of photosystem I. As was mentioned above, no esr signals typical for Fe-S centers on the acceptor side of photosystem I were found in preparations of subchloroplast particles of photosystem II. Nevertheless, these preparations contain iron in significant amounts (190). This is not surprising in itself, since heme iron is contained in photosystem II as cytochrome b_{559}. However, a part of the iron in these preparations is clearly not contained in a heme complex. This is shown by the formation of the nitrosyl-Fe complex in the presence of nitrite and cysteine with characteristic esr signals at $g \approx 2.03$ at 77 K (191).

V. ESR SIGNAL II AND THE REACTIONS ON THE OXIDIZING SIDE OF PHOTOSYSTEM II

The esr signal II at $g \approx 2.0046$ with partially resolved hyperfine structure (Figure 3) is observed in all photosynthesizing organisms capable of oxygen evolution. The most convenient particles for studying this signal are subchloroplast fragments enriched in photosystem II and purified from P700, algae mutants inactive in photosystem I reaction, and chloroplasts adapted to darkness, especially in anaerobic conditions. The esr signal II is not observed in photosynthesizing bacteria and algae mutants incapable of oxygen evolution. These are the facts that support the assertion that the esr signal II comes from photosystem II.

A whole range of modern methods has been used in the study of the paramagnetic centers responsible for the esr signal II. Methods have involved comparison of various mutants and fractions of photosynthetic organisms, variations in the illumination and composition of the medium, the use of isotopically pure media for growing algae, the application of inhibitors, electron

donors and acceptors, the use of kinetic measurements and models *in vitro*. Earlier experiments have been reviewed by Kohl (192). It has been shown that the partially resolved hyperfine structure of the esr signal II is caused by protons, since it disappears for algae grown on D_2O. It now seems sure that the sources of the esr signal II are plastosemiquinone radicals that arise from a certain fraction of the plastoquinones present in chloroplasts and algae in great excess compared with the other electron carriers in thylakoid membranes. The most convincing argument in favor of this identification is the fact that the esr signal II from chloroplasts, which disappears when plastoquinone is extracted, is restored to its original shape when exogenous plastoquinone is introduced. If fully deuterated exogenous plastoquinone is introduced, the signal becomes narrower and structureless (193). Nevertheless, it was found impossible to reproduce completely the asymmetric shape of the esr signal observed *in vivo* by using isolated plastosemiquinone *in vitro*.

The esr signal II is not related to noncyclic electron transport between the two photosystems, as it is stable in darkness for many hours, nor is it related to the early reactions in the photochemical centers. This is because when preparations adapted to darkness are excited at room temperature by short light pulses, the esr signal II increases within seconds. Recently, however, the appearance of a light-induced esr signal with the characteristics of signal II has been reported from subchloroplast particles enriched in photosystem II at cryogenic temperatures.*

The plastoquinones, the source of this signal, are a group of structurally related compounds (Figure 20). In chloroplasts, plastoquinone A is the main component of this group, about 50 moles per mole of reaction center (194). About 6-10 plastoquinone molecules take part in the electron transfer between the two photosystems. (This process will be considered in the next section.) The functions of the remaining part of the plastoquinone pool are not yet clear. Plastoquinone is easily extracted from chloroplasts. This destroys some obviously secondary electron-transport reactions. Plastoquinone, which seems to be the only low-molecular-weight carrier in thylakoid membranes, is thought to be capable of transferring electrons between donors and acceptors fixed in the membrane and separated by a hydrophobic barrier. This property of plastoquinone has been recently illustrated in model membranes separating donor and acceptor solutions (195).

Semiquinones are a traditional object of esr research. Since Michaelis (196), it has been accepted that the

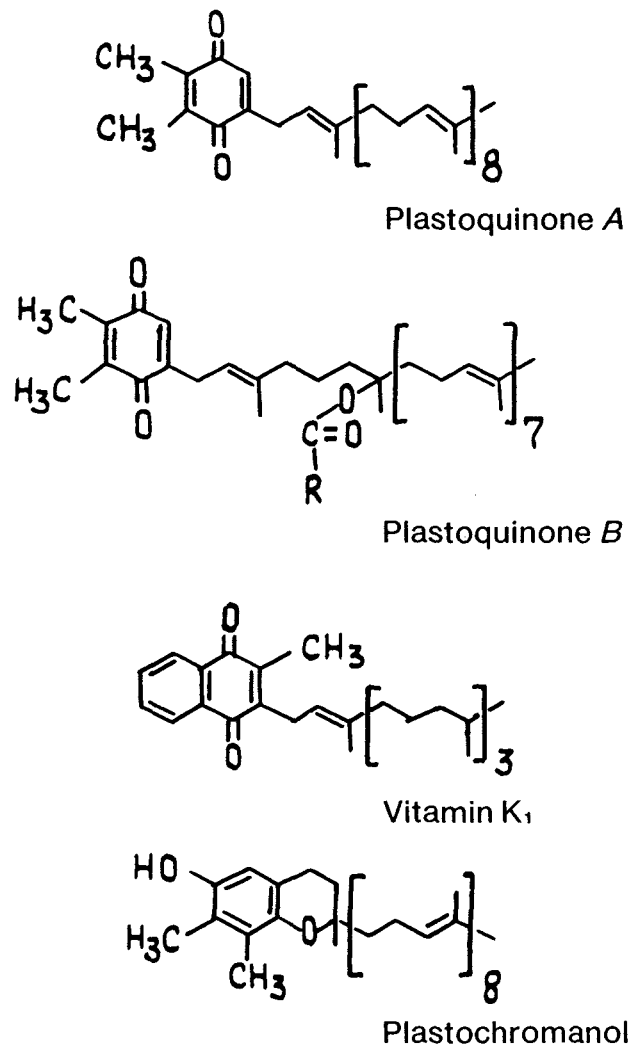


Figure 20. Structures of some prenylquinones.

redox reactions involving quinones proceed by a one-electron mechanism, i.e., semiquinone radicals or radical anions are formed as intermediates. The observation of the redox transformation *in situ* is made difficult, however, because of the absence of intense absorption bands in both the oxidized and the reduced form. The esr signals from plastosemiquinone radicals *in situ* overlap with the strong esr signals from oxidized chlorophyll. The lifetime and the steady-state concentration of plastosemiquinone radicals are rather small since equilibrium in their dismutation reaction is shifted far towards the diamagnetic products, unless there are special reasons, such as limited mobility in membranes, that interfere with the establishment of this equilibrium.

Due to the recent work of Sauer and co-workers (197-204), significant progress has been made in the

*J. H. A. Nugent and M. C. W. Evans, *FEBS Lett.* **101,** 101 (1979).

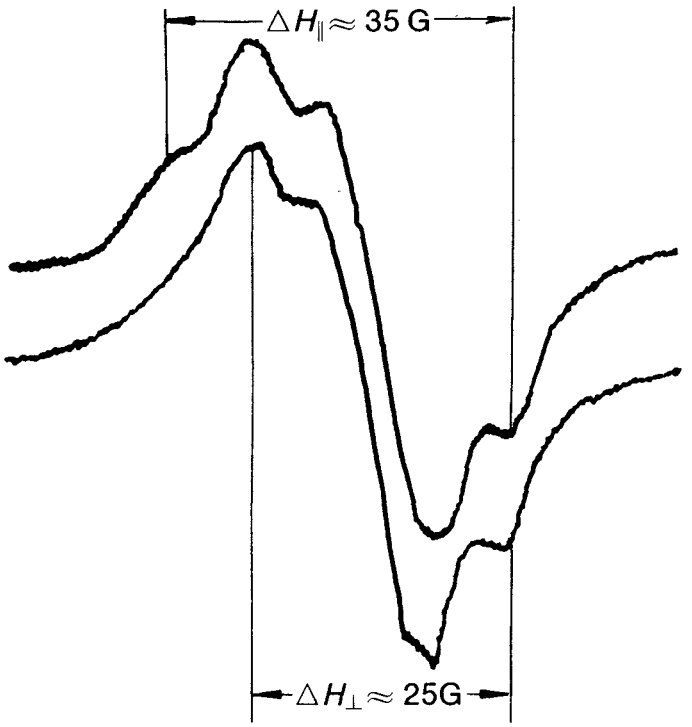


Figure 21. The esr signal II from bean chloroplasts cooled to −120°C in a magnetic field of ca 10 kG in the initial orientation, and after rotation of the sample by 90°.

understanding of plastoquinone functions and the nature of the esr signal II. Three kinetic components of the signal II were found: S_{const}, S_{slow}, and S_{fast}. The esr signal II S_{const} is observed in darkness and does not decay even after leaves are aged for several days in darkness. Chloroplast aging, heating with Cl-CCP [carbonyl cyanide *m*-chlorophenyl hydrazone], incubation with 0.8 *M* tris-HCl and divalent cations, higher alcohols (205-208), other treatments causing the inactivation of the system of water decomposition, and other reactions on the oxidizing side of photosystem II, induce the decay of signal II S_{const}.

