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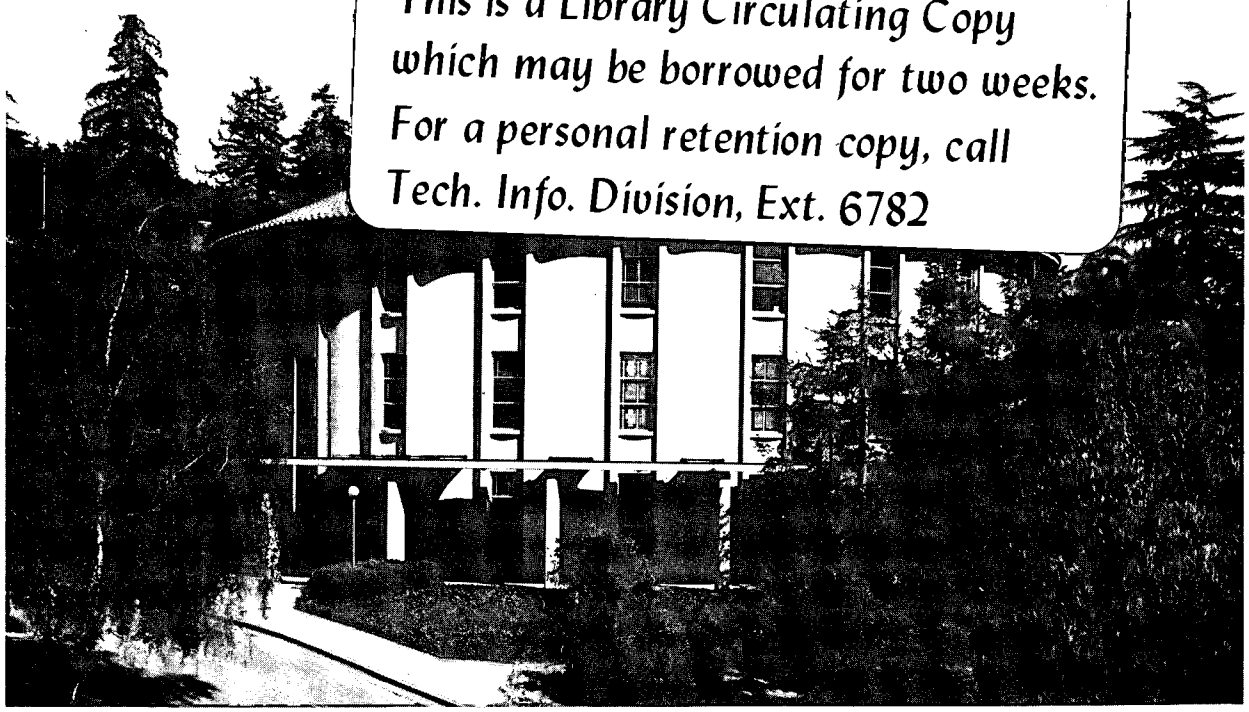

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TRIPLET STATES IN PHOTOSYSTEM I OF SPINACH CHLOROPLASTS AND SUBCHLOROPLAST PARTICLES

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ABSTRACT

We report light-induced EPR triplet spectra from samples of chloroplasts or digitonin Photosystem I particles which depend upon the dark redox state of the bound acceptors of Photosystem I. If the reaction centers are prepared in the redox state P700 A X^- Fd_B^- Fd_A^-, then upon illumination at 11K we observe a polarized chlorophyll triplet species which we interpret as arising from radical pair recombination between $P700^+$ and A^-. This chlorophyll triplet is apparently the analog of the P_R state of photosynthetic bacteria [Parsons, W.W. and Cogdell, R.J. (1975) Biochim. Biophys `. Acta 416 105-149]. If the reaction centers are prepared in the dark redox state P700 A X Fd_B^- Fd_A^-, then upon illumination at 11K we observe a different triplet species of uncertain origin, possibly pheophytin or carotenoid. This species is closely associated with the Photosystem I reaction center, and it traps excitation when P700 is oxidized.

