EPR measurements on the effects of bicarbonate and triazine resistance on the acceptor side of Photosystem II

Wim F.J. Vermaas* and A. William Rutherford+

+*Service de Biophysique, Département de Biologie, Centre d'Études Nucléaires de Saclay, 91191 Gif-sur-Yvette, Cédex, France* and **Laboratory of Plant Physiological Research, Agricultural University, Gen. Foulkesweg 72, 6703 BW Wageningen, The Netherlands*

Received 6 July 1984

CO_2 depletion leads to an approximately 10-fold increase in the light-induced EPR signal at $g = 1.82$, attributed to the $Q_A^- \cdot Fe^{2+}$ complex, in Photosystem II-enriched thylakoid membrane fragments. Upon reconstitution with HCO_3^- the signal decreases to the size in control samples. The split pheophytin$^-$ signal is broader in control or reconstituted than in CO_2-depleted samples. It is concluded that HCO_3^- strongly influences the localization and conformation of the $Q_A^- \cdot Fe^{2+}$ complex. The $Q_A^- \cdot Fe^{2+}$ and split pheophytin$^-$ EPR signals from triazine-resistant *Brassica napus* were virtually identical to those from triazine-susceptible samples, indicating that the change in the 32-kDa azidoatrazine-binding protein does not lead to a conformational change of the $Q_A^- \cdot Fe^{2+}$ complex.

Semiquinone-iron complex *Photosystem II* *Bicarbonate effect* *Triazine resistance* *Photosynthesis* *Herbicide*

1. INTRODUCTION

Recently, EPR signals attributed to the reduced form of the primary quinone-type electron acceptor interacting with a neighbouring Fe^{2+} atom have been reported in Photosystem II (PS II) of plants [1,2]. The signals are similar in shape and g value ($g = 1.82$, or $g = 1.90$, $g \simeq 1.68$) to those attributed to the analogous acceptor in purple bacteria [3,4]. In PS II, the EPR signal at $g = 1.82$ can be converted into the broader signal at $g \simeq 1.9$ when the pH is raised, and vice versa [5,6]. The $g = 1.90$ and $g = 1.82$ signals have been attributed to different resonance forms of $Q_A^- \cdot Fe^{2+}$ [5,6]. Both resonance forms are affected by herbicides which block electron transfer between the primary and secondary quinone acceptors [5–7].

When the pheophytin acceptor of PS II is photoreduced at low temperature in the presence of $Q_A^- \cdot Fe^{2+}$ an EPR signal is formed that is split by $\simeq$50 G around $g \simeq 2.00$ [8]. This signal (split Pheo$^-$) is almost identical to the analogous signal previously observed in purple bacteria [9]. In PS II the split Pheo$^-$ signal is also affected by pH [5] and the presence of herbicides [7].

It has been shown that the EPR signals from $Q_A^- \cdot Fe^{2+}$ and split Pheo$^-$ are sensitive to changes in or near the acceptor complex [5–7] and thus they can be used as a way of monitoring such changes.

It is known that absence of CO_2 (in the presence of formate) leads to a blockage of electron flow between Q_A and the plastoquinone pool [10], whereas steady-state electron flow from H_2O to Q_A^- does not appear to be impaired [11,12]. However, the Q_A^- oxidation by the water splitting system is slowed down in the absence of HCO_3^- [13,14], whereas also the oxidation of Q_A^- by the one-electron acceptor C400 [15] was interpreted to be blocked in the absence of HCO_3^- [16], although this interpretation was questioned recently [17]. In addition, CO_2, and probably also the redox state of Q_A, can modulate herbicide affinity [12,18,19]. In this work the effects of CO_2 depletion (in the

+ To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

presence of formate) on reaction center components measured by EPR are reported.

As reviewed in [19], resistance to triazine herbicides is often caused by a change in a single amino acid in the azidoatrazine binding 32-kDa protein (ABP-32). In this work we also report the results of a study comparing the $Q_A^- \cdot Fe^{2+}$ and split $Pheo^-$ EPR signals in PS II particles isolated from triazine-resistant and susceptible plants.

2. MATERIALS AND METHODS

Oxygen-evolving PS II-enriched thylakoid fragments were prepared from spinach, or from triazine-resistant and -susceptible rape seed (*Brassica napus* L.) according to [20] with modifications as in [7]. The thylakoid fragments used were prepared freshly, or had been stored at high concentrations (5–10 $mg \cdot ml^{-1}$) at −80°C in darkness.

