SEMI-QUANTITATIVE DETECTION OF FREE RADICALS USING SPIN TRAPPING AND PHOSPHORUS-31 NMR SPECTROSCOPY

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ABSTRACT

In this study the effectiveness of quantitative ^{31}P nuclear magnetic resonance (NMR) spectroscopy in conjunction with spin trapping to detect and quantify various free radical species was investigated. Initially the research was focussed on assigning the ^{31}P NMR signals for hydroxyl and superoxide radicals and on identifying the quantitative reliability of the technique. The radical adduct reaction products (diamagnetic species), of hydroxyl ($HO^{\bullet}$) and superoxide ($O_2^{-\bullet}$) radicals with phosphorus containing nitroxides, were determined to be relatively stable and as a result the ^{31}P NMR chemical shifts for these species were determined. Their structures were verified and quantified using ^{31}P NMR in the presence of a phosphorus containing internal standard. The ^{31}P NMR chemical shifts for 5- diisopropylphosphoryl-5-methyl-1-pyrroline-N-oxide (DIPPMPO) and 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO) spin traps with a variety of carbon centered radicals were also determined.

A novel equilibrium reaction involving molecular oxygen and alkaline hydrogen peroxide was investigated and it was found that the concentration of superoxide radicals present in a system of alkaline hydrogen peroxide is affected by the nature of gas with which it is in equilibrium. The concentration of superoxide radicals was determined to be significantly larger than that of hydroxyl radicals at elevated pH values.

Experiments have shown that NMR in conjunction with spin trapping can be a valuable tool for the detection and quantification of radical species.

RESUME

Cette étude examine la détection et la quantification de divers radicaux libres par le moyen de la spectroscopie de résonance magnétique nucléaire (RMN) combinée avec "spin trapping". Au départ, ce projet visait à identifier les signaux de RMN ^{31}P des radicaux hydroxyle et superoxyde, et à étudier la fiabilité quantitative de la méthode. Les produits de la réaction entre les radicaux hydroxyle ($HO^{\bullet}$) et superoxyde ($O_2^{-\bullet}$) et des nitroxydes comportant un atome de phosphore se sont avérés relativement stables. En conséquence, les résonances chimiques pour ces espèces ont été déterminées. Leur structure a été vérifiée et quantifiée par RMN ^{31}P en présence d'un étalon interne comportant un atome de phosphore. Les résonances chimiques RMN ^{31}P des "spin traps" de 5-diisopropylphosphoryl-5-methyl-1-pyrroline-N-oxide (DIPPMPO) et de 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO) avec divers radicaux de carbone ont aussi été déterminés.

Une nouvelle réaction d'équilibre qui met en jeu l'oxygène moléculaire et le peroxyde d'hydrogène à pH élevé a été examinée. Les résultats montrent que la nature du gaz en contact avec le peroxyde influence la concentration de radicaux superoxyde présents dans le système. Par ailleurs, la concentration de radicaux superoxyde est supérieure à celle des radicaux hydroxyle, en particulier à des pH élevés.

Ces expériences ont donc montré que la RMN en combinaison avec "spin trapping" est une méthode valable pour la détection et la quantification de radicaux.

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CHAPTER 1

SCOPE AND AIM OF THESIS

To date there have been various methods used to detect and quantify free radical species. Electron paramagnetic resonance (EPR) spectroscopy has been the main method for free radical detection. Although the information that is provided by this spectroscopic technique has been invaluable, the short lifetime of free radical species is a major limitation of this technique. The objective of this thesis is to use phosphorus containing spin traps to detect and quantify various free radical species using ^{31}P NMR spectroscopy.

In order to detect these free radical species the phosphorus containing spin traps DEPMPO and DIPPMPO were employed. The free radical species examined in this work were generated either by ultraviolet irradiation of a variety of solutions, or by reacting the spin trap in buffered solutions of hydrogen peroxide. Quantitative information was obtained through the use of an internal standard and ^{31}P NMR spectroscopy.

This thesis commences with an introduction about the important oxygen-centred free radicals and then discusses the most common methods of free radical detection. A brief discussion as to the importance of free radical detection for biology, medicine and the pulp and paper industry is also presented. Chapter 3 outlines the reagents and the procedures used to synthesise spin traps as well as to generate, trap and obtain quantitative information about a variety of free radical species.

Chapter 4 begins with a discussion about the synthesis of the DIPPMPO spin trap, followed by a brief summary of the similarities in the electronics of the two spin traps

used in this thesis. The spin trapping of important oxygen centred radicals, hydroxyl and superoxide radicals, as well as a variety of carbon centred free radicals is then presented. The quantitative reliability of the NMR-spin trapping technique, when ultraviolet irradiation was used as a free radical generator is also discussed in this chapter. Chapter 4 provides initial evidence into the significance of an equilibrium reaction between hydrogen peroxide and oxygen. This chapter also describes the effect of pH on the concentration of hydroxyl and superoxide radicals. Chapter 5 presents an application of the NMR/spin trapping technique to a complex biological system. More specifically, the technique was used to detect the formation of hydroxyl radicals in astrocytes transfected with the HO-1 gene. Chapter 6 summarises the conclusions of this thesis while Chapter 7 provides suggestions for future work.

CHAPTER 2

INTRODUCTION

2.1 FREE RADICALS

A free radical species is defined as "a highly reactive chemical species that contains an odd number of valence electrons and thus has a single, unpaired electron in one of its orbitals."[1] Free radicals are classified according to their kinetic properties as well as orbital occupation of the odd electron on the radical.[2] Kinetically, radicals can be transient or persistent. Transient free radicals undergo bimolecular self-reactions at the diffusion-controlled limit, whereas persistent radicals are radicals that have kinetic stability. Radical species are also characterised as either π or σ species depending upon where the odd electron is located. Pi-localized radicals have an unpaired electron localized in a single *p* orbital, while in π delocalized radicals the unpaired electron can delocalize to neighbouring *p* orbitals. Sigma radicals have unpaired electrons in an *sp* or sp^2 orbital[2].

There are three main types of "radical reactions": 1) reactions that result in radical formation, 2) reactions in which the radical site is transferred, and 3) reactions in which radicals are destroyed.[3] Radical formation involves the cleavage of two-electron bonds (e.g. peroxide dissociation). Radicals can be destroyed via combination reactions with other radicals (e.g. $2\ Cl^{\bullet} \rightarrow Cl_2$) or though disproportionation reactions (i.e. $2\ CH_3{-}CH_2^{\bullet} \rightarrow CH_3{-}CH_3 + CH_2{=}CH_2$). Disproportion is the process in which a hydrogen atom is transferred from one radical to another radical.[4]

2.1.1 Significance and Chemistry of Hydroxyl Radicals

The hydroxyl radical is the most reactive oxygen centred radical. Radiation chemists performed the earliest studies of this radical. The most important methods of generating hydroxyl radicals are from hydrogen peroxide by either bond homolysis using ultraviolet light or by electron capture.[5] The hydroxyl radical has a high electron affinity and its reactions are often referred to as 'electrophilic' in nature.[5]

2.1.1.1 Generation of Hydroxyl Radicals

Ionizing Radiation

The hydroxyl radical can be formed from water by electron loss using ionizing radiation. In this process the oxygen-hydrogen covalent bond is fragmented leaving a single electron on hydrogen and one on oxygen (Equation 1).[6]

$$H-O-H \xrightarrow{radiation} H^{\bullet} + {}^{\bullet}OH \qquad \text{Equation 1}$$

Fenton Reaction

The Fenton reaction refers to the generation of hydroxyl radicals from hydrogen peroxide by metal containing reducing agents.[7] Equation 2 gives the Fenton reaction:

$$M^{2+} + H_2O_2 \rightarrow M^{3+} + {}^{\bullet}OH + OH^{-} \qquad \text{Equation 2}$$

In the original Fenton reaction M^{2+} was iron, however variants of the reaction exist in which the ferrous ions are replaced by cuprous or titanous ions.[8] In the typical Fenton reaction, high concentrations of hydrogen peroxide and ferrous ions are used in acidic media. Fenton reactions carried out at neutral pH suffer from the precipitation of the iron salts to form hydroxides. In order to alleviate this problem metal ion chelators such as ethylenediaminetetraacetate (EDTA) or diethylenetriaminepentaacetate (DTPA) are needed.

Photochemical Generation of Hydroxyl Radicals

Hydroxyl radicals can also be generated by the vacuum-UV photolysis of alkaline water.[9]

$$H_2O \xrightarrow{h\nu} H^{\bullet} + {}^{\bullet}OH \qquad \text{Equation 3}$$

Another method that can be employed to generate •OH is the photolysis of H_2O_2. The photolysis wavelength needed to generate •OH from hydrogen peroxide is very accessible since hydrogen peroxide has a broad absorption band that extends into the red ~360 nm, with mercury lamps irradiating at 253.7 nm.[9]

$$H_2O_2 \xrightarrow{h\nu} 2{}^{\bullet}OH \qquad \text{Equation 4}$$

2.1.2 Significance and Chemistry of Superoxide/Hydroperoxyl Radicals

Marshall[10] first proposed the presence of the hydroperoxyl radical in the gas phase in 1926. A few years later, in 1931 Haber and Willstatter[11] first proposed its presence in aqueous solution. They proposed that the hydroperoxyl radical was an intermediate in the decomposition of hydrogen peroxide by the enzyme catalase. In 1934, potassium superoxide (KO_2) was found to be very paramagnetic and composed of K^+ and $O_2^{\bullet-}$ ions.[12] Weiss subsequently noted that in aqueous solution $O_2^{\bullet-}$ would be in the basic form of $HOO^{\bullet}$.[13]

Superoxide is a relatively small univalent anion with an O-O bond distance that is intermediate between that of molecular oxygen and hydrogen peroxide. Superoxide forms strong hydrogen bonds with water and is a powerful nucleophile only in non-hydrogen bonding media.[14] The most dominant reaction of superoxide is its ability to act as a Bronsted base. It can remove protons from water and weakly acidic substrates, which results in its disproportionation to give molecular oxygen and hydrogen peroxide (Equation 5). In aqueous media at pH 7 the equilibrium is far to the right. Superoxide/hydroperoxyl radicals are not very reactive towards organic compounds when in aqueous solution, the exception being quinones, which react rapidly with superoxide.[9] However, in aprotic solvents the superoxide radical is very stable since disproportionation to give the dianion, $O_2^{\bullet-}$ is very unfavorable.[14]

$$2O_2^{\bullet-} + 2H^+ \leftrightarrow O_2 + H_2O_2 \qquad \text{Equation 5}$$

2.1.2.1 Generation of Superoxide/Hydroperoxyl Radicals

Photochemical Generation of Superoxide/Hydroperoxyl Radicals

$O_2^{\bullet-}$/HOO$^\bullet$ can be produced by the photolysis of an aqueous solution. One method is by the vacuum-UV photolysis of water at alkaline pH.[9] In this method the energy of the photons from photolysis converts the water molecules into H$^\bullet$ and $^\bullet$OH:

$$H_2O \xrightarrow{h\nu} H^\bullet + {}^\bullet OH \qquad \text{Equation 6}$$

In the presence of O_2 and alcohols, the primary radicals produced (H$^\bullet$and $^\bullet$OH) can be used to generate $O_2^{\bullet-}$/HOO$^\bullet$as follows:

$$\begin{aligned} &H^\bullet + O_2 \rightarrow HOO^\bullet \\ &{}^\bullet OH + HCO_2^- \rightarrow CO_2^{\bullet-} + H_2O \\ &CO_2^{\bullet-} + O_2 \rightarrow O_2^{\bullet-} + CO_2 \end{aligned} \qquad \text{Equation Set 7}$$

Superoxide/hydroperoxyl radicals can also be produced photolytically using ketones, such as benzophenone or acetone. This procedure described by McDowell[15] and coworkers involves the excitation of ketones to the n-π* state. This photoexcited state is quenched by reaction with either primary of secondary alcohols. An advantage of this technique to generate radicals is that it is carried out at higher wavelengths (lower energy) than vacuum-UV photolysis, therefore mercury lamps or sunlight may be used.

$$(CH_3)_2CO \xrightarrow{h\nu} {}^3(CH_3)_2CO$$
$${}^3(CH_3)_2CO + CH_3CH_2OH \rightarrow (CH_3)_2{}^{\bullet}COH + CH_3{}^{\bullet}CHOH$$
$$O_2 + (CH_3)_2{}^{\bullet}COH \rightarrow (CH_3)_2C(O_2^{\bullet})OH$$
$$O_2 + CH_3{}^{\bullet}CHOH \rightarrow CH_3C(O_2^{\bullet})HOH$$ Equation Set 8
$$OH^- + (CH_3)_2C(O_2^{\bullet})OH \rightarrow O_2^{\bullet -} + (CH_3)_2CO + H_2O$$
$$OH^- + CH_3C(O_2^{\bullet})HOH \rightarrow O_2^{\bullet -} + CH_3CHO + H_2O$$

Also, as shown previously (Equation 4) photolysis of hydrogen peroxide by UV light results in the splitting of the H_2O_2 molecule into two hydroxyl radicals. The hydroxyl radicals generated in this process can then lead to the generation of $O^{\bullet -}{}_2$/$HOO^{\bullet}$.

$$HO^{\bullet} + H_2O_2 \rightarrow H_2O + HOO^{\bullet}$$ Equation 9

Electrolysis

Stable solutions of $O_2^{\bullet -}$ can be prepared by electrolysis of oxygen in liquid ammonia and in aprotic organic solvents[16,17], however in water the reduction of oxygen does not generate superoxide but goes all the way to hydrogen peroxide or even water. However, in alkaline solutions of surface-active materials molecular oxygen can be reduced to superoxide radical[18]. In situations where there is no surfactant available, the $O_2^{\bullet -}$ formed at the electrode can pick up a second electron from the cathode and with a proton from the water form HOO^-:[18]

$$H_2O + O_2^{\bullet -} + e^- \rightarrow HOO^- + {}^-OH$$ Equation 10

Chemical Generation of Superoxide/Hydroperoxyl Radicals

Potassium Superoxide (KO_2)

Potassium superoxide (KO_2) in aprotic solvents can be used as a source of generating superoxide radicals. Stable solutions of $O_2^{\bullet-}$ in aprotic solvents such as dimethyl sulfoxide (DMSO), which contains hydrogen but cannot donate protons to other substances, has been reported.[16] Aprotic solutions are often used due to the rapid disproportionation of superoxide radical in aqueous solutions.[9] Crown ethers are often used to solubilize KO_2 and allow for the preparation of relatively concentrated solutions prepared in this manner.

KO_2 can be dissolved in aqueous solutions at a pH above 9.[9] Metal chelators such as ethylenediaminetetraacetate (EDTA) or diethylenetriaminetetraacetate (DTPA) along with near freezing temperatures are required to stabilise the superoxide radical.[9]

Enzymatic Generation by Xanthine/Xanthine Oxidase

The most common method of generating superoxide radicals is by using the enzymatic system, xanthine/xanthine oxidase. The mechanism of this reaction involves the oxidation of xanthine by xanthine oxidase.[9] The reduced xanthine oxidase then loses electrons via two different pathways (Equation Set 11), a univalent reaction producing superoxide radicals and a divalent reaction, which leads directly to hydrogen peroxide formation.[7] The end result is the production of two superoxide radicals for every molecule of xanthine oxidase that is reduced.

$$Enzyme - H_2 + 2O_2 \rightarrow Enzyme + 2H^+ + 2O_2^{\bullet -}$$
$$Enzyme - H_2 + O_2 \rightarrow Enzyme + H_2O_2$$

Equation Set 11

2.2 DETECTION OF RADICALS

The magnetic property of free radical species, due to their unpaired electron, is a property that can be used for their detection. Unlike most species, which are diamagnetic, free radicals are paramagnetic. Diamagnetic species when placed in a magnetic exert a force to move out of the magnetic field. This force is the result of paired electrons in the molecule aligning themselves in a direction opposite to the external magnetic field. Conversely, paramagnetic species are drawn towards the field as a result of the odd electron opposing the diamagnetic contribution of all of the paired electrons.[3]

There is a variety of methods to detect free radical species. Two methods that utilize the paramagnetic property of radicals are electron paramagnetic resonance spectroscopy and a relatively newer technique; namely nuclear magnetic resonance (NMR) spectroscopy in conjunction with spin trapping. These radical detection methods will be discussed in detail in the following sections.

2.2.1 EPR

2.2.1.1 The Fundamentals of EPR

The most common method to detect paramagnetism directly was developed in 1945.[3] This technique is called electron paramagnetic resonance (EPR) or electron spin resonance (ESR). EPR spectra are produced due to the fact that electrons have a spin, and this spin is associated with a magnetic moment. When an external magnetic field is applied, the magnetic moment of the electron can adopt one of two orientations, with (parallel) or against (anti-parallel) the magnetic field. This creates distinct energy levels for the unpaired electrons making the net absorption of electromagnetic radiation possible. When the magnetic field and the microwave frequency are matched, (i.e. the energy of the microwaves equals that of the energy difference of the pair of involved spin states), the resonance condition has been met.[3] Figure 2-1 illustrates the simplest EPR transition for an electron in a magnetic field H.

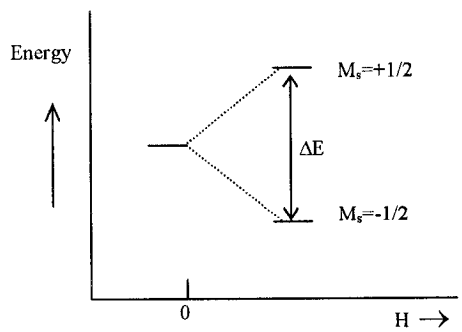


Figure 2-1: Energy level diagram for the transition of an electron in a magnetic field H

The absorption (or emission) of microwave energy between the spin states is given by the equation: $\Delta E = h\nu = g\beta H$

where:	ΔE is the energy difference between the two spin states
	h is Planck constant
	ν is the microwave frequency
	g is the Zeeman splitting factor
	β is the Bohr magneton
and	H is the applied magnetic field

Transitions between these two levels result in an EPR spectrum. An electron in a magnetic field thus gives rise to a single line in the EPR spectrum. Typically an EPR spectrum for most free radicals contains more than just one line, due to hyperfine splitting. When the odd electron in a radical is located on an atom that has a nucleus with a magnetic moment, the magnetic moment interacts with the electron and causes further splitting of its energy levels. Splitting also occurs when the odd electron interacts with a proton. In this case, the magnetic field of the proton produces an additional field, which is either added or subtracted from the external magnetic field. This results in each energy level in Figure 2-1 being split in two by the interaction with the proton.[3]

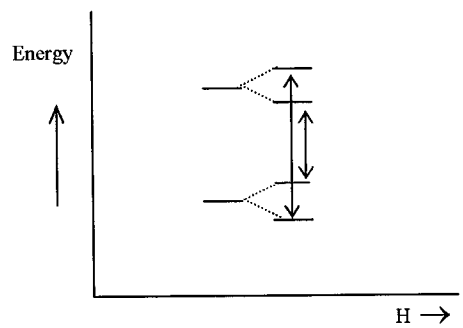


Figure 2-2: Transition for an electron interacting with a proton in a magnetic field H

Two transitions occur in Figure 2-2, and now the electron produces a pair of lines. $H^{\bullet}$ is an example of a radical that would give a two line spectrum. The number of lines in an EPR spectrum is given by $2I+1$ where I is the nuclear spin. When n equivalent protons interact with the electron, $n+1$ lines appear in the spectrum. For instance, $CH_3^{\bullet}$ gives a spectrum with four lines due to the three equivalent protons. Figure 2-3 shows the EPR spectra of the hydrogen atom and the methyl radical.[3]

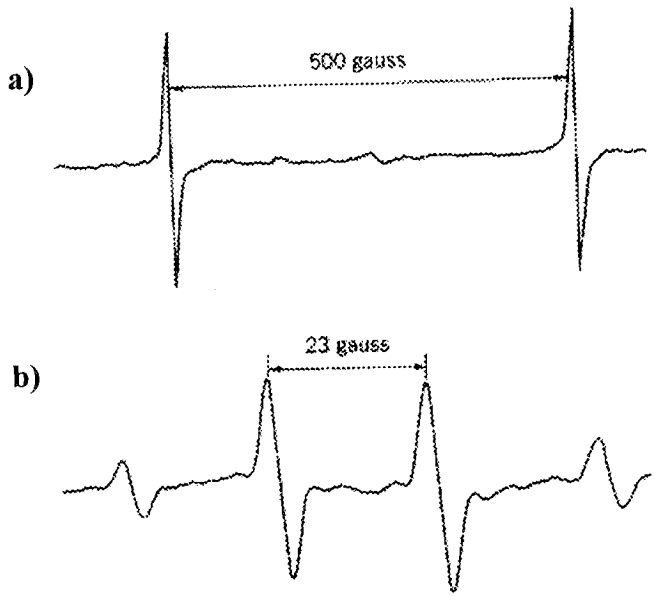


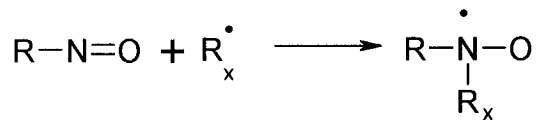
Figure 2-3. The EPR spectra of a) the hydrogen atom and b) the methyl radical.

