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Ribonuclease A modification induced by 1,2-Naphthoquinone and 2-Hydroxy-1,4-Naphthoquinone

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Ribonuclease A Modification Induced by 1,2-Naphthoquinone and 2-Hydroxy-1,4-Naphthoquinone

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Departmental Honors Thesis

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Abstract

Protein modifications may occur upon exposure to environmental toxins such as polycyclic aromatic hydrocarbon (PAH) molecules or their metabolites. In this context, our laboratory was interested in investigating protein modifications in the presence of select naphthoquinones, which were 2-hydroxy-1,4-naphthoquinone (HNQ) and 1,2-naphthoquinone (*o*-NQ). The effects of HNQ and *o*-NQ on the protein Ribonuclease A (RNase) were investigated through a variety of conditions. These modified incubation conditions included pH variation and the addition of metal ions to further mimic physiological conditions. Documentation of results was carried out through sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis (SDS-PAGE). Of the two quinones, *o*-NQ exhibited a greater level of activity toward RNase. HNQ was found to be stable under the studied conditions, resulting in almost no observed RNase modification. Linking environmentally abundant compounds to toxicological effects on biologically significant molecules can help to set precedents toward their usage and disposal that have a positive ripple effect.

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Glossary

PAH……..Polycyclic Aromatic Hydrocarbon

RNase…….Ribonuclease A

p-BQ……..…….1,4-Benzoquinone

SDS-PAGE……Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

o-NQ……..…….1,2-Naphthoquinone

HNQ…….2-Hydroxy-1,4-Naphthoquinone

Fe^{2+}……..Iron (II)

Cu^{+}…….Copper (I)

Mn^{2+}…….Manganese (II)

p-NQ……..…….1,4-Naphthoquinone

GPDH……..Glyceraldehyde-3-Phosphate Dehydrogenase

GSH……...Glutathione

GSSG……..Glutathione Disulfide (Oxidized Glutathione)

kDa……...Kilodaltons (equivalent to 1000 amu)

RNA…….Ribonucleic Acid

LYS…….Lysine Residue on RNase

G6PD……...Glucose-6-Phosphate Dehydrogenase

THN……1,2,4-Trihydroxynaphthoquinone

hr(s)…….…….hour(s)

min(s)……....minute(s)

Chapter 1: Introduction and Background

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Before the invention of central heat and air, people depended on burning fires for warmth. Frequent fires prompted the need for chimney sweeps to come and clean away soot. Many of the people who worked in this career later developed cancer, which was related to their exposure to the soot [1]. What about soot might have caused the illness? The answer lies in the fact that soot contains reactive chemical substances that negatively interact with biological molecules. The cause and effect relationship of soot has been realized, but there are many more toxins like soot present in the environment from various industrial sources. Through recent history, many environmental toxins have been defined by recording the reactions they go through once in the body. In this context, the present study will dive into the investigation of two quinone form polycyclic aromatic hydrocarbon (PAH) molecules that are suspected to have adverse effects on biological molecules. Their interaction with the protein Ribonuclease A (RNase) will be outlined to help better understand what reactions they may induce in the physiological system.

1.1: PAH Molecules and their Metabolites

The term PAH encompasses a large variety of molecules with some common defining characteristics. These common characteristics are cyclic rings with aromatic character. This basic structure can be modified through various natural and man-made processes that give rise to the large variety of molecules that fall under the umbrella term of the PAH family [2]. This variety includes modifications such as oxygen atoms being bonded onto differing locations of the rings, which in some cases gives rise to quinone molecules. One characteristic of PAH molecules is that through change, they retain their aromatic nature. This factor is important to the molecules' ability to continually be

modified, through both redox reactions and some bond delocalization [2]. This aspect of these molecules will be important to many things discussed in this work, in both background and findings.

1.1.2: Exposure to PAH molecules

PAH molecules develop prevalence in their broad use in the manufacturing of many goods that people use frequently. Some very important resources whose production or use involves PAH molecules include gasoline, coal, and other fossil fuels. There are products that also may lead to the direct consumption of these molecules, and some of the most prevalent are cigarettes and food preservatives [2]. It is difficult to fully explore everywhere that PAH molecules are used, but future chapters will survey the use of the molecules of interest. This ubiquitous presence has presented the need for PAH metabolism to be examined for any potential adverse health implications.

