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Hydrogen Peroxide and Hydroxyl Radical Formation by Methylene Blue in the Presence of Ascorbic Acid

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Summary. Using ESR we have demonstrated the formation of the ascorbate free radical from sodium ascorbate, methylene blue and light. In oxygen uptake experiments we have observed the production of hydrogen peroxide while spin trapping experiments have revealed the iron catalyzed production of the hydroxyl free radical in this system. The presence of this highly reactive radical suggests that it could be the radical that initiates free radical damage in this photodynamic system.

Introduction

It has been brought to light that ophthalmic drugs (eye drops) containing methylene blue (MB) are available, without medical prescription, in certain areas [4]. MB-mediated DNA damage was produced using two of these readily available products [4].

Methylene blue is a well-known singlet oxygen sensitizer [11, 23]. There is a great deal of evidence suggesting that the photosensitized production of singlet oxygen by MB can cause damage to biochemical [8] and biological systems [4, 13, 14, 20, 21, 36]. However, there is also evidence that photosensitizers can produce free radicals [2, 6, 24, 26, 37] and that in the presence of ascorbate, methylene blue photosensitizes the production of free radicals that can result in damage to biological systems [4, 8, 24]. Oxygen free radicals have been shown to mediate an intense uveal and retinal inflamation [35]. Here we report the formation of hydrogen peroxide and hydroxyl free radicals by methylene blue in the presence of ascorbate. These observations, in conjuction with the fact that the levels of vitamin C in the lens and aqueous humor of the eye are 1.0 ± 0.2 mM [38], suggest that the use of such ophthalmic products containing MB should be questioned.

Materials and Methods

Sodium ascorbate, catalase, superoxide dismutase, bovine serum albumin and methylene blue were products of Sigma Chemical Co., St. Louis, MO and were used without further purification. The spin trapping agent, DMPO (5,5-dimethylpyrroline-N-oxide), was a product of Aldrich Chemical Co., Milwaukee, WI. The DMPO was purified as in [6] and stored at 4° C as an aqueous solution. The concentration of the stock solution was determined using $\varepsilon_{232} = 7700$ $M^{-1}\,cm^{-1}$ in ethanol [16]. All solutions were prepared in 50 mM phosphate buffer, pH 7, and all concentration given below are final concentrations.

Electron spin resonance spectra were obtained with a Varian E-4 ESR spectrometer using the aqueous sample cell accessory. Oxygen uptake was monitored with a Yellow Springs Instrument Co., Inc. Model 53 Biological Oxygen Monitor. A slide projector equipped with a 3200 K tungsten bulb was employed as a white light source. The light incident on the sample was filtered through an Oriel IR blocking filter (#5205) and an Oriel long pass filter (#5149, 50% transmission cut at 480 nm). Using a Yellow Springs Instrument Model 65A radiometer and the model 6551 probe, the filtered light intensity was determined to be 115 Jm^{-2} for the oxygen uptake experiments and 175 Jm^{-2} for the electron spin resonance experiments, assuming the cavity grid transmits 50% of the incident light.

Results and Discussion

In a 1 mM aqueous sodium ascorbate solution (pH = 7.6, pKa of AH_2 = 4.2 [29]) there exists, in equilibrium with AH^- and A, an ESR detectable concentration of $A^{\cdot -}$ [10, 28]. (AH_2 = ascorbic acid, AH^- = ascorbate anion, A = dehydroascorbic, and $A^{\cdot -}$ = ascorbate free radical, pKa = −0.45 [28].)

$$AH^- + A \rightleftharpoons 2\,A^{\cdot -} + H^+ \tag{1}$$

The intensity of the $A^{\cdot -}$ ESR signal was not effected by illumination of this solution with filtered white light.

However, when MB was introduced at 9×10^{-5} the $A^{\cdot -}$ ESR signal increased by 400−500% without illumination in an air saturated solution. Illumination of this solution with a one second pulse of filtered white light resulted in an additional immediate increase of about 50% in the $A^{\cdot -}$ ESR signal intensity.

$$AH^- \xrightarrow{(\mathrm{MB} + h\nu)} A^{\cdot -}\,. \tag{2}$$

As seen in Fig. 1, continued illumination resulted in a decrease in signal intensity, and cessation of illumination resulted in a dramatic decrease in signal intensity. A visible bleaching of the MB color resulted with continued illumination of the solution, while cessation of illumination brought about a restoration of the MB color as observed in [8, 21, 26]. When the sample was