2.2.1.2 EPR/Spin Trapping

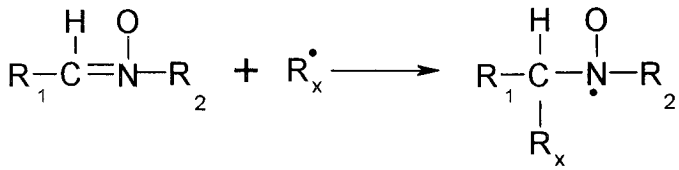
The technology to detect free radicals has drastically improved over the past 30 years. There are a variety of detection methods, including the use of fluorescent probes

and the detection of chemical reaction products. However, of all the techniques that have been devised, none is as specific as EPR. EPR when coupled with spin trapping can provide verification for the presence of free radical species.[19]

Most free radical species are highly reactive and therefore short lived. EPR spectroscopy can be used to detect these free radicals, however in some cases it is necessary to detect these radicals indirectly using spin traps. Spin trapping involves the introduction of a nitrone or nitroso diamagnetic species into an unstable free radical system, the end result of which is the formation of a relatively stable radical adduct that can be detected by EPR.[20,21]



Scheme 2-4. Spin trapping with a nitroso compound.



Scheme 2-5. Spin trapping with a nitrone

Nitroso compounds often can provide more information than nitrones, since the free radical trapped adds directly to the nitroso nitrogen (Scheme 2-4), increasing the hyperfine splitting parameters. However, for oxygen centred radicals nitrones are often

the spin traps of choice due to the fact that most oxygen centred radical adducts of nitroso compounds are unstable. With the use of nitrones some information is lost, since the radical trapped adds to the carbon adjacent to the nitrogen (Scheme 2-5).[22] Spin traps that have a β hydrogen provide a wealth of information about the nature of the free radical trapped as a result of additional hyperfine splitting.

Spin trapping is often used when the concentration of free radicals is below the level of detection threshold of the EPR spectrometer. Spin trapping allows for the detection of free radicals that are too short-lived to be observed directly. The spin adduct, which accumulates to a high concentration, can be detected with high sensitivity by EPR spectroscopy. In most cases the resulting EPR spectrum allows for the identification of the original reactive radical, using the spectral parameters g and hyperfine splitting, which serve as a fingerprint for species trapped. In instances where no unique assignment is possible, spin trapping still provides information about the nature of the radical being trapped (i.e. carbon-centred, oxygen-centred, nitrogen-centred, etc). Spin trapping has allowed for valuable information to be obtained on the production of free radicals in biological and biochemical systems. This technique is often employed as it can indicate the presence of more than one free radical or the formation of secondary radicals during an experiment. Spin traps have been used quite extensively in the detection of oxygen centred radicals, namely hydroxyl and superoxide radicals[23,24,25,26]. Spin trapping was originally envisioned as a technique that would allow for the detection and the quantification of these radicals. Detection and quantification of hydroxyl and superoxide radicals has been of utmost importance in the fields of biology and medicine. This is because, oxygen centred free radicals have been shown to have an important role

in a variety of clinical diseases including heart attack, stroke, respiratory distress syndrome and cancer[25,27,28]. EPR spectroscopy coupled with spin trapping is the most direct method of detecting these oxygen centred radicals in biological cells and tissues.[28]

Nitrones are the most commonly used spin traps, especially for biological applications[27], and the cyclic nitrone 5,5-dimethyl-1-pyrroline N-oxide (DMPO) can be considered as the most widely used spin trap due to its ability to yield distinct spin adducts when reacted with hydroxyl or superoxide radicals.[29] When both spin-trapped adducts are formed in the presence of DMPO the ESR signal is a composite of the two spin- trapped adducts. The difference in hyperfine splitting pattern of each spin-trapped adduct allows one to distinguish between these free radicals. Unfortunately the use of DMPO to trap superoxide is very complex. The spin adduct that results from trapping experiments with DMPO and superoxide radical is 2-hydroperoxy-5, 5-dimethyl-1-pyrrolidinyloxyl (DMPO-OOH). This radical adduct is unstable and rapidly undergoes chemical conversion to give 2-hydroxy-5, 5-dimethyl-1-pyrrolidinyloxyl (DMPO-OH). This means that the hydroxyl radical spectrum may be produced through a pathway that does not involve the direct spin trapping of the hydroxyl radical. Thus the appearance of a spectrum characteristic of DMPO-OH does not necessarily mean that this radical was present in the reaction system.[29,30,31] DMPO-OOH is also susceptible to metal ion-catalyzed addition of water, leading via a non-radical reaction, to many unwanted pyrrolidinoxyl radicals including DMPO-OH.[27,28,30] It is for these reasons why new spin traps have been synthesised in order to study oxygen centred radicals. Recently, new phosphorus-containing analogues of DMPO have been synthesised, which have radical adducts that are more persistent. Frejaville *et al.*[28, 32] and Barbati *et al.*[27] reported on the

synthesis of the new α-phosphorus containing spin trap, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide (DEPMPO). The incorporation of ^{31}P into the spin trap provides useful EPR information since the ^{31}P hyperfine splitting constant (^{31}P-hfsc or a_p) is quite large.[27] This parameter is sensitive and characteristic to the nature of the added radical. DEPMPO has been shown to trap a variety of free radicals such as $^{\bullet}OH$, $^{\bullet}O_2^-$, $R^{\bullet}$, $RS^{\bullet}$ and $RO^{\bullet}$.[25, ,32,33,34] Barbati *et al.*[27] have synthesized, examined the kinetics, the EPR characteristics and the relative persistencies of the radical adducts of DEPMPO with hydroxyl and superoxide radicals. Their study showed that the reaction of hydroxyl radicals with DEPMPO produced a persistent 2-(diethoxyphosphoryl)-2-methyl-5-hydroxy-1-pyrrolidinoxyl (DEPMPO-OH) radical adducts. The reaction was shown to be stereospecific and the reaction rate is approximately twice that of with the DMPO spin trap.[27] An advantage of using DEPMPO compared to DMPO is that the superoxide radical adduct, DEPMPO-OOH, does not spontaneously decompose to give DEPMPO-OH. The radical adduct is quite persistent with a half life for DEPMPO-OOH in pH 7 phosphate buffer reported as 780 s.[27] Also, artefacts due to transition metal impurities in water are also not significant with this phosphorus-containing compound.[27]

Despite the fact that the superoxide radical adduct of DEPMPO is more stable than that of DMPO/OOH, the reaction of $O_2^{\bullet-}$/$HOO^{\bullet}$ with these nitroxide based spin traps, DMPO and DEPMPO is relatively slow. More specifically, the rate constants for the reaction of superoxide and hydroperoxyl radical with DMPO are 10 M/sec. and 6.6 x 10^3 M/sec. [35] respectively, and with DEPMPO 90 M/sec for the reaction with superoxide. Due to the low rates for these reactions, spontaneous dismutation of superoxide is a major

complication of any quantitative studies except at very high pH.[9] In order to circumvent this problem a high concentration of spin traps must be used.

2.2.1.3 Advantages and Disadvantages of EPR

EPR spectrometers are very sensitive and claims of thresholds of 10^{-8}M spin for signals with 1 G line width have been reported.[22] This threshold concentration is rarely reached because side-reactions, such as dimerization, disproportionation, abstraction, and oxidation or reduction may occur at a much faster rate than the rates of radical production.[2] For some radicals, even if they are present in concentrations greater than 10^{-8} M, the EPR signal will not be observed at room temperature due to their short spin relaxation times. The short lifetimes and broad line widths of hydroxyl, superoxide and alkoxyl radicals make their detection by EPR spectroscopy difficult if not impossible.[22] The practical application of in vivo EPR to systems which involve oxygen centred radicals has proven to be challenging due to limitations in instrument sensitivity and the relatively instability of these radicals and their nitrone adducts in biological media. [36]

2.2.3. NMR

2.2.3.1 NMR/Spin Trapping

In its earliest application, spin trapping was a technique based solely on the detection of radical adducts in an EPR spectrometer. Unfortunately, in many of the reactions in which spin traps are used, namely nitrone spin traps, loss of the paramagnetic property of the radical occurs. The consequence of this is the formation of species that are "EPR silent".

Recently, Khramstov *et al.*[19,37] have demonstrated that nuclear magnetic resonance (NMR) spectroscopy can be used in conjunction with spin trapping to detect a variety of free radical species. This technique was termed "NMR spin trapping" and by utilizing a phosphorus containing spin trap they were able to detect EPR silent species. NMR spectroscopy was first proposed for free radical detection in order for the degradation/decomposition reaction products of spin trapping experiments to be observed. Many of these products are diamagnetic and therefore can be detected by NMR spectroscopy. Proton and carbon-13 NMR are not very useful in these cases, as the resulting spectrum is too rich in resonance lines. Thus, the use of nuclei that are NMR active, stable and sensitive such as ^{31}P and ^{19}F can make information regarding the diamagnetic reaction products simpler to obtain[37,38]. The use of either ^{31}P and ^{19}F NMR spectroscopy in conjunction with spin traps containing these nuclei eliminates the problem of overlapping resonance lines that would undoubtedly occur if proton or carbon NMR spectroscopy was used. Similarly to EPR, NMR-spin trapping retains information pertaining to the structure of the radical being trapped. [37,38]

2.2.3.2 Advantages and Disadvantages of NMR/ Spin Trapping

The main drawback of this technique is the reduced sensitivity of NMR compared to EPR spectroscopy. This limitation is overcome with NMR spin trapping through the accumulation of the stable diamagnetic reduction products of spin adduct degradation products. This accumulation markedly decreases the sensitivity gap between NMR and EPR.[19,37] NMR spin trapping is more advantageous than EPR in biological systems, due to the stability of the diamagnetic products in reducing environments. In many biological

cases, the medium is highly reducing therefore EPR spin trapping cannot be used due to the rapid reduction of the nitroxide moiety (spin adducts) of the spin trap (< 1 sec.) which creates EPR silent products.[37]

Recently, Khramstov *et al.*[37], have measured the ^{31}P chemical shifts for the diamagnetic products of DEPMPO/adduct degradation for $^{\bullet}OH$, $^{\bullet}OOH$, $^{\bullet}CH_2OH$ and $^{\bullet}CH_3$ radicals. The ^{31}P resonance lines for these products are in the range of 27 to 33 ppm, which makes this technique suitable for biological work in which most of the main phosphorus-containing compounds range from -3 to 21 ppm (i.e. for ATP, ADP, AMP, NAD, inorganic phosphate, etc.).[37]

NMR does not detect paramagnetic radicals or radical adducts due to paramagnetically broadened spectra, but it does detect the diamagnetic products of radical adduct degradation. The ability to distinguish and quantify the many diamagnetic degradation products by NMR spectroscopy would be valuable in determining degradation pathways.

2.3 IMPORTANCE OF FREE RADICAL DETECTION

2.3.1 Biological Importance

Many researchers have devoted a great amount of effort in order to address the role played by hydroxyl and superoxide radicals in mediating a variety of pathological conditions including ischemia/reperfusion injury[39] as well as chronic diseases and normal physiological processes.[40] The hydroxyl radical is generated within cells and tissues where it can attack proteins, lipids and DNA, thus initiating secondary radical reactions that can lead to irreparable cellular damage.[41] These radical species can readily pass

through membranes and cannot be kept out of the cell. Hydroxyl radicals are very reactive species that react with the first bio-molecule that they encounter. Since hydroxyl radical damage is "diffusion rate limited" damage can be widespread and very indiscriminate.[5] Hydroxyl radicals react mainly by hydrogen abstraction or by addition to double bonds. Their ability to propagate damage is due to their interaction with substrates such as DNA, which results in widespread damage. DNA can be oxidatively damaged at the nucleic bases or at the sugars that link the bases. This damage results in the degradation of the bases and breaking of the DNA strands, and results in mutations or cell death.[42]

The superoxide radical can be formed from the reduction of triplet oxygen. Superoxide can act as both an oxidant and reductant. Superoxide is not very reactive in biological systems and does not by itself cause much oxidative damage. It acts mainly as a precursor to other oxidizing agents such as singlet oxygen and peroxynitrite.[42] In some cells superoxide is produced to act as an antibiotic weapon to kill invading micro organisms. When cells are injured or stressed, the normal metabolism of oxygen changes resulting in an increase in superoxide radical concentration. In addition, white blood cells that are activated by trauma and inflammation intentionally produce superoxide in order to kill microorganisms. Therefore, almost all diseases result in the production of large quantities of superoxide radicals. These free radical species will propagate damage throughout the body until there is another radical species that can couple and quench the superoxide radical (termination). In the body, this termination of superoxide is achieved by the enzyme superoxide dismutase (SOD). The biological function of this enzyme is to protect living cells against the toxicity of superoxide by controlling the levels in the cell.

Superoxide dismutase takes two superoxide radicals and dismutates them (takes an electron from one and adds it to the other) to generate molecular oxygen and with the addition of two protons, hydrogen peroxide (Equation 12).

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2 \qquad \text{Equation 12}$$

The central role of hydroxyl and superoxide radicals in many diseases has necessitated that methods for their detection and quantification be developed. Thus the development of a quantitative tool to measure hydroxyl and superoxide radicals would be beneficial to a variety of chemical and biological applications.

2.3.2 Industrial Significance

Free radical detection is of importance to many industries; however it is of particular interest to the pulp and paper industry. For many years the most widely used bleaching agent for chemical pulps was chlorine and its compounds. Due to regulatory and economic pressures however, the pulp and paper industry has been forced to implement new delignification and bleaching practices in order to produce high quality printing and writing papers. There has been a major effort towards the partial replacement of chlorine and chlorine dioxide delignification and bleaching stages with oxygen delignification and bleaching. The environmental, technical and economic benefits of oxygen delignification are numerous and include lower chemical requirements in subsequent bleaching sequences, higher brightness with equivalent amount of chemicals, lower rejects and lower water consumption.

Despite these advantages, the effectiveness of an oxygen delignification stage is limited at the 50% level. Beyond this level, severe cellulose degradation takes place, resulting in the deterioration of paper strength characteristics. This selectivity issue is due to free radical side-reactions interacting with the cellulose and represents the most significant drawback of oxygen delignification. Oxygen centred radicals, such as superoxide $O_2^{-\bullet}$, hydroxyl HO• and hydroperoxy (HOO•) radicals have been shown to play an important role in the oxygen delignification processes. The extremely short half-lives of these free radical species has necessitated that techniques such as spin trapping be developed for their detection in oxidative processes.

These species have been shown to interact with the phenolic moieties of lignin producing phenoxy radicals, which can then undergo rearrangement and coupling reactions. During oxygen bleaching, hydroxyl radicals are thought to interact with lignin to form substrate radicals and initiate C_α-C_β bond and aryl-alkyl ether bond cleavage, while superoxide radicals in conjunction with hydroxyl radicals result in the oxidative degradation of lignin and carbohydrates during oxidative bleaching.[43]

The profound complexity of the lignocellulosic substrate and the complexity of the various bleaching reactions have precluded the detailed structural identification and quantification of the radical species involved. In this respect, this thesis attempts to focus on detecting and quantifying various radical species involved in a number of important processes in the pulp and paper industry.

CHAPTER 3

EXPERIMENTAL

3.1 REAGENTS

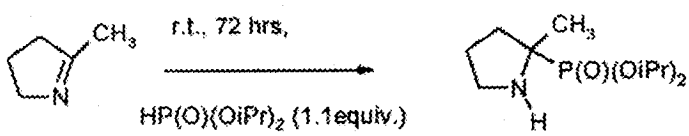
2-methyl-1-pyrroline, boron trifluoride diethyl etherate, HCl, methylene chloride, sodium carbonate, chloroform, sodium sulfate, $Na_2WO_4 \cdot 2H_2O$ were of reagent grade and purchased from Aldrich. Diisopropyl phosphite was purchased from TCI America.

Spin Trapping Experiments

Diethylenetriaminepentaacetic acid (DTPA), potassium superoxide (KO_2), 30%w/w hydrogen peroxide, 3% w/w hydrogen peroxide, methanol, ethanol (100%), dimethylsulfoxide (DMSO) and acetone were purchased from Aldrich. Bovine erythrocyte superoxide dismutase (SOD), and *tert*-butyl hydroperoxide was obtained from Sigma. DEPMPO (5-diethoxy-phosphoryl-5-methyl-1-pyrroline-N-oxide) was obtained from Oxis International (Portland, OR) and DIPPMPO (5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-N-oxide) were synthesised as described below. Water used in sample preparation was distilled and deionized.

3.2 SYNTHESIS OF DIPPMPO

Compound 1: Diisopropyl(2-methylpyrrolidin-2-yl)phosphonate

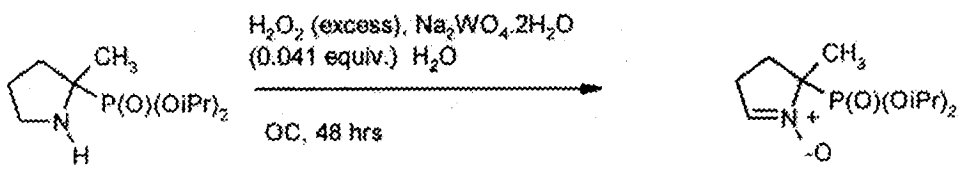


Scheme 3-1. Formation of diisopropyl(2-methylpyrrolidin-2-yl)phosphonate from 2-methyl-1-pyrroline.

