

Article

Reactivity of Tertiary Amines with Singlet Oxygen and as Electron Donors in Riboflavin-Mediated Quinone Photoreduction

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Abstract

Singlet oxygen is produced by light-dependent excitation of molecular oxygen using photosensitizers. Using blue light and riboflavin (RF) or riboflavin phosphate (RFP) as photosensitizers, we studied the reaction of singlet oxygen with tertiary amines in aqueous solution because many are used as biochemical buffers and as electron donors in photoreduction reactions. The reactions of singlet oxygen with multiple tertiary amines, including ethylenediamine tetraacetic acid (EDTA), bicine, and triethanolamine (TEOA), produced micromolar hydrogen peroxide (H_2O_2) as the stable end product. For bicine and TEOA, but not EDTA, H_2O_2 yield increased as pH increased due to their higher amine pKa values. A white LED used in conjunction with riboflavin and tertiary amines also produced H_2O_2 , a contaminant likely to form during tissue culture manipulations. Direct photoreduction of 2,6-dichlorophenolindophenol and 2,3-dimethoxy-5-methyl-p-benzoquinone was achieved using blue light, RF or RFP, and tertiary amines as electron donors. With RF, EDTA was the optimal electron donor for both substrates, whereas with RFP, bicine and TEOA were superior to EDTA. Photochemical redox cycling of both quinones produced H_2O_2 via singlet oxygen-dependent re-oxidation of reduced quinols. Several amine buffers, including HEPES and PIPES, reacted with singlet oxygen to produce H_2O_2 but did not function as electron donors in quinone photoreduction assays.

Keywords: singlet oxygen; riboflavin; tertiary amine; hydrogen peroxide; photoreduction; benzoquinone; 2,6-dichlorophenolindophenol

1. Introduction

Visible light and numerous dye photosensitizers produce singlet oxygen (1O_2), an excited state of molecular oxygen (O_2) [1,2]. Foote and Wexler verified photosensitized production of 1O_2 , a reactive intermediate, in 1964 [3]. Methylene blue, Rose Bengal and phenosafranine are commonly used photosensitizers that produce 1O_2 [1,4,5]. Biological photosensitizers include chlorophyll and its metabolites as well as riboflavin [6–8]. Most 1O_2 research focuses on its light-dependent generation, with applications in synthetic chemistry, environmental settings, and living organisms [1,9,10].

Chlorophyll is the light harvesting pigment of photosynthesis, a process whereby red light initiates electron transfer from the excited state of chlorophyll to plastoquinone, a benzoquinone embedded in chloroplast membranes [11,12]. When red light is overabundant, the photoexcited state of chlorophyll initiates 1O_2 formation [6,7]. To limit cellular damage, plants contain antioxidants that scavenge 1O_2 and any resulting reactive oxygen



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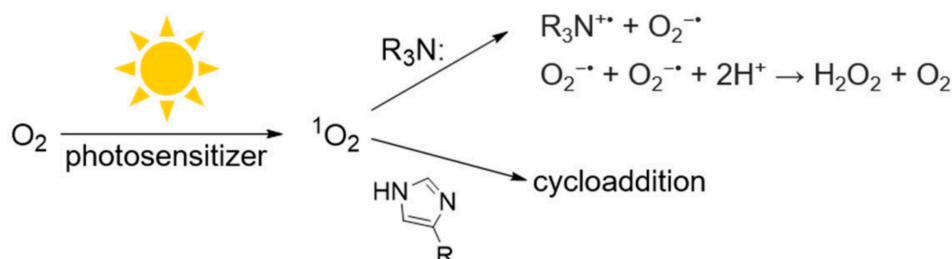
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species (ROS) [13]. Thus, the ability of a photosensitizer to initiate photoreduction and photo-oxidation concurrently is well established.

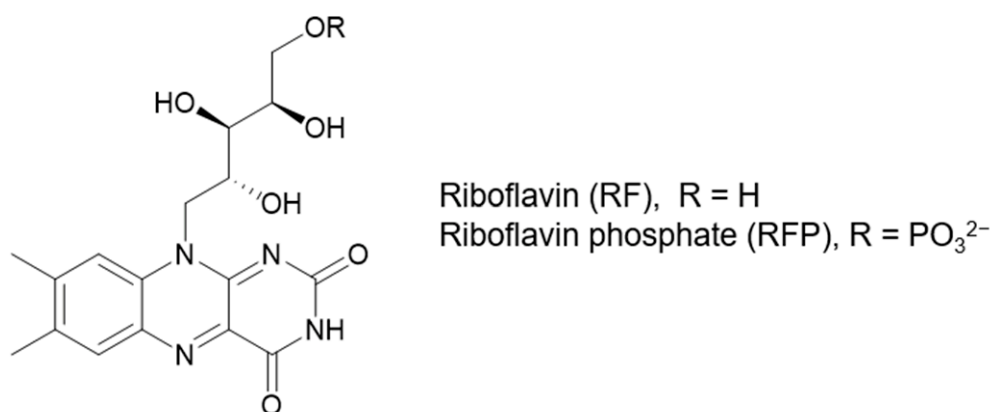
Because our lab employs tertiary amines (R_3N) as electron donors in quinone photoreduction reactions, we are also interested in the direct reaction of tertiary amines with 1O_2 [14,15]. The initial reaction of 1O_2 and R_3N produces superoxide anion and an amine radical cation, as shown in Scheme 1 [16]. Two superoxide anions disproportionate to H_2O_2 and regenerate O_2 .



Scheme 1. Photochemical generation of 1O_2 and its reaction with tertiary amines.

The reaction of tertiary amines with 1O_2 has received little attention, and to our knowledge, only one report determined a rate constant in water for the reaction of 1O_2 with EDTA, a commonly used tertiary amine, and with some metal–EDTA complexes [17]. Recently, we reported that the combination of either methylene blue or chlorin e6, multiple tertiary amines, and red light generated H_2O_2 [14]. Our goal was to determine relative rate constants for the reaction of the amines with 1O_2 by using histidine as a competing 1O_2 scavenger. Rate constants for the reaction of histidine with 1O_2 in aqueous solution at multiple pHs have been reported [18–20] (Scheme 1). The histidine concentrations required to scavenge 1O_2 varied for each tertiary amine but also depended on the photosensitizer employed [14]. We hypothesized that ionic interactions between the amines, histidine and the photosensitizer were responsible.

Our interest in riboflavin stems from its essential biological roles as a redox cofactor in enzyme catalyzed reactions and as a photosensor in organisms ranging from bacteria and plants to humans [21–23]. Riboflavin is an essential micronutrient synthesized by plants, fungi and many microorganisms that animals and humans obtain from their diet. Riboflavin can be phosphorylated to form riboflavin phosphate (RFP), which is also known as flavin mononucleotide (FMN) (Scheme 2).



Scheme 2. Structures of riboflavin (RF) and riboflavin phosphate (RFP).

The riboflavin component of the redox cofactor, flavin adenine dinucleotide (FAD), of the Krebs cycle enzyme succinate dehydrogenase is essential for catalysis (light inde-

pendent) [22]. Riboflavin as RFP cycles between its oxidized and reduced forms in the electron transport chain of mitochondria where ATP is produced. Notable light-dependent riboflavin-containing proteins include DNA photolyase, a bacterial enzyme that repairs thymine photodamage and cryptochromes in plants that detect blue light and initiate phototropism [24,25]. Mammalian cryptochromes that bind flavins also regulate circadian rhythms [26].

Riboflavin and its derivatives are of interest in photodynamic therapy (PDT) because they are nontoxic in biological settings and produce $^1\text{O}_2$ and other ROS upon activation by blue light [8]. Ongoing research is evaluating their uses in dermatology, dentistry and ophthalmology [27].

