# The effect of herbicides on components of the PS II reaction centre measured by EPR

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Received 10 October 1983

Incubation of PS II membranes with herbicides results in changes in EPR signals arising from reaction centre components. Dinoseb, a phenolic herbicide which binds to the reaction centre polypeptide, changes the width and form of the EPR signal arising from photoreduced $Q_A^-Fe$. *o*-Phenanthroline slightly broadens the $Q_A^-Fe$ signal. These effects are attributed to changes in the interaction between the semiquinone and the iron. DCMU, which binds to the 32 kDa protein, has virtually no effect on the width of the $Q_A^-Fe$ signal but does give rise to an increase in its amplitude. This could result from a change in redox state of an interacting component. Herbicide effects can also be seen when $Q_A^-Fe$ is chemically reduced and these seen to be reflected by changes in splitting and amplitude of the split pheophytin$^-$ signal. Dinoseb also results in the loss of 'Signal II dark', the conversion of reduced high-potential cytochrome $b_{559}$ to its oxidized low-potential form and the presence of transiently photooxidized carotenoid after a flash at 25°C; these effects indicate that dinoseb may also act as an ADRY reagent.

*Herbicide* *Photosystem II* *Plastoquinone* *EPR* *Pheophytin* *Photosynthesis* *ADRY reagent* *Cytochrome* $b_{559}$

## 1. INTRODUCTION

Recent progress in the study of Photosystem II (PS II) by EPR (review [1]) has relied heavily on the analogy with the acceptor side of purple bacteria (review [2]). It has been demonstrated biochemically [3] and spectroscopically [4,5] that $Q_A$, the primary quinone acceptor of PS II, is associated with an iron atom and gives rise to an unusual EPR signal almost identical to that well-known in bacteria [6,7]. In bacteria, the secondary quinone acceptor [8,9] is also associated with the iron atom and also gives rise to characteristic EPR signals [9–11]. Preliminary data [12] indicating that a semiquinone iron signal may arise from the secondary quinone acceptor $Q_B^-$ [13,14], in PS II have also been reported.

A number of inhibitors of electron transfer, including important classes of herbicides, act by preventing electron transport between $Q_A$ and $Q_B$. A current theory of herbicide action is that $Q_B$ is excluded from its binding site on the reaction centre by competitive binding of the herbicide molecule [15–18]. Different classes of herbicides apparently have different binding sites (review [19]). DCMU-type herbicides bind to a 32 kDa polypeptide [20–23] thought to be a regulatory protein associated with $Q_A$ to $Q_B$ electron transfer [24,25] while phenolic herbicides bind to a 42 kDa polypeptide [23,26], which is probably a reaction centre protein [27]. Herbicide-induced perturbations of these polypeptides might be expected to modify the EPR signals arising from reaction

*Abbreviations:* Adry, reagents which accelerate the deactivation reactions of the water splitting enzyme; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; dinoseb, 2,4-dinitro-6-*s*-butylphenol; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; Mes, 2-(*N*-morpholino)ethanesulphonic acid; *o*-phen, *o*-phenanthroline



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centre components. In particular, changes in the signal arising from $Q_A^-Fe$ might be expected since it arises from an interaction between two components, the semiquinone and the iron, and since the components are expected to be located close to the herbicide binding sites. In bacteria, an effect of *o*-phen upon the $Q_A^-Fe$ signal has already been briefly noted [28]. Here, some effects of herbicide inhibitors upon EPR signals arising from PS II reaction centre components are described.

## 2. MATERIALS AND METHODS

Oxygen evolving PS II membranes were prepared from spinach chloroplasts as in [29] but with the following modifications: (1) Triton digestions and resuspensions were carried out at pH 6.0 [50 mM Mes replaced Hepes (pH 7.5), Yocum, C. personal communication]; (2) 10 mM $MgCl_2$ was used instead of 5 mM $MgCl_2$ in all steps; (3) the second digestion in low concentration Triton was missed out. The membranes were resuspended at high concentration (≈12 mg Chl/ml) in buffer containing sucrose (400 mM), Mes (20 mM, pH 6.0), NaCl (15 mM), $MgCl_2$ (5 mM) and EDTA (2 mM).

