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Electron Spin Resonance Detection of Singlet Oxygen Produced in Irradiated Acriflavine Solutions

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When a solution of acriflavine was irradiated, singlet oxygen was detected by electron spin resonance (ESR). When a 2,2,6,6-tetramethyl-4-piperidone solution (TEMP) was irradiated in the presence of acriflavine (AF) or hematoporphyrin (HP), a signal was detected and identified as being due to produced 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl (TEMPO) from a comparison of the *g* values and the hyperfine splitting constants with the nitrogen nuclear spin with those of an authentic sample. HP produced TEMPO more efficiently than AF at the same concentration (10^{-4} M). Prolonged irradiation brought about rapid destruction of TEMPO as indicated by the disappearance of the TEMPO signal. This phenomenon may be due to the quenching of nitroxide radical by the decomposition product of TEMP. However, TEMPO itself was very stable in the presence or absence of AF even under such drastic conditions as irradiating the sample with ultraviolet (UV) light at a distance of 0.5 cm for 60 min.

Maximum TEMPO production was obtained when the TEMP solution was irradiated by the UV lamp for 25 min from 3 cm distance in the presence of 10^{-5} M AF.

Keywords——singlet oxygen; ESR; acriflavine; photo-sensitization; nitroxide radical; hematoporphyrin; tungsten lamp; UV lamp

Acridine dyes have been demonstrated to be active photo-sensitizers in a variety of organisms.[1] Acridine orange (AO),[2] proflavine (PF),[2b,c] acriflavine (AF)[2c] and acridine yellow (AY)[2b] induce cell inactivation and gene conversion in yeast, *Saccharomyces cerevisiae*. We found that some acridines[3] induce cytoplasmic respiration-deficient mutants ("rho$^-$" or "petite") in addition to causing cell inactivation and nuclear mutagenesis by a photodynamic action. These photo-biological effects of acridines were inhibited by the presence of sodium azide[1a,2c,d,3] or the absence of molecular oxygen[3] and were enhanced in the presence of deuterium oxide.[2c,d,3a] These results suggested that the photo-biological effects of acridines are driven by singlet oxygen-dependent type II photodynamic action.

We tried to demonstrate that singlet oxygen was produced by the photo-sensitization of AF *in vitro*. Lion and coworkers[4] have previously shown that sterically hindered amines can be converted into nitroxide radicals by reaction with singlet oxygen. Moan and Wold[5] improved this method and reported that 2,2,6,6-tetramethyl-4-piperidone (TEMP) was the best compound for this purpose. 2,2,6,6-Tetramethyl-4-piperidone-*N*-oxyl (TEMPO) generated from TEMP and singlet oxygen is a stable nitroxide radical detectable by electron spin resonance (ESR) spectrometry. Using this method, we could demonstrate that the singlet oxygen was produced by the photo-sensitization of AF.

Experimental

Detection of Singlet Oxygen——Singlet oxygen was detected according to the method described by Moan and

Wold.[5]

Preparation of Reaction Mixture——All the solutions were prepared in glass using redistilled water. The reaction mixture contained 2×10^{-2} M TEMP and 10^{-5} M sensitizer in 1/15 M phosphate buffer, pH 8.0. Fifty microliters of the air-saturated reaction mixture was taken in a glass pipet (Clay-Adams, Micropet 100 μl). Both ends of the pipet were heat-sealed carefully in order to avoid thermal decomposition of the contents.

Irradiation of Sample——Samples were irradiated with a fluorescent lamp (National 15 W), a tungsten lamp (Toshiba Photoreflector lamp 500 W) or a ultraviolet (UV) lamp (Toshiba SHL-100 UV-2 type 100 W). Irradiation time and distance between the sample and lamp were varied with each experiment.

Measurement of ESR Spectra——Spectra were recorded on a JEOL JES-3BS X spectrometer (X band) with 100 kHz field modulation (0.8 gauss). When the line shape is Lorentzian, the product of the peak-to-peak height and the square of the peak-to-peak width of the signal is proportional to the radical concentration. The widths of the spectra obtained here were same. Therefore the mean $\pm$ S.D. (mm) values of the three peak-to-peak heights were calculated for each signal and used as a measure of the relative concentration. Irradiation and ESR spectral measurements were performed at room temperature.

