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A PHOTOCHEMICAL SYSTEM FOR GENERATING FREE RADICALS: SUPEROXIDE, PHENOXYL, FERRYL AND METHYL

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The photosensitizer flavin mononucleotide (FMN), in conjunction with the reducing agents diethylenetriaminepentaacetic acid (DTPA), hydrazine and hydroxylamines derived from nitroxides, generates superoxide radicals in a strictly light-dependent reaction in aerobic solution. Addition of superoxide dismutase (SOD) converts this system to a hydrogen peroxide generator. In the presence of horseradish peroxidase the latter system becomes a phenoxyl radical generator with appropriate phenolic substrates. Under anaerobic conditions FMN, hydrogen peroxide and an iron chelate generate ferryl and when this system is combined with dimethylsulfoxide, methyl radicals are produced. All the radicals can be generated with little contamination from other radicals, in high yields and the reaction can be terminated immediately upon cessation of illumination. Useful applications of this photochemical system include ESR studies of transient free radical species.

KEY WORDS: Superoxide, ferryl, hydroxyl, phenoxyl, methyl, FMN.

INTRODUCTION

Researchers have relied heavily upon model systems to elucidate the mechanisms responsible for biological free radical damage. These have included a variety of approaches for producing known oxidants, e.g., superoxide has been generated enzymically by xanthine oxidase,[1] singlet oxygen has been produced chemically and photochemically,[2,3] and hydroxyl (or ferryl) has been generated by Fenton reagents.[4] Many of these systems are unreliable or cumbersome. For example, the substrates for xanthine oxidase are poorly water soluble and useful concentrations cannot readily be achieved in biological preparations by dilution of aqueous stock solutions. Fenton reagents, often utilizing aliquots of hydrogen peroxide, usually produce a burst of "hydroxyl" radicals, and quantitation of these is difficult, and often cannot yield reproducible data. Another problem with chemical or enzymic methods is that free radical production occurs as soon as reaction components are mixed, often cannot readily be terminated or may be transient, which frequently produces "all-or-none" effects. Quantitative radical production can also be achieved through water radiolysis, but the many inconveniences of using a radiation source and its limited availability make this system far from ideal. Many of these problems can be overcome with a

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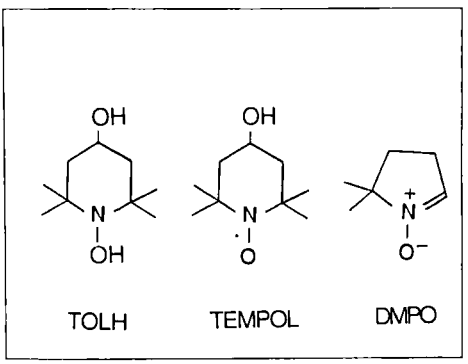
photochemical system, since illumination can easily be controlled. A negative aspect of working with light is the abundance of pigments in most biological preparations, indeed, photodamage is a familiar phenomenon, which spans the ultraviolet and visible (blue) wavelength range.[5,6] These limitations can be minimized by choosing flavin photosensitizers, utilizing only wavelengths absorbed by them and working with high concentrations of both flavins and photoreductants, so that intrinsic photochemcial reactions of the system under investigation are negligible.

Photoexcited flavins, e.g., FMN, are known to become efficiently reduced by a variety of organic molecules. Previously this fact was exploited to study the photochemical reduction of nitroxides and to demonstrate that free flavins, upon being released from their enzyme binding sites are potent catalysts of light-dependent biological damage.[7] Photoexcited flavins in the presence of hyrogen donors have been used to induce cell damage and the damage is considered to result from superoxide production followed by dismutation and H_2O_2 generation.[8] However, a careful study of optimum conditions for superoxide production and of alternative radical species that could be generated by this system has not been performed to our knowlege. We have now demonstrated that the continuous generation of reduced flavins can be used as a powerful tool in the generation of many radical species.