As some of the most abundant dietary photosensitizers that can be excited by visible light, chlorophyll- and riboflavin-based molecules are of interest because they will be photoreactive when consumed [28,29]. Our work with chlorophyll metabolites and tertiary amine electron donors confirmed that they mediate benzo- and naphthoquinone photoreduction [15,30,31]. Only limited research has addressed photoreaction reactions mediated by RF and its derivatives. One notable example is work by Alonso-de Castro et al., where they studied RF-dependent two-electron photoreduction of platinum IV complexes using a tertiary amine as the electron donor [32–34].

Herein, our objectives were to measure H_2O_2 produced photochemically from the reactions of RF and RFP with multiple tertiary amines in aqueous solution. Further, we tested the tertiary amines for their ability to serve as electron donors to RF and RFP during catalytic cycles of quinone photoreduction. Photoreduction of the quinones DCPIP and CoQ_0 is readily monitored because the oxidized forms have visible colors, whereas the reduced forms do not.

2. Materials and Methods

Materials: All chemicals were from Fisher Scientific or Sigma (St. Louis, MO, USA) and were of the highest purity available. CoQ_0 stock solutions were prepared in DMF and used immediately or stored at $-20\text{ }^\circ\text{C}$ for up to a month. For RF, 5 mM stock solutions contained up to 15 mM NaOH to ensure solubility. RF and RFP solutions were prepared fresh daily, diluted with water prior to use (to 0.5 or 0.1 mM) and stored in the dark when not in use. All other solutions were prepared in water or 10 mM phosphate buffer (PB) pH 7.4. Horseradish peroxidase (HRP) Type I and bovine liver catalase from Sigma were dissolved to 2 mg/mL in 10 mM PB pH 7.4, and aliquots were stored at $-80\text{ }^\circ\text{C}$. HRP concentration was determined from its absorbance at 403 nm ($\epsilon = 100,000\text{ M}^{-1}\text{ cm}^{-1}$). Tertiary amine stock solutions in water (100 mM) were adjusted to pH 7.4 with NaOH or HCl. All phosphate buffers were equilibrated to room temperature ($20\text{--}22\text{ }^\circ\text{C}$) to ensure no variation in dissolved O_2 .

Concentration measurements: All absorbance scans were performed using a Perkin-Elmer Lambda 35 UV/Visible spectrophotometer (PerkinElmer Life and Analytical Sciences, Inc., Shelton, CT, USA). For CoQ_0 , absorbance at 405 nm was used to calculate its concentration ($740\text{ M}^{-1}\text{ cm}^{-1}$ in 10 mM PB pH 7.4). DCPIP standard curves from 0 to 100 μM in 10 mM PB pH 7.4 or 6.4 were linear. For RF and RFP, absorbance at 450 nm was used to calculate concentrations ($12,500\text{ M}^{-1}\text{ cm}^{-1}$ in water or 10 mM PB pH 7.4) [23,35].

Light specifications: For blue light irradiation, a strip of blue LED lights ($450 \pm 10\text{ nm}$, 5 W total) was affixed to a foam board mounted on a black foam core box (3 sides). The base of the box was covered in aluminum foil. The distance from the LEDs to the samples was 14 cm. Light was measured in lux with a light meter, and mW/cm^2 at 450 nm was measured with a ThorLabs S120C power meter (ThorLabs, Newton, NJ, USA). Samples were positioned at the point of maximum light intensity, where lux readings were

1200–1300 and mW/cm^2 was 0.68–0.70. Blue light irradiation times were optimized to limit photobleaching of RF or RFP and did not exceed 10 min. For white light irradiation, a 60-watt equivalent LED bulb was mounted 18 inches above the samples. Typical lux readings were 2350–2400.

Detection of H_2O_2 with TMB and HRP: For reactions containing only photosensitizers and tertiary amines, TMB and HRP were used to detect H_2O_2 . Aliquots of photochemical reactions (20 μL) were combined with 0.5 mM TMB and 0.5 μM HRP in 10 mM PB pH 7.4 (100 μL total volume). After blue color development, 100 μL 1 M HCl was added [36]. Absorbance was read at 450 nm in a 96-well plate. A standard curve from 0 to 40 μM H_2O_2 was used to calculate H_2O_2 concentrations in the photochemical reactions.

DCPIP photoreduction assays: DCPIP (100 μM final) in 10 mM PB of varying pHs was combined with 2.5 μM RF or RFP and varying concentrations of tertiary amines. DCPIP photoreduction was performed in either a semimicro cuvette (1 mL) or a 96-well plate (200 μL). Absorbance (UV/Vis) scans for the cuvette or absorbance at 605 nm in the 96-well plate (200 μL total) was measured prior to irradiation and at time intervals. Dark samples containing all reactants served as controls. Prior to the initial absorbance reading, the cuvette was inverted three times to normalize dissolved O_2 .

Detection of H_2O_2 in photoreduction samples: HRP (8 μL , 50 μM) was added directly into the cuvette solution (1 mL) after irradiation and mixed gently to avoid introducing O_2 . Absorbance increases with HRP (0.4 μM final) indicative of re-oxidation of photoreduced DCPIP were monitored for up to 3 min until no further increase occurred. Controls were performed, where only 8 μL of buffer was added and mixed under identical conditions.

CoQ₀ photoreduction assays: CoQ₀ (0.5 mM) was combined with RF or RFP and tertiary amines in a semimicro cuvette (1 mL). Typical concentrations of CoQ₀, RF or RFP and EDTA were 0.5 mM, 10 μM and 8–12 mM, respectively, in 10 mM PB pH 7.4. Absorbance (full UV/Vis) scans were measured prior to irradiation and at time intervals. Dark samples containing all reactants served as controls. Typical molar ratio of quinone to photosensitizer was 1000:1. Equivalent reactions (200 μL) were also performed in a 96-well plate, and absorbance at 405 nm was measured. As for DCPIP, HRP was added to determine H_2O_2 produced during irradiation.

Data analysis: For each experiment, at least two–three independent experiments were performed in duplicate or triplicate. Mean values were calculated using all data (typically 6–9 replicates). For figures showing error bars, error is the standard deviation. Details for each are stated in figure legends.

3. Results

3.1. Detection of H_2O_2

Figure 1 shows that the combination of RF or RFP, tertiary amines and blue light generated H_2O_2 as the stable end product. The structures of the amines tested are shown in Scheme 3. To detect H_2O_2 , we used horseradish peroxidase (HRP) and a colorimetric co-substrate, tetramethylbenzidine (TMB), that generates a stable yellow product under acidic conditions (Supplemental Figure S1) [36]. In the absence of blue light or photosensitizer, no H_2O_2 was detected. If catalase, an enzyme that scavenges H_2O_2 according to Equation (1), was added prior to HRP and TMB, no yellow color was detected.



Phosphate buffers (PB) were used because phosphate does not react with $^1\text{O}_2$ or other ROS. Two $^1\text{O}_2$ are required to generate one H_2O_2 , but because one O_2 is regenerated, the stoichiometry is one O_2 consumed per H_2O_2 (Scheme 1). The concentration of dissolved

O₂ is ~300 µM in 10 mM PB at 20–22 °C (measured with an oxygen electrode). The H₂O₂ concentrations shown in Figure 1 are all below 300 µM, confirming that dissolved O₂ was not limiting.