Electron paramagnetic resonance (EPR) detection of triplet states has provided an effective probe of both the mechanism of the primary light reaction of bacterial photosynthesis and the structure and geometry of reaction center components (1). The first observation of a triplet state in photosynthetic bacteria by EPR methods by Dutton, Leigh and Seibert (2) has led to a significant advance in our understanding of the initial charge separation process. They showed that intense, spin polarized triplet EPR signals arise upon illumination at low temperatures when normal photochemistry is inhibited. Further work in this area has revealed that this triplet state, designated P_R, forms on the primary electron donor, P860, as the result of a charge recombination reaction between the photoreduced electron acceptor, I^-, and the photooxidized primary donor $P860^+$ (3). However, in green plant and algal preparations, only low intensity EPR triplet signals which most likely originate outside the reaction center have been reported, and no unambiguous assignment of a triplet state to the primary photoreactions has been made (4-9). A component of delayed luminescence observed optically by Shuvalov et. al. (10,11) was attributed to triplet formation dependent upon the redox state of the reaction center of Photosystem I.

Our choice of sample conditions for studying EPR triplet spectra was guided by the current model of the Photosystem I reaction center, which may be represented

$$P700\ A\ X\ Fd_B\ Fd_A$$

P700 is the primary electron donor (12). Fd_B and Fd_A are iron sulfur centers (13,14). X is a species of unknown chemical composition which can be observed in its reduced state by EPR at liquid helium temperatures (15). In samples containing reduced Fd_B and Fd_A, illumination at liquid helium temperature reduces X to X^-, which then undergoes a back reaction with $P700^+$ with a decay time of 130 msec (16). A is an acceptor species which participates as an intermediate in the light-induced charge separation between P700 and X (16,17). When X has been reduced prior to illumination (16,17) or when X and the iron sulfur centers are chemically inactivated (18,19), a back reaction attributed to $P700^+$ and A^- occurs with a decay time of 1 msec at liquid helium temperatures in Photosystem I subchloroplast particles. Under the same redox conditions in chloroplasts at liquid helium temperatures, two components with halftimes of 122 μsec and 1.7 msec contribute to the decay (20). Motivated by these new findings we have designed experiments to detect triplet states by EPR in samples where the normal photochemistry of Photosystem I is blocked immediately beyond the initial electron acceptor.

MATERIALS AND METHODS

Chloroplasts were isolated from market spinach in a medium containing 0.4 M sucrose, 0.01 M NaCl, 0.05 M Tris buffer (pH 8.0) and 10^{-5} M EDTA, and collected by centrifugation at 5000 x g for 5 minutes.

To prepare digitonin Photosystem I subchloroplast particles, chloroplasts were resuspended to a chlorophyll concentration of 0.3-0.4 mg ml^{-1} in 50 mM Tris buffer (pH 8.0) containing 10 mM Mg^{+2} to assure clean fractionation of Photosystem I from Photosystem II (21). Digitonin was added to the chloroplast suspension as a 10% (w/v) solution to give 0.5% (w/v) digitonin. After incubation for 2 hr at 4C the detergent incubate was centrifuged 0.5 hr at 30,000 X g. The supernatant contained Photosystem I particles with the characteristics: Chla/Chlb = 5.6 and Chl/P700 = 175.

The supernatant was concentrated for EPR studies by precipitation with protamine sulfate as described by Nelson, et al (22).

Treatment with dithionite: Chloroplasts or digitonin Photosystem I pellets were degassed under vacuum and mixed with sodium dithionite in 100 mM glycine buffer (pH 10) under a nitrogen atmosphere to give 12 mM dithionite.