MATERIALS AND METHODS

Spin Probes

Probe structures. Tempol (2,2,6,6-tetramethyl-4-hydroxypiperidin-1-oxyl) and the nitrone spin trap 5,5-dimethyl-l-pyrroline-N-oxide (DMPO) were purchased from Sigma Chemical Company. The latter was redistilled under reduced pressure, maintaining a temperature of less than 50°C. 2,2,6,6-tetramethyl-4-hydroxypiperidin-1-ol (TOLH) was synthesized by reduction of 0.5 M Tempol with 0.6 mole equivalents of isoascorbic acid (reaction for 2 min at room temperature), removal of solids by filtration, adjustment of the pH to 7.4, extraction of the TOLH into ice-cold diethyl ether, and drying over anhydrous magnesium sulfate, followed by solvent removal under reduced pressure. The concentration of the product was determined by ESR after oxidation with ferricyanide.



ESR spectroscopy. First derivative ESR spectra (100 kHz modulation) were recorded at ambient temperature (22°C) on a IBM ESR spectrometer, model # ER 200D-SRC, equipped with an IBM-AT and a Data Translation DT2800 series data acquisition board. Spectral acquisition and analysis programs were developed using ASYST, a FORTH based compiler and subroutine library package. Instrument settings: modulation amplitude 1.25 G, microwave power 10 mW, gain of 4.0×10^5 unless otherwise specified. Experiments were carried out with the probe-containing samples (50 μl) placed either in 75 μl glass capillaries or, where indicated, in gas-permeable tubing, 0.032 in. i.d 0.005-in. wall thickness (Zeus Industrial Products, Inc, Raritan, NJ). In the latter case a N_2 gas flow at a rate of 1.2 liter. min^{-1} was maintained over the samples if anaerobic conditions were used. For monitoring probe oxidation and/or destruction rates the line height of the mid-field peak of the nitroxide ESR signal was followed. For monitoring the rate of formation of DMPO-OH adducts the rate of increase of the peak immediately left to the center of the magnetic field was followed. Rates of DMPO-OH adduct formation were converted into quantitative rates (mM/minute) by reference to a TEMPOL standard as follows: double integration of a TEMPOL spectrum of known concentration was compared with the double integratal of a DMPO-OH adduct spectrum and this information was used to calculate a calibration factor for converting DMPO-OH lines heights to concentrations. All values shown for reduction or oxidation rates are the average of 2 or 3 experiments. Variability between any two experimental results did not exceed 10%.

Light source. Samples were illuminated within the ESR cavity with a 1000 W Universal Xenon Arc lamp, Model 6140 (Oriel Corp, CT). An interference filter (445 nm maximal transmission, band width 50 nm at half intensity) was placed in front of the cavity of the ESR instrument, which gave a light intensity at the sample of approximately 1.12 mW/cm^2.

Polarographic measurements. Polarographic measurements were performed with a Rank Brothers LTD Oxygen polarograph Model 10.

Chemicals. Chemicals and enzymes were purchased from Sigma Chemical Company, with the exception of catalase which was purchased from Boehringer Mannhein, West Germany.

RESULTS

Flavin Photoreduction

Flavin photoreduction involves the reactions:

$$\text{FMN} \rightarrow \text{FMN}^* \text{ (excited flavin)} \quad (1)$$

$$\text{FMN}^* + \text{Photoreductant} \rightarrow \text{FMNH}_2 + \text{Products} \quad (2)$$

Several known reductants for photoexcited flavins were compared in terms of rates of reduced nitroxide oxidation. TOLH and hydrazine were found to be the most efficient photoreductants (Table 1). The method used for the determination of reductant efficiency was an indirect method based on competition experiments, which exploited the fact that the oxidized form of TOLH (TEMPOL) can be directly

TABLE 1
Efficiency of various photoreductants as compared to TOLH

Photoreductant	Efficiency*
Hydrazine	0.25
EDTA	0.17
DTPA	0.17
Ascorbate	0.06
TOLH	1.0

*Photoreductant efficiency was estimated with competition experiments between TOLH and the photoreductants. Solutions containing 100 μM FMN, 500 μM TOLH and an equimolar amount of photoreductant were illuminated and the rate of TOLH oxidation measured by ESR. Comparison of the rates of TOLH oxidation in the presence and absence of photoreductants allows the determination of the % FMN reaction with the photoreductant and therefore photoreductant efficiency.

detected by ESR. Because of the absence of alternative reactants, the initial rate of anaerobic TOLH oxidation of saturating TOLH concentration is expected to correspond with the rate of reduction of the excited flavin to its one-electron reduction product. In principle, it would be possible to measure the formation of reduced flavins under identical conditions, and this could serve as a strategy to obtain absolute rate constants for both steps of the flavin reduction by TOLH, as well as the flavin radical dismutation rates. However, the complexity of these processes precluded such an analysis at this time and only relative efficiencies have therefore been provided. It must also be considered that reaction products of the photoreductant will accumulate, but this was judged not to exert a significant effect on the TOLH oxidation rates because[1] these rates were linear for prolonged periods (even when many equivalents of TOLH were oxidized relative to the FMN concentration), and[2] initial TOLH oxidation rates were not affected by TEMPOL, up to the concentrations that would have been formed at the end of the measurement (data not shown).