After long adaptation to darkness the signal S_{slow} appears from chloroplasts in light. When illumination is by a series of flashes there is a periodic component in the increase of the esr signal II, with maxima at the second and sixth flashes. It follows that the free radical arises from the interaction of the diamagnetic precursor with certain of the intermediate states of the water decomposition system (states S_2, S_3 in Kok's terminology) (210). At the same time a monotonic increase of the signal in response to the flashes shows that it cannot be identified with any particular S_i state, since the concentration of each of them must oscillate with a period of four flashes.

The anisotropy of the esr signal II (S_{const} and S_{slow}) is in itself an indication of the fact that the corresponding free radical centers are not in a state of rapid rotation, i.e., the anisotropic hyperfine structure is not averaged. Experiments with magnetically oriented chloroplasts show that free radical centers are not only immobilized but also rigidly oriented relative to the thylakoid membrane (98,211). Figure 21 shows the esr signals from pea chloroplasts in suspensions cooled to −120°C in a magnetic field of about 10 kG both at the initial orientation and after a 90° rotation. The disappearance of the low-field shoulder when the sample is rotated seems to mean that the *A*-tensor component corresponding to maximal splitting is oriented normal to the plane of the thylakoid membrane. Similar results were obtained with whole cells of the alga *Chlamydomonas reinhardi* (mutant deficient in photosystem II (212)).

There are no orientation effects in photosystem II subchloroplast particles because shape asymmetry is lost upon fragmentation. The dependence of the shape of the esr signal II on chloroplast orientation in the magnetic field was also observed at room temperature for the chloroplasts oriented by hydrodynamic flow (98).

In chloroplasts with water splitting inactivated by removal of Mn^{2+}, an esr signal was observed whose parameters were close to those of the steady-state esr signal II. The signal appears in light in less than 100 μs and decays in darkness after about 2 s (II S_{fast} (201)). This signal is inhibited by diuron, which is an inhibitor of noncyclic electron transport. In its intensity this fast signal II approximately corresponds to the content of the P700 centers. In fully active chloroplasts a much more short-lived signal of roughly similar shape ($\tau_{1/2} \leq 100$ μs for decay) was observed instead of II S_{fast} (203-204). This signal is saturated by low microwave power. At such a power no saturation of other components of the esr signal II has been observed. The changes in relaxation and kinetic characteristics seem to be caused by the removal of paramagnetic Mn^{2+} ions. The substitution of Ni^{2+} for Mn^{2+} partially restored the signal's initial dependence on microwave power without any change in its lifetime. The data obtained make it possible to attribute the function of the physiological electron donor of photosystem II to the diamagnetic precursor of the center responsible for the fast or very fast esr signal II. It does not seem to be the direct electron donor for $P680^+$ pigment, but it acts on the later stages of electron transport on the oxidation side of photosystem II.

The problem of the nonhomogeneity of the paramagnetic centers responsible for the esr signal II has also been tackled by means of radiospectroscopy. In highly native samples (whole leaves of higher plants (213)) the signal is saturated at room temperature at a microwave power of about 25 mW, and the saturation is accompanied by a change in the signal shape. The shape change under saturation was also observed for chloroplasts from various sources (214).

The electron spin-echo resonance technique has

been recently used by Nishimura et al (209) for the investigation of esr signal II. They also concluded that signal II consists of at least three "dark" components that differ in their relaxation times.

The centers responsible for the esr signal II do not seem to be readily accessible for exogenous electron donors and acceptors. Nevertheless, in the presence of certain electron-transport mediators and in combination with other treatments destabilizing the intermediate states of the water-splitting system, electron donors tend to inhibit the esr signal II. In this connection it is natural to suppose that it is formed by plastohydroquinone oxidation and not by plastoquinone reduction. In photosystem II fragments dichlorophenolindophenol, an effective electron-transport mediator, inhibits the esr signal II in darkness, but does not interfere with its increase in light. A characteristic feature of the esr signal II is its inhibition in light and in darkness in various photosynthetic preparations (chloroplasts, photosystem II fragments, algae) in the presence of silicomolybdate (179,182). Evidently, redox agents can inhibit the esr signal II both by the oxidation of plastosemiquinone radicals (silicomolybdate) and by their reduction (reduced dichlorophenolindophenol). The high reactivity of silicomolybdate towards plastosemiquinone radicals is in agreement with its ability to accept electrons from the reduced bound acceptor Q of photosystem II, which is also a plastosemiquinone radical. Hydrophilic redox agents and redox agents not permeating the membrane (ferricyanide, ascorbate) do not affect the intensity of the esr signal II in darkness. In light, ferricyanide induces an increase of the esr signal II. This light-induced signal quickly decays in darkness. When the kinetics of the light-dark transitions of the esr signal II in the presence of dichlorophenolindophenol and ferricyanide, are compared, it becomes evident that the reactions in the presence of both these acceptors are quite independent from each other and involve different centers contributing to the total esr signal II (Figure 22). It is interesting that the relaxation characteristics of these two types of paramagnetic centers are also different, as follows from a comparison of the shape and microwave saturation of corresponding signals at 77 K.

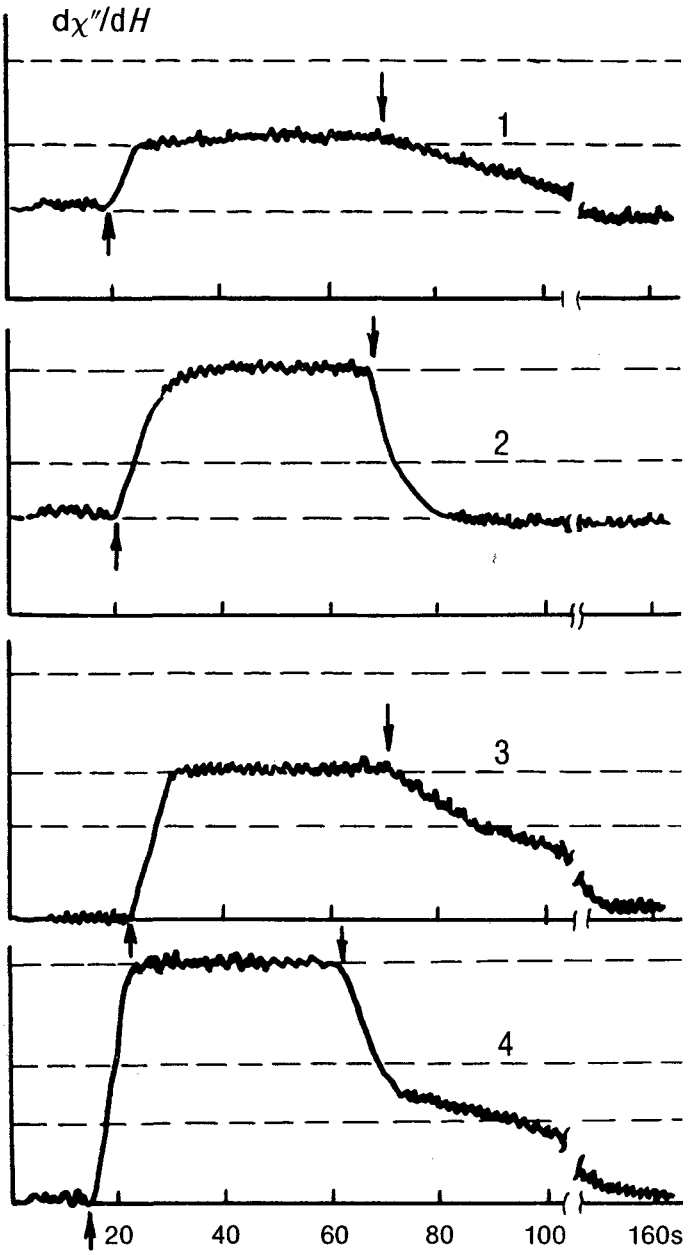


Figure 22. Some typical recordings of the increase of the esr signal II from photosystem II particles under illumination and its dark decay: *1*) without additives; *2*) in the presence of 1 m*M* ferricyanide, *3*) in the presence of 1 m*M* 2,6-dichlorophenolindophenol; *4*) with both oxidants in the medium.

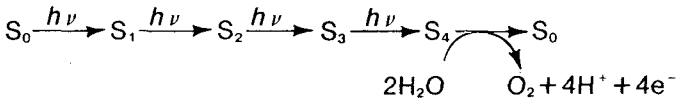
VI. Mn(II) IONS AND THE WATER-SPLITTING SYSTEM

The distribution of metals in photosynthetic membrane fractions (168, 170, 190, 215), the effect of chelating agents on O_2 evolution by chloroplasts in the presence of silicomolybdate (216, 217), and some other facts indicate that transition ions participate in reactions on the oxidizing side of photosystem II. Most attention has been given to the study of the function of Mn in the water-splitting system.