Treatment with ferricyanide Chloroplast or digitonin Photosystem I pellets were mixed with $K_3Fe(CN)_6$ in 50 mM Tris buffer (pH 8) to give 1 mM ferricyanide.

The treated pellets were then combined with an equal volume of ethylene glycol, sealed in EPR tubes and stored at 77K. Final chlorophyll concentration of the samples was about 2 mg ml^{-1}. Samples prepared in Condition [1] described below were illuminated by a tungsten lamp for approximately 3 minutes and frozen to dry ice-acetone temperature under illumination before storing at 77K.

EPR measurements were accomplished using a Varian E-109 spectrometer at X-band with 100 kHz field modulation and equipped with an Air Products Helitron cryostat. The triplet state signals were detected using a light modulation technique where the exciting light was chopped at 33.5 Hz. The output of the EPR system was fed directly to a PAR (Princeton Applied Research) Model 210 selective amplifier and then to a PAR Model 220 lock-in amplifier which was referenced to the chopper.

Excitation was provided by an Oriel 1000W xenon lamp filtered by 5 cm of water. Temperature measurements were performed using a gold/chromel thermocouple. No change in the relative signs of the EPR signal occurred upon reducing the microwave power to 1 μw, lowering the chopper frequency to 11 Hz or altering the detector phase angle on the lock-in amplifier.

RESULTS

Condition [1] Dithionite, pH 10, frozen under illumination. Chloroplast or digitonin Photosystem I particles which were frozen under illumination in the presence of dithionite give the dark EPR spectrum shown in Fig. 1a. In the light-modulated triplet state spectra for chloroplasts (Fig. 2a) and digitonin Photosystem I particles (Fig. 3a) prepared in condition [1] one major triplet, denoted triplet I, is observed. Triplet I has the spin polarization pattern aee aae, where a denotes a signal in absorption and e denotes a signal in emission. The zero-field splitting parameters of

triplet I are |D| = 0.0278 ± 0.0009 cm^{-1} and |E| = 0.0039 ± 0.0009 cm^{-1} (Table 1).

Condition [2] Dithionite, pH 10, frozen dark.

Chloroplast and digitonin Photosystem I particles which were frozen dark in the presence of dithionite give the 11K dark EPR spectrum shown in Fig. 1b. Figs. 2b and 3b show the light-induced triplet signals obtained in Condition [2] from chloroplasts and digitonin Photosystem I particles, respectively. One major triplet state, denoted triplet II, is observed. Triplet II has the spin polarization pattern eae aea and zero-field splitting parameters |D| = 0.0383 ± 0.0013 cm^{-1} and |E| = 0.0040 ± 0.0013 cm^{-1} (Table 1).

Condition [3] Ferricyanide, pH 8.

Figs. 2c .and 3c show the light-induced triplet signals obtained in condition [3] for chloroplasts and digitonin Photosystem I particles, respectively. Once again we observe triplet II as the major species, with the polarization pattern eae aea and zero-field splitting parameters |D| = 0.0383 ± 0.0013 cm^{-1} and |E| = 0.0040 ± 0.0013 cm^{-1} (Table 1).

DISCUSSION

The dark signals at g = 1.78 and g = 1.89 in Fig. 1a indicate that X and Fd_B become reduced in samples frozen under illumination in the presence of dithionite (23). During subsequent light-modulation experiments in this redox state we observe light-induced signals which accompany the process of charge separation and recombination (16,17,20)

which we interpret as

$$P700\ A\ X^-\ Fd_B^-\ Fd_A^- \rightleftharpoons P700^+\ A^-\ X^-\ Fd_A^-$$
$$\tau_{1/2} = 1 \text{ msec}$$

The aee aae polarization pattern of triplet I is characteristic of triplets formed via a charge recombination reaction, and is best explained by the radical pair mechanism (1). According to this mechanism, the system is initially prepared in an excited singlet state. After one electron is transferred from a donor to an acceptor, a change in spin correlation occurs between the electron localized on the acceptor and the one remaining on the donor. This mixes the singlet state, S, predominantly with the middle energy high field triplet spin sublevel, $T_\emptyset$. The effect of this process is to drive population into the $T_\emptyset$ level selectively. Hence, the aee aae pair polarization pattern is observed in the triplet state EPR spectrum.