1.1.3: Metabolic Pathways

Just like any other substance that is introduced to the physiological system, PAH molecules have a pathway by which they are metabolized. It has been outlined that these molecules are greeted by certain promiscuous enzymes that work to process them into a product that can be excreted from the body. Pre-metabolized PAH molecules tend to be non-polar hydrocarbons, which are not soluble in urine. The pathway that the body takes to incorporate these molecules into urine is to oxidize them. This cascade starts with Cytochrome P450, which oxidizes the PAH into an epoxide form that is then further oxidized by the proteins Epoxide Hydrolase, Dihydrodiol Degydrogenase, and Tyrosinase [3] [4] [5] [6]. Through this process, reactive species such as epoxides and

quinones are produced. Two specific quinone forms will be the subject of this investigation, and because of the above pathway, some of their precursor molecules will also be discussed.

1.2 Overview of Quinones

Although a significant portion of exposure to quinones is caused by the metabolism of PAH molecules, some quinones occur naturally in the physiological system. For organisms whose metabolism depends on cellular respiration, ubiquinone plays a very important role in the electron transport chain. Organisms whose metabolism depends on photosynthesis utilize plastoquinone in their electron transport chains, giving another notable example of quinones present in the normally functioning biological system [7]. Quinones exhibit ease in delocalizing their bonds. This ease in delocalization is also related to their ability to easily be oxidized and reduced. This redox cycling fuels the purpose that quinones serve in their natural occurrence, which is to accept and pass on electrons. The quinones that have their place in nature's machinery show no harm to the physiological system, but what happens when this reactive species exists in the body with no defined function? This interaction is outlined by what was documented from studies performed with 1,4-benzoquinone (*p*-BQ) and RNase [8].

1.3 Prior Evidence with *p*-BQ

Through multiple experimental methods, Kim and colleagues have determined that *p*-BQ is an agent responsible for inducing protein crosslinking, aggregation, and adduct formation through lysine residues within the protein RNase. The study focused on observing the modification of RNase as a result of exposure to the quinone. Assays were

conducted and were investigated with the following methods: SDS-PAGE, fluorescence spectroscopy, UV-Vis and microscopy. The SDS-PAGE analysis revealed that the molecular weight of some of the protein increased as a result of exposure, showing banding that indicated the presence of dimers, trimers, other oligomers and aggregates [8].

The fluorescence spectroscopy portion of the experimentation confirmed that conformational changes had taken place as a result of RNase being exposed to *p*-BQ. The fluorescent behavior of RNase is due to its tyrosine residues. As the concentration of *p*-BQ was increased, the fluorescence signal that RNase produced was decreased [8]. A logical explanation that would support the other findings of the study is that as modification takes place through lysine residues, the fluorescent tyrosine residues are moved within the protein. This change could be responsible for the signal quenching observed [9] [10]. Although this portion of the experiment does not absolutely confirm the modification pathway being suggested, it does confirm that the structure of the protein is experiencing morphological changes upon exposure to *p*-BQ.

Absorbance plots from UV-Vis spectroscopy also indicated that protein modification took place. The landscape of the absorbance spectra changed as the concentration of quinone was increased. The supporting evidence could be found in an additional peak being found in the absorbance spectrum after exposure to the quinone. A last method for confirming this modification was confocal microscopy. The protein aggregates were able to be visualized with this method [8].

Through all this data, a pathway by which this occurs was developed. Kim and colleagues proposed that the activity of the quinone revolves around the redox cycling of the quinone and the nucleophilic ability of the protein to attack the quinone. Their mechanism suggests that when the quinone is in the oxidized form, it has the ability to oxidize the lysine residue of an RNase protein. This oxidation, followed by a pathway of acid-base catalysis leaves the lysine residue in an oxidized form. This oxidized form of lysine is reactive, and is suspected to form protein crosslinks. This process would explain the construction of the dimers, trimers, and other oligomers documented after exposure by SDS-PAGE. The decrease in fluorescent behavior has its explanation in protein crosslinking as well, which was mentioned earlier.

The other portion of modification suggested to be taking place is adduct formation. Adducts might be formed if the nucleophilic lysine attacks the quinone and creates a bond. Although this interaction is difficult to quantify, it is very evident that modification took place and the given mechanism includes this interaction. Through many data points, the evidence suggests that the proposed mechanism leading to protein modification is being followed [8]. This background is the inspiration and general method by which the quinone forms of this study will be investigated.

