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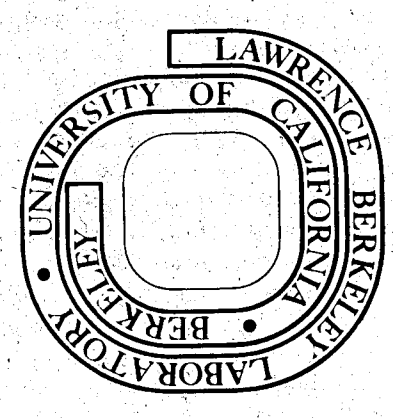

DISCLAIMER

STABILIZATION OF ELECTRON SPIN RESONANCE PROBES FOR PHOTOSYNTHESIS STUDIES

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Summary

The major obstacle to the study of functional/structural interrelationships of spinach chloroplasts by using spin labels has been the rapid loss of the epr signals upon illumination with visible light. The present study demonstrates that the addition of ferredoxin and $NADP^+$ in the presence of tricine buffer at pH 7.1 or higher mitigates the rapid loss of Biradical X (N,N'-Bis(1-oxyl-2,2,5,5-tetramethylpyrroline-3-carboxy)-1,2-diaminoethane) and Monoradical A (2,2,5,5-tetramethyl-3-carbamidopyrroline-1-oxyl). However, the 5-line epr spectrum characteristic of Biradical X in aqueous solution was changed to a dominantly 3-line spectrum within a few minutes after illumination in the presence of ferredoxin and $NADP^+$. Analysis of the double integration of the first derivative epr spectrum revealed no decrease in Biradical X concentration for more than 30 minutes of illumination. Our data suggest that Biradical X attaches to some soluble macromolecule(s) and that illumination of chloroplasts promotes such an attachment.

Introduction

Light-scattering and electron micrographic techniques have demonstrated that gross structural changes are associated with functional changes occurring in the chloroplasts.[1-4] These changes would be better understood at the supramolecular level if a spin label, a probe useful in elucidating the structure of biological membranes, were used. However, only a few reports[5-8] on its application to chloroplasts have appeared since it was introduced more than a decade ago. Weaver and Chon[5] first reported their spin label study in Chlamydomonas and found that the electron paramagnetic resonance (epr) signal of Monoradical A was rapidly decreased during irradiation with visible light, suggesting that Monoradical A was reduced at a specific site in Chlamydomonas. Corker, et al.[6], using di-tertiarybutyl nitroxide as a spin label to study spinach chloroplast function, also found the epr signal lost completely within 2 minutes after illumination. Tzapin et al.[7], using 2,2,6,6-tetramethyl-4-carpiloiloxypiperidine-1-oxyl, had to first fix chloroplasts with glutaraldehyde in different conformational states and then apply the spin label in the dark. Torres-Perreira et al.[8], applied several different spin labels to chloroplasts, and while they were able to find some change in the line shape of the epr spectrum of 2,2,-dimethyl-5,5-dipentyl-N-oxyloxazolidine during illumination, this change was accompanied by 30% loss of the epr signal within 10 minutes. This photoreduction of free radicals by chloroplasts during illumination has been an obstacle to the use of spin labels in studying chloroplast function.

In the present study ferredoxin and $NADP^+$ were used because, as physiological electron acceptors in chloroplasts, they not only compete favorably with other acceptors for electrons, but also provide a very active photosynthetic electron transport reaction.[9] For this reason, they not only protect

spin labels but assure that the work is being done with active photosynthetic systems.

Results

Spinach (Spinacia oleracea var. early hybrid No. 7) was planted in a growth chamber and chloroplasts were isolated as previously reported.[9] In very dim green light, chloroplasts were added to the reaction mixture (described in Fig. 1), mixed in a test tube for 5 sec, then introduced into a flat quartz epr sample tube. The sample tube was placed in the epr cavity of a Varian E-3 epr spectrometer, the cavity being kept completely in the dark. Even a brief exposure of the reaction mixture to light after the chloroplasts were added would cause the Biradical X to lose its characteristic 5-line spectrum. The sample was illuminated by an American Optical microscope lamp and a Corning IR filter I-60 placed in front of the epr sample cavity.