The radical pair mechanism explains the observation in the photosynthetic bacteria of the triplet state, P_R, which has the radical pair polarization pattern (3,24). Also, the radical pair mechanism is known to be operating in Photosystem I from recent studies of chemically induced dynamic electron polarization (CIDEP) observed in green plant preparations (25,26), which were interpreted by Friesner, et al (27) to arise from a dynamic interaction between P700, A and X. We believe that triplet I, whose spectrum is shown in

Figs. 2a and 3a is the Photosystem I analog of the bacterial P_R state.

The zero-field splitting parameters for triplet I (see Table 1) are precisely those observed for monomeric chlorophyll a (24,28). The bacterial P_R state zero-field splitting parameters are about 20% smaller than the corresponding monomeric bacteriochlorophyll a values measured in vitro (1,24). This is consistent with the idea that the triplet state, P_R, is delocalized over more than molecule; i.e. the bacterial reaction center primary donor, P860, is a bacteriochlorophyll dimer (1,24). In view of the fact that P700 is thought to be a chlorophyll a dimer (1,29), the observation of monomeric chlorophyll a |D| and |E| values for triplet I is puzzling.

If triplet I is not localized on P700, it could be centered on a chlorophyll a monomer closely associated with the reaction center and on which the charge recombination reaction energy is trapped. Such a process would have to occur coherently so that the spin polarization is preserved. Alternatively, triplet I may remain on the acceptor A, after charge recombination between $P700^+$ and A^- takes place. It has been suggested that A is a chlorophyll species (27). We are not aware, however, of any precedent for this kind of event.

If triplet I is localized on P700, then we must explain the fact that the zero-field splitting parameters correspond to monomeric chlorophyll a values. Clarke et.al. (30,31)

have proposed a simple exciton model which they use to calculate the angle between the chlorophyll planes of the bacterial dimer, P860, based on a comparison between monomeric bacteriochlorophyll zero-field splittings measured *in vitro* and those obtained for several species *in vivo*. Following this reasoning our results could be explained by a plane-parallel P700 dimer structure where the monomeric magnetic axes are all parallel. This possibility was suggested as the model for P700 by Fong (32). However, this interpretation is not fully consistent with the P700 circular dichroism spectrum obtained by Philipson, Sato and Sauer (33). Further experimental and theoretical investigations will be needed to corroborate structural models based on observed triplet state parameters with those obtained from optical measurements.

The signal at g = 1.89 in Fig 1b indicates that Fd_A and Fd_B are reduced (23), but the absence of a signal at g = 1.78 shows that X is not reduced when samples are frozen dark in the presence of dithionite. Subsequent illumination at liquid helium temperatures of samples in this redox state causes the rapid transfer of an electron from P700 to X. Because the donor system to $P700^+$ does not function at low temperatures (20) and the charge recombination time between $P700^+$ and X^- is 100 msec (16), illumination produces the redox state

$$P700^+ \; A \; X^- \; Fd_B^- \; Fd_A^-$$

during light modulation experiments.

The eae aea polarization pattern seen in triplet II is one among many patterns which can arise from a molecular intersystem crossing mechanism populating the lowest triplet state of an aromatic molecule. Such a mechanism has been studied in great detail for aromatic hydrocarbons (34-36) and chlorophylls (37-39). The eae aea polarization pattern indicates that the most populated triplet spin sublevel is the middle energy zero-field level (38). The population is driven into this level as the result of spin-orbit and vibronic coupling between the singlet and triplet manifolds of states of an isolated molecule (40,41). In contrast to the radical pair mechanism, no charge transfer-recombination process need be operating for the triplet to be formed. Consequently, spin polarization patterns distinct from the radical pair mechanism pattern are observed.