EPR samples in calibrated quartz tubes were incubated in darkness for 10 min at 20°C in the presence or absence of inhibitors (see legends for concentrations), before being frozen to 77 K. EPR spectra were recorded on a Bruker ER-200t-X-band spectrometer using an Oxford Instruments liquid helium cryostat and temperature control system. Illumination was provided by an 800 W projector in an unsilvered Dewar flask containing liquid $N_2$. Dinoseb was a gift from Dr Van Assche (Procida, Marseille). The stock (30 mM) was dissolved in ethanol. DCMU, recrystallized by Dr J. Farineau, was dissolved in isopropanol (10 mM). *o*-Phen was dissolved in ethanol.

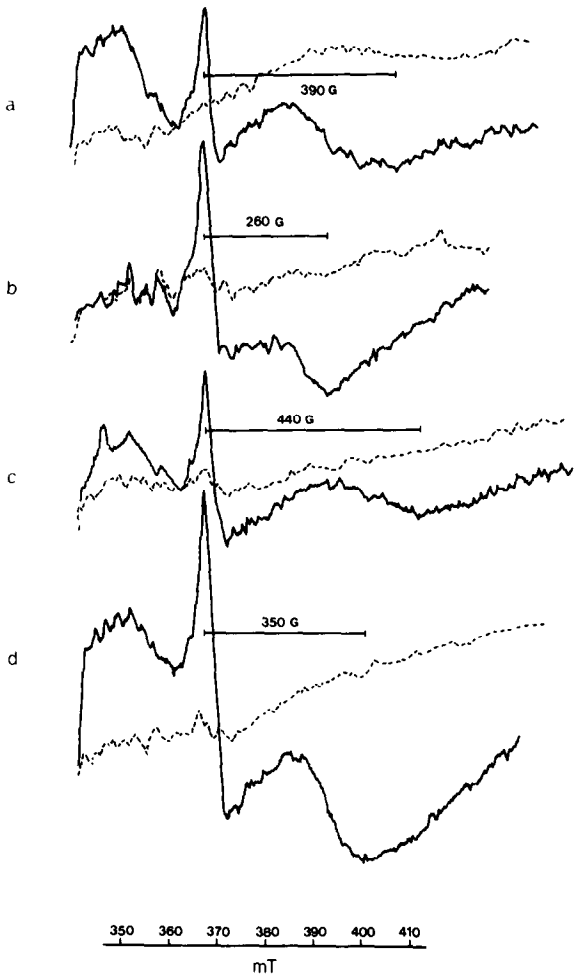


Fig.1. EPR spectra of the photoinduced $Q_A^-Fe$ signal in PS II membranes and the effect of incubation at 20°C with PS II inhibitors. Broken lines, dark; solid lines, after illumination for 10 min at 77 K. (a) No additions, (b) dinoseb 1 mM, (c) *o*-phen 60 mM, (d) DCMU 1.8 mM. Instrument settings: temperature, 4.8 K; microwave power, 8 dB down from 200 mW; frequency, 9.465 GHz; modulation amplitude, 20 G.

## 3. RESULTS AND DISCUSSION

Fig.1a shows a high field spectrum of a sample of PS II membranes frozen in darkness in the absence of inhibitors. In darkness (the broken line), no signal was present in the region of $g = 1.82$ but after 10 min illumination at 77 K a large, well-resolved signal was observed. This signal is attributed to $Q_A^-Fe$ as previously observed in this kind of PS II peparation [5]. No significant increase in signal amplitude could be produced by further illumination at 200 K. This result contrasts with our previous preliminary report where 200 K illumination was required to obtain the maximal $Q_A^-Fe$ signal [5]. This discrepancy is probably due to the improved intactness of the donor side of PS II in the preparation used in the current work.

Of note in fig.1a is the presence of a broad signal at $g = 1.87$ which is also photoinduced under these

conditions. This signal has been attributed to an alternative resonance form of $Q_A^-Fe$ [12].

Incubation of the PS II membranes with inhibitors resulted in slight changes in the EPR spectra observed in the dark (fig.1b,d, broken lines). With dinoseb, *o*-phen and DCMU a small $Q_A^-Fe$ signal at $g = 1.82$ was present in the dark while, in the control samples with no additions or with 10% ethanol (not shown), no signal was present in the dark at $g = 1.82$. This inhibitor-induced formation of $Q_A^-Fe$ is attributed to back transfer of electrons from the small percentage of stable $Q_B^-$ normally present in the dark [30].