A. Charge Accumulation in the Water-Splitting System

Water oxidation with O_2 formation requires the accumulation of four oxidizing equivalents. Joliot's experiments (218) showed that when chloroplasts and algae are illuminated by short light flashes, O_2 release is a periodic function of the number of the flashes (with a

period of four flashes). Hence it follows unambiguously that oxidizing equivalents are accumulated one by one in every electron-transport chain and that there is no cooperation among different photosystem II centers in the process of water splitting. These postulates form the basis of the phenomenological scheme due to Kok et al (210), according to which water splitting goes through a sequence of five states with the absorption of four light quanta:



Many experimental results have given more information about the details of this scheme, such as the individual properties of the S_i states, their lifetimes, their dependence on the redox potentials of the medium, and so on. However, the nature of these states is still not clear, the chemical composition of the complexes in which the positive charges accumulate is not known, and attempts to isolate the water-splitting complex from the photosynthetic membranes have failed. Mn participation in photosynthetic O_2 production and the independence of other electron-transport steps on the presence of Mn is reviewed in ref. (219). Mn redox reactions are thought to play a central role in the suggested models of water splitting (220).

B. ESR Spectra of Hydrated Mn(II) Ions

ESR signals from hydrated Mn(II) ions in photosynthetic systems are observable at room temperature in highly native specimens (leaves, algae, chloroplasts). Almost all the experimental data obtained show that this signal does not come from functionally active Mn in the water-splitting system. Thus, functionally active Mn does not give any esr signal in normal observation conditions, and its presence can be proved only after cell (chloroplast) destruction and Mn extraction by treatments that destroy the water-splitting system. Among these are treatments with acids (down to *p*H 4), cyanide, 0.8 *M* tris-HCl, and high concentrations of divalent cations (221-223).

The esr spectrum of hydrated Mn(II) ions in solution is a sextet of lines of approximately equal intensity, caused by hyperfine splitting on the ^{55}Mn nucleus with nuclear spin $I = 5/2$. The Mn^{2+} esr spectrum is described by a spin-Hamiltonian

$$\mathcal{H} = g\beta H + AIS$$

where $g = 2.003$, $A = 94.5$ G. If the symmetry of the system is lower than octahedral, a quadratic term is added to this spin-Hamiltonian, corresponding to zero field splitting

$$\mathcal{H}_{zFS} = D[S_z^2 - \tfrac{1}{3}S(S+1)] + E(S_x^2 - S_y^2)$$

where D and E are the zero-field-splitting parameters. It leads to the appearance of five groups of lines due to the fine structure, corresponding to transitions $\Delta M_s = \pm 1$ between the levels $M_s = \pm 5/2, \pm 3/2, \pm 1/2$. However, these transitions, except between $M_s = \pm 1/2$, are highly anisotropic and the corresponding lines are broadened in powder samples so that they become unobservable. In this case, the spectrum looks like the familiar sextet, caused by the transitions between the levels $M_s = \pm 1/2$.

In biological systems, as well as in solutions of Mn(II) complexes, molecular rotation can average the anisotropy of the zero field splitting, if $\tau_c^{-1} > \mathcal{H}_{zFS}$ (where τ_c is the rotational correlation time and $\mathcal{H}_{zFS}$ is in frequency units). In this case the whole spectrum collapses to the center, and its observed intensity is increased due to the contribution of the transitions between other spin levels. This situation occurs also with hydrated Mn(II) ions. For the complexes with macromolecules τ_c^{-1} is often less than $\mathcal{H}_{zFS}$. In this case there is no averaging of the anisotropy of the zero field splitting, and only the lines corresponding to the transitions between the levels $M_s = \pm 1/2$ are observed, i.e., the spectrum decreases to about one fifth of its total intensity. Siderer et al (224) have obtained data that indicate that at least a part of the Mn(II) bound in chloroplasts is in the form of molecular complexes with $\tau_c < \mathcal{H}_{zFS}$. The intensity of the Mn(II) sextet in lettuce chloroplasts was four to five times less than the signal from the same quantity of Mn(II) in water solution. In our laboratory, measurements have been made of the Mn(II) esr signal from chloroplast suspensions, from supernatants of centrifuged chloroplasts, and from bound Mn in chloroplasts (in the last case measurements were made after chloroplasts were treated with acid). These measurements did not reveal significant contribution of bound Mn to the total signal from the suspension (225).

The above-mentioned approach is not applicable in the case of highly effective spin-orbital coupling. For this case intense groups of lines occur typically in the low field region (226, 227). This type of signal has been observed at room temperature in concentrated chloroplast preparations from bean leaves (225).

C. Displacement, Binding, and Photooxidation of Mn(II) Ions by Chloroplasts

The study of the state and the functions of Mn(II) in photosynthetic systems follows different lines, viz., 1)

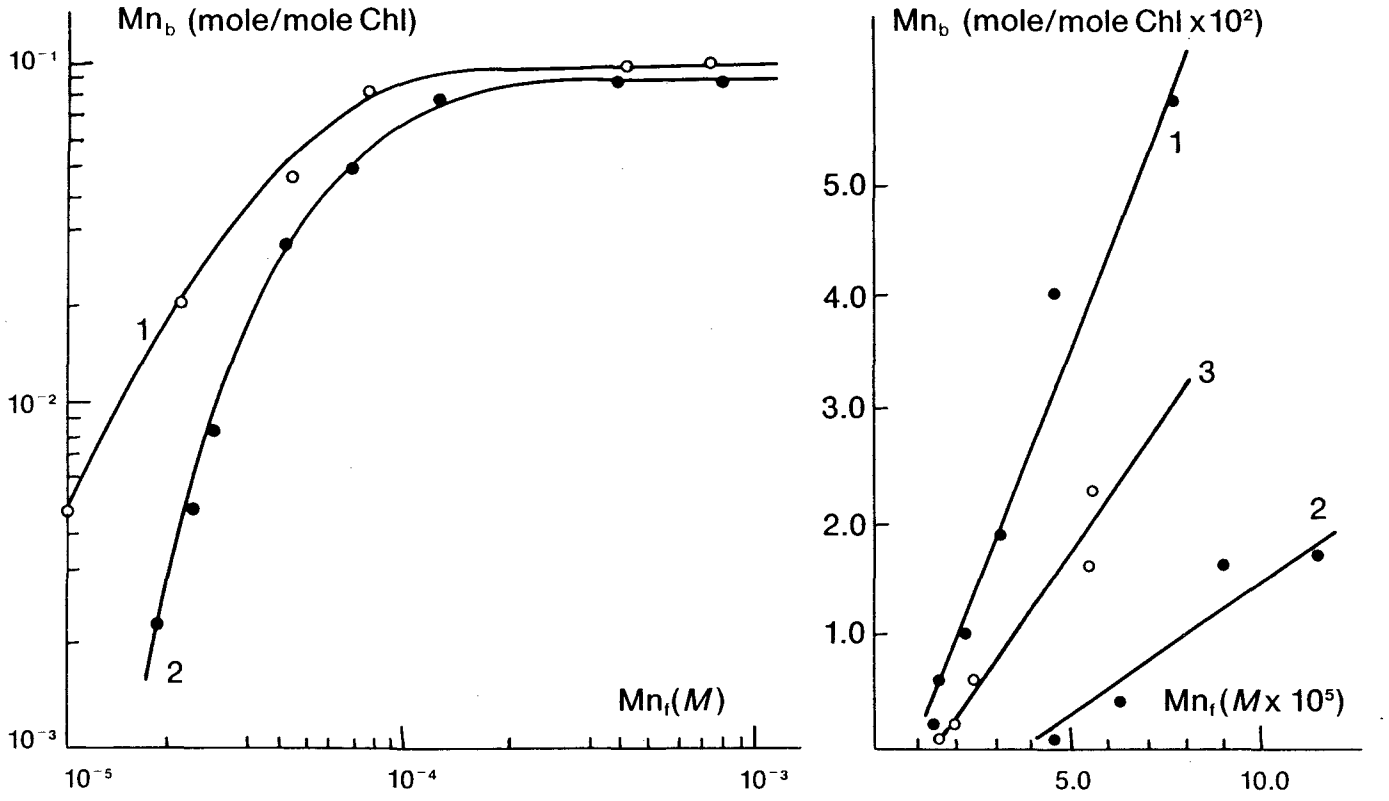


Figure 23. Binding curves of exogeneous Mn(II) by chloroplasts from bean leaves; *1*) chloroplasts treated with 0.8 *M* tris-HCl, *p*H 7.8; *2*) intact chloroplasts; *3*) tris-pretreated chloroplasts with a constant total cation concentration, $[Mn^{2+}] + [Mg^{2+}] = 0.8$ m*M*.

the binding of exogenous Mn(II) by chloroplasts, either intact or after displacement of bound Mn by mild treatments, which allow many types of photosynthetic activity to be preserved, 2) the electron-donor function of Mn in chloroplasts, 3) the search for conditions that enable observation of intrinsic Mn esr signals in photosynthetic systems. In addition, the technique of nmr relaxation of water protons has recently been suggested for the study of the Mn state in chloroplasts (228).