2-methylpyrroline (16.0 mL, 151.6 mmoles, 1.0 equiv.) was placed into an oven dried, nitrogen filled, and septa sealed flask via syringe. Diisopropyl phoshite (28.4 mL, 166.8 mmoles, 1.1 equiv.) was then added and a catalytic amount of boron trifluoride diethyl etherate (1.76 mL, 14.1 mmoles, 9.3 mol%) also via syringe. This mixture was then allowed to stir at room temperature for 72 hours under nitrogen gas, after which the nitrogen was turned off and the product worked up.

Product work up: The product was then poured into a 1N HCl solution (120 mL) and mixed by gentle agitation. This solution was then washed with 200 mL methylene chloride using a separatory funnel. The organic phase was then conserved and the aqueous phase basified using 120 mL saturated sodium carbonate solution. The now basic solution was then extracted a second time using 120 mL of chloroform and added to the previously conserved organic phase. The organic solution was then dried using sodium sulfate, and concentrated under vacuum using a vacuum pump. Diisopropyl (2-methylpyrrolidin-2-yl) phosphonate was obtained as a light coloured oil (96%) and used without further purification in the second step of the synthetic procedure.

Compound 2: 5-Diisopropylphosphoryl-5-methyl-1-pyrroline-N-oxide (DIPPMPO)



Scheme 3-2. Formation of 5-Diisopropylphosphoryl-5-methyl-pyrroline-N-oxide.

An aqueous solution of $Na_2WO_4 \cdot 2H_2O$ (4.12 g, 12.48 mmoles, in 1600 mL of water) was mixed with compound 1(32 mL, 303.2 mmoles) in a 3.0 L reactor at 0°C. A 30% solution of hydrogen peroxide (60 mL, 921 mmoles) was added dropwise over 1.5 hours with stirring at 0°C. The sample was then kept for an additional 48 hours at a temperature of 0°C in the refrigerator. After this time, the mixture was then extracted into chloroform (500 mL) and set aside. The remaining aqueous layer was saturated with brine and subsequently extracted using an additional 500 mL of chloroform. The organic phases were then combined and dried over sodium sulfate for a period of 12 hours at 0°C. The solvent was then removed under vacuum and the resulting amber oil (50% yield by weight) chromatographed on silica using 1:9 ethanol/methylene chloride mixture. The oil was then analysed using ^{31}P NMR and ^{1}H NMR spectroscopy. The ^{31}P NMR spectra showed a single resonance at 22.2 ppm, which is in agreement with the chemical shift for the commercially available DIPPMPO spin trap. The chemical shifts as well as the multiplicities for the resonances in the ^{1}H NMR spectrum were also consistent with the commercial DIPPMPO spin trap. The spin trap was stored under nitrogen at -70°C.

3.3 COMPUTATIONAL METHODS

The initial structures of all compounds were produced using a model builder (HyperChem 5.11 Professional, HyperCube, Inc.). The structures were then subjected to preliminary geometry optimization with the MM+ molecular mechanics[44] force field (to root mean square, RMS, gradient $kJ \cdot mol^{-1}Å^{-1}$). A single-point semi-empirical PM3 (3^{rd} parametrization method;[45,46]) computation was then performed to assign partial charges on all atoms. The charges permitted the use of an improved MM+ force field

(HyperChem), based on non-bonded electrostatic interactions. Using this force field, we generated the starting structures by simulated annealing (molecular dynamics under increasing and then decreasing temperature). The starting geometries were then optimized with MM+ and finally with the PM3 semi-empirical method[45,46] with self-consistent-field convergence set to 0.01 and the optimization convergence to RMS gradient 0.06 $kJ \cdot mol^{-1} Å^{-1}$. The unrestricted Hartree-Fock (UHF) algorithm was used. The atomic charges, atomic orbital electron populations, and heats of formation were computed from these energy-minimized structures.

3.4 GENERATION OF FREE RADICALS FOR NMR MEASUREMENTS

3.4.1 Hydroxyl Radicals

Photolysis of H_2O_2 was used to generate hydroxyl radicals. A solution of DIPPMPO/DEPMPO (68mM) and H_2O_2 (2.5M) in sodium hydrogen phosphate buffer (30mM, pH 7.4) containing DTPA (4mM) was prepared and the reaction mixture transferred to a quartz cuvette. The sample was then irradiated with a 450W medium pressure mercury-vapour lamp (Ace Glass Incorporated, Serial No. 7825-34, 222.4 - 1367.3 nm), which was used as the UV light source. The formation of radical species was monitored as a function of irradiation time.

3.4.2 Superoxide/Hydroperoxyl Radicals

Photolysis of 30% H_2O_2 was also used to generate superoxide radicals. The procedure is the same as above. When UV irradiation was not required, solid KO_2 dissolved in water was used as a source of superoxide.

3.4.3 Methyl Radicals

Photolysis of *tert*-butylhydroperoxide (70% in water) or dimethylsulfoxide (DMSO) in 3% hydrogen peroxide was used to generate methyl radicals. DIPPMPO/DEPMPO (68mM) was added to either *tert*-butylhydroperoxide or 3% H_2O_2 containing DMSO and the reaction mixture transferred to a quartz cuvette. The sample was then irradiated with a 450W medium pressure mercury-vapour lamp. The formation of radical species was monitored as a function of irradiation time.

3.4.4 Hydroxyalkyl Radicals

Hydroxymethyl and hydroxyethyl radicals were made by hydroxyl radical induced hydrogen abstraction from methanol and ethanol respectively. Hydroxyl radicals were produced by UV irradiation (450W medium pressure mercury-vapour lamp), of the appropriate alcohol in 3% H_2O_2.

3.5 FREE RADICALS IN SOLUTIONS OF HYDROGEN PEROXIDE

Solutions of 30% hydrogen peroxide containing DIPPMPO (68mM) purged with either nitrogen, oxygen or left untreated were stirred at room temperature for 90 minutes.

3.6 TRAPPING FREE RADICALS AS A FUNCTION OF pH

DIPPMPO (68mM) was introduced into aqueous solutions of peroxide in acidic, neutral or alkaline pH. Buffer solutions were used in all experiments: pH 4 (0.05M potassium biphthlate), pH 6 (0.05M potassium phosphate monobasic sodium hydroxide), pH 7 (0.03M sodium hydrogen phosphate), pH 9 (0.05M sodium bicarbonate, 0.1M sodium hydroxide and hydrochloric acid), pH 10 and 11(0.05M sodium bicarbonate and

0.1M sodium hydroxide). These reaction systems were purged of air with nitrogen, and stirred at room temperature for 90 minutes. The samples were then analysed using ^{31}P NMR spectroscopy.

3.7 ^{31}P NMR SPECTRA

^{31}P NMR spectra were acquired on a Varian XL-200 MHz spectrometer (operating at 80.989 MHz). The chemical shifts reported are relative to external *ortho*phosphoric acid (85%). All spectra were acquired with proton decoupling. The total number of scans for all experiments was 1024 with an acquisition time of 1.60 seconds. Trimethylphosphate was used as the internal standard for quantification and was added to the sample prior to measurement. The relaxation time (T_1) of the internal standard was measured and was determined to be approximately 13.5 seconds. In order to decrease the relaxation time a relaxation agent (chromium chloride) was added to the mixture. With the addition of chromium chloride, (30 – 35mM), to the samples prior to NMR measurement the relaxation time of the phosphorus nuclei was decreased to ~200ms. $5T_1$ was used for the pulse delay.

3.8 GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS)

Structural analyses were performed using a Hewlett Packard 5972 mass spectrometer interfaced to a Hewlett Packard 5890-A gas chromatograph equipped with a DB-5 30 m x 0.25 mm packed silica capillary column.

3.9 MASS SPECTROMETRY (MS)

MS analyses were carried out on a KRATOS analytical MS25RFA mass spectrometer at 70 eV with a source temperature of 200°C. In some cases, a Finnigan LC Q Duo (ESI) mass spectrometer was utilized. Samples were freeze dried prior to MS analysis.

CHAPTER 4

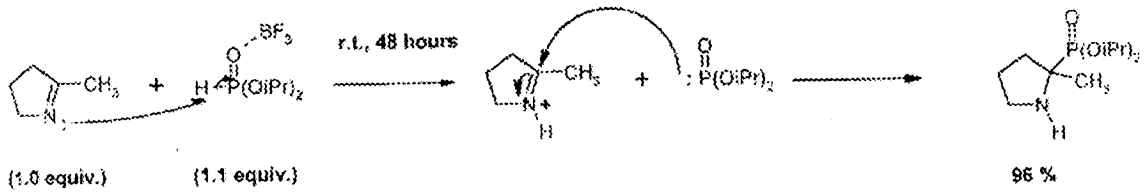
RESULTS AND DISCUSSION

4.1 SYNTHESIS OF DIPPMPO SPIN TRAP

The spin trap 5-Diisopropylphosporyl-5-methyl-1-pyrroline-N-oxide (DIPPMPO) was synthesized according to a previously published procedure by Barbarti *et al.*[47] The synthetic route involved the nucleophilic addition of diisopropyl phosphite to 2-methylpyrroline in the presence of a catalytic amount of boron trifluoride diethyl etherate. The oxidation of the intermediate produced was carried out using a variety of oxidants including *meta*-chloroperoxy benzoic acid (m-CPBA), dimethyldioxirane (DMDO), and hydrogen peroxide/Na_2WO_4.2 H_2O. Of all the oxidants employed, only the sodium tungstate catalysed oxidation using hydrogen peroxide gave the desired product in ~ 50% yield.

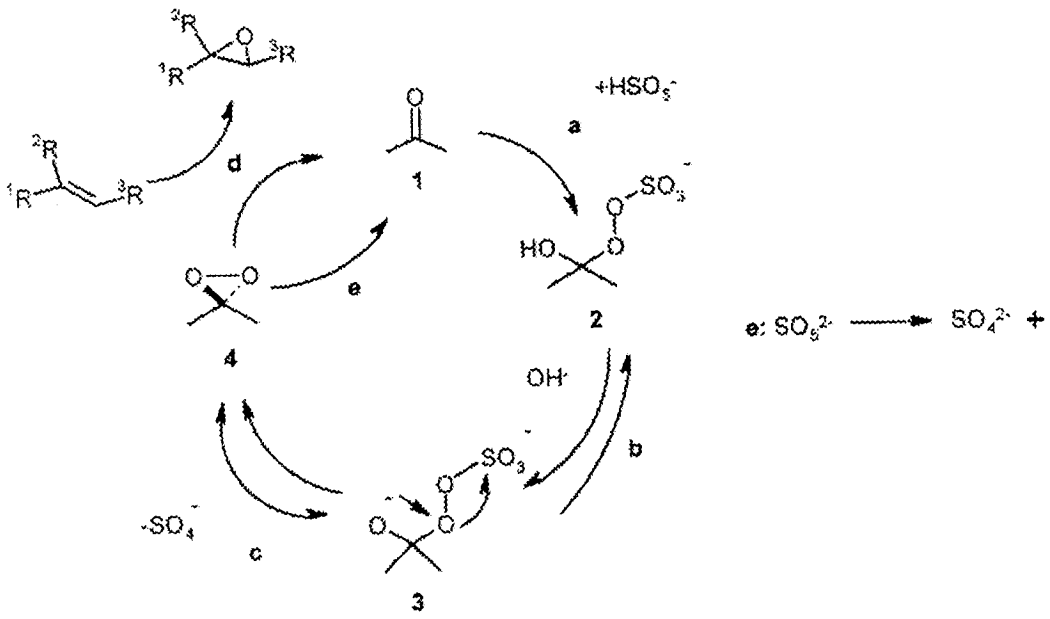
The first step in the synthesis of DIPPMPO involves stirring diisopropyl phosphite in 2-methyl-1-pyrroline for seven days. In this reaction the alkylphosphite is added onto the double bond of the imine. The end result of this reaction, is the phosphonate, diisopropyl-(2-methylpyrrolindin-2-yl)phosphonate in 95% yield. The reaction rate for this initial reaction is increased drastically, upon the addition of a catalytic amount of the Lewis acid, boron trifluoride diethyl etherate. The boron trifluoride diethyl etherate accelerates the overall reaction most likely by coordinating with the lone pairs on the oxygen of the diisopropyl phosphite thereby making the phosphorus moiety more electrophilic. An increase in the electrophilic character of the phosphorus results in an increase in the acidity of the hydrogen on the phosphorus, thus

making hydrogen abstraction by the pyrroline more facile. The nucleophilic attack of the phosphorus onto the double bond completes the reaction mechanism. (Scheme 4-1)



Scheme 4-1. Mechanism of phosphite addition to 2-methyl-1-pyrroline.

In attempting to synthesize DIPPMPO several oxidants were used. The first oxidant employed was dimethyldioxirane (DMDO). DMDO was generated *in situ* according to the method by Frohn *et al.*[48] The catalytic cycle (Scheme 4-2) for the formation of dimethyldioxirane involves the formation of an oxy-anion intermediate **3**. The first step in the cycle (a) involves the nucleophilic addition by potassium monoperoxy sulfate; 2 $KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$ (Oxone) to the carbonyl carbon of acetone **1** to produce **2**. The next step, b involves the removal of the proton forming the oxyanion **3**. This then allows for the formation of the dimethyldioxirane **c**. Steps d and e both involve the loss of dimethyldioxirane and the formation of acetone either through reactions with a substrate **d**, or directly **e**.

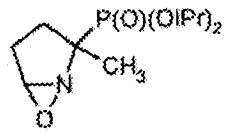


Scheme 4-2

The use of DMDO to oxidize diisopropyl-(2-methylpyrrolindin-2-yl)phosphonate resulted in the production of two over oxidation products. These products identified using GC/MS had parent peaks at *m/z* = 279 and 311. The structures for these compounds are shown below (Figure 4-3).

Figure 4-3. Over-oxidation products formed when DMDO is used as an oxidant.

The second oxidation reagent used was *meta*-chloroperoxy benzoic acid (m-CPBA). The oxidation of diisopropyl(2-methylpyrrolidin-2-yl) phosphonate to generate DIPPMPO was unsuccessful using this oxidant. The species formed from this reaction was the oxaziridine (Figure 4-4).

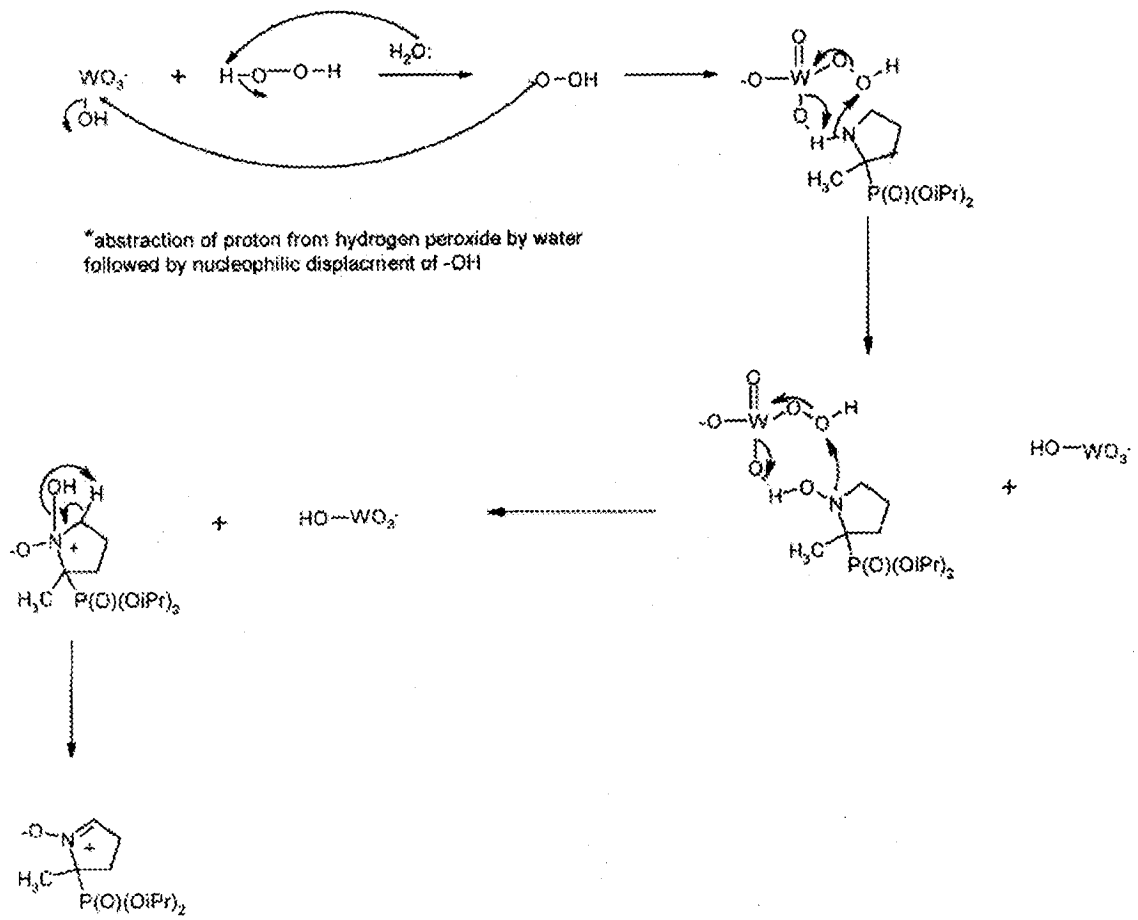


oxaziridine

Figure 4-4. Oxaziridine formed using m-CPBA as an oxidant.

The most effective oxidation reagent was sodium tungstate and hydrogen peroxide. The mechanism proposed by Murahashi *et al.*[49] for the oxidation involves the formation of a peroxytungstate species that arises due to the reaction of sodium tungstate and hydrogen peroxide (Scheme 4-5). The peroxytungstates HWO_5 and HWO_8 are believed to be formed in this reaction, (as seen by polographic studies),[49] and these species act as catalysts. In their study the presence of a hydroxylamine intermediate was detected in the oxidation of secondary amines.[49] This intermediate is thought to be involved in the conversion of secondary amines to N-oxides. Secondary amines undergo a nucleophilic reaction with the peroxytungstate to generate the hydroxylamine, which is subsequently oxidized and dehydrated to give the corresponding N-oxide.

In the proposed mechanism[49] (Scheme 4-5) for oxidation involving sodium tungstate and hydrogen peroxide, there is the nucleophilic attack of tungstate by the hydroperoxyl anion, (deprotonated form of hydrogen peroxide), forming a peroxytungstate. One oxygen atom from the peroxytungstate is then transferred to the phosphite generating an hydroxylamine. A second peroxytungstate molecule then oxidizes the hydroxylamine intermediate in a similar manner. The di-oxy intermediate then loses water to afford the final product, 5-diisopropylphosporyl-5-methyl-1-pyrroline-N-oxide (DIPPMPO).



Scheme 4-5. Proposed mechanism of oxidation using sodium tungstate and hydrogen peroxide.