The presence of herbicides has significant effects on the photoinduced $Q_A^-Fe$ signals (fig.1b,d). Although accurate width measurements are difficult to make due to the broadness of the high field dip, it can be seen that marked differences in width are induced by dinoseb and *o*-phen while DCMU and ethanol (not shown) have virtually no effect on the width. Dinoseb induces a narrowing of the $Q_A^-Fe$ signal by about 33% and the $g = 1.87$ resonance form of $Q_A^-Fe$ is almost completely suppressed. Since the $g = 1.87$ signal is associated with a low field dip at $g \simeq 1.63$ [12], the suppression of the signal may at least partially explain the observed narrowing effect. The amplitude of the $g = 1.82$ signal is enhanced by the presence of dinoseb. This could be due to the narrowing of the signal and/or conversation of the $g = 1.87$ resonance form to the $g = 1.82$ form.

The presence of *o*-phen results in a small yet significant broadening of the light-induced signal ($\simeq$13%). The amplitude of the $g = 1.82$ signal is not significantly changed by *o*-phen incubation. DCMU has a very slight effect on the width of the $g = 1.82$ signal but close comparison of the signal in the presence of DCMU with that in control samples in several experiments indicates that the slight narrowing present in fig.1 is probably not significant. A large increase in the amplitude of the $Q_A^-Fe$ signal is induced by DCMU incubation. Since the form and width of the signal are hardly modified, the increase in the intensity of the signal is probably due to an increase in spins contributing to the signal. An explanation for this effect can be postulated using the analogy with the purple bacteria. In bacteria, it has been demonstrated that if $Q_A^-Fe$ is generated in the presence of $Q_B^-Fe$, the two components interact and this results in a diminution and even the disappearance of the signals at $g = 1.82$ [10,30]. This kind of interaction would be predicted to occur in PS II if $Q_B^-$ is associated with the iron atom as suggested in [12]. In dark-adapted chloroplasts a significant amount of $Q_B^-$ is present [31,32] and the addition of DCMU results in the loss of $Q_B^-$ due to back transfer of electrons to $Q_A^-$ [30]. Thus the effect of DCMU could be the removal of dark-stable $Q_B^-Fe$ and the consequent disappearance of a magnetic interaction with the photoinduced $Q_A^-Fe$. In agreement with this idea is the fact that the addition of dichlorodicyanobenzoquinone (1.5 mM), an oxidizing agent, also resulted in a comparable increase in the photoinduced $Q_A^-Fe$ signal amplitude (not shown), just as would be expected if $Q_B^-$ was lost by oxidation. However the addition of dithionite, which was also expected to remove $Q_B^-$ by reducing it to $Q_BH_2$, had no effect on the signal amplitude (see fig.2b). The effect of DCMU could still be due to the removal of an interaction by a change in redox state of a nearby component, but, if so, the interacting component is probably not $Q_B^-$. In bacteria, the existence of a component other than $Q_B^-Fe$ which diminishes the $Q_A^-Fe$ signal has been suggested [33]. When samples incubated with inhibitors were reduced with sodium dithionite, the $Q_A^-Fe$ signals present in the dark showed some features similar to those of the signals photoinduced in non-reduced samples (fig.2b). The changes in the width of the signal are more difficult to discern under these conditions. This is partly due to lack of appropriate baselines but may also reflect diminished inhibitor binding at low potentials.

When reduced samples were illuminated at 200 K, the split signal attributed to reduced pheophytin (Ph) interacting with the semiquinone–iron complex (split $Ph^-$) [34] was photoinduced (fig.2a). The splitting of the $Ph^-$ signal was slightly modified by the presence of the herbicides. Only slight differences between the control ($\simeq$40 G) and *o*-phen ($\simeq$41 G) and DCMU ($\simeq$44 G) were observed. However dinoseb reduced the splitting to only $\simeq$33 G. Whether this effect is a reflection of the narrowing of the $g = 1.82$ signal itself or of the loss of the $g = 1.87$ resonance form has not yet been determined. With all 3 inhibitors an increase in the amplitude of the split $Ph^-$ was observed.

