DIRECT MEASUREMENT OF THE REDOX POTENTIAL OF THE PRIMARY AND SECONDARY QUINONE ELECTRON ACCEPTORS IN *RHODOPSEUDOMONAS SPHAEROIDES* (WILD-TYPE) BY EPR SPECTROMETRY

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1. Introduction

The secondary acceptor in bacterial photosynthesis is a specialized quinone molecule, Q_2, which is bound to the reaction centre (reviewed [1]). By carrying out flash experiments upon reaction centres of *Rhodopseudomonas sphaeroides* it was demonstrated that Q_2 can accept two electrons (e^-) consecutively, in a process which involves the formation of the stable semiquinone on odd-numbered flashes and the donation of a pair of electrons (and protons) to exogenous ubiquinone on even-numbered flashes [2,3].

Subsequently binary oscillations of the semiquinone were reported in chromatophores of *Rps. sphaeroides* [4,5], *Rhodopseudomonas capsulata* [6] and in whole cells of *Rhodospirillum rubrum* [5]. Out of phase oscillations of the slow phase of the carotenoid bandshift [5] and of cytochrome *b* reductions [6] have also been reported in chromatophore preparations. Since the presence of the ubiquinone pool is not necessary for cyclic electron transport to occur [7] and cytochrome b_{50}, a $1e^-$ acceptor, is thought to be the oxidant of Q_2 (e.g. [8]), mechanisms of electron transfer from Q_2 have had to be invoked for chromatophores [5,6] which are more complicated than that indicated from the reaction centre data [2,3]. These models were formulated in the absence of *Em* values for the redox changes of Q_2.

We have reported the presence of a semiquinone-iron signal attributable to Q_2^--Fe in *Rhodopseudomonas viridis* [9,10]. Redox titrations were carried out measuring this signal and $Em_{8.0}$ values for the $Q_2/Q_2^{\cdot}H$ and $Q_2^{\cdot}H/Q_2H_2$ couples were estimated to be +67 mV and −15 mV, respectively. Little work on the electron transport chain of this species has been done, so application of these values to the formulation of an electron-transport model is purely speculative. Here we report semiquinone-iron EPR signals in chromatophores of *Rps. sphaeroides* which are attributable to the primary and secondary acceptors. Directly measured *Em* values have been obtained for the $Q_1/Q_1^{\cdot}H$, $Q_2/Q_2^{\cdot}H$ and $Q_2^{\cdot}H/Q_2H_2$ couples.

2. Materials and methods

Rhodopseudomonas sphaeroides wild-type strain 2.4.1 was grown to late log phase in a modified Hutner's medium and chromatophores were prepared as in [11]. Concentrated chromatophore suspensions (5–12 mM Bchl) were used for redox titrations and flash experiments. Bacteriochlorophyll (Bchl) concentration was estimated by the method in [12].

Redox titrations and EPR sample preparation were carried out as in [11]. Samples were illuminated at liquid He temperatures using a 1000 W projector (intensity $\simeq$4.57 mE . m^{-2}. s^{-1}). Flash illumination at room temperature was provided by a 20 J Xe flash lamp (flashlength $\simeq$10 μs). Samples were frozen quickly to liquid nitrogen temperature as in [10]. EPR spectroscopy was done at liquid He temperatures using a Jeol Felx spectrometer with a cyclindrical cavity as in [11]. Conditions of higher gain (× 2000), higher power (50 mW) and lower temperature (5 K) were needed to detect the $g = 1.82$ signals in chromatophores of this species. Difference spectra were obtained using a Nicolet 1020A signal averager.

Theoretical curves for a $1e^-$ acceptor were fitted using a Tektronix 4051 computer.

3. Results

Figure 1(a) shows redox titrations, at pH 8, measuring the extent of the $g = 1.82$ signal. The results indicate the presence of 2 components, a low potential component attributable to Q_1^--Fe and a high potential component attributable to Q_2^--Fe. The EPR spectra of the two components can be distinguished by the positions of their high field resonances (fig.2). The Q_1^--Fe signal (fig.2(a)) has g-values of 1.82 and ~1.70, while the Q_2^--Fe signal (fig.2(c)) has g-values of 1.82 and ~1.62. These values correspond closely to those reported for Q_1^--Fe and Q_2^--Fe, respectively, in reaction centres of this species [13,14]. Figure 2(b) shows the Q_1^--Fe signal which is reversibly photo-induced at 5 K in a sample poised at +200 mV; it can be seen that this signal is similar to the chemically reduced Q_1^--Fe signal (fig.2(a)).