Thus, we believe that triplet II forms while the Photosystem I reaction center is in the charge separated $P700^+$ A X^- Fd_B^- Fd_A^- state. Because the back reaction time between X^- and $P700^+$ is sufficiently slow (see above), the P700 trap remains closed to excitation during the light-modulation time period. The excess excitation energy incident on the sample is funnelled into a different trap where molecular intersystem crossing takes place to form triplet II.

The zero-field splitting parameters of triplet II (see Table 1) are substantially larger than those of either

chlorophyll or pheophytin monomers (24,42). Similar triplet state parameters were obtained by Hoff, et al. (9) using optical detection of magnetic resonance techniques on reduced chloroplasts or digitonin particles prepared without intense illumination while freezing (our condition [2]). They suggested that the observed signals arose from a non-chlorophyll species, possibly pheophytin. We feel that it is also possible that the signals arise from carotenoids. Numerous optical experiments on green plants and bacteria have revealed that carotenoid triplet states serve as sinks for excess energy (43-47). However, triplet state EPR spectra of these systems are sparse, presumably due to the difficulty of photoexciting carotenoids directly into their triplet states (48). Chlorophyll sensitization greatly enhances the carotenoid triplet population (48).

Our studies of chloroplasts and digitonin Photosystem I particles treated with ferricyanide confirm the hypothesis that triplet II appears when the P700 traps are closed. In the presence of ferricyanide we have the redox state

$$P700^{+} \; A \; X \; Fd_B \; Fd_A$$

in the dark. One can see from Figs 2b,2c,3b, and 3c that the same triplet state spectrum, triplet II, arises either when all P700 traps are closed by chemical oxidation (condition (3) or when X is not reduced (condition [2]). This result provides evidence that triplet II is not localized on one of the main components active in the primary charge transfer

events of Photosystem I, but serves as an energy sink for excess excitation which never reaches P700.

Our observation of the radical pair polarization of triplet I is consistent with the observation of a radical pair mechanism polarizing the EPR signal from $P700^+$ and giving rise to CIDEP during normal forward photochemistry at room temperature in chloroplasts (26,27). Furthermore, the observation of triplet I provides independent corroboration that an electron carrier A mediates in the charge transfer froom P700 to X (17,16).

Our conclusion that triplets I and II are associated with P700 in the Photosystem I reaction center is supported by [1] the light-induced appearance of triplet I only when X is reduced prior to the EPR experiment; [2] the disappearance of triplet I and the appearance of triplet II when P700 is oxidized; and [3] the observation of these events in a Photosystem I -enriched digitonin fraction.

ACKNOWLEDGEMENTS

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FIGURE CAPTIONS

1. EPR spectra of reduced chloroplasts observed in the dark at 11K. The conditions under which the samples were frozen are: a) dithionite, illumination while cooling; b) dithionite, dark. The EPR conditions for both spectra are: microwave power, 10 mW; sweep time, 2 min; time constant, 0.5 sec; modulation amplitude 16 G; modulation frequency, 100 kHz; microwave frequency, 9.079 GHz; receiver gain, 2500.

2. Light modulated triplet state spectra of chloroplasts. The conditions under which the samples were frozen are a) dithionite, illumination while cooling; b) dithionite, dark; c) ferricyanide. The EPR conditions for all three spectra are: microwave power, 1 mW; sweep time, 1 hr; recorder time constant, 30 sec; modulation amplitude, 16 G; modulation frequency, 100 kHz; receiver gain, 80; microwave frequency, 9.075 GHz; light modulation frequency 33.5 Hz.

