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Detection of a Free Radical in the Primary Reaction of Chloroplast Photosystem II

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ABSTRACT Electron paramagnetic resonance (EPR) spectroscopy has revealed a new free radical signal produced by illumination of spinach chloroplasts at 77°K. This signal is observed only when an oxidant (ferricyanide) is added to the chloroplast suspension in the dark prior to illumination. The EPR signal is produced at 77°K by illumination with 645-nm monochromatic light capable of activating Photosystem II but not with 715-nm illumination capable of activating Photosystem I. Furthermore, since the signal shows a relative increase in chloroplast fragments enriched in Photosystem II but is absent in chloroplast fragments enriched in Photosystem I, we conclude that this new free radical EPR signal is associated with the primary photoact of Photosystem II in chloroplasts. On the basis of the measured EPR parameters ($g = 2.0026 \pm 0.0002$, linewidth = 8 gauss), it is suggested that the signal may be associated with the reaction center chlorophyll of Photosystem II.

The mechanism of oxygen evolution is one of the major unresolved problems in chloroplast photosynthesis. Oxygen evolution has been found to proceed more efficiently in short-wavelength light ($\lambda < 700$ nm), associated with Photosystem II, than in long-wavelength light ($\lambda > 700$ nm), associated with Photosystem I (1-3) but the nature of the primary reactants of Photosystem II and the subsequent dark reactions involved in the process of oxygen evolution have not yet been fully characterized.

Two different light-induced absorbance changes appear to be related to the primary photochemical event of Photosystem II. A light-induced absorbance change at 550 nm was discovered by Knaff and Arnon (4) and was found to be due to the Photosystem II photoreduction of a chloroplast component, designated C550. The photoreduction of C550 was found to proceed not only at physiological temperature but also at 77°K, a finding which indicated a possible relation with a primary photochemical event. On the basis of this work, which was confirmed and extended in other laboratories (5-10), C550 was proposed (4-8) as the primary electron acceptor of Photosystem II. Döring et al. (11,12) observed a different light-induced absorbance change at physiological temperature in chloroplast fragments enriched in Photosystem II. This absorbance change was very rapid and had a spectrum with peaks at 435 nm and 682 nm. These workers suggested this change was due to a form of chlorophyll a which functioned as the reaction center chlorophyll of Photosystem II. Later measurements of the photoinduced absorbance change at 682 nm showed the reaction occurred at 77°K (13). The possible relation of this absorbance change and C550 to the primary photoact of Photosystem II has been discussed by Butler (14).

Although electron paramagnetic resonance (EPR) spectroscopy has been used to study the primary photoact in photosynthetic bacteria (15-18) and to study the primary reaction of chloroplast Photosystem I (19-22), EPR signals

specifically associated with the primary reactants of chloroplast Photosystem II have not heretofore been detected. An EPR signal, now known as "Signal II" (23-25), has been associated with Photosystem II but the nature of the component responsible for this signal and its role in photosynthetic electron transport is not clear (24,25).

In this paper we report an EPR spectroscopic examination of chloroplasts and chloroplast fragments at 77°K for changes which are attributable to the primary photoreaction of Photosystem II. We have detected a new free radical EPR signal which is different from other previously described chloroplast free radical signals and which may be associated with the reaction center chlorophyll of Photosystem II.

MATERIALS AND METHODS

Whole spinach chloroplasts and washed, broken chloroplasts were prepared from greenhouse spinach as previously described (26,27). Digitonin chloroplast fragments enriched in Photosystem II (D-10) and Photosystem I (D-144) were prepared by the procedure of Hauska et al. (28). Triton chloroplast fragments enriched in Photosystem II were prepared from whole chloroplasts by the procedure of Malkin (26). Chlorophyll concentrations and the chlorophyll a:b ratio were measured by the method of Arnon (29).

Oxidation-reduction potentials of chloroplast suspensions at 5°C in the presence of 10 mM ferricyanide were measured with a Radiometer PK-149 combined platinum-calomel electrode and a Corning digital pH meter (Model 110). The oxidation-reduction potentials are reported relative to the standard hydrogen electrode.