Spinach and bean chloroplasts, washed out by the usual incubation solution and actively evolving O_2 in light, contain six to ten Mn atoms per electron-transport chain. Further extraction of Mn leads to inactivation of O_2 release, though the ability to oxidize various artificial electron donors to photosystem II is still preserved. Treatments mild enough to allow reactivation by the introduction of exogenous Mn(II) do not lead to the displacement of all endogenous Mn. However, photosynthetic membranes can bind much greater amounts of Mn(II) (225). Figure 23 shows the binding of exogenous Mn(II) by intact chloroplasts and by chloroplasts inactivated by treatment with tris. The maximum amount of bound Mn is about 80 atoms per electron-transport chain, i.e., it is much higher than the amount of Mn(II) necessary for O_2 evolution. The characteristic feature of the binding curve is its *S* shape, which indicates the cooperativity of the binding. For chloroplasts treated with 0.8 *M* tris-HCl, the point of inflection shifts in the direction of smaller free Mn(II) concentrations, but it is still distinctly seen. The *S* shape is not a nonspecific cation effect; in fact, the Mn(II) sorption curve in the presence of Mg^{2+}, providing constant total cation concentration in the solution, has the same *S* shape as for the binding of Mn(II) in the absence of Mg. The nature of the cooperativity is not yet clear. At the present stage of research two hypotheses can be suggested: 1) The binding of the first Mn(II) ions causes a change in the membrane structure that is favorable for further binding. 2) Binding is accompanied by a change in the oxidation state of Mn, and stable oxidation and binding take place only when there are no less than two Mn ions at the given membrane locus. For example, Mn(II) is oxidized to Mn(III), but the latter is strongly held by membranes only after dismutation into Mn(II) and Mn(IV). This process is probable because in neutral solutions the Mn(IV) state is more stable than the Mn(III) state. For the dismutation reaction to be possible at a given membrane locus there must be at least two Mn(II) ions present. This explains the inflection of the binding curve. The oxidation of Mn during its binding under conditions

of chloroplast reactivation seems to take place. It requires illumination of short duration at least (229).

Conclusions about the electron-donor properties of Mn(II) in chloroplasts were first made from observations of the activation of electron transfer onto an exogenous acceptor after addition of Mn(II) salts (230, 231). The esr technique has made it possible to observe directly Mn(II) disappearance in illuminated chloroplasts or chloroplast particles of photosystem II both under steady-rate and pulsed illumination (182, 232). The quantitative correspondence between the Mn consumed and the reduced electron acceptor in steady-state experiments or between the Mn signal and esr signal I decay in pulse experiments, and also the competitive inhibition of Mn(II) oxidation by other electron donors of photosystem II, show that in these experiments actual Mn(II) oxidation takes place rather than nonspecific cation binding. The free Mn(II), but not the bound Mn, is oxidized upon illumination, and the decrease in the Mn(II) esr signal is partially reversible in darkness after a flash. This effect is caused by the removal of a part of the bound and esr-inactive Mn from chloroplasts. The donor function of exogenous Mn(II) manifests itself only after the degradation of the activity of the water-splitting system (by aging or tris-HCl treatment of chloroplasts or in photosystem II particles). This agrees with the suggestion that the binding of exogenous Mn(II) during the reactivation of the O_2-releasing system of chloroplasts is accompanied by its oxidation and with the observations that for the tight binding of Mn in the O_2-releasing system it is necessary that at least some of the atoms forming the catalytic complex should be oxidized. However, these arguments are all indirect ones, and there is *no unambiguous information about the valence state of* Mn in chloroplasts in the rest state.

D. Mn in Chloroplasts as a Relaxant of Water Protons

Mn (II), as a paramagnetic ion, enhances the magnetic relaxation of water protons. This phenomenon is used in some recent experiments (228, 233-235). The data obtained can be summarized as follows. Chloroplasts increase the rate of relaxation of water protons as shown by increase in T_1^{-1} and T_2^{-1}, where T_1 and T_2 are the spin-lattice and spin-spin relaxation times respectively. This effect is caused by endogenous Mn in the water-splitting system. There is a correlation between the degree of displacement of Mn by Mg, the decrease in O_2-releasing activity, and the decrease in the enhancement effect of proton relaxation. Other chloroplast paramagnetic centers do not give any significant contribution to this effect because they are well shielded from water. The dependence of T_1^{-1} on the resonance frequency (233) shows that the relaxation rate is determined by the electron spin-relaxation time (τ_s about 1 ns). This agrees with the suggestion that the proton relaxant is Mn(II), since Mn(III), Fe(II), and Fe(III) (for which $\tau_s \approx 10^{-10}$ to 10^{-11} s (236)) cannot effectively enhance proton relaxation. When chloroplasts were excited by light pulses, changes in T_2^{-1} have been observed with a period of four flashes (228), which indicates that the state of the Mn in the system also changes with the same period. The reason for this change is not known: it may be caused by a Mn valency change and the change in its accessibility for water protons. Thus, these measurements have also made it possible to establish correlation between the states of the water-splitting system (S_0-S_4) and the state of Mn in chloroplasts. It should be noted that the proton-exchange rate between bulk water and water in the coordination sphere of endogenous Mn is quite high (the free induction decay is simply described by an exponential function). Thus T_2^{-1} changes cannot be explained by variations in the exchange rate.

To summarize the information available for Mn in chloroplasts, four pools of Mn in chloroplasts can be distinguished. The "zero" pool, which cannot be isolated by nondestructive treatments, consists of two or three Mn atoms per electron-transport chain. It is not clear whether this tightly bound Mn has anything to do with the water splitting.* There is also a Mn pool in normal chloroplasts, which can be isolated by comparatively mild treatments that provide reversible inactivation of the O_2-releasing system. Two more pools are filled only in the presence of Mn(II) ions in the medium. *The filling of one of them is specific for Mn and is characterized* by the cooperativity mentioned above, while the filling of the other one is a nonspecific effect of *divalent cations. There exists a slow Mn(II)-ion exchange* among the last three pools. At high concentration of divalent cations other than Mn(II) in the medium, this *exchange causes the loss of Mn(II) from the pool* specifically related to O_2 evolution. In complete absence of Mn^{2+} in the medium, this pool loses Mn, which also leads to the inactivation of O_2 release upon chloroplast aging. This inactivation occurs more quickly than with other types of electron-transport activity. Evidently, the water-splitting system can function for a long time only when a certain background concentration of Mn^{2+} ions is present in water, since there exists a dynamic equilibrium between the Mn in the catalytically active complex and Mn in the solution or in other membrane loci (207).

*It may be related to the superoxide dismutase activity of the chloroplast membrane [C. H. Foyer and D. O. Hall, *FEBS Lett.* **101**, 324 (1979)].

E. The Cluster Nature of the Mn Centers in Chloroplasts

Water oxidation, as was mentioned above, requires the accumulation of four oxidative equivalents in the one locus. That is why in different models of this process it is assumed that several Mn ions form a complex, and it is natural to suppose that a magnetic coupling can occur among these ions. The properties of Mn as a paramagnetic proton relaxant must change if the Mn ions form a cluster with correlated spins. ESR signals of quite a new type were observed in bean chloroplasts at liquid-helium temperature with g ca 3, which depends on the room-temperature preillumination (171). A similar signal was found independently in spinach chloroplasts by Slabas and Evans (237). The signal with g ca 3 appears in chloroplasts adapted to darkness at observation temperatures below 40 K. The signal characteristics (g-factor, line width, intensity) depended on the number of pulses at preillumination of the sample and, what is particularly interesting, on the orientation of the sample relative to the magnetic field in the esr spectrometer. The temperature dependence of the g-factor is consistent with the conclusion that the signal is caused by a certain complex, evidently containing Mn, in which ferromagnetic coupling among the metal ions takes place (237). Further experiments (238) show that the signal with these properties appears only when the sample is gradually cooled to a temperature of about 23 K in a magnetic field of $H = 2300$ G. The dependence of the signal on temperature and magnetic field during freezing indicates that this process is of the phase-transition type. The system examined belongs to the class of spin glasses, i.e., magnetically diluted structures with cooperative magnetic coupling, which occur within a certain interval of temperatures and external magnetic fields (239). This effect should give research workers a new technique for studying cluster structures formed by paramagnetic ions, which play an important role in many biocatalytic processes, including photosynthesis.

F. Alternative Hypotheses on the Structure of the Water-Splitting Complex

Some data indicate that Mn is not the only, and probably not the indispensable, component of the water-splitting system. There is evidence that Cu ions participate in the reactions of photosystem II, including water splitting. This possibility is especially interesting if O_2 release is regarded as the reverse of terminal oxidation in respiration. It is well known that the enzyme that catalyzes O_2 binding contains Cu in its active center. Holdsworth and Arshad (240) isolated a pigment-protein complex from the diatomic alga *Phaeodactylum tricornutum,* which contained 8 moles of Cu and 2 moles of Mn per 40 moles of chlorophyll. This photochemically active complex displayed a number of reactions typical of photosystem II, and its esr spectrum contained components that were identified as signals of Mn^{2+} and Cu^{2+}. There is other information about the isolation of Mn-containing complexes (241, 242), but none of the experiments proved that the formation of these complexes is not an artifact caused by the treatment of the initial material, and in any case these complexes did not show catalytic activity in the water-splitting reaction. Finally, recent experiments have shown that the introduction of Cu ions (in the form of an albumin complex) into potato chloroplasts, pretreated with 0.8 *M* tris-HCl, restores the O_2-evolving activity (243).