Thylakoid fragments were CO_2 depleted by incubation in darkness under Ar atmosphere at room temperature in a buffer which was previously bubbled with Ar, at pH = 6.0 containing 50 mM MES/NaOH, 25 mM HCO_2Na, 10 mM NaCl, 5 mM $MgCl_2$ and 0.15 M sorbitol, at $\simeq 100\ \mu g \cdot ml^{-1}$ chlorophyll. To part of the sample 10 mM $NaHCO_3$ and 5 mM HCl was added after this incubation ('reconstituted' PS II particles). As a control, samples of thylakoid fragments were incubated under identical conditions, but after replacement of 25 mM $NaHCO_2$ by 25 mM additional NaCl. After incubation, the membrane fragments were pelletted by centrifugation at 35000 × *g* for 30 min, and the pellet was resuspended in a small quantity of the buffer in which they were incubated. To the reconstituted PS II particles, 10 mM $NaHCO_3$ and 5 mM HCl was added, and the concentrated suspensions (5–10 $mg \cdot ml^{-1}$ chlorophyll) were transferred to CO_2-free EPR tubes. The resuspension and transfer were carried out under Ar atmosphere, in the dark or under green safelight. The EPR tubes were capped by serum flask stoppers. The samples were subsequently frozen in liquid N_2, and kept in the dark until the EPR spectra were recorded.

EPR measurements were made using a Bruker ER-200t-X-band spectrometer fitted with an Oxford Instruments liquid He temperature cryostat and control system. Illuminations at 77 K and 200 K were done in an unsilvered dewar containing liquid N_2 or an ethanol/solid CO_2 mixture, respectively, using an 800 W projector. Chemical reduction of samples was carried out by addition of sodium dithionite (end concentration $\simeq 7\ mg \cdot ml^{-1}$) from a CO_2-free stock in MES-buffer at pH = 6.0 to the sample in the EPR tube in the dark at 0°C.

3. RESULTS

3.1. *The effect of CO_2-depletion*

Fig.1 shows EPR spectra recorded in spinach PS II membranes in control, CO_2-depleted and HCO_3^--reconstituted samples. Spectra were recorded in the dark (broken lines) and after illumination at 77 K. Illumination at this temperature results in stable photoreduction of $Q_A^- \cdot Fe^{2+}$ and photooxidation of cytochrome b_{559}. It can be seen that CO_2 depletion leads to a number of changes in the EPR spectrum of $Q_A^- \cdot Fe^{2+}$. (1) There is a dramatic increase (about 10-fold) in the amplitude of the light-induced $Q_A^- \cdot Fe^{2+}$ signal at $g = 1.82$ in CO_2-depleted samples. (2) The light-induced $g \simeq 1.9$ signal present in control samples cannot be discerned in the CO_2-depleted sample. (3) The linewidth of the signal (measured from the peak of the $g = 1.82$ signal to the trough at $g \simeq 1.7$) is only 250 G in CO_2-depleted samples whereas in controls the signal is clearly broader (≃450 G) (although accurate measurements were difficult due to the broadness of the high field trough), in accordance with previously reported values [4–7]. (4) A large $Q_A^- \cdot Fe^{2+}$ signal is present in the dark in CO_2-depleted samples, but whether this is due to an increased stability of $Q_A^- \cdot Fe^{2+}$ or simply to the 10-fold increase in signal size is unclear, since it is difficult to estimate what proportion of $Q_A^- \cdot Fe^{2+}$ signal was present in the dark in control samples. All of these effects are reversed by addition of HCO_3^- to CO_2-depleted samples. Spectra of reconstituted samples were virtually identical to control samples.

In all samples cytochrome b_{559} and the Signal II present in the dark were monitored before and after 77 K illumination using EPR conditions as previously reported [7]. Depletion of CO_2 had no effect on either of these signals (not shown); the amplitudes of the signals were similar to those reported in this kind of PS II preparation [7].

