## SHORT COMMUNICATIONS

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### Electron paramagnetic resonance signal produced by ferricyanide in photosynthetic plant materials

It has been found that the absorption change at 700 nm produced by ferricyanide oxidation of subcellular photosynthetic particles derived from plants removes the typical light-induced change at this wavelength[1]. Ferricyanide also induces an EPR signal in chloroplasts which is similar in shape, width and $g$ value to that produced by light; the same midpoint redox potential has been demonstrated for the optical change and EPR signal, as well as a reciprocal relationship between the light-induced EPR signal and oxidation potential of a chloroplast suspension[2,3]. All these facts support the conclusion that the EPR signal I is identical to the oxidized form of the photoreactive center, P700 (refs. 4, 5).

When one wishes to make a quantitative determination of the maximum number of spins in signal I of a photosynthetic system, it is necessary to saturate the signal. Using light alone, this can be difficult; although a thin suspension (*e.g.* with an absorbance at 680 nm of 0.5 or less in the EPR cuvette) of fresh cells or chloroplasts can be saturated with easily obtainable intensities of white illumination, *e.g.* less than $10^5$ erg·cm$^{-2}$·sec$^{-1}$, the signal-to-noise ratio of such thin aqueous suspensions is likely to preclude an accurate determination of signal intensity. A dense suspension provides more favorable signal-to-noise, but is more difficult, perhaps impossible, to light-saturate. If all the potential spin sites can be oxidized with ferricyanide, then, this problem is side-stepped and a determination can be made at room temperature on the "chemical" signal which is presumably identical to the light-induced signal. Such studies have been made[6] and it is probable that further work will be done with ferricyanide on EPR and optical changes. It is the purpose of this note to call attention to the fact that apparently not every potential spin site in plant material is accessible to the oxidizing action of ferricyanide; determinations made using an excess of ferricyanide will result in a figure for number of spin sites which is low.

The effects noted in this paper were observed with a variety of materials, all prepared in standard ways. Since details of the preparations did not affect the result, they will not be given minutely here, but indicated in the legend to the appropriate figure.

Chlorophyll was determined by extraction with 80 % acetone[7]. $K_3Fe(CN)_6$ or $K_4Fe(CN)_6$ was used the same day it was prepared. The indicated addition to the suspension of photosynthetic material was made in dim light, pipetted into the quartz EPR cuvette, and scanned immediately.

EPR measurements were made with a standard Varian V-4500 spectrometer with 6-inch magnet and 100 kcycles field modulation at ambient temperature. Modu-

lation amplitude was 4.6 gauss, sweep rate 40 gauss/min and response times 0.1, 0.3 or 1 sec. One set of measurements was made on the Varian E-3 EPR spectrometer, with a 25-mW He–Ne laser (6328 Å) defocussed with a lens, as light source (Fig. 1). For all other measurements, white light was provided by a 500-W projector, cooled by passage through three Balzers heat filters. White light energy incident on the sample was $5 \cdot 10^4$ erg·cm$^{-2}$·sec$^{-1}$.

For a given concentration of cellular material, the ferricyanide-induced signal size was independent of ferricyanide concentration over a range from $10^{-4}$ M, which BEINERT, KOK AND HOCH[2] state is sufficient to saturate all spin sites, to $10^{-2}$ M, where the signal soon vanished with a loss of color (browning) by the preparations. We used chlorophyll concentrations between 0.2 and 1 mM, and 2 mM ferricyanide. Ferricyanide alone has no EPR signal.

Fig. 1 illustrates the signals produced by light and by ferricyanide. They have identical widths (7.5 gauss), $g$ values (2.0025) and line shapes.

We found in every case that the signal which was produced by an excess of ferricyanide could be increased by illumination. In most preparations, the signal

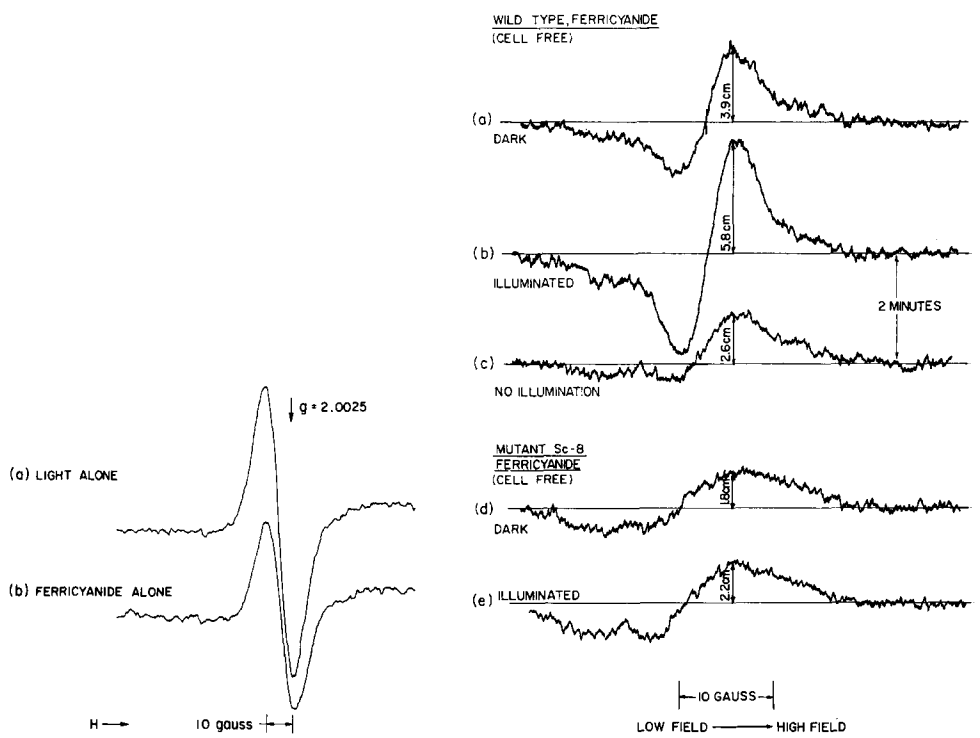


Fig. 1. Traces made from small subchloroplast particles derived from spinach showing primarily photosystem I activity[4]. Lyophilized particles were resuspended in 0.1 M phosphate buffer, pH 7.8. Chlorophyll *a* concentration, 0.25 mM. Trace a, illuminated suspension; b, ferricyanide added to 2 mM, but no illumination.

Fig. 2. Traces a, b and c are made with cell-free Scenedesmus, 2 mM ferricyanide. Total chlorophyll concentration, 1.12 mM for both preparations. Traces d and e are from a similar preparation derived from the mutant, Sc-8, which has no optically detectable P700 and almost no signal I (ref. 8).

produced by saturating light alone was as large as the signal resulting from ferricyanide *plus* light. Moreover, the "dark" (*i.e.* ferricyanide induced) signal was invariably smaller after illumination, recovering slowly in the dark. These facts are illustrated in Fig. 2. The smaller post-illumination signal (c) would suggest a light-induced reduction of the ferricyanide. A qualitatively similar sequence was observed with spinach chloroplasts and with cell-free preparations of Chlamydomonas and Anacystis, as well as with lyophilized and resuspended "System I" (PD-10) particles of VERNON, KE AND SHAW[4]. A mutant strain of Scenedesmus (Sc-8) which has no light-induced signal I and no optically detectable P700 (ref. 8) also produces no signal I in the presence of ferricyanide (Figs. 2d and 2e), although the broader signal II, also produced by ferricyanide oxidation, is evident. Addition of Triton X-100 did not alter the size of the ferricyanide-induced signals in Scenedesmus preparations.

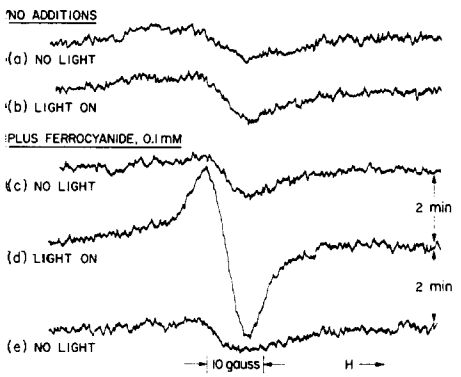


Fig. 3. Wild type ($D_3$) Scenedesmus, 1.1 mM chlorophyll, cell-free, frozen 7 days, then thawed. Traces a and b, no additions. Traces c to e, made 1 mM ferrocyanide.

One more comment may be made and illustrated: cell-free preparations of wild-type Scenedesmus were frozen, and upon thawing did not exhibit the expected light-induced signal I although they had good Hill activity. The addition of ferricyanide in this case did elicit a signal which behaved as described above. The addition of ferrocyanide produced no signal I, in accordance with the results of others. However, when the ferrocyanide-containing suspension was illuminated, a typical signal I appeared, as illustrated in Fig. 3 (*cf.* results in Table I of BEINERT AND KOK[6] with TX-27). We cannot offer any explanation for this observation.

We conclude, therefore, that some of the oxidizable sites remain inaccessible to ferricyanide, and that this agent alone cannot be used to determine the maximum number of spins in a photosynthetic preparation. These results, however, are still compatible with the theory that the light-induced spin signal and ferricyanide-induced spin signal both arise from $P700^+$. We recommend, then, that when one wishes to determine the maximum signal I in a subcellular preparation, that aliquots both with and without ferricyanide be illuminated in the EPR spectrometer, and the largest signal used as a basis for calculation. It will be interesting to see whether sensitive optical methods for detecting the absorption change at 700 nm with light or ferricyanide will reveal the same relationship as has been demonstrated for the EPR signal.

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## Absence of NADH:NAD$^+$ oxidoreductase activity in mitochondrial NAD(P) transhydrogenase

NAD(P) transhydrogenase (NADPH:NAD$^+$ oxidoreductase, EC 1.6.1.1, also known as TD-transhydrogenase) was first found in animal tissues by KAPLAN, COLOWICK AND NEUFELD[1]. Preparations of this enzyme always contain various amounts of NADH:NAD$^+$ oxidoreductase (also known as DD-transhydrogenase) activity[1,2].

It was assumed that a single lipoprotein was responsible for both activities[3]. However, the present communication shows that by treatment with a detergent, followed by gel filtration, fractions could be obtained containing the two activities separately.

Rat liver mitochondria (about 260 mg protein) prepared by WEINBACH's[4] method were treated with 7 ml of an ice-cold 0.016 M solution of Triton X-100 (0.05 M potassium phosphate, pH 7.4, 0.001 M EDTA) for 1 h, and centrifuged for 2 h at 38000 $\times$ *g*. In the clear supernatant both activities were assayed with oxidized 3-acetylpyridine–adenine dinucleotide (APAD$^+$) as hydrogen acceptor[5]. The specific activities were found to be 0.040 and 0.190 $\mu$mole/min per mg protein for the NADPH:APAD$^+$ and NADH:APAD$^+$ oxidoreductase reactions, respectively*. The supernatant was chromatographed on a Sephadex G-100 column, which was eluted

Abbreviations: APAD$^+$ and APADH, oxidized and reduced forms of 3-acetylpyridine–adenine dinucleotide.

* Mitochondria disintegrated in an analogous manner with 0.016 M digitonin[6] showed specific activities of 0.084 and 0.159, respectively.