1.4: Significance of Study

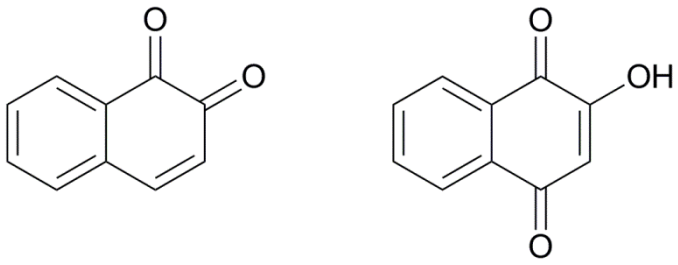
Quinones playing a part in the production of protein crosslinking leading to possible aggregation are a very significant correlation to be considered. Inducing a protein to form an aggregate means that the substance could play a part in diseases that have devastating effects. Protein aggregates have a role in the way Alzheimer's,

Parkinson's, and other neurodegenerative diseases cause their adversary effects [11]. Many neurodegenerative diseases occur with the aggregation of amyloidal proteins. RNase is not amyloidal, but could still serve as a model of what might happen with other proteins that are amyloidal [9]. Regardless of amyloidosis, anytime a protein exists in a modified form, some of the integrity of its function can be lost [9]. In this way, any interaction that quinones have with proteins is significant.

1.5 Direction of the present study

In the context of the prior evidence presented, the present study will be focused on two naphthoquinone species. Naphthoquinones differ from benzoquinones in that they have an additional benzene ring attached to the substituted benzene. This extra benzene creates a molecule with different properties than benzoquinones, and their interactions with biological molecules such as proteins could be different in nature. Two specific naphthoquinone species will be investigated in this present study, which are 1,2-naphthoquinone (*o*-NQ) and 2-hydroxy-1,4-naphthoquinone (HNQ). The interaction between these two potentially reactive species and the protein RNase will be documented. The structures of *o*-NQ and HNQ are presented in Figure 1.4.

Figure 1.4: Structures of *o*-NQ and HNQ



o-NQ HNQ

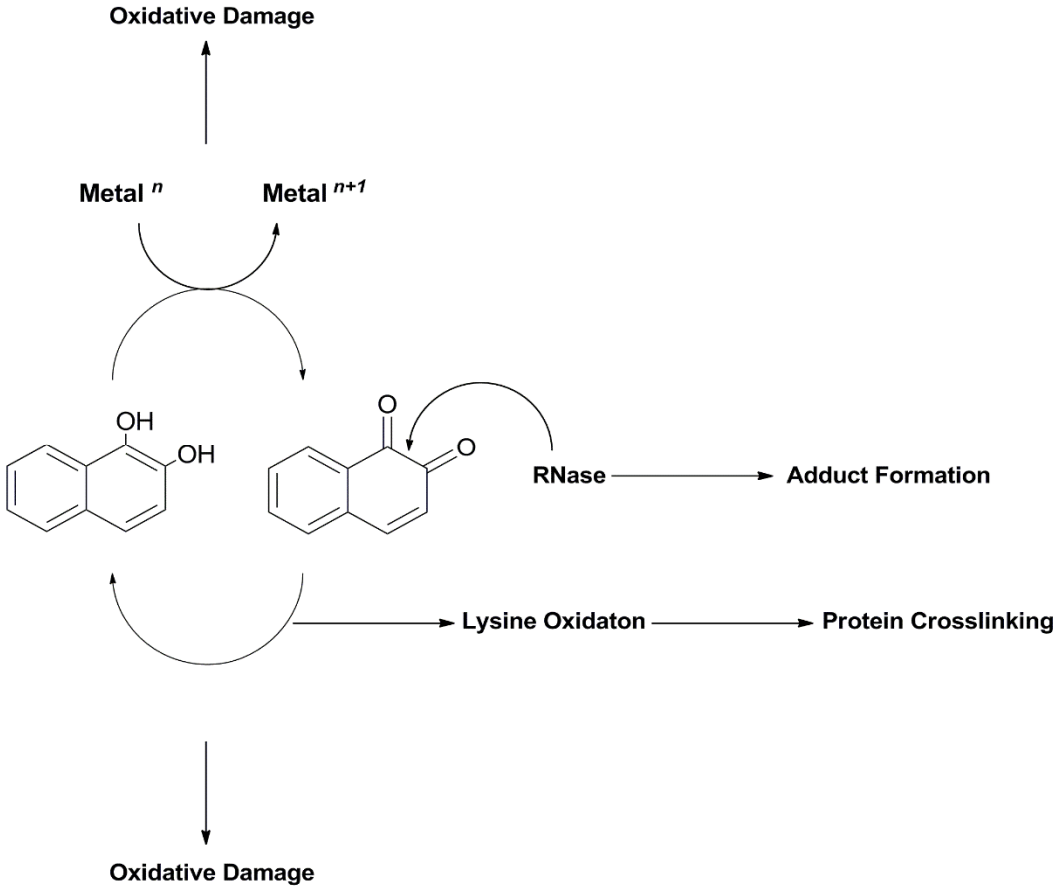
1.5.1: Why RNase?

