ESR Characterization of Induced Reactive Oxygen Species The Antibiotic Mechanism of Action Controversy

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INTRODUCTION

The goal of this research project was to examine the capacity of literature-described methods to radicalize certain organic and biological molecules. One such method is the Fenton method. In most cases, Fenton reactions are used to dismantle organic compounds. These reactions are an intricate series of interactions between hydrogen peroxide (H_2O_2) and the ferrous ion (Fe^{2+}), otherwise known as Fenton's reagent. Generally, in a simple Fenton reaction, hydrogen peroxide would serve to repeatedly oxidize and reduce the ferrous and ferric (Fe^{3+}) ions, respectively, effectively generating hydroxyl radicals.

Hydroxyl radicals may also be described, in a more comprehensive sense, as reactive oxygen species (ROS). ROS, as the name suggests, are highly reactive molecules that contain oxygen. These molecules, typically formed as natural byproducts of oxygen metabolism, serve crucial roles in both homeostasis and cell signaling. Under typical physiological conditions, in which ROS formation and depletion are regulated, ROS are essential to driving regulatory pathways, however stressful environmental conditions may result in an accumulation of ROS, in turn causing oxidative damage.

The scope of my project dealt with a, presently, ongoing controversy circling antibiotics. There have been disputes about the mechanism by which antibiotics kill microbial organisms. A 2007 study by Collins (*et.al*) [1] presented evidence that antibiotics kill bacteria by inducing reactive oxygen species (ROS). However two subsequent studies brought forth evidence effectively declining Collins' theory. One study, by Lewis (*et.al.*) [13] measured ROS by use of hydroxyphenyl fluorescein (HPF), similarly to the Collins study, and determined that HPF does not fluoresce under the formation of ROS, thereby having no coloration to between HPF fluorescence and bacterial cell death. Lewis, and coworkers, discovered that HPF only fluoresces under anaerobic conditions, making it impossible for ROS to be the cause.

A second study headed by Imlay and Liu, from the University of Illinois, underwent several different experiments to test the ROS theory and found: there was no formation of hydrogen peroxide ("a hallmark ROS"), no activation of bacteria to fight oxidative damage, and no oxidation of bacterial DNA. Essentially both studies, by Lewis and Imlay, assert that antibiotics kill bacteria under ROS- hostile conditions, and under anaerobic conditions; their conclusion relying on the assumption that the generation of ROS requires a source of atmospheric oxygen (O_2) [13].

For the purpose of this project, *in vitro* biological compounds were used. This simply means that I observed and experimented with organic substances outside of a biological system, or organism. I also gained more experience with a technique, used for qualitative analysis, called Electron Spin Resonance Spectroscopy (ESR). Essentially, this technique allowed me to determine the presence of radicals in a sample. This allowed me to be able to qualify the effectiveness of various methods, in lit, in radicalizing the given substances.

It is known that reactive oxygen species (ROS), specifically hydroxyl radicals, are extremely volatile molecules with the potential to cause devastating damage to biological macromolecules. The high reactivity of the hydroxyl radical also ensures an extremely short lifespan, leaving it difficult to directly observe. Superoxide (O_2^-) radicals, in contrast, are slightly less reactive, than their hydroxyl counterparts. Nonetheless, O_2^- remained the object, in this study, with which we sought to induce oxidative damage.

It's possible that ROS production in the presence of guanine may result in a guanine-radical adduct. We sought to capture some form guanine- O_2^- interaction using ESR spectroscopy; in addition, the use of O_2^- may potentially allow for a greater chance to capture any interaction between ROS and guanine due to its lower reactivity. *In vitro* generation of O_2^- was accomplished using hypoxanthine-xanthine oxidase incubations based from literature-cited systems [17, 18], in which xanthine oxidase catalyzes the oxidation of substrates such as xanthine and, the more relevant, hypoxanthine. In addition, these systems may also be utilized to produce hydroxyl radicals via superoxide mediated Fenton chemistry.

It is important to note that O_2^- radicals alone typically do not emit signal when characterized using ESR. This characteristic of superoxide, due to its strong spin orbital coupling, makes it difficult to utilize ESR spectroscopy as a form of characterization, even at higher concentrations. In these instances, spin traps serve as useful tools, with which short-lived radicals form stable paramagnetic radical adducts, to allow for ESR detection of unstable/ highly reactive radicals. However, there are also caveats to using spin trapping techniques with superoxide. Superoxide

adducts decay rapidly in non-radical species, and require high concentrations of spin trap due to the low rate constant of reaction, relative to the dismutation of superoxide. Spin traps for superoxide are usually nitrones (18). Nitroso spin traps, namely 5, 5-Dimethyl-1-pyrroline N-oxide (DMPO), may contain colored impurities which lead to artifactual signal. Purification of the DMPO spin trap was, therefore, paramount to the accuracy of this study.

