Formation of 2,5-Dihydroxybenzoic Acid during the Reaction between $^1O_2$ and Salicylic Acid: Analysis by ESR Oximetry and HPLC with Electrochemical Detection†

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Abstract: The oxygen consumption, vis-a-vis $^1O_2$ production during irradiation of dyes such as rose bengal, merocyanine-540, and aluminum phthalocyaninetetrasulfonate, in the presence of salicylic acid was measured by electron spin resonance (ESR) oximetry. Concomitantly, formation of 2,5-dihydroxybenzoic acid (2,5-DHBA) in the same sample was analyzed by HPLC–EC. Both O_2 consumption and 2,5-DHBA formation were stimulated by D_2O, quenched by azide, unaffected in the presence of catalase, superoxide dismutase, and hydroxyl radical scavengers (ethanol, formate, etc.), and vastly diminished under N_2. The stoichiometry between $^1O_2$ consumption and 2,5-DHBA formation was determined to be ca. 0.5. On the basis of experiments using histidine, the chemical rate constant for the reaction between $^1O_2$ and salicylic acid was determined to be 0.20×10^6 M^{-1} s^{-1}. Furthermore, $^1O_2$ generated from the thermal decomposition of the water-soluble endoperoxide of 3,3′-(1,4-naphthylidene)dipropionate ($NDPO_2$) was shown to react with salicylic acid to form 2,5-DHBA as the major product. We conclude that exclusive formation of 2,5-DHBA is highly diagnostic of $^1O_2$ intermediacy in photochemical systems and in biochemical systems lacking metabolic activity. HPLC–EC is thus a valuable adjunct to ESR oximetry in the characterization of $^1O_2$ and may, on the basis of the selectivity of this reaction, provide a sensitive analytical method for detecting $^1O_2$ intermediacy.

Introduction

Salicylic acid (SA), a commonly-used radical scavenger, has been shown to react rapidly with hydroxyl radical (•OH) ($k = 5 \times 10^9$ M^{-1} s^{-1}) to form 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) as major products and catechol as a minor product.[1-5] The relative stability of these products combined with the ease with which they can be detected in picomolar concentrations by HPLC with electrochemical detection (HPLC–EC) have made this technique highly suitable for measuring •OH in biological tissues under in vitro and in vivo conditions. For example, using salicylate as a trap, both 2,3- and 2,5-DHBA have been detected in myocardial tissues and effluents from isolated rat hearts subjected to ischemia and reperfusion.[6-8] Inhibition of these products by radical scavengers implicated the intermediacy of •OH. Recently, Ingelman-Sundberg et al.[9] have shown that 2,5-DHBA can also be produced via the microsomal metabolism of SA and thus cautioned against the use of 2,5-DHBA per se as diagnostic marker of •OH formation. These investigators have also suggested that monitoring of 2,3-DHBA is a more reliable indicator of •OH in experiments where SA is used as an oxy-radical scavenger. Feix and Kalyanaraman[10] have recently reported that singlet oxygen ($^1O_2$) also reacts with SA to form 2,5-DHBA as the only product.

The purpose of this study is to determine the stoichiometry between O_2 consumption and 2,5-DHBA production during the reaction between $^1O_2$ and SA and to measure the rate constant for physical and chemical quenching of $^1O_2$ by salicylate. The singlet oxygen was photodynamically generated by irradiation of either rose bengal (RB), aluminum phthalocyaninetetrasulfonate (AlPcTS), or merocyanine 540 (MC540) associated with dilauroylphosphatidylcholine (DLPC) liposomes.

The photodynamic mechanism of action involves absorption of visible light by the sensitizer such as RB etc. to form the electronically excited triplet state of the sensitizer, which undergoes either a type I or type II reaction. In type I reactions, the triplet-state dye reacts directly with a substrate (i.e., lipid) to form radicals via electron-transfer reactions.[11] In type II reactions, the triplet-state dye transfers energy to molecular oxygen to form $^1O_2$. Since most triplet excited dyes react with molecular oxygen at nearly diffusion-controlled rates (10^9 to 10^{10} M^{-1} s^{-1}), the type II reaction is presumably most favored at ambient oxygen concentrations.

The first detectable event following the production of $^1O_2$ in a type II reaction is rapid consumption of molecular oxygen that occurs as a result of reactions of $^1O_2$ with suitable traps. The measurement of O_2 uptake in a type II reaction may therefore be used to examine the kinetics of $^1O_2$ production.

Electron spin resonance (ESR) oximetry[12,13] is a highly sensitive methodology for measuring O_2 consumption and allows continuous

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† Abbreviations: DLPC, dilauroylphosphatidylcholine; DHBA, dihydroxybenzoic acid; ESR, electron spin resonance; HPLC, high-performance liquid chromatography; EC, electrochemical detection; $^1O_2$, singlet oxygen; •OH, hydroxyl radical; MC540, merocyanine 540; AlPcTS, aluminum phthalocyaninetetrasulfonate; RB, rose bengal; LUV, large unilamellar vesicles; CTPO, 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy; SA, salicylic acid; $NDPO_2$, endoperoxide of disodium 3,3′-(1,4-naphthylidene)dipropionate; NDP, disodium 3,3′-(1,4-naphthylidene)dipropionate; SOD, superoxide dismutase.