What is the nature of the other Mn ligands, apart from water, in the water-splitting complex? This question is closely connected with the existing models of the catalytic complex (220). It is usually assumed that a catalytic complex contains two Mn(II) ions oxidized to Mn(IV), or four Mn(II) ions oxidized to Mn(III). In this case it is also assumed that Mn(III) or Mn(IV) oxidizes the ligand water molecules into H_2O^+ or $OH^{\cdot}$. As the formation of reactive particles like OH radicals in the organic medium would be quite toxic (any organic molecules would have to hydroxylate in the presence of OH radicals), it is assumed that these particles are stabilized in the coordination shell of Mn until their number becomes large enough for O_2 release to be possible. In some variants of the model it is supposed that H_2O_2 is the intermediate form (this suggestion is indirectly supported by the ability of chloroplasts to use H_2O_2 as an electron donor in photosystem II (244)).

It should be noted that the redox potential of the Mn(II)/Mn(III) pair is 1.5 V, and although a quantum of red light has sufficient energy to transport an electron against this potential, the formation of such a strong oxidant in a biological system is highly improbable, and the potential is still not enough for a reaction such as $H_2O \rightarrow OH$ ($\triangle E_m$ ca 2V). Evidently the ligand shell contains components that lower the potential of this pair. Data indicate that electron-transport reactions in higher-plant chloroplasts on the oxidizing side of photosystem II are inhibited by the removal of plastoquinones. Along with the properties of the transient esr signal II, this fact leads to the hypothesis that the plastoquinone in photosystem II, as in the noncyclic chain, is an electron carrier across the hydrophobic barrier separating Mn complex from the reaction center. One can suppose (245) that plastoquinone, which acts as electron-transfer mediator between Mn^{2+} and $P680^+$, forms a charge-transfer complex (an ion pair)

$Mn^{3+} - Pq^{-\cdot}$ whose potential (only slightly more than 0.8 V) is not sufficient to oxidize water into OH radicals. Hence these ion pairs can exist long enough for all four Mn ions to form similar ion pairs during consecutive absorption of four quanta by photosystem II. Only at the last step, when an energy of more than 3.2 eV (sufficient for the transformation $2H_2O \rightarrow O_2 + 4H^+ + 4e^-$) has accumulated in the center, does water oxidation take place. In this case no superreactive particles are formed; hence some of the above problems disappear.

The tight interaction of semiquinone radicals with paramagnetic Mn^{3+} ions explains the absence of an esr signal corresponding to this paramagnetic product. The spin-lattice relaxation time decreases so much that the line width of the semiquinone radical signal, according to the Heisenberg principle, increases to values that make resonant absorption impossible to observe. This mechanism keeps the reaction center of photosystem II in a state that is practically independent of the state of the water-splitting complex. Thus, the principle of spatial separation is realized here. It is generally quite characteristic of membrane biochemical systems and provides the stabilization of the reaction products after the reaction event occurs.

G. Cl^- Ions in the Water-Splitting System and the Mn(II) Signal with Superhyperfine Structure

Physiological experiments show that for O_2 evolution in chloroplasts chloride ions must be present, as must Mn^{2+} ions (246, 247). It is not known whether these requirements are related and whether any direct interaction between Mn^{2+} and Cl^- ions takes place in the catalytic complex, although recent results make this interaction probable.

Figure 24 shows the esr spectrum of Mn in cypress needles (248). The main feature of this spectrum is the quartet super-splitting presumably caused by interaction with a nucleus with $I = 3/2$. The usual six-component esr signal from Mn^{2+} ions is also observed. The quartet superhyperfine structure is connected with the appearance of weak lines in the low field, which are apparently caused by $g_{\parallel}$. Evidently, the quartet split Mn signal, unlike the signal of free Mn^{2+}, is characterized by a certain anisotropy of its g-tensor. Similar signals have been found in many other photosynthesizing species, in the leaves of some citrus plants, black currant, thuja, and pine. In at least two cases (thuja and pine) the presence of these paramagnetic centers in chloroplasts has been proved. Superhyperfine structure of this type has been observed in the signal from photosynthetically active lettuce chloroplasts (224). This signal is not observed in ordinary preparations from standard photosynthetic species, such as bean or spinach chloroplasts and leaves. However, if the chloroplasts are

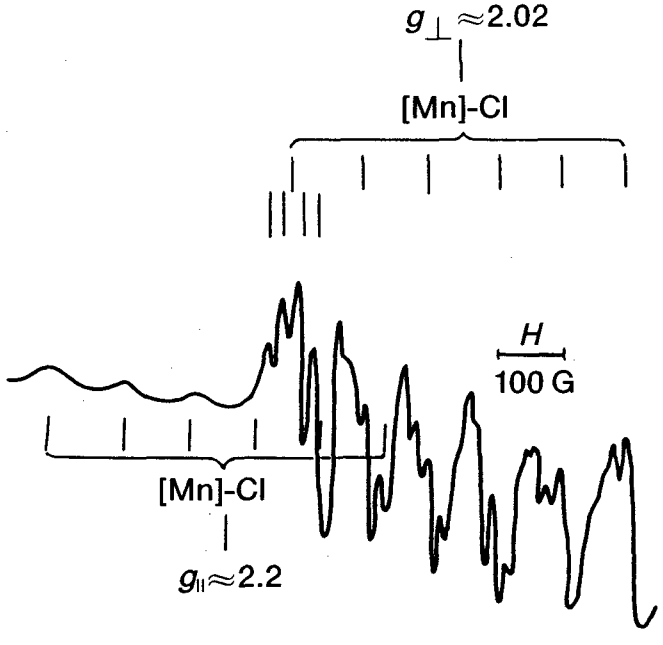


Figure 24. The esr signal from cypress needles (*Cupressus sempervirens*, var. *pyramidalis*) at 77 K.

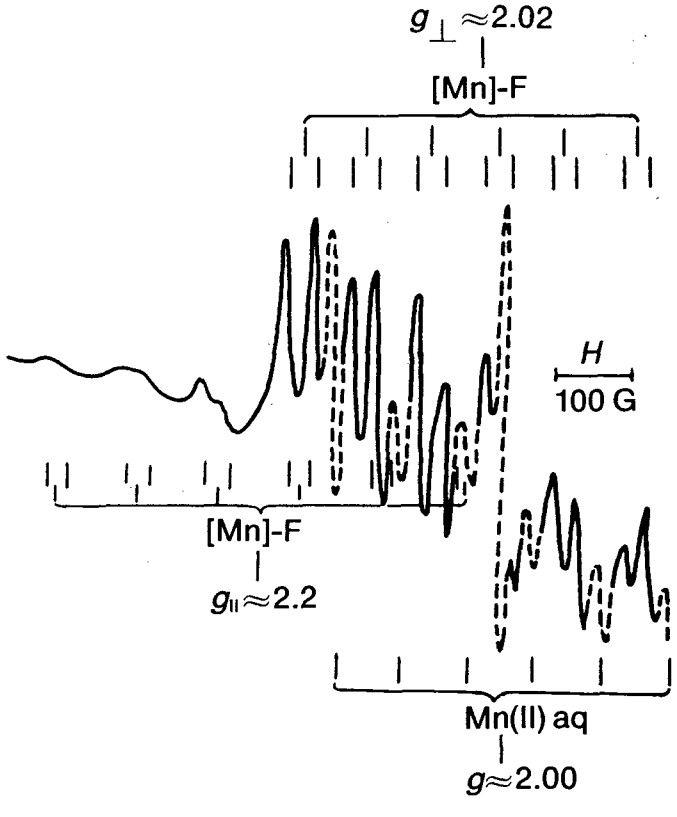


Figure 25. The esr signal from cypress needles pretreated to substitute F ligand for Cl ligand.

isolated from aging bean plants, quartet superhyperfine structure is also observed. It should be mentioned that for Mn super-splitting of the hyperfine structure lines on other nuclei is generally not at all typical, as it is very rare that Mn forms strong high-spin complexes with ligands other than water. Quartet superhyperfine structure can indeed be caused only by a Cl ligand, because only Cl of the common elements has an isotope with a nuclear spin of 3/2. The nuclear spin of Cu is also 3/2, but binuclear complexes containing magnetically interacting Mn and Cu produce signals of quite a different type (as does, for example, the binuclear complex of Mn and Cu with pyridine oxide, due to the antiferromagnetic interaction between Mn^{2+} and Cu^{2+} ions) (249).