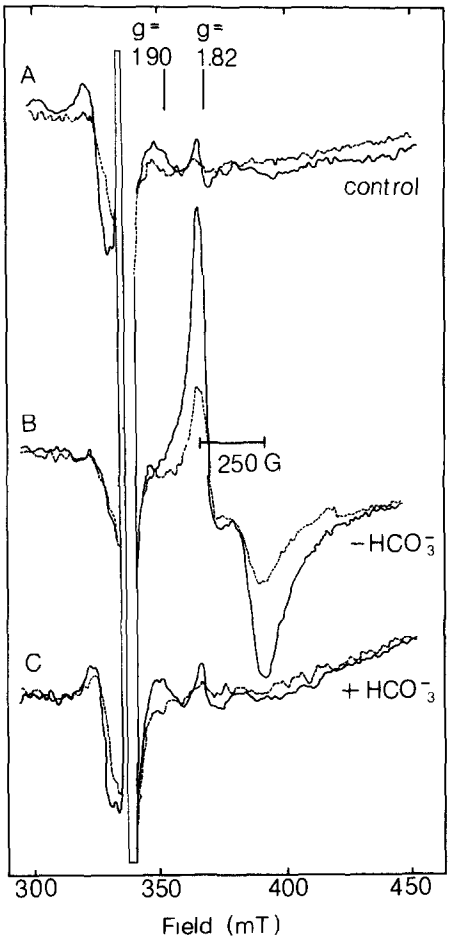

Fig.1. EPR spectra of the photoinduced $Q_A^- \cdot Fe^{2+}$ signal in PS II-enriched thylakoid membranes, after control treatment (A) or after CO_2 depletion without ($-HCO_3^-$; B) or with ($+HCO_3^-$; C) subsequent addition of 10 mM $NaHCO_3$ and 5 mM HCl. Broken lines, dark; solid lines: after illumination for 20 min at 77 K. Instrument settings: temperature, 4.5 K; microwave power: 32 mW (8 dB down from 200 mW); frequency: 9.44 GHz; modulation amplitude: 20 G; gain: 1.25×10^5.

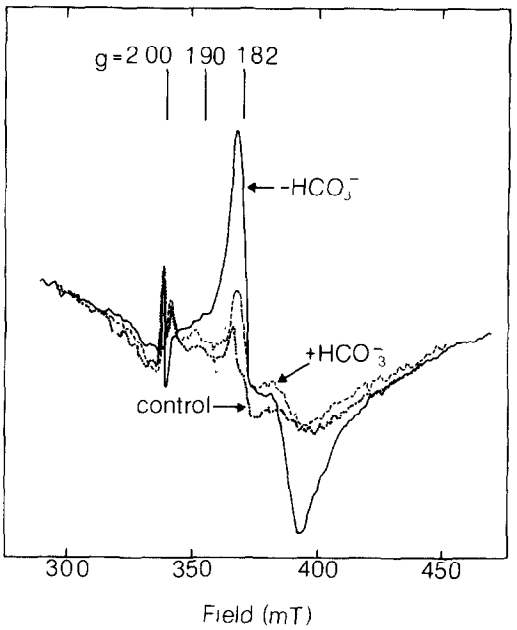

Fig.2. EPR spectra of the chemically reduced $Q_A^- \cdot Fe^{2+}$ signal without CO_2 depletion (···) or after CO_2 depletion without (——) or with (– – –) subsequent addition of 10 mM $NaHCO_3$ and 5 mM HCl. Instrument settings as in fig.1.

When samples were reduced with sodium dithionite in darkness before freezing, EPR spectra from $Q_A^- \cdot Fe^{2+}$ were obtained (fig.2) which were similar to those photoinduced in fig.1. CO_2-depleted samples showed chemically reduced $Q_A^- \cdot Fe^{2+}$ EPR signals that were comparable in both amplitude and width to those obtained by photoreduction in the absence of dithionite. The chemically reduced $Q_A^- \cdot Fe^{2+}$ signal in the HCO_3^--reconstituted sample was twice as big as in the photoreduced sample. This is probably due to incomplete reconstitution or to reversal of the reconstitution due to gassing of the anaerobic sample with argon upon dithionite addition.

Also of note in fig.1 is that CO_2 depletion also affects a light-induced signal on the low field side of the large $g \simeq 2.00$ free radical. In control samples and in samples reconstituted with HCO_3^-, illumination at 77 K results in the formation of a signal at $g \simeq 2.045$. In CO_2-depleted samples this signal was absent. Since chemical reduction of samples by dithionite induced the high field $Q_A^- \cdot Fe^{2+}$ signals but not the $g \simeq 2.045$ signal (fig.2), it seems unlikely that this signal is due to $Q_A^- \cdot Fe^{2+}$ itself. In a previous report [21] it was suggested that the $g \simeq 2045$ signal might be due to an acceptor more primary (or perhaps on a side path) than $Q_A^- \cdot Fe^{2+}$ in PS II. If this is the case it has to be assumed that dithionite treatment at pH 6 does not achieve a potential sufficiently low to reduce this component.