Materials——Acriflavine and 2,2,6,6-tetramethyl-4-piperidone were purchased from Aldrich, and hematoporphyrin from Wako. 2,2,6,6-Tetramethyl-4-piperidone-*N*-oxyl was synthesized as described elsewhere.[6]

Results and Discussion

Detection of Singlet Oxygen Production

In order to detect singlet oxygen, an aqueous solution of TEMP was irradiated with two 15 W fluorescent lamps for 60 min from 5 cm distance in the presence of 10^{-4} M AF or HP. Small signals attributable to nitroxide radical were detected by ESR in the HP photosensitized sample but not in the AF sample. To obtain more distinct signals, the samples were irradiated with a 500 W tungsten lamp for 15 min from 10 cm distance. As shown in Fig. 1, a small but clear triplet signal was obtained from the AF sample and a large signal was observed for the HP sample. No ESR signal was detected for the irradiated controls which contained no

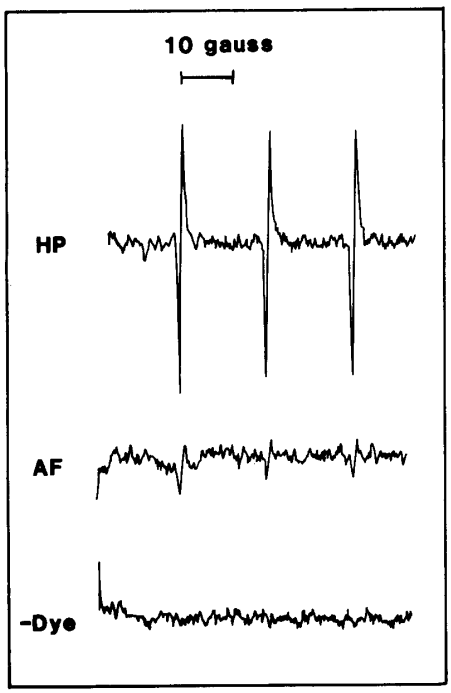

Fig. 1. ESR Spectra of Nitroxide Radical Produced by Photo-Sensitization by Acriflavine or Hematoporphyrin

Samples containing 2×10^{-2} M TEMP and 10^{-4} M sensitizer in 1/15 M phosphate buffer (pH 8.0) were irradiated with a tungsten lamp (Toshiba Photoreflector lamp 500 W) for 15 min from 10 cm.

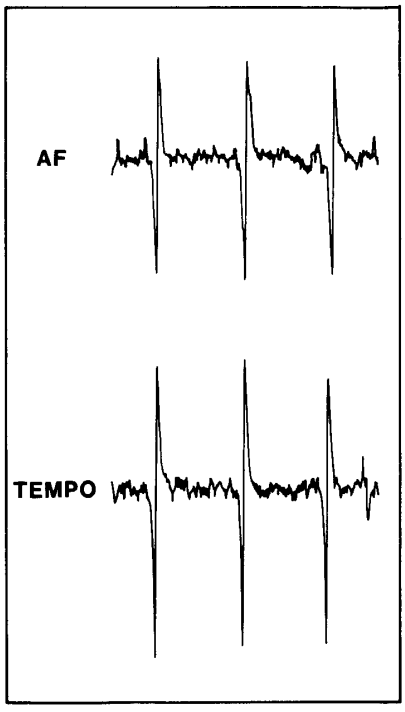

Fig. 2. ESR Spectra of Nitroxide Radical Produced by Photo-Sensitization by AF, and of Authentic TEMPO

The AF sample was irradiated with a UV lamp (Toshiba SHL-100 UV-2 type 100 W) for 18 min from 3 cm. TEMPO was dissolved in 1/15 M phosphate buffer (pH 8.0) at the concentration of 1.4×10^{-6} M.

