Plant Physiol. (1969) 44, 1439–1445

On the Molecular Identity of ESR Signal II Observed in Photosynthetic Systems: The Effect of Heptane Extraction and Reconstitution With Plastoquinone and Deuterated Plastoquinone[1]

Daniel H. Kohl and Patrick M. Wood
Department of Botany, Washington University, St. Louis, Missouri 63130

Received May 14, 1969.

Abstract. Speculation as to the identity of Signal II, the light-induced, broad, slow decaying electron spin resonance signal with hyperfine structure observed in photosynthetic materials, has tended to center on the semiquinone of plastoquinone. Experiments reported here were designed to give direct evidence bearing on that speculation. Heptane extraction of lipids from lyophilized spinach and tobacco chloroplast fragments reduced the amplitude of Signal II and increased the ratio of Signal I:Signal II. Reconstitution of the system by the addition of plastoquinone partially restored Signal II as well as the ratio of Signal I:Signal II to its pre-extraction condition. Addition of totally deuterated plastoquinone to extracted chloroplasts in which considerable Signal II had survived heptane extraction resulted in a spectrum which showed the characteristic hallmarks of Signal II observed in totally deuterated organisms. These results establish that a free radical immediately derived from plastoquinone contributes to Signal II. The data taken by themselves are consistent with plastochromanoxyl as well as plastosemiquinone free radicals giving rise to Signal II. Other contributors to Signal II are not ruled out.

Two light-induced ESR signals generated by O_2-evolving photosynthetic systems have been observed for many years (1). Signal I decays rapidly in the dark (although small amounts may persist in the dark in samples which have been frozen), has a g-value of 2.0025, a half-width of about 7.5 gauss, and reveals no hyperfine lines although the unpaired electron clearly interacts with protons (2). Signal II persists for many min in the dark, has a g-value of 2.0046, a half-width of about 19 gauss and displays partially resolved hyperfine structure which is not present when the signal is generated by fully deuterated organisms (2). Speculation as to the molecular identity of Signal II has centered on PSQ[2]. However, the issue is not settled as may be seen in the conclusion of a recent review of the subject: "Although conclusive correlation of electron flow through a plastoquinone pool with EPR (ESR) Signal II has not yet been demonstrated, there is no evidence to the contrary" (3). In fact not even that strong a statement is appropriate since there is one observation which strongly argues against PSQ as the source of Signal II: namely, the spectrum of PSQ immobilized at 77°K, as well as in solution, is symmetrical while Signal II is decidedly asymmetric (4). Thus it is clear that experiments designed to identify the molecular origin of Signal II are still very much in order.

The experiments reported here are based on 2 types of previously reported experiments.

A) Well-established techniques exist for the extraction of plant lipids (with a consequent decrease in Hill activity) and the readdition of lipids, particularly PQ (with a consequent, at least partial, restoration of Hill activity) (5) and B) Signal II is considerably narrower (half-width *ca.* 5–6 gauss) when fully deuterated cultures of O_2-evolving photosynthetic microorganisms are used to generate the signal (2) as compared with the more usual spectrum (half-width *ca.* 19 gauss).

If then, ESR Signal II is directly derived from PQ, lipid extraction ought to remove it and PQ readdition might be expected to at least partially restore Signal II. This result by itself would not, however, establish that PQ was the parent compound of the observed free radical since removal of PQ might result in signal loss due to the interruption of photosynthetic electron transport. Readdition of

[1] This work was supported by NSF Grant No. GB-7536.

[2] Abbreviations used: ESR = electron spin (paramagnetic) resonance; PQ = plastoquinone; D-PQ = totally deuterated PQ; PSQ = semiquinone of plastoquinone; H-II = ESR Signal II generated by photosynthetic systems in which all hydrogen nuclei are protons; H-I = ESR Signal I generated by photosynthetic systems in which all hydrogen nuclei are protons; D-II = ESR Signal II generated by photosynthetic systems in which all hydrogen nuclei are deuterons.

D-PQ should allow a choice between the 2 alternatives in that the resulting ESR signal would be narrow if PQ were the parent compound of the observed free radical and broad if the only role of PQ were to maintain the integrity of the transport chain.