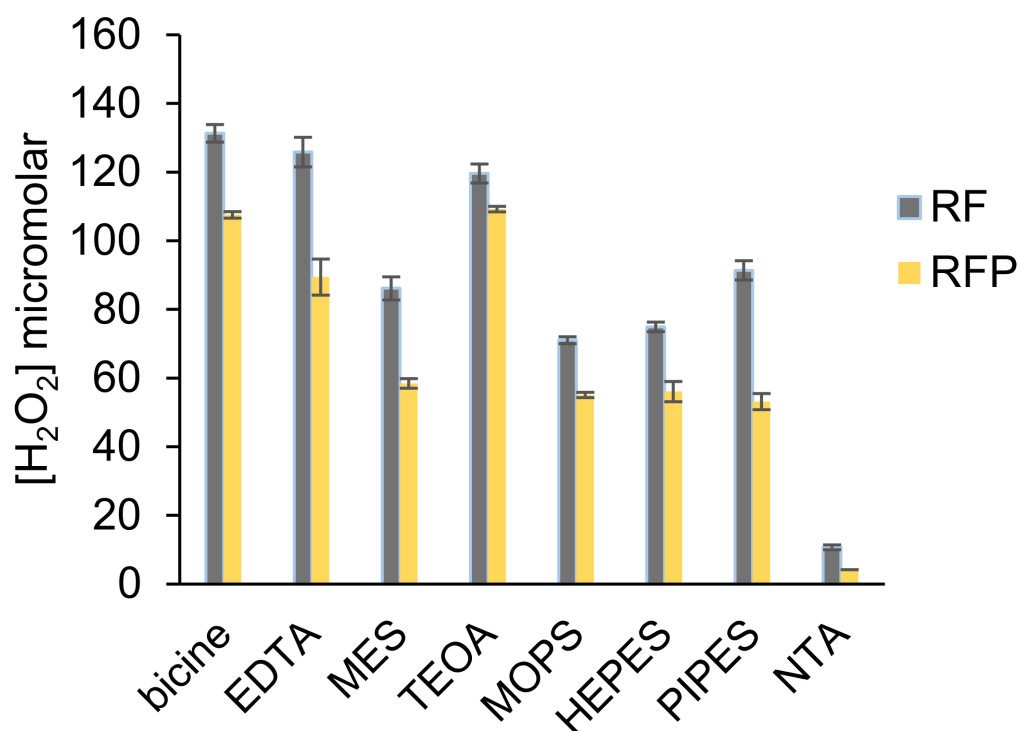
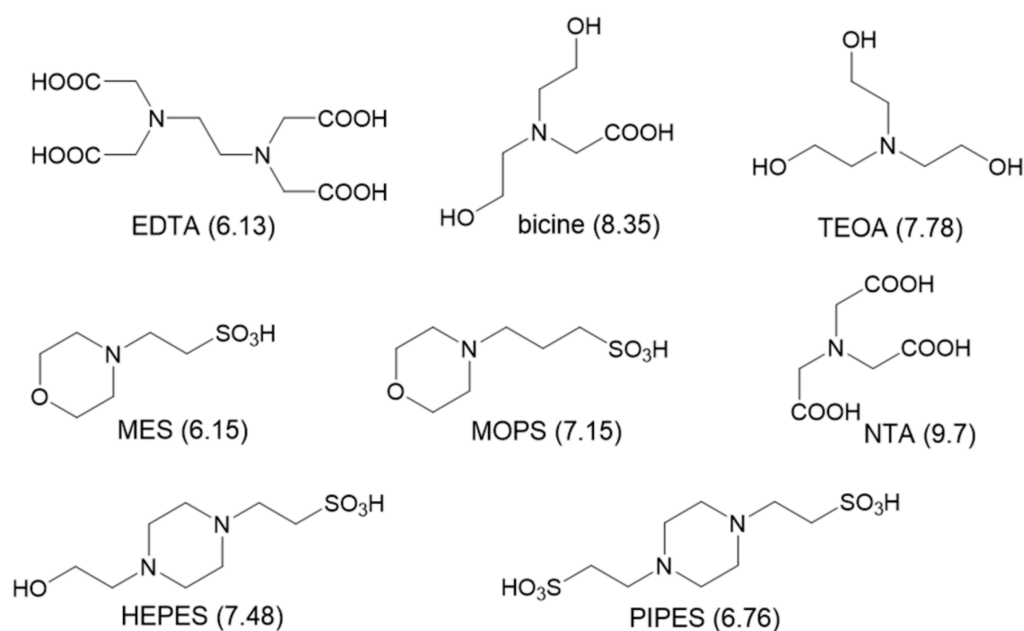


Figure 1. H₂O₂ yield by RF or RFP, tertiary amines and blue light. Reactions (100 µL) contained 5 µM RF or RFP and 5 mM tertiary amine in 10 mM PB pH 7.4. Samples were irradiated for 5 min. Aliquots (20 µL) were combined with 10 mM PB pH 7.4, 0.5 mM TMB and 0.5 µM HRP (100 µL total). Reactions were quenched with 100 µL 1 M HCl, and absorbance was measured at 450 nm. [H₂O₂] was determined from a standard curve. These data are the average of three independent experiments performed in duplicate.



Scheme 3. Structures of tertiary amines and their amine pK_a values.

When RF was used as the photosensitizer, reactions with bicine, EDTA and TEOA and blue light generated the most H_2O_2 , followed by PIPES, MES, HEPES and MOPS (Figure 1). With RFP, all H_2O_2 yields were lower than RF, and TEOA and bicine generated more H_2O_2 than EDTA. H_2O_2 yields with NTA were negligible regardless of photosensitizer, despite similarities in structure to both EDTA and bicine (Scheme 3).

3.2. pH Dependence of H_2O_2 Yield

To react with $^1\text{O}_2$ as shown in Scheme 1, tertiary amines must be deprotonated. Therefore, we examined the pH dependence of H_2O_2 formation using the most reactive tertiary amines in Figure 1 (pH 7.4). In addition to bicine, EDTA, and TEOA, MES was also tested at multiple pH values because Alonso-de Castro et al. employed it as an electron donor in their work on platinum complex photoreduction at pH 6.0 [32–34].

In Figure 2A, using RF as the photosensitizer, bicine showed the greatest pH dependence, with a 20-fold increase in H_2O_2 as phosphate buffer pH increased from 5 to 8. For TEOA, H_2O_2 yield increased 6-fold as pH increased. The H_2O_2 yield did not change when EDTA was assayed across this pH range, and MES showed only a modest increase in H_2O_2 . For MES, once pH was 6, H_2O_2 yield was constant (Figure 2A).

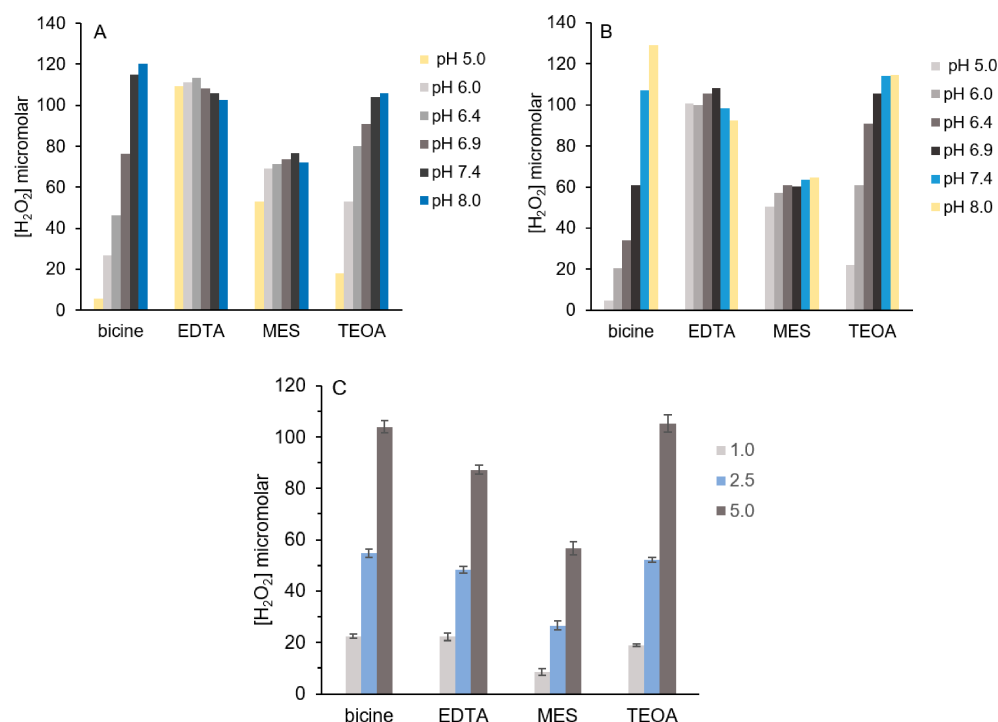


Figure 2. pH- and time-dependence of tertiary amine reactions with RF or RFP and blue light. Reactions (100 μL) containing 5 μM RF or RFP and 5 mM tertiary amine in 10 mM PB of varying pHs were irradiated for 5 min. (A) RF. (B) RFP. (C) Reactions contained 5 μM RFP and were irradiated for 1–5 min. These data are the average of three independent experiments performed in duplicate.

