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Ozone Degrades into Hydroxyl Radical under Physiological Conditions[1]

A SPIN TRAPPING STUDY

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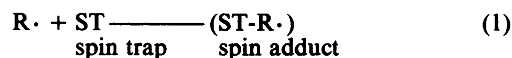
ABSTRACT

Defining the reactants is a critical step towards elucidating the mechanism of ozone toxicity to biomembranes. To document ozone-induced HO• radicals, the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide was used and the resulting spin adduct was monitored with electron spin resonance spectroscopy. Chelexed potassium phosphate buffer (10 millimolar and 0.2 molar) at pH 7.2 and 7.8 was exposed to ozone (1–40 microliters per liter) by directing a stream of ozone over the surface for 60 seconds. Under these conditions, no HO• was detected. Using 0.5×10^{-4} molar caffeic acid in phosphate buffer, strong DMPO•OH electron spin resonance signals were obtained, indicating HO• production. Air controls yielded no signal. High pH (7.8) enhanced signal strength. Furthermore, with sorbitol (0.4 osmolal final concentration), a net HO• signal loss of 28% was observed, while a carbon-centered sorbitol radical adduct appeared. Although HO• radicals were produced, no breakage of *Daucus carota* protoplast plasma membranes was observed nor were differences in membrane fluidity observed as determined by 5-doxyl stearic acid.

Ozone is an important constituent of photochemical air pollution and the resultant damage can readily be observed subsequently as lesions on leaves of many plant species. The mechanism of ozone damage to plants, however, is uncertain. To understand the nature of ozone toxicity, it is imperative to first understand the chemistry of ozone and its decomposition. Based on kinetic data, Hoigné and Bader (13) have reported that ozone decomposes in solution to hydroxyl and superoxide radicals with the superoxide radicals reacting to yield more hydroxyl radicals. Other workers have suggested that H_2O_2 may be another intermediate of ozone decomposition (1, 11, 13, 28). It is generally accepted that these reported free radicals are the reactant species in ozone toxicity (1, 12, 13, 15–17). Evidence for radical production however, usually was obtained under nonphysiological conditions using very high ozone concentrations and acidic (pH 2.0–4.5) or basic (concentrated KOH) conditions (15, 28).

In the present study, mild ozone conditions were used at physiological pH values and ozone concentrations to facilitate the understanding of ozone degradation in biological systems. The short-lived free radicals were identified through the technique of spin trapping (for reviews, see 4, 25) whereby the free radicals were transformed into more persistent parmagnetic species. In this technique, a small quantity of a diamagnetic substance (the spin trap) having a particularly high affinity for free radicals is added to the system. The product of this trapping reaction is a more stable free radical (spin adduct) whose concentration increases to a readily detectable level (> about 10^{-7} to 10^{-8} M). The general reaction is represented by equation 1.



$$\underset{\text{}}{R\cdot} + \underset{\text{spin trap}}{ST} \longrightarrow \underset{\text{spin adduct}}{(ST\text{-}R\cdot)} \qquad (1)$$

If a nitrone or nitroso spin trap is used, a stable nitroxide will be formed which can easily be detected by ESR[4] spectroscopy. Furthermore, the spin adduct has a characteristic set of hyperfine coupling constants which depend on the structure of the trap itself, the nature of the initial unstable radical, and the chemical environment of the spin adduct.

MATERIALS AND METHODS

Chemicals. The spin trap DMPO (Aldrich) was vacuum distilled twice and stored at −70°C under N_2 until used. Two µl (1.8×10^{-4} M final concentration) of DMPO were added to 100 µl of either a K-phosphate (pH 7.2 and 7.8) or dilute HCl (pH 4.5). Unless otherwise indicated, all buffers were passed through a chelex-100 (Bio-Rad) column to remove trace metal impurities, including iron.

Catalase (EC 1.11.1.6) and SOD (EC 1.15.1.1) were obtained from Sigma. Activity of these enzymes was verified by the method of Gregory and Fridovich for catalase (9) and Winterbourn for SOD (30). DETAPAC was a gift from Dr. J. N. Siedow, Duke University.