MATERIALS AND METHODS

The xanthine oxidase used in this study was purchased from Sigma-Aldrich. The Hypoxanthine, DMPO, DETAPAC, and guanine were already available for use in house but were also obtained from Sigma-Aldrich, although several years prior. Chelexed phosphate buffer and electrolyticly purified (e pure) was produced in house, within the chemistry and biology departments respectively.

Reagent Preparation: Commercially obtained 5, 5-Dimethyl-1-pyrroline *N*-oxide (DMPO) spin trap was purified in order to prevent the occurrence of any misleading artifactual signal. The DMPO (1.0g) was diluted with electrolyticlly-purified (e-pure) water, yielding a dark yellow-brown solution. After the addition of Activated charcoal (1.25g) the solution was vortexed, for 1-2 minutes, then centrifuged at *1000 x g* for a range of 5-10 minutes. Once the activated charcoal was separated from the solution, resulting in a pellet-like accumulation, the DMPO solution was decanted. Aside from the initial dilution, all steps were repeated twice. The final product was a clear liquid filtered through a filter syringe removing any excess charcoal, and stored at 0^oC. Concentration of the final product was determined using the molar absorptivity coefficient: $7.2 mM^{-1}cm^{-1}$ at *236nm*.

Solutions of hypoxanthine (2.0mM) and diethylenetriaminepentaacetic acid (DETAPAC: 1mM) were made with a chelexd phosphate buffer solvent (pH 7.4). 1.361mg hypoxanthine was dissolved in 5ml of 0.5M phosphate buffer; whereas 3.9mg DETAPAC was solvated in 10ml of 50mM phosphate buffer. All reagents necessary for the *in vitro* spin trapping and production of superoxide were stored at 0° C.

In order to find a proper solvent for guanine, multiple solubility tests were performed. Different solvents were saturated with guanine and placed in an ultrasonic bath for several minutes to hasten the dissolution of guanine. Once samples were taken out and arrived at equilibrium, 0.1ml of were left to dry on a watch glass. Concentrations were confirmed by weighing the amount of solute residue left behind. Among the solvents tested were: 95% ethanol, absolute ethanol, 100mM HCl, 10mM HCl, and 1mM HCl.

Electron Spin Resonance (ESR) Experiments: All EPR experiments were conducted using a *10µl* aspirating flat cell. This apparatus, also known as a filling flat cell, used vacuum suction (~23mm Hg) to transfer samples into the flat cell. Prior to suctioning, samples were placed in an ice bath to aid in the potential improvement of the spectra's signal to noise ratio, and to help capture short lived radicals by conceivably stifling quick reactions.

ESR spectrometry was performed on the DMPO aqueous solution in order to determine optimal parameters, under which following experiments would be performed, and to allow for further qualification of the spin trap's purity. *0.56ml* of the DMPO solution (178.9mM) was diluted with phosphate buffer (50mM) arriving at a final sample volume of *1.0ml*. The best signal was obtained at a microwave frequency and power of 9.400GHZ and 20mW, respectively, a field modulation of 0.100mT, a receiver gain at 1000, with a time constant of 0.1 seconds, sweep width and time of ±5.00mT and 2 minutes respectively, and a 337.70mT center field.

Incubations of Hypoxanthine-Xanthine Oxidase systems were prepared for the initial generation of superoxide in the presence of the DMPO spin trap. Reaction mixtures (~1.00ml) contained concentrations of 0.5mM Hypoxanthine, 0.610mM DETAPAC, 24.9mM DMPO, and 55.5mM phosphate buffer. Superoxide production was initiated with the addition of xanthine-oxidase (7µl). An ESR spectrum was taken immediately following reaction initiation.

Similar incubations containing an additional guanine reagent were prepared with the intention of generating superoxide radicals in the presence of both solvated guanine and DMPO. In general, reaction mixtures contained concentrations of: 0.50mM hypoxanthine, 24.9mM DMPO, 25mM guanine, 0.47mM DETAPAC, and 36mM phosphate buffer. Solvents for guanine used are 10mM HCl and phosphate buffer. The reaction mixture was initiated with the addition of xanthine oxidase (7µl). An ESR spectrum was taken immediately following reaction initiation.