All the amines tested with RFP in Figure 2B showed the same pH dependence as for RF. We attribute the results in Figure 2A,B to differences in amine pK_a values (given in Scheme 3). Bicine and TEOA have amine pK_a values of 8.35 and 7.78, respectively, whereas those of EDTA and MES are 6.13 and 6.15. While EDTA has two amines and therefore two pK_a values, the second value of 10.37 renders that amine unreactive in this pH range. Further, it is unlikely that both amines of an EDTA molecule would react with $^1\text{O}_2$. For bicine and TEOA, as pH increases, their amines are deprotonated and consequently capable of reacting with $^1\text{O}_2$.

Amine pKa values also explain H₂O₂ yields in Figure 1 for PIPES and HEPES using RF. Although they are similar in structure (Scheme 3), PIPES has a lower pKa of 6.76 vs. 7.48 for HEPES. Consequently, more H₂O₂ was generated by the PIPES reaction. Likewise, MES and MOPS are similar in structure, but MES, with its lower pKa of 6.15 vs. 7.15 for MOPS, yielded more H₂O₂. NTA has the highest pKa of the amines tested (9.7) and consequently yielded the least H₂O₂ at pH 7.4 regardless of photosensitizer.

The difference in HEPES and PIPES reactivity in Figure 1 using RF was not observed with RFP. PIPES has two negative charges at pH 7.4 due to its sulfonates; HEPES has only one. Because RFP is also negatively charged, charge repulsion between it and PIPES is likely responsible for the lower H₂O₂ yield in Figure 1 relative to HEPES. This observation reinforces that ionic interactions between the photosensitizer and the tertiary amine influence reaction outcome.

H₂O₂ yields in Figures 1 and 2A,B were measured after 5 min of blue light irradiation. A time course of H₂O₂ production in Figure 2C shows no noteworthy differences in H₂O₂ production after 1 or 2.5 min, and results for bicine, EDTA and TEOA are identical. Results for the other amines were all comparable with time-dependent increases in H₂O₂.

Histidine is a well-characterized ¹O₂ scavenger that effectively traps O₂ via a 4 + 2 cycloaddition reaction [20,37]. This differs from other ¹O₂ scavengers like azide that regenerates O₂ and produces azide-based radicals [38,39]. When millimolar histidine was added to reactions with select tertiary amines as described in Figure 1, H₂O₂ yields decreased (Supplemental Figure S2). This was observed when both RF and RFP were employed.

Many of the tertiary amines shown in Scheme 3 are biochemical buffers, including HEPES, MES, MOPS, PIPES and TEOA. EDTA is routinely added to buffers to scavenge redox-active metal cations that are required for ROS generation. Further, tissue culture media is supplemented with low micromolar concentrations of RF because it is essential for cell growth and survival. Figures 1 and 2 confirm that blue light irradiation produces up to 120 µM H₂O₂ in 5 min using only 5 µM RF or RFP. Amines used as buffers are commonly at millimolar concentrations equal to or greater than 5 mM used in Figures 1 and 2.

To mimic reaction conditions during tissue culture manipulations, we tested the combinations of RF and tertiary amines for H₂O₂ production using a 60-watt equivalent white LED bulb. RF concentrations of 2.5 or 5 µM were assayed with 5 mM bicine or EDTA for 10 min. Additionally, samples were either irradiated on a foil-lined surface or a black lab bench. Figure 3A shows that H₂O₂ was detected under all conditions, and the highest H₂O₂ was detected with 5 µM RF on the foil-lined surface.

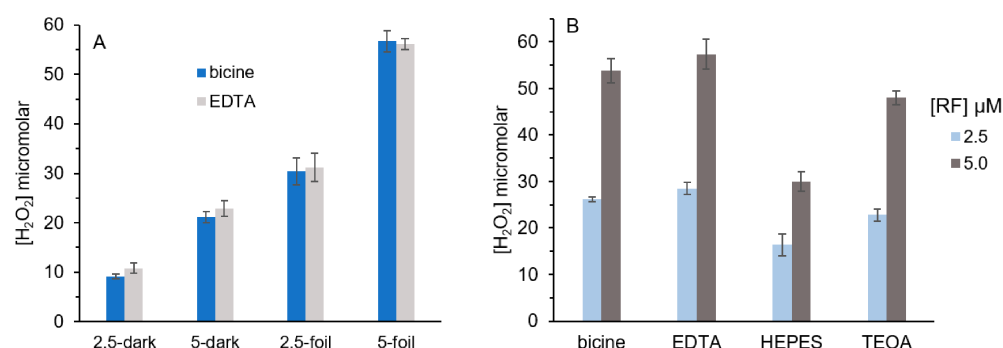
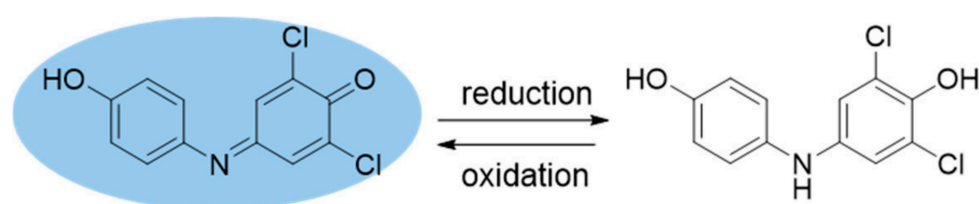


Figure 3. H₂O₂ yield by RF and tertiary amines under white LED light. Reactions (100 µL) contained varying RF and 5 mM tertiary amine in 10 mM PB pH 7.4. Samples were irradiated for 10 min. (A) Samples containing 2.5 or 5 µM RF were positioned 18 inches from a white LED on a foil-lined or black surface. (B) All samples were on a foil-lined surface 18 inches from a white LED. These data are the average of two independent experiments performed in triplicate.

When HEPES and TEOA in addition to bicine and EDTA were tested at 2.5 or 5 μM RF under the white light for 10 min, similar micromolar H_2O_2 was detected (Figure 3B). HEPES is a very common buffer used in biochemical experiments near physiological pH. Further, Zigler et al. reported H_2O_2 production in tissue culture media that contained riboflavin and HEPES after exposure to visible light. Data in Figure 3A,B supports their findings but also confirms that H_2O_2 generation is not limited to HEPES. All tertiary amines in Scheme 3 were tested under the white light, and H_2O_2 was detected with all except NTA.

Our interest in tertiary amine reactivity stems from our quinone photoreduction work. Tertiary amines are routinely used as sacrificial electron donors in photoreduction experiments [40]. In our work on para-benzoquinone and naphthoquinone photoreduction, we routinely use EDTA in combination with methylene blue and red light [15,30,31]. Likewise, we have reported that the primary dietary chlorophyll metabolite pheophorbide A catalyzes quinone photoreduction with TEOA as the best electron donor (red light dependent) [15,41]. In those studies, quinone photoreduction was optimized using different combinations of photosensitizer and electron donor.

Recently, we reported that DCPIP, a quinoneimine, was readily reduced using methylene blue, EDTA and red light. DCPIP is a well-characterized redox indicator that is blue in its oxidized form and colorless when reduced (Scheme 4) [42]. Oxidized DCPIP absorbs strongly at 605 nm and is water soluble; the molar absorptivity is pH-dependent (Supplemental Figures S3–S6). DCPIP is also used to measure ascorbic acid [43]. In the Hill reaction of chloroplasts, DCPIP serves as the terminal electron acceptor [44].



Scheme 4. Redox reaction of DCPIP.