The changes induced in the $Q_A^-Fe$ and split $Ph^-$ signals indicate that, in the dark, significant in-

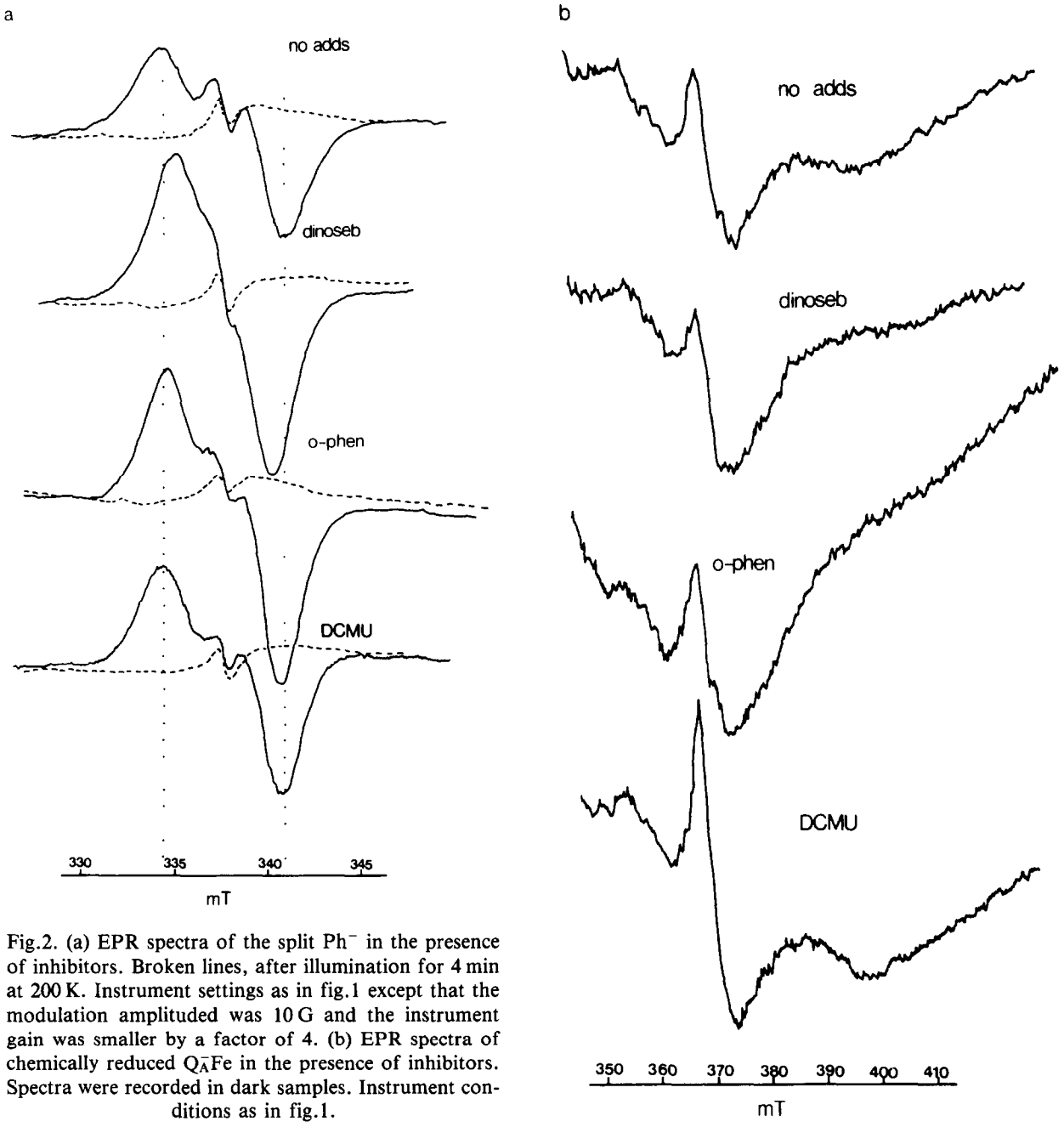


Fig.2. (a) EPR spectra of the split $Ph^-$ in the presence of inhibitors. Broken lines, after illumination for 4 min at 200 K. Instrument settings as in fig.1 except that the modulation amplituded was 10 G and the instrument gain was smaller by a factor of 4. (b) EPR spectra of chemically reduced $Q_A^-Fe$ in the presence of inhibitors. Spectra were recorded in dark samples. Instrument conditions as in fig.1.

hibitor binding occurs under reducing conditions.