Methods and Materials

Chloroplast Preparation. Preparations containing chloroplasts and chloroplast fragments were made from either market spinach or greenhouse grown tobacco. The deveined leaves were briefly macerated in a blender in 0.35 M NaCl, 0.02 M tris-HCl buffer (pH 7.3), and then filtered through several thicknesses of cheesecloth or Miracloth. Five min of centrifugation at 500*g* served to collect starch, chunks of leaf tissue and nuclei. The supernatant was then centrifuged for 10 min at 2000*g* and the pellet containing chloroplasts and chloroplast fragments was resuspended in deionized water. This preparation was processed as indicated in Fig. 1 to produce the samples used in these experiments. Lyophilization was carried out in absolute darkness. All other steps were carried out in a darkened room.

Heptane Extraction and PQ Readdition. The chloroplast preparations were extracted with heptane in an ice bath for 3 min with the aid of a motor driven pestle. About 0.5 μmole PQ/50 mg lyophilized chloroplasts (*ca.* 1.8 mg chlorophyll) was used in the readdition experiments. Notice that the chloroplast preparations were resuspended in water before ESR spectra were recorded.

PQ Isolation and Assay. PQ was isolated from spinach chloroplasts as described elsewhere (4). Deuterated PQ was isolated from deuterated *Synecoccus lividus* (6), 7 grams of lyophilized organisms yielded about 4.5 μmoles of D-PQ. PQ was assayed spectrophotometrically (7) using

$$\Delta\epsilon^{1\,cm}_{255}\ (\text{oxidized-reduced}) = 15 \text{ liter mmole}^{-1}$$

The PQ was reduced in the spectrophotometer cuvette by shaking in the presence of a chip of KBH_4.

Assay of Ferricyanide Hill Activity. The reaction mixture contained the following in a volume of 2.0 ml: 50 μmole tris-HCl buffer, pH 7.3; 2 μmole potassium ferricyanide; 5 μmoles ammonium chloride; and chloroplasts containing 0.050 to 0.100 mg chlorophyll. The reaction mixture was placed in a water-jacketed cell and illuminated for 3.0 min with white light of saturating intensity. After illumination, the reaction mixture was deproteinized in 5 % trichloroacetic acid and the optical density of the filtrate was measured at 420 mμ.

Assay of Chlorophyll. Chlorophyll was determined by the method of Arnon (8).

ESR Spectroscopy. Chloroplast samples were suspended in water at a chlorophyll concentration of 0.2 to 0.5 mg/ml and 2 milliliters were transferred into a quartz cuvette. A 23 mm length of the cuvette, containing about 0.15 ml, is ultimately within the spectrometer cavity. The chloroplasts were packed into the cuvette by centrifuging at 1000*g* for 10 min in a swing-out rotor. ESR spectra were recorded on a 9GHz spectrometer with automatic frequency control similar to the spectrometer previously briefly described (1).

While it is difficult to obtain absolute quantitative data from ESR, it is often possible to calculate the ratio of free radical concentrations in 2 samples with much less uncertainty. Even that proved not possible in some cases in these experiments. For example, the lyophilized sample in Fig. 2 did not pack down in the ESR cuvette in the same way as did those samples which had been extracted with heptane. Since both signals are light induced and since the light beam did not illuminate the entire cavity, the signal amplitude depends not so much on the amount of chlorophyll in the ESR cavity, but rather on how it is distributed within the cavity. The complicating factors of relatively optically dense packing of chloroplasts and the saturation of Signal II at low light intensities (9) make it impossible in most cases to correct for sample size and sample packing.

Illumination of Sample. The sample was illuminated through a 0.6 cm diameter port in the rear of the cavity by a miniature 23 watt bulb (GE 1073) placed 6 cm from the cuvette. This intensity is