RNase is ideal for the sought out type of protein work because it is small and well understood. This protein is also present in more species on the planet than humans. For example, the protein investigated in this study was from bovine pancreas. For these reasons, it is a significant candidate for observing modification due to some environmental toxin. The primary function of the protein is to digest RNA strands. RNase exhibits endonuclease activity and can cut RNA molecules in the centers as well as the ends [9]. It was also the first protein to be synthesized in a lab, by R. Bruce Merrifield [12]. His discovery that proteins are nothing more than chemically constructed molecules opened the doors to years of studies in labs on how proteins interact with other molecules. This is what the present study revolves around: the modification of a protein as a result of exposure to some other molecule. Along with all these points, the previous investigation of *p*-BQ was carried out with RNase. Looking at modification with another quinone PAH will help build a profile of modification induced by this type of molecule.

1.5.2: Metal Ion Assays

In addition to observing the behavior of the naphthoquinones at a very simple recreation of physiological settings, this study looked at the way metal ions can change the interaction with both naphthoquinones. It is pertinent to add that all buffers used in the assays were made in lab using NaH_2PO_4 and Na_2HPO_4. The metals used in the study were Fe^{2+}, Cu^{+}, and Mn^{2+}. These choices of metals are relevant to the study, because they can all be found in the physiological systems of most life forms. Iron and copper are commonly found in the electron transport chains of many organisms. Iron is found in the center of hemoglobin, which is a very important molecule of the body. [7]. Manganese is present in most tissues of the human body, and is described as being present in an even distribution through the system [13]. The addition of metal ions is expected to render the mechanism presented in Figure 1.5.2, in which metal ions intervene with the quinone's redox cycling. It has been shown with copper that hydroquinone form benzoquinones are rapidly oxidized back to the quinone form [14] . The quinone species is the one that has the ability to create the oxidized lysine side chain needed for protein crosslinking, and also to form adducts with the quinone. In this context, the activity of the naphthoquinones might be stimulated with the addition of metals. A general mechanism for the proposed action of the metals can be seen in Figure 1.5.2. The exact conditions of each assay will be discussed in greater detail in the materials and methods chapter immediately following this.

Figure 1.5.2: General Mechanism between *o*-NQ and Metals



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Chapter 2: Materials and Methods

Chapter 2: Materials and Methods

A general process was followed to investigate the protein modification of interest. Incubations at varying conditions were carried out in 24 hr intervals and physiologically relevant conditions (pH 7.0, 37 °C). This incubation was followed by immediate freezing at 0 °C to halt the reaction. SDS-PAGE analysis was then carried out, followed by staining and then de-staining of the gels. Photos were immediately taken in high quality, and then the gels were stored.

2.1: Materials

The RNase used in these assays was of bovine pancreas and was purchased from Sigma. The naphthoquinone samples used came from differing distributors. The HNQ was purchased from Acros Organics, and the *o*-NQ used was purchased from Aldrich. All naphthoquinones were 95% or higher purity, and use was mediated by glass vials. The vials were used because the naphthoquinones were labelled as air sensitive, and the goal was to open the main stock as little as possible. All stock bottles were flushed with argon after use. The metal samples used in the metal ion assays were all purchased from Acros Organics. They were all metal chlorides of the appropriate oxidation state. These samples were also mediated in their use, this time with Eppendorf tubes. The stocks and single use working stocks were flushed with argon to maintain the integrity of the oxidation state. All chemicals used to make gels, protein dye, gel staining solution, buffers and any other reagent through the experiment were reagent grade. The protein ladder used was purchased from Fisher, with the lot number 130579. Finally, the

electrodes used in SDS-PAGE analysis were purchased from Fisher Biotech and Thermo Scientific. The power supply for the electrode was a Thermo Scientific EC 300XL.