EDTA (ethylenediaminetetraacetic acid) was not suitable for use as a photoreductant specifically because of its high affinity for transition metal ions, coupled with facile redox cycling, which introduced complications from secondary (e.g., Fenton-type) free radical reactions. Even with considerable care to remove transition

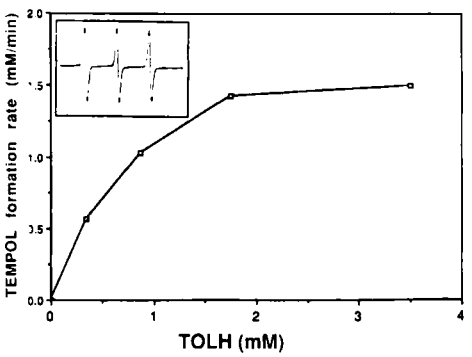


FIGURE 1 FMN photoreduction by TOLH. The reaction mixture contained 100 μM FMN and varying concentrations of TOLH. Rates are the initial TOLH oxidation rates upon illumination, measured by ESR spectroscopy as described under Materials and Methods. Insert: Spectrum of Tempol – midline peak height was used for rate data.

metal ions from buffers this was reflected in a high rate oxidation of TOLH in the dark by millimolar solutions of EDTA in chelexed phosphate buffer (data not shown). At a given concentration of FMN, reaction 2 is dependent on the photoreductant concentration, until saturation is reached. For example, at 100 μM FMN near-saturation rates are attained with 3.5 mM TOLH as a photoreductant (Figure 1). All subsequent experiments, which used 100 μM FMN or less were done with saturating concentrations of the photoreductants.

Superoxide and Hydrogen Peroxide Generation

When an aerobic system containing flavins and photoreductants is illuminated the reduced flavins ($FMNH_2$) reduce oxygen in one-electron steps producing superoxide:

$$FMNH_2 + O_2 \rightarrow FMNH^{\cdot} + O_2^{\cdot -} + H^+ \quad (3)$$

$$FMNH^{\cdot} + O_2 \rightarrow FMN + O_2^{\cdot -} + H^+ \quad (4)$$

Adding a nitroxide ESR probe to this system results in nitroxide reduction with biphasic kinetics (Figure 2). The slow initial rates can be explained by a competition between the nitroxide and O_2 for $FMNH_2$. When the oxygen is depleted through superoxide generation the solution becomes anaerobic and the rate of nitroxide reduction becomes maximal. In fact, the same maximal reduction rates can be obtained by removing the oxygen in the sample by flusing it with nitrogen in gas permeable tubing (data not shown). Therefore the time elapsed before the fast nitroxide reduction rate starts can be used to estimate the oxygen consumption rates (Figure 3). The rates of oxygen consumption are in agreement with the rates of reduced flavin formation under saturating conditions of photoreductant (Figure 4) indicating that reactions 3 and 4 (and therefore oxygen availability) are rate limiting for the superoxide generating system.

It has been demonstrated that superoxide forms a complex with nitroxides[9] which will result in probe reduction only in the presence of thiols. We have confirmed that