Ozone Measurement and Spin Trapping. Ozone was generated by passing dry air (calcium sulfate) through an electric spark gap generator. The concentration of ozone in solution was measured by reduction of indigotrisulfonate (Aldrich) (14). Concentrations of the O_3 in the gaseous form and liquid form were presented as µl/l or µg/g, respectively. Both units are equivalent to ppm, but follow the metric system. After all solutions and glassware had been degassed, they were subjected to ozone (5–35 µl/l·min) by blowing the ozone through a capillary tube, thereby streaming it

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[4] Abbreviations: ESR, electron spin resonance; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; SOD, superoxide dismutase; EDU, (*N*-[2-(2-oxy-1-imidazolidinyl)ethyl]-*N*′-phenylurea; DETAPAC, diethylenetriamine pentaacetic acid; 5DS, 5-doxyl stearic acid.

over the surface of the solution for various times. The flow rate was fast enough so that a vortex was created throughout the solution. The sample was then quickly transferrred to the ESR capillary cell and the signal recorded on a Varian E-109B ESR spectrometer equipped with a TE231-2 cavity. The ESR spectra were run under the following conditions: room temperature, 14 to 16 dB microwave power, 9.52 GHz microwave frequency, 100 G scan range, 2 G modulation, 0.25 s time constant, 4 to 8 min scan time, and a variable gain usually at 2×10^4. Each experiment was performed at least seven times, on different days, and consistent results were obtained.

UV light from a mercury lamp and 3% H_2O_2 were used to generate HO· and superoxide by shining the UV beam onto the ESR spectrometer cell and recording the signal. Radical concentration was estimated by comparing the spin trap signal to a 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-yloxy (Aldrich) standard at 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} M concentration at instrument settings as above. Increasing the radical concentration yields a stronger signal or higher amplitude, hence the relative peak heights are a reflection of the free radical concentration. This technique is sensitive down to 10^{-7} M free radical concentration.

HPLC was performed on a Waters 6000A solvent delivery system equipped with a 254 nm fixed wavelength detector and a Waters C18 column. For separation of the phenolic compounds, methanol:acetic acid:ethylacetate:H_2O (25:2:1:72) was used as the eluant. Peak areas were integrated against an internal standard to yield quantitative changes in phenolic compounds.

Protoplast Isolation. *Daucus carota* L. cells of two types were maintained on either a modified Murashige and Skoog medium with 1 mg/l naphthaleneacetic acid and 0.2 mg/l kinetin (5) for the larger (32 μm diameter) cells or modified Whites medium with 0.5 mg/l 2,4-D (29). Protoplasts from these latter cells were smaller (14 μm diameter) and hence yielded better ESR resolution. Cells were transferred every 6 d and used while in log phase (3–4 d) of growth. Protoplasts were isolated in 2% Driselase (lot 051), 0.4 molal sorbitol, and 1 mM Mes at pH 5.8 for about 3 h. After release, the protoplasts were washed three times by suspending in 0.4 molal sorbitol/1 mM Mes (pH 5.8) and centrifuging at 40*g*. Both protoplast types responded to ozone in a similar fashion.

Spin Labeling. Washed protoplasts were spin labeled with 5DS (3 μg label/0.1 ml packed volume) while in sorbitol/Mes at pH 6.0 unless otherwise indicated (3). Exposure to ozone was performed both before and after addition of the spin label as indicated in the results section.

RESULTS

Ozone Degradation. No free radicals were detected after passing ozone (8 μl/l) over a simple aqueous solution consisting of 10 mM K-phosphate and DMPO (pH 7.2) (Fig. 1a). Increasing the buffer concentration to 200 mM did not result in a signal, nor was it found to alter results in other experiments significantly. Hence, 10 mM buffer was used. A weak DMPO·OH ESR signal was obtained if the ozone was bubbled into this solution for 90 s. If, however, the phosphate buffer/DMPO solution was mixed 1:1 with 10^{-4} M caffeic acid, phenol, ferulic acid, or cinnamic acid, a DMPO·OH adduct was easily detectable after simply blowing the ozone over the solution for 15 to 30 s. Strong ESR signals were obtained after 60 s of blowing ozone over the solution which resulted in 0.3 μg/g dissolved ozone (Fig. 1b). Inasmuch as similar results were obtained from any of these phenolic compounds, caffeic acid was used for the remainder of the experiments. The lowest concentration of caffeic acid which gave a detectable DMPO·OH signal when ozone was blown over the solution was 0.5×10^{-6} M caffeic acid. HPLC data, using 0.5×10^{-3} M caffeic acid, indicated that only 10% of the caffeic acid was degraded under these conditions (data not shown). Also, no significant change in pH was observed, again indicating no major abstraction of the caffeic acid side-chain. Air controls gave no free radical signal nor any loss of caffeic acid. Hence, phenolic compounds dramatically enhanced the ozone induction of HO·radicals. The fact that the observed 1:2:2:1 signal was due to trapped HO· was substantiated by forming secondary radicals. When the HO· radical is formed in solution, it will react with other compounds