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monitoring of dissolved oxygen without stirring or agitation of the sample. This methodology has been used (as an alternate approach to the conventional Clarke oxygen electrode technique) in photochemical studies.[13,14] The nitroxide spin probe 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy (CTPO) used in this technique does not react with $^1O_2$ to an appreciable extent and absorbs light only in the near-UV region. These factors make this probe highly suitable for photodynamic studies.

In this paper we use ESR oximetry in conjunction with HPLC–EC to investigate the following: (1) trapping of $^1O_2$ by salicylic acid to form 2,5-DHBA as the major product, (2) the stoichiometry between oxygen consumption and 2,5-DHBA formation, and (3) the rate constant for physical and chemical quenching of $^1O_2$ by salicylic acid. We have also obtained unequivocal evidence for the formation of 2,5-DHBA as the major product during the reaction between salicylic acid and $^1O_2$ in a purely chemical system where $^1O_2$ was generated by thermolysis of the water-soluble endoperoxide of 3,3′-(1,4-naphthylidene)dipropionate ($NDPO_2$).

Materials and Methods

Materials. Merocyanine 540 (MC540) from Sigma Chemical Co. (St. Louis, MO) was prepared as a stock 1 mg/mL solution in a 50% ethanol/water mixture and stored at −20 °C in the dark. Both rose bengal (RB) (Aldrich Chemical Co., Milwaukee, WI) and aluminum phthalocyaninetetrasulfonate (AlPcTS) (Porphyrin Products, Logan, UT) were prepared as stock solutions (1 mg/mL) in deionized water and stored in the dark at 4 °C. Dilauroylphosphatidylcholine (DLPC) was purchased from Sigma Chemical Co. and stored as stock solutions (10–20 mg/mL) in chloroform at −20 °C. 3-Carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxy (CTPO) was obtained from Molecular Probes, Inc. (Eugene, OR). Catalase and superoxide dismutase were obtained from Boehringer-Mannheim (Indianapolis, IN). Salicylic acid, 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,4-dihydroxybenzoic acid (2,4-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA), and citric acid were from Aldrich Chemical Co. (Milwaukee, WI).

Perdeuterated 50 mM phosphate buffer (PB–D_2O) was prepared by addition of perdeuterated phosphoric acid (D_3PO_4) to D_2O and titration with deuterated sodium hydroxide (NaOD) to pH 7.8 (pD 7.4). D_2O, D_3PO_4, and NaOD were obtained from Aldrich Chemical Co.

Synthesis of $NDPO_2$. Diethyl α,α'-dicarbethoxy-1,4-naphthalenedipropionate was prepared from 1,4-bis(bromomethyl)naphthalene and malonic ester by following the procedure of Marvel and Wilson.[15] Saponification and decarboxylation was achieved according to the method of Lock and Walter,[16] and photochemical oxidation of the disodium salt of naphthalene-1,4-dipropionate using RB immobilized on Dowex-1 gave the corresponding endoperoxide in good yield.

Rose bengal was immobilized on Dowex-1 strong anion exchange resin. The dye-anchored resin was washed with water until there was no further elution of free dye. In a typical experiment, 0.5 g of the disodium salt was dissolved in 1 mL water to which 20 mL of methanol and 100 mg of rose bengal–Dowex resin were added. While oxygen was being bubbled through the suspension, it was irradiated with a 500-W mercury lamp at 5 °C for 3 h. The resin was filtered off, and the solution was dried over Na_2SO_4 in an ice bath. The solvent was removed on the vacuum line at ambient temperature. The resulting white powder (≈0.5 g) was stored at −20 °C. The conversion of NDP to $NDPO_2$ was monitored by UV spectroscopy. The endoperoxide has no absorption maximum in the range 250–350 nm, whereas the starting material has a maximum at 290 nm.[17]

Liposome Preparations. Stock solutions of dilauroylphosphatidylcholine (DLPC) in chloroform were dried under a stream of dry nitrogen gas and then further dried under vacuum for at least 1 h. Multilamellar vesicles (MLV) were prepared by hydration of the dried lipid film in 20 mM phosphate, pH 7.5 at 37 °C, with vigorous vortexing to produce a homogeneous suspension. Large unilamellar vesicles (LUV) were formed by extrusion of the MLV through 0.2-μm polycarbonate filters (Nucleopore, Pleasanton, CA), using an extruder (Lipex Biomembranes, Inc., Vancouver, BC). Five cycles of freezing and thawing preceded the extrusion, as previously described.[18] Stock solutions of liposomes were prepared at a concentration of 50 mM DLPC and stored at 4 °C.

Sample Preparation and Irradiation Procedure. A typical reaction mixture contained the following components: 50 μL of RB (10 μg/mL), MC540 (1 mg/mL), or AlPcS (100 μg/mL), 50 μL of CTPO (1.14 mM), 50 μL of DLPC (10 mM), 50 μL of SA (20 mM), and $^1O_2$-specific quenchers and enzymatic scavengers at concentrations indicated in the figure legends. The final volume of the reaction mixture was increased to 0.5 mL by addition of chelex-treated phosphate buffer (20 mM, pH 7.5).