3. Light modulated triplet state spectra of digitonin Photosystem I particles. The conditions under which the samples were frozen are: a) dithionite, illumination while cooling; b) dithionite, dark; c) ferricyanide. The EPR conditions for spectra a) and b) are: microwave power, 1 mM; sweep time, 1 hr; recorder time constant, 30

sec; modulation amplitude, 16 G modulation frequency, 100 kHz; receiver gain, 20; microwave frequency, 9.075 GHz; light modulation frequency, 33.5 Hz. EPR conditions for spectrum c) are the same as in a) and b) except for receiver gain, 80.

TABLE I Zero-field splitting parameters and electron spin polarization patterns of the observed triplet state signals. The errors represent the uncertainty in the parameters as deduced from the repeatability of the field position measurements. a = absorption, e = emission.

	|D|	|E|	Polarization pattern
Triplet I	.0278± .0009cm^{-1}	.0039± .0009cm^{-1}	aee aae
Triplet II	.0383± .0013cm^{-1}	.0040± .0013cm^{-1}	eae aea

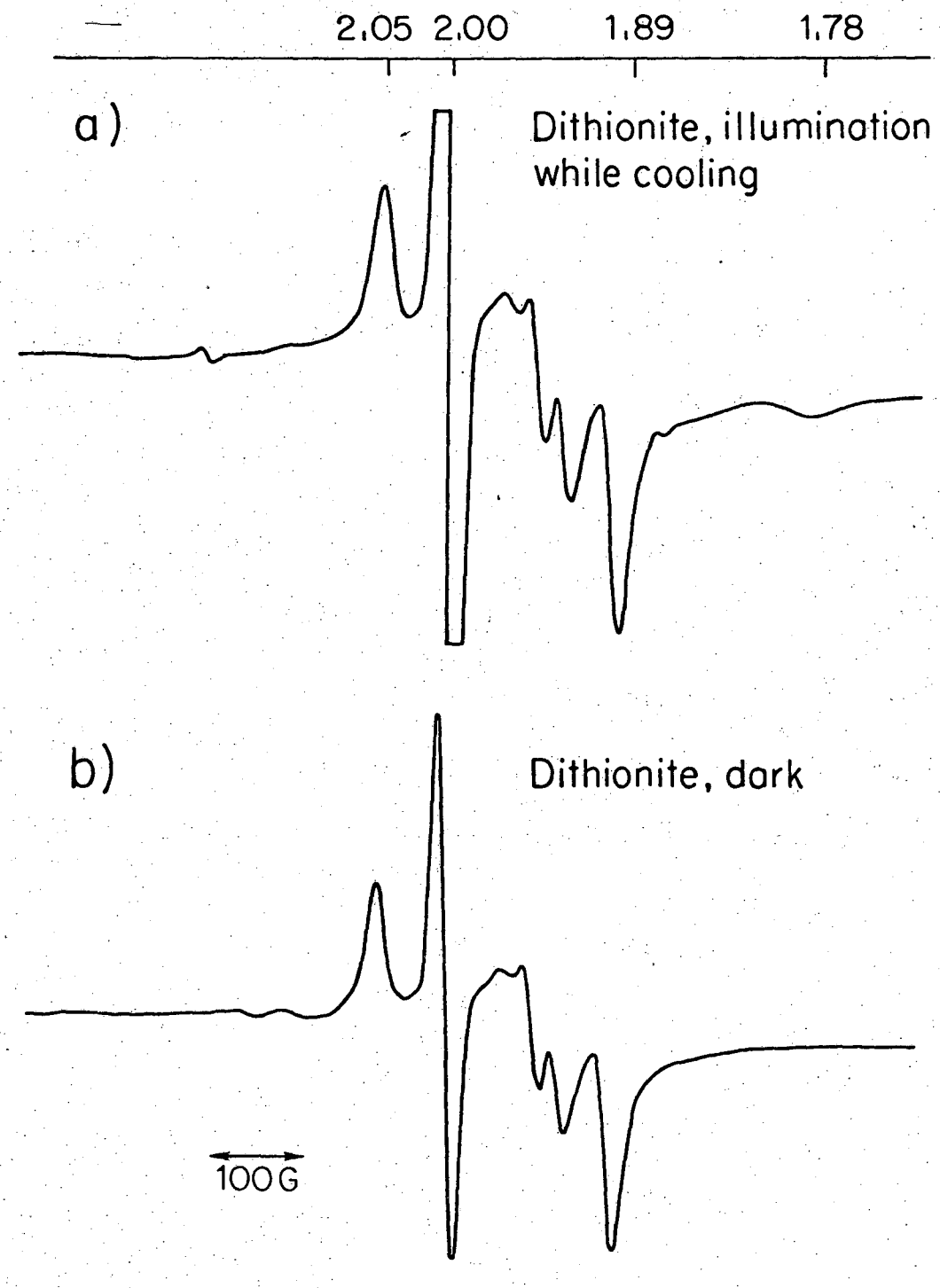

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Figure 1

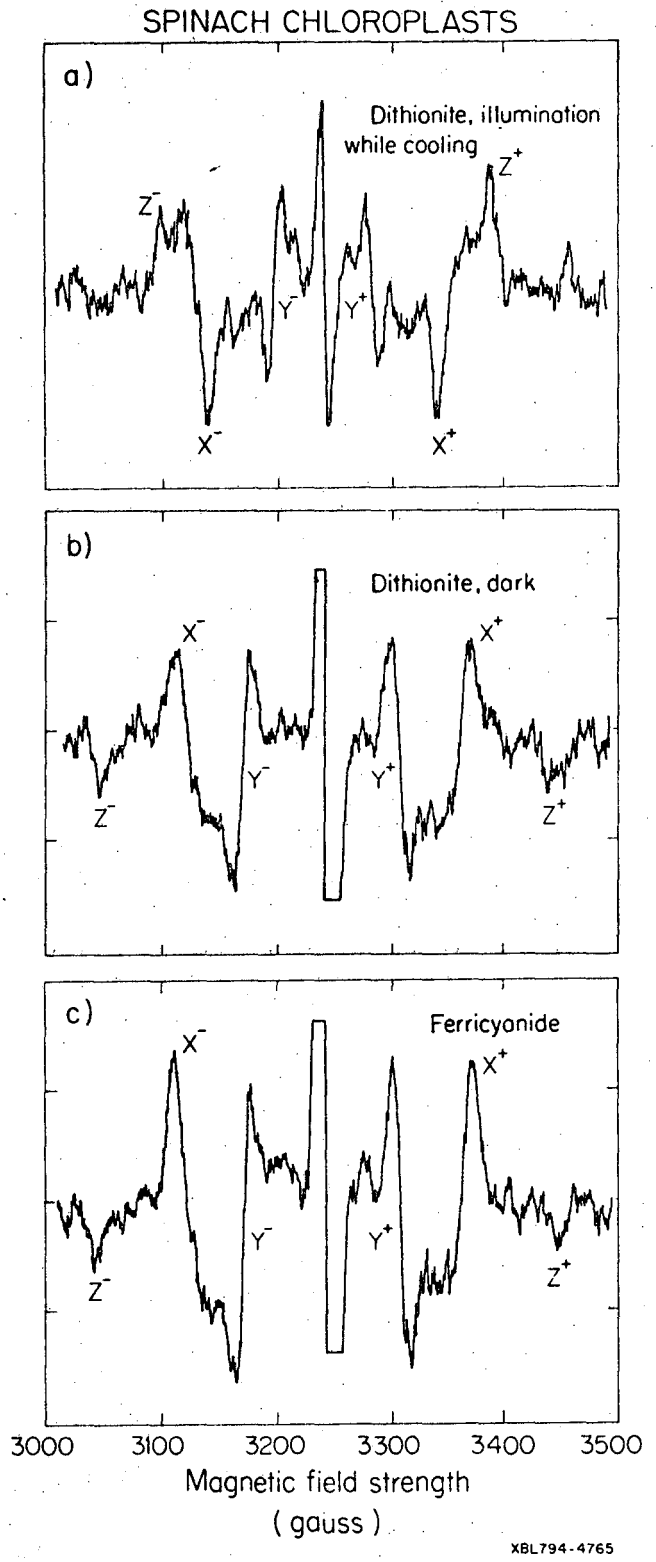

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Figure 2

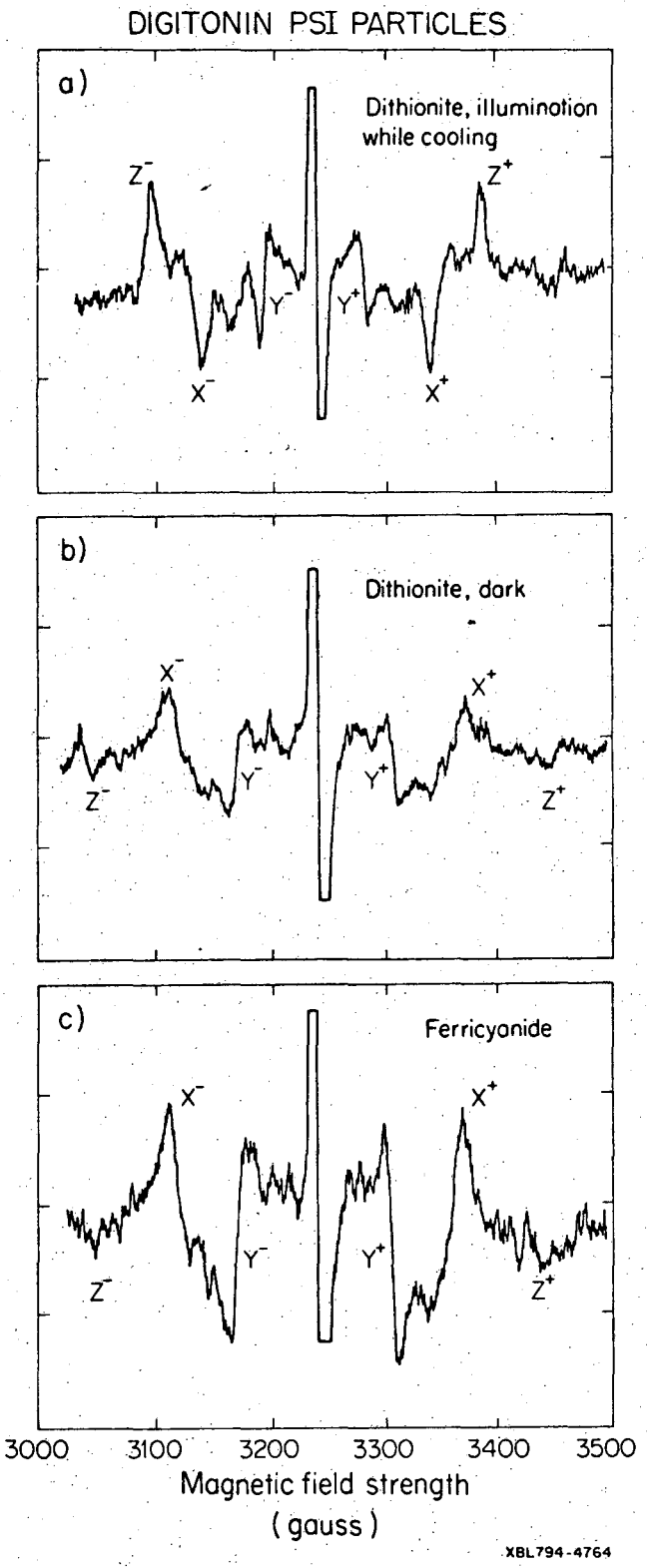

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Figure 3

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