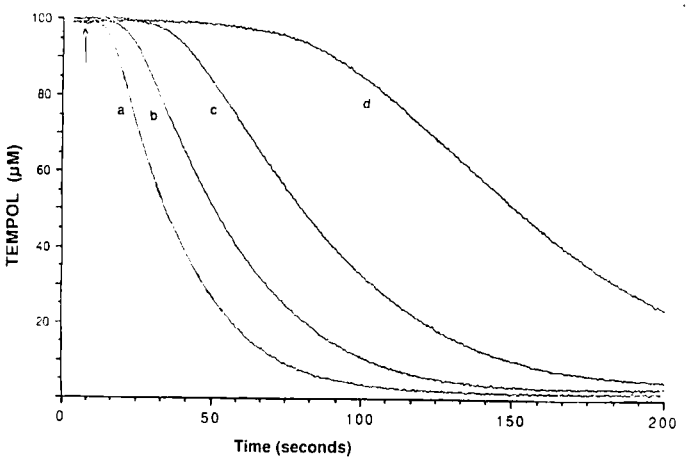


FIGURE 2 Photochemical reduction of nitroxides. Illumination was started at the arrow. Sample containing 20 mM DTPA was placed in a 75 μl glass capillary tube as described under Materials and Methods. FMN: a) 100 μM, b) 50 μM, c) 25 μM and d) 10 μM.

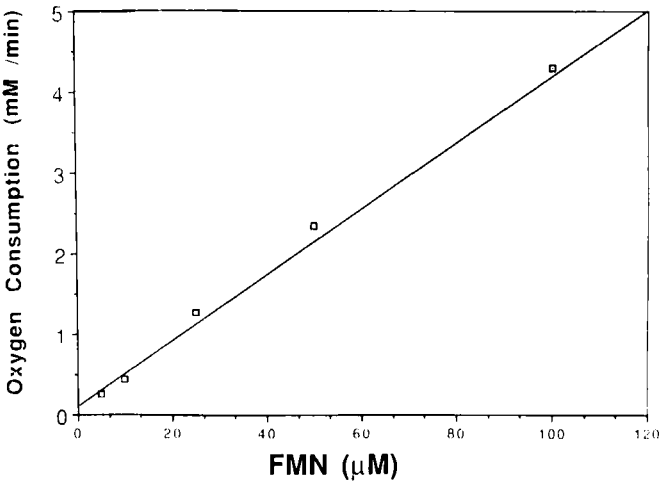


FIGURE 3 Oxygen consumption rates for superoxide generating system. Rates were estimated assuming linearity of oxygen consumption and using the time elapsed before the onset of the rapid phase of nitroxide reduction (see Figure 2).

our superoxide generating system results in TEMPOL reduction in the presence of GSH (Figure 5). Addition of SOD to the reaction mixture results in a 30 fold decrease in the rate of probe signal loss. The remaining rate is that of direct probe signal loss. The remaining rate is that of direct probe reduction by $FMNH_2$. Therefore we conclude that the rates of oxygen consumption in Figure 3 are rates of superoxide production. The production of superoxide can also be inferred by the accumulation of H_2O_2, through the reaction:

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (5)$$

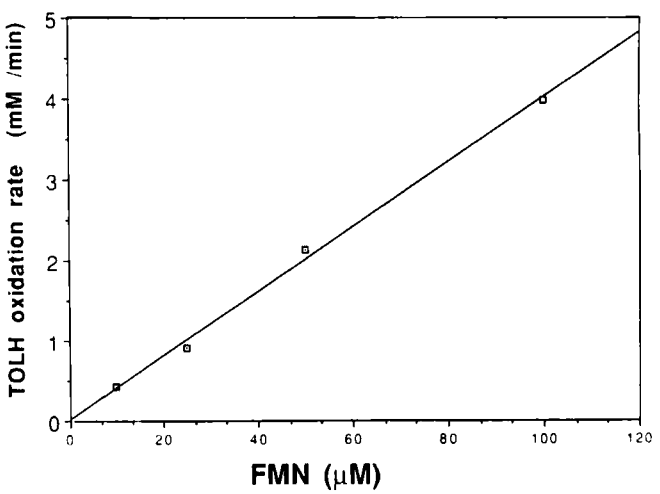


FIGURE 4 Initial TOLH oxidation rates vs. FMN concentrations. Reaction mixture: 0.14 M NaPi buffer, pH 7.4, 3.5 mM TOLH, FMN as indicated.