Dinoseb also affected EPR signals from other PS II reaction centre components. Signal II dark, which arises from a component which acts as a donor to PS II under some circumstances (e.g. [35]) and which may be due to a cationic semiquinone [36], is almost completely absent in samples incubated with dinoseb (fig.3a). Other treatments which have this effect are reducing agents and some ADRY reagents [35,37].

Oxidized cytochrome $b_{559}$ gives rise to EPR signals at $g \simeq 3.1$ and $g \simeq 3.0$ when it is in its high-

and low-potential form, respectively [38,39]. Fig. 3b shows that cytochrome $b_{559}$, which was mostly in its reduced high-potential form in dark-adapted PS II membranes, was converted to the oxidized low-potential form by the addition of dinoseb. Subsequent illumination of PS II particles at 77 K results in photooxidation of high-potential cytochrome $b_{559}$ in the control sample and very little change in the sample containing dinoseb.

Dark oxidation of cytochrome $b_{559}$ [40,41] and removal of signal II dark [37] are properties of some ADRY reagents. The presence of ADRY reagents is known to result in the transient photooxidation of carotenoid in PS II [41,42]. This effect was used as a test of the putative ADRY properties of dinoseb. Fig.3c shows that dinoseb addition does lead to the photooxidation of carotenoid as measured by the characteristic absorbance increase at 990 nm after a flash [42]. These results indicate that dinoseb has ADRY reagent characteristics as well as its well-known inhibitory effect on the electron acceptor side of PS II. It is of note that previous work with dinoseb has indicated a donor side effect [43,44].

The herbicide-induced changes in the semiquinone–iron interaction and the consequent effect on the split $Ph^-$ signal are of interest because they could lead to a better understanding of the structural relationship between these two acceptors and the iron atom.