Table I. ESR Parameters for the Photo-Produced Radicals and for Authentic TEMPO

	g value	a_N (gauss)
Photo-produced radical by AF sensitization	2.0061	16.12 ± 0.05
Authentic TEMPO	2.0061	16.09 ± 0.10
TAN[a] (TEMPO)	2.0068[a]	16.10 ± 0.10[a]

a) J. Moan and E. Wold, *Nature* (London), **279**, 450 (1979).

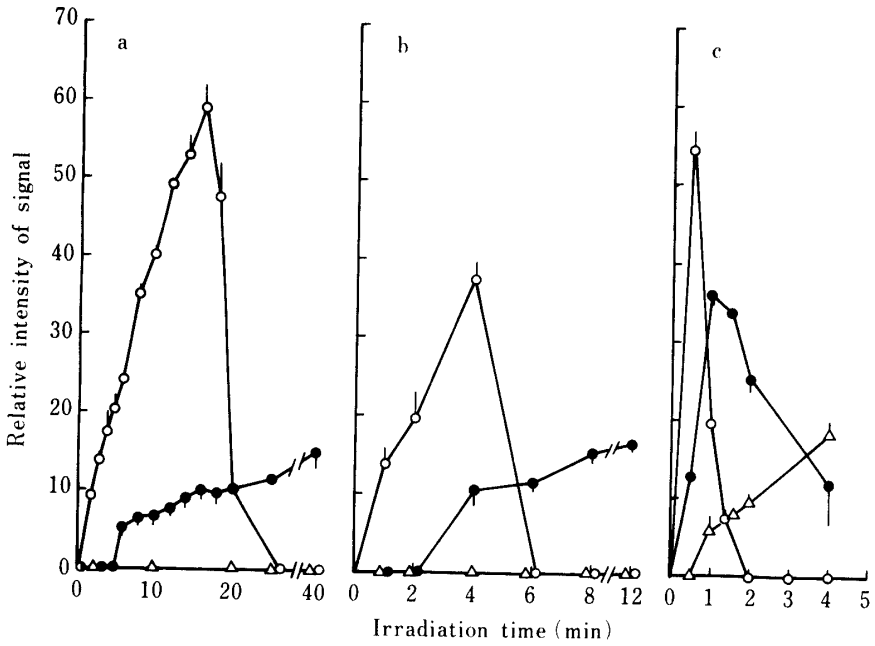

Fig. 3. Time Course of TEMPO Production by AF or HP Photo-Sensitization

Samples containing 2×10^{-2} M TEMP and 10^{-4} M sensitizer in 1/15 M phosphate buffer (pH 8.0) were irradiated with a tungsten lamp from 0.5 cm (a) or a UV lamp from 5 cm (b) or 0.5 cm (c). The ESR results are expressed as the mean $\pm$ S.D. (mm) of the peak-to-peak height: ○—○, HP; ●—●, AF; △—△, no sensitizer.

dye or in the un-irradiated samples containing AF or HP. Since the signal obtained for AF was too small to characterize, a higher energy irradiation source was tested. As shown in Fig. 2, when a UV source was used, a larger signal was obtained for AF photo-sensitization. This signal was quite similar to that of authentic TEMPO. The g values and the hyperfine splitting constants (a_N) were identical (Table I). These results suggested that a sensitizer such as AF or HP activates $^3O_2$ to $^1O_2$ using light energy and finally produces TEMPO from TEMP.

Time Course of TEMPO Radical Production

TEMPO was effectively generated by irradiation with a tungsten lamp when HP was used as a sensitizer. The signal increased sharply in the first 15 min of irradiation (Fig. 3a), but decreased suddenly after 15 min. In the AF photo-sensitization, the generation of TEMPO was delayed. The amplitude of the signal was increased with the irradiation time, then reached a plateau and did not diminish even after 40 min of irradiation. The yield of TEMPO was much higher in the HP photo-sensitized sample than in the AF photo-sensitized sample. UV irradiation from 5 cm decreased the signal produced by HP but did not alter the signal produced by AF except for shortening the time scale (Fig. 3b). However, irradiation from 0.5 cm distance brought about a more rapid production and destruction of nitroxide radical in