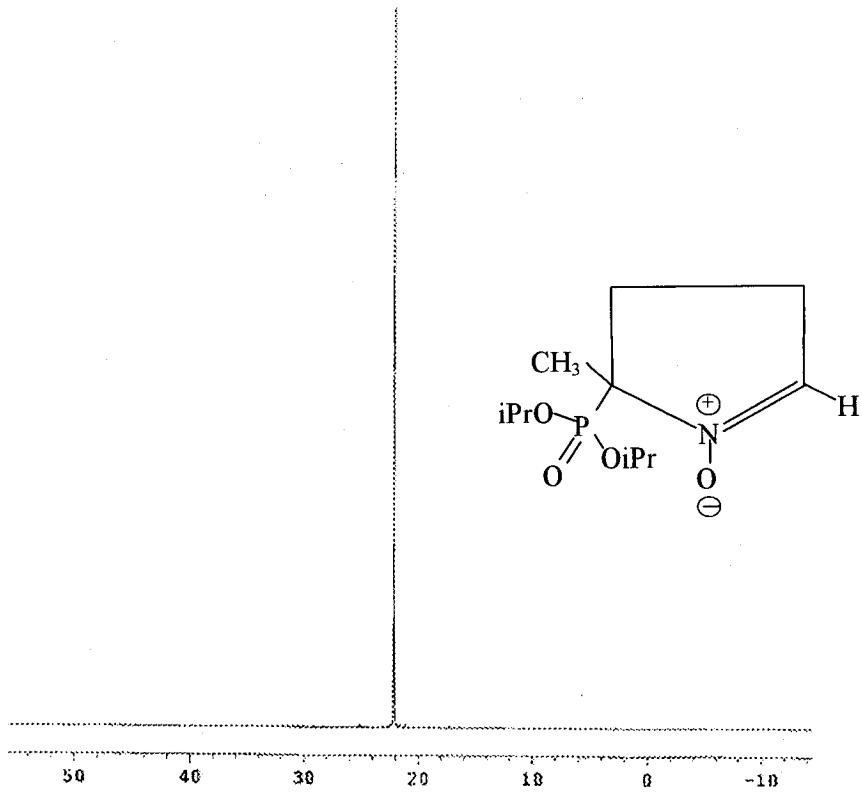


Figure 4-6. ^{31}P NMR spectrum of synthesized DIPPMPO in deuterium oxide.

In order to ensure that the DIPPMPO spin trap had been prepared successfully, ^{1}H and ^{31}P NMR spectroscopy were performed and the spectra compared to the spectra of commercially available DIPPMPO. The ^{31}P chemical shift for the prepared DIPPMPO spin trap in deuterium oxide using *ortho*-phosphoric acid as an external references was 22.2 ppm (Figure 4-6), which is in agreement with the chemical shift obtained for commercial DIPPMPO.

The ^1H NMR spectrum for the DIPPMPO spin trap prepared in this work is shown below (Figure 4-7). The single resonance labelled H_a that appears downfield at approximately 7.1 ppm corresponds to the double bond proton that is adjacent to nitrogen. The multiplet centred at 4.6 ppm (H_h) is attributed to the proton of the isopropxy group that is split by the methyl groups of the isopropoxy moiety. This resonance is shifted downfield due to the electronegative oxygen. The signals that appear upfield at 2.1 and 2.6 ppm are assigned to cyclic protons H_b, H_c, H_d, H_e. The doublet centred at 1.5 ppm (H_f) is assigned to the methyl group located on the ring and the resonance at 1.2 ppm (H_g) is to the four methyl groups of the isoproxy moiety. The ^1H NMR spectrum obtained for the DIPPMPO spin trap agrees with the ^1H NMR spectrum obtained from commercial sources.

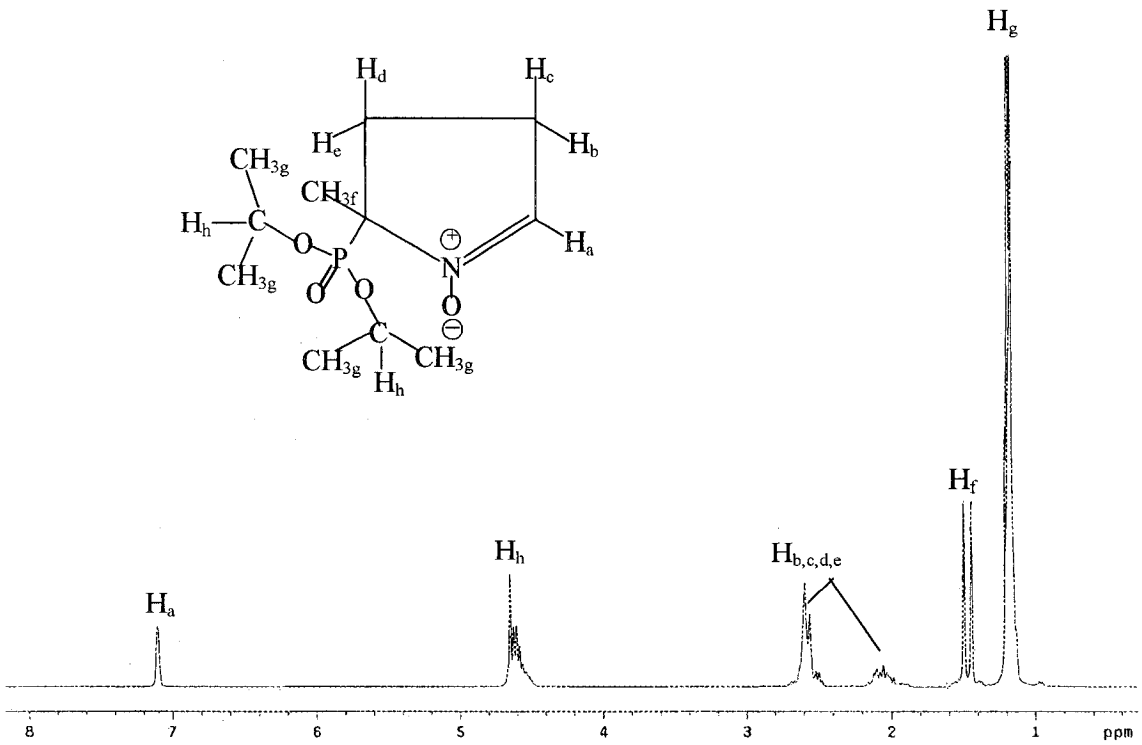


Figure 4-7. ^1H NMR spectrum of DIPPMPO spin trap.

4.2 COMPUTATIONAL CHEMISTRY – COMPARISON OF SPIN TRAPS

Computational chemistry using HyperChem 5.11 Professional® was performed in order to compare the two spin traps used in this research. Spin density, partial charge and singularly occupied molecular orbital (SOMO) energy values were obtained for both the DEPMPO and DIPPMPO spin trap using molecular mechanics. The molecular mechanics method involves calculating the potential energy of molecular systems as a function of coordinates of their atomic nuclei. MM^+ is the most general method for molecular mechanics calculations. This method, which prepares structures generated from calculations for semi-empirical treatment, was used for spin trap calculations.

Semi-empirical calculations are a form of quantum mechanics chemical calculation that uses the parameters derived from experiments to simplify the calculation process. In HyperChem®, semi-empirical calculations use approximate solutions of the Schrödinger equation in addition to empirical parameters to predict the electronic properties of molecular systems.

Literature accounts have shown that differences exist in radical adduct stability depending on the nitrone spin trap used. For example the half-life for DEPMPO/OOH and DIPPMPO/OOH radical adducts are respectively, and ~13-18 minutes[32,34] and 21 minutes[50] for DEPMPO/OOH and DIPPMPO/OOH in pH 7 buffer. Radical adduct stability is dependent upon either electronic or steric influences. Computational analyses were performed in order to determine whether differences in radical adduct stability for the spin traps were due to electronic influences. DEPMPO and DIPPMPO spin traps were compared to the non-phosphorylated spin trap 5, 5-dimethylpyrroline - N- oxide (DMPO). Calculations revealed that the highest spin densities and partial charges are

centred on the nitrogen and the oxygen of the nitroxide moiety. All of the other nuclei present in the molecule show similar if not identical values for the total spin density, partial charge and SOMO energy for both of the phosphorus containing radical adducts, (DEPMPO/OH and DIPPMPO/OH). Figure 4-8 therefore shows only the values for spin density for DMPO, DEPMPO and DIPPMPO for the nitrogen and oxygen atoms. It is important to note that the partial charges and SOMO energy values for these atoms were similar for all three of the spin traps.

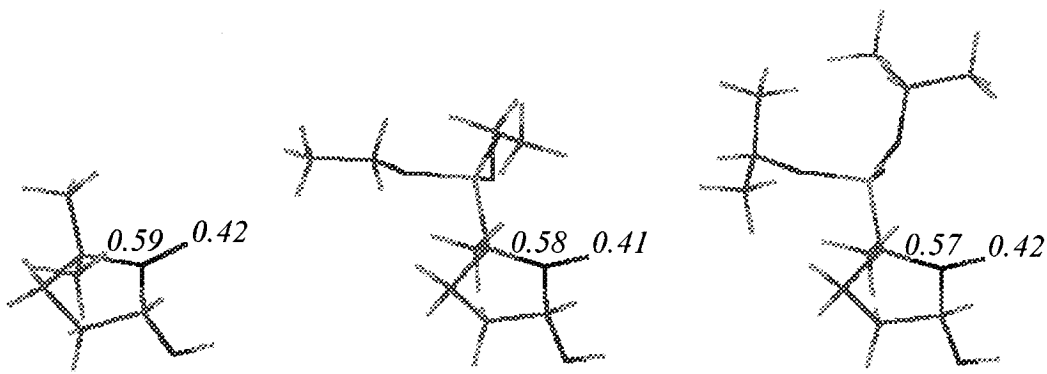


Figure 4-8. Ball and stick models of DMPO, DEPMPO and DIPPMPO spin traps. The values shown correspond to partial charges on nitrogen and oxygen atoms.

These computational results were in agreement with what was predicted. From the calculations performed it was established that the two spin traps are similar electronically, and therefore the major difference in radical adduct stability is most likely due to steric influences. In inert solvents and in the absence of scavengers, the lifetimes of radical intermediates are limited by either radical-radical coupling or radical-radical

disproportionation reactions.[51] Radical-radical reactions are typically minimized or even eliminated by imposing significant steric effects on the radical center.[52] From the space-filling models (Figure 4-9) it is clear that the DIPPMPO spin trap has a larger surface area than DEPMPO. Therefore, it can be expected that fewer random collisions, which lead to disproportionation, will occur with the DIPPMPO spin trap. This leads in a slight increase in stability for DIPPMPO radical adducts compared to DEPMPO radical adducts.

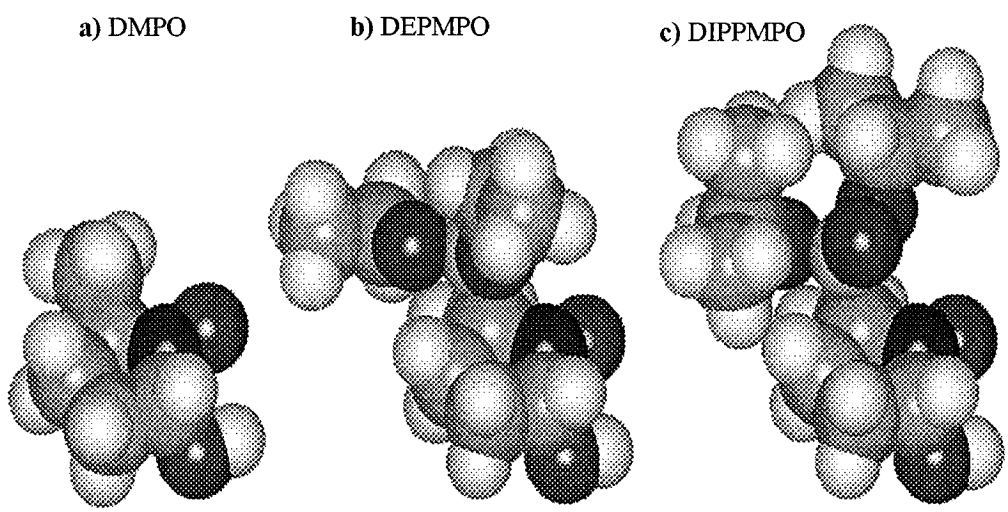


Figure 4-9. Space filling model of a) DMPO, b) DEPMPO and c) DIPPMPO spin traps.

4.3 QUANTITATIVE ^{31}P NMR SPECTROSCOPY

The concentration of free radicals trapped by the spin trapping method was determined through the use of an internal standard. The internal standard used in all experiments was trimethylphosphate. This compound has a single resonance line in the ^{31}P NMR spectrum, upfield from any signals of interest. The chemical shift for this internal standard was in the range of 3.7 to 4.3 ppm, depending upon the solvent used (i.e. deuterium oxide, DMSO, *tert*-butylhydroperoxide) in the experiment. With the use of known concentration of internal standard and integration, the concentration of the peaks in the ^{31}P NMR spectrum was determined. The area under each peak in the NMR spectrum is proportional to the quantity of compound contained in the NMR sample.

The technique employed in this work is termed "semi- quantitative" to reflect the fact that although the concentrations obtained were reproducible within a given reaction system, however the values stated are not absolute. Quantification of the free radicals studied was not verified using an independent method. This lack of supportive evidence was not an oversight, but merely due to the fact that an EPR spectrometer was not available to carry out comparative studies.

4.4 SPIN TRAPPING OF OXYGEN CENTERED RADICALS

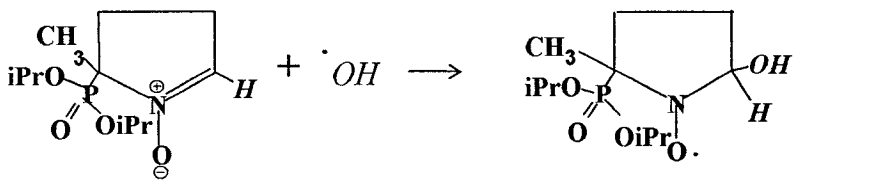
The spin trapping reactions whose details are explored in this work using ultraviolet photolysis of peroxide solutions can be described as follows:

$$HOOH \xrightarrow{h\nu} 2HO^{\cdot}$$

$$HO^{\cdot} + Trap \rightarrow RA(HO^{\cdot})$$

Equation Set 13

Where RA(HO$^{\bullet}$) is the radical adduct obtained upon trapping of the hydroxyl radical. Scheme 4-10 illustrates the spin trapping reaction between DIPPMPO and hydroxyl radical.



DIPPMPO (1)

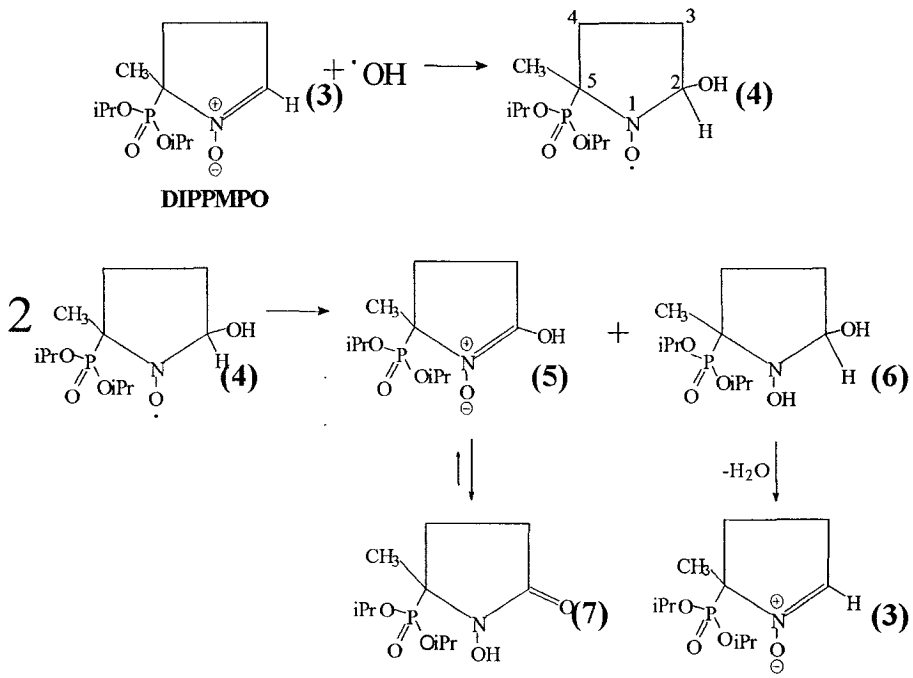
DIPPMPO-OH (2)
(Radical Adduct)

Scheme 4-10. Spin trapping reaction between DIPPMPO and hydroxyl radicals.

For the spin trapping of hydroxyl radicals, Khramstov *et al.*[37] have suggested that the amount of new nitrone observed in the ^{31}P NMR spectrum represents only half of the total spin trapped radical. Half of the radical adducts initially formed are transformed back into the original spin trap via the loss of water of the hydroxylamine product (Scheme 4-11). Assuming the same ratio for DIPPMPO as for the ethoxy analogue (DEPMPO), in order to obtain the real value for the quantity of radicals trapped; one must integrate the ^{31}P NMR signal due to the hydroxyl radical and multiply by a factor of two and compare this to a known concentration of internal standard.

As proposed by Khramstov *et al.*[37], spin trapping by DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide), the ethoxy analogue of DIPPMPO, results in the formation of a radical adduct. When the concentration of radical adducts is high, disproportionation and rearrangement reactions occur, affording a new nitrone (7)

and the original spin trap (Scheme 4-11). The nitrone (**7**), which is the disproportionation product of trapping of hydroxyl radicals, has a ^{31}P NMR chemical shift of 25.3 ppm (a chemical shift difference of 3.4 ppm from the parent compound).



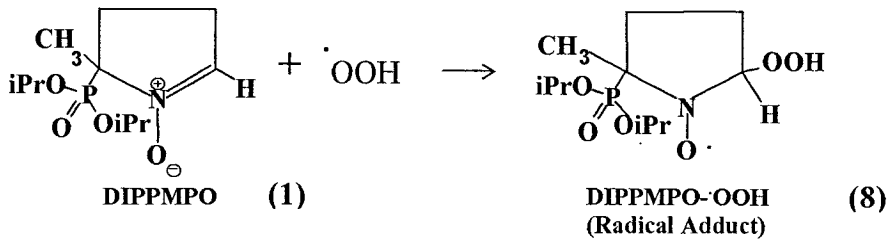
Scheme 4-11. Mechanism for the spin trapping of hydroxyl radicals using DIPPMPO. The reactions shown are similar to those proposed for DEPMPO.[37]

Hydroperoxyl radicals can readily be formed via the reaction between hydroxyl radicals and hydrogen peroxide:

$$^{\bullet}OH + H_2O_2 \rightarrow H_2O + {}^{\bullet}OOH$$
$$^{\bullet}OOH + Trap \rightarrow RA(^{\bullet}OOH)$$

Equation Set 14

The DIPPMPO spin trap may also trap the hydroperoxyl radical. Scheme 4-12 illustrates the spin trapping reaction between DIPPMPO and hydroperoxyl radicals.



DIPPMPO (1) DIPPMPO-OOH (Radical Adduct) (8)

Scheme 4-12. Spin trapping reaction between DIPPMPO and superoxide radicals.

4.4.1 Detection of DIPPMPO/ •OH and DIPPMPO/ •OOH

A typical ^{31}P NMR spectrum obtained from the UV photolysis reaction of hydrogen peroxide (2.5M) in the presence of DIPPMPO (68mM) is shown in Figure 4-13 (numbers shown refer to structures shown in Scheme 4-11). Measurements were taken 5-10 minutes after the irradiation of the sample was completed. The spectrum shows many peaks that correspond to radical disproportionation products of •OH and O_2^{-} •. The full assignment of the ^{31}P NMR signals observed in this work is presented in Table 1. The DIPPMPO/ •OH product appears at a chemical shift of 25.3ppm while the peak at 17.1

ppm is attributed to DIPPMPO/OOH reduction product. The ^{31}P NMR signal present at 25.3ppm can unambiguously be assigned to the hydroxyl radical reaction product. The chemical shift difference between this signal and the DIPPMPO resonance is 3.1 ppm. This chemical shift difference closely agrees with the difference observed by Khramstov *et al.*[37] for DEPMPO and the DEPMPO/adduct reduction product that arises upon the trapping of hydroxyl radicals (3.4 ppm). A small chemical shift difference between this work and the literature is expected since the systems under investigation were not identical. The hydroxyl radicals generated in this work were produced via the photolysis of hydrogen peroxide, while in the work of Khramstov[37] and co-workers the Fenton reaction was used as a source of $^{\bullet}$OH. The assignment of the signal due to the DIPPMPO/OOH disproportionation product is described in Section 4.5. The ^{31} P NMR signal at 18 ppm to date is an unidentified reaction product.