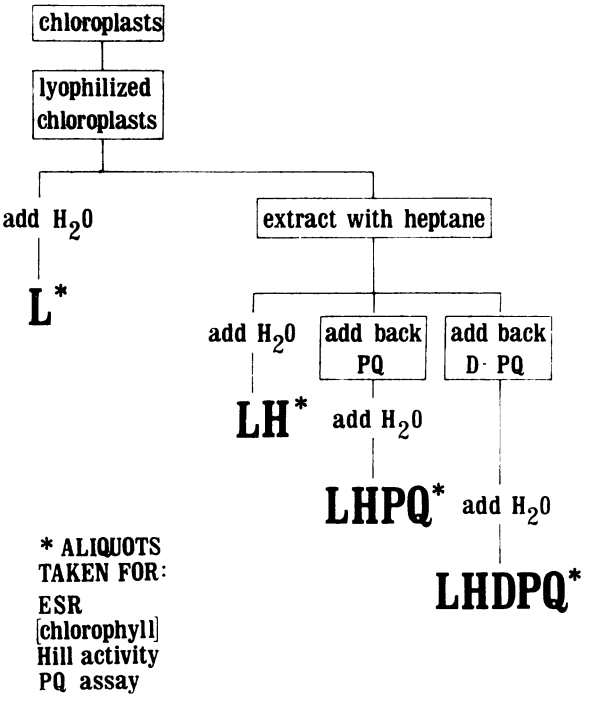
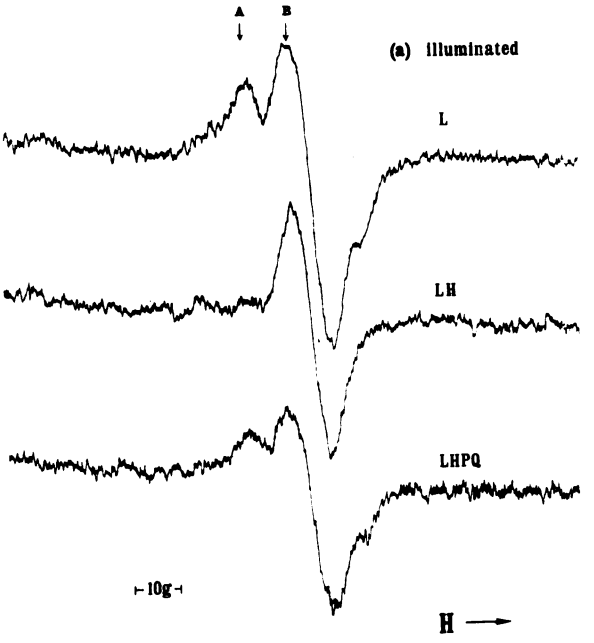


FIG. 1. Flow chart for sample preparation.



nearly sufficient to light saturate Signal II but Signal I amplitude is a linear function of light intensity at these values.

Results

The effect of heptane extraction was observed on the ESR signal recorded during illumination and in the dark following illumination. Spectra recorded in the light normally are composites containing both Signals I and II. The rapid decay of Signal I after cessation of illumination normally leaves only Signal II. Vigorous extraction of lyophilized chloroplasts with heptane usually sharply reduced the magnitude of Signal II. This can be seen in Fig. 2(a) and 2(b) as a decrease at field point A where only Signal II contributes (*cf.* spectrum "L" *vs.* "LH"). The non-decaying amplitude at field point B in spectra "LH" (Fig. 2a, 2b) is due to Signal I as can be seen from the line shape, line width and g-value. While dark persistent Signal I is anomalous when compared to the expected behavior of that signal in fresh chloroplasts, in fact some fraction of Signal I produced in the light is often observed in material which has been lyophilized and even more