The typical 5-line epr spectrum of Biradical X is shown in Fig. 1. In the dark, the 5-line spectrum is similar to the A type epr spectrum described previously;[11] i.e., biradicals undergo rapid and unhindered tumbling and bending through conformations, many of which are favorable to spin exchange. After illuminating for 2-6 min, peaks 1,3, and 5 of the 5-line epr spectrum increased in intensity while peaks 2 and 4 decreased. This spectrum is similar to the B-G type epr spectrum of Biradical XIX, N,N'-Bis(Trimethylammoniumiodide ethylamide) of N,N'-Bis(1-oxyl-2,2,5,5-tetramethylpyrroline-3-carboxy)ethylenediamine-N,N'-diacetic acid, in glycerol at 160°C, where these biradicals undergo slower tumbling and bending in a more viscous environment and the spin-exchange is less favorable than that of

Type A.[11] The double integration of the first derivative epr spectra (to correspond to the area under a simple absorption spectrum) remained constant for more than 30 min after illumination, as shown in Fig. 1, suggesting that the total number of unpaired electrons remained unchanged. Because the epr absorption is easily saturated and also depends on the geometry of the sample tube in the cavity, we used very low power and did not change the instrumental settings on the epr spectrometer throughout the course of the experiment. Repeated measurements of Biradical X in the reaction mixture without chloroplasts indicated the average standard deviation of double integrations to be $\pm$ 10%. When the sample was illuminated for 30 min and then placed in the dark for 36 hours, or when chloroplasts were removed from the sample following 30 min of illumination and then allowed to remain in the light for 36 hours, the 3-line spectrum returned to its original 5-line spectrum (see Fig. 1.).

After illumination of the sample, as shown in the 3-line spectrum in Fig. 1, the intensity of hyperfine components at low field (peaks 1 or 3) was higher than that in the high field (peak 5), and two side peaks developed outside of peak 1 and 5. No attempt was made in this study to interpret these changes.

Fig. 2 shows the height ratio of peak 2 to peak 1 as a function of the time of illumination. The minimum ratio, i.e., $H_2/H_1 = 0.2$, was achieved within 6 min after illumination, after which the ratio remains relatively constant for about 30 min of illumination. The rapid change of the H_2/H_1 ratio to its minimum indicates a conformational change of Biradical X rather than photoreduction, which would be manifested as a continuous reaction since chloroplasts remain active for more than 20

min under illumination, as shown previously[9] and discussed later in the text.

When we mixed the Monoradical A with Biradical X in aqueous solution of different molar ratios but kept the concentration of total nitroxide constant, we could simulate the epr spectra of those in Fig. 1, but the hyperfine component intensities in the high and low field are not completely identical with those in Fig. 1 which is made in the presence of chloroplasts. For approximation, the ratio of H_2/H_1 as a function of the molar ratio of Monoradical A and Biradical X is shown in Fig. 2. The steady H_2/H_1 ratio produced by illumination is 0.2 and this would be equivalent to 60% of Biradicals X being in conformations in which spin exchange is unfavorable using the aqueous Monoradical A-Biradical X mixture as calibration. Our data could not distinguish whether Biradical X lost 60% of its spin-exchangeability or 60% of Biradicals X lost their spin exchange completely.