The assignment of the superhyperfine structure as that caused by splitting on Cl is confirmed by the possibility of substitution of F^- for Cl^- leading to the doublet splitting on the ^{19}F nucleus (Figure 25) (250). It is reasonable to assume that the Mn-Cl complex in photosynthetic membranes is a product of the partial catabolism of the water-splitting active structure, which can accumulate in sufficient quantity only in certain species. Certainly, this product is not identical with the water-splitting complex itself. However, this end product of degradation preserves some features of the initial structure and indicates the presence of the Mn-Cl link.

VII. NONCYCLIC ELECTRON TRANSPORT BETWEEN THE TWO PHOTOSYSTEMS

A. Spectral-Kinetic Separation of the Two Photosystems

Electron transfer between the reaction centers of the two photosystems is the slowest process of the entire light stage of photosynthesis and so limits its rate. This process, in which P680 in photosystem II centers is an electron donor and $P700^+$ in the photosystem I center is an acceptor, proceeds with the participation of plastoquinone, cytochrome *f*, plastocyanin, and probably one other component, a high-potential Fe-S center (251, 252).

The reaction of plastocyanin with $P700^+$ takes place at high rate ($\tau_{1/2} \approx 20$ μs at room temperature), whereas the reduction of plastocyanin and cytochrome *f* upon pulse excitation of photosystem II is characterized by $\tau_{1/2}$ = 6-150 ms (253). In the study of the kinetics and mechanism of the slowest steps of noncyclic transport, $P700^+$ can be considered a direct electron acceptor from plastohydroquinone because of the significant difference between these times. This is the basis for the application of the esr signal I from $P700^+$ to study the kinetics of noncyclic electron transport. The other participant in this process, plastoquinone, as was mentioned above, does not reveal itself in the esr spectra, though there is no doubt that during its one-electron reduction and reoxidation, semiquinone radicals or radical anions are formed.

The oxidation of $P700^+$, which can be observed by the esr technique in any photosynthetic system of higher-plant type exposed to far-red light, and its reduction in near-red light, which mainly excites photosystem II, provide one of the most simple, direct, and easily observable confirmations of spectral separation of the two photosystems and of sequential electron transfer (Figure 26) (254-257). The dependence of the esr signal on the intensity of near-red or of white light (Figure 27) indicates that the limiting stage of the whole electron-transport system is that between the two photosystems. In weak light the electrons from photosystem II completely cancel the positive charge on the P700 that appears upon photosystem I excitation. In bright light, when the interval between two consecutive excitations of photosystem I is smaller than the time required for electron transfer in the limiting step, P700 centers are in the oxidized state most of the time. This causes the appearance of the steady-state esr signal I.

The kinetics of the increase of the esr signal in far-red light depend mainly on the preillumination of the photosynthetic system. In bright light, which excites both photosystems, the plastoquinone pool between them is reduced because the limiting step of the electron transport is between plastohydroquinone and $P700^+$. As a result, with subsequent far-red illumination the increase in the esr signal I is retarded. Diuron, an inhibitor of noncyclic electron transport between the two photosystems, removes the qualitative differences in the effects of red and far-red light on the esr signal I, as well as the lag phase. The "memory" of the preceding illumination in intact systems such as higher-plant leaves is preserved in darkness for tens of seconds. Hence the steady state of leaves and chloroplasts is usually far from equilibrium under the medium's conditions (255).

Noncyclic electron transport is not the only factor that determines the steady-state intensity of esr signals. Cyclic electron flow in photosystem I and reactions of these centers with endogenous reductants (which form an inner redox buffer and react with $P700^+$ via plastoquinone, plastocyanin and cytochrome *f*) also lead to the reduction of $P700^+$ (258-262). They slow down the increase of the esr signal I in far-red light and accelerate its dark decay. Both these processes of P700 reduction are inactivated when chloroplasts are exposed for a long time in aerobic conditions, which result from the exhaustion of the reductants and the transfer of all the components of the cyclic chain to the oxidized state. With long dark incubation the lag phase of the signal rises because of the reduction of the plastoquinone pool by near-red

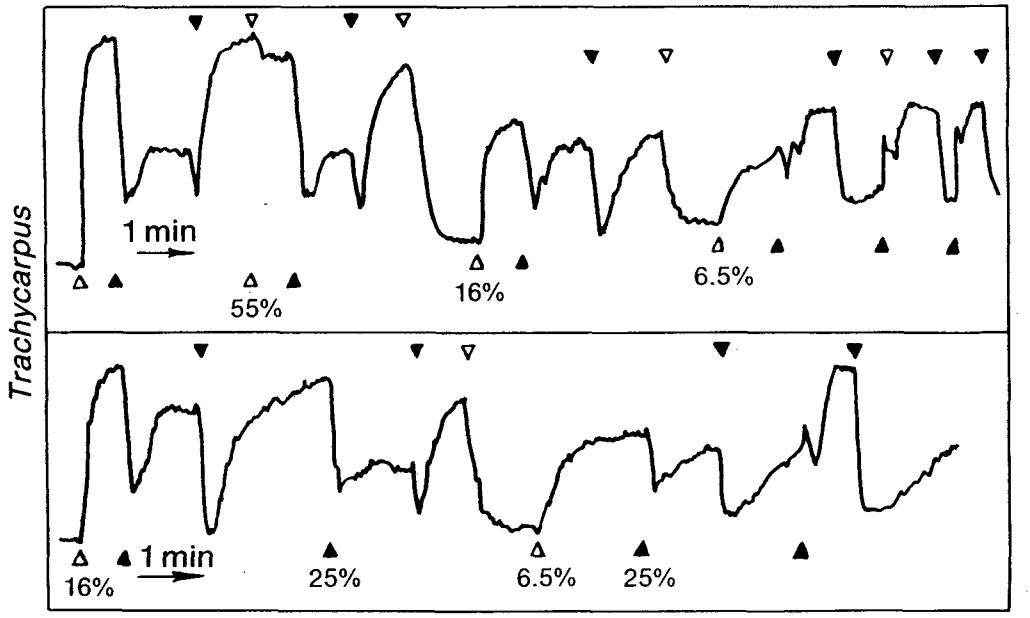


Figure 26. Chromatic transitions in the esr signal I intensities from the leaf of *Trachycarpus fortunei* sp. Open triangles: 710 nm light switching on and off. Solid triangles: the same for 634 nm light. The light intensities are given as percentages of the maximum intensity (2.0 W/m² for 710 nm, 40 mW/m² for 634 nm).

light and decreases due to plastohydroquinone interaction with atmospheric oxygen. As a result, the excitation of chloroplasts by a single pulse of less than 1 μs duration is sufficient for P700 oxidation. This proves the one-quantum mechanism of charge separation in photosystem I (262).

There are some typical transitory effects that accompany changes in the spectral composition of light acting on the leaves of higher plants (255, 256). Switching on 634 nm light (which excites both photosystem I and photosystem II) over a constant 710 nm background (which excites mostly photosystem I) induces a rapid decrease in the esr signal, with a subsequent transition to a plateau (Figure 26). The times of nonmonotonic transition effects are of the order of seconds, which is much more than the characteristic times of any elementary steps of electron transfer. Analysis of these effects and comparison with other data such as induction phenomena in chlorophyll *a* fluorescence, changes of accessibility of the donor centers of photosystem II for silicomolybdate (178), and the inhibitors of electron-transport reactions and membrane modifiers in photosystem II (263, 264), lead to the conclusion that these long-duration transitory processes are at least partially caused by structural changes in the photosynthetic membranes. These influence the efficiency of the energy transfer between the two photosystems and/or the rate constants of the slowest elementary steps of electron transfer, which depend on the spectral composition of the light and the degree of reduction of the components of the electron-transport chain (255). Detailed computer calculations that assume all the kinetic constants to be invariable do not give a quantitative description of the transitory effects observed under changed illumination (265, 266). The structural-kinetic lability of the electron-transport chain increases the adaptation and regulatory abilities of photosynthetic membranes to changing external conditions.

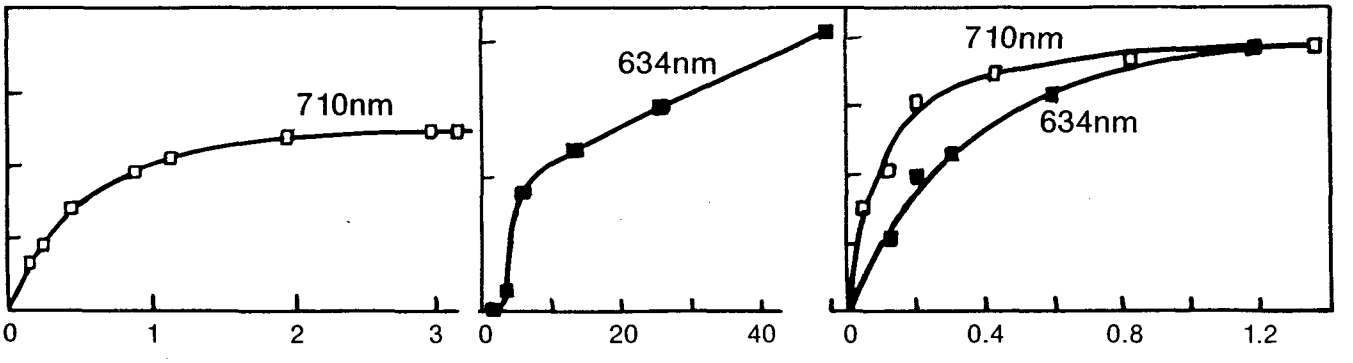


Figure 27. The esr signal I intensity (arbitrary units) from *Hibiscus* sp. leaf as function of light intensity (W/m²). Right curves: the leaf pretreated with diuron.