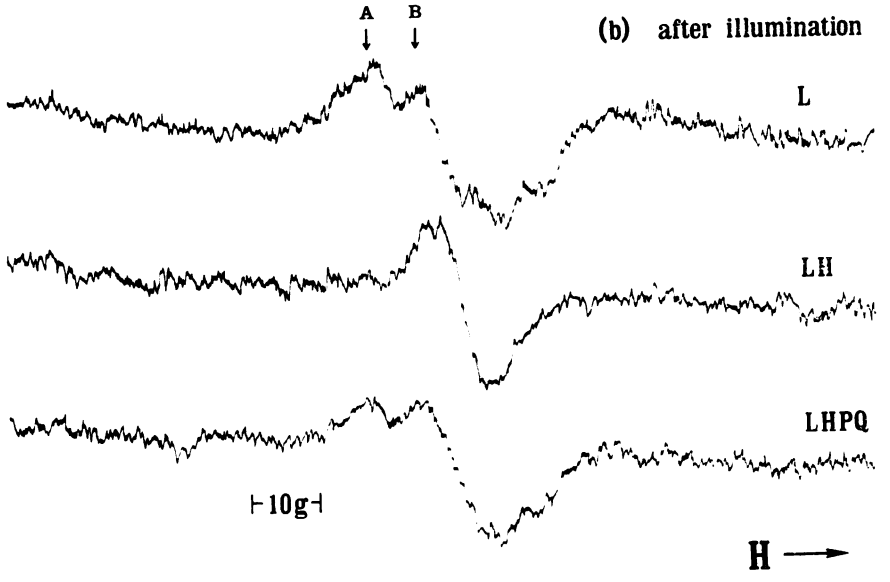


FIG. 2. Complete extraction of Signal II. ESR signals recorded from spinach chloroplasts a) in the light and b) in the dark after cessation of illumination. See Fig. 1 for meaning of L, LH, LHPQ. Sample L) 0.53 mg chlorophyll, 0.025 μmole PQ:μmole chlorophyll. (b) 73 min in dark. Sample LH) 0.18 mg chlorophyll, 0.0012 μmole PQ:μmole chlorophyll. (b) 28 min in dark. Sample LHPQ) 0.36 mg chlorophyll, 0.5 μmole PQ added back to sample. (b) 25 min in dark. It was not possible to normalize the signal amplitudes to permit direct comparison of signal intensity (see Methods and Materials). Spectra virtually identical to those displayed here were recorded over the time interval, 3 min to 73 min in the dark. In the case of "L" and "LHPQ," there was some decay in amplitude during this time, but "LH" remained unchanged. Modulation amplitude = 3.0 gauss, T = 20°.

often observed if the material has been heptane-extracted as well. The effect of the readdition of PQ to the extracted chloroplasts is 2-fold (spectra "LHPQ" in Fig. 2a and 2b). The signal amplitude at field point A in the light and in dark following light is at least partially restored, and the ratio of signal amplitudes at field point B as compared to A is restored to a value close to that seen prior to heptane extraction (*cf.* spectrum "LHPQ" *vs.* "L"). In the experiment depicted in Fig. 2a and 2b the heptane extraction very nearly eliminated Signal II. However, most often some residual Signal II was left, despite the fact that at least 90 % of the PQ had been extracted.

The ratio of signal amplitude observed in the light at magnetic field points B *vs.* A accurately reflects the relative contributions of Signals I and II to the composite spectrum. (For Signal II alone, B:A~3:4). Heptane extraction clearly influenced the ratio of Signals I to II even when it did not completely remove Signal II (Fig. 3, "L" *vs.* "LH"). The increase of this ratio was repeatedly observed and was usually due both to the reduction of the amplitude at A and to an increase at B. The re-addition of PQ restored the ratio of Signal I to II to that seen prior to heptane extraction (Fig. 3). However, the increase at B (reflecting predominantly an increased concentration of the free radical molecules producing Signal I) was often greater than the decrease at A (reflecting a decreased concentration of II). This increase in the ratio B:A in the light upon heptane extraction (samples LH) is taken as 1 condition for considering the experiment to be valid. The other criterion is that the ratio B:A be at least partially restored to the ratio observed before heptane extraction (samples L) upon the readdition of plastoquinone (samples LHPQ). By these criteria, 7 experiments were unquestionally valid, 3 probably valid and 1 was discarded.

These results indicate only that plastoquinone is necessary for the production of the free radical of Signal II. They still do not establish that the free radical is necessarily generated from plastoquinone. For example, the free radical may be an electron carrier down the chain from PQ. In that case, removing PQ would remove the signal since it is light induced (from which we infer that it depends on electron transport) and PQ would be necessary only for the maintenance of the integrity of the transport chain. However, if the free radical is generated from PQ, then when D-PQ is used to replace the PQ removed by extraction the spectrum generated by the reactivated system will be that of "deuterated Signal II" (half-width *ca.* 5–6 gauss) and without observable hyperfine structure (2). If on the other hand the PQ serves only to maintain the integrity of the transport system and the radical is a carrier further down the chain, then only the usual "protonated Signal II" will be observed (half-width *ca.* 19 gauss, partially resolved hyperfine structure).