Figure 1 also shows that the redox change attributed to the reduction of Q_1^--Fe in fig.1(a) corresponds closely to the reduction of the primary acceptor as measured indirectly by the attenuation of the reversibly photo-induced $[Bchl]_2^+$ signal (fig.1(b)) and the appearance of the reversibly photo-induced $[Bchl]_2$ triplet in the same samples. (The apparent change in the $[Bchl]_2^+$ signal size in fig.1(b) over −50 to +50 mV is due to the interference of an unidentified signal at $g = 2.02$ which disappears as the potential is lowered).

The $Em_{8,0}$ values for the reduction of the primary acceptor measured directly in fig.1(a) (≃−75 mV) and indirectly in fig.1(b,c) (≃−80 mV) are in good

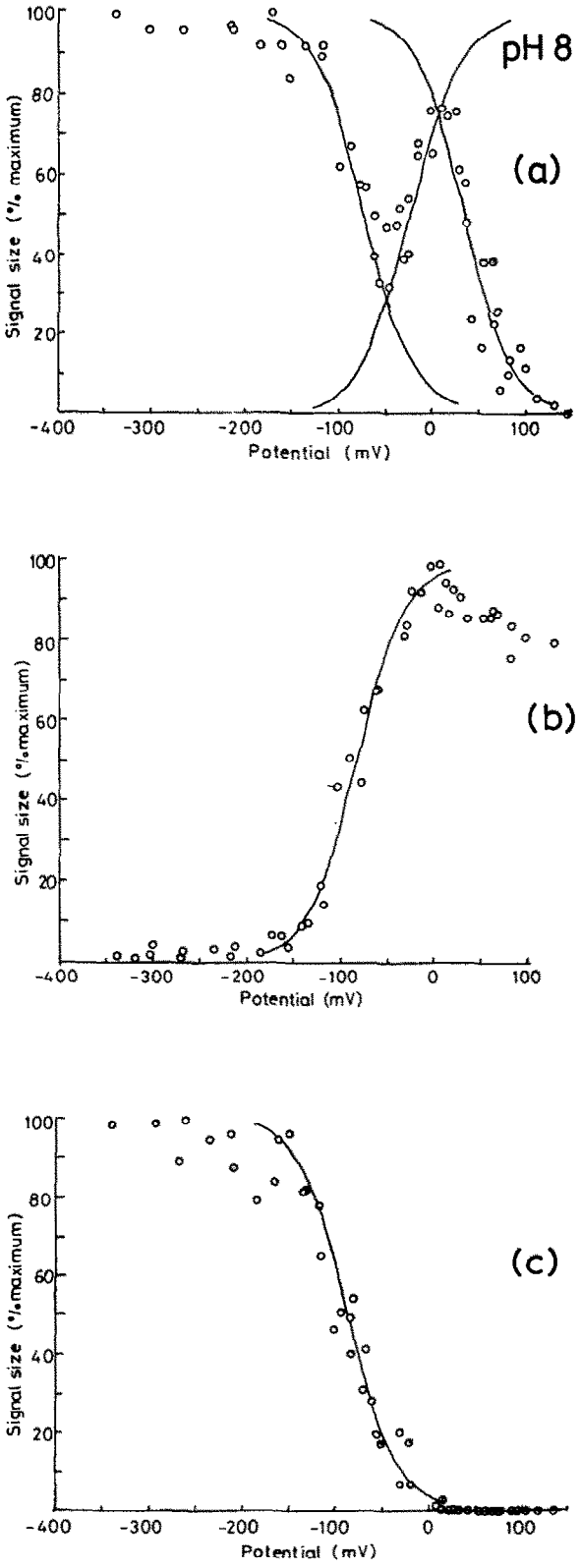

Fig.1. Redox titrations, at pH 8, on chromatophores of *Rps. sphaeroides* (5–12 mM Bchl *a*). The points shown at potentials >−50 mV were obtained from titrations done in oxidizing and reducing directions. The points shown at potentials <−50 mV were obtained from titrations in the oxidizing direction only (see text). Titrations were done with and without Triton X-100 (0.2%, w/v). The curves fitted are computer drawn for 1 e^- acceptors. (a) The extent of the $g = 1.82$ signal measured in dark samples with EPR conditions as follows: temperature 5 K; modulation amplitude 10 G (1.0 mT); microwave power 50 mW; frequency, 9.09 GHz; instrument gain 2000. (b) The extent of reversibly photo-induced $g = 2.00$ signal attributed to $[Bchl]_2^+$. (c) The extent of the reversibly photo-induced $g \simeq 2.15$ signal, the low field peak of the $[Bchl]_2^+$ triplet signal. EPR conditions for (b,c) were as follows: temperature, 7 K; modulation amplitude, 10 G (1 mT); microwave power 1 mW; frequency, 9.09 GHz; instrument gain 125 for (b) and 1000 for (c). $Em_{8,0}$ values of the curves fitted are as follows: (a) 40 mV, −40 mV, −75 mV; (b) −80 mV; (c) −80 mV.

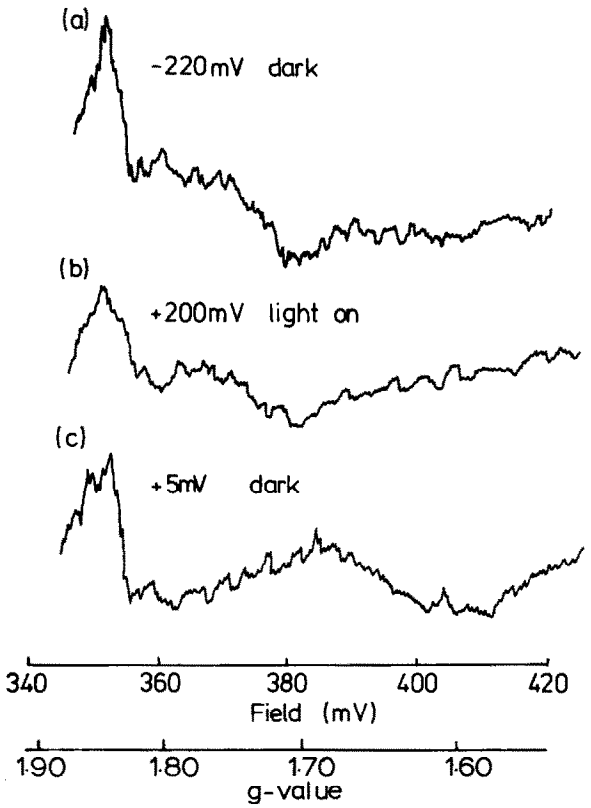

Fig.2. EPR signals in the $g = 1.82$ region in chromatophores of *Rps. sphaeroides* poised at different redox potentials. The spectra are of samples from a redox titration, at pH 8.0, in the absence of Triton X-100. (a,c) The spectra after subtraction of the base-line. (b) The light–dark spectrum. EPR conditions were as in fig.1(a).

agreement with indirectly measured values [15]. This redox change was found to show hysteresis when too little time was allowed for equilibration when titrating in the reducing direction (not shown). This hysteresis effect was identical whether measured indirectly as the extent of the $g = 1.82$ signal or indirectly by the extent of $[Bchl]_2^+$ or the $[Bchl]_2$ triplet. No hysteresis was observed in redox changes affecting the Q_2^--Fe signal. This behaviour indicates that Q_1 is less accessible to reduction than Q_2.

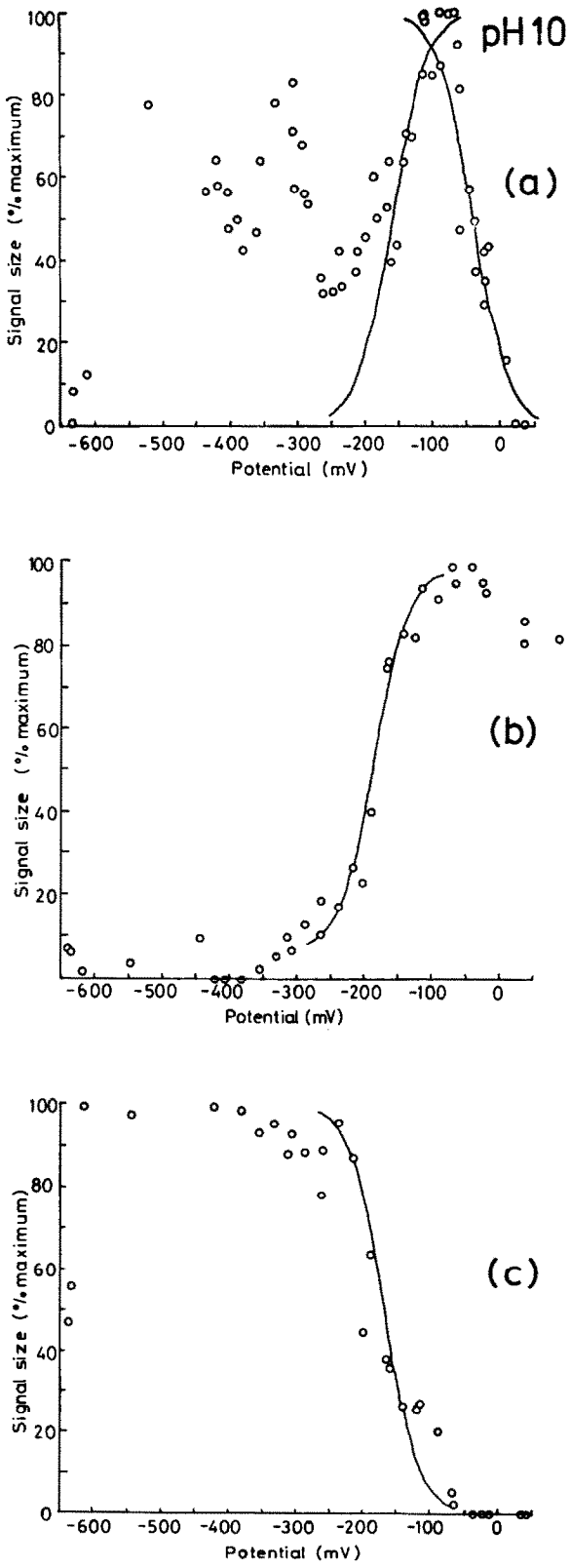

Fig.3. The titrations were as in fig.1 but at pH 10. The points were obtained from titrations done in both oxidizing and reducing directions except those shown at potentials <-500 mV which were obtained from a titration in the reducing direction only. The *Em* values of the curves fitted are as follows: (a) −50 mV, −165 mV; (b) −185 mV; (c) −165 mV.

Figure 1(a) shows that Q_2 undergoes a reduction to the semiquinone with an $Em_{8.0}$ of ~+40 mV followed by a second reduction to form the EPR silent quinol with an $Em_{8.0}$ of ~–40 mV. The two redox changes overlap making the *Em* estimations less accurate.

Figure 3 shows redox titrations at pH 10. Assuming a –60 mV/pH unit dependency, a p*k* on the semiquinone of Q_2 at pH ~9.5 can be extrapolated (*Empk* ≃–50 mV). At pH values more alkaline than the p*k* on the semiquinone a –120 mV/pH unit dependency would be expected for the second reduction assuming the $Q_2^{\cdot}/Q_2H_2$ couple is operating. In fig.3(a) however, the Q_2^--Fe signal attenuates at an *Em* which corresponds to the reduction of the primary acceptor as measured indirectly in fig.3(b,c) (*Empk* ≃–180 mV, p*k* ≃ pH 9.8). By analogy to [9,13] the fall in the $g = 1.82$ signal may be due to interaction between the two semiquinones. If this is the case then the appearance of the Q_1-Fe signal at anomalously low potentials may be due to the second reduction of Q_2 and the consequent relaxation of this reaction.

$$\underset{\text{EPR silent}}{Q_1\,Fe\,Q_2} \xrightarrow{-50\text{ mV}} \underset{g=1.82\text{ signal}}{Q_1\,Fe\,Q_2^-} \xrightarrow{-180\text{ mV}} \underset{\substack{\text{No signal at}\\ g=1.82}}{Q_1^-\,Fe^-\,Q_2^-} \rightarrow \underset{g=1.82\text{ signal}}{Q_1^-\,Fe^-\,Q_2^-}$$

The scatter of points around –400 mV and the small size of the Q_1^--Fe signal (compared to Q_2^--Fe) remain, however, unexplained and a more complex situation, possibly involving another component like that postulated in *Rps. viridis* [11] may be present.

Figure 3(a) also shows the disappearance of the Q_1^--Fe signal at very low potentials. This redox change is slow (the signal is completely absent only after 2 h incubation with excess dithionite) and consequently exhibits considerable hysteresis (not shown). This change is attributed to the second reduction of the primary acceptor to form the fully reduced quinol and has also been observed in *Rps. viridis* [11] and *Chromatium vinosum* [16].

In fig.3(c) at the lowest potential, the light-induced $[Bchl]_2$ triplet signal is still ~50% of the maximum signal. This indicates that the *Em* for the reduction of I, the intermediate, is at a lower potential than the *Em* for the reduction of Q_1^--Fe to the fully reduced quinol and is in agreement with the observation that

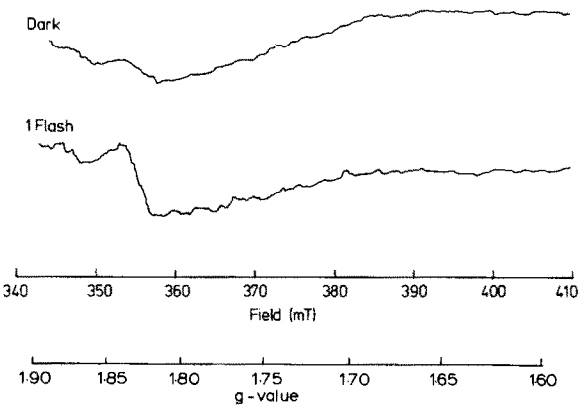

Fig.4. The effect of short (10 μs) flash illumination on the $g = 1.82$ signals in *Rps. sphaeroides* chromatophores. Samples were dark adapted for 5 min in the presence of 2 mM potassium ferricyanide before the flash and then rapidly frozen. EPR conditions were as in fig.1(a).

I^- can donate to Q_1^--Fe under conditions of strong illumination at room temperature [17].

The Q_2^--Fe signal could not be photo-induced at low temperature but its role as a photosynthetic electron acceptor was demonstrated by experiments involving flash illumination at room temperature followed by rapid freezing essentially as in [10,13]. Figure 4 shows a $g = 1.82$ signal induced by 1 flash at room temperature in a sample poised with ferricyanide at a potential where Q_2 was just oxidized. The position of the high-field line is not well defined but the shape is more like that of the Q_2^--Fe signal than the Q_1^--Fe signal. The absence of any light-inducible $[Bchl]_2$ triplet in this sample indicates that no Q_1^--Fe is present and therefore that the flash-induced signal is probably due solely to Q_2^--Fe.

The flash-induced signal proved stable for 2–3 min at room temperature not disappearing on a second flash even in the presence of exogenous donors (i.e., reduced cytochrome *c* and/or reduced DAD). The lack of oscillatory behaviour may be due to the high concentration of the chromatophore suspensions causing the flash to be non-saturating but further investigation of this phenomenon is required.

4. Discussion

The $Em_{8.0}$ values obtained here for the redox changes of Q_2 in *Rps. sphaeroides* are similar to those

obtained in *Rps. viridis* [9,10] and indicate that the semiquinone is thermodynamically stable in this species also. The directly measured *Em* values can be correlated with other changes reported to occur over these ranges of potential. Assuming a –60 mV/pH unit dependency ($Em_{7.0} \simeq +100$ mV for $Q_2/Q_2^{\cdot}H$ and $Em_{7.0} \simeq +20$ mV for $Q_2^{\cdot}H/Q_2H_2$) these results agree with the idea that formation of the Q_2 semiquinone may result in the uptake of the antimycin-insensitive proton ($Em_{7.0} = +85$ mV) [18]. Similarly the reduction of Q_2 to $Q_2^{\cdot}H$ in the dark may be responsible for the unexplained increase in the initial rate of flash-induced cytochrome *b* reduction reported in *Rps. capsulata* chromatophores in the presence of antimycin A as the potential is lowered ($Em_{7.0} \simeq +110$ mV) [19,20].