2.1: Incubation of RNase ± Naphthoquinones ± Metals

Several incubations at variable conditions were carried out. The simplest condition was carried out at 37 °C and pH 7.0. As a first step, pH 7.0 phosphate buffer and RNase were allowed to warm to room temperature. The appropriate amount of the buffer was then added to an Eppendorf tube, and combined with a predetermined amount of protein. The buffer and protein were vortexed and then placed in a heating block at 37 °C. While the protein was heating to physiological temperature, the quinone solution was prepared. The naphthoquinones were introduced into a solution of 10% methanol and 90% of the same phosphate buffer, for dissolution. The naphthoquinone solution was then sonicated for approximately 10 mins, and then immediately introduced into solution with the protein. After being vortexed again, the solution containing all components was placed in the heating block and a timer was set. Aliquots were taken slightly before the specified time, so that the reaction would be stopped in the freezer at the incubation time suggested. Aliquots were taken in a consistent manner through all experiments.

The metal ion assays were carried out in much the same manner. The only significant difference in these incubations was the preparation of the metal ions. They were introduced into a complete phosphate buffer solution, and sonicated for approximately 10 mins. The preparation of metals took place simultaneously with the naphthoquinone preparation. The metals were introduced at the same time as the quinones.

There were some incubations that utilized different buffers than phosphate, and this was specific to HNQ. Acetic acid buffers were used to alter pH in some trials. When these different buffers were used, they were integrated consistently through the experiment. The buffer used with the initial addition of protein, along with the buffer used to make the naphthoquinone solutions, and the metal ion solutions were all changed to acetic acid buffer. The methanol was still introduced to the naphthoquinone solutions for consistency and dissolution. The pH levels tested were 5.8, 4.8, and 3.8, and the relevance of this is discussed in Chapter 4's introduction.

The general time allotment for all incubations was 24 hrs. Aliquots were taken at specified time periods to view the progress of the reaction taking place. There was one incubation that ran for 72 hrs to view the extended activity of HNQ, and it is the only one with time segments out of character from the rest. All specific information regarding concentrations of components, aliquot draw times, and other relevant information to the gels presented in the next chapters can be found in the appendix of this work.

2.2: SDS-PAGE Analysis

To begin the electrophoretic analysis, the protein solutions were subjected to some denaturation processes. The incubation samples were first allowed to warm to room temperature. This was followed by the addition of dye that included β-mercaptoethanol and SDS. This was the first denaturation agent used. Immediately following the addition of the dye, the samples were subjected to heat denaturation. They were heated at 100 °C for 5 mins. After heat denaturation, the solutions were well mixed and loaded into the wells of the gel.

The gel contained SDS detergent, and this is was third denaturation method. The marker was added to the well farthest away from the unmodified RNase sample. This was done to improve the appearance of the ladder in the separation of the bottom bands. It was thought that by doing this, less interaction would occur between the negatively charged proteins and allow for better separation. All samples were loaded into the wells in order of the timed aliquots, and then the electrode was turned on. Most gels were run at 100V, 30mA, and 150 mins.

Immediately following the running, the gel was put into staining solution, with Coomassie G-250 blue dye. The gel staining solution was used overnight on a shaker. In the morning, the gels were de-stained for approximately 45 mins to 1 hr. Kimwipes were introduced to the de-staining solution to absorb some of the lost dye molecules. The gel in the de-staining solution was also left on the shaker for the duration of the process. Once the gel had been de-stained to an appropriate level of color, a picture was taken through a scanner, always in .tiff format for later analysis.

2.3: ImageJ Quantification

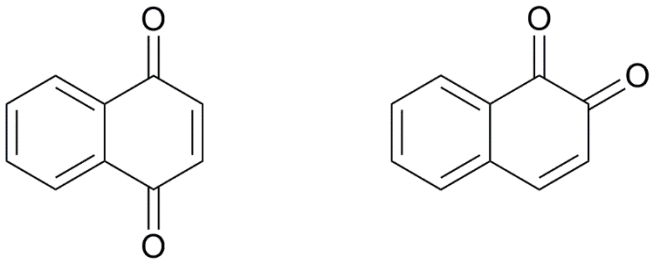
One representative gel was chosen for its overall quality. This gel is displayed in a later chapter, and was also analyzed with ImageJ software. The lanes were selected in full, and pixilation was plotted as a function of area. The areas were normalized, so that the modification could be viewed as a percentage of total protein in each level of the gel. Looking at this quantification allowed for a more accurate view than that of the eye to observe the modification that took place. This method was performed for *o*-NQ, on Figure 3.2.1.