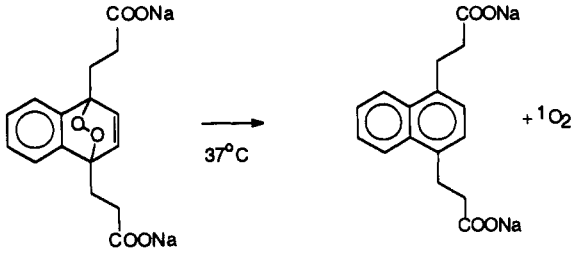
Samples (~40 μL) were taken up in a 100-μL micropipet (Fisher Scientific Co., Pittsburgh, PA) and placed in a 4-mm quartz tube, and the tubes were inserted into the ESR cavity at 37 °C. For experiments under anaerobic conditions, samples (~20 μL) were taken up in a capillary made of the gas-permeable methylpentene polymer TPX (Westlake Plastics, Lenni, PA), directly inserted into the ESR cavity, and deoxygenated by flowing N_2 gas over the TPX sample capillary for at least 10 min prior to irradiation.

Samples were irradiated in situ through the slotted window on the front of the ESR cavity. Light from a 300-W Hg-arc lamp source was passed first through a 5-cm $CuSO_4$ solution (100 g/L) and then through a long pass filter (Oriel), which cut all wavelengths below 345 nm. Light intensity was measured at the front of the ESR cavity using a YSI Radiometer Model 65A. No appreciable variation was found over the time period necessary to carry out ESR experiments.

Following irradiation and ESR measurements, 20 μL of the sample was taken using a Hamilton syringe and diluted 10 times with phosphate buffer for analysis of 2,3- and 2,5-DHBA by HPLC–EC (see the HPLC–EC Analysis).

Generation of Singlet Oxygen by $NDPO_2$. Singlet oxygen was generated by thermal dissociation of $NDPO_2$, forming disodium 3,3′-(1,4-naphthylidene)dipropionate (NDP) (Scheme I).[19] To a 0.9-mL solution containing salicylic acid (10 mM) in phosphate buffer (pH 7.4, 50 mM) at 37 °C was added 100 μL of $NDPO_2$. The stock solution of $NDPO_2$ (100 mM) was prepared by adding solid $NDPO_2$ to a chelex-treated phosphate buffer kept in ice. Aliquots of samples were then periodically taken for HPLC–EC analysis.

Scheme I. Thermolysis of $NDPO_2$ to NDP



ESR Oximetry. ESR oximetric measurements were made on an ER200D Brucker spectrometer equipped with a temperature controller. Typically, the superhyperfine structure of the low-field ($M_I = +1$) line is analyzed using a microwave power level of 0.98 mW, 10-G sweep width, and a field modulation amplitude of 0.04 G.

ESR oximetry operates as folllows: In air, dissolved oxygen interacts with nitroxide free radicals through Heisenberg spin exchange (via bimolecular collision with paramagnetic ground state O_2) to produce broadening of the ESR lines that is proportional to O_2 concentration. As O_2 is removed from the system, Heisenberg exchange is reduced and spectral resolution increases.

The spectral resolution is measured in terms of the parameter K, which is calibrated against $[O_2]$. The parameter K has been schematically defined in previous publications.[12,13] For aqueous solution at 37 °C in a capillary, the following relationship is valid:

$$K = 0.413 - 3.197 \times 10^3[O_2] + 8.154 \times 10^6[O_2]^2 \qquad (1)$$

By simply substituting the measured K value into this equation, the actual $[O_2]$ is determined. Routinely, this is calculated using dO/dt, a computer

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program written by Dr. Christopher Felix at the National Biomedical ESR Center. For other sample geometries (i.e., flat cells and temperatures), the above relationship is appropriately modified on the basis of standard calibration curves.[12]

Measurement of Rate Constants. The quantitative relationship between oxygen consumption and $^1O_2$ production during light treatment of dye in the presence of salicylic acid in a type II mechanism can be derived from the following equations:

$$^1O_2 \xrightarrow{k_d} {}^3O_2 \tag{2}$$

$$^1O_2 + SA \xrightarrow{\text{chemical quenching } (k_r)} \text{products} \quad ^1O_2 + SA \xrightarrow{\text{physical quenching } (k_q)} SA \tag{3}$$

From eqs 2 and 3, one can obtain

$$\left(\frac{-d[O_2]}{dt}\right)^{-1} = \frac{1}{R}\left(1 + \left(\frac{k_d}{(k_r + k_q)}\right)\frac{1}{[SA]}\right) \tag{4}$$

where R is the rate of $^1O_2$ production. Using ESR oximetry, $-d[O_2]/dt$ can be obtained. From a plot of $(-d[O_2]/dt)^{-1}$ versus $[SA]^{-1}$ is obtained a gradient to intercept ratio, which equals $k_d/(k_r + k_q)$, where k_d is the rate constant for decay of $^1O_2$ to $^3O_2$. Using a value of 2.5×10^5 s^{-1} for k_d, $k_r + k_q$ (i.e., the sum of physical and chemical quenching of $^1O_2$ by SA) is calculated. The absolute value of the chemical quenching of $^1O_2$ by SA is calculated through competition experiments using another $^1O_2$-specific trap, namely histidine (eq 5).