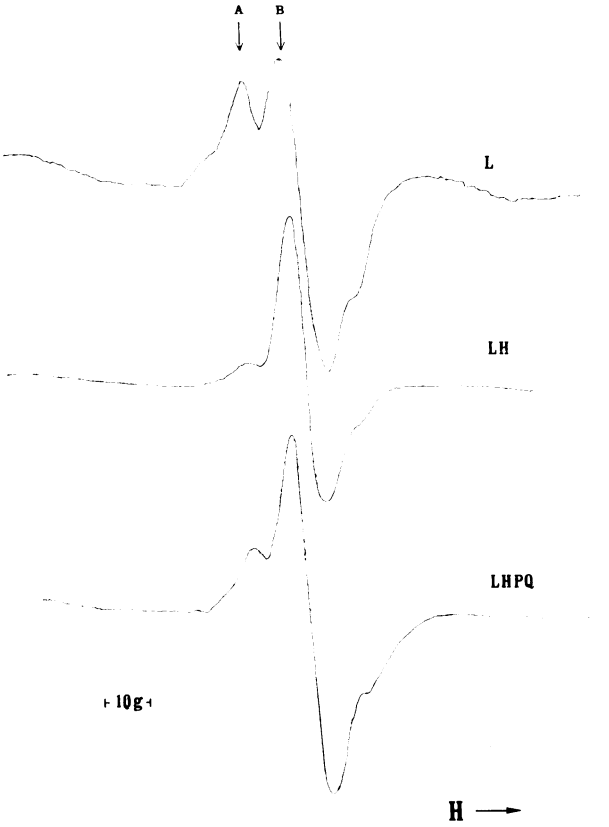


FIG. 3. Incomplete extraction of Signal II. A substantial quantity of Signal II survived heptane extraction ("LH") despite the fact that only 7 % of the PQ remained. In most experiments, the ratio B/A in sample "LHPQ" was even closer to the value in "L" than in this one. Sample L) 2.2 mg chlorophyll, 0.029 μmole PQ: μmole chlorophyll, B:A~1.4. Sample LH) 2.2 mg chlorophyll, 0.0020 μmole PQ:μmole chlorophyll, B:A~7.4. Sample LHPQ: 2.0 mg chlorophyll, 0.16 μmole PQ added, B:A~2.7, amplitude reduced by 1.5. It was not possible to normalize the signal amplitudes to permit direct comparison of signal intensity (see Methods and Materials).

Unfortunately, the actual experiments were not as neat as the foregoing analysis would suggest owing mainly to the fact that some Signal II often remains after vigorous extraction with heptane. In 2 of the 4 experiments in which D-PQ was added back after the heptane extraction some Signal I was also present, having failed to decay completely in the dark. The situation then was that "protonated Signal II" (H-II) and, to a lesser extent, "protonated Signal I" (H-I) provided background ESR signals against which any contribution of "deuterated Signal II" (D-II) had to be assessed.

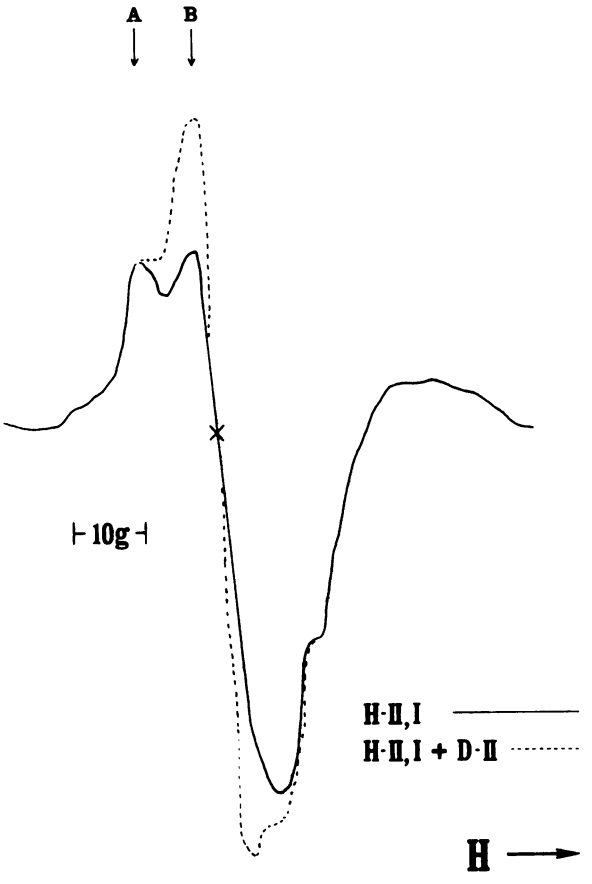


FIG. 4. The effect of adding "deuterated Signal II" to a background of "protonated Signals II and I." The spectrum labelled "H-II, I" was constructed by adding together experimental spectra in which Signals II and I from protonated organisms occurred separately. Enough H-I was added to give the ratio of signal amplitude at magnetic field points B:A seen in sample "LHPQ" in the experiment presented in Fig. 5. Signal II from deuterated organisms was added to this to give the spectrum "H-II, I + D-II." D-II was added to "H-II, I" until the B:A ratio was that seen in sample "LHDPQ" in the experiment presented in Fig. 5. They were constructed in this way since the experimental spectra to which they must be compared contain some Signal I which did not decay in the dark (a common situation when the sample has been lyophilized) as indicated by excess amplitude at B. The fact that D-II has the same g-value as H-II but is much narrower results in the contribution of D-II to the composite spectrum being clearly observed a) as an increase in the signal amplitude ratio B:A and b) by the appearance of a new local minimum 4 to 5 gauss downfield from the usual peak.