When the same experiment, described in Fig. 1, was performed without adding chloroplasts, no change in the epr spectrum of Biradical X could be observed. When chloroplasts were first added to the reaction mixture in the dark and then removed by centrifugation at 1,000 g for 10 min, the Biradicals X remain in the supernatant. Illumination of the supernatant produced no change in the epr spectrum. Thus both chloroplasts and light are essential for inducing the change from a 5-line to a 3-line epr spectrum of Biradical X. To determine whether stable chemical products from photosynthesis will induce the change in epr spectrum of Biradical X in the dark, immediately (< 30 sec) after a reaction mixture (without Biradical X) had been illuminated for 20 min, we added Biradical X to this reaction mixture. No change in the epr spectrum could be observed for 20 min in

the dark, but when this same sample was illuminated the typical change from a 5-line to a 3-line epr spectrum of Biradical X could again be obtained. Thus this change is directly induced by chloroplasts during illumination.

Three possibilities are offered to explain the change of a 5-line to a 3-line epr spectrum of Biradical X. The first is that biradicals may be attached to macromolecules or to membranes and thus constrained in their mobility.[11,12]

A second possibility is that half of the Biradical X became reduced. In this case, however, the total intensity of absorption of the epr spectrum should have decreased. Because the double integration of the first derivative epr spectra remained unchanged for more than 30 min of illumination as shown in Fig. 1, the reduction of half of Biradical X should be negligible. if there is any, especially when it is compared with the change from 5-line to 3-line spectrum which could be accomplished within a few minutes. When sodium dithionite was added to the reaction mixture we found that the 5-line epr spectrum of Biradical X decreases its intensity evenly at all 5 peaks. No change from 5-line to 3-line spectrum was observed. The reduction of half of Biradical X chemically is thus unlikely.

The third type of change could be that the covalent bonds between the two nitroxides were broken in the presence of light and chloroplasts. Again this process should be a continuous reaction. The rapid change of H_2/H_1 ratio to its minimum and the return from 3-line to 5-line spectrum in the presence or in the absence of chloroplasts does not favor this type of change.

When Monoradical A was used instead of Biradical X, no drastic change in the shape of the epr spectrum was observed, although the ratio of the

correlation time in the light to that in the dark was 1.9 after 6 min of illumination, inating some small degree of immobilization. The double integration of this first derivative 3-line epr spectrum also remained constant for more than 30 min after illumination. Lack of reduction of monoradicals further supports the conclusion that the photoreduction of nitroxide free radicals is negligible under our experimental conditions.

As in the early reports cited,[5-8] using pH 6.5 and without adding $NADP^+$ and ferredoxin (Fd) spin label signals were rapidly lost during illumination with chloroplasts. We also found that Biradical X signals were completely lost within 3 min after illumination under the above conditions, as shown in Fig. 3. Furthermore, at pH 6.3 and 6.7 Biradical X signals were also lost even in the presence of $NADP^+$ and ferredoxin. Because the ($H_2O \xrightarrow{e^-} NADP^+$) reaction approaches a maximum[9] at pH 7.5 and most of the electrons produced by chloroplasts during ilumination reduce $NADP^+$, Biradical X is protected. Other work in this laboratory[6,13] also demonstrated that the photoreduction of nitroxide free radicals could be inhibited by adding 3-(3,4-dichloro-phenyl)-1,1-dimethylurea at 3×10^{-6} M or higher concentration, which is known to inhibit the ($H_2O \xrightarrow{e^-} NADP^+$) reaction. This protection of Biradical X by $NADP^+$ and ferredoxin indicates that the loss of epr in their absence is due to the photoreduction by chloroplasts during illumination. The site of photoreduction of Biradical X might be expected to be close to where $NADP^+$ is reduced.

When the reaction mixture was illuminated for 6 min and the typical 3-line epr spectrum of photo-transformed Biradical X was observed, we

removed the chloroplasts by centrifugation. We found almost all the Biradicals X remained in theupernatant and the same 3-line epr spectrum was retained there. When we passed the supernatant through a Sephadex G-25 column, equilibrated with 0.04 M of tricine at pH 7.5 and 0.1 M NaCl, all of Biradical X showing a 5-line spectrum was recovered in fractions 30-33 where Biradical X is found hen it is applied to the column alone. This suggests that the binding is not very strong.