$$\left(\frac{-d[O_2]}{dt}\right)_{\text{histidine}} \Big/ \left(\frac{-d[O_2]}{dt}\right)_{SA} = \frac{(k_r)_{\text{histidine}}}{(k_r)_{SA}} \tag{5}$$

In the presence of a physical quencher such as azide, the rate of spontaneous deactivation of $^1O_2$ is increased

$$^1O_2 \xrightarrow{k_d'} {}^3O_2 \tag{6}$$

where k_q' is the rate constant for physical quenching. In order to further confirm that 2,5-DHBA formation is diagnostic for $^1O_2$ production, a Stern–Volmer plot is constructed for the quenching of 2,5-DHBA by azide, and from the slope of eq 7, k_q' is calculated and compared with the literature value.

$$\frac{[2,5\text{-DHBA}]_{-\text{azide}}}{[2,5\text{-DHBA}]_{+\text{azide}}} = 1 + \left(\frac{k_q'}{k_d}\right)[N_3^-] \tag{7}$$

HPLC–EC Analysis. HPLC was run on a Beckman system with a 25 cm × 4.6 mm Whatman Partisil 10 ODS-3 C18 reverse-phase column and an Alltech macrosphere 300 C18 guard column. The electrochemical detector (EG & G Princeton Applied Research model 400) was set at 600 mV in the oxidative mode and was interfaced to a Beckman 427 integrator. A 20-μL sample was injected by overfilling a 20-μL sample loop and eluted at either 1.0 or 1.3 mL/min with a mobile phase of 30 mM citrate/27 mM acetate, pH 4.75.[9] Elution times and the detector/integrator response to 2,3-DHBA and 2,5-DHBA were calibrated each day with authentic standards.

Results

HPLC–EC Analysis of Products Formed during Irradiation of Rose Bengal and Salicylic Acid. Hydroxylated products of SA, such as 2,3-, 2,5-, 2,4-, and 2,6-DHBA, are readily analyzed by HPLC with electrochemical detection.[1] Of these, only 2,3- and 2,5-DHBA have been used as reliable diagnostics for ·OH production. The HPLC chromatogram obtained with equimolar amounts of 2,3- and 2,5-DHBA is shown in Figure 1 (top). The retention times of 2,3- and 2,5-DHBA are dependent on the type of columns used, eluant composition and eluant rates, etc.[5,6] Figure 1 (middle) shows the chromatogram obtained following irradiation of RB and SA with visible light under aerobic conditions. As can be seen, 2,5-DHBA is formed as a major product and 2,3-DHBA as a minor product. Irradiation of the same sample under N_2 atmosphere vastly diminished formation of 2,5-DHBA (Figure 1 (bottom)). Similar chromatographic patterns were obtained using MC540 and AlPcTS (data not shown). Formation of 2,3-DHBA was not dependent on oxygen. These results indicate that the photodynamic activation of dyes such as RB, MC540, or AlPcTS in the presence of SA produces 2,5-DHBA as the major product.

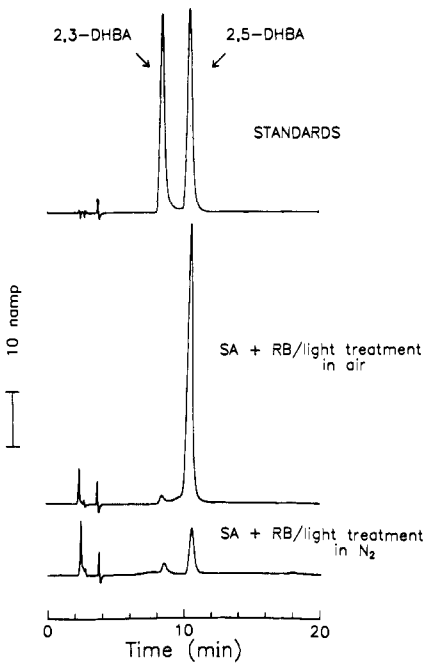

Figure 1. HPLC with electrochemical detection of products formed during irradiation of rose bengal and salicylic acid with visible light: (top) a standard sample containing 20 pmol each of 2,3- and 2,5-DHBA; (middle) products formed during irradiation of RB (10 μg/mL) in phosphate buffer (pH 7.5, 50 mM) containing 1 mM DLPC and 20 mM SA (Samples were irradiated in a TPX capillary placed inside an ESR cavity (kept at 37 °C) with continued air purging.); (bottom) same conditions as above except that samples were purged with N_2 gas for about 10 min prior to irradiation. HPLC–EC profiles were obtained by injecting 20 μL of irradiated sample that had been diluted by 10-fold with phosphate buffer and eluting at 1.3 mL/min with citrate/acetate buffer as described in Materials and Methods. The amount of light incident on the cavity was 50 W/m^2.