As seen previously (Schemes 4-10 and 4-12) the initial species formed after the spin trap reacts with the radical is a radical adduct (paramagnetic). The lifetime of this paramagnetic species is increased due to the incorporation of phosphorus in the spin trap; however it is not indefinitely stable. The disproportionation product (diamagnetic) is observed in the ^{31}P NMR spectrum as a sharp signal (Figure 4-13).

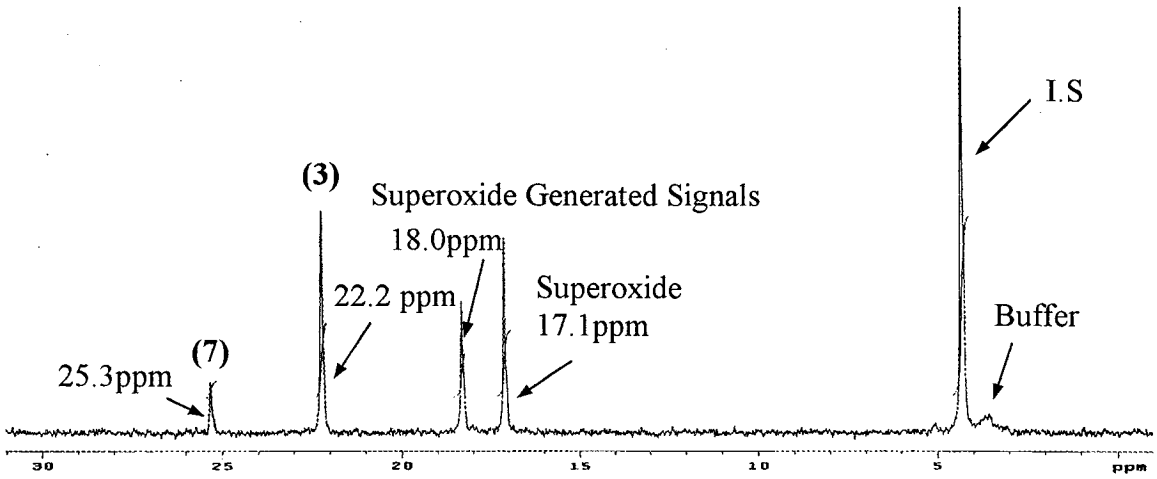


Figure 4-13 Quantitative 31P NMR spectrum of the products formed from the UV photolysis of H2O2 in a sodium hydrogen phosphate buffer solution (pH 7.4). I.S. (internal standard) =trimethylphosphate.

At shorter ultraviolet irradiation times only singlets at 17.1 and 18.0 ppm were observed, however as the duration of irradiation increased two new signals appeared in the NMR spectrum at 16.9 and 18.3 ppm. The ^{31}P NMR signals at 18.0 and 18.3 ppm are to date unidentified. The signals at 16.9 and 17.1 ppm have been assigned to superoxide reaction products; the rationale for their assignment will be subsequently explained (Section 4.5).

Table I. ^{31}P NMR Signals for DIPPMPO Reaction Products

Species	Chemical Shift (ppm)
DIPPMPO (3)	22.2[a,b]
DIPPMPO/OH (7)	25.3[b]
DIPPMPO/OOH (9, 10)	16.9[b], 17.1[b]
Superoxide Generated Species	18.0[b], 18.3[b]

a -deuterium oxide, b- 30mM sodium hydrogen phosphate buffer (pH 7.4)

In order to further confirm and establish that the signal at 25.3 ppm in the ^{31}P NMR spectrum is indeed the result of the spin trapping of hydroxyl radicals; GC/MS was performed on the sample. Figure 4-14 indicates that the molecular ion has an m/z of 279, which is what is expected if hydroxyl radicals have been trapped by DIPPMPO.

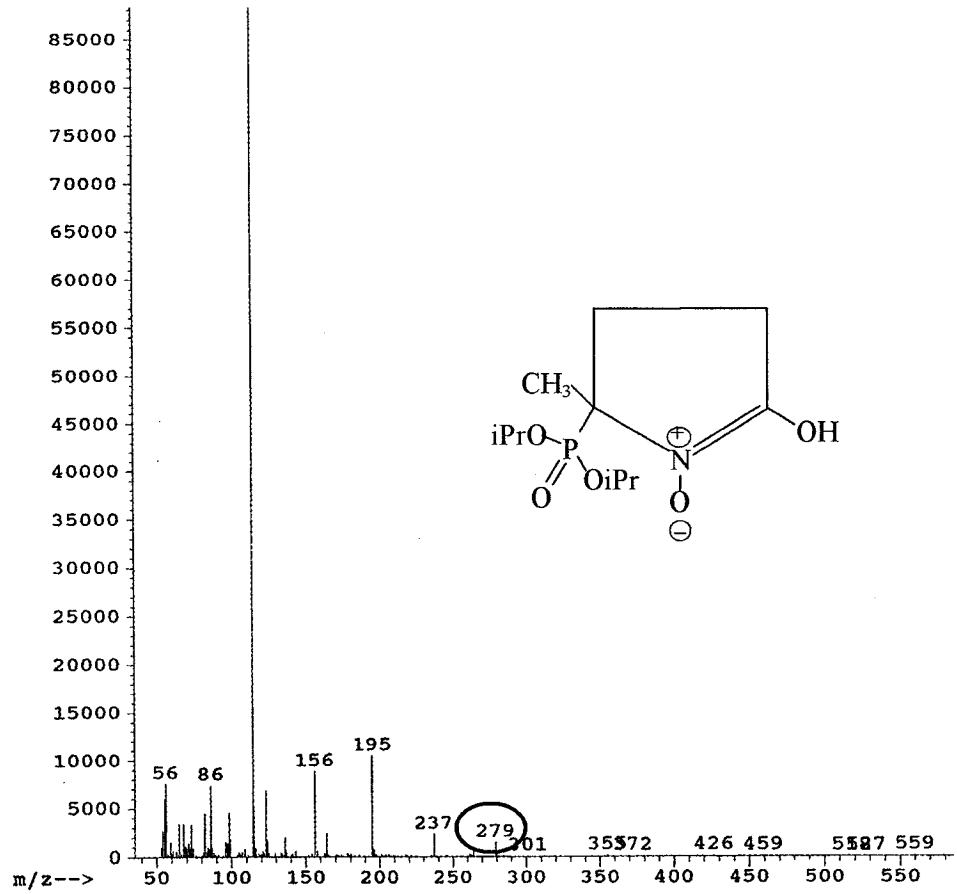


Figure 4-14. Mass spectrum of the nitrone formed upon trapping hydroxyl radical with DIPPMPO after disproportionation and rearrangement have occurred.

4.4.2 Detection of Carbon Centred Radicals

In order to generate carbon centred radicals solvents such as ethanol and dimethylsulfoxide can be added to a system in which $^{\bullet}OH$ is present.[20] Methyl radicals

were generated by either the photolysis of *tert*-butylhydroperoxide or through scavenging reactions of hydroxyl radicals with dimethylsulfoxide (DMSO). The formation of methyl radicals from the hydroperoxide is explained in Section 4.6.3. The methyl radicals generated using the •OH dependent oxidation of DMSO in dilute hydrogen peroxide[24] are formed in the following manner (Equation Set 15):

$$^{\bullet}OH + (CH_3)_2SO \rightarrow {}^{\bullet}CH_3 + CH_3SO_2^-$$
$$^{\bullet}CH_3 + Trap \rightarrow Trap - CH_3$$

Equation Set 15

These radicals were trapped using both DEPMPO and DIPPMPO spin traps. The GC/MS spectrum for the spin trapping of $^{\bullet}CH_3$ with DIPPMPO is presented below (Figure 4-15). The molecular ion for this product is observed at an *m/z* 277.

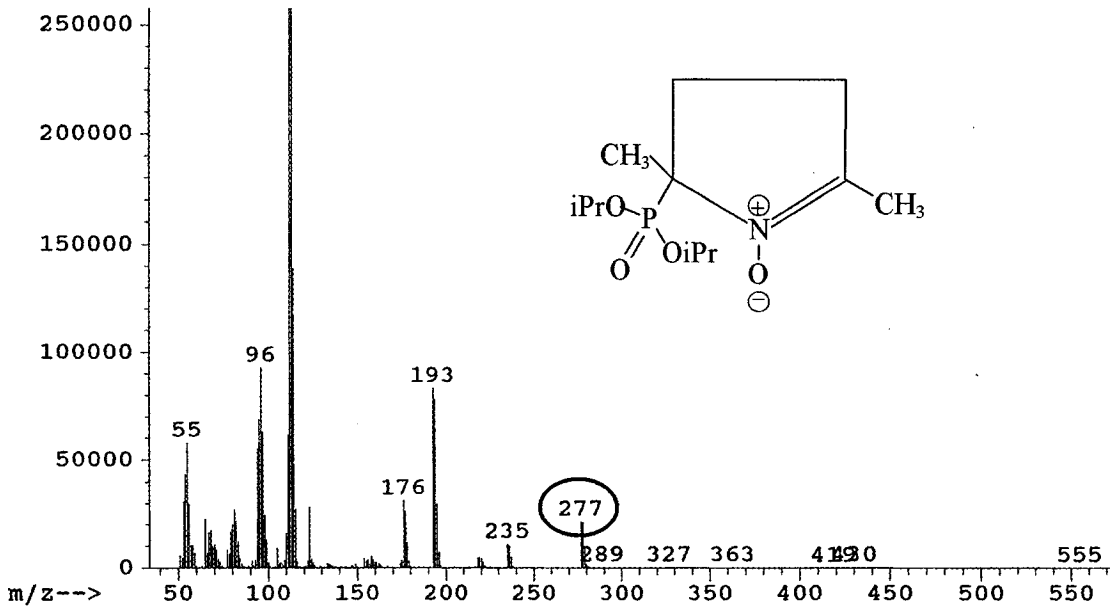


Figure 4-15. Mass spectrum of the nitrone formed upon trapping methyl radical with DIPPMPO after disproportionation and rearrangement have occurred.

In order to produce hydroxyalkyl radicals, hydrogen peroxide was combined with the appropriate alcohol and the sample irradiated with ultraviolet light.[24] These alcohols are hydroxyl radical scavengers that react to form carbon centred radicals. Hydroxymethyl ($^{\bullet}CH_2OH$) and α-hydroxyethyl ($^{\bullet}CH(OH)CH_3$) [53,54] radicals can be readily formed if methanol and ethanol are introduced into a reaction system in which hydroxyl radicals are present. The carbon centred radicals, $^{\bullet}CH_2OH$ and $^{\bullet}CH(OH)CH_3$ were generated by photolysing dilute solutions of hydrogen peroxide in the presence of methanol and ethanol respectively. The ^{31}P NMR chemical shifts for diamagnetic disproportionation products were 22.6 and 27.3 ppm respectively. In this work the ^{31}P NMR signals for the hydroxylamines that are formed when disproportionation occurs after the hydroxyalkyl radical has been trapped were not detected. In previous work done by Frejaville[32], nitrone and two stereoisomeric hydroxylamine signals were observed in the ^{31} P NMR spectra for reactions in which $^{\bullet}CH_2OH$ was trapped. In their work the nitrone degradation product for this radical was shifted 0.4 ppm downfield from the parent spin trap (DEPMPO). In this work the nitrone formed by trapping $^{\bullet}CH_2OH$ also appears at a chemical shift difference of 0.4 ppm from the DIPPMPO spin trap.

It has been reported that spin trapping of the α - hydroxyethyl radical in organic solvents does result in the production of two EPR spectra that correspond to diastereomeric isomers.[54,55] However, it has been observed by Pou *et al.*[56] that in EPR experiments in which the α - hydroxyethyl radical was generated in aqueous solution, diastereomeric isomers were not observed. In this work only one resonance at 27.3 ppm was observed for the spin trapping of $^{\bullet}CH(OH)CH_3$.

Aldehydes and ketones absorb in the 230 to 330 nm region. This is due to the π-π* singlet – singlet transition.[57] Thus aldehydes and ketones excited by ultraviolet light can cleave and generate free radical species:

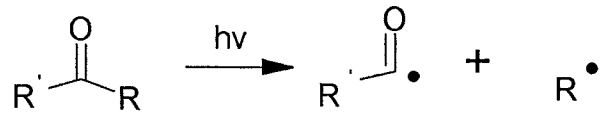


Figure 4-16. Cleavage of aldehyde or ketone using ultraviolet light.

For ketones the cleavage reaction is called the Norrish Type I cleavage. It is possible for R$^{\bullet}$' to be formed via secondary processes that result in the acyl radical (R'-CO$^{\bullet}$) losing carbon monoxide (Equation Set 16).[57]

$$CH_3COCH_3 \xrightarrow{h\nu} CH_3CO^{\bullet} + {}^{\bullet}CH_3$$
$$CH_3CO^{\bullet} \rightarrow {}^{\bullet}CH_3 + CO$$
$$2\,{}^{\bullet}CH_3 \rightarrow CH_3CH_3$$

Equation Set 16

In order to produce acetyl radical ($^{\bullet}$C (O) CH_3), acetone was irradiated with ultraviolet light in the presence of DIPPMPO and the ^{31}P NMR spectrum subsequently obtained. Figure 4-17 clearly indicates the presence of two ^{31}P NMR signals that correspond to the DIPPMPO spin trap (22.2 ppm) and most likely the nitrone formed

when acetyl radical is trapped. Mass spectroscopy was used in an attempt to prove that the signal observed at 30.2 ppm corresponds to the structure shown in Figure 4-17. Unfortunately, MS/MS as well as GC/MS did not reveal any species that had a molecular ion at 305. The only molecular ion observed was 263, which corresponds to the original DIPPMPO spin trap. It is likely that during MS analysis, under the conditions used, the structure shown in Figure 4-17 readily fragments producing a species with a m/z of 263 (DIPPMPO) and 43 (acyl group). The presence of a distinct ^{31}P NMR signal at 30.2 ppm is therefore only assumed to be that of the nitrone formed when the acetyl radical is trapped by DIPPMPO, since ultraviolet irradiation of the symmetrical ketone acetone should afford $^{\bullet}C(O)CH_3$.[57] Further work in this area with acetone and other ketones is consequently essential in clarifying this important issue.

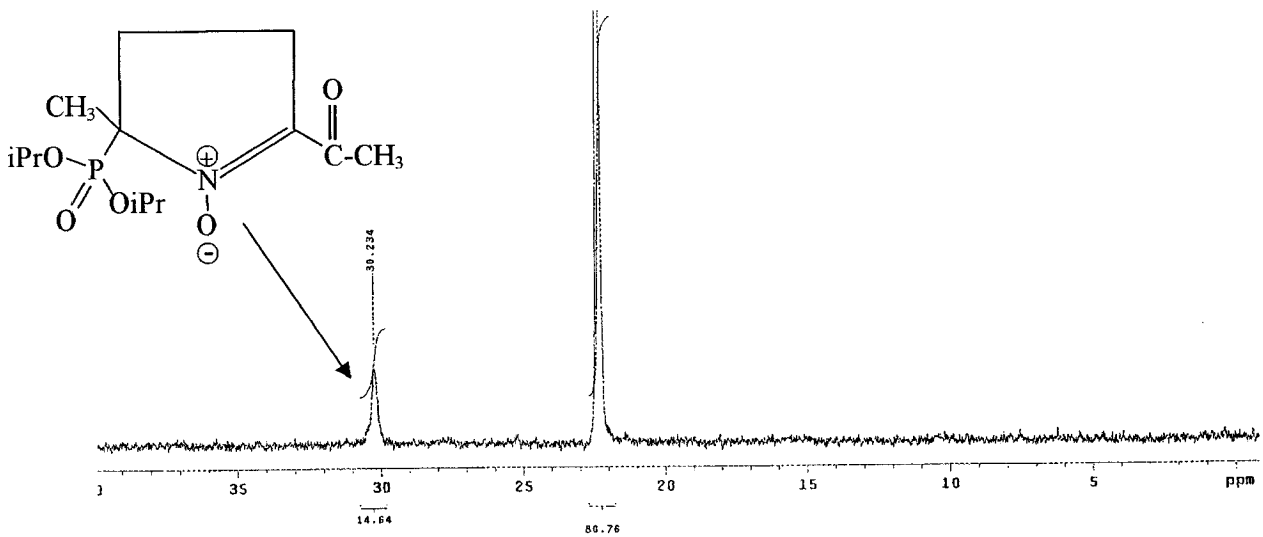


Figure 4-17. ^{31}P NMR spectrum of the nitrone formed from the UV photolysis of acetone with DIPPMPO spin trap.

Table II presents ^{31}P NMR signals for the products of DIPPMPO and DEPMPO with various free radical species observed in this work.

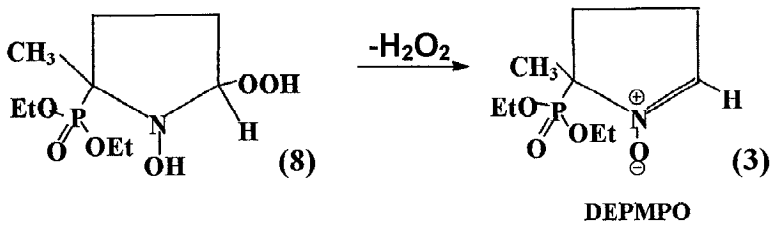
Table II. ^{31}P NMR Signals for DIPPMPO/DEPMPO Reaction Products

Species	Chemical Shift (ppm)
DEPMPO	24.5[a], 23.4[b]
DEPMPO/$^{\bullet}CH_3$	24.2[d]
DEPMPO/$^{\bullet}$ CH_2OH	24.0[c]
DIPPMPO/$^{\bullet}CH_3$	23.1[d,e]
DIPPMPO/$^{\bullet}$ CH_2OH	22.6[c]
DIPPMPO/$^{\bullet}CH$ (OH) CH_3	27.3[f]
DIPPMPO/$^{\bullet}C(O)CH_3$	30.2[g]

a- deuterium oxide, b – 30% H_2O_2+ UV light c- methanol and 3% H_2O_2 + UV light, d- dimethylsulfoxide, 3 % H_2O_2+ UV light, e – tert-butylhydroperoxide+ UV light, f- ethanol, 3% H_2O_2+ UV light, g - acetone+ UV light.

4.5 ASSIGNMENT OF SUPEROXIDE SIGNALS

Literature accounts indicate that the trapping of superoxide with DEPMPO results in the formation of a signal that cannot be distinguished from that of the hydroxyl reaction product.[37] This finding was based on the fact that no new phosphorus resonances were observed in the spectrum even though ESR measurements showed that the DEPMPO spin trap did indeed trap superoxide. The weak signals reported by these researchers, as a result of trapping superoxide, were explained to be a consequence of superoxide radical adducts being reduced to their corresponding hydroxylamines and converted back into the original DEPMPO spin trap via the loss of hydrogen peroxide (Scheme 4-18).[37]



Scheme 4-18. Loss of hydrogen peroxide from hydroxylamine.