Under similar conditions we also found that Monoradical A remained in the supernatant, which differs from an earlier report[5] in which Monoradical A was reported to be attached to a specific site in Chlamydomonas.

Our data suggest that (1) at a pH higher than 7.1 and in the presence of $NADP^+$ and ferredoxin, the photoreduction of Biradical X by chloroplasts can be slowed down during illumination; (2) Because $NADP^+$ coupled through ferredoxin competes with Biradical X for electrons from chloroplasts, the loss of epr signals of spin labels must be due to their photoreduction in the absence of $NADP^+$ and Fd. The site of reduction should be close to where $NADP^+$ is reduced. (3) Our finding that chloroplasts and light are essential for promoting the attachment of Biradicals to some soluble macromolecules suggests that the role of these macromolecules in photosynthesis should be further investigated.

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Figure Legends

Fig. 1. The change of the first derivative epr spectrum of Biradical X during illumination.

The reaction mixture contained the following in μmoles per ml: Tricine 90, at pH 7.5 or as indicated; $MgCl_2$, 7.5; $NADP^+$, 0.67; ferredoxin, 33 μg; Biradical X, 16.7; Monoradical A, 33.4; chloroplasts, 167 μg chlorophyll. Biradical X and Monoradical A were synthetsized by Dr. P. Ferruti in this laboratory.[11] Ferredoxin was isolated from spinach as reported previously.[9] EPR spectra were measured at X-band in a Varian E-3 spectrometer. Scanning rate, 50 gauss/min; field modulation, 1 gauss; time constant, 0.3 sec; microwave power 2 mwatts. Chlorophyll concentration was determined according to Mackinney.[10] White light of intensity 45 mwatts/cm^2 at the surface of the sample tube. A = double integration (absorption area) of the first derivative epr spectra normalized to be unity in the dark. (a) in the dark, (b) scan started 2 min after light was turned on, (c) 6 min of illumination, (d) 30 min of illumination, (e) 30 min of illumination followed by 36 hr in the dark.

Fig. 2. The ratio of height at peak 2 to peak 1 as a function of the time of illumination and of the molar ratio of Biradical X and Monoradical A.

(a) H_1 = height of peak 1, H_2 =height of peak 2. Experimental conditions same as described in Fig. 2.

(b) Biradical X is mixed with Monoradical A at different molar ratios in aqueous solution. The total concentration of nitroxide free radical is 1.0 milliequivalent.

Fig. 3. The effect of pH, $NADP^+$ and ferredoxin on the photo-transformation of Biradical X by chloroplasts during illumination.

The reaction conditions are the same as in Fig. 1, except that

the pH was varied and no $NADP^+$ or ferredoxin was added as indicated in the figure. The double integration of the first derivative epr spectra of Biradical X was normalized with respect to that in the dark.

(A) $NADP^+$ and ferredoxin were added: (a) —+—+— pH at 7.1, 7.5, 8.0 and 8.5, only data obtained at pH 7.1 is shown. (b) —×—×— pH 6.7. (c) —○—○— pH 6.3.

(B) No $NADP^+$ or ferredoxin was added; (d) - - -□- - -□ pH 7.5.

(e) - - -△- - -△ pH 6.3.