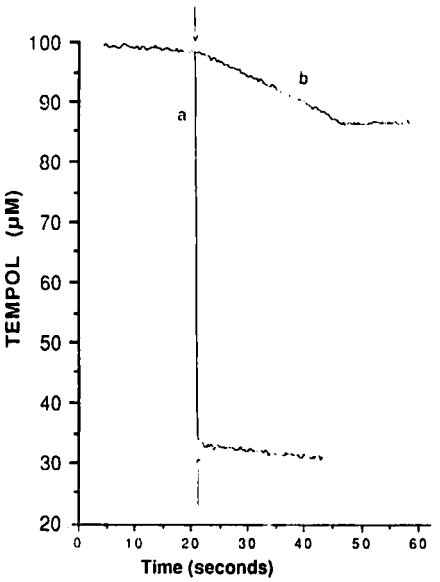


FIGURE 5 Superoxide- and glutathione-dependent reduction of TEMPOL. Illumination was started and terminated at the arrows. a) Control: 20 mM DTPA, 3.5 mM glutathione, 100 μM FMN, 100 μM Tempol, 0.14 M NaPi, pH 7.4, b) Control plus 150 IU/ml SOD.

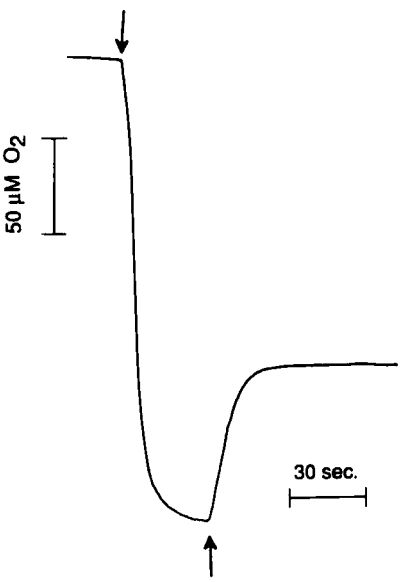


FIGURE 6 Photoproduction of hydrogen peroxide. Reaction mixture contained 20 mM DTPA, 20 μM FMN. Illumination started at downward arrow and ended at upward arrow, when catalase was added to a final concentration of 1750 U/ml.

Polarographic measurements were used to measure oxygen consumption with the photochemical superoxide generating system. After all the oxygen is consumed addition of catalase which catalizes the reaction

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

results in oxygen evolution to approximately half of the initial value as would be expected from the reaction stoichiometry (Figure 6).

Generator of Hydrogen Peroxide and Phenoxyl Radicals

Horseradish peroxidase catalyzes the reaction:

$$\text{phenol} + H_2O_2 \rightarrow 2 \text{ phenoxyl radicals}$$

When the superoxide generating system described above was treated with SOD, phenol and horseradish peroxidase oxidation of TOLH resulting in TEMPOL formation was strongly stimulated (Figure 7). The observed rate was two times higher than that observed in the presence of catalase. In the presence of catalase the production of phenoxyl radicals by this system is inhibited and the remaining TOLH oxidation rate is that of FMN photoreduction.

Ferryl ("Hydroxyl") Radical Generation

Under anaerobic conditions the rapid reaction of photochemically reduced flavin with oxygen does not occur and other reductions can be carried out. Addition of Fe^{3+}-

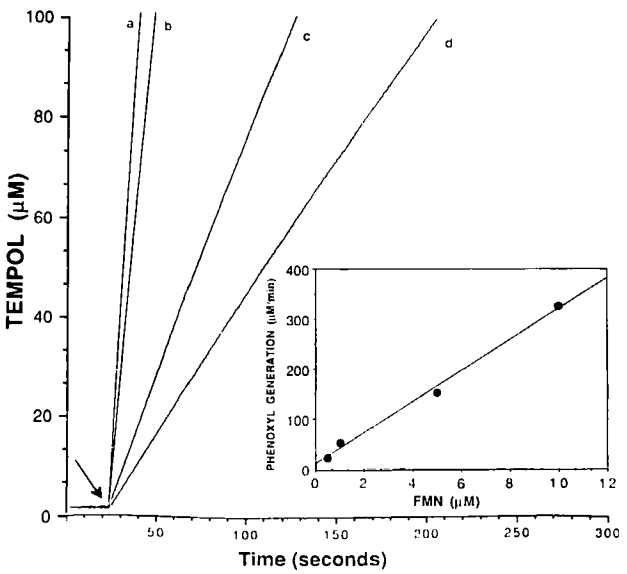


FIGURE 7 Photochemical generation of phenoxyl radicals. Illumination started at the arrow. Reaction mixtures were assayed in gas-permeable tubing and contained 100 IU/ml horseradish peroxidase, 400 μM phenol, 3.5 mM TOLH and the following amounts of FMN: a) 10 μM, b) 5 μM, c) 1.0 μM and d) 0.5 μM. Phenoxyl radical generation rates were calculated from the difference of the rates of TEMPOL formation in the absence and presence of catalase (1750 U/ml).