Next we examined whether the selective formation of 2,5-DHBA is in fact due to the selective destruction of 2,3-DHBA during photodynamic action. To this end, we monitored the stability of 2,3-DHBA in the presence of RB and light. During light treatment, 2,3-DHBA underwent some (<20%) photochemical destruction (data not shown). However, in the presence of 2 mM SA, the extent of photochemical destruction was decreased (data not shown). These results clearly suggest that, under our experimental conditions, the photochemical destruction of 2,3-DHBA is minimal and cannot be the basis for the almost exclusive observation of 2,5-DHBA.

Effect of ·OH- and $^1O_2$-Specific Scavengers on 2,5-DHBA Formation. Addition of catalase did not significantly inhibit 2,5-DHBA formation (Table I). Since it has been shown previously that the activity of catalase is not destroyed during such photodynamic treatment,[10] a lack of inhibition by catalase implies that neither H_2O_2 nor ·OH or perferryl iron is responsible for the formation of 2,5-DHBA. Consistent with this, the presence of ethanol—even in large excess—did not have any effect (Table I). As reported previously, SOD did not have an appreciable effect on 2,5-DHBA, suggesting that $O_2^{\cdot-}$ is not involved in the hydroxylation of SA. Under these experimental conditions, D_2O

Table I. Effect of Inhibitors on 2,5-DHBA Formation and O_2 Consumption during RB-Sensitized Oxidation of SA

incubation conditions[a]	irradiation time (min)	O_2 consumed (μM)	2,5-DHBA (μM)	[2,5-DHBA]/[O_2]
RB (10 μg/mL) and SA (20 mM) in phosphate buffer (H_2O)	20	194	110.9	0.57
+ D_2O	10	218	114.3	0.53
+ azide (1 mM)	20	75	40.6	0.54
+ catalase (20 μg/mL)	20	149	88.1	0.59
+ N_2 purging	20		1.35	
+ ethanol (1%)	20	183.5	96.3	0.53

[a] Irradiation conditions identical to those described in Figure 1. Because of the rapid rate of oxygen uptake in D_2O, the irradiation time was shorter (10 min).

enhanced formation of 2,5-DHBA by about 2-fold (Table I). Azide at low concentration (~1 mM) inhibited the rate of reaction by more than 2-fold (Table I). These results indicate that 2,5-DHBA formation is probably mediated by $^1O_2$.

Further proof of involvement of $^1O_2$ in the selective hydroxylation of SA was obtained by kinetic analysis using azide as a competitive quencher. As shown in Figure 2A, formation of 2,5-DHBA was inhibited by sodium azide in a dose-dependent fashion. From a Stern–Volmer quenching analysis (Figure 2B), the rate constant (k_q') for physical quenching of $^1O_2$ by azide was calculated to be ca. 3.6×10^8 M^{-1} s^{-1}, using the value for k_d as 2.5×10^5 s^{-1}, which is in reasonably good agreement with the literature rate constant for physical quenching of $^1O_2$ by azide.[20] The formation of 2,3-DHBA, albeit in small amounts, was not inhibited by azide (data not shown), nor was it stimulated in the presence of D_2O.[10] It is very likely that 2,3-DHBA is formed via direct interaction between the triplet state and SA, as reported by Rizzuto and Spikes.[21]

Stoichiometry between O_2 Consumption and 2,5-DHBA Formation. As discussed under Materials and Methods, ESR oximetry (using the spin probe CTPO) was employed to measure O_2 consumption during visible irradiation of RB and SA in air. The spin probe CTPO (~100 μM) did not have any effect on 2,5-DHBA formation. When an aqueous solution containing RB (10 μg/mL), SA (10 mM), and CTPO (100 μM) was irradiated with visible light, a steady uptake of O_2 was observed. Figure 3 shows the temporal relationship between O_2 consumption and 2,5-DHBA formation. The stoichiometry between 2,5-DHBA formation and O_2 consumption was measured to be ca. 0.57, again indicating that 2,5-DHBA is the major product formed during the reaction between $^1O_2$ and SA. Figure 3 also shows that, when the system became anaerobic, formation of 2,5-DHBA plateaued, indicating that the continued production of $^1O_2$ is essential to formation of 2,5-DHBA.

After 20 min of irradiation, the amount of O_2 consumed was calculated to be 194 μM (Table I). In D_2O buffer, the rate of O_2 uptake was increased by 2-fold (Table I). Azide decreased the O_2 uptake by 2-fold (Table I). Catalase did not have an appreciable effect on O_2 uptake (Table I). The effects of these agents on O_2 uptake paralleled those observed for 2,5-DHBA formation (Table I).