When the chemically reduced samples were illuminated at 200 K, the split signal attributed to

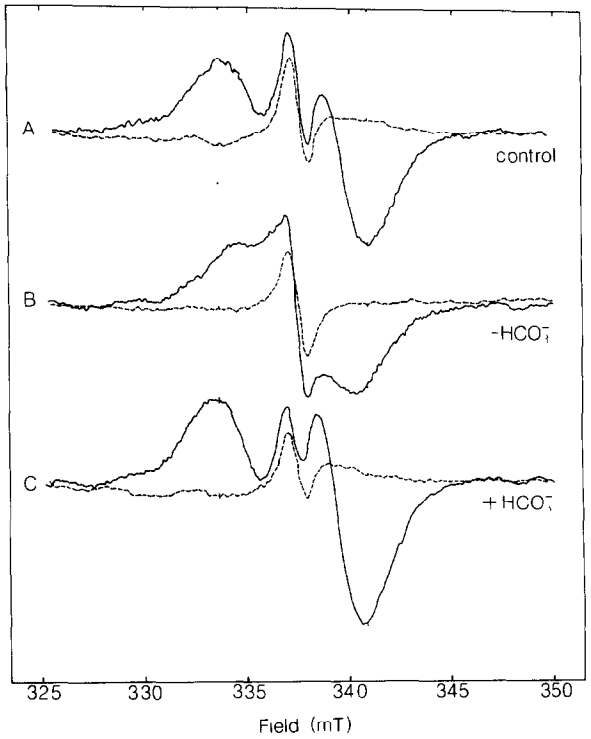

Fig.3. EPR spectra of the photoinduced split Pheo$^-$ signal in control samples (A), or after CO_2 depletion without (B) or with (C) subsequent reconstitution with 10 mM $NaHCO_3$ and 5 mM HCl. Broken lines, dark; solid lines, after illumination for 6 min at 200 K. Instrument settings were as in fig.1, except that the temperature was 4.7 K and the modulation amplitude was 10 G. The dotted lines are to emphasize splitting differences.

Pheo$^-$ interacting with the semiquinone–iron complex [8] was photoinduced (fig.3). The splitting of the Pheo$^-$ signal was ≃40 G in control and reconstituted samples, but was found to be decreased to ≃31 G in CO_2-depleted samples. Earlier results [5–7] show that when the $Q_A^- \cdot Fe^{2+}$ signal at $g = 1.82$ increases compared to that at $g \simeq 1.90$ (at low pH [5] or in the presence of dinoseb [7]) the width of the split Pheo$^-$ signal decreases. The minimal width reported was 33 G in the presence of dinoseb, where a large $g = 1.82$ but no $g = 1.90$ signal was detectable. This indicates that in CO_2-depleted samples only the $g = 1.82$ form is present, and that no significant amount of $g = 1.90$ signal has been 'hidden' under the greatly increased $g = 1.82$ signal.

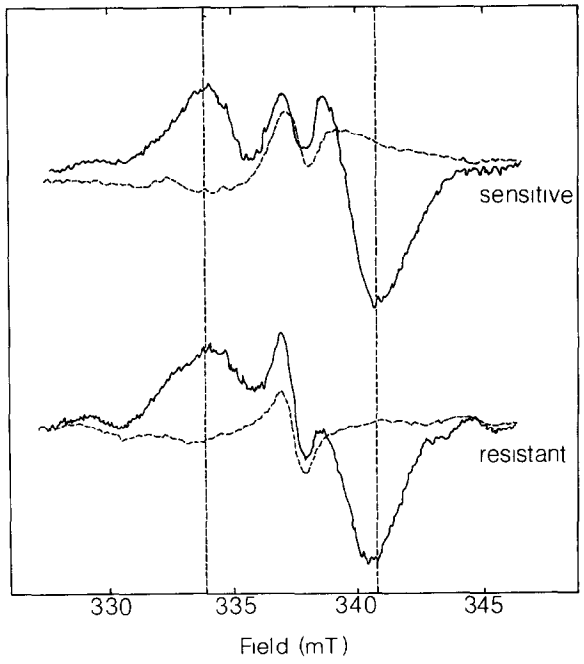

Fig.4. EPR spectra of the photoinduced split Pheo$^-$ signal in PS II-enriched subchloroplast particles from triazine-sensitive (upper) and triazine-resistant (lower) *Brassica napus*. Broken lines, dark; solid lines, after illumination for 6 min at 200 K. Instrument settings were as in fig.3.