We sought to test RF and RFP as photosensitizers for DCPIP photoreduction in a manner analogous to our prior work. Figure 4A shows that the combination of RF, EDTA and blue light reduced DCPIP. We performed absorbance scans in a 1 mL semimicro cuvette so that it could be irradiated and scanned directly at the time intervals to limit mixing and O_2 exposure. After 7 min total of light exposure in the cuvette, 95% of the 100 μM DCPIP was reduced using 2.5 μM RF and 2 mM EDTA. For photocatalysis studies, both the ratio of substrate to photosensitizer (100 μM to 2.5 μM , 40 $\times$) and the ratio of electron donor to photosensitizer (2 mM to 2.5 μM ; 800:1) are routinely reported.

After irradiation, horseradish peroxidase (HRP) was added, and an increase in DCPIP absorbance was detected as a result of its re-oxidation (Figure 4A). HRP is an enzyme that requires H_2O_2 to oxidize organic substrates, including catechols and quinols [45–47]. Because reduced DCPIP is only oxidized by HRP when H_2O_2 is available, oxidized, blue DCPIP was regenerated when HRP was added after irradiation (Scheme 5). The increase in DCPIP absorbance in Figure 4A confirms that H_2O_2 was produced as DCPIP was photoreduced. When an equivalent volume of buffer was added to the cuvette instead of HRP, no increase in DCPIP was observed. If catalase was added prior to HRP, DCPIP absorbance did not increase.

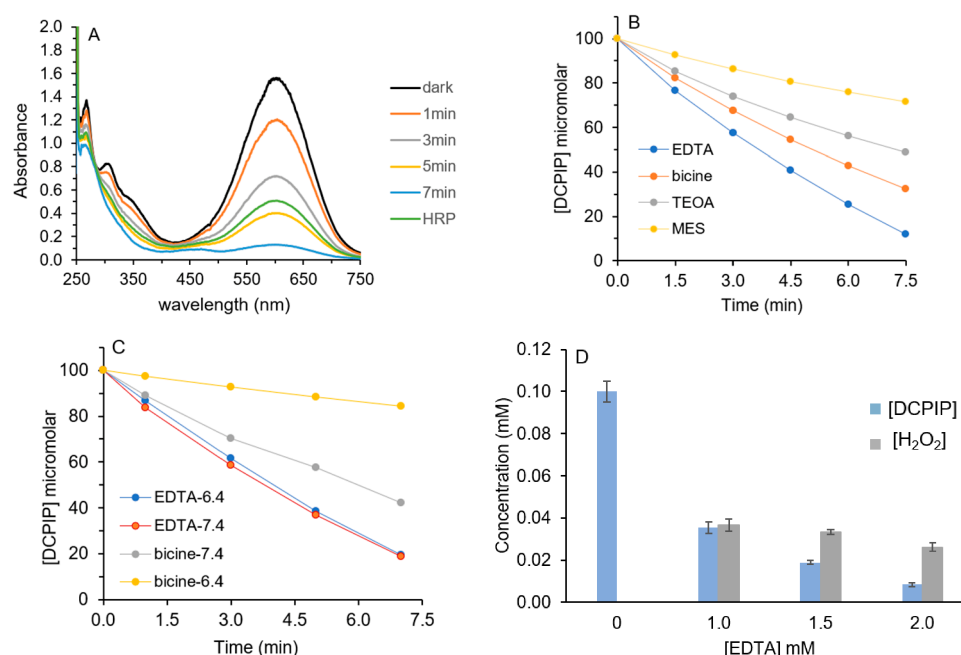
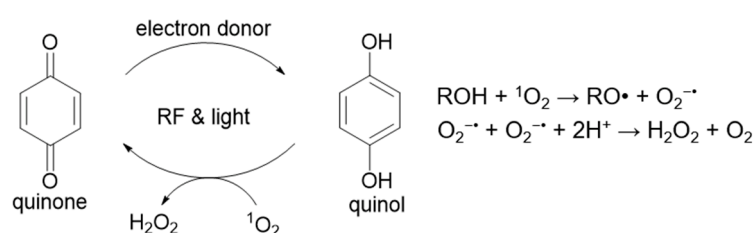


Figure 4. DCPIP photoreduction by RF and tertiary amines. (A) Reactions (1 mL in a quartz cuvette) contained 100 μ M DCPIP, 2.5 μ M RF and 2 mM EDTA in 10 mM PB pH 7.4 in a quartz cuvette. A blue light source with maximum output at 450 nm was employed, and DCPIP photoreduction was monitored at 605 nm. The increase in A_{605} after HRP addition (0.4 μ M final) was used to calculate $[H_2O_2]$. (B) Samples (200 μ L) contained 100 μ M DCPIP, 2.5 μ M RF and 2 mM amine in 10 mM PB pH 7.4. Samples were irradiated in a 96-well plate for up to 7.5 min. The decrease in A_{605} was used to calculate [DCPIP] at each time. These data are the average of three independent experiments. (C) Samples were prepared as in (B) with 1.5 mM amine and 10 mM PB pH 7.4 or 6.4. These data are the average of two independent experiments performed in duplicate. (D) Samples were prepared as in (A), except samples were irradiated for 7.5 min and [EDTA] varied. These data are the average of three independent experiments.



Scheme 5. Photochemical redox cycling of quinones to produce H_2O_2 .

There are two possible sources of H_2O_2 during irradiation of DCPIP, RF and EDTA. The combination of RF and EDTA generates H_2O_2 from 1O_2 , as described in Figures 1–3. H_2O_2 will also form if 1O_2 reacts with reduced quinol (ROH in Scheme 5) [48]. Hydrogen atom abstraction from the quinol by 1O_2 produces a semiquinone and peroxy radical ($\bullet OOH$). The pKa of $\bullet OOH$ is 4.8; therefore, it will deprotonate at neutral pH to a superoxide anion that becomes H_2O_2 [49]. Two semiquinones ($RO\cdot$) disproportionate rapidly to yield a quinol and regenerate a quinone (blue DCPIP). These results confirm that both photo-oxidation of O_2 to 1O_2 and photoreduction of DCPIP via an electron transfer mechanism are mediated by the excited state of RF.

All tertiary amines in Scheme 3 were tested for their ability to serve as electron donors for DCPIP photoreduction by RF. Figure 4B shows the time courses of DCPIP photoreduction by RF using EDTA, bicine, TEOA and MES at pH 7.4. Of the other amines in Scheme 3,

MOPS and NTA showed minimal DCPIP reduction by RF. No DCPIP photoreduction was detected using HEPES and PIPES under the reaction conditions employed.

Given the pH dependence observed in Figure 2A, EDTA and bicine were also tested at pH 6.4 as electron donors for DCPIP photoreduction (Figure 4C). There was no difference in the rate of DCPIP photoreduction at pH 7.4 vs. pH 6.4 with EDTA. However, for bicine, the rate was $\sim 4\times$ slower at pH 6.4 vs. pH 7.4. We attribute this difference to the amine pKa values of 6.16 and 8.35 because the amines must be deprotonated to serve as electron donors.

Table 1 summarizes DCPIP photoreduction rates mediated by EDTA and MES at pH 7.4 and 6.4 using three different concentrations of each electron donor. Regardless of the amine concentration, reactions with bicine were $\sim 4\times$ slower at pH 6.4 vs. 7.4, consistent with Figure 4C. When additional experiments were performed at pH 8, a further increase in the rate of DCPIP photoreduction was observed with bicine but not with EDTA.

Table 1. Rates of DCPIP reduction by RF and bicine or EDTA.

[Amine] mM	Bicine pH 6.4	Bicine pH 7.4	EDTA pH 6.4	EDTA pH 7.4
1.0	1.3 ± 0.1	5.5 ± 0.3	8.9 ± 0.4	9.2 ± 0.3
1.5 ¹	1.7 ± 0.2	6.7 ± 0.4	11.6 ± 0.5	11.5 ± 0.4
2.0	2.2 ± 0.2	8.2 ± 0.4	12.8 ± 0.3	13.0 ± 0.5

Rates of DCPIP photoreduction are in $\mu\text{M}/\text{min}$; ¹ Data from Figure 4C at 1.5 mM amine is included.