In this work DIPPMPO, the isopropoxy analogue of DEPMPO, has been used to detect and quantify products generated through the reaction of the spin trap and superoxide radicals. It has been shown that superoxide radicals can be formed in good yield by the photolysis of hydrogen peroxide solutions.[58] Therefore, signals due to the spin trapping of hydroxyl radicals and superoxide radicals were expected in the ^{31}P NMR spectrum. The signals that appear at 16.9 and 17.1 ppm are thought to correspond to the two stereoisomeric forms of the reaction product of DIPPMPO with superoxide, since the carbon in position 2 is chiral (Scheme 4-19).

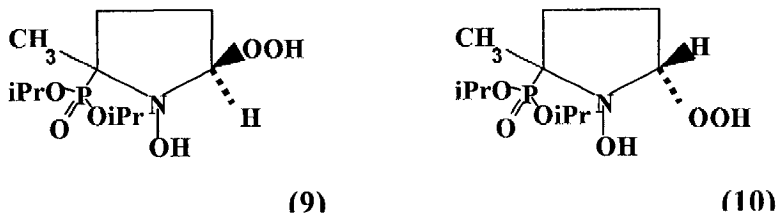


Figure 4-19. Hydroxylamines formed from the reaction of superoxide with DIPPMPO spin trap.

The stereoselectivity and stereospecifity of radical addition to nitroxides has been mentioned in the literature.[59] It has been reported that superoxide radical additions with DEPMPO are stereoselective, while hydroxyl radicals are trapped stereospecifically with

DEPMPO.[27,59,60 6162] Frejaville *et al.*[28] have also reported on the stereospecificity of DEPMPO with a variety of other radicals such as $CO_2^{\bullet-}$, $SO_3^{\bullet-}$, $^{\bullet}CH_3$ and α-hydroxyalkyl in aqueous solution. These researchers have also reported that in phosphate buffers at pH 5.6 and 7.0 the superoxide adduct of DEPMPO is stereoselective.[28] From this, it follows that spin trapping experiments should result in the formation of both superoxide radical adducts and only one isomer of the hydroxyl spin adduct. If this reasoning is extended to the DIPPMPO spin trap, then one would expect to observe a single resonance that corresponds to the trapping of hydroxyl radicals and two signals for superoxide trapped products, this being the case in the present work. The appearance of only one peak, at shorter reaction times most likely has to do with the fact that at shorter irradiation times only the major stereoisomer of the superoxide adduct is prevalent, but at longer times there is accumulation of the minor stereoisomer, which allows for its detection.

GC/MS analyses were also carried out in order to elucidate the structure of the signals that appear at 16.9 and 17.1ppm. The major mass spectrometric fragmentation peaks were as follows (Figure 4-20): *m/z* (relative intensity %): 296(M-1, 10%), 254(100%), 212(8%). The major peak at *m/z* =254 corresponds to the loss of one isopropyl group.

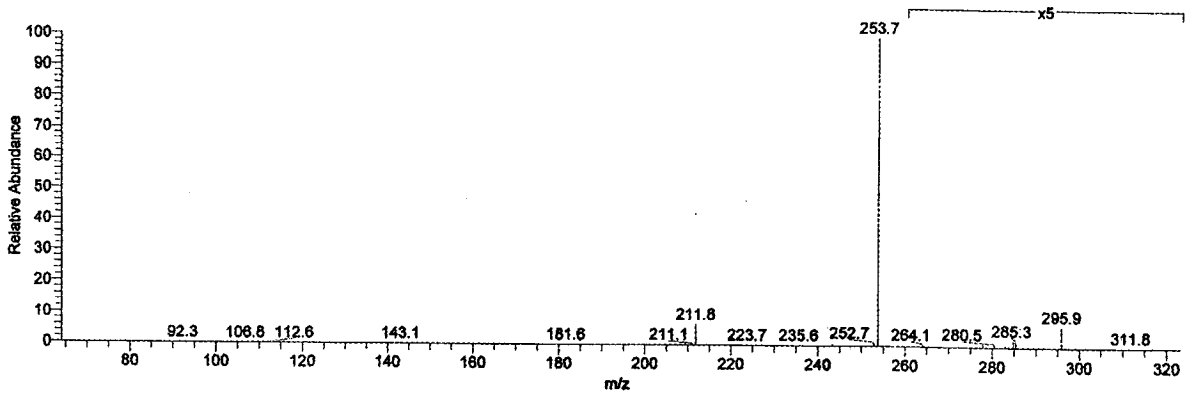


Figure 4-20. Mass spectrum of the superoxide reaction product (hydroxylamines) obtained by GC/MS analysis.

MS/MS analysis was also performed on these samples and the m/z for the molecular ion was determined to be 297, in agreement with the results obtained from GC/MS analysis (Figure 4-21).

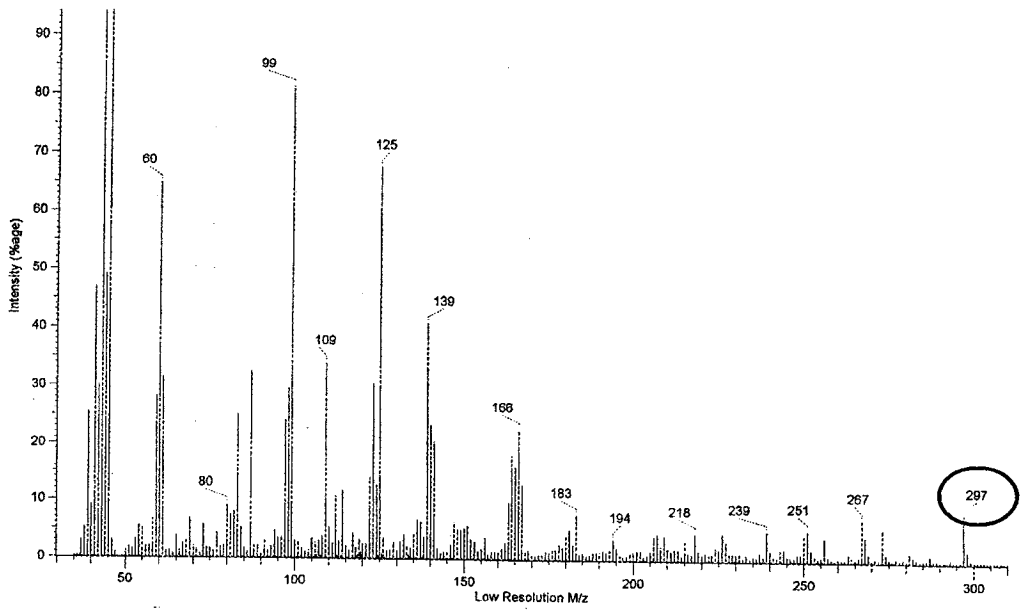


Figure 4-21. Mass spectrum of the superoxide reaction product (hydroxylamines) obtained from MS/MS analysis. *m/z* =297.

The superoxide free radical will add to the activated double bond of the spin trap resulting in the formation of hydroperoxide.[20] However, the primary reaction of superoxide in aqueous solutions, disproportionation, occurs at a faster rate than its addition to a double bond.[53] Disproportionation via hydrogen abstraction from solvents or substrates produces HOO$^{\bullet}$ which then can add to the double bond of the nitrone. It is therefore expected that the reaction product of superoxide with DIPPMPO should indeed be an hydroperoxide.

Superoxide radical anion is a very strong nucleophile in aprotic solvents.[14] However, in protic solvents such as water, it does not react as a nucleophile. Thus nucleophilic addition of the superoxide radical anion to the double bond of the spin trap is not likely.

The assignment of these signals to superoxide products was further verified by introducing DIPPMPO into a system that generates superoxide. Potassium superoxide (KO_2), when dissolved in water is known to generate superoxide[63] which can then be trapped and its phosphorus signal detected. The spectrum that resulted from this reaction contained a ^{31}P NMR resonance at 17.1 ppm along with several unidentified peaks whose identity is currently under examination. The presence of this signal in the superoxide generating system, along with the observation that this signal was drastically reduced when the enzyme superoxide dismutase (SOD) was introduced into the system, further confirms that the peak at 17.1 ppm is related to the generation of the $O^{\bullet -}_2$. Superoxide dismutase accelerates the rate of superoxide radical dismutation to hydrogen peroxide, therefore a dose dependent diminution in the spectrum should be observed if the signal is due to the presence of superoxide radical anion.[23]

4.6 EXAMINING THE QUANTITATIVE RELIABILTY OF THE TECHNIQUE

Spin trapping experiments used to determine the concentrations of free radicals were performed a minimum of three times in order to examine the quantitative reliability of the technique. UV irradiations of 30% hydrogen peroxide solutions were used to generate hydroxyl and superoxide radicals, while methyl radicals were produced by the irradiation of *tert*-butyl hydroperoxide. All of these experiments were performed at neutral pH, (pH 7.4 phosphate buffer solution), and the concentration of radical species monitored as a function of time using an internal standard and ^{31}P NMR spectroscopy.

A major source of error associated with this spin trapping is the presence of trace metal impurities, especially in buffered solutions.[20] To reduce many of the problems caused by these metals, metal-chelating agents such as diethylenetriamine pentaacetic acid, (DTPA), can be used. The chelating agent is usually present in concentrations of 0.1 to 1 mM[20] and is added prior to reaction initiation to ensure that all metals present in the system have been bound. In this work the 4 mM of DTPA was used to rid samples of metal impurities.

The amount of dissolved oxygen in the samples is another possible source of error. As will be shown in this work, the concentration of free radicals, namely superoxide radicals, in the peroxide solutions is influenced by the concentration of molecular oxygen present in the sample. The concentration of oxygen in the samples used for quantitative reliability studies was not monitored or kept constant during sample preparation.

4.6.1 Hydroxyl Radicals

The concentration of hydroxyl radicals produced and trapped by DIPPMPO was measured as a function of total irradiation time. Previous ESR experiments have shown that when high concentrations of DEPMPO are used the rate of spin trapping is equal to the rate of hydroxyl radical generation.[28] Figure 4-22 illustrates that under the UV photolysis conditions reported in the experimental section, the concentration of hydroxyl radicals produced and trapped is linear with time. The rate of hydroxyl radical production for these experiments was found to be 200μM/min.

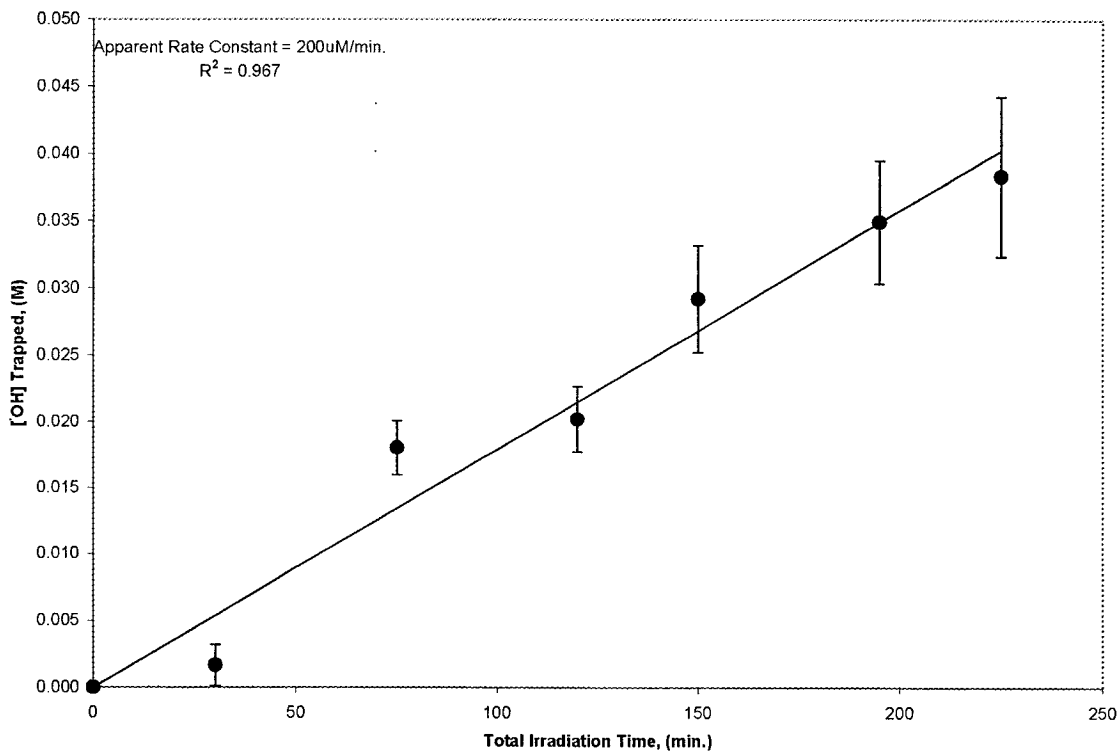


Figure 4-22. Concentration of hydroxyl radicals trapped as a function of total ultraviolet irradiation of 30% hydrogen peroxide solutions containing DIPPMPO spin trap.

It is important to note that the hydroxyl radicals formed from UV photolysis experiments of 30% H_2O_2 were derived from hydrogen peroxide and not from solvent water. Lloyd *et al.*[63] have carried out spin trapping experiments with 30% hydrogen peroxide and labelled water (^{17}O) in which they have proven that exchange between solvent water and H_2O_2 does not occur.[63]

4.6.2 Superoxide Radicals

During ultraviolet irradiation of hydrogen peroxide solutions, superoxide radicals were also generated. The concentration of trapped superoxide radicals varied dramatically from experiment to experiment as is reflected in Figure 4-23. There is no correlation in the total concentration of radicals formed as a function of total irradiation time. However, as would be expected, there is an overall trend, which is an increase in superoxide radical concentration with increasing time.

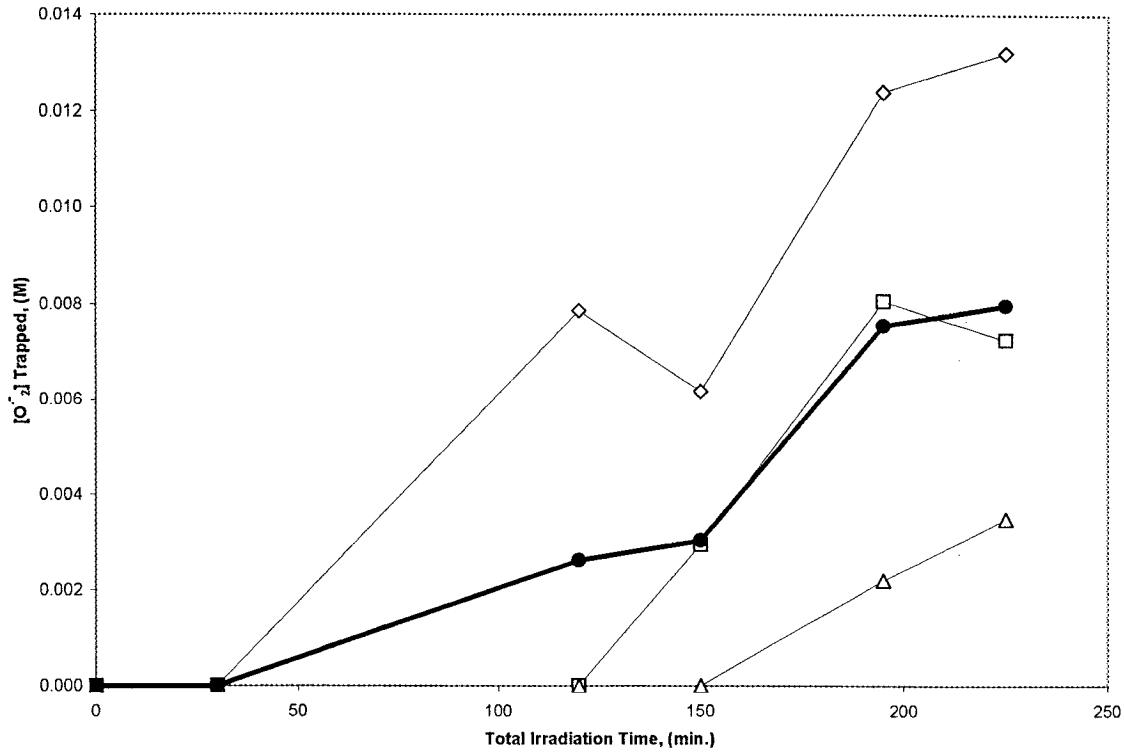
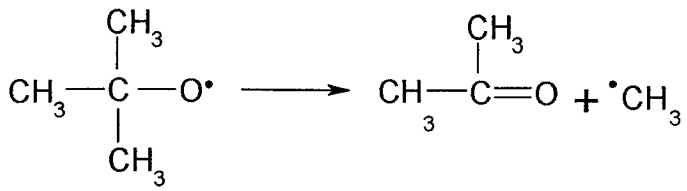


Figure 4-23. Concentration of superoxide radicals trapped as a function of total ultraviolet irradiation of 30% hydrogen peroxide solutions containing DIPPMPO spin trap (pH 6). ◊ - trial 1 data points, □- trial 2 data points, Δ- trial 3 data points and • - average data points.

4.6.3 Methyl Radicals

UV photolysis of the organic peroxide, tert-butylhydroperoxide, in 70 % water (30% tert-butylhydroperoxide by weight), was also carried out as a function of time. This reaction did not generate the tert-butoxy radical, $^{\bullet}O(CH_3)_3$, but rather the methyl radical, $^{\bullet}CH_3$. This is expected since tertiary alkoxy radicals predominantly fragment into alkyl radicals and carbonyl species via intramolecular rearrangements.[64]:



Equation Set 17

Figure 4-24 illustrates that the formation of methyl radicals is linear with total irradiation time. The apparent rate constant for this reaction was determined to be 20 μM/min. It is apparent from the graph that the linear regression line does not go through the zero. This is most likely due to the fact that the methyl radicals formed are the result of secondary reactions. The tert-butoxy radical is formed initially; however, the rearrangement of this radical into acetone and the methyl radical then occurs. From Figure 4-24 it appears that detectable levels of methyl radicals from the rearrangement reactions of $^{\bullet}O(CH_3)_3$ do not occur until approximately 100 minutes.