Measurement of the Rate Constant between $^1O_2$ and SA. The O_2 uptake measured during irradiation of RB and SA increased with increasing concentration of SA and plateaued at approximately 50 mM of SA (data not shown). A double-reciprocal plot between $(d[O_2]/dt)^{-1}$ and $[SA]^{-1}$ was constructed (data not shown), and from the ratio of slope to intercept (i.e., β value), the rate constant ($k_q + k_r$) for total quenching of $^1O_2$ was calculated (Table II, see also Materials and Methods). The rate constant

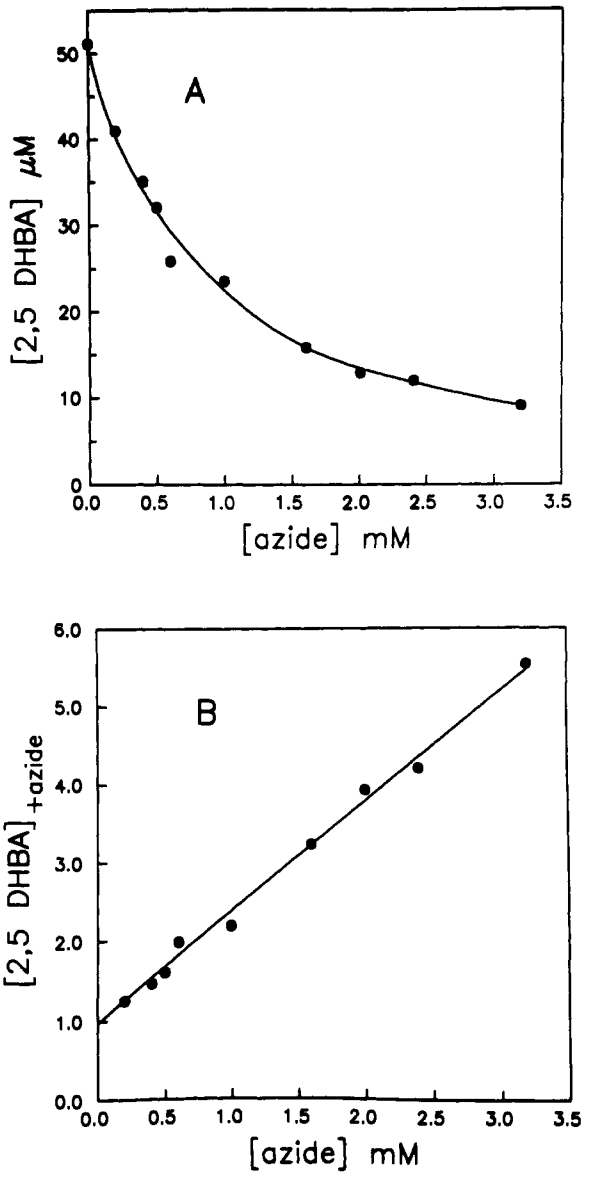

Figure 2. Effects of azide on 2,5-DHBA formation. (A) Solutions containing RB (10 μg/mL), SA (20 mM), and various concentrations of azide irradiated at room temperature with visible light of wavelengths greater than 345 nm for 10 min. A 20-μL aliquot of the reaction mixture was analyzed by HPLC–EC. (B) A Stern–Volmer plot of [2,5-DHBA] produced with and without azide vs [azide].

for chemical quenching was then calculated by comparison with the O_2 uptake observed with histidine (eq 5). Similarly, rate constants for the reaction between $^1O_2$ and SA were also obtained using MC540 or AlPcTS as a sensitizer (Table II). These rate constants are reasonably independent of the sensitizers used and are in agreement with the literature values.[22]

HPLC–EC Analysis of Products Formed during Thermolysis of $NDPO_2$ and Salicylic Acid. Next we demonstrated that 2,5-DHBA is produced as the major product during the reaction between $^1O_2$ and SA, using a purely chemical system. Figure 4A,B shows the HPLC chromatograms obtained from reaction mixtures containing $NDPO_2$ and SA, incubated at 37 °C. The predominant product was again 2,5-DHBA. Formation of 2,5-DHBA was enhanced in D_2O buffer (Figure 4A) and vastly diminished in the presence of azide (Figure 4B). At these concentrations of SA and $NDPO_2$, the yield of 2,5-DHBA in D_2O buffer was enhanced approximately 8-fold relative to H_2O

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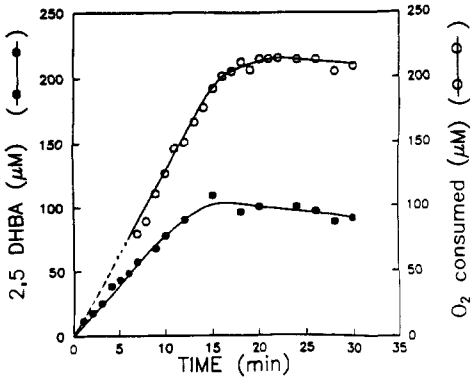

Figure 3. Stoichiometric relationship between the rate of O_2 consumption and 2,5-DHBA formation. A 50-μL sample containing RB (10 μg/mL), CTPO (100 μM), DLPC (1 mM), and SA (20 mM) was taken up in a 100-μL micropipet and placed inside a 4-mm quartz tube which was kept inside the ESR cavity at 37 °C. Samples were irradiated in situ. Oxygen consumption (○) was determined by measuring the resolution of superhyperfine splittings of CTPO as described in Materials and Methods. For comparison, samples were removed at regular intervals and analyzed for 2,5-DHBA (●) by HPLC–EC. The dotted region represents O_2 concentration in which the K parameter could not be measured due to poor resolution. The light intensity incident on the cavity was 50 W/m².