As shown in Figure 4A, HRP addition and the subsequent increase in DCPIP absorbance confirmed that H_2O_2 was produced. Additional samples containing different EDTA concentrations were irradiated and monitored in a cuvette to determine the change in DCPIP absorbance after HRP addition. This increase can be converted to a H_2O_2 concentration because, for each DCPIP that is re-oxidized, one H_2O_2 is consumed (Scheme 5).

Figure 4D summarizes the decrease in DCPIP and the increase in H_2O_2 at three EDTA concentrations. In the absence of EDTA, no DCPIP was photoreduced. The highest concentration of H_2O_2 detected was $37 \mu\text{M}$ with 1 mM EDTA, and H_2O_2 decreased as EDTA increased (Figure 4D). If EDTA were reacting with $^1\text{O}_2$, H_2O_2 would be expected to increase as EDTA increased.

Controls were performed, where equivalent concentrations of RF ($2.5 \mu\text{M}$) and EDTA (1–2 mM) were irradiated for 7.5 min either in a cuvette or in a plate well (200 μL vol) but without DCPIP. Under these conditions, H_2O_2 concentrations increased from $61 \mu\text{M}$ to $79.5 \mu\text{M}$ as EDTA increased from 1 to 2 mM. H_2O_2 concentrations in the absence of DCPIP are greater than those in Figure 4D at all EDTA concentrations tested. Increased electron donor favored the photoreduction pathway, thereby limiting $^1\text{O}_2$ formation and its reaction with EDTA or quinol, the two possible sources of H_2O_2 .

The rates summarized in Table 1 and Figure 4B,C were measured using 200 μL samples in a 96-well plate, whereas those in Figure 4A,D were obtained in a 1 mL cuvette. The plate format is more amenable to obtaining replicate data, but O_2 exposure is greater in the plate well vs. in a narrow microcuvette. For this reason, DCPIP photoreduction rates in Figure 4A–D and Table 1 are not identical.

DCPIP photoreduction was monitored using RFP as the photosensitizer with different amine electron donors. Figure 5A shows that, with RFP, bicine and TEOA were equivalent and more effective than EDTA at pH 7.4. Although reaction conditions were identical to those in Figure 4B, photoreduction rates with RFP were slower than with RF, consistent with the results in Figure 1. Only 56% of the $100 \mu\text{M}$ DCPIP was photoreduced with RFP and either bicine or TEOA vs. 90% in Figure 4B using RF and EDTA. MES was the least effective of the electron donors in Figure 4B, and only minimal DCPIP photoreduction

was detected with equal concentrations of MOPS and NTA. HEPES and PIPES were not effective as electron donors under the reaction conditions used, consistent with the results obtained with RF.

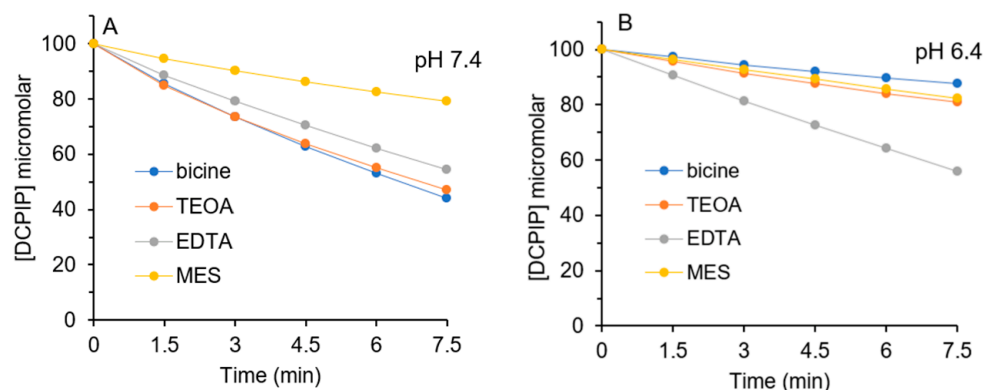


Figure 5. DCPIP photoreduction by RFP and tertiary amines. Samples (200 μ L) contained 100 μ M DCPIP, 2.5 μ M RFP and 2 mM amine in 10 mM PB. Samples were irradiated in a 96-well plate for up to 7.5 min. The decrease in A_{605} was used to calculate [DCPIP] at each time. (A) pH 7.4; (B) pH 6.4. These data are the average of three independent experiments. All errors were less than 5%.

When DCPIP photoreduction with RFP and select electron donors was performed at pH 6.4, the pH dependence of bicine and TEOA was evident because the time course of DCPIP photoreduction slowed precipitously. At pH 6.4, EDTA was the best electron donor with RFP (Figure 5B).

In addition to DCPIP, we routinely use 2,3-dimethoxy-5-methyl-p-benzoquinone (CoQ_0) as a photoreduction substrate [15,41]. CoQ_0 is a more water-soluble analog of CoQ_{10} , the benzoquinone that cycles between its oxidized and reduced forms in the electron transport chain in mitochondria. Oxidized CoQ_0 absorbs at 405 nm, whereas its reduced form does not. Because the molar absorptivity of CoQ_0 is much lower than that of DCPIP, higher CoQ_0 concentrations were necessary for reliable data.

Prior to any photoreduction experiments, UV/visible spectra of RF and CoQ_0 and the combination of the two were obtained because of considerable overlap between 350 and 500 nm (Figure 6A). The concentrations of RF (10 μ M) and CoQ_0 (0.5 mM) were optimized to monitor CoQ_0 photoreduction in a 1 mL cuvette.

Figure 6B shows that the combination of RF, EDTA and blue light photoreduced CoQ_0 because absorbance centered at 405 nm decreased. After 10 min, HRP was added, and oxidized CoQ_0 absorbance increased, confirming that H_2O_2 was produced during CoQ_0 photoreduction. Because RF also absorbs at 405 nm, full reduction of 0.5 mM CoQ_0 would not decrease absorbance to zero. From the absorbance of RF at 405 nm in Figure 6A, we can estimate that CoQ_0 absorbance would decrease to ~ 0.08 if all CoQ_0 were reduced. However, some photobleaching occurred when RF alone was exposed to equivalent blue light over 10 min (Supplemental Figure S7). While photobleaching decreased RF absorbance at 450 nm, absorbance at 403–408 nm did not decrease. It is unclear if the same level of RF photobleaching occurs during photoreduction when electron donors and CoQ_0 are present. For this reason, we can only estimate turnover numbers for RF or RFP as photosensitizers using yellow CoQ_0 as a photoreduction substrate.