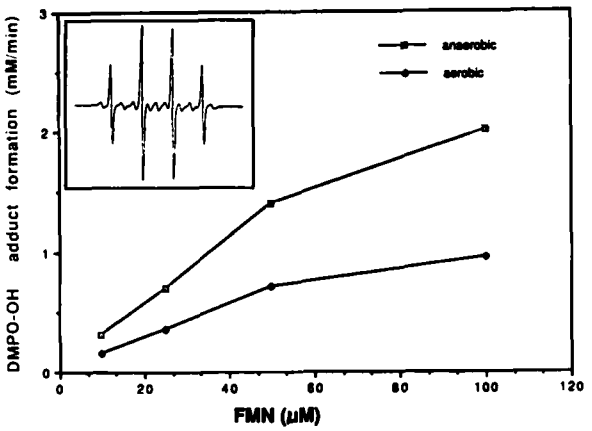


FIGURE 8 Photochemical ferryl radical generation. Reaction mixtures contained 200 mM DMPO, 2 mM DTPA, 100 μM Fe-EDTA and FMN as shown. Samples were placed in gas-permeable tubing under a stream of N_2 for at least 8 min to render them anaerobic prior to illumination. Rates were obtained by measuring the rate of DMPO-OH adduct formation as described under Materials and Methods. The DMPO-OH adduct spectrum shown (Insert) was obtained with 10 mM H_2, 2 mM hydrazine, 10 μM Fe-EDTA, 50 μM FMN and 100 mM DMPO.

EDTA generates the ferrous form of this chelate, which can be used, in conjunction with H_2O_2 to produce strongly oxidizing radicals, with properties similar to those of hydroxyl radicals and referred to here as ferryl radicals:

$$FE^{3+}\text{-EDTA} + FMNH_2 \rightarrow Fe^{2+}\text{-EDTA} + FMNH^{\cdot}$$

$$Fe^{2+}\text{-EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{-EDTA} + OH^{\cdot} + OH^{-}$$

In the presence of the spin trap DMPO, substantial $OH^{\cdot}$ adduct signals are observed. Under conditions of high DMPO concentrations the rate of adduct formation can be used to estimate the rates of ferryl generation (Figure 8). The rates are significantly decreased under aerobic conditions. Under such conditions the reduced flavin can react with either O^2 or Fe^{3+}-EDTA. Therefore superoxide and ferryl are simultaneously produced under aerobic conditions. Rates of ferryl generation are dependent on Fe-EDTA concentrations. With 100 μM FMN, saturating concentrations of photoreductants and H_2O_2 the maximum velocity for ferryl production is obtained at Fe-EDTA levels of about 100 μM.

It has been suggested that formation of hydroxyl radicals in a H_2O_2/Fe-EDTA reaction mixture can arise only via reaction of hydrogen peroxide with ferryl radicals.[10] Since the ferryl/hydroxyl radical generating system we have described contains H_2O_2, we cannot ascertain if the observed DMPO–OH adducts are due to ferryl, hydroxyl or both radicals. However, it may be possible in future experiments to resolve the two possibilities because ferryl and hydroxyl radicals differ in their reactivity with many organic compounds.[10] This differential reactivity will also affect the reactions of the photochemical ferryl/hydroxyl-generating system we have described, depending on the type of organic compounds present in the reaction mixture used.

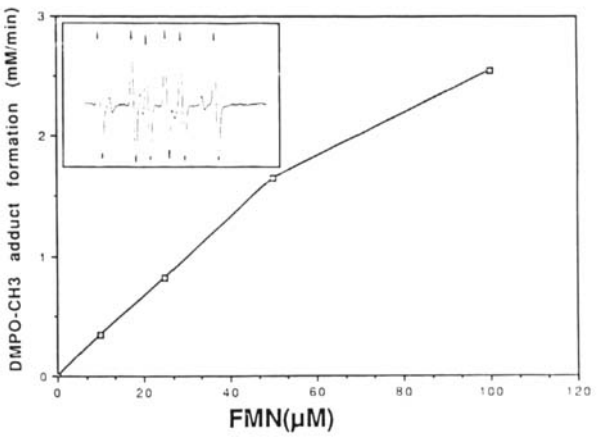


FIGURE 9 Photochemical methyl radical production. Reaction mixtures contained 1 mM TEMPOL, 0.6 M DMSO, 2 mM DTPA, 100 μM Fe-EDTA and FMN as indicated. Samples were placed in a gas permeable tubing under a stream of N_2 as described under Materials and Methods. Rates refer to TEMPOL destruction. The DMPO-CH_3 adduct spectrum shown (Insert) was obtained after a 10 min illumination of 0.6 M DMSO, 100 mM DMPO, 10 mM H_2O_2, 100 μM FMN, 2 mM DTPA and 100 μM Fe-EDTA.