Table II. Physical (k_q) and Chemical (k_r) Quenching Rate Constants of $^1O_2$ and SA

$^1O_2$ generating system	β value[a] (mM)	$10^{-7}(k_r + k_q)$ (M^{-1} s^{-1})	$10^{-5}k_r$[b] (M^{-1} s^{-1})
MC540 (200 μM) in DLPC (20 mM) and SA	12.43	2.01	2.5
RB (10 μg/mL) in DLPC (1 mM) and SA	22.0	1.13	1.4
AlPcTS (100 μg/mL) in DLPC (10 mM) and SA	15.8	1.58	2.1

[a] $\beta = k_d/(k_r + k_q)$ where k_d is the rate constant for decay of $^1O_2$ to $^3O_2$. k_d was taken to be 2.5×10^5 s^{-1}.[14,16] [b] Calculated by comparison of O_2 consumption in the presence of histidine using the value for $(k_r)_{histidine}$ as 2.8×10^7 M^{-1} s^{-1}.[16]

buffer (Figure 4A). For samples containing 10 mM SA and 10 mM $NDPO_2$, a 50% reduction in 2,5-DHBA yield was obtained with 0.5 mM azide and 5 mM quenched 2,5-DHBA production by >95% (Figure 4B). Neither catalase nor superoxide dismutase had any effect on 2,5-DHBA formation (data not shown). Hydroxyl radical scavengers such as formate and ethanol exhibited little or no effect. These results are consistent with $^1O_2$ intermediacy in the formation of 2,5-DHBA.

Discussion

Floyd et al.[1] had originally reported that the reaction between ·OH and SA yields 2,3- and 2,5-DHBA as major products, which can be readily isolated and identified by HPLC–EC. The mechanism of this reaction involves formation of radical intermediates.[5] In accord, the ratios of 2,3-DHBA to 2,5-DHBA varied anywhere between 5 and 1 depending upon the oxygen tension, the nature of the oxidant, and the presence of redox-active metal ions.[5] Ingelman-Sundberg et al.[9] have suggested that 2,5-DHBA can also be formed via enzymatic hydroxylation. Selective formation of 2,5-DHBA during ischemia- and reperfusion-induced myocardial injury has been attributed to such a reaction.[5]

We have previously reported that the reaction between $^1O_2$ and SA yields primarily 2,5-DHBA and relatively little 2,3-DHBA.[10] Our interpretations were based on D_2O stimulation, azide inhibition and lack of effect in the presence of catalase and

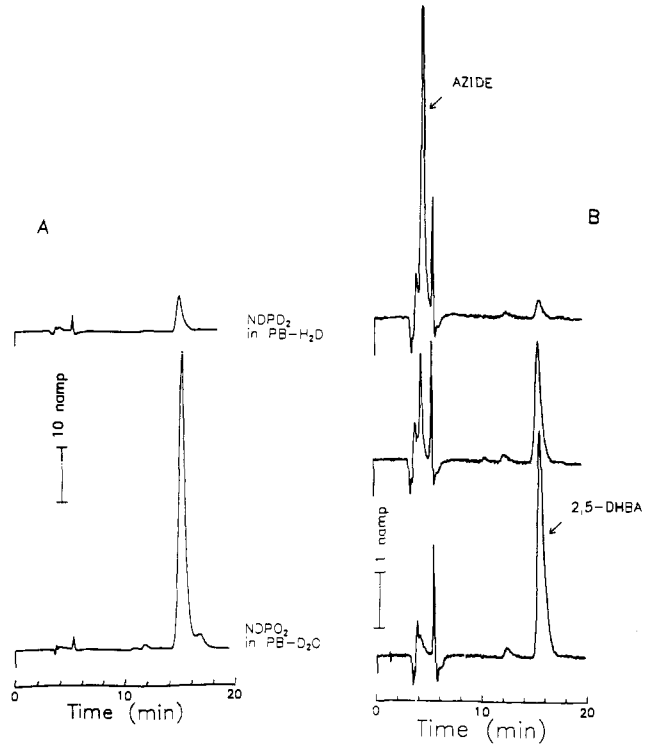

Figure 4. (A) Effects of solvent deuteration on product formation during $NDPO_2$ thermodecomposition: (top) HPLC–EC chromatogram obtained from salicylate trapping in 20 mM phosphate buffered H_2O; (bottom) same as above except in phosphate buffer–D_2O. HPLC–EC chromatograms of products formed during thermolysis of $NDPO_2$ (10 mM) in phosphate buffer (20 mM) in H_2O or in D_2O containing salicylic acid (10 mM) at 37 °C. (B) Effects of azide on product formation during the thermodecomposition of $NDPO_2$: (bottom) HPLC–EC chromatogram of products formed during thermolysis of $NDPO_2$ (10 mM) in aqueous phosphate buffer (pH 7.0, 20 mM) containing salicylic acid (10 mM) at 37 °C; (middle) same as above but in the presence of 0.5 mM sodium azide; (top) same as bottom chromatogram but in the presence of 5 mM azide. The elution rate was 1.0 mL/min. Under these conditions, unreacted $NDPO_2$ elutes at approximately 28 min.