3.2. *Effects of triazine-resistance*

The $Q_A^- \cdot Fe^{2+}$ and split Pheo$^-$EPR signals of PS II-enriched subchloroplast particles from triazine-resistant and triazine-sensitive *B. napus* were compared. No difference in the signal size or the signal shape in the $g = 1.8$–1.9 region could be detected (data not shown). Interestingly, in this species the $g \simeq 1.9$ resonance form of $Q_A^- \cdot Fe^{2+}$ dominated even at pH 6.0 in both triazine-resistant and -sensitive samples. The split Pheo$^-$ signal was virtually identical in triazine-resistant and -susceptible samples (fig.4) although the splitting of the Pheo$^-$ signal was consistently found to be slightly smaller in triazine-resistant samples (≃38 G in triazine-sensitive and 34 G in -resistant samples). This may reflect a slight relative decrease of the $g = 1.90$ resonance form of the $Q_A^- \cdot Fe^{2+}$ complex in triazine-resistant samples under these conditions, not detectable by direct monitoring of the $Q_A^- \cdot Fe^{2+}$ signal in the $g = 1.8$–1.9 region [5].

4. DISCUSSION

The marked effect of CO_2 depletion on the $Q_A^- \cdot Fe^{2+}$ and the split $Pheo^-$ signal indicates that this treatment modifies the interaction between the primary semiquinone and the iron atom. Interestingly, the narrowing of the linewidth of the $Q_A^- \cdot Fe^{2+}$ signal, the loss of the $g \simeq 1.9$ resonance form and the associated decrease in splitting of the split $Pheo^-$ signal were all previously observed as effects induced by the herbicide, dinoseb [6,7], or by lowering the pH [5]. However, dinoseb addition increased the amplitude of the $g = 1.82$ signal by only 50% [6,7] while CO_2 depletion induces an amplitude increase by ≃10-fold.

The enormous increase in signal amplitude is surprising and difficult to explain. Previously, to explain the DCMU-induced increase (2–3-fold) in the size of the $g \simeq 1.82$ signal an interaction with another component had been suggested which might be removed by DCMU binding [7,22]. The much larger effect of CO_2 depletion might be similarly explained. Recently, however, advances in the understanding of the analogous semiquinone–iron signal in purple bacteria have been made [23,24]. It seems clear that the EPR signal is extremely broad and spread over several thousand gauss [24]. A redistribution of the absorption towards the $g = 1.8$ region due to changes in the magnetic interaction between the semiquinone and the iron might account for the startling increase in signal size induced by CO_2 depletion without having to invoke the involvement of an interaction with another component. The amplitude changes induced by herbicides [6,7,22] could also be due to this kind of phenomenon.

The effects of triazine-resistance on the EPR properties of the $Q_A^- \cdot Fe^{2+}$ complex and of the coupling of $Pheo^-$ with $Q_A^- \cdot Fe^{2+}$ are very slight, indicating that the change in the ABP-32 does not affect the conformation of the $Pheo^- \cdot Q_A^- \cdot Fe^{2+}$ complex. Since it is known that triazine-resistance brings about a large change in the semiquinone equilibrium of Q_A and Q_B, and in binding of PS II herbicides [19,25], it appears that the change in the ABP-32 causing triazine-resistance only affects the properties of Q_B and of herbicides, but not those of Q_A coupled to Fe^{2+}, nor of Pheo. Therefore, the ABP-32 is part of the binding environment of Q_B and PS II herbicides, but probably not of Q_A, Fe^{2+} and Pheo, in agreement with previous assumptions [19]. However, this does not mean that herbicides and/or Q_B do not interact with the protein(s) to which Q_A, Fe^{2+} and Pheo are bound: herbicides are able to change the EPR signals of $Q_A^- \cdot Fe^{2+}$ and the split $Pheo^-$ signal in the presence of $Q_A^- \cdot Fe^{2+}$ [6,7]. This confirms the notion that more proteins than the ABP-32 only are involved in herbicide binding [19,26].

In support of our conclusion that the Q_A properties are not changed in triazine resistant compared to -sensitive samples, the back reaction rate between Q_A^- and the S_2 state of the oxygen-evolving complex (in the presence of diuron) is the same. In contrast, the back reaction rate between Q_A^- and S_2 in the presence of diuron is dependent on HCO_3^-: in CO_2-depleted thylakoids the back reaction was slowed down [14], which could be taken as evidence that HCO_3^- not only affect the properties of Q_B, but might also change the Q_A^- properties, although the E_m of the Q_A/Q_A^- couple does not change upon CO_2 depletion [27].