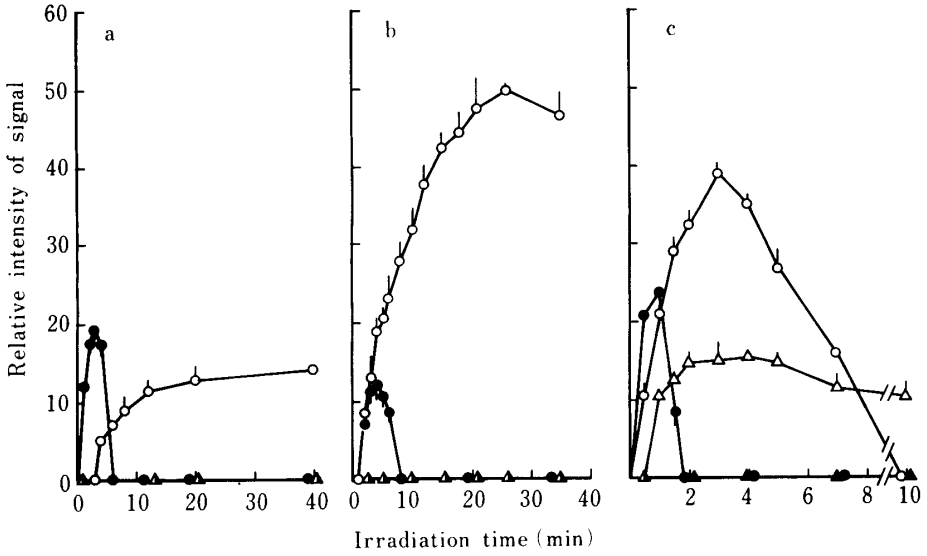

Fig. 4. TEMPO Production at Various Concentrations of AF

Samples containing various concentrations of AF were irradiated with a tungsten lamp from 0.5 cm (a) or a UV lamp from 3 cm (b) or 0.5 cm (c): △—△, no AF; ▲—▲, AF 10^{-3} M; ●—●, AF 10^{-4} M; ○—○, AF 10^{-5} M.

both the AF and HP samples. Under these extreme conditions where the sample was irradiated by intense UV light, the samples boiled within the capillary tube. Furthermore TEMPO was generated in the control sample containing only TEMP and no sensitizer.

TEMPO Production at Various Concentrations of AF

The concentration of AF may be an important parameter in the generation of the nitroxide radical. Thus, the time course of TEMPO production was compared at various concentrations of AF. As shown in Fig. 4, there was no TEMPO production with 10^{-3} M AF. At 10^{-4} M AF, the signal of TEMPO was produced at short irradiation times under all irradiation conditions used here. The maximum yield of TEMPO which was stable for up to 40 min of irradiation was obtained using a UV source at 3 cm distance with 10^{-5} M AF. Irradiation in the presence of 10^{-5} M AF from 0.5 cm with a UV source brought about the destruction of TEMPO within 10 min.

Stability of TEMPO on Irradiation

The stability of TEMPO was examined to determine whether or not TEMPO is destroyed during prolonged irradiation. TEMPO itself was very stable even on UV irradiation from 0.5 cm for 60 min. The addition of 10^{-5} M or 10^{-4} M AF did not influence its stability. However, when TEMP was also present, the TEMPO signal was eliminated within 12 min (data not shown). These results suggest that either the presence of TEMP with AF and/or TEMPO caused the decrease in the TEMPO radical signal.

Many chemical[7] and physical[8] methods for detecting singlet oxygen have been reported. Some of them are not specific for singlet oxygen and some are of low sensitivity. However, the ESR method used here is thought to be specific and convenient, since it can detect less than 10^{-7} M nitroxide radical. However, the signal for the TEMPO radical produced in this photochemical reaction was eliminated in the presence of TEMP and AF. The mechanism underlying this process is stable under the most drastic conditions.

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