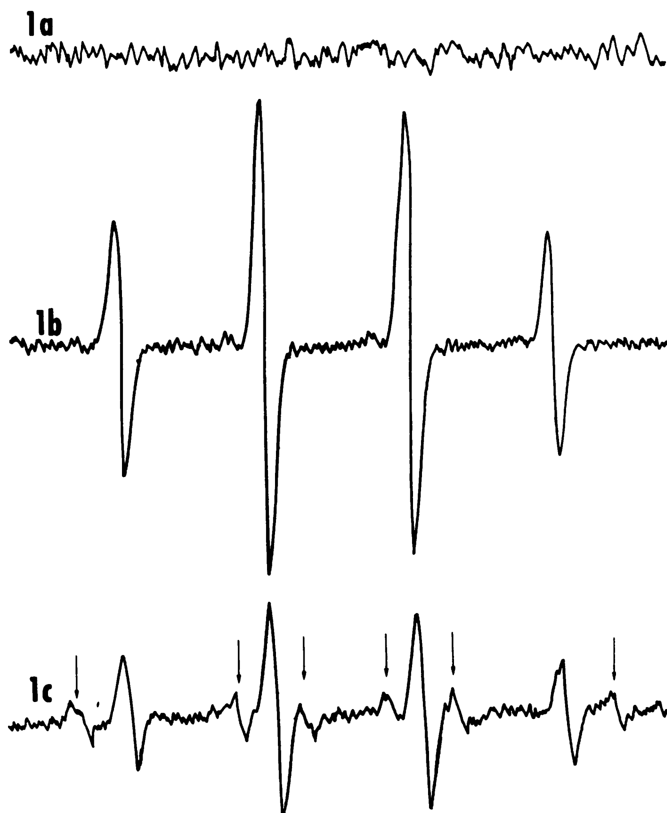

FIG. 1. First derivative ESR spectra of the spin-trapped free radicals produced when ozone is blown over a 10 mM phosphate buffer (pH 7.2). a, Typical noise generated when no radicals were produced. Ozone was blown over the phosphate buffer but no caffeic acid was present. b, 1:2:2:1 spectrum obtained with 0.5×10^{-4} M caffeic acid present and 60 s of ozone exposure (0.3 μg/g dissolved ozone). c, Spectrum obtained under same conditions as 'b', but with 0.45 M sorbitol present. Arrows indicate a separate six-line spectrum which is a sorbitol adduct.

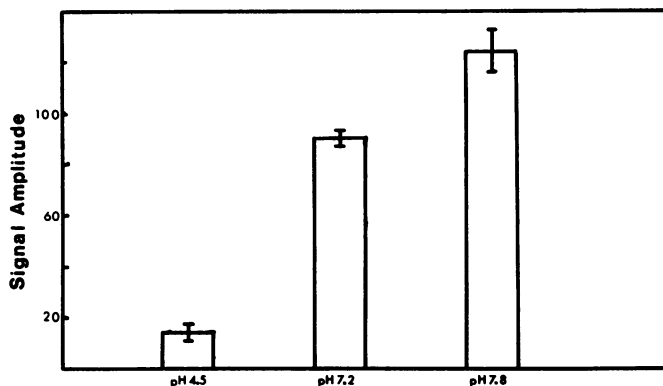

FIG. 2. Effects of various pH values on DMPO·OH signal production. Data for pH 4.5 was obtained in a dilute HCl solution while pH 7.2 and pH 7.8 were obtained with a 10 mM phosphate buffer. SD is indicated by error bars. Signal amplitude refers to the height of the second peak of the 1:2:2:1 signal. This signal height is proportional to the concentration of radical trapped, see "Materials and Methods."