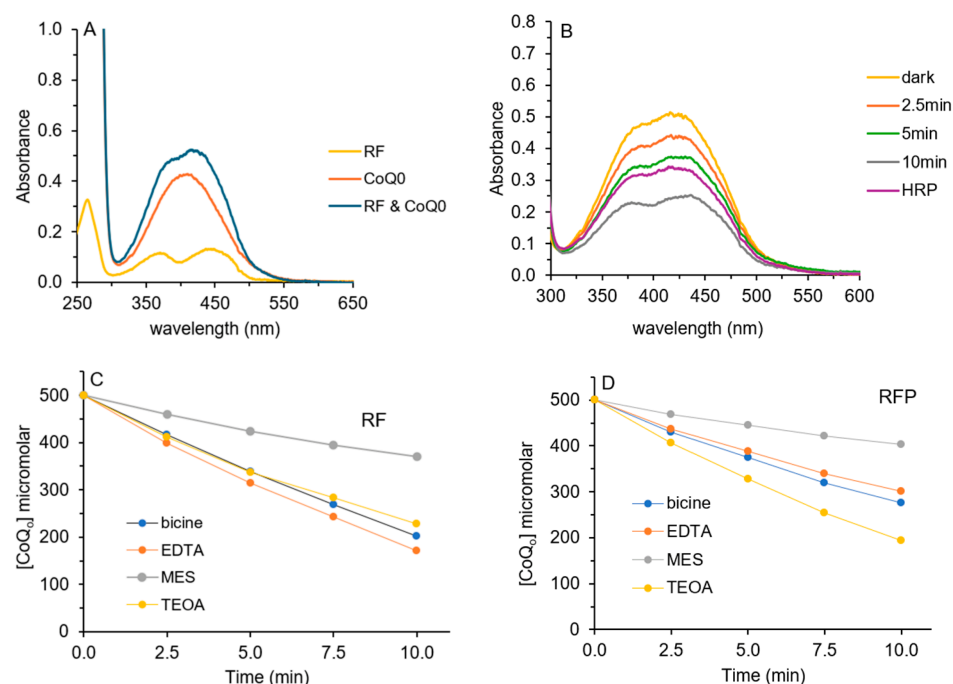


Figure 6. CoQ₀ photoreduction by RF or RFP and amines. **(A)** Samples (1 mL) contained 10 μ M RF, 0.5 mM CoQ₀ or both in 10 mM PB pH 7.4. **(B)** 10 μ M RF, 10 mM EDTA and 0.5 mM CoQ₀ in 10 mM PB pH 7.4 (1 mL) were irradiated for up to 10 min. CoQ₀ photoreduction was monitored at 405 nm. The increase in A_{405} after HRP addition was used to calculate $[H_2O_2]$. **(C)** Samples (200 μ L) contained 0.5 mM CoQ₀, 10 μ M RF and 10 mM amine in 10 mM PB pH 7.4. **(D)** samples were prepared as in (C), except 10 μ M RFP was used. Samples were irradiated in a 96-well plate for up to 10 min. The decrease in A_{405} was used to calculate $[CoQ_0]$ at each time. Data in (C,D) are the averages of three independent experiments. Error did not exceed 5% for any sample.

Turnover numbers with CoQ₀ (mol quinone reduced/mol photosensitizer) are similar to those observed with DCPIP (Figures 4 and 5). For DCPIP, the maximum turnover number was 40 (100 μ M DCPIP and 2.5 μ M RF or RFP), whereas with CoQ₀, the maximum possible is 50 (0.5 mM CoQ₀ and 10 μ M RF or RFP). These values are similar to those measured in our photoreduction studies using these quinones and red light with MB or chlorophyll metabolites [15,30,31]. The ratios of amine to photosensitizer were also consistent with our prior work. For DCPIP photoreduction, the highest ratios were 800:1 (2 mM amine and 2.5 μ M RF or RFP) and 1200:1 for CoQ₀ (12 mM amine and 10 μ M RF or RFP).

In Figure 6C, different amine electron donors were tested for their ability to support CoQ₀ photoreduction at pH 7.4. Here, only absorbance at 405 nm was measured (96-well plate format). To calculate CoQ₀ concentrations, the absorbance of RF at 405 nm was subtracted from that of CoQ₀ (dark). EDTA, bicine and TEOA were equally effective. As for DCPIP photoreduction using RF, CoQ₀ photoreduction was detected using MES and, to a lesser extent, MOPS and NTA. No photoreduction was detected using HEPES or PIPES. With RFP, TEOA was superior to bicine or EDTA as the electron donor for CoQ₀ photoreduction (Figure 6D).

4. Discussion

Our results confirm that the combination of RF or RFP, tertiary amines and blue light produces H₂O₂ in neutral, aqueous solution. Numerous tertiary amines, including several that are used routinely as biochemical buffers, produced 60–130 μ M H₂O₂ under ambient oxygen conditions (Figure 1). For all amines tested, RF was superior to RFP as the photosensitizer. H₂O₂ yields in Figure 1 are comparable to those we obtained using red

light, tertiary amines and the photosensitizers methylene blue (MB) and chlorin e6 in our prior work [14].

The pH dependence of the photochemical reaction of tertiary amines with $^1\text{O}_2$ reinforced the importance of amine pKa values (Figure 2 and Scheme 3). Increased H_2O_2 yields at higher pH for bicine and TEOA regardless of photosensitizer were evident because those amines, unlike EDTA or MES, have higher pKa values, and only the deprotonated amines react with $^1\text{O}_2$.

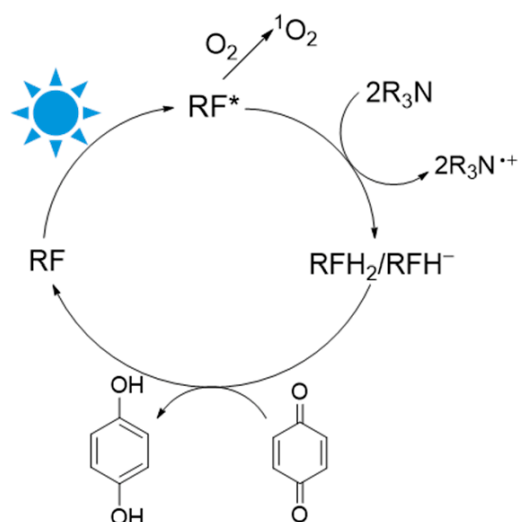
In our prior work, EDTA showed a modest two-fold increase in H_2O_2 with MB across the same phosphate buffer range (pH 5–8) but not with chlorin e6 [14]. Although TEOA showed pH-dependent increases in H_2O_2 with both MB and chlorin e6, neither increase was comparable to the six-fold increase shown in Figure 2A,B with RF or RFP. MB has a positive charge in this pH range, and chlorin e6 has three negative charges [50]. RF is neutral, while RFP has a negatively charged phosphate (Scheme 2). Thus, the identity and chemical properties of the photosensitizer, in addition to the amine pKa values, are likely to influence H_2O_2 yields.

To determine a rate constant for the reaction of $^1\text{O}_2$ and EDTA and its metal complexes, Hessler et al. used Rose Bengal as the photosensitizer, and they measured quenching of $^1\text{O}_2$ phosphorescence at 1270 nm [17]. Their reported rate constant on the order of $\sim 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for $^1\text{O}_2$ and EDTA is likely dependent on the photosensitizer in addition to the identity of the tertiary amine. It is expected that optimal scavenging of $^1\text{O}_2$ by tertiary amines requires $^1\text{O}_2$ generation in close proximity to the photosensitizer.

Although histidine scavenged $^1\text{O}_2$ and decreased H_2O_2 yields in reactions of RF and RFP with tertiary amines, higher concentrations were required than in our prior work with MB and chlorin e6 (Supplemental Figure S2), even though 5 mM amine was used in both. Up to 5 mM histidine was used herein, whereas we observed more complete $^1\text{O}_2$ scavenging with only 1.0–1.5 mM histidine when MB or chlorin e6 were used as the photosensitizers [14]. As for the different photosensitizer/amine combinations, optimal scavenging of $^1\text{O}_2$ by histidine may depend on its interaction with photosensitizers of variable charge and polarity.

Our work using a white LED is important because we detected micromolar H_2O_2 from the reaction of RF, tertiary amines, and visible light. The stainless steel surfaces of laminar flow hoods are reflective, and exposure to light can extend well beyond the 10 min employed in Figure 3A,B. Zigler et al. reported H_2O_2 production in tissue culture media that contained RF and HEPES after exposure to visible light using 25 mM HEPES vs. only 5 mM in Figure 3B [51]. Recently, Liu et al. tested some primary, secondary and tertiary amines (all at 25 mM) for H_2O_2 generation in RPMI 1640, a common tissue culture medium [52]. While they detected micromolar H_2O_2 with tertiary amines, they did not test bicine, EDTA or TEOA, the most reactive amines at pH 7.4 in Figure 1. Further, none of these studies addressed the pH dependence of the photochemical reaction between RF or RFP and tertiary amines.