The measurements reported here indicate that the conformation of the $Q_A^- \cdot Fe^{2+}$ complex and/or its direct vicinity depends on HCO_3^-, indicating that HCO_3^- action at the PS II acceptor side is not limited to processes involving Q_B and herbicide binding, but extend to those in the whole $Q_A \cdot Q_B$ protein complex. On the other hand, changes associated with triazine-resistance are limited to the ABP-32 and the ligand interactions with this protein and do not involve marked modification of the $Q_A^- \cdot Fe^{2+}$ complex.

ACKNOWLEDGEMENTS

We would like to thank Dr D.M. Tiede for useful discussion. W.V. would like to thank Dr P. Mathis for his hospitality. A.W.R. is supported in part by the CNRS.

REFERENCES

[1] Nugent, J.H.A., Diner, B.A. and Evans, M.C.W. (1981) FEBS Lett. 124, 241–244.
[2] Rutherford, A.W. and Mathis, P. (1983) FEBS Lett. 154, 328–334.
[3] Feher, G. and Okamura, M.Y. (1978) in: The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R. eds) pp.349–386, Plenum, New York.

[4] Dutton, P.L., Prince, R.C. and Tiede, D.M. (1978) Photochem. Photobiol. 28, 939–946.
[5] Rutherford, A.W. and Zimmermann, J.L. (1984) Biochim. Biophys. Acta, in press.
[6] Rutherford, A.W., Zimmermann, J.L. and Mathis, P. (1984) in: Advances in Photosynthesis Research (Sybesma, C. ed.) vol.1, pp,445–448, Nijhoff/W. Junk, The Hague.
[7] Rutherford, A.W., Zimmermann, J.L. and Mathis, P. (1984) FEBS Lett. 165, 156–162.
[8] Klimov, V.V., Dolan, E. and Ke, B. (1980) FEBS Lett. 112, 97–100.
[9] Tiede, D.M., Prince, R.C., Reed, G.H. and Dutton, P.L. (1976) FEBS Lett. 65, 301–304.
[10] Vermaas, W.F.J. and Govindjee (1981) Photochem. Photobiol. 34, 775–793.
[11] Van Rensen, J.J.S. and Vermaas, W.F.J. (1981) Physiol. Plant. 51, 106–110.
[12] Khanna, R., Pfister, K., Keresztes, A., Van Rensen, J.J.S. and Govindjee (1981) Biochim. Biophys. Acta 634, 105–116.
[13] Stemler, A. and Govindjee (1974) Photochem. Photobiol. 19, 227–232.
[14] Vermaas, W.F.J., Renger, G. and Dohnt, G. (1984) Biochim. Biophys. Acta 764, 194–202.
[15] Bowes, J.M. and Crofts, A.R. (1980) Biochim. Biophys. Acta 590, 373–384.
[16] Radmer, R. and Ollinger, O. (1980) FEBS Lett. 110, 57–61.
[17] Jursinic, P.A. and Stemler, A. (1984) Biochim. Biophys. Acta 764, 170–178.
[18] Vermaas, W.F.J., Van Rensen, J.J.S. and Govindjee (1982) Biochim. Biophys. Acta 681, 242–247.
[19] Vermaas, W.F.J. (1984) PhD Thesis, Agricultural University Wageningen, The Netherlands.
[20] Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234.
[21] Rutherford, A.W. (1984) Biochim. Biophys. Acta, submitted.
[22] Atkinson, Y.E. and Evans, M.C.W. (1983) FEBS Lett. 159, 141–144.
[23] Dismukes, G.C., Frank, H.A., Friesner, R. and Sauer, K. (1984) Biochim. Biophys. Acta 764, 253–271.
[24] Butler, W.F., Calvo, R., Fredkin, D.R., Isaacson, R.A., Okamura, M.Y. and Feher, G. (1984) Biophys. J. 45, 947–973.
[25] LeBaron, H.M. and Gressel, J. (1982) Herbicide Resistance in Plants, Wiley, New York.
[26] Renger, G., Hagemann, R. and Vermaas, W.F.J. (1984) Z. Naturforsch. 39c, 362–367.
[27] Vermaas, W.F.J. and Govindjee (1982) Biochim. Biophys. Acta 680, 202–209.