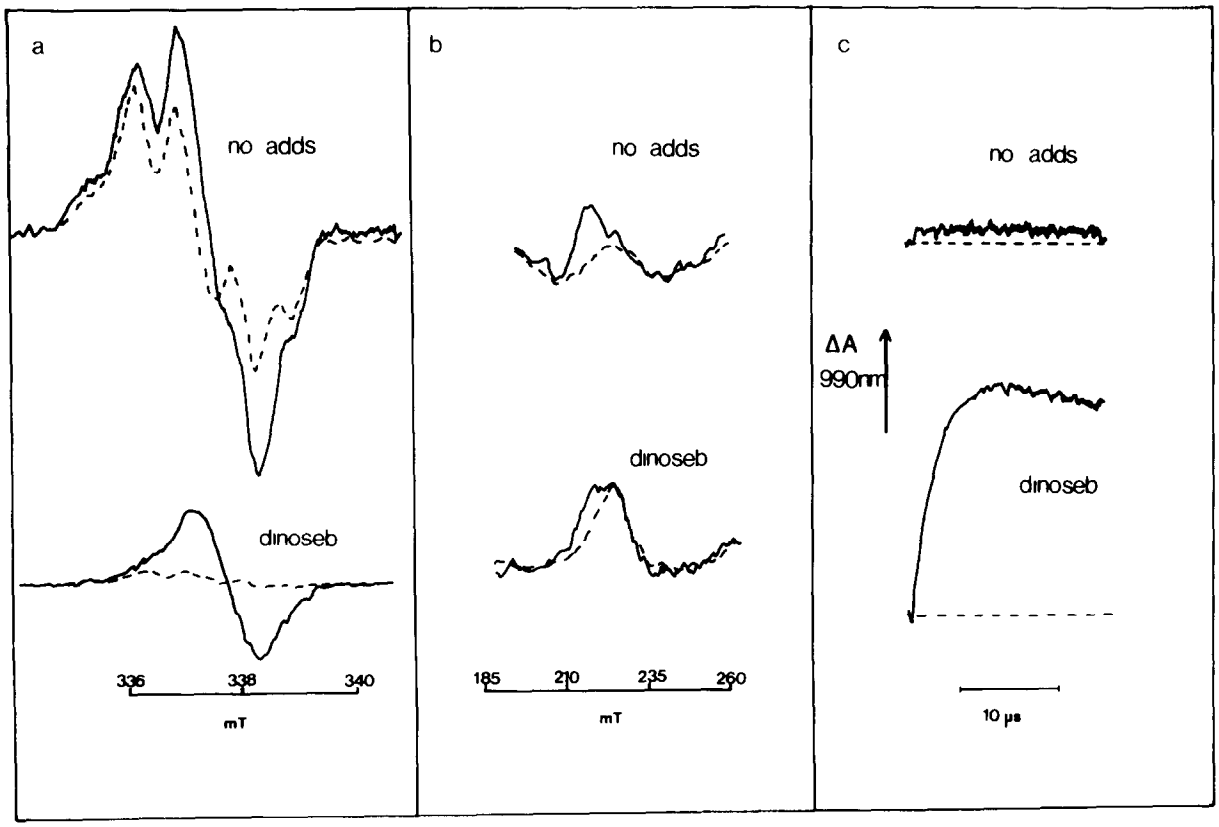


Fig.3. (a) The effect of dinoseb on Signal II dark. Broken lines, dark; solid lines, after 10 min illumination at 5 K. Instrument settings: temperature, 15 K; microwave power, 65 dB down from 200 mW; frequency, 9.465 GHz; modulation amplitude, 1 G. (b) The effect of dinoseb on cytochrome $b_{559}$. Broken lines, dark; solid lines, after 10 min illumination at 5 K. Instrument conditions as in fig.1 except that the temperature was 15 K. (c) Absorption change at 990 nm induced by one laser flash (20 ns, 600 nm) in PS II membranes (chlorophyll concentration, $4.2 \times 10^{-5}$ M; optical path, 10 nm; 21°C) in buffer with no further addition (upper trace) or with 33 $\mu$M i-dinoseb (maximum $\Delta A = 1.1 \times 10^{-3}$).

The effects of dinoseb and *o*-phen on the semiquinone–iron interaction are different to that of DCMU. This difference might correlate with current ideas of their different sites of binding . Dinoseb is representative of phenolic herbicides which bind to a reaction centre polypeptide [23,26]. It has been suggested that *o*-phen binds to a reaction centre polypeptide [45]. In contrast, DCMU-type herbicides bind to the 32 kDa polypeptide [20–23].

While this manuscript was in preparation an apparent effect of DCMU on the amplitude of the $Q_A^-Fe$ signal was noted in a cyanobacterium [46].

## ACKNOWLEDGEMENTS

A.W.R. would like to thank Professor A.R. Crofts, Dr C.T. Yerkes and especially Dr John R. Bowyer for useful discussion. We thank Dr J. Farineau for purifying the DCMU and Dr Van Assche for providing the dinoseb. This work was supported in part by the Commission of the European Community, Solar Energy Program ESD-015-5.