B. The Two-Electron Shutter in the Noncyclic Electron-Transport Chain

Under steady-state illumination the pigment P700 is an intermediate electron carrier, and the fraction of oxidized P700 centers is determined by the ratio of the oxidation and reduction rates. Thus, the esr signal I cannot be used to obtain a direct estimate of the electron-transport rate. However, short light pulses provide a single excitation of the reaction centers of each of the two photosystems, and the kinetics of the changes in the esr signal I induced by a flash allow the evaluation of the rate of $P700^+$ reduction due to noncyclic electron transport and other reductive processes, and also the determination of the quantity of reducing equivalents reaching the $P700^+$ centers at a single excitation of photosystem II. This approach, called esr-flash photolysis (258), was used for studying the various reactions of $P700^+$ reduction. In this section we shall consider one aspect of noncyclic electron transport, which concerns the coupling of one- and two-electron reactions in a noncyclic chain.

All the carriers acting in a noncyclic chain can be divided into one-electron carriers (reaction centers, cytochromes, plastocyanin) and two-electron carriers (plastoquinones). Plastoquinone forms the pool between the two photosystems (6-10 moles of plastoquinone per electron transport chain). Optical absorption data indicate that in the process of electron transport plastoquinone forms a product of twofold reduction, plastohydroquinone. Under steady-state illumination semiquinone radicals are not accumulated in any significant amounts. The problem is: How does twofold reduction of plastoquinone occur by its interaction with a one-electron donor, the photosystem II reaction center? In principle, two ways of coupling are possible:

1) Electron-transport chains are joined together in pairs at the level of the photosystem II secondary acceptor, so that a flash of saturating intensity (i.e., one that excites all the photosystems II simultaneously) leads to synchronized two-electron reduction of a plastoquinone molecule, which forms a complex with two reaction centers of photosystem II. A variant of such cooperation between the chains is a simultaneous reduction of two plastoquinone molecules to plastosemiquinone radicals in the neighboring chains and subsequent dismutation of these plastosemiquinone radicals into a pair of diamagnetic particles, plastohydroquinone and plastoquinone:

$$2Pq^{-\cdot} + 2H^+ = Pq + PqH_2$$

If the system is excited by a sequence of short flashes, a similar quantity of reducing equivalents in the form of plastohydroquinone molecules appears in response to every flash.

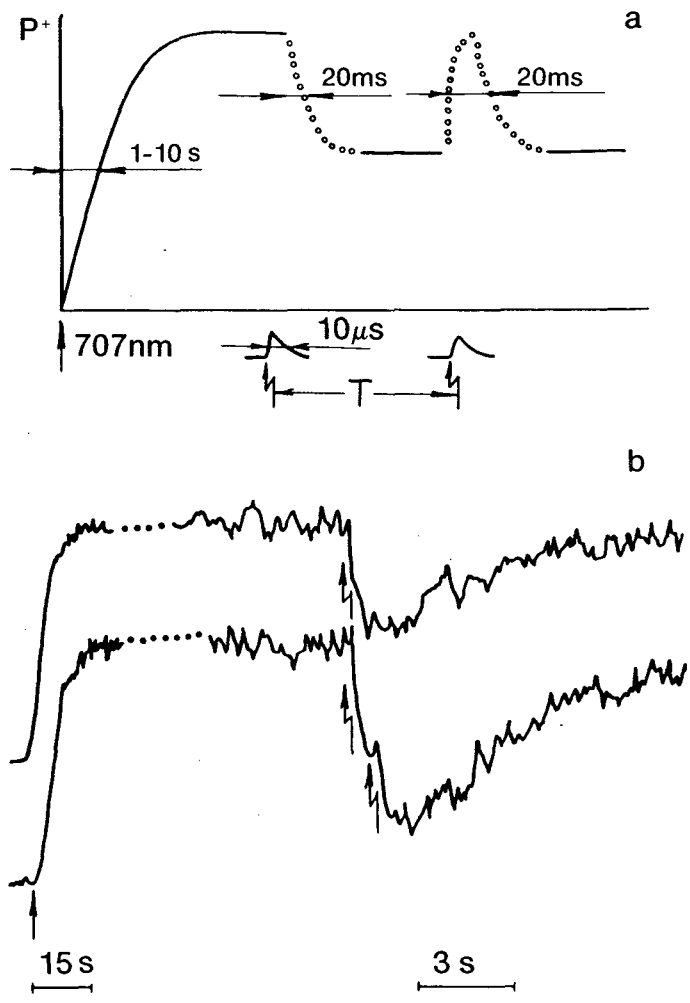


Figure 28. The reduction of P700 centers measured by esr spectroscopy. 710 nm background is 2.0 W/m². Zigzag arrows indicate flashes of 1 μs duration. Curve *a* shows the expected behavior of the signal (not to scale) and curve *b* the experimental result.

2) A one-electron donor and a two-electron acceptor are coupled in a different way if the chains do not cooperate at the acceptor level. In this case, the result of excitation by a single short flash depends on the environment of the acceptor at the moment before the flash. If at that moment there is a plastosemiquinone radical close to the acceptor (plastoquinone), the flash will cause the formation of a second semiquinone, which dismutates with the first one into a quinone-hydroquinone pair. If initially there are no semiquinone radicals close to the acceptor, the flash will cause the formation of a single plastosemiquinone.

The results of the flash are quite different for the two cases because the reactivity, mobility, charge, and other properties of plastohydroquinone and plastosemiquin-

one radicals (or radical anions) are very different. The situation is, to some degree, similar to that of the water-splitting system; the periodicity in O_2 evolution indicates consecutive charge accumulation on the oxidizing side of photosystem II, while a lack of periodicity would correspond to the cooperation of several photosystem II reaction centers in the synchronized oxidation of two water molecules. The observation of changes in the esr signal I from $P700^+$ centers exposed to short light pulses enabled the correct alternative to be selected (267-269). Experiments of two types were performed, one with a weak red background constantly supporting P700 in its oxidized state and the other in the absence of the far-red background. In the first case, at the instant of the flash the P700 centers are initially oxidized, and the only result of the flash can be $P700^+$ reduction by the electrons injected into the chain by photosystem II. This is shown in Figure 28. The second flash, which occurs about 1 s after the first one, oxidizes all the reduced P700 centers and excites photosystem II. Thus, the net result of the two flashes would not be different from that of one flash if the amounts of reducing equivalents coming to the P700 center in response to each flash were the same. However, the experiment showed that the second flash is always more effective than the first one. The criterion of the reducing efficiency of the flash is the difference between the level of the esr signal I in saturating far-red light (with complete oxidation of P700) and its level immediately after the flash. This result does not depend on the water-splitting system (water as a donor can be replaced by diphenylcarbazide or Mn(II) ions). Oxidation of the exogenous donor (Mn^{2+}), also observable by the esr technique, occurs uniformly in response to each light flash (232). The reactions on the oxidizing side of photosystem I have nothing to do with the effect, since all the experiments were conducted in saturating concentration of the photosystem I electron acceptor (methylviologene).

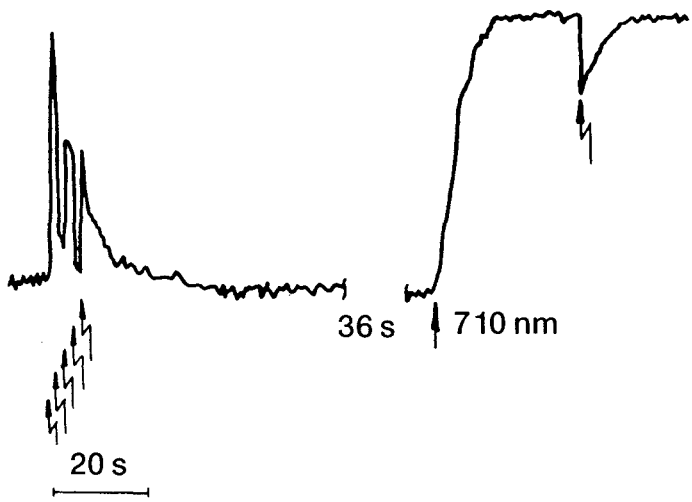


Figure 29. Oscillations of the esr signal I from bean chloroplasts under pulsed illumination. Right trace is the signal variation for flash fired with constant 710 nm background (2.0 W/m²).