Chloroplast samples were placed in standard X-band quartz EPR tubes (3 mm i.d.) and illuminated for 30 sec at 77°K directly in the EPR cavity with the apparatus previously described (20). Baird-Atomic interference filters

(715 nm or 645 nm) of half-band width 10 nm were used for the monochromatic illuminations. The incident light intensity on the sample was approximately 5×10^4 ergs·cm^{-2}·sec^{-1}. EPR spectra were recorded at 9.22 GHz at 77°K. Details of the EPR methods are the same as given in previous publications (19,22, 27).

RESULTS AND DISCUSSION

As shown in Fig. 1A, an EPR free radical signal, identical to "Signal II" (24,25), is present in washed, broken chloroplasts at 77°K in the dark. The addition of 10 mM ferricyanide to the chloroplasts at 5°C in the dark followed by EPR examination at 77°K (Fig. 1B) results in the appearance of a second narrow free radical signal centered at g = 2.002 and having a linewidth of approximately 8 gauss. This signal, referred to as "Signal I" (24,25), has been shown to be due to the oxidized form of P700, the reaction center chlorophyll of Photosystem I (24,25,30,31). The concentration of ferricyanide added to these samples was sufficient to raise the ambient oxidation-reduction potential of the suspension to approximately +540 mV. If the chloroplast sample is illuminated at 77°K in the presence of ferricyanide with far-red light (715 nm), which activates primarily Photosystem I, there is no increase in the observed EPR signal (Fig. 1C). The intensity of far-red light used for the illumination was sufficient to completely photooxidize P700 at 77°K when ferricyanide was omitted from the reaction mixture. This finding indicates that P700 was completely oxidized chemically prior to illumination. If, however, the same sample is subsequently illuminated with red light (645 nm), which activates Photosystem II, a large increase in the EPR signal is now observed (Fig. 1D). Since this light-induced change occurs at an oxidation-reduction potential where P700 is already fully oxidized, is produced by monochromatic illumination which

Fig. 1

activates Photosystem II, and is not produced by illumination which activates Photosystem I, it is clear that the additional EPR signal must be due to a photoreaction of a Photosystem II component.

The observation that the EPR signal appears when the chloroplast sample is illuminated at low temperatures (77°K or lower), temperatures where chemical reactions would be strongly inhibited, is consistent with an association of the component responsible for the signal with a primary photochemical event.

The light-induced EPR signal observed in the presence of ferricyanide was also detected in whole spinach chloroplasts, although broken chloroplasts were mainly used in these studies to facilitate the interaction of ferricyanide with membrane-bound components.

Primary photoreactions in photosynthetic bacteria are reversible on the cessation of illumination even at temperatures as low as 1.7°K (32,15,17). In contrast, EPR signals from chloroplasts or chloroplast fragments are irreversible up to temperatures of at least 77°K (17,19,20). The light-induced change observed in the presence of ferricyanide is similar to other light-induced chloroplast EPR signals in this respect.

Fig. 2 shows EPR difference spectra obtained from the data with washed, broken chloroplasts. (The signals have been multiplied by a factor of two to facilitate comparison.). Fig. 2A presents the difference between the spectrum of the dark sample treated with ferricyanide and the spectrum of the sample with no addition. This free radical signal is due to the oxidized form of P700. Fig. 2B shows the difference spectrum between the sample illuminated with 645-nm light at 77°K (in the presence of ferricyanide) and the sample illuminated with 715-nm light at 77°K (in the presence of ferricyanide). This difference spectrum indicates that a free radical is photoinduced by 645-nm light. The free radical

Fig. 2

has a g value of 2.0026 ± 0.0002 and a peak-to-peak width of approximately 8 gauss. An estimation of the concentration of the new light-induced free radical, assuming $S = 1/2$, gives a value of one radical per 330 chlorophyll molecules. Although this value is approximate, it indicates that the species producing the new signal is present at a concentration comparable to that of other electron transfer carriers of the photosynthetic electron transport chain.