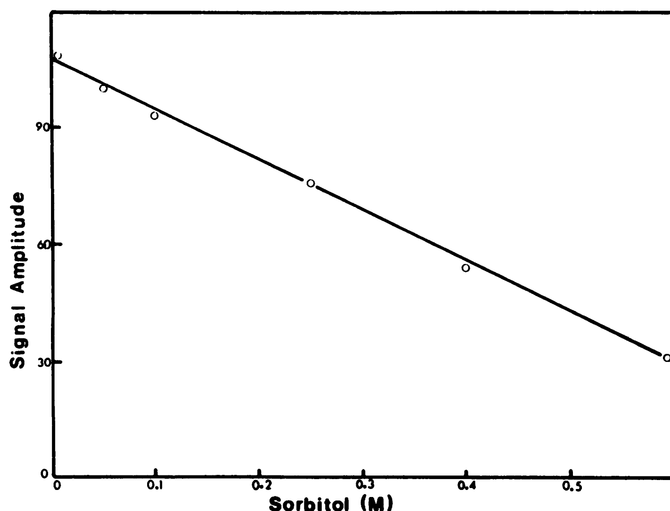

FIG. 3. Effect of increasing sorbitol concentration on relative amounts of DMPO·OH radical adduct produced. Reaction conditions were 10 mM phosphate buffer (pH 7.2)/DMPO/0.5 × 10^{-4} M caffeic acid with various concentrations of sorbitol subjected to ozone (5 μl/l) for 60 s.

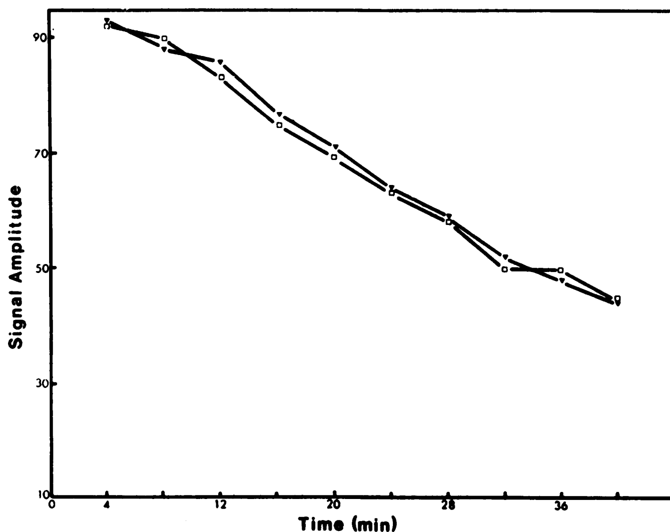

FIG. 4. Rate of degradation of the 5DS spin label *versus* time. Protoplasts were labeled with the 5DS spin label, subjected to 30 min of ozone (5 μl/l), and placed in the ESR cavity. (▼), Ozone-treated protoplasts; (□), air control.

such as methanol, ethanol, and azide to form further free radicals. These secondary radicals can also be trapped by DMPO. When either ethanol, methanol, or azide was added to phosphate buffer/caffeic acid/DMPO solutions, typical carbon-centered (ethanol, methanol) (6) or nitrogen-centered (NaN_3) (18) DMPO adducts were observed (data not shown).

The acidity of the aqueous environment affected production of HO· radicals (Fig. 2). Higher pH values could not be examined due to degradation of the DMPO trap above pH 8.0. The decreased signal height at low pH was not due to inability of DMPO to trap HO· at low pH since experiments performed with DMPO trapping of HO· generated from 3% H_2O_2 and exposed to UV light at pH 4.5 yielded a strong DMPO·OH signal (data not shown). Furthermore, when the DMPO was analyzed optically at A_{234}, no significant degradation was observed after 30 min. Water purification data (13) suggested that ozone was more stable in solution at low pH, hence eight separate 8-min time scans of the phosphate/caffeic acid/DMPO were performed after treatment with ozone. No increase in the DMPO·OH signal height was observed after 60 min (data not shown), thus indicating acidic conditions reduced the quantity of HO· radicals induced by ozone. These data should be considered when studying the interactions of acid rain and ozone on crop productivity.