## REFERENCES

[1] Rutherford, A.W. (1983) in: The Oxygen-Evolving System of Photosynthesis (Inoue, Y. et al. eds) Academic Press, Tokyo, in press.
[2] Dutton, P.L., Prince, R.C. and Tiede, D.M. (1978) Photochem. Photobiol. 28, 939–949.
[3] Klimov, V.V., Dolan, E., Shaw, E.R. and Ke, B. (1980) Proc. Natl. Acad. Sci. USA 77, 7227–7231.
[4] Nugent, J.H.A., Diner, B.A. and Evans, M.C.W. (1981) FEBS Lett. 124, 241–244.
[5] Rutherford, A.W. and Mathis, P. (1983) FEBS Lett. 154, 328–334.
[6] Feher, G. and Okamura, M.Y. (1976) Brookhaven Symp. Biol. 28, 183–194.
[7] Dutton, P.L., Leigh, J.S. and Reed, D.W. (1973) Biochim. Biophys. Acta 292, 654–664.
[8] Vermeglio, A. (1977) Biochim. Biophys. Acta 459, 516–524.
[9] Wraight, C.A. (1977) Biochim. Biophys. Acta 459, 525–531.
[10] Rutherford, A.W. and Evans, M.C.W. (1979) FEBS Lett. 100, 305–308.
[11] Rutherford, A.W. and Evans, M.C.W. (1979) FEBS Lett. 104, 227–230.
[12] Rutherford, A.W., Zimmermann, J.L. and Mathis, P. (1983) in: Proc. 6th Int. Congr. Photosynth. (Sybesma, C. ed.) Martin Nijhoff, The Hague, in press.
[13] Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 314, 250–256.
[14] Velthuys, B.R. and Amesz, J. (1974) Biochim. Biophys. Acta 333, 85–94.
[15] Velthuys, B.R. (1981) FEBS Lett. 126, 277–281.
[16] Wraight, C.A. (1981) Isr. J. Chem. 21, 348–354.
[17] Bowes, J.M. and Crofts, A.R. (1981) Arch. Biochem. Biophys. 209, 682–686.
[18] Lavergne, J. (1982) Biochim. Biophys. Acta 682, 345–353.
[19] Trebst, A. and Draber, W. (1979) in: Advances in Pesticide Science (Geissbühler, H. ed.) part 2, pp. 223–234, Pergamon Press, Oxford.
[20] Mullet, J.E. and Arntzen, C.J. (1981) Biochim. Biophys. Acta 635, 236–248.
[21] Pfister, K., Steinback, K.E., Gardner, G. and Arntzen, C.J. (1981) Proc. Natl. Acad. Sci. USA 78, 981–985.
[22] Gardner, G. (1981) Science 211, 937–940.
[23] Oettmeier, W., Masson, K. and Johanningmeier, U. (1982) Biochim. Biophys. Acta 679, 376–383.
[24] Renger, G. (1976) Biochim. Biophys. Acta 440, 287–300.
[25] Regitz, G. and Ohad, I. (1976) J. Biol. Chem. 251, 247–252.
[26] Oettmeier, W., Masson, K. and Johanningmeier, U. (1980) FEBS Lett. 118, 267–270.
[27] Kaplan, S. and Arntzen, C.J. (1982) in: Photosynthesis, vol. I (Govindjee, ed.) pp. 65–151, Academic Press, New York.
[28] Feher, G. and Okamura, M.Y. (1978) in: The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R. eds) pp. 349–386, Plenum Press, New York.
[29] Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234.
[30] Wraight, C.A. (1978) FEBS Lett. 92, 283–288.
[31] Mathis, P. and Haveman, J. (1977) Biochim. Biophys. Acta 461, 167–181.
[32] Wollman, F.A. (1978) Biochim. Biophys. Acta 503, 263–273.
[33] Rutherford, A.W., Heathcote, P. and Evans, M.C.W. (1979) Biochim. Biophys. Acta 182, 515–523.
[34] Klimov, V.V., Dolan, E. and Ke, B. (1980) FEBS Lett. 112, 97–100.
[35] Velthuys, B.R. and Visser, J.W.M. (1975) FEBS Lett. 55, 109–113.
[36] O'Malley, P.J. and Babcock, G.T. (1983) Biophys. J. 41, 315a.
[37] Babcock, G.T. and Sauer, K. (1973) Biochim. Biophys. Acta 325, 504–519.

[38] Malkin, R. and Vänngård, T. (1980) FEBS Lett. 111, 228–231.
[39] Nugent, J.H.A. and Evans, M.C.W. (1980) FEBS Lett. 112, 1–4.
[40] Cramer, W.A., Fan, H.M. and Böhme, H. (1971) Bioenergetics 2, 289–303.
[41] Velthuys, B.R. (1981) FEBS Lett. 126, 272–276.
[42] Schenck, C.C., Diner, B.A., Mathis, P. and Satoh, K. (1982) Biochim. Biophys. Acta 680, 216–227.
[43] Lavorel, J. (1980) Biochim. Biophys. Acta 590, 385–399.
[44] Van Assche, C.J. and Carles, P.M. (1982) in: Biochemical Responses Induced by Herbicides, ACS Symp. Ser. no. 181 (Moreland, D.E. et al. eds) pp. 1–21.
[45] Laval-Martin, D., Grizeau, D. and Calvayrac, R. (1983) Plant Sci. Lett. 29, 155–167.
[46] Atkinson, Y.E. and Evans, M.C.W. (1983) FEBS Lett. 159, 141–144.