Fortunately, the large difference in line width between H-II *vs.* D-II makes it possible to detect small relative contributions of D-II to the composite line (Fig. 4).

A comparison of a spectrum recorded from a lyophilized, heptane extracted sample which had protonated plastoquinone added back (LHPQ) with a spectrum from a similar sample to which deuterated plastoquinone was added back (LHDPQ) is seen in Fig. 5. In Fig. 6, the LHDPQ sample is compared to the constructed composite spectrum of Fig. 4 to emphasize the fact that the alterations of the spectrum of LHDPQ in comparison with LHPQ are exactly those which are predicted when Signal D-II is superimposed on a background of Signals H-II and H-I. Four experiments were done in which deuterated plastoquinone was added back to heptane extracted chloroplasts. One experiment was inconclusive while the other 3 show results consistent with Fig. 5 and 6. The increase in the number of free radicals obtained on addition of deuterated

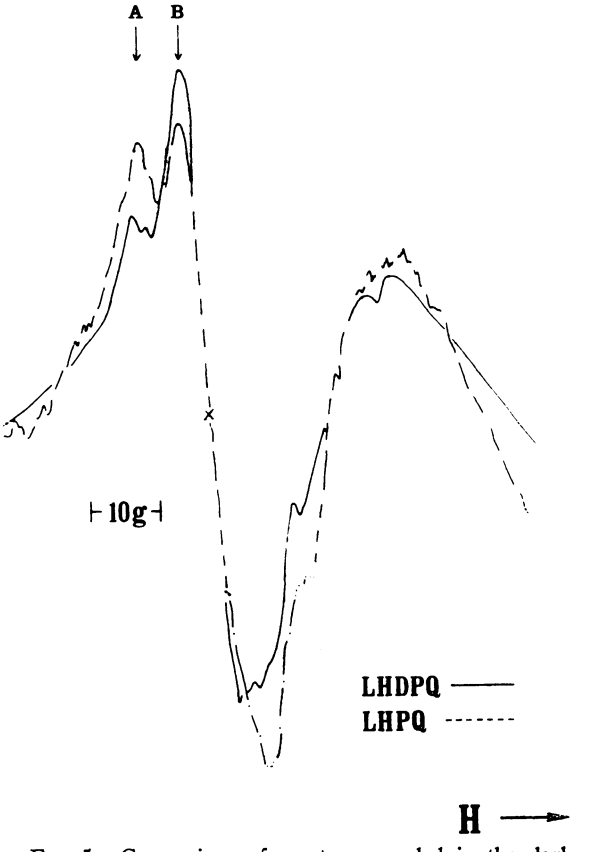


FIG. 5. Comparison of spectra recorded in the dark following illumination from heptane extracted tobacco chloroplasts to which protonated vs. deuterated plastoquinone was added. Note that the ratio of amplitudes at B:A is greater in the sample to which deuterated PQ has been added (LHDPQ) and that the first local minimum is displaced 4 to 5 gauss downfield in the LHDPQ compared to the LHPQ sample. Modulation amplitude = 3.0 gauss, T = 20°.