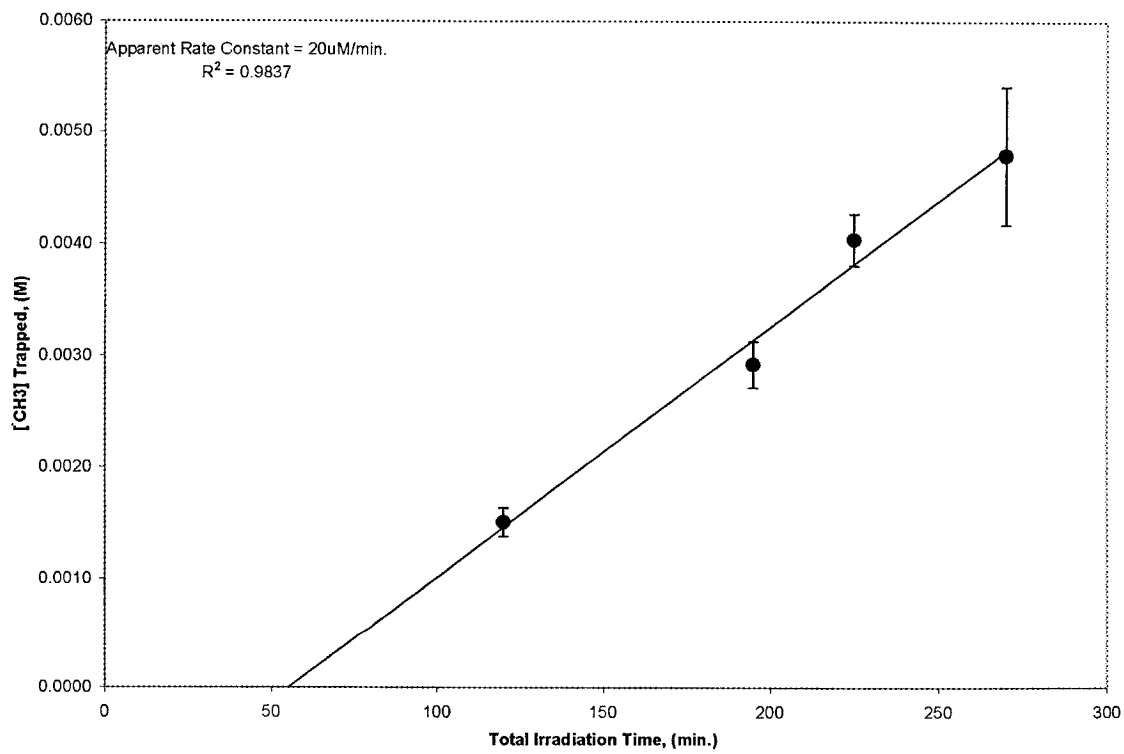


Figure 4-24. Concentration of methyl radicals trapped as a function of total ultraviolet irradiation of tert-butyl hydroperoxide solutions containing DIPPMPO spin trap.

4.7 FREE RADICALS IN SOLUTIONS OF HYDROGEN PEROXIDE

The chemistry of hydrogen peroxide is a topic that has been investigated by numerous researchers. Hydrogen peroxide is readily formed in any oxidative processes that involve oxygen and water. As reported by many researchers reactive free radical species are formed during the decomposition of aqueous hydrogen peroxide, as shown by the following set of equations: [65].

$$H_2O_2 + OH^- \leftrightarrow HOO^- + H_2O$$
$$H_2O_2 + HOO^- \rightarrow HO^{\cdot} + O_2^{\cdot-} + H_2O \qquad \text{Equation Set 18}$$
$$HO^{\cdot} + O_2^{\cdot-} \rightarrow OH^- + O_2$$

Hydroxyl and superoxide radicals are the free radical intermediates involved in the decomposition reactions and are more prevalent when peroxide decomposition is catalysed by transition metals.[65, 66] It is important to mention that, although the reactions in Equation Set 18 have been reported by several researchers[65,66], there is some debate about whether or not these reactions occur in the absence of transition metals. Thermodynamics have shown that the second reaction (the spontaneous formation of hydroxyl and superoxide radicals) is not favourable and that the reaction does not happen if not in the presence of metal catalysts.

In this part of our work, the concentration of hydroxyl and superoxide radicals formed in peroxide solutions has been quantified using ^{31}P NMR spectroscopy.

4.7.1 A Novel Equilibrium Reaction

Recently, Petlicki and Van de Ven[67] have proposed that superoxide radicals seem to exist in equilibrium with alkaline hydrogen peroxide when in contact with either air or oxygen. (Equation 19)

$$O_2 + HOO^{(-)} +^{(-)} OH \rightarrow 2O_2^{(-)\bullet} + H_2O \qquad \text{Equation 19}$$

Aqueous solutions of alkaline hydrogen peroxide in contact with air and oxygen should result in the production of varying quantities of superoxide radicals, while alkaline hydrogen peroxide in contact with nitrogen should not produce any superoxide radicals. In this work the concentration of superoxide radicals generated was found to depend upon the atmosphere with which the peroxide was in equilibrium (Figure 4-25). It is interesting to observe that non-irradiated solutions of hydrogen peroxide can readily produce superoxide radicals via the interaction of oxygen with alkaline hydrogen peroxide (Equation 19). According to Petlicki's and van de Ven's theory, an increase in superoxide radical anion concentration should be observed if aqueous peroxide is in contact with oxygen rather than air. Figure 4-25 shows an increase, albeit slight, in the concentration of superoxide radicals trapped in the presence of oxygen. Conversely, a decrease in the quantity of superoxide radicals trapped is expected if oxygen is purged by nitrogen in the sample. This decrease is apparent in Figure 4-25 and supports the argument that H_2O_2 can be oxidised by oxygen to afford superoxide.[67]

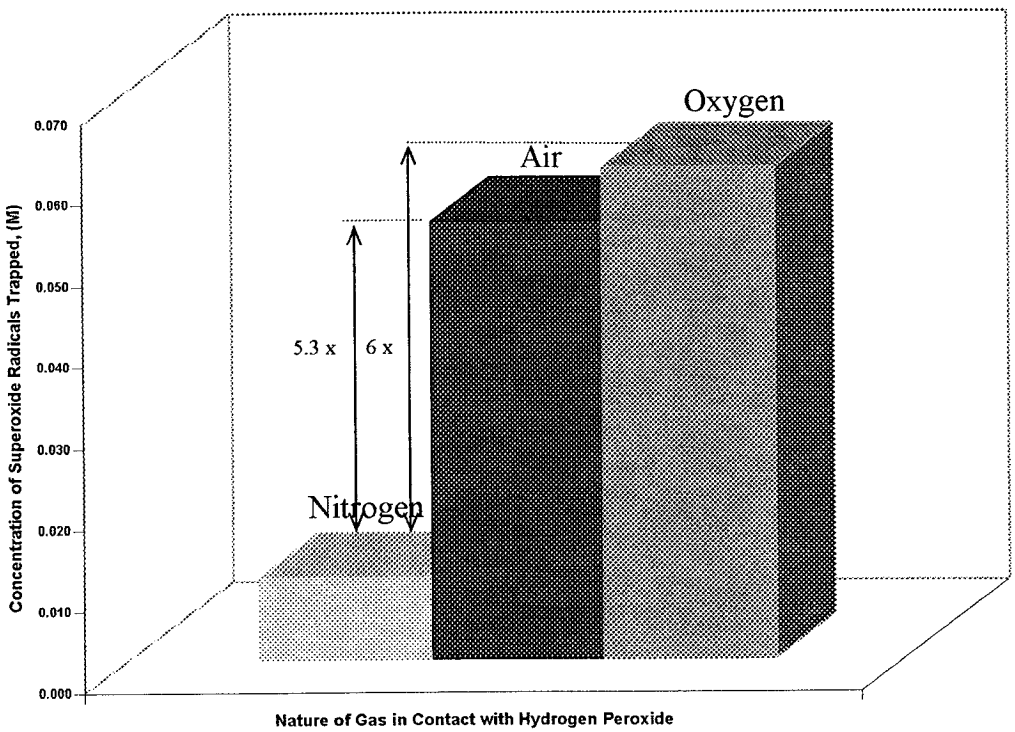


Figure 4-25. Concentration of superoxide radicals present in solutions of hydrogen peroxide when in contact with nitrogen, air and oxygen.

According to Equation 19, hydrogen peroxide in contact with nitrogen should not generate superoxide radicals. Under the experimental conditions used it is clear, (Figure 4-25); that some superoxide radicals exist when alkaline hydrogen peroxide is in contact with nitrogen gas. The presence of superoxide radicals is most likely due to the incomplete replacement of nitrogen gas for air during the freeze/thaw cycles used to rid samples of air/oxygen. Additional sequential freeze and thaw cycles under nitrogen to completely eliminate the oxygen from peroxide solutions should further substantiate these conclusions.

A prelimary look into the dependence of the measured concentration of superoxide radicals as a function of spin trap concentration was investigated. According

to Equation 19, the addition of spin trap should result in a shift in the equilibrium to the right resulting in the formation of more superoxide radicals. In an irreversible reaction the concentration of superoxide radicals should be equal to or less than the spin trap concentration. A look at Figure 4-25 clearly illustrates that the concentration of superoxide radicals trapped when alkaline peroxide is in contact with oxygen is approximately 70 mM, which is equal to the concentration of the DIPPMPO spin trap used in these experiments (68 mM). This result suggests that the spin trapping reaction of superoxide radicals may indeed be irreversible.

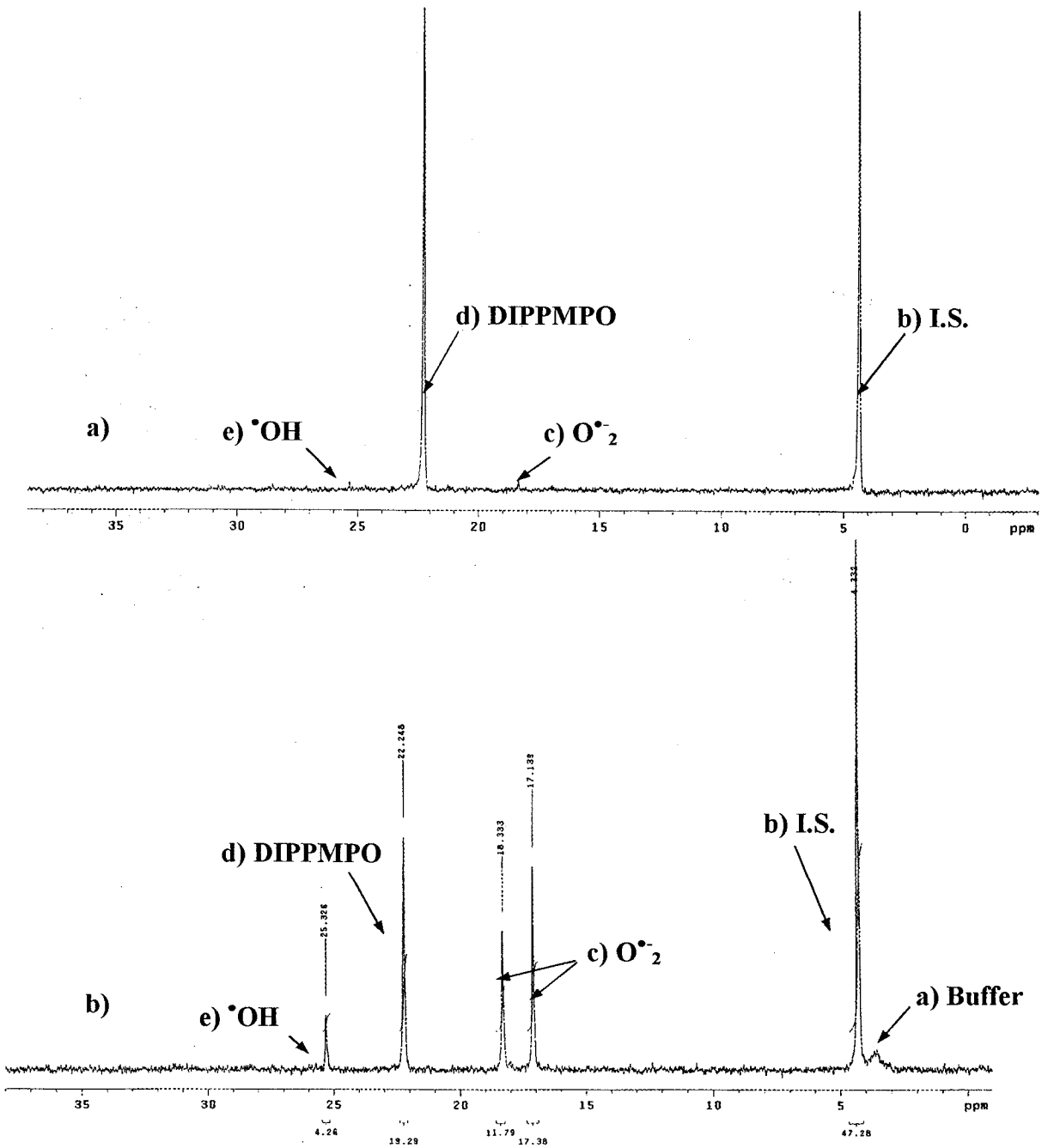
4.7.2 Radicals in Peroxide Solutions as a Function of pH

The free radicals present in solutions of hydrogen peroxide were examined as a function of pH. Buffered solutions of hydrogen peroxide were stirred under ambient conditions and the concentrations of hydroxyl and superoxide radicals were determined.

Figure 4-26 shows typical ^{31}P NMR spectra that were obtained when the concentration of hydroxyl and superoxide radicals were determined as a function of pH. It is clear by looking at these spectra that there is a variation in signal intensity, (i.e. variation in concentration), for the radical species as a function of pH. The incorporation of a known concentration of trimethylphosphate (internal standard) into the samples prior to ^{31}P NMR measurement allows for the concentration of radical species to be determined as the pH is varied.

At acidic pH (pH 4), the concentrations of superoxide and hydroxyl radicals are barely above the level of detection in the ^{31}P NMR spectrum. However, as the pH is increased to neutral, there is an increase in the concentration of superoxide and hydroxyl

radicals, and the simultaneous decrease in DIPPMPO concentration. Surprisingly, as the pH becomes more alkaline, the superoxide signals begin to collapse into each other, with the total convergences of the two signals becoming complete at ~ pH 11. The collapse of these two ^{31}P NMR signals into a sharp resonance at 17.1 ppm and a tail at 16.9 ppm is surprising. A possible explanation for this convergence may be a shift in the isomeric forms of DIPPMPO-OOH as pH increases.



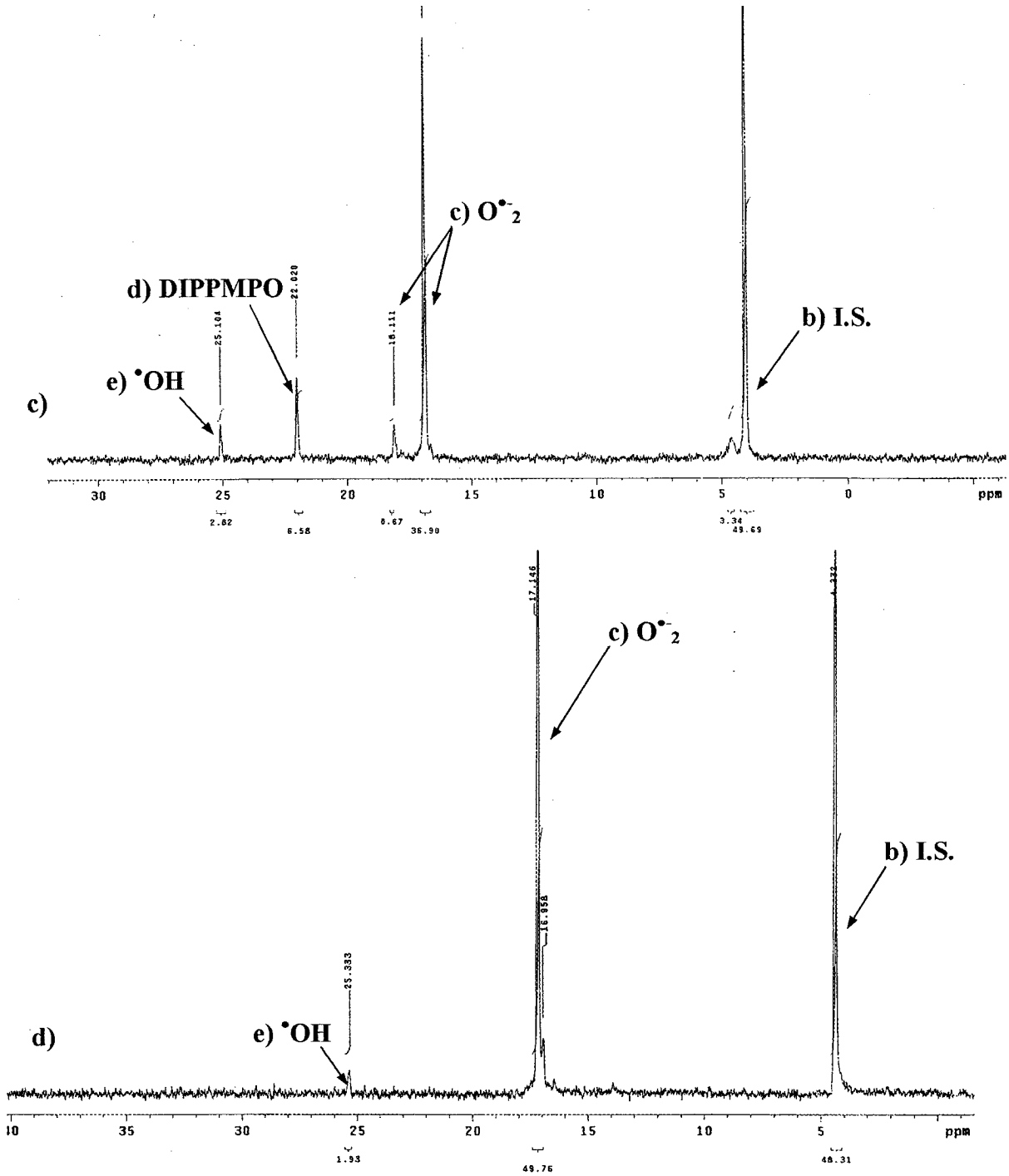


Figure 4-26. ^{31}P NMR spectra a) pH 4 buffer solution, b) pH 7 buffer solution, c) pH 9 buffer solution and d) pH 11 buffer solution. a-sodium hydrogen phosphate buffer, b-trimethylphosphate internal standard, c-superoxide radical reduction products signals, d-DIPPMPO, e- hydroxyl radical reduction product signal.