The findings with chloroplasts reported above indicate that the new light-induced free radical signal is associated with Photosystem II. Additional support for this conclusion is based on experiments with chloroplast fragments enriched in either Photosystem I or Photosystem II. As shown in Fig. 3, digitonin chloroplast fragments enriched in Photosystem II (D-10) show, in the presence of ferricyanide, no light-induced changes after illumination with 715-nm light (Fig. 3B), but a subsequent illumination with 645-nm light (Fig. 3C) results in a large light-induced free radical signal. In contrast, the digitonin chloroplast fragments enriched in Photosystem I (D-144) show no light-induced changes after the addition of ferricyanide with either red or far-red illumination (Fig. 4B and 4C). Quantitative estimates of the spin concentration of the free radical component photoinduced in the presence of ferricyanide in the D-10 fragments show an approximately two-fold increase when compared to chloroplasts. The P700 content of these fragments, estimated from chemical difference spectra, was one P700 per 2000 chlorophyll molecules. This amount of P700 is far too low to account for the free radical signal observed in the preparation after low-temperature illumination. An increase in the concentration of the new light-induced signal relative to chloroplasts was also observed with chloroplast fragments enriched in Photosystem II prepared by Triton treatment. The results with chloroplast fragments enriched in Photosystem II strongly support the conclusion that this free radical is indeed associated with the photochemical activity of Photosystem II.

Fig. 3

Fig. 4

CONCLUDING REMARKS

The results presented indicate the existence of a light-induced EPR signal associated with the primary photochemical reaction of chloroplast Photosystem II. Previously, the only known EPR signal in chloroplasts related to this photosystem was that known as "Signal II" (24,25); this component, however, does not undergo photoreactions at low temperatures and therefore is not likely to be associated with a primary photochemical event. In addition, the EPR parameters of the new low-temperature, light-induced signal ($g = 2.0026$, linewidth = 8 gauss) clearly distinguish this signal from Signal II ($g = 2.004$, linewidth = 19 gauss).

A light-induced free radical signal in chloroplasts at room temperature has previously been observed at oxidation-reduction potentials greater than +550 mV (33) but no results were reported to indicate an association of the component with a primary reactant of Photosystem II. Since we have not made measurements of our light-induced change at physiological temperatures, the relation of the reported radical signal and our newly detected signal is not clear.

Although we cannot assign this new free radical signal to either the donor or the acceptor of Photosystem II, the similarity between the EPR parameters (g value and linewidth) of the new free radical signal and the EPR signals of chlorophyll free radicals (34,35) suggests the signal may originate from the reaction center chlorophyll of Photosystem II. We have been unable to detect any corresponding EPR change associated with the second reactant (the electron acceptor) of the primary photoact of Photosystem II. Additional experiments are currently in progress to clarify the identity of the signal-producing component in the photosynthetic electron transport pathway and to further elucidate the nature of the primary photochemical act of chloroplast Photosystem II.