·OH-specific scavengers. In contrast to ·OH addition to SA, which is mediated by a cyclohexadienyl radical, $^1O_2$ reaction with SA is presumably mediated by an endoperoxide intermediate.[22] SA also is an effective physical quencher of $^1O_2$ (Table II), which is in agreement with the literature report for phenols.[23] There is precedence in the literature for the selective formation of 2,5-DHBA in systems producting $^1O_2$. Galaris et al.[24] reported that the reaction between metmyoglobin and hydrogen peroxide in the presence of SA produces predominantly 2,5-DHBA. When cholesterol was used instead of SA, 5α-cholesterol hydroperoxide, which is highly diagnostic of $^1O_2$ formation,[25] was reported as a product. The investigators concluded that either ·OH or $^1O_2$ was produced during the metmyoglobin/H_2O_2 reaction. It is also likely that a higher oxidation state of myoglobin is responsible for the oxidation of SA to 2,5-DHBA.[26] Bruce Davis et al.[27] recently reported hydroxylation of salicylic acid to 2,5-DHBA

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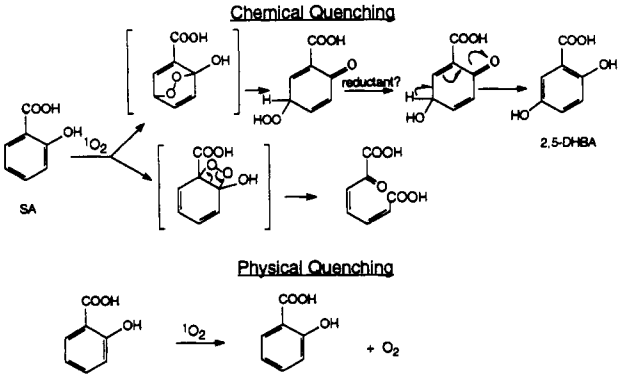
(major product) and 2,3-DHBA (minor product) in the presence of activated neutrophils. Although the exact nature of the oxidant responsible for salicylate hydroxylation was not known, the authors suggested that $^1O_2$ might be involved. Recently, it has been suggested that $^1O_2$ is a mediator of postanoxic reoxygenation injury, as evidenced by the exclusive formation of 2,5-DHBA, which was significantly inhibited by histidine.[28]

The discrepancy in D_2O enhancement between $^1O_2$ generated from chemical and photochemical systems needs to be addressed. Both rose bengal[29] and merocyanine-540[30] were reported to undergo rapid photobleaching mediated by $^1O_2$. It has also been reported that the sensitizer AlPcTS is capable of physical quenching of $^1O_2$ at a rapid rate ($k_q = 10^9$ M^{-1} s^{-1}).[31] Thus, it appears that either a physical or chemical quenching of $^1O_2$ by the sensitizers themselves may be the reason that the D_2O effect is considerably less in photochemical systems as compared with the chemical system. However, the fact that a nearly 8-fold D_2O enhancement in 2,5-DHBA formation is observed in the chemical system proves beyond any doubt the intermediacy of the $^1O_2$ in the present study.

On the basis of the stoichiometry between O_2 consumption and 2,5-DHBA yield (Table II), it is evident that 2,5-DHBA is formed as the major product during the reaction between $^1O_2$ and SA. The carboxylate group in SA is meta-directing and the hydroxyl group ortho- or para-directing. Therefore, one would expect to detect both 2,3- and 2,5-DHBA as a result of $^1O_2$ addition to SA. However, the 2,3-endoperoxide, being very unstable, probably undergoes a ring-cleavage reaction to form an open-chain aldehyde (Scheme II).[32] Further studies, specifically designed to isolate and determine ring-fission products formed during $^1O_2$ reaction with SA are clearly warranted.

Scheme II



The present HPLC–EC assay for 2,5-DHBA formed from selective trapping of $^1O_2$ by SA may be used to monitor $^1O_2$ leakage into the cytosolic compartment during photodynamic reactions in membranes. This assay may be used as a valuable adjunct to the cholesterol product analysis (i.e., formation of the 5α- and 7α,7β-cholesterol hydroperoxides), which measures $^1O_2$ and oxy-radicals formed in membranes.[25]

In conclusion, the present data has clearly established trapping with salicylate as a valuable method for the detection and quantitation of $^1O_2$. Differences in DHBA product profiles, notably the almost exclusive production of 2,5-DHBA from the reaction of $^1O_2$ and SA, allow ready discrimination between $^1O_2$-mediated and $^{\bullet}OH$-mediated reactions. The HPLC methodology employed is relatively routine. The HPLC–EC in combination with ESR oximetry enables one to obtain stoichiometry between O_2 consumption and product formation using exceedingly small amounts of sample. These methods are of particular value in photochemical systems and other instances where there is a need to elucidate the role of activated oxygen species.

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