RESULTS AND DISCUSSION

Treatment with activated charcoal proved to be a valuable method to purify the spin trap [17, 18]. DMPO was treated multiple times with charcoal before the final purified product was obtained. Following purification, an ESR background was performed on DMPO (Figure **1**). From the spectra we

were able to distinguish four visible peaks characteristic of DMPO line splitting. Among the two spectra, the former (Figure 1A) was obtained with a scan length of two minutes while the later (Figure 1B) was obtained with a four minute scan length. Although subsequent ESR experimentation would require scan lengths of no more than two minutes, making (Figure 1A) a more accurate representation in terms of parameters, (Figure 1B) yields a more conclusive spectra; underlining the purity of the final product.

To investigate potential interactions between O_2^- and the nucleotide guanine, incubations containing three major components were made. Each reaction mixture, unless otherwise specified, contained the spin trap DMPO, a Hypoxanthine-xanthine oxidase system, and a guanine component. This experimental design was constructed in order to generate superoxide radicals in the presence of both DMPO and guanine, and to compare any EPR signal obtained from the incubation with a DMPO control. We hypothesized that if the guanine-containing system produced unique and replicable spectra, it would serve as a means to indirectly view a guanine-O_2^- adduct; providing evidence as to the ROS vulnerability of guanine.

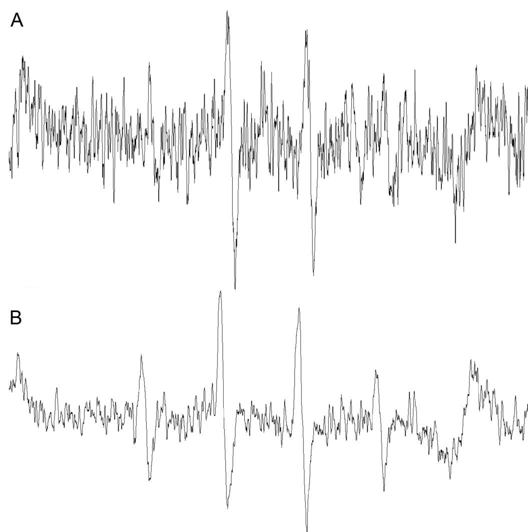

Figure 1: ESR spectra of the 5, 5-Dimethyl-1-pyrroline *N*-oxide (DMPO) spin trap after purification with active charcoal. (A) Spectrum was obtained from a sample (1ml) of DMPO (*100 mM*) in phosphate buffer with a 2 minute scan length.(B) Spectrum taken from the same sample with a 4 minute scan length; all other parameters remained identical.

Hypoxanthine-xanthine oxidase systems were tested in order to determine if, and under what, conditions incubations would produce spin trapped O_2^- (Figure 2). Upon review of the spectrum (Figure 2A), the presence of DMPO spin trapped superoxide (DMPO-OOH) appears evident. The line splitting resembles those characteristic to DMPO-OOH. Furthermore, it is appropriate to point out that the second scan (Figure 2B) was initiated immediately after the prior 2 minute scan. Although slight signal peaks are observed, the majority of the signal appeared to be lost in noise and less prominent in magnitude when compared to the former. This suggests that the *in vitro* spin-adduct formed is short lived; a characteristic which coincides with the determination that this signal was produced due to the presence of DMPO-OOH adducts.

Guanine containing systems were scrutinized and compared to the DMPO control (Figure 3). The initial system tested (figure 3A), however, did not appear to exhibit any comprehensible signal. A following scan, in which the guanine component was replaced with an equal volume of buffer, (figure 3B) exhibited the familiar DMPO-OOH signal; suggesting that the incubation was capable of producing superoxide and that the guanine component may have hindered its ability to do so.

We believed that the lack of signal (Figure 3A) may have been due to guanine's limited solubility in an aqueous phosphate buffer medium. The guanine, in a prepared stock mixture, may have not completely dissolved but rather formed a suspension. It, therefore, remained possible that the source of the interference was the non-solvated guanine.