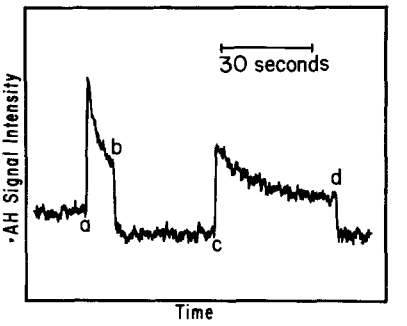

Fig. 1. ESR signal intensity for the ascorbate free radical when an air saturated sample of 9×10^{-5} M MB and 1 mM sodium ascorbate was illuminated with filtered light. The ESR spectrometer was set on the low field peak of the $A^{\cdot -}$ doublet, the modulation amplitude was 0.4 gauss and the time constant was 0.125 seconds. At points "a" and "c" the light was turned on and at points "b" and "d" the light was turned off. The initial rise observed corresponds to an increase of approximately 50% in the $A^{\cdot -}$ signal intensity

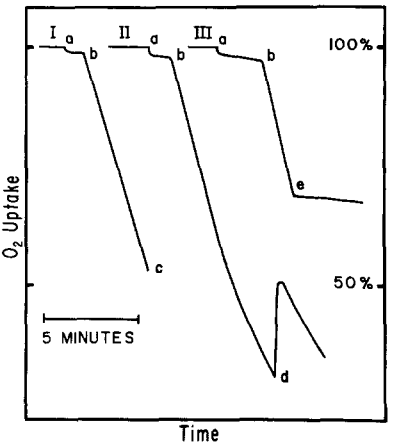

Fig. 2. Oxygen consumption of air saturated solutions of 9×10^{-5} M MB. On all three traces, "a" marks the point at which 1 mM sodium ascorbate was introduced, and "b" the point at which illumination of the samples commenced. Sample I contained in addition to MB, 150 units/ml of catalase at point "a". Trace II shows the change in slope and the return of oxygen to the solution when 150 units/ml of catalase is added at "d" rather than at time zero. Trace III demonstrates the effect on oxygen consumption when illumination was stopped at "e"

illuminated for sufficient time such that nearly all the oxygen was consumed, or when the solution was bubbled with nitrogen gas to remove oxygen prior to illumination, the initial $A^{\cdot -}$ concentration was much lower, but with illumination the increase in the $A^{\cdot -}$ signal intensity was still produced, and as with the oxygenated solution, this signal decreased in intensity with constant illumination. The addition of N_3^- at 20 mM (an efficient $^1O_2$ quencher) to the oxygenated solution produced no significant change in signal intensity with illumination.

When 1.25×10^{-6} M Fe(III)EDTA was added to a 1 mM ascorbate solution, a gradual increase in the $A^{\cdot -}$ signal was observed, in agreement with Halliwell's observation that ascorbate can reduce Fe(III)EDTA [15] and the observations of Winterbourn [39].

$$\text{Fe(III)EDTA} + AH^- \rightarrow \text{Fe(II)EDTA} + A^{\cdot -} + H^+ . \quad (3)$$

In the absence of light, oxygen was slowly consumed by an aqueous solution of 9×10^{-5} M MB and 1 mM sodium ascorbate. As can be seen in Fig. 2, the rate of consumption increased dramatically upon illumination. Addition of 150 units/ml

of catalase to the illuminated solution resulted in the return of 25–30% of the O_2 that had been consumed up to that point.

$$2\ H_2O_2 \xrightarrow{\text{CATALASE}} 2\ H_2O + O_2\ . \tag{4}$$

Thus, the return of oxygen to the system with the introduction of catalase indicates the presence of H_2O_2 in the solution. The return of 25–30% of the oxygen suggests that 50–60% of the oxygen consumed had been converted to H_2O_2. In oxygen uptake experiments (data not shown) the inclusion of N_3^-, a singlet oxygen quenching agent, at 15 mM decreased the rate of oxygen consumption to approximately 55–60% of the rate without N_3^-. This observation is consistant with that of [8, 36] and is also consistent with $\approx$ 40% of the oxygen consumed being the result of singlet oxygen reactions, while $\approx$ 60% is converted to H_2O_2. Thus,

$$AH^- + O_2 \rightarrow A + H_2O_2 \quad \text{(final products)} \tag{5}$$

and as argued in [8, 19]

$$AH^- + {}^1O_2 \xrightarrow{(\text{MB} + h\nu)} \text{AHOO}^-\ . \tag{6}$$

The presence of H_2O_2 may result from the production of superoxide by this photodynamic system. However, we were unable to gather evidence for its presence. The addition of superoxide dismutase at 150 units/ml^{-1} in the oxygen uptake experiments resulted in the same decrease in the rate of oxygen consumption as when bovine serum albumin was added at the same protein concentration. The spin trapping experiments (see below) provided no evidence for the DMPO-superoxide spin adduct. However, it should be kept in mind that it will be extremely difficult to detect the presence of $O_2^-\cdot$ (or $HO_2\cdot$) in a system containing 1 mm AH^- as well as substantial $A^{\cdot -}$. Cabelli and Bielski [7] have extended the work of Nishikimi [32] and have determined that