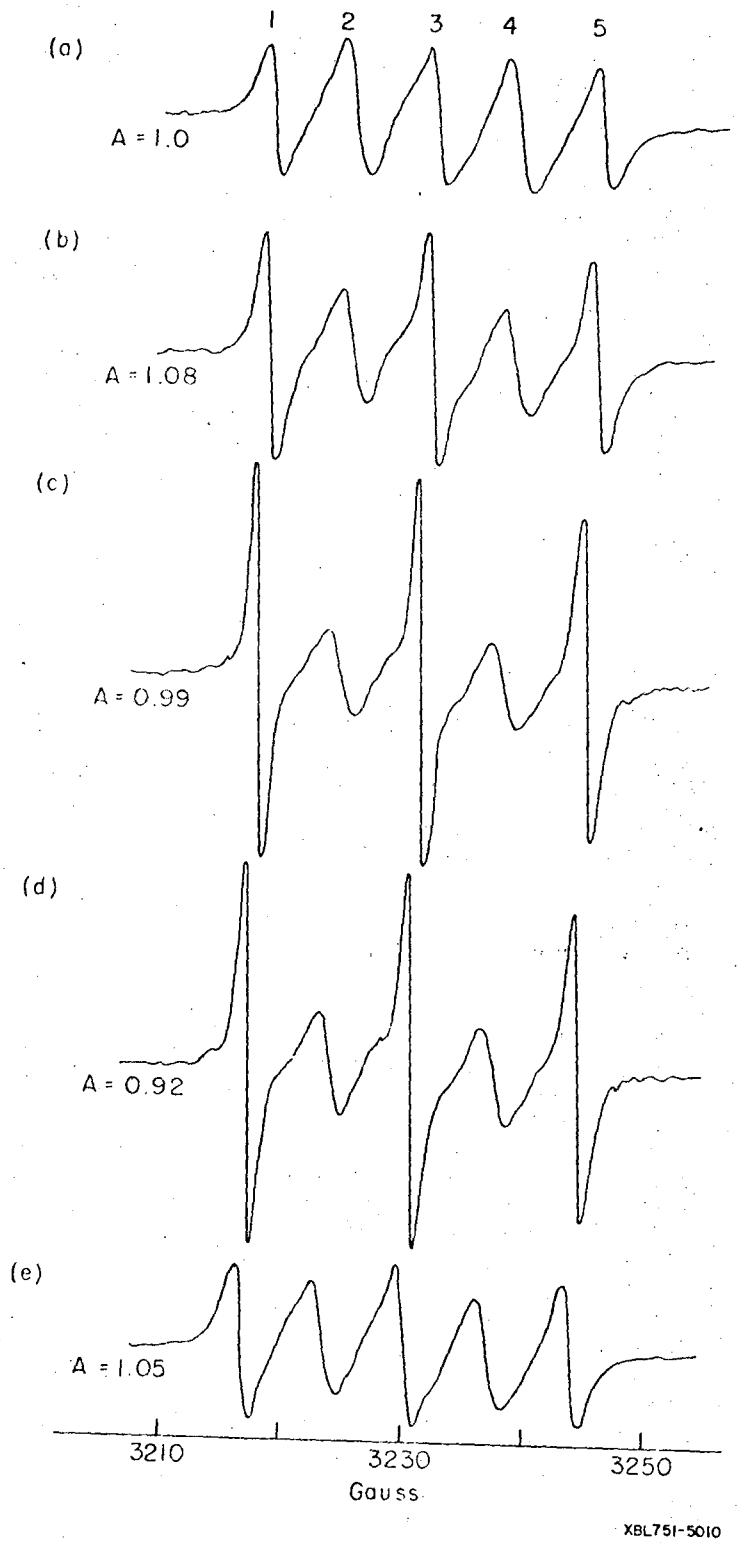
1
2
3
4
5
(a)
A = 1.0
(b)
A = 1.08
(c)
A = 0.99
(d)
A = 0.92
(e)
A = 1.05
3210
3230
3250
Gauss
XBL751-5010

Fig. 1.