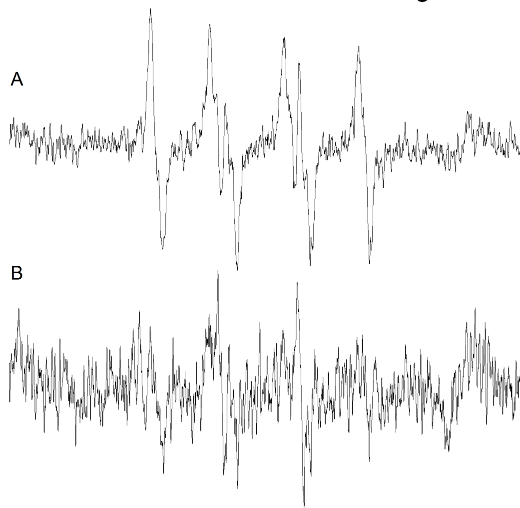

Figure 2: Hypoxnthine-Xanthine oxidase system. (A) spectrum of what appears to be superoxide (B) spectrum taken immediately following the one above, underlining the shortness of the lifespan of the superoxide radical adduct.

To remedy this problem solubility tests were performed. Among the solvents studied dilute hydrochloric acid (HCl) was determined to be the most adequate. ESR experiments were then performed using incubations containing dilute HCl. Spectra were obtained from an HCl-solvated guanine sample (Figure 3C), and a control, in which the guanine solution was replaced with an equal volume of diluted HCl (Figure 3E). The spectrum obtained

from the HCl-solvated guanine incubation (Figure 3E) was identified as DMPO spin trapped hydroxyl radical (DMPO-OH).. Although signal was observed from the HCl-solvated guanine sample, a similar spectrum was obtained from its control (Figure 3C). Not only do the spectra exhibit line splitting characteristic to DMPO-OH, the radical adduct in the control sample appeared more stable than the DMPO-OOH adduct. A second spectrum of the HCl-control system was taken (Figure 3D) immediately following its predecessor (Figure 3C). No apparent loss of signal was observed. This suggests that the radical adduct observed is more stable than DMPO-OOH; a feature not uncommon to DMPO-OH.

However, EPR spectra of the HCl-solvated guanine and the HCl-control suggest that the DMPO-OH signal observed was a result of HCl interference; not the guanine. It remains possible that the HCl may have engendered the protonation of O_2^- by increasing the concentration of hydron (H^+) in solution; also understood as lowering pH. A feature not uncommon to acids.

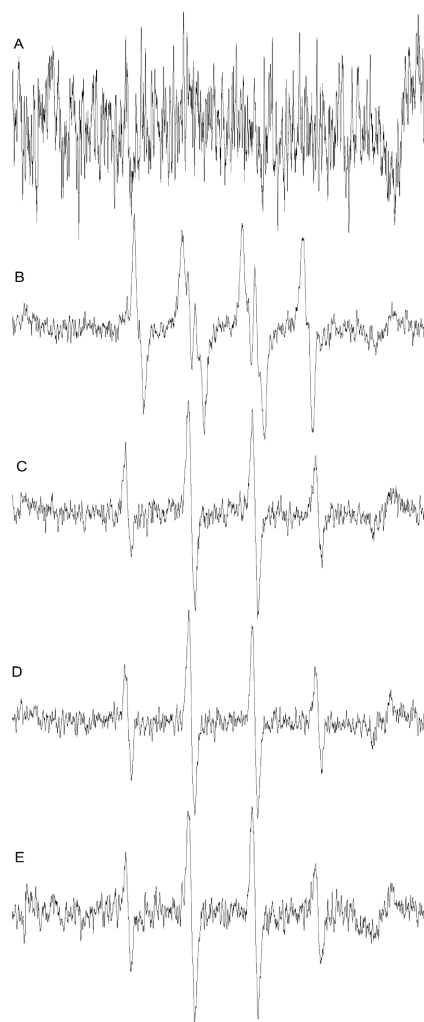

Figure 3: ESR spectra obtained when testing for guanine reactivity with superoxide, relative to DMPO. In general, reaction mixtures contained guanine (25mM), DETAPAC (0.47mM), DMPO (24.9mM), hypoxanthine (0.50mM), chelexed phosphate buffer (36mM), and were initiated by xanthine oxidase (7µl); summing to ~1.00ml incubations.
(A) Typical incubation, composition already specified.
(B) Similar composition to [A]; guanine was replaced with and equal amount of phosphate buffer
(C) Guanine replaced with dilute HCl (10mM), otherwise identical to [A]
(D) Spectra taken immediately after [C], using the same sample
(E) Reaction mixture containing guanine dissolved in HCl (10mM)

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