Chapter 3: Modification Induced by 1,2-Naphthoquinone

3.1: Introduction

Although the present study's findings will focus on *o*-NQ, much of this background discussion will cover 1,4-naphthoquinone (*p*-NQ) as well. These two molecules are very similar in structure. Both molecules have a basic structure of two rings linked together with two oxygen atoms bonded on one of them at differing locations. Though very similar, the difference in the positioning of the oxygen atoms likely results in different levels of activity and defines the importance of eventually studying each of them.

Figure 3.1.1: Structures of *p*-NQ and *o*-NQ



p-NQ *o*-NQ

3.1.1: Sources of Exposure

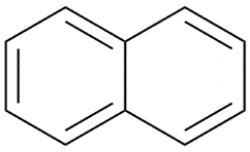
A prominent source of exposure to *o*-NQ and *p*-NQ is ambient air, which was documented by the investigation by Cho and colleagues [1]. It has been found that the two quinones are products of diesel emissions. Air samples were taken from the Los Angeles, California area and analyzed for their content. Significant amounts of four quinones, two of which are the quinones discussed in this chapter, were quantified in

relatively significant levels [1]. The commuter culture of Los Angeles would give this area a higher level of any automobile exhaust than a less urban region. This factor is irrelevant, however, when considering the biological significance of exhaust particles. Diesel emissions are present all around the world, even if in lower volumes. Along with this consideration, the presence of naphthoquinones in ambient air suggests that all organisms are exposed continually in small doses to these molecules that are known to have some biological activity. This concept warrants the need for these molecules to be further investigated to elucidate exactly what effect they have on biological molecules.

3.1.2: Metabolic Production of *o*-NQ and *p*-NQ

A common precursor molecule to *o*-NQ and *p*-NQ is naphthalene, whose structure is shown in Figure 3.1.2. Naphthalene is a common PAH molecule that is used in a number of things that people are exposed to each day. The emission from jet fuels, mothballs, production of phthalic anhydride, and all the products that come from this intermediate are sources of naphthalene [2].

Figure 3.1.2: Structure of Naphthalene



In Chapter 1.1 PAH metabolism was discussed, and the same mechanism that converts benzene to quinone forms is employed in the metabolism of naphthalene. There are many oxidized forms of the molecule which include *o*-NQ and *p*-NQ [3]. This metabolic

pathway ensures that both of these molecules would be present after a person has been working with some substance that exposes them to naphthalene.

3.1.3: Toxicity Associated with Naphthalene

The Agency for Toxic Substances and Disease Registry has documented a large amount of information about naphthalene. This agency is headed up by the United States Department of Health and Human Services, and the studies discussed throughout this section were all reported in the summary published. The International Agency for Research on Cancer carried out a 13 week trial of feeding naphthalene to rats that did not result in cancer, and because of this finding, *o*-NQ was classified as a "possible carcinogen". Although there was no incidence of cancer, many other adverse health issues developed in this study that helped outline all the conditions that naphthalene exposure is responsible for. The report concluded that exposure caused the development of cataracts, and also sometimes caused hemolytic anemia. Both conditions had been previously noticed in human patients that had worked in environments in which they were exposed to naphthalene, but these studies finally made the direct correlation between substance and condition [2].

As mentioned before, when a PAH molecule enters the physiological system, it is oxidized into products that can be excreted. This factor means that when naphthalene enters the body, it goes through this process and becomes the epoxide and quinone form metabolites [3] [4]. When one considers this, it seem very logical that the quinone forms could be the actual molecule that is directly responsible for the physiological damage incurred by people who've been exposed to naphthalene.

3.1.4: Prior Knowledge of Significance of *o*-NQ

To continue the previous section's ideas, *o*-NQ has been directly investigated for its biological significance before this current study. The modification of biological molecules has been investigated with some proteins, and it was discovered that glyceraldehyde-3-phosphate dehydrogenase (GPDH) is able to form adducts with *o*-NQ [17]. This experiment also found that GPDH is able to go through a reverse reaction with glutathione (GSH), and release the quinone to restore functionality to the protein. It is also true that GPDH's ability to undergo reactions like this could implicate it as a protector protein against the toxicity of quinones [6]. This protective mechanism works by the quinone being reduced to the hydroquinone form in the release. Hydroquinone forms are not electrophilic, and thus do not react with the nucleophilic residues on proteins that they would otherwise be inclