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Quantitative Electron-Paramagnetic-Resonance Measurements of the Electron-Transfer Components of the Photosystem-I Reaction Centre

THE FREE-RADICAL SIGNAL I AND THE BOUND IRON–SULPHUR CENTRE A

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E.p.r. spectrometry was used to investigate the quantitative relationships between the oxidized chlorophyll free-radical signal I and the reduced iron–sulphur centre-A signal generated on illuminating Photosystem-I particles at cryogenic temperatures. In Photosystem-I particles prepared by using the French press or Triton X-100, at pH 8.0 in the presence and absence of ascorbate and at pH 10.0 in the presence of ascorbate, the size of the light-induced signal I and iron–sulphur centre-A signals, corresponded to equal numbers of unpaired electron spins in each component. At 77K the spin–lattice relaxation time, T_1, of the free-radical signal I in samples of Photosystem-I particles prepared with Triton X-100 in the absence of ascorbate was 0.68 times the T_1 value in the presence of ascorbate. Such changes in relaxation time can account for the different quantitative conclusions incorrectly arrived at from measurements made at saturating microwave powers [Bearden & Malkin (1976) *Biochim. Biophys. Acta* **430**, 538–547; Malkin & Bearden (1976) *FEBS Lett.* **69**, 216–220]. In the presence of benzoquinone and ferricyanide the ratio of free radical to centre A was 2.96:1, and at 77K the T_1 was 0.50 times the T_1 for ascorbate-treated samples. Here free radicals from bulk chlorophyll are generated in addition to those from the reaction-centre chlorophyll.

Absorption of light and the subsequent transfer of an electron from a reaction-centre chlorophyll to electron acceptors is the primary event in the conversion of light-energy into chemical reductants in plants. Kok (1956) proposed that an optical change at 703nm induced by illumination or oxidation of chloroplasts represented the oxidation of the reaction-centre chlorophyll of Photosystem I (P700). An e.p.r.-detectable light-induced free-radical signal (signal I) was observed in chloroplasts by Commoner *et al.* (1956) and associated with centre P700 by Beinert *et al.* (1962). That signal I and the optical change at 703nm represented detection of the same component was confirmed by quantitative measurements at room temperature (Warden & Bolton, 1973; Baker & Weaver, 1973). However, although the oxidation or illumination of chloroplasts at cryogenic temperatures does give rise to both 703nm optical changes and signal I, no apparent quantitative relationship between the two signals at low temperatures was found (Beinert & Kok, 1964).

Malkin & Bearden (1971) demonstrated that at cryogenic temperatures the photo-oxidation of the reaction-centre chlorophyll giving rise to signal I is irreversibly coupled to the reduction of a bound iron–sulphur centre (centre A). If centre A is reduced before illumination of the Photosystem-I preparations at cryogenic temperatures the electron from the chlorophyll electron donor is transferred to a second bound iron–sulphur centre B (Evans *et al.*, 1974). Bearden & Malkin (1972) found a quantitative relationship between the number of electrons transferred from the species giving rise to signal I and the number reducing the bound iron–sulphur centre A. Mayne & Rubinstein (1966) and Warden *et al.* (1974) first reported observations of a partial reversibility in the dark of the light-induced signal I, and subsequently Evans & Cammack (1975) demonstrated that the appearance of this signal could become totally reversible if both bound iron–sulphur centres A and B were reduced before illumination. The reversible signal I was kinetically linked with a possible primary electron acceptor X (McIntosh *et al.*, 1975), which was shown to be a stable chemical entity by fully reducing photosystem-I particle preparations before freezing them (Evans *et al.*, 1975, 1976). However, Bearden & Malkin (1976) and Malkin & Bearden (1976) have argued that the electron transfer between P700 and component X cannot play a significant part in electron transport in

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Photosystem I. This suggestion is based on the observation that, although the free radical quantified in relation to centre A appears 'large' under the conditions used in measurements of the iron–sulphur centre A, the reversible free-radical appears 'small' under these conditions. Malkin & Bearden (1976) and Bearden & Malkin (1976) have argued that the small reversible signal represents the photo-oxidation of only a small proportion of the reaction-centre chlorophyll and is seen as a result of damage to the reaction centre by sodium dithionite or by alkaline pH. Evans *et al.* (1976, 1977) had demonstrated that illumination of Photosystem-I particles at cryogenic temperatures would produce a normal centre-A e.p.r. spectrum and a relatively 'small' free radical, comparable with the signal intensity of the reversible free radical associated with component X, if the particles were prepared in the dark at pH 8 or pH 10 under various reducing conditions before freezing. Evans *et al.* (1977) have also proposed that an even 'larger' free radical seen when Photosystem-I particles are incubated in the presence of ferricyanide is mainly due to oxidation of bulk rather than reaction-centre chlorophyll.

As all the free-radical signal intensities being discussed have been measured at temperatures and microwave powers suitable for observation of the bound iron–sulphur centre A, but saturating for signal I, quantitative measurements of the relationship between the various free-radical signals and centre A are necessary to resolve the different interpretations of these results. Such measurements should also establish the relative significance of the light-induced reversible signal I and possible primary electron acceptor X.

Materials and Methods

Materials

Tris, Triton X-100 and sodium ascorbate were from Sigma (London) Chemical Co. (Kingston-upon-Thames, Surrey, U.K.). Other chemicals were from BDH Chemicals (Poole, Dorset, U.K.), AnalaR-grade reagents being used where possible.

Preparation of spinach photosystem-I particles

Washed broken spinach (*Spinacea oleracea*) chloroplasts were prepared by the procedure of Whatley & Arnon (1963). Particles enriched in Photosystem I were isolated by using a French press as described by Sane *et al.* (1971) or with the non-ionic detergent Triton X-100. The Triton X-100 treatment was performed as described by Vernon & Shaw (1971) to the stage where the Photosystem-II particles were removed by centrifugation. The Photosystem-I particles were then purified by chromatography on hydroxyapatite as described by Evans *et al.* (1977), with an additional wash of the column with 2 litres of 0.02M-Tris/HCl buffer, pH 8.0, containing 0.2M-NaCl and 0.5% Triton X-100, to remove bulk chlorophyll. The preparation normally had a molar P700:bulk chlorophyll ratio of between 1:30 and 1:50. The particles were concentrated and stored frozen in liquid N_2. Samples for e.p.r. were prepared as described previously (Evans *et al.*, 1972). Chlorophyll was measured by the method of Arnon (1949).

E.p.r. spectroscopy

E.p.r. spectra were recorded on a Varian E9 spectrometer operating at X-band frequencies (approx. 9GHz). Samples were maintained at 77K in a liquid-N_2 finger Dewar, or cooled to 20.0K in an Oxford Instruments (Oxford OX2 0DX, U.K.) liquid-He cryostat. The sample temperature was measured by a gold–iron/chromel thermocouple (Oxford Instruments), situated 0.5cm below the sample. The thermocouple was calibrated before each run against a carbon resistor of known characteristics, inside a 3mm e.p.r. tube filled with silicone oil. This resistance in turn was measured with a cryo bridge model S72 (Nuclear Physics Institute, Prague, Czechoslovakia) Wheatstone bridge, which has an extremely low power dissipation.

Line-shape simulations

Simulations of the line-shapes of the free-radical signal I and centre A were performed by using a computer program which sums the interactions of a random distribution of spins with the applied field. The program incorporated the re-evaluation of the intensity factors required to correct for the field-dependence of the transition probability in a field-swept spectrometer (Aasa & Vänngård, 1975), and the linewidth was varied as a function of θ and ϕ, the azimuthal and polar angles of the applied field in the molecular co-ordinate system, in the same way as a first-order hyperfine interaction (Leigh, 1970). These simulations are valid for $S = \frac{1}{2}$ systems that are unoriented and show no electron-spin–electron-spin interactions. Signal I and centre A in our samples behaved as systems of this type and both could be adequately simulated in this way on the basis of a Gaussian line-shape.

Quantification

The simulation procedure generates plots of the first derivative of the microwave absorption, Y_m', versus magnetic field for a fixed total number of spins, so the ratio of the simulated peak-to-trough height at $g = 2.003$ (signal I) and $g = 1.94$ (centre A) could then be used to quantify the experimental signals.

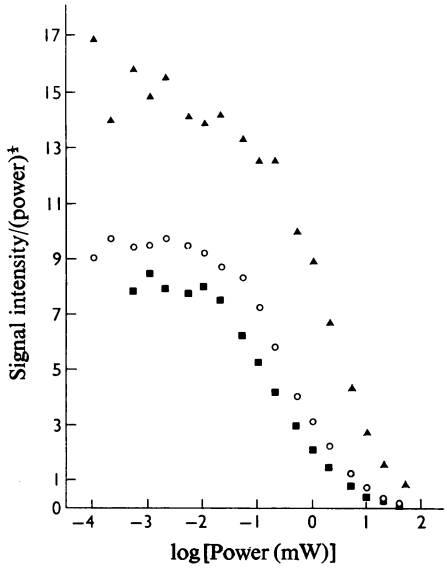


Fig. 1. *Saturation curves for signal I in Photosystem-I particles prepared under different conditions*

Photosystem-I particles prepared with Triton X-100 in 0.1M-Tris/HCl buffer, pH8.0, were preincubated in the dark (before freezing in the dark) under the following conditions: ○, 2min with no additions; ■, 30min with 10mM-sodium ascorbate; ▲, 120min with 40mM-ferricyanide and 66μM-benzoquinone. After illumination at 77K the e.p.r. spectra were recorded at 77K and the peak-to-peak signal amplitudes of signal I measured at various microwave powers with the following instrument settings: frequency 9.165GHz; modulation amplitude 0.1mT; scan rate 12.5mT/min; instrument gain 5×10^3.

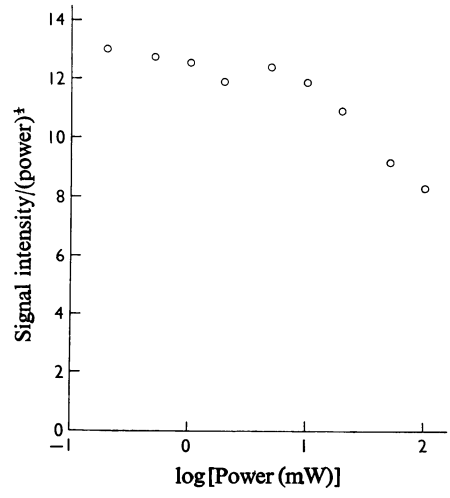


Fig. 2. *Saturation curve for $g = 1.94$ component of the bound iron–sulphur centre A*

Photosystem-I particles prepared by using Triton X-100 (1.37mg of chlorophyll/ml) were preincubated in the dark with 10mM-sodium ascorbate and then frozen in the dark. The sample was illuminated at 77K for 30s, and the signal intensity of the $g = 1.94$ component recorded at 20.0K under various microwave-power conditions and the following instrument settings: frequency 9.06GHz; modulation amplitude 1mT; scan rate 100mT/min; instrument gain 5×10^2.

Quantitative measurements must be made at non-saturating microwave powers. We found that signal I saturates at the minimum power value that we could obtain (50nW) at the ideal measuring temperature for the bound iron–sulphur centre A, 20.0K. So we were obliged to record the spectra from signal I and centre A at the (differing) temperatures of 77K and 20.0K respectively. On the basis of the plots of signal intensity/(power)$^{\frac{1}{2}}$ versus log(power) shown in Figs. 1 and 2, non-saturating power values of 5mW (centre A) and 5μW (signal I) were used. To eliminate errors that could arise from changes in the microwave magnetic field at the sample in the two cryostats, and to correct at the same time for the difference in measuring temperatures, a paramagnetic standard, here 1mM-$CuSO_4$/10mM-EDTA, of identical physical dimensions was run immediately after each sample at both temperatures. Non-saturating microwave powers of 0.1mW at 20K and 5μW at 77K were used for measurement of the Cu/EDTA standard.

This quantification method assumes that both signals show absorption intensities expected of a true $S = \frac{1}{2}$ system at these temperatures. This would seem reasonable, as discussed in the following paper (Heathcote *et al.*, 1978).

Spin–lattice relaxation times

The ratios of the electron-spin–lattice relaxation times, T_1, of the free-radical signal I at 77K in different preparations of Photosystem-I particles were calculated by using the method described by Poole & Farach (1971).

Results and Discussion

Figs. 3 and 4 present the simulated and experimental e.p.r. spectra of the free-radical signal I and the bound iron–sulphur centre A. The free-radical simulation is an exact fit to the recorded spectrum, but the centre-A simulation differs from the recorded spectrum in the region between the $g = 1.94$ and $g = 1.89$ components. This could be due to a contribution in this region of

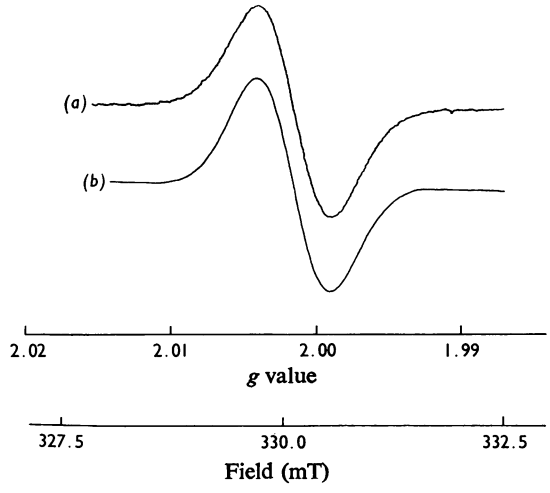


Fig. 3. *Comparison of the experimental e.p.r. signal-*I (*P*700) *spectrum* (*a*) *and the Gaussian line-shape simulation* (*b*)

Photosystem-I particles prepared by using Triton X-100 (1.10mg of chlorophyll/ml) were preincubated for 30min in the dark with 10mM-sodium ascorbate before freezing, and illuminated for 30s at cryogenic temperatures. The e.p.r. spectrum of signal I was recorded at 77K and the following instrument settings: frequency 9.25GHz; microwave power 5μW; modulation amplitude 0.1mT/min; instrument gain 10^3. The experimental spectrum has $g = 2.0047$ and $\Delta H = 6.00$. For the simulation the input values were $g = 2.005$ and $\Delta H = 6.00$, and the output values were $g = 2.0047$ and $\Delta H = 6.00$.

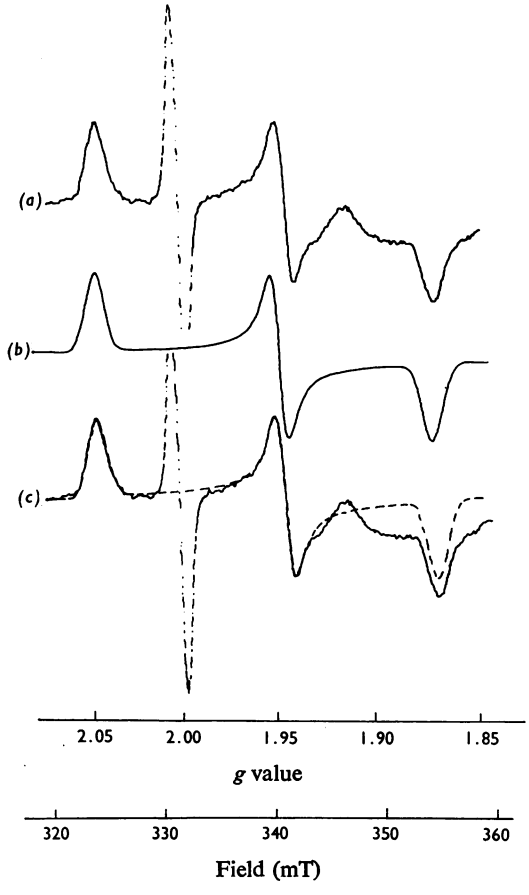
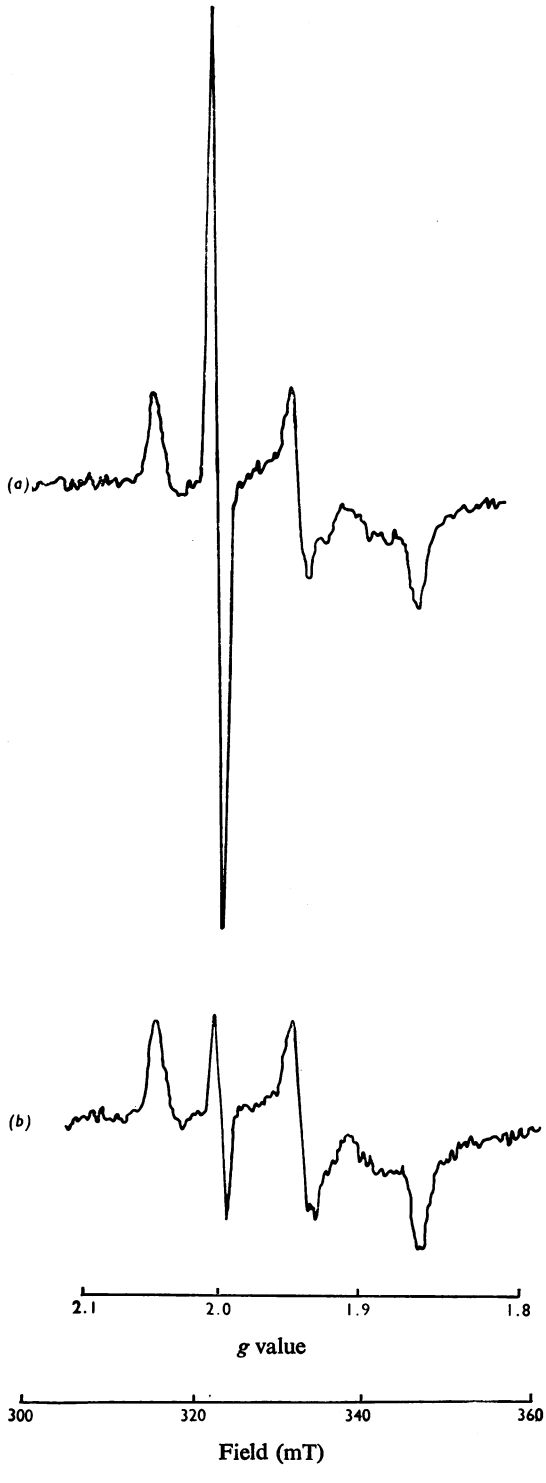


Fig. 4. *Comparison of the experimental e.p.r. spectrum of centre A and the Gaussian line-shape simulation*

(*a*) Experimental spectrum; (*b*) simulated spectrum; (*c*) simulated (----) and experimental (——) spectra superimposed. The experimental spectrum has $g_x = 2.050$, $g_y = 1.946$ and $g_z = 1.868$ with $\Delta H_x = 19.4$, $\Delta H_y = 18.8$, $\Delta H_z = 21.4$. For the simulation the input parameters were $g_x = 2.050$, $g_y = 1.946$, $g_z = 1.868$, $\Delta H_x = 16.7$, $\Delta H_y = 14.4$ and $\Delta H_z = 17.9$ and the output parameters were $g_x = 2.050$, $g_y = 1.946$, $g_z = 1.869$, $\Delta H_x = 20.0$, $\Delta H_y = 19.0$ and $\Delta H_z = 20.9$. Photosystem-I particles prepared by using Triton X-100 (1.10mg of chlorophyll/ml) were prepared as in Fig. 3. The e.p.r. spectrum of centre A was recorded at 20.0K with the following instrument settings: frequency 9.25GHz; microwave power 20mW; modulation amplitude 1mT; scan rate 100mT/min; instrument gain 5×10^2.

the recorded spectrum by a Rieske protein described by Malkin & Aparicio (1975), which has a mid-point potential of +290mV. However since this signal is not decreased when the particles have been pre-incubated in the dark with ascorbate, it is more likely that it is due to a small amount of reduced centre B. Double integration of a measured centre-A spectrum containing this component and a simulated centre-A spectrum which lacks it, after the low-field regions of the two spectra have been exactly matched, indicates that this extra signal corresponds to less than 10% of the number of spins in the simulated centre-A spectrum.

Photosystem-I particles prepared by using Triton X-100 or French-press fractionation were incubated in e.p.r. tubes in the dark in the presence or absence of ascorbate, and frozen in liquid N_2 in the dark. On illumination at cryogenic temperatures both signal I and the e.p.r. spectrum of centre A are irreversibly generated. However, at the e.p.r. microwave power (20mW) and temperature (20K) normally used for observation of the e.p.r. spectrum of centre A, the signal intensity of signal I in the sample prepared without ascorbate was as much as 5 times that of the sample incubated with ascorbate (Fig. 5). The particles were incubated in e.p.r. tubes under the conditions shown in Table 1. The samples were then frozen in liquid N_2 in the dark. Under these con-



ditions the samples had no signals in the dark. So, before measurement of the signal intensity of the $g = 1.94$ components of the centre-A spectrum, the samples were illuminated for 30s at liquid-N_2 temperature. The minimum signal-I size was observed only after 30min exposure to ascorbate at pH8.0. As the sample was then stored until the centre-A spectra of all the samples had been recorded, signal I was measured at liquid-N_2 (77K) temperatures both before and after a second period of illumination. From these measurements the ratios of electron spins in signal I to those in centre A were calculated (Table 1). Re-illumination of the samples had only a small effect on the signal intensity of signal I (relative to centre A), demonstrating that even over a period of several hours the photo-oxidation of the reaction-centre chlorophyll and the photoreduction of centre A is essentially irreversible. In contrast with the difference in the relative signal intensities of signal I in particles prepared in the absence or presence of ascorbate when measured under saturating microwave-power conditions, the size of signal I measured under non-saturating microwave-power conditions was the same in all samples. This is reflected in the observation that in both types of photosystem-I particles, and under all conditions, the ratio of electron spins represented by signal I to those represented by centre A is close to unity. The variability in the results is remarkably small in measurements on Photosystem-I particles purified with Triton X-100, and only slightly greater in particles made with a French press, where some margin of error is introduced owing to the relatively small signal intensity of the centre-A signals.

The intensity of signal I decreases more rapidly at high microwave powers in samples preincubated with ascorbate than in those prepared without reducing agents. This difference is due to changes in the saturation characteristics of signal I. This difference in saturation characteristics is expressed more rigorously in terms of the ratios of the electron-spin–lattice relaxation times, T_1, of signal I in the

Fig. 5. *Effect of preincubation with ascorbate on the apparent size of signal I relative to the signal intensity of the centre-A e.p.r. spectrum*

Photosystem-I particles prepared by using Triton X-100 (0.5mg of chlorophyll/ml) in 0.1M-Tris/HCl buffer, pH8.0, were either (*a*) preincubated for 15min in the dark or (*b*) preincubated with 10mM-sodium ascorbate for 30min, before freezing in the dark. The light-minus-dark difference spectra were recorded at 20.0K in the dark and after illumination for 30s at 20.0K with the following instrument settings: frequency 9.06GHz; microwave power 20mW; modulation amplitude 1mT; scan rate 500mT/min; instrument gain 10^3.

Table 1. *Measured ratio of signal I (P700) to bound iron–sulphur centre-A signal in Photosystem-I samples pre-incubated in the dark under various conditions*

Photosystem-I particles prepared by using the French press (2.0mg of chlorophyll/ml) or Triton X-100 (1.37mg of chlorophyll/ml) were preincubated in the dark for (*a*) 2min at pH8.0, (*b*) 30min at pH8.0 plus 10mM-sodium ascorbate and (*c*) 10min at pH10.0 plus 10mM-sodium ascorbate before freezing. After illumination for 30s at liquid-N_2 temperatures the peak-to-peak signal intensity of signal I (P700) and the size of the $g = 1.94$ component of the e.p.r. spectrum of centre A were measured at 77K and 20.0K respectively and the following instrument settings: signal I, frequency 9.07GHz; modulation amplitude 0.1mT; scan rate 12.5mT/min; centre A, frequency 9.07GHz; modulation amplitude 0.4mT; scan rate 25mT/min. The relative signal sizes were calculated as described in the text.

	Signal-I/centre-A ratio		
	French-press particles		Triton X-100 particles
(*a*)	0.82	(*a*)	1.12
	0.85		1.16
	0.81		1.18
(*b*)	0.81	(*b*)	1.05
	1.38		1.18
	1.37		0.97
(*c*)	1.77	(*c*)	0.94
	1.09		0.92
			1.18

different preparations [T_1 (without reducing agents)/T_1 (ascorbate-reduced) = 0.68]. The observed increase in T_1 for the particles incubated with ascorbate can account for lowering in intensity of this signal relative to the untreated particles when observed under saturating conditions at 20K. As shown in Table 1, at non-saturating powers our quantification procedures reveal that the signal-I/centre-A-signal ratio is identical in the presence or absence of ascorbate, at pH8.0 and pH10.0.

Although this phenomenon accounts for the observed difference of a factor of 5 between the 'large' and 'small' light-induced signal I (Fig. 5), it does not explain the very large free-radical signals observed when Photosystem-I particles are chemically oxidized by incubation with mediators (benzoquinone) and high (20mM) concentrations of ferricyanide for long periods (Evans *et al.*, 1977). Illumination of an e.p.r. sample containing only benzoquinone and ferricyanide produced no free-radical e.p.r. signals, excluding the possibility that the free radical was contributed by chemical or photochemical reaction of the quinone. Measurement of the saturation characteristics of signal I from Photosystem-I particles incubated with benzoquinone and ferricyanide (Fig. 1) demonstrated that the saturation characteristics of this free radical differ from those previously measured for the 'large' and 'small' radicals. Essentially the signal I seen in the presence of benzoquinone and ferricyanide does not saturate as easily as signal I observed from untreated Photosystem-I particles. In fact, when compared under non-saturating conditions with the $g = 1.94$ signal intensity of a centre-A spectrum from a duplicate untreated Photosystem-I particle sample, the ratio of electrons represented by signal I to centre A is 2.96:1. The hypothesis put forward by Evans *et al.* (1977), proposing that the large signal I seen in the presence of benzoquinone and ferricyanide may represent oxidation of both P700 and bulk chlorophyll, is supported by these observations. We find an identical peak-to-peak linewidth, 0.63mT, in all three preparations.

These results show that in samples prepared at pH8.0 or pH10.0 in the presence or absence of reducing agents there is a quantitative relationship between the photo-oxidation of the reaction-centre chlorophyll measured as signal I and the photoreduction of iron–sulphur centre A. It is clearly shown that the variations observed in the size of signal I when it is measured under the conditions used to measure centre A are due to variations in the electron-spin–lattice relaxation time, T_1, and hence the saturation characteristics of this signal, presumably resulting from changes in the environment of the reaction centre on reduction of the sample. This shows that the size of signal I under saturating microwave-power conditions cannot be used to provide even relative information about the amount of oxidized reaction-centre chlorophyll present. Malkin & Bearden (1976) and Bearden & Malkin (1976) have drawn a number of conclusions about the amount of reaction-centre chlorophyll involved in reversible photochemical reactions, and on the possible existence of an electron donor to the reaction centre, based on measurements of signal I made under saturating conditions. It is clear that in fact there is no variation in the amount of reaction-centre chlorophyll photo-oxidized under the different conditions. This result supports the conclusion that all of the reaction-centre chlorophyll involved in the reduction of centre A can also undergo a reversible photo-oxidation coupled to the reduction of the component X and that there is no evidence for the operation of an electron donor to the reaction centre at low temperatures. The quantitative relationship between the reversible signal I and component X, and between component X and centre A, is fully investigated in the following paper (Heathcote *et al.*, 1978).

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