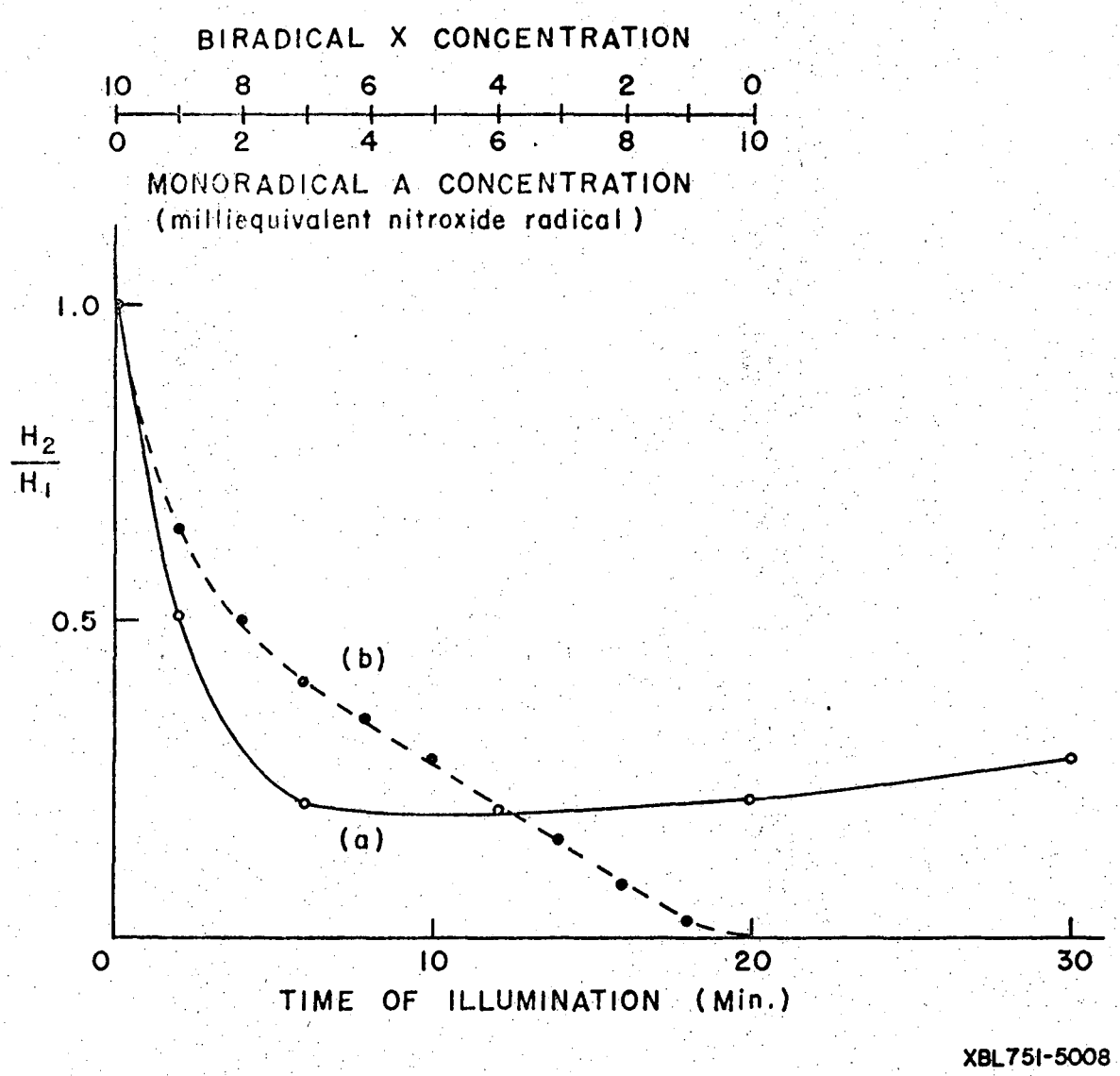

Fig. 2.