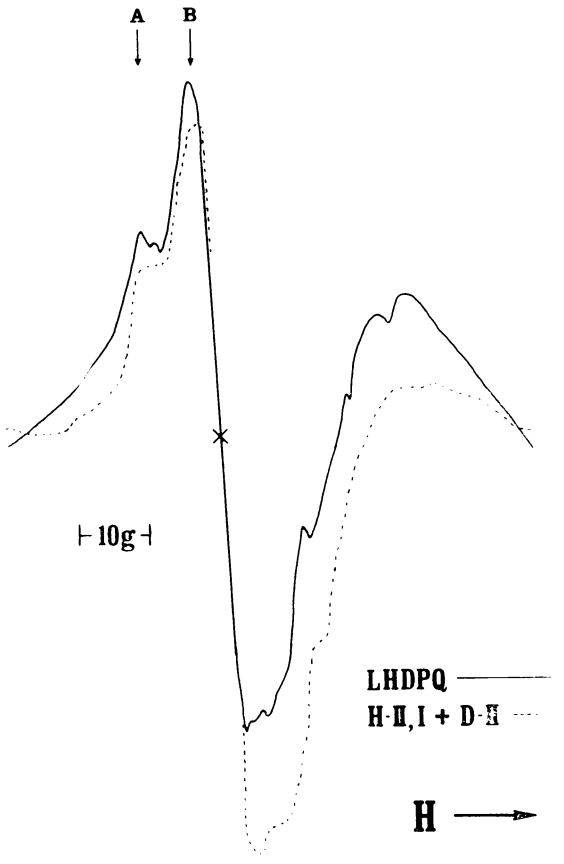


FIG. 6. LHDPQ *vs.* constructed spectrum of Fig. 5. Comparison of spectrum recorded in the dark following illumination from heptane extracted tobacco chloroplasts to which deuterated PQ was added with the composite constructed spectrum (H-II, I + D-II) of Fig. 5. This comparison emphasizes the fact that the modifications of the LHDPQ spectrum are exactly those expected when deuterated Signal II is added to the "background" spectrum which remained in this particular experiment after heptane extraction.

plastoquinone appears to be small but the interaction of the unpaired electrons with deuterons present only in PQ, or molecules directly derived from PQ, is unmistakable. This interaction is seen in Fig. 5 as (a) the increase in the ratio of the signal amplitude at magnetic field point B to that at A when deuterated plastoquinone was added back (LHDPQ) as compared with the readdition of protonated plastoquinone (LHPQ) and (b) the 4 to 5 gauss shift to lower magnetic field values of the first high field extremum in LHDPQ *vs.* LHPQ. The composite results of this series of experiments clearly show that the increase in the ratio B:A is associated with the readdition of deuterated plastoquinone. In only 1 of the 7 valid experiments do the spectra of LHPQ show a B:A ratio close to that seen in the LHDPQ spectrum of Fig. 5, and in this 1 case it is clear that the elevated ratio is due to residual, non-decaying Signal I rather than any contribution due to the readdition of plastoquinone. Likewise, there is no case in which the readdition of protonated plastoquinone resulted in the downfield shift of the high field extremum seen in spectrum LHDPQ in Fig. 5.

Discussion

The main conclusion derived from this work is based on the data of Fig. 5 and 6. These data make it clear, as pointed out in the "Results" section, that there is interaction between unpaired electrons and hydrogens which are originally present on the plastoquinone which is added back to the heptane extracted chloroplasts. The question remains whether these radicals reflect normal *in vivo* processes or are artifacts. The fact that the line shape of the sample to which PQ had been added back (LHPQ) is identical to that which is generated by the lyophilized sample (L) (Fig. 2b) supports the proposal that the same radical is involved. This fact makes it clear, for example, that the "LHPQ" spectrum cannot be the result of the added PQ remaining in solution and being reduced there since then the line shape would be altogether different (4). This evidence by itself is not sufficient, however, because the line shape could result from the non-specific adsorption of PQ in a reducing environment. In this hypothetical case the immobilization of the PQ would result in the observed line shape as well as would the presumably more specific binding of PQ in the unextracted "L" sample. However, if the exogenous PQ in the reconstituted system were simply non-specifically adsorbed, then the readdition of PQ would be unlikely to affect the relative ratio of Signals I to II consistently seen and demonstrated in Fig. 2 and 3. Taken together, the facts that the line shape in the reconstituted sample is the same as before extraction (Fig. 2b) and that the readdition of PQ tends to restore the relative ratio of Signals I to II to the value seen in the lyophilized sample (L) compared with the heptane extracted one (LH) (Fig. 3) strongly support the conclusion that the exogenous PQ is functioning in the manner of the endogenous PQ before its extraction. The alternative proposal of an artificial shunt created by the non-specific participation of the exogenous PQ would be subject to the restrictions imposed by these results; namely, the shunt cannot be created by molecules in solution, the shunt must be able to supply electrons to the molecules producing Signal I and the free radical generated in the shunt must be directly deriveable from PQ. We wish to emphasize that the interaction of the unpaired electron with deuterons introduced only in the plastoquinone which we believe these data demonstrate (Fig. 5 and 6) does not necessarily mean that the observed free radical is the semiquinone of plastoquinone. The

identification of plastochromanoxyl free radical as a contributor to Signal II is also consistent with the result that the unpaired electrons interact with deuterons introduced in PQ only since plastochromanoxyl free radical is the product of the one electron reduction of solanachromene, a cyclical isomer of PQ. We have presented evidence elsewhere (4) which, when taken in conjunction with the results reported here, leads us to believe that Signal II is of the substituted phenoxy-type (plastochromanoxyl) rather than a semiquinone.