A major goal of this work was to assess the effectiveness of tertiary amines as electron donors in quinone photoreduction reactions mediated by RF and RFP. In our prior work, the parallel reaction of tertiary amines with $^1\text{O}_2$ as benzo- and naphthoquinones were photoreduced was observed regardless of the photosensitizer and amine electron donor employed [15,30,31]. The photoexcited, triplet state RF^* will either react with O_2 to form $^1\text{O}_2$ or accept electrons from tertiary amines (Scheme 6). Once reduced to RFH_2 , electron donation to quinone substrates occurs to regenerate RF. RFH_2 has a pKa of 6.7; consequently, some RFH^- will be present at neutral pH [53]. Any $^1\text{O}_2$ produced has two reaction paths that produce H_2O_2 : reaction with a tertiary amine as shown in Scheme 1 or with the quinol photoreduction product (Scheme 5).



Scheme 6. Photochemical oxidation and reduction pathways of RF.

Both DCPIP and CoQ₀ are excellent model substrates for photoreduction because their oxidized forms have a visible color that is lost upon reduction. Further, their reduced forms are co-substrates for HRP, providing a simple method to quantify H₂O₂ (Scheme 5). Results in Figures 4A and 6B confirm that H₂O₂ was generated during photoreduction of both DCPIP and CoQ₀. Although both of these experiments used EDTA, H₂O₂ was detected in all DCPIP and CoQ₀ photoreduction experiments with RF and RFP regardless of the tertiary amine used.

When irradiation is first initiated, only the amine can react with ¹O₂ because no quinol is present. As the reduced quinol forms, it becomes the likely substrate for hydrogen atom abstraction by ¹O₂. Rate constants for the reaction of ¹O₂ with quinols in aqueous solution are ~10⁹ M⁻¹ s⁻¹, several orders of magnitude greater than the published rate constant of ~10⁵ M⁻¹ s⁻¹ for ¹O₂ and EDTA [48]. It is not possible to differentiate the source of H₂O₂ in our experiments, and amine cation radicals R₃N^{•+} are the initial products of both amine photo-oxidation (Scheme 1) and electron donation in photoreduction (Scheme 6). Further decomposition of R₃N^{•+} to form well-characterized, inert products would be identical [40].

In addition to concurrent quinone photoreduction and ¹O₂ generation, we determined the best tertiary amine donors for RF and RFP by comparing rates of photoreduction for DCPIP and CoQ₀. Figures 4B and 5A show differences in donor compatibility for DCPIP photoreduction at pH 7.4 with RF and RFP, respectively. While EDTA was best with RF, TEOA and bicine were superior to EDTA with RFP. Electrostatics likely play a role because EDTA has four negative charges at pH 7.4, but bicine has only one, and TEOA is uncharged. Any charge repulsion between EDTA and RFP would be expected to diminish electron donation (Scheme 6).

For CoQ₀ photoreduction with RF, the same amine ranking as for DCPIP was observed (Figure 4B vs. Figure 6C). This observation supports the catalytic cycle depicted in Scheme 6, where photoexcited RF receives electrons from the amine, followed by electron donation to the quinone substrate. Likewise for RFP, TEOA and bicine were superior to EDTA (Figure 5A vs. Figure 6D).

DCPIP photoreduction rates using RF and either EDTA or bicine at pH 7.4 vs. 6.4 (Figure 4C and Table 1) were consistent with H₂O₂ yields in Figure 2A at different pH values. As in their reaction with ¹O₂, amines must be deprotonated to donate electrons to RF*. Bicine showed pH-dependence, but EDTA did not (Figure 4C and Table 1).

EDTA, bicine and TEOA were the most reactive amines with ¹O₂ (Figure 1) and also the best electron donors at pH 7.4. Although MES, MOPS, HEPES and PIPES reacted with

$^1\text{O}_2$ to produce H_2O_2 , only modest photoreduction was observed with MES and MOPS. No photoreduction was observed with HEPES or PIPES. All four have sulfonate groups that would be negatively charged, and their pKa values are similar to EDTA, bicine and TEOA. Given that negatively charged amines like EDTA and bicine were very effective electron donors, optimal electron donation requirements are not clear.

Photoreduction turnover numbers (mol DCPIP reduced/mol RF) of ~35 were achieved with 100 μM DCPIP, 2.5 μM RF, and 2 mM EDTA (Figure 4) at pH 7.4. For RFP, turnover numbers were 20–25 with 100 μM DCPIP, 2.5 μM RFP, and 2 mM bicine (Figure 5A). These are comparable to values of 40–45 with MB and EDTA in prior work using DCPIP. Increased electron donor increased photoreduction and decreased H_2O_2 formation (Figure 4D), which is consistent with Scheme 6. For CoQ_0 , measurements were complicated by overlapping absorbances of RF or RFP and CoQ_0 (Figure 6A,B). Nonetheless, we estimate ~30–35 turnovers in Figure 6B with EDTA as the electron donor. While tertiary amines are not biologically relevant electron donors for photoreduction, it is important to show that photoreduced flavins can donate electrons to substrates like CoQ_0 , an analog of CoQ_{10} , a key component of the electron transport chain in mitochondria.

The photocatalytic cycle depicted in Scheme 6 mirrors the work of Alonso-de Castro et al., where they used blue light, RF, and the tertiary amine MES at pH 6.0 to photoreduce platinum (IV) prodrugs to activated platinum (II) complexes [32–34]. This methodology was used successfully to induce DNA damage in Capan-1 cells because the DNA targeted-platinum (II) drugs were produced, and ROS were generated simultaneously [32]. Their work is one of the only detailed examples of photoreduction using RF or RFP in conjunction with a tertiary amine electron donor. However, they did not evaluate multiple tertiary amines as we have, and their reasoning for performing the reaction at pH 6.0 was not clear.

Understanding RF photoreactivity in a human nutritional context is essential because RF is added to foods, taken as a vitamin supplement, and consumed as a natural food dye. While most RF is consumed as FMN or FAD that are in many of foods, free RF circulates through the blood stream mostly bound to transporter proteins [21]. At least 90 proteins in humans require riboflavin and its derivatives for activity [22]. Blue light penetrates skin, and there are specific photoreceptor proteins in the eye that utilize RF [27].

While photochemical H_2O_2 generation by RF and amines under cell culture conditions is undesirable, our work suggests a photochemical method to generate H_2O_2 . This work is an example of green chemistry that uses biocompatible RF, common tertiary amines like EDTA, blue light, and ambient O_2 in neutral, aqueous solution. While dissolved O_2 will become limiting, this can be rectified by bubbling solutions with air or O_2 gas. Unlike current H_2O_2 industrial processes that rely on a Pd catalyst, H_2 and O_2 gases, anthraquinone as a redox cyler, and a mixture of organic solvents, our reactants are nontoxic, and no organic solvents are required [54,55].

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/oxygen6030021/s1>, Figure S1: H_2O_2 standard curve with TMB and HRP—0 to 40 μM linear; Figure S2: Effect of histidine on H_2O_2 yields in photochemical reactions; Figure S3: UV/Vis scans of DCPIP (20–100 μM) in 10 mM PB pH 7.4; Figure S4: DCPIP standard curve in 10 mM PB pH 7.4; Figure S5: UV/Vis scans of DCPIP (20–100 μM) in 10 mM PB pH 6.4; Figure S6: DCPIP standard curve in 10 mM PB pH 6.4; Figure S7: Photobleaching of RF.

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Abbreviations

The following abbreviations are used in this manuscript:

bicine	Bis(2-hydroxyethyl)amino]acetic acid
CoQ ₀	2,3-dimethoxy-5-methyl-p-benzoquinone
DCPIP	2,6-dichlorophenolindophenol
DMF	dimethylformamide
EDTA	ethylenediaminetetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
H ₂ O ₂	hydrogen peroxide
HRP	horseradish peroxidase
MES	2-(N-morpholino)ethanesulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
NTA	nitrilotriacetic acid
PB	phosphate buffer
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
RF	riboflavin
RFP	riboflavin phosphate
TEOA	triethanolamine
TMB	3,3',5,5'-tetramethylbenzidine

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