Methyl Radical Production

When DMSO is added to the ferryl radical generating system methyl radicals are produced through the reaction:

$$CH_3(S=O)CH_3 + OH^{\cdot} \rightarrow CH_3^{\cdot} + CH_3(S=O)OH$$

The methyl radicals can be detected by the formation of DMPO–CH_3 adducts (Figure 9). We had previously demonstrated that methyl radicals destroy nitoxide probes.[11] We have used the rates of nitroxide probe signals loss to quantitate methyl radical production in this system under anaerobic conditions. Under saturating concentrations of DMSO the observed rates are similar to those for ferryl radical production (Figure 9).

DISCUSSION

We have developed and characterized the kinetics of a photochemical system for generating a variety of radicals, using the blue-light sensitizer FMN. This system provides a high degree of control over both the initiation and termination of free radical reactions and should facilitate comparisons of free radical studies among different investigators who will utilize it in their research. The results demonstrate that superoxide, phenoxyl, ferryl and methyl radicals as well as hydrogen peroxide can be generated quantitatively and conveniently with readily available light sources. The free radical fluxes can be substantial — for example, all of the oxygen dissolved in aqueous solution can be converted to superoxide radicals in about one minute (Figure 3). FMN is charged and would not be expected to cross most biological or model membranes; hence this system can be employed as an extracellular free radical source

in investigations of free radical-mediated biological damage. A potential problem of using flavins as a source of specific free radicals is the fact that excited flavins readily react with oxygen forming singlet oxygen.[3] However, the use of saturating concentrations of photoreductants will minimize singlet oxygen production, since these efficiently compete with oxygen for the excited flavins. This expectation was confirmed by experiments measuring oxygen consumption rates under various photoreductant concentrations (data not shown). We observed that for low photoreductant concentrations oxygen consumption rates are slower initially, and increase as the oxygen is consumed. The data suggest singlet formation under high oxygen concentrations, which decays to the ground-state without reacting at low concentrations of organic molecules. At saturating concentrations of photoreductants the rates of oxygen consumption are linear, as would be expected if singlet production is insignificant. Reduced nitroxides are the most efficient photoreductants found among the compounds we screened and should be ideal for many applications of this system due to the lack of reactivity of the nitroxide radicals formed. However, special care should be taken with the use of nitroxides in biological systems, since they are known to be reduced, e.g., by the electron transport chain of mitochondria,[12] and this could exert effects independent of photooxidative damage. Dark controls containing the nitroxide should be used as to ensure that possible effects due to interference with electron transport systems or other biological processes are negligible compared to the photochemically generated free radical effects. Alternatively, membrane impermeable charged reduced nitroxides may be used as extracellular reductants for excited flavins.

Each of the free radical species we have described is likely to play a role in at least some biological oxidative damage processes, and many applications of the system can be envisioned. Carbon-centered radicals may be involved in the toxicity of hydrazines and organic peroxides.[13,14] phenoxyl radicals may be formed in biological tissues through the action of peroxidases[15] since benzene hydroxylation is involved in its toxicity. Studies to elucidate the membrane permeability and reactivity of various free radicals are feasible with this system and have been used to demonstrate that methyl radicals readily pass through bilayer membranes (manuscript in preparation). Phenoxyl radicals can be used as precursors to other radicals species, e.g. the tocopheroxyl radical, as we have recently demonstrated.[17] Perhaps one of the most significant applications of the photochemical system described here is the analysis of the potential of ferryl radical production by various iron complexing molecules. When coupled with DMSO-dependent methyl radical production and the resultant nitroxide destruction, the capacity of iron chelates to activate peroxides and to generate secondary organic free radical species can readily be quantitated.

Acknowledgement

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