The result is not related to interchain interaction since the inhibition of noncyclic transport in some of the chains by varying the concentration of the photosystem II inhibitor, diuron, had no effect on the ratio of the reducing efficiencies of the first and second flashes. Typical recordings that correspond to the second type of experiment without the far-red background are shown in Figure 29. With chloroplast excitation by several light flashes, the esr signal I oscillates with a period of 2 flashes. Since in this case also the difference between the levels of the esr signal I in saturating far-red light and the level after the flash corresponds to the quantity of reducing equivalents that reach the $P700^+$ centers, this result shows that the conductivity of the electron-transport chain oscillates. The first flash, which acts after chloroplast adaptation to far-red light (for complete oxidation of the plastoquinone pool) and a short adaptation to darkness (for lowering the esr signal I), does not induce the reduction of $P700^+$ centers. Thus the reducing equivalents generated by photosystem II do not reach the $P700^+$ centers. The second flash causes the complete reduction of the $P700^+$, the third causes its partial oxidation, etc. The oscillations are damped quickly. These observations indicate the existence of a two-electron shutter in the electron-transport chain, i.e., a component that accepts electrons one by one and that is able to transfer them further to the chain only after twofold reduction. Independent data confirming the existence of the two-electron shutter were obtained by the coulometric technique (270) and by chlorophyll *a* fluorescence measurements (271).

What is this component? Some authors have suggested the existence of a special unidentified carrier with unusual electron-transport properties (271). In the model proposed (267-269) the differences in the properties of plastoquinone in its three possible redox states are used. The one-electron reduction of plastoquinone induced by a flash causes the formation of a plastosemiquinone radical bound with the reaction center of photosystem II. This radical with p K ca 5-6.5 (the usual p K interval of benzosemiquinone radicals, slightly dependent on the substituents) is not protonated at the expected values of p H, i.e., it is a radical anion. The plastosemiquinone radical anion, because of its charge, remains bound at the site where it is formed. Its mobility in the hydrophobic region of the membrane is appreciably lower than that of the unchanged plastoquinone species, and it is unable to transfer a reducing equivalent to the photosystem I reaction center. As a result, the first flash causes $P700^+$ oxidation, which is

not compensated by its reduction by electron transfer from photosystem II. The second flash generates a second radical anion that by dismutation with the first one, produces a quinone-hydroquinone pair. Noncharged plastohydroquinone (the p K of substituted hydroquinones is always higher than 10) freely passes through a hydrophobic barrier and reduces the $P700^+$ center. The process then repeats itself, but the accumulation of unused reducing equivalents on the inner side of a membrane (in the form of plastosemiquinone radical anions) leads to rapid damping of the oscillations of esr signal I.

This model is somewhat oversimplified. The interaction of plastohydroquinone and plastosemiquinone with atmospheric oxygen and with the redox buffer of the stroma together with the nonzero rate of recombination of semiquinone radicals simultaneously formed in neighboring electron-transport chains, makes the amplitude of oscillations rather variable. The amplitude value depends on the redox potential of the medium, the chloroplast state, and the p H value (272, 273). The identification of oscillations as the stepwise reduction of plastoquinone is in accord with the oscillations of the optical absorption at the maximum of the plastoquinone absorption band (274). This model also enables periodic effects of the proton uptake under pulse illumination of chloroplasts to be explained (275) because the dismutation reaction is accompanied by the uptake of two protons from the external medium, and the reoxidation of plastohydroquinone on the inner side of the membrane must be accompanied by the injection of two protons into the inner thylakoid space.

The two-electron shutter based on the properties of the prenylquinones is a quite common phenomenon. Some indications of two-electron oscillations of electron-transport-chain conductivity have also been observed in photosynthetic bacteria (276, 277). The nonobservability of the esr signal from plastosemiquinone radicals at the sites of photoreduction on the external side of the membrane and of reoxidation on the internal side of the membrane probably occurs in both cases because these species are coupled to other paramagnetic centers, most probably containing iron. The presence of iron in the photosystem II reaction centers, where plastoquinone photoreduction takes place, has already been discussed. Now we shall consider some data that indicate that plastohydroquinone is reoxidized also at the site containing iron.

C. The High-Potential Fe-S Center in a Noncyclic Chain

Malkin and Aparicio (251) found in spinach chloroplasts an esr signal with $g_z \approx 2.02$, $g_y \approx 1.89$, and $g_x \approx 1.78$, observable at liquid-helium temperature. As two of these lines overlap with the signals from other paramagnetic centers, this center can be most conveniently identified by the $g \approx 1.89$ line. The esr properties and redox characteristics of the 1.89 center are similar to those of the paramagnetic center discovered earlier by Rieske et al (278) in mitochondria and submitochondrial particles. As with other Fe-S centers, the reduced form of the 1.89 center in chloroplasts is paramagnetic. Reduction is achieved by using mild reductants such as hydroquinone. The center's redox potential is +290 mV (252) and does not depend on the p H in the p H interval 6.0-8.0.

The exact location of this Rieske-type center in chloroplasts has not yet been determined. By analogy with the Rieske center in mitochondria, which interacts with cytochrome c, it is suggested that the 1.89 center in chloroplasts also takes part in electron transport in the region of cytochrome f.

Durohydroquinone effectively reduces the 1.89 center, and this reaction is inhibited by dibromothymoquinone, a plastoquinone antagonist, but it is not sensitive to diuron (279). Cytochrome f is reduced simultaneously. As the cytochrome f redox potential is much higher than that of the 1.89 center (350-380 mV), its position in the electron-transport chain can be expected to be as follows:

plastohydroquinone → 1.89 center → cytochrome f

This localization of the 1.89 center between plastohydroquinone and cytochrome f is also supported by the fact that in mutants of duckweed (genus *Lemna*) in which electron transport between plastohydroquinone and cytochrome f is blocked (252), the chloroplasts are capable of photooxidation, but not of photoreduction of cytochrome f, and no Rieske-type-center reduction is observed, whereas in chloroplasts from the wild strain these reactions occur in the same way as in spinach chloroplasts. A Rieske-type center has been discovered also in etioplasts before greening (280), as have cytochrome f, plastocyanin, soluble ferredoxin, and ferredoxin-NADP reductase, but low-potential Fe-S centers A and B have not been reduced in etioplasts. Their photoreduction is observed only after several hours of greening in light, and simultaneously etioplasts acquire the ability to oxidize P700 at 15 K. It should be mentioned that the site between plastohydroquinone and cytochrome f is one of the coupling sites between chloroplast electron transport and photophoshorylation. Hence it is quite probable that the process of biological energy transformation takes place in chloroplasts with the direct involvement of the 1.89 center. This center is rigidly fixed in the thylakoid membrane, and the corresponding esr signal depends on the chloroplast orientation in the magnetic field (87).

The Fe-S centers of the Green N2 complex of mitochondria reduced by excess substrate in anaerobic conditions differ in their conformation states in coupled and uncoupled organelles. This difference, which cannot be detected by comparing the shapes of the esr spectra from the reduced centers at any fixed temperature, can be detected by comparing the temperature dependences of the esr spectra from the Fe-S centers in the interval 10-40 K. This effect probably reflects the participation of conformationally constrained protein states in the coupling site during the biological energy transformation (150), as considered in Section III A. It may be expected that the same differences will be observed for the 1.89 center in coupled and uncoupled chloroplasts, if this center is really situated at the coupling site between the electron transport and the phosphorylation.

VIII. CONCLUSIONS

The information contained in this review clearly shows that the esr technique has played a most important role at all stages of investigation of the light processes in photosynthesis. With its help the fundamental problem of establishing the nature of the primary electron donors and acceptors in photochemical reaction centers has been solved. The discovery and investigation of Fe-S centers, a most important class of electron carriers, have become possible only due to the application of the esr technique. Many features of the secondary electron-transport processes, including the mechanisms of their individual steps and the structural-kinetic lability of the process as a whole can also be studied by esr spectroscopy. The investigation of photosynthesis by the esr technique is at present going through a period of intense development. The sensitivity of esr spectral parameters to subtle variations of the local environment of paramagnetic centers opens up the possibility of studying the nonequilibrium states of individual electron carriers *in situ*. This approach, which has already given initial results in the study of respiratory metabolism, can also be expected to be successful in photosynthetic investigations. The properties of some recently discovered paramagnetic centers, the composition and physiological functions of which are still unknown, also require further consideration.

A retrospective glance at the development of esr research into photosynthesis shows that not only has the esr technique made it possible to achieve valuable results in this area, but the problems themselves stimulated the development of esr spectroscopy. This happened both in the experimental field (the decrease of the dead time of the equipment, the increase of sensitivity, the widening of the temperature interval of measurements, etc.) and in the theoretical interpretation of spectral data, relaxation effects, and kinetic results. This makes the study of photosynthesis an extremely satisfying field of application of esr spectroscopy.

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