Superoxide and H_2O_2 are reportedly degradation products of ozone (13). If they are major intermediates of ozone decomposition, then SOD or catalase should decrease the DMPO·OH signal strength. Either SOD or catalase (both at 0.1 mg/ml final concentration) was added to the phosphate/caffeic acid/DMPO solution and the ozone-induced HO· measured. BSA (0.1 mg/ml) was used as a control. All of these proteins reduced the DMPO·OH signal height approximately 10% (data not shown). This 10% signal loss was not significant within the limits of the assay. Also, inasmuch as BSA reduced DMPO·OH signal height to the same extent as SOD and catalase, we concluded that superoxide and H_2O_2 were not major intermediates in HO· production.

EDU, a compound which has been shown to reduce ozone damage to bean plants (20) and a possible free radical scavenger (19), was added to the phosphate/caffeic acid/DMPO solution at a final concentration of 1 mg/ml. This treatment resulted in no significant signal loss (data not shown) indicating that EDU is not a HO· scavenger.

Dugger and Ting (5) have reported that plants sprayed with free sugars exhibit reduced ozone damage. Hence, it was decided to investigate the effect of sorbitol, glucose, and sucrose on ozone decomposition. All sugars gave similar results. Figure 3 demonstrates that sorbitol (0.6 M) resulted in approximately a 50% reduction in relative DMPO·OH peak height. As the concentration of sorbitol was increased, a six-line spectrum appeared and became more prominent at higher sorbitol concentrations (Fig. 1c). This six-line spectrum showed hyperfine splittings of A_N = 15.9 G and A_H = 22.5 G and was observed only when sorbitol was present. Addition of 1 mM DETAPAC to the phosphate/caffeic acid/sorbitol/DMPO solution showed no reduction of the six-line spectrum, suggesting that it was not due to iron-catalyzed formation of the DMPO-dimer (7). When higher amounts of HO· were generated from HCl (pH 4.5)/3% H_2O_2/sorbitol/DMPO and UV light, the six-line spectrum became more pronounced (data not shown) while the DMPO·OH signal decreased. Attempts to trap this radical with 2-methyl-2-nitrosopropane were unsuccessful.

Ozone Interaction with Membranes *In Vivo*. Initially, experiments were performed to determine if ozone exposure would damage the protoplast plasma membrane enough to result in observable changes or breakage. Protoplasts isolated from carrot suspension culture cells were exposed to gaseous ozone (40 μl/l) for up to 3 h. Protoplasts were incubated in 0.45 M sorbitol for these treatments. Ozone in solution was assayed by indigotrisulfonate reduction to be 0.1 μg/g. No visible change in the protoplasts was observed. Neutral red and fluorescein diacetate staining indicated that 95% of the protoplasts remained viable.

Caffeic acid (10^{-5} M) was added to enhance free radical presence. Controls demonstrated that this level of caffeic acid had no significant effect on the protoplasts during the time course of the experiment. After treating with gaseous ozone, the HO· concentration was estimated to be 10^{-5} M, as described in "Materials and Methods." Again, no observable change in the protoplasts occurred, nor did viability decrease.

In order to increase the concentration of ozone and HO· in solution, ozone was bubbled into ionic medium (250 mM KCl, 20 mM $MgCl_2$, 10 mM K-phosphate, pH 6.0) for 2 to 4 h. Ozone concentration in solution was 10 μg/g. With caffeic acid present, the HO· concentration was estimated to be 10^{-4} M. Washed protoplasts were suspended in the ionic medium with and without ozone, and with and without HO· for up to 60 min. No visible change nor breakage was observed as a result of any of these

treatments. Neutral red and fluorescein diacetate staining demonstrated that approximately 95% of protoplasts from all treatments remained viable. Furthermore, respiration, as measured on a Clark electrode, showed no detectable changes after 60 min of ozone exposure.

To understand better the HO· radical or ozone capacity to penetrate the membrane bilayer, ESR studies were undertaken to determine membrane fluidity changes using the 5DS fatty acid spin label. The doxyl group was on the fifth carbon of the fatty acid spin label and probes the glycerol backbone region close to the surface of the plasma membrane. Protoplasts were suspended in ionic medium with and without ozone (5 μg/g) for 30 min and washed two times. The final wash was sorbitol/Mes (pH 6.0), and the supernatant was removed. The sorbitol wash was necessary for efficient labeling with 5DS. Maximum hyperfine splitting as determined with 5DS was $2A_{max} = 51.5 \pm 1$ G and was not altered by the ozone treatment (data not shown).