The increase in superoxide radical concentration as pH increases can be readily explained by Equation 19, which predicts that there will be an increase of superoxide radicals with increasing ^{-}OH concentrations. As one moves to more alkaline pH's more of the hydrogen peroxide in the system will be in the form of it's conjugate base ($pKa(H_2O_2)$= 11.8), therefore the formation of superoxide radical anion should be more significant. When the solution is buffered at high pH the following equilibrium is shifted to the right:

$$HOO^{\bullet} \rightleftarrows H^{+} + O_2^{(-)\bullet} \qquad \text{Equation 20}$$

The lifetime of superoxide radical anion is thereby increased since superoxide does not react with itself at any appreciable rate ($O_2^{(-)\bullet} + O_2^{(-)\bullet}$ in water <0.3$M^{-1}s^{-1}$). [68] The hydroxyl radicals formed during the decomposition of peroxide (i.e. catalyzed by the presence of trace amounts of metal impurities in the buffer solution) can subsequently react with hydroperoxyl anions to give hydroperoxyl and superoxide radicals:

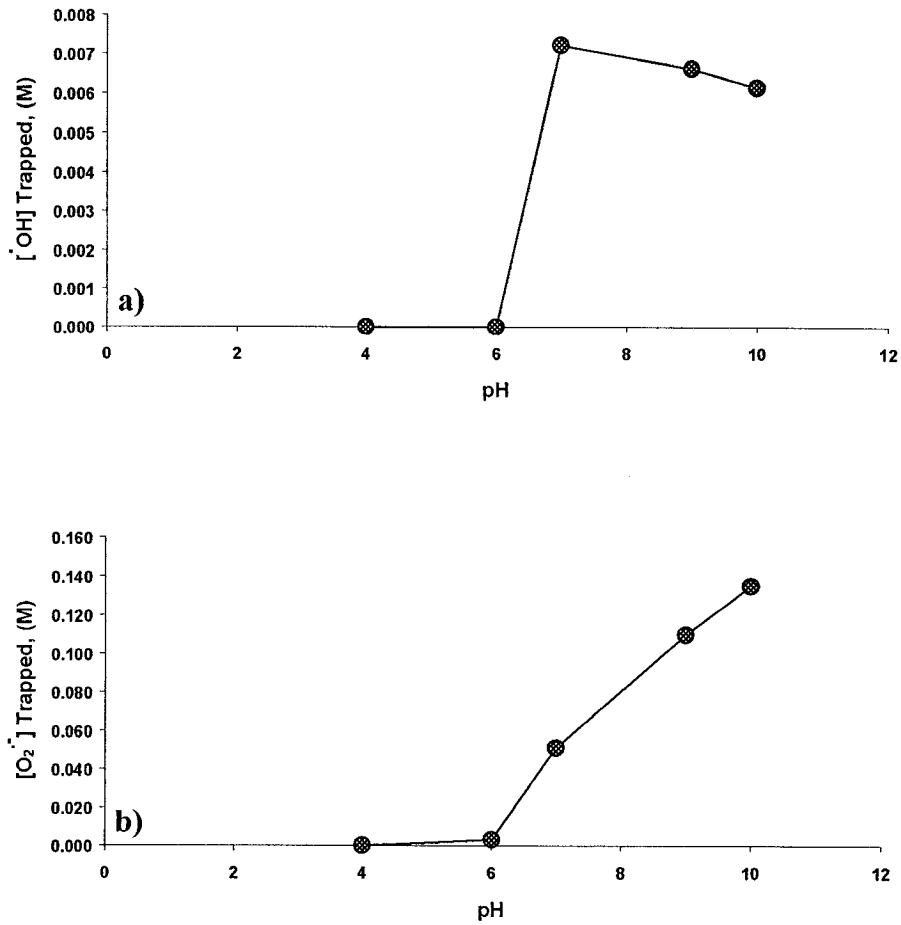
$$HOO^{(-)} + {}^{\bullet}OH \rightarrow {}^{(-)}OH + HOO^{\bullet} \rightleftarrows O_2^{(-)\bullet} + H_2O \qquad \text{Equation 21}$$

Figure 4-27b illustrates the anticipated increase in superoxide radical concentration with increasing pH. Superoxide radicals were not detectable under acidic conditions. However, with increasing pH their formation was found to markedly increase. In contrast, the concentration of hydroxyl radicals trapped did not vary as profoundly with pH compared to superoxide (Figure 4-27a). Interestingly, there were no detectable

amounts of hydroxyl radicals trapped at pH 4 or 6. However, the maximum quantity of hydroxyl radicals trapped was at neutral pH, followed by a slight decrease as the alkalinity was increased. The low concentration of hydroxyl radicals trapped at higher pH values is not due to the scavenging capabilities of the hydroperoxyl anion. This is evident by the low concentration of hydroxyl radicals that are trapped at acidic pH when hydrogen peroxide is not in its deprotonated form.

It is important to note that the large difference between the concentration of hydroxyl radical and superoxide radical trapped by the spin trap is not the result of preferential reaction rates between $O_2^{(-)\bullet}$ and $^{\bullet}OH$ and the trap . The spin trapping rate is 10^8 times faster[32] for $^{\bullet}OH$ than for $O_2^{(-)\bullet}$ therefore the concentration of hydroxyl radicals is expected to be greater than that of superoxide radical.

The increase in superoxide radical concentration observed in this work can also be explained by the work done by Finkelstein *et al.*[30] These researchers investigated the pH dependence on the apparent rate constant for superoxide trapping in aqueous solutions with the dimethylated analogue of DIPPMPO. Finkelstein *et al.*[30] have shown that the apparent rate constant for superoxide trapping increases as the pH is lowered; however this increase is offset by the increase in the rate of spontaneous dismutation of superoxide that occurs as pH decreases. At higher pH values, it was observed that the spontaneous dismutation of superoxide decreased linearly with pH and that the spin trapping reaction became less sensitive to pH. As a result of this, the ability of the spin trap to compete with the spontaneous dismutation of superoxide is enhanced at higher pH.



Figures 4-27. a) Concentration of hydroxyl radicals as a function of pH. b) Concentration of superoxide radicals as a function of pH.

As stated previously, higher concentrations of superoxide radical should be expected as pH increases. However, the decrease in hydroxyl radical concentration at elevated pH values can be explained by the following sets of equations:

Indirect

$$O_2^{(-)\bullet} + {}^{\bullet}OH \rightarrow {}^{(-)}OH + O_2$$
$$O_2 + {}^{(-)}OOH + {}^{(-)}OH \rightarrow 2O_2^{(-)\bullet} + H_2O$$

Equation 22 (c.f. Equation 19)

Direct

$$HOO^{(-)} + {}^{\bullet}OH \rightarrow {}^{(-)}OH + HOO^{\bullet} \leftrightarrow O_2^{(-)\bullet} + H_2O$$

(c.f. Equation 21)

In both of these equations, hydroxyl radicals are lost via rapid reactions with superoxide radical ($k = 8.0 \times 10^9 M^{-1}s^{-1}$)[68] and hydroperoxyl anion ($k = 7.5 \times 10^9 M^{-1}s^{-1}$)[68] respectively. The end result of either one of these reaction pathways is the generation of superoxide radical either indirectly, (through the formation of molecular oxygen), or directly (Equation 21).

Similarly to the ultraviolet irradiation experiments, (Section 4.6.2), hydroxyl and superoxide radicals were generated in the ambient condition hydrogen peroxide experiments (unchelated buffer solutions, refer to page 28). The source of these radicals is due to transition metal impurities in the buffer solutions which promote hydrogen peroxide decomposition. However, unlike the ultraviolet case, the superoxide concentration was found to be reproducible. Figure 4-26 shows the concentration of both hydroxyl radicals and superoxide radicals for triplicate experiments carried out at a pH value of 7. From these figures it is apparent that these experiments are indeed reproducible. These results are typical of all the pH ranges examined.

The concentration of hydroxyl and superoxide radicals generated during the stirring of hydrogen peroxide at pH 7 is expectedly lower than that obtained during UV photolysis experiments of hydrogen peroxide at pH 7. From Figure 4-26 it is clear that the concentration of these free radical species is surprisingly high in systems in which hydrogen peroxide is not subjected to thermal or photolytic dissociation.

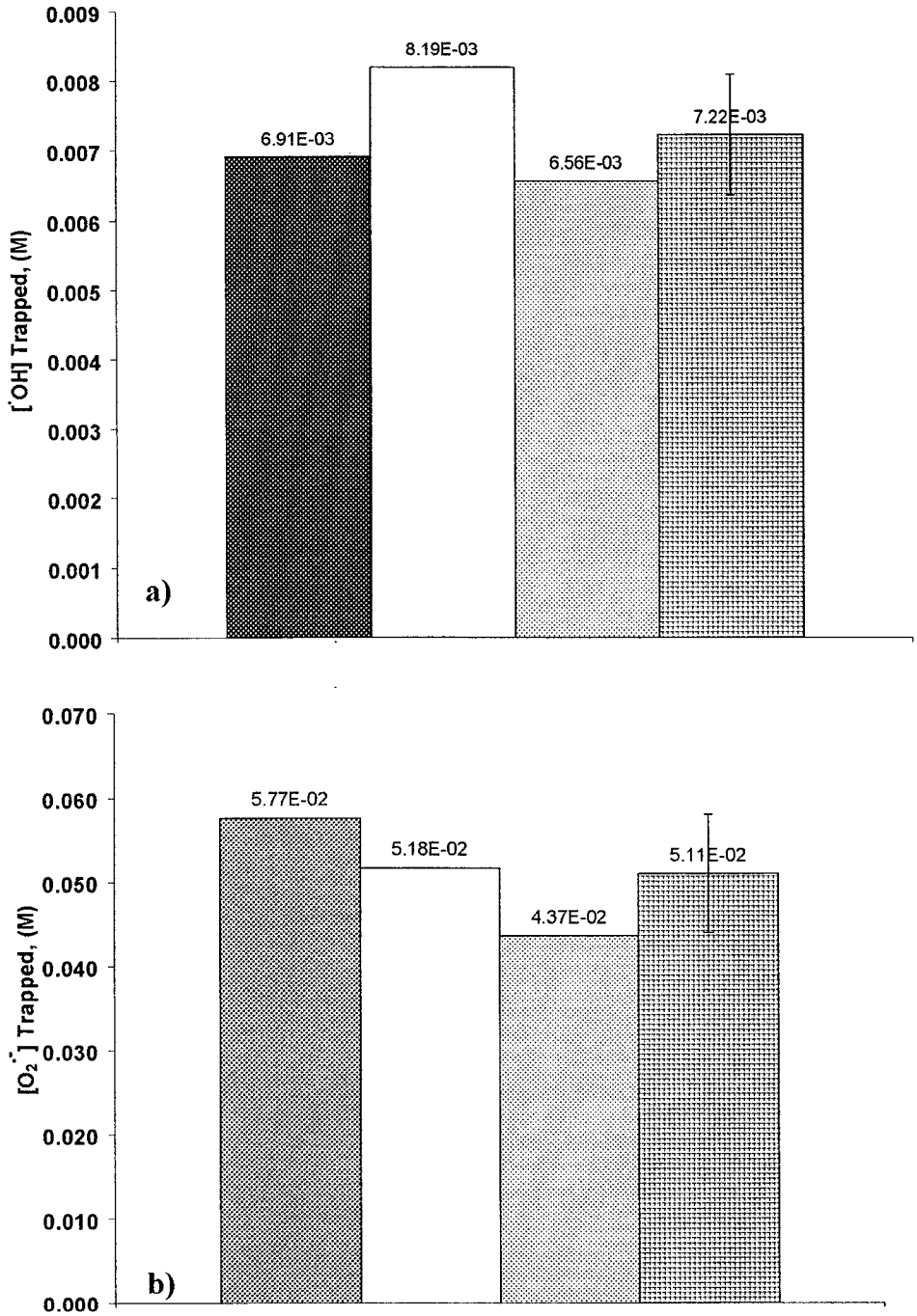


Figure 4-26. Bar graphs for triplicate experiments done at pH 7 for a) concentration of hydroxyl radicals and b) concentration of superoxide radicals.

CHAPTER 5

APPLICATIONS

5.1 A BIOMEDICAL APPLICATION OF THE DEVELOPED TECHNIQUE

The importance of detecting and monitoring reactive oxygen species or related radicals in medicine is imperative due to their participation in pathology and disease.[25] The goal of in vivo electron paramagnetic (EPR) resonance spin trapping is to allow for the characterization and quantification of free radicals with time within living organisms.[36] However due to limitations associated with this technique; toxicity limitations with dosing and most importantly the relative instability of the radical adducts, novel approaches have been used that combine the strengths of spin trapping with methodologies which aim to overcome the issue of radical adduct decomposition. A new technique, NMR spin trapping, although in infancy has shown promise as a tool that can be used to measure oxidative stress in living organisms in real time.

In deciding on a technique for the detection of free radical species in vivo certain criteria must be considered. These criteria include: i) the sensitivity of the measuring device, ii) the stability of the reaction products, iii) the specificity of the free radical or oxidant reactions, iv) localization and v) toxicity and invasiveness. To date no single probe or methodology exists that meets all of these criteria.[36]

Since most free radicals have short half-lives as a result of their reactivity with other reactive species and occur at low concentrations, sensitive measuring techniques are crucial. This parameter directly relates to the intrinsic sensitivity of the spectroscopic method. In order to compensate for instrument sensitivity levels, product accumulation

within tissues is necessary. In order for this accumulation to occur requires that the reaction products be relatively stable. The spin trap or probe used must be specific for the oxidants or radicals of interest and must not be affected by normal cellular processes (e.g. enzymatic and metabolic). Localization of the spin trap/probe is also essential in order for product accumulation to be achieved. Ideally, the spin trap/probe will target specific cells or body compartments, remain and accumulate. Lastly, the spin trap/probe must not interfere with normal cell or organism function, which affects free radical or oxidant production.[36]

The role that partially reduced oxygen species such as superoxide and hydroxyl radicals play in mediating degenerative diseases such as immunodeficiencies and aging has been investigated.[69,70,71,72] Using EPR, it has been shown that the DEPMPO spin trap can be used for cellular quantification of superoxide radicals generated from stimulated polymorphonuclear leukocytes. [73] In particular, phosphorus containing spin traps and NMR spectroscopy have been used in vivo to detect the presence of hydroxyl radicals after ischemia/ reperfusion injury, the cytotoxicity occurring when tissues subjected to partial or total oxygen deprivation are reoxygenated, in isolated rat liver.[74] The observation of free radical generation in this model supports the potential use of NMR spin trapping in biomedicine.

In this study the use of phosphorus containing spin traps was extended for the detection of cellular hydroxyl radical production with experiments performed on $^{\bullet}OH$ formation from rat brain cells transfected with the Heme Oxygenase-1 (HO-1) gene. It has been shown that over expression of HO-1, a stress protein that degrades heme to biliverdin, free iron and carbon monoxide, contributes to processes that are related to

neurodegenerative disorders such as Alzheimer's[75] and can be used as a biological marker for sporadic Alzheimer's disease. The HO-1 gene also stimulates the formation of free radicals in cells, among them hydroxyl radicals. In collaboration with researchers at the Jewish General Hospital, astrocytes transfected with the HO-1 gene were prepared and the presence of hydroxyl radicals detected using ^{31}P NMR spectroscopy. Astrocytes are a class of non-neuronal brain cells and they are the most abundant of all cell types in the mammalian nervous system. Problems with these cells are linked to a variety of aging-related neurodegenerative disorders including Alzheimer's disease, Parkinson's disease and Huntington's disease.[75]

To study this system, two control experiments were performed: The positive control was a system that was expected to generate hydroxyl radicals- astrocytes plus 1.5% hydrogen peroxide solution. The negative control consisted of astrocytes transfected with DNA free from HO-1 or any hydroxyl radical generating system. The DIPPMPO spin trap was introduced into both of these samples and ^{31}P NMR spectroscopy performed. The sample of interest, astrocytes transfected with HO-1 gene, was then prepared and analysed.

Preliminary results indicate that all of the spectra, (positive control, negative control and astrocytes transfected with HO-1 gene), have ^{31}P NMR resonances that correspond to that of the phosphate buffer, spin trap signal and a signal at 27.3 ppm. To date, the structure of the species that generated the signal at 27.3 ppm has not been determined. However, based on the fact that all of the samples, produced similar spectra, it appears that the spin trapping experiment did not work under the conditions used. Further investigations into sample preparation and spin trap concentration are needed in

order for the presence of free radicals in astrocytes cells using ^{31}P NMR spectroscopy and spin trapping to be determined.

CHAPTER 6

CONCLUSIONS

The spin trap 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-N-oxide (DIPPMPO) was synthesised in reasonably good yield (~50%) using a two-step procedure. The first step generated diisopropyl(2-methylpyrrolidin-2-yl)phosphonate in 95% yield. The second step, which involved the oxidation of the phosphonate, was best-achieved using hydrogen peroxide/sodium tungstate as the oxidant. This oxidation resulted in the formation the DIPPMPO spin trap. Preparative column chromatography was found to yield a product of adequate purity for further spin trapping and ^{31}P NMR experiments.

Computational analysis of the two spin traps used in this research, DEPMPO and DIPPMPO, showed that the spin density, partial charge and singularly occupied molecular orbital (SOMO) energy values for these spin traps were indeed very similar. Any difference in radical adduct stability is therefore attributed to steric influences and not electronic considerations.

This effort demonstrates that DIPPMPO spin trapping when used in conjunction with quantitative ^{31}P NMR is an effective tool for the identification and quantification of free radical species. Using this technique, the radical adduct reduction products of a variety of free radical species can be detected. Hydroxyl, superoxide and methyl radicals were detected and quantified with the use of a phosphorus-containing internal standard. The chemical shifts for hydroxymethyl and hydroxyethyl radicals were also determined using the NMR spin trapping technique. The concentration of hydroxyl and methyl

radicals was determined as a function of total ultraviolet irradiation time of hydrogen peroxide and *tert*-butyl hydroperoxide respectively, and the results were shown to be reproducible, while the concentration of superoxide radicals produced by this method was found to be irreproducible. Apparent rate constants for hydroxyl and methyl radicals generated from the photolysis of hydrogen peroxide and *tert*-butyl hydroperoxide were determined to be 200μM/min. and 20μM/min. respectively

The possibility of an equilibrium between oxygen and superoxide in peroxide solutions was also investigated. It was established that the hypothesis that superoxide radicals exist in equilibrium with aqueous peroxide when in contact with air or oxygen might indeed be valid. This statement is based on preliminary observations that illustrated a dependence on superoxide radical concentration with the nature of gas that it was in contact with. The effect of pH on the concentration of radical species trapped was also examined and, it was shown that there was an increase in the quantity of superoxide radical trapped with increasing pH. This increase in superoxide radical concentration is most likely related with the increase in hydrogen peroxide decomposition that occurs at higher pH values. The concentration of hydroxyl radicals was shown experimentally to be lower than that of superoxide radicals especially at elevated pH values. These data suggest that superoxide radicals are key species in aqueous hydrogen peroxide chemistry.

A preliminary look at the biomedical application of the developed technique was investigated. Astrocytes transfected with the HO-1 gene were examined in order to determine whether hydroxyl radicals could be detected. Unfortunately, this technique did not result in the detection of signals due to the trapping of hydroxyl radicals. The absence of hydroxyl radical signal may be due to the manner in which the samples were

prepared. In order for the hydroxyl radicals to be trapped, the concentration of spin trap and the spin trapping conditions must be suitable. In this case, it is not clear if either of these criteria have been met.

CHAPTER 7

RECOMMENDATIONS FOR FUTURE WORK

This thesis has presented the effective application of a novel technique to detect and quantify various free radical species. In order to establish the developed technique as quantitative, as opposed to semi-quantitative, further research is necessary.

The absolute concentration of free radical species present in the samples examined is unknown due to the lack of corroborative evidence. To this end, electron paramagnetic resonance (EPR) spectroscopy, which can be used for quantitation, could be employed. EPR quantitation is often very difficult due to sample related and instrument related factors.[76] The biggest challenge of quantitative EPR is finding a spin standard that is similar in EPR behavior to the sample of interest but for nitroxides TEPMOL can be used. The application of spin trapping in conjunction with EPR spectroscopy provides direct evidence for the production of free radicals and when used properly it can confirm that the formation of radical adducts was indeed due to free radical production in your reaction system. It would therefore be useful to use this spectroscopic technique to validate the results obtained from NMR spin trapping experiments.

In order to obtain significant quantitative information kinetic studies are also required. The kinetics of radical adduct formation and decay have not been measured in this work. It would therefore be useful to determine the rate constants for all of the possible reactions that may occur during the spin trapping experiment.

It has been reported that transition metals play an important role in the decomposition of hydrogen peroxide[64, 65] and that their presence can affect the concentration of free radical species present in solution. Therefore, it would be useful to study the formation of various radical species in the presence of a variety of transition metals such as Fe^{3+}, Cu^{2+} and Mn^{2+} and hydrogen peroxide using the developed spin trapping technique.

In this work the majority of free radicals investigated were generated through ultraviolet photolysis of aqueous solutions. Future work could therefore included an investigation into the effectiveness of the NMR/spin trapping technique using radical generating systems that do not involve ultraviolet irradiation of the samples.

CHAPTER 8
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