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LEGENDS TO FIGURES

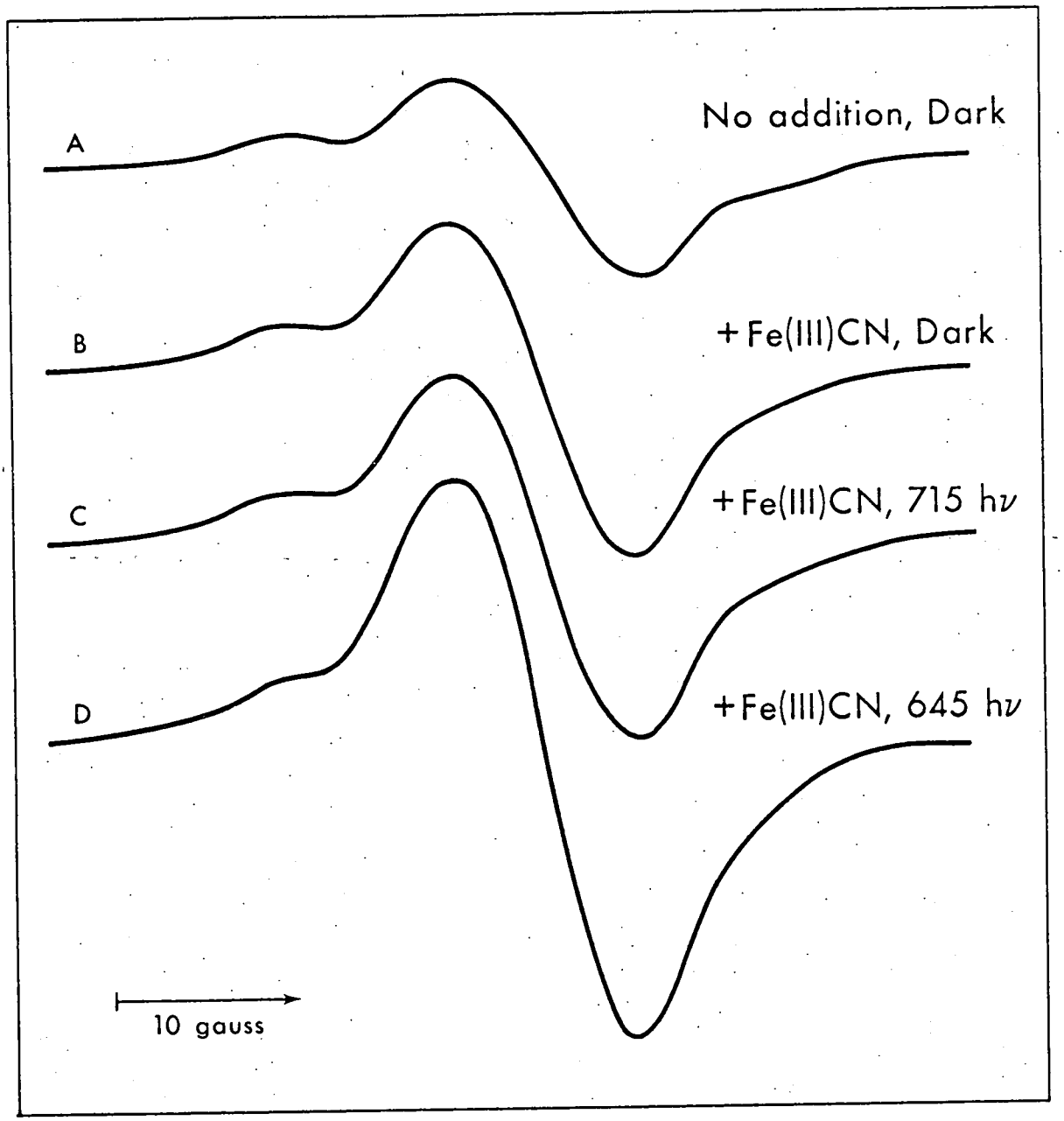
Fig. 1. Low-temperature, light-induced free radical signal in chloroplasts in the presence of ferricyanide. The reaction mixture contained 50 mM Tricine (pH 7.8), 20 mM NaCl, washed, broken chloroplasts (0.3 mg chlorophyll per ml) and, where present, 10 mM potassium ferricyanide. (A) No additions, dark; (B) Plus ferricyanide, dark; (C) Plus ferricyanide, illuminated at 77°K with 715-nm light; (D) Plus ferricyanide, illuminated at 77°K with 645-nm light. First derivative EPR spectra were recorded at 77°K with the following instrument settings: Frequency, 9.22GHz; power, 0.5 mW; modulation amplitude, 2 gauss; scan rate, 5 gauss/sec.

Fig. 2. EPR difference spectra of chloroplast free radical signals. (A) The difference spectrum in the dark between the sample treated with ferricyanide and the sample with no addition. (B) The difference spectrum of the sample illuminated with 645-nm light (in the presence of ferricyanide) at 77°K and the sample illuminated with 715-nm light at 77°K (in the presence of ferricyanide). Both difference spectra have been multiplied by a factor of two. EPR spectra were recorded at 77°K as described in Fig. 1.

Fig. 3. Low-temperature, light-induced EPR free radical signal in Photosystem II chloroplast fragments in the presence of ferricyanide. The reaction mixture contained 50 mM Tricine (pH 7.8), 20 mM NaCl, 10 mM potassium ferricyanide, and D-10 chloroplast fragments (0.3 mg chlorophyll per ml). (A) Plus ferricyanide, dark; (B) Plus ferricyanide, illuminated at 77°K with 715-nm light; (C) Plus ferricyanide, illuminated at 77°K with 645-nm light. EPR spectra were recorded at 77°K as described in Fig. 1.