$$A^{\cdot -} + HO_2\cdot \xrightarrow{k = 5 \times 10^8\ \text{M}^{-1}\text{s}^{-1}} A + HO_2^- \xrightarrow{(H^+)} A + H_2O_2 \tag{7}$$

$$A^{\cdot -} + O_2^-\cdot \xrightarrow{k = 2.6 \times 10^8\ \text{M}^{-1}\text{s}^{-1}} A + HO_2^-$$

$$+ OH^- \xrightarrow{(H^+)} A + H_2O_2 + H_2O \tag{8}$$

while

$$AH^- + HO_2\cdot \tag{9}$$

or

$$\xrightarrow{k = 1.4 \times 10^7\ \text{M}^{-1}\text{s}^{-1}} A^{\cdot -} + H_2O_2$$

$$AH_2 + O_2^-\cdot \tag{10}$$

and

$$AH_2 + O_2^{-}\cdot \xrightarrow{k = 5 \times 10^4\ M^{-1}s^{-1}} (+\ H^+) \rightarrow A^{\bullet -} + H_2O_2\ . \tag{11}$$

Thus, even though the superoxide-SOD reaction is very fast [25, 31]

$$2\ O_2^{-}\cdot + 2\ H^+ \xrightarrow[(SOD)]{k = 2 \times 10^9\ M^{-1}s^{-1}} H_2O_2 + O_2\ . \tag{12}$$

AH^- and $A^{\bullet -}$ will be able to compete very effectively in this system for any $O_2^{-}\cdot$ ($HO_2\cdot$) present.

In addition DMPO reacts much more slowly with superoxide [9] than $A^{\bullet -}$ and AH^-

$$DMPO + O_2^{-}\cdot \xrightarrow{k = 10\ M^{-1}s^{-1}} DMPO/O_2H \tag{13}$$

$$DMPO + HO_2\cdot \xrightarrow{k = 6.6 \times 10^3\ M^{-1}s^{-1}} DMPO/O_2H\ . \tag{14}$$

Thus, it is seems reasonable that superoxide can not be detected by these techniques as reactions 7–11 are very effective in removing $O_2^{-}\cdot/HO_2\cdot$ from the system.

A plausible mechanism for the production of H_2O_2 in this system consistent with the observations of [8, 20, 24, 26, 36] and the observations made here is (S_0, S*, and T are the ground state singlet, excited singlet and excited triplet states of MB, respectively.)

$$MB(S_0) + h\nu \rightarrow MB(S^*) \tag{15}$$

$$MB(S^*) \rightarrow MB(T) \tag{16}$$

$$MB(T) + AH^- \rightarrow MBH + A^{\bullet -} \tag{17}$$

$$MBH + O_2 \rightarrow HO_2\cdot + MB(S_0) \tag{18}$$

$$HO_2\cdot + O_2^{-}\cdot \rightarrow H_2O_2 + O_2 \tag{19}$$

The $^{\cdot}OH$ free radical is very short-lived because of its extreme reactivity. Thus, it is not possible to observe its presence directly in this ESR experiment. However, we have employed the spin trapping technique [5, 22, 30] in which short-lived radicals are reacted with a spin trap producing a much longer lived free radical spin adduct, which will accumulate to a concentration sufficient to be observed by ESR.

$$DMPO + R^{\bullet} \rightarrow DMPO/R \tag{20}$$

or

spin trap + radical → spin adduct

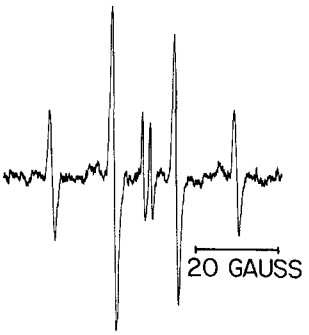

Fig. 3. The signal with the 1 : 2 : 2 : 1 intensity ratio is an example of the hydroxyl radical spin adduct of DMPO ($<a_N = a_H = 15.0$ gauss). This spectrum was generated upon illumination of a solution that contained 9×10^{-5} M MB, 50 mM DMPO, 0.5 mM sodium ascorbate, 1 μM Fe(III) and 2 μM EDTA. The less intense doublet signal in the center corresponds to the ascorbate free radical (overmodulated). Spectrometer settings were: scan rate = 25 gauss/min, modulation amplitude = 0.4 gauss, and the time constant = 0.5 s