The observed increase in the ratio of the signal amplitude at B:signal amplitude at A in heptane extracted, as compared with lyophilized, chloroplasts is consistent with a system in which the electrons removed from component II when the system is illuminated serve to re-reduce component I which has been oxidized to its free radical state. Thus, if Signal I is a chlorophyll radical in O_2-evolving systems as it is in bacterial photosynthetic systems (10, 11) and assuming that Signal II feeds electrons to Signal I, then the relationship observed here between Signals I and II is evidence that this chlorophyll free radical is the positive ion.

Correlation between Hill activity and Signal II has been cited as evidence that Signal II is due to plastosemiquinone (3). Ferricyanide Hill activity was often measured on portions of the samples used for ESR in these experiments. As expected, the Hill activity was drastically reduced by the relatively vigorous heptane extraction, yet the chloroplasts often generated a substantial Signal II. Signal II can also be observed in lyophilized chloroplasts which have been aged for several weeks before resuspending. No Hill activity would be expected under these conditions. However, plastoquinone reduction has been observed in frozen-thawed and sonicated chloroplast preparations in which ferricyanide Hill activity has been destroyed (12). Thus, the destruction of Hill activity with the retention of at least some Signal II does not rule out free radicals derived from plastoquinone as the source of Signal II.

The results reported here will be confirmed by doing the inverse experiment; *i.e.*, by preparing chloroplasts from deuterated organisms and adding back protonated PQ. In this case, any residual Signal II (D-II) will be confined to a relatively narrow region of the magnetic field and any contribution of H-II to the composite spectrum will be easily observed and more easily quantitated.

The fact that a free radical derived from PQ contributes to Signal II does not exclude contributions from other molecular species. Experiments with α-tocopherol analogous to those reported here will be done to directly assess whether free radicals derived from it contribute to Signal II. γ-tocopherol cannot be a major contributor since it is not found in *Scenedesmus* cultures which give normal Signal II (N. I. Bishop, private communication).

Acknowledgment

We thank John R. Wright for his extremely capable assistance and Ruth G. Alscher for valuable discussions.

Literature Cited

1. COMMONER, B., J. J. HEISE, B. B. LIPPINCOTT, R. E. NORBERG, J. V. PASSONNEAU, AND J. TOWNSEND. 1957. Biological activity of free radicals. Science 126: 57–63.

2. KOHL, D. H., J. TOWNSEND, B. COMMONER, H. L. CRESPI, R. C. DOUGHERTY, AND J. J. KATZ. 1965. Effects of isotopic substitution on electron spin resonance signals in photosynthetic organisms. Nature 206: 1105–10.

3. WEAVER, E. C. 1968. EPR studies of free radicals in photosynthetic systems. Ann. Rev. Plant Physiol. 19: 288–94.

4. KOHL, D. H., J. R. WRIGHT, AND M. WEISSMAN. 1969. Electron spin resonance studies of free radicals derived from plastoquinone, α- and γ-tocopherol and their relation to free radicals observed in photosynthetic material. Biochim. Biophys. Acta 180: 536–44.

5. BISHOP, N. I. 1961. The possible role of plastoquinone (Q-254) in the electron transport system of photosynthesis. In: Quinones in Electron Transport. G. E. W. Wolstenholme, and C. M. O'Connor, eds. Little, Brown and Company, Boston, Massachusetts. p 385.

6. DABOLL, H. F., H. L. CRESPI, AND J. J. KATZ. 1962. Mass cultivation of algae in pure heavy water. Biotech. Bioeng. 4: 281–97.

7. CRANE, F. L. 1961. Isolation and characterization of the co-enzyme Q (Ubiquinone) group and plastoquinone. In: Quinones in Electron Transport. G. E. W. Wolstenholme and C. M. O'Connor, eds. Little, Brown and Company, Boston, Massachusetts. p 36.

8. ARNON, D. I. 1949. Copper enzyme in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15.

9. HEISE, J. J. 1962. Electron spin resonance studies in photosynthetic systems. Ph.D. Dissertation, Washington University, St. Louis, Missouri.

10. MCELROY, J. D., G. FEHER, AND D. C. MAUZERALL. 1969. On the nature of the free radical formed during the primary process of bacterial photosynthesis. Biochim. Biophys. Acta 172: 180–83.

11. BOLTON, J. R., D. W. REED, AND R. K. CLAYTON. 1968. An identification of the radical giving rise to the light-induced ESR signal in photosynthetic bacteria. Photochem. Photobiol. 9: 209–18.

12. WOOD, P. M. 1968. A study of the redox state of plastoquinone A and the importance of the redox state in photosynthetic reactions. Dissertation, Purdue University, Lafayette, Indiana