Fig. 4. Low-temperature, light-induced EPR free radical signal in Photosystem I chloroplast fragments in the presence of ferricyanide. The reaction mixture contained 50 mM Tricine (pH 7.8), 20 mM NaCl, 10 mM potassium

ferricyanide, and D-144 chloroplast fragments (0.3 mg chlorophyll per ml). (A) Plus ferricyanide, dark; (B) Plus ferricyanide, illuminated at 77°K with 715-nm light; (C) Plus ferricyanide, illuminated at 77°K with 645-nm light. EPR spectra were recorded at 77°K as described in Fig. 1.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B
+Fe(III)CN, Dark
C
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D
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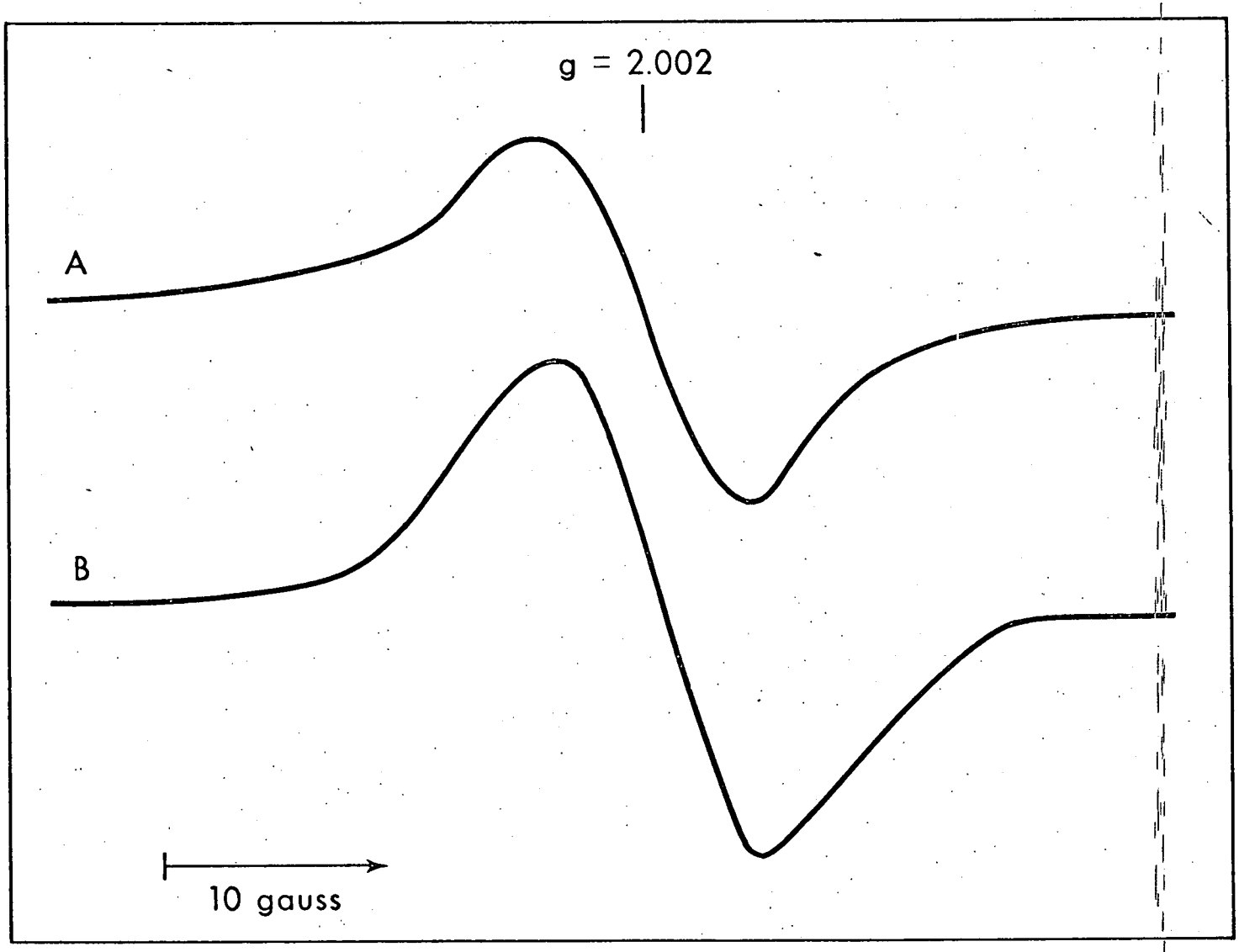
10 gauss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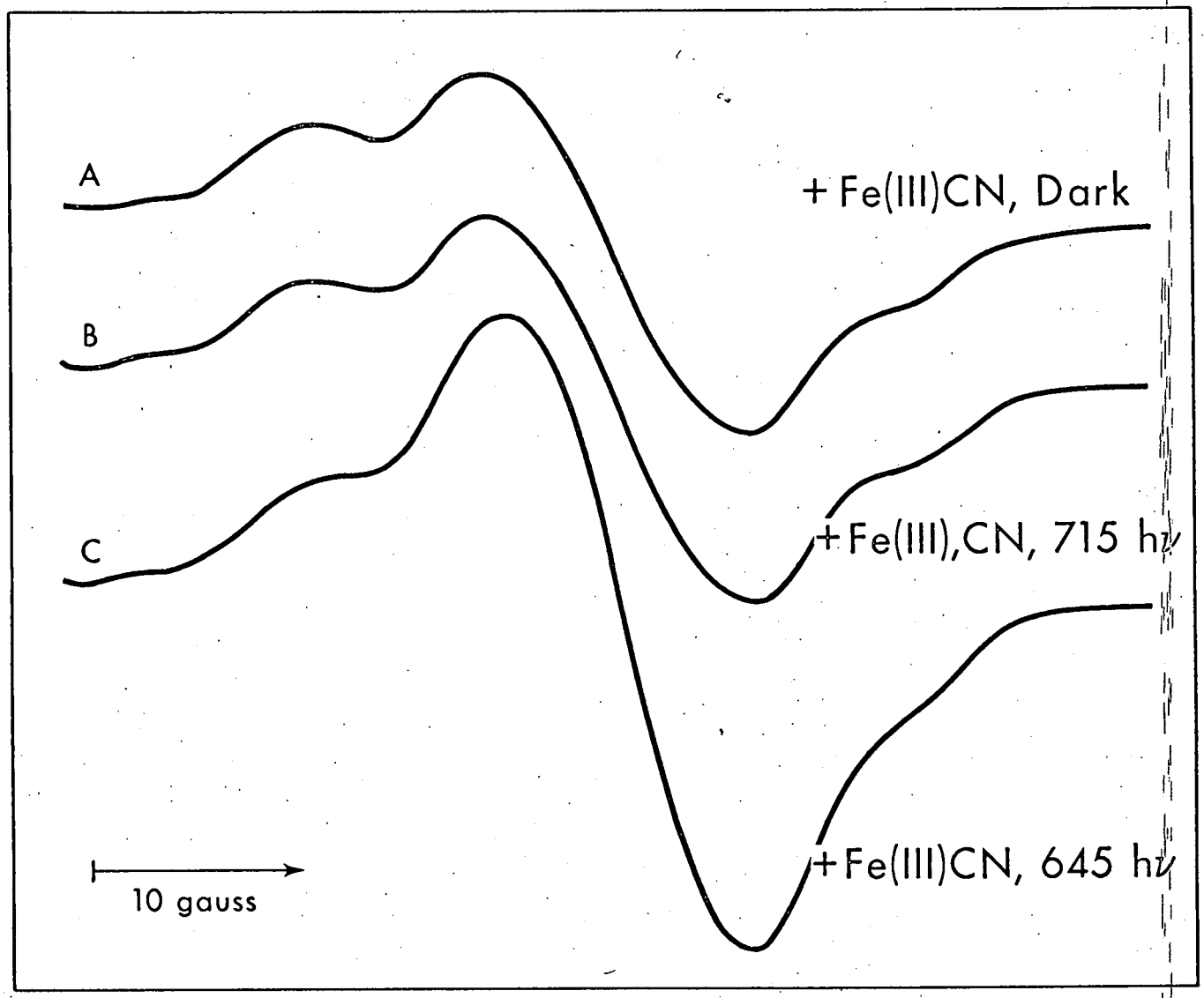
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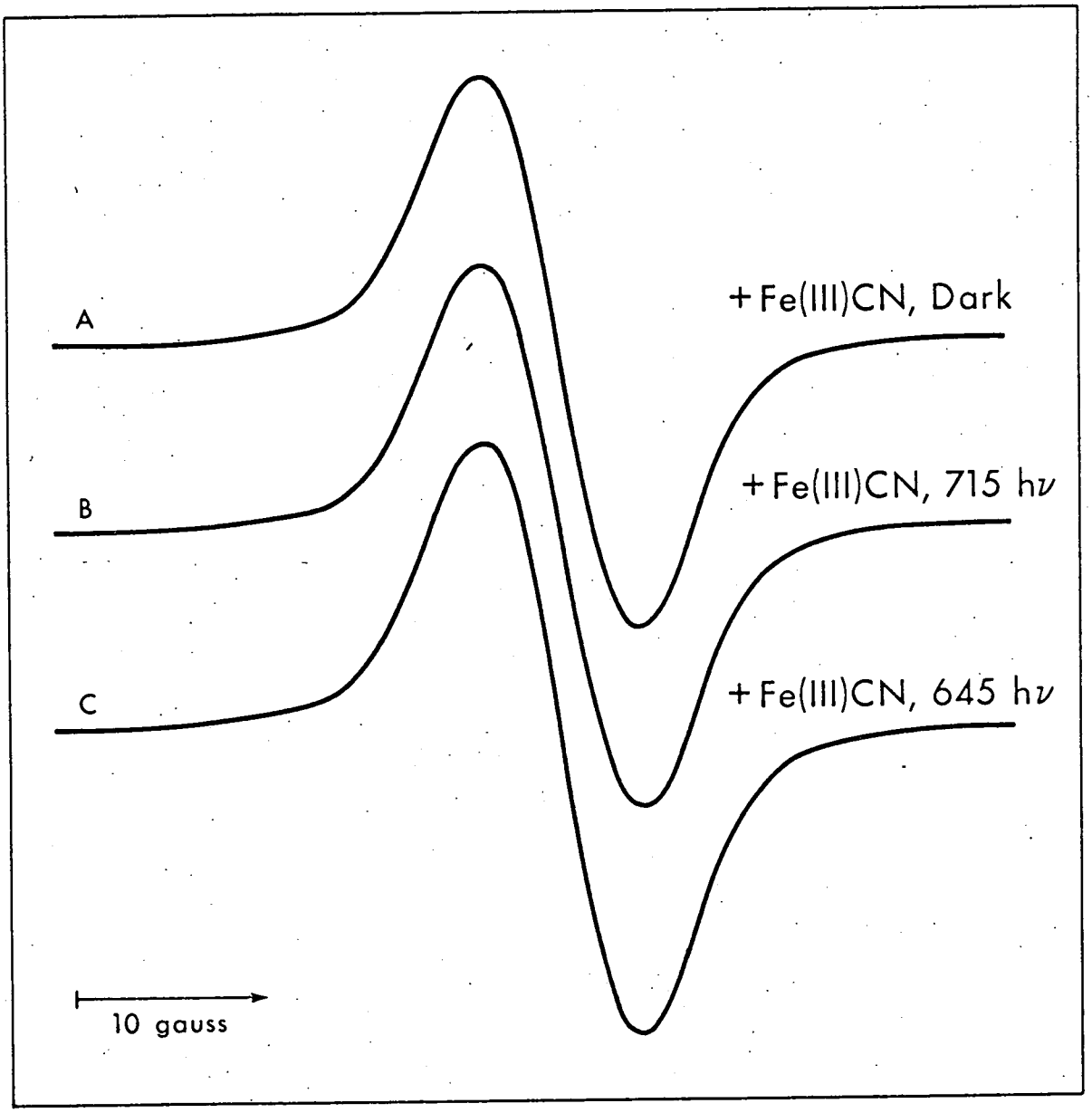
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