These *Em* values also help to explain the oscillation data in chromatophores. It can be seen that donation to cytochrome *b* from $Q_2^{\cdot}H$ formed on the first flash would be thermodynamically unfavourable while Q_2H_2 formed on the second flash would be able to donate to either cytochrome *b* in a 1 e^- process or to the ubiquinone pool ($Em_{7.0} \simeq 90$ mV [21], in a 2 e^- process. (NB: $Em_{7.0}$ of Q_2/Q_2H_2 can be estimated from these results as +60 mV.) A situation in which damped oscillations of semiquinone formation and out of phase cytochrome *b* reduction is observed [6] can be explained if Q_2H_2 can donate to either cytochrome *b* or the ubiquinone pool in an equally favourable manner. Such a mechanism obviates the need to invoke another 1 e^- acceptor as postulated in [6].

Several anomalies remain, however:

(1) Semiquinone, stably flash-induced in odd-numbered flashes, is the unprotonated form [6]. The $Q_2/Q_2^{\cdot -}$ couple measured here has an *Empk* of –50 mV. Therefore in order to explain the oscillations in chromatophores it is necessary to postulate an inhibitory effect (possibly involving proton uptake) which prevents $Q_2^{\cdot -}$ reducing cytochrome *b*, despite the favourability of the reaction.

(2) The disappearance of oscillations as the potential is lowered at ~+300 mV, may be due to the reduction of a component which relaxes the inhibitory effect on $Q_2^{\cdot -}$ allowing it to reduce cytochrome *b* on the first and following flashes. This area of electron transport is not yet understood.

Em values for the redox changes of Q_2 could be fitted to a scheme in which Q_2H_2 is the reductant of cytochrome *b* while Q_2 could act as the oxidant of cytochrome *b*. Thus a situation where the same quinone molecule, or quinone molecules with same binding site-controlled redox properties, can act as Q_2 and Z can be envisaged.

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References

[1] Wraight, C. A. (1980) Photochem. Photobiol. in press.
[2] Vermeglio, A. (1977) Biochim. Biophys. Acta 459, 519–524.
[3] Wraight, C. A. (1977) Biochim. Biophys. Acta 459, 525–531.
[4] Barouch, Y. and Clayton, R. K. (1977) Biochim. Biophys. Acta 462, 731–747.
[5] De Grooth, B. C., Van Grondelle, R., Romijn, J. C. and Pulles, M. P. J. (1978) Biochim. Biophys. Acta 503, 480–490.
[6] Bowyer, J. R., Tierney, G. V. and Crofts, A. R. (1979) FEBS Lett. 101, 201–206.
[7] Takamiya, K., Prince, R. C. and Dutton, P. C. (1980) J. Biol. Chem. in press.
[8] Crofts, A. R. and Bowyer, J. R. (1978) in: The Proton and Calcium Pumps (Azzone, G. F. et al. eds) pp. 55–64, Elsevier/North-Holland, Amsterdam, New York.
[9] Rutherford, A. W. and Evans, M. C. W. (1979) FEBS Lett. 100, 305–308.
[10] Rutherford, A. W. and Evans, M. C. W. (1979) FEBS Lett. 104, 227–230.
[11] Rutherford, A. W., Heathcote, P. and Evans, M. C. W. (1979) Biochem. J. 182, 515–523.
[12] Clayton, R. K. (1963) in: Bacterial Photosynthesis (Gest, H. et al. eds) p. 498, Antioch Press, Yellow Springs, Ohio.
[13] Wraight, C. A. (1978) FEBS Lett. 93, 283–288.
[14] Okamura, M. Y., Isaacson, R. A. and Feher, G. (1978) Biophys. J. 21, 8a.
[15] Prince, R. C. and Dutton, P. L. (1976) Arch. Biochem. Biophys. 172, 328–338.
[16] Rutherford, A. W. (1979) PhD Thesis, University College London.
[17] Okamura, M. Y., Isaacson, R. A. and Feher, G. (1979) Biochim. Biophys. Acta 546, 394–417.
[18] Petty, K. M., Jackson, J. B. and Dutton, P. L. (1979) Biochim. Biophys. Acta 546, 17–42.
[19] Evans, E. H. and Crofts, A. R. (1974) Biochim. Biophys. Acta 357, 89–102.
[20] Bowyer, J. R. (1979) PhD Thesis, University of Bristol.
[21] Takamiya, K. and Dutton, P. L. (1979) Biochim. Biophys. Acta 546, 1–16.