If ozone or its degradation products penetrate the membrane bilayer *in vivo*, then a change in the 5DS label signal amplitude should be evident as a result of ozone treatment. To test this hypothesis, protoplasts were labeled with 5DS and then exposed to ozone (0.3 μg/g) in solution for 30 min. In separate experiments, the 5DS label was added before and after the ozone treatment. Also, the caffeic acid was added on several trials to enhance HO· concentration as opposed to ozone. Figure 4 shows one representative experiment of label degradation with time. As can be seen, the 5DS probe was not oxidized at a faster rate with ozone or the air control. These data strongly implied that ozone, or the free radical degradation products, did not penetrate significantly the lipid bilayer in the region of the spin label fatty acid after 30 min exposure.

DISCUSSION

When determining the chemistry of ozone degradation, it was decided to pass ozone over the surface of the aqueous solution, thereby mimicking field conditions. Ozone decomposition into HO· radical could be detected with no phenolics present only if ozone was bubbled directly into the solution. With phenolic compounds present, however, the DMPO·OH signal was detected by simply blowing the ozone over the surface of the solution. Phenolic compounds, then, played a major role in the catalyzing the breakdown of ozone.

Although superoxide may be an intermediate in ozone degradation, it was not detected down to the 10^{-7} M detection limit of this technique. Furthermore, SOD did not significantly affect the production of HO· radical providing evidence that superoxide, or its protonated form, was not a major intermediate in this system. Since catalase also did not affect DMPO·OH signal strength, we concluded that H_2O_2 was not a significant intermediate of ozone decomposition in phosphate buffer. This datum supports earlier data of Heath (11). Hence, either ozone or HO· radical should be considered as primary toxic agents in ozone injury.

The sugar sorbitol significantly reduced the strength of the DMPO·OH signal. This datum offers one possible explanation for the observations of Dugger and Ting (5) and Amthor and Fong (2) that increased sugar levels on field plants reduced the amount of ozone damage.

Furthermore, sorbitol reacted with the HO· radical to form a carbon-centered radical. We assign the observed six-line spectrum of the DMPO adduct to a carbon-centered DMPO-sorbitol adduct because: (a) the splittings are similar to other known carbon-centered DMPO adduct splittings such as DMPO-ethanol; (b) the same six-line spectrum has been generated with sorbitol/H_2O_2/DMPO and UV light; (c) the spectrum was observed only when sugars were present; (d) as the concentration of sorbitol was increased, the six-line spectrum became more prominent while the DMPO·OH spectrum was diminished; and (e) the addition of DETAPAC to chelate Fe^{2+} did not affect the six-line spectrum, nor did the hyperfine splittings correspond to those of the Fe^{2+}-catalyzed DMPO dimer (7). The addition of the HO· radical to the sorbitol was nonspecific, meaning that the free electron could have attached to any of the carbon atoms (8). Whether sugar acts as a free radical terminator (or trap) or simply as an intermediate for further free radical transfer is unknown at this time. The possibility is raised, however, that the cell wall may be a site of extensive free radical chemistry during ozone exposure.

Attempts to injure carrot suspension cells or protoplasts proved unsuccessful. Tobacco and bean mesophyll protoplasts were also resistant to ozone damage (24). Conditions were such that *Daucus* protoplasts were subjected to at least 10^{-5} M HO· radical and 0.3 to 40 μl/l ozone. To increase the level of resolution for assaying ozone damage, the spin label 5DS was added to the protoplasts. This method also failed to show any damage due to ozone. This datum would seem to be in conflict with data obtained by many workers (10, 21–23, 26) who have shown that free lipids or microsomal fractions were sensitive to ozone-induced oxidation. It must be remembered that this system is an intact protoplast as opposed to free lipids or microsomal fractions, and also that the spin probe may not react in the same manner as membrane phospholipids to oxidation.

In summary, it has been shown that in aqueous solution ozone decomposes into primarily HO· radical and that phenolic compounds enhance this decomposition. Furthermore, by manipulating the chemical conditions of the system one can control the amount of decomposition occurring and, indeed, whether ozone or HO· is the prevalent component in the system. This should be helpful in gaining insight as to whether ozone or its decomposition products are the toxic agents in ozone damage.

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