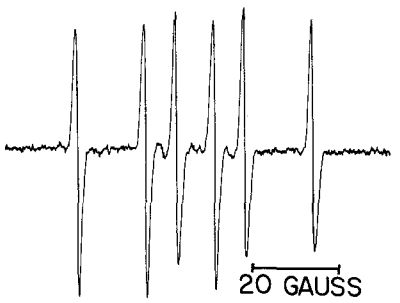

Fig. 4. An example of the alpha-hydroxyethyl radical spin adduct of DMPO ($a_n = 15.8$ gauss, $a_H = 22.8$ gauss). This reaction mixture was the same as Fig. 3 except ethyl alcohol was included at 1 M. The gain was decreased by a factor of three, and all other instrument settings were the same as in Fig. 3. The same spectrum is observed if the ethyl alcohol concentration is reduced to 0.1 M, except there is also a small amount of DMPO/OH signal present in the spectrum

The inclusion of the spin trap DMPO in an illuminated solution of 9×10^{-5} M MB and 1 mM AH^- resulted in the spin trapping of a spin adduct whose characteristics are consistent with the hydroxyl radical spin adduct of DMPO ($a_N = a_H = 15.0$ gauss [5, 18], see Fig. 3. The inclusion of Fe(III)EDTA at catalytic levels, 1 μM Fe(III) and 2 μM EDTA, resulted in a significant increase in the DMPO/OH spin adduct signal intensity. When the solution was bubbled with oxygen gas for approximately 5 min prior to illumination there was also an increase in the DMPO/OH signal intensity. The inclusion of hydrogen peroxide in the reaction mixture at 1 mM in addition to Fe(III)EDTA, ascorbate and methylene blue also produced a large increase in the DMPO/OH signal intensity, both in the dark and when illuminated. When catalase was included at 80 or 200 units per ml the rate at which the DMPO/OH signal appeared was decreased. When 0.1 M ethyl alcohol was included in the spin trapping solution of 9×10^{-5} M MB, 1 mM sodium ascorbate, 0.05 M DMPO and 1 μM Fe(III)EDTA the spectrum of Fig. 4 was obtained. This is characteristic of the spin trapping of the alpha-hydroxyethyl free radical ($a_N = 15.8$ gauss and $a_H = 23.0$ gauss [5, 27]). Thus, we have observed the appropriate carbon-centered radical that is consistent with the following mechanism:

$$\cdot OH + CH_3CH_2OH \rightarrow H_2O + CH_3\dot{C}HOH \quad (21)$$

$$DMPO + CH_3\dot{C}HOH \rightarrow DMPO/CH_3CHOH\,. \quad (22)$$

When sodium formate was substituted for ethyl alcohol, a radical with properties consistent with those of the formate radical spin adduct of DMPO was seen (a_N = 15.8 gauss, a_H = 18.6 gauss [5, 17]). These observations are consistent with the formation of the ·OH free radical by this photosensitizing system. Catalase was able to slow the rate at which the DMPO/OH spin adduct appeared, demonstrating the role of hydrogen peroxide in the formation of ·OH. It would appear that ascorbate reduces Fe(III)EDTA to Fe(II)EDTA which then reacts with H_2O_2 to form ·OH *via* the Fenton reaction.

$$\text{Fe(III)EDTA} + AH^- \rightarrow A^{\bullet -} + \text{Fe(II)EDTA} + H^+ \quad (23)$$

$$\text{Fe(II)EDTA} + H_2O_2 \rightarrow \cdot OH + OH^- + \text{Fe(III)EDTA}\,. \quad (24)$$

Conclusions

The observed formation of ·OH in this photodynamic system suggests that the free radical mechanism for the inactivation of yeast cells [20] and the inactivation of ribosomes [36] as well as the depolymerization of hyaluronic acid [8] is viable. MB has been shown to be capable of diverting the electron flow in *Escherichia coli* resulting in an increased cellular production of $O_2^{-}\cdot$ and H_2O_2 [12]. ·OH is perhaps the most reactive free radical that can arise in a biological system and has been demonstrated to be quite capable of cell killing and cell function inactivation [1, 33, 34]. In addition, recent findings [3] indicate that H_2O_2 impedes the mechanism of DNA repair in cells.

The observed production of H_2O_2 and hydroxyl free radical by MB, AH^- and light suggests that the use of MB in ophthalmalic products should be questioned. In addition, the free radical component to photodynamic action must be carefully considered when appropriate reducing agents such as ascorbate are present in the system.

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