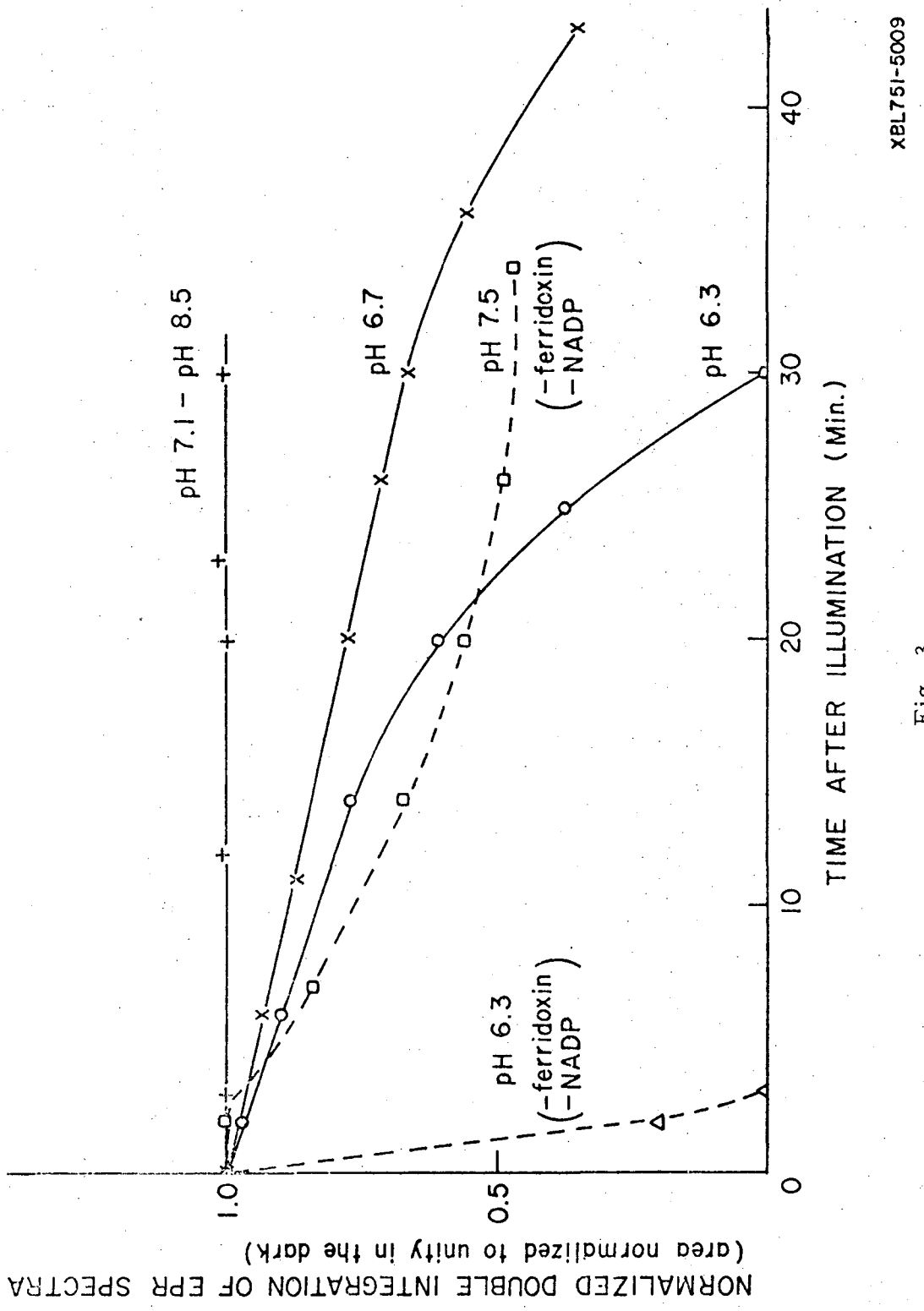
NORMALIZED DOUBLE INTEGRATION OF EPR SPECTRA
(area normalized to unity in the dark)
1.0
0.5
0
10
20
30
40
TIME AFTER ILLUMINATION (Min.)
pH 7.1 – pH 8.5
pH 6.7
pH 7.5
(-ferridoxin
-NADP)
pH 6.3
pH 6.3
(-ferridoxin
-NADP)
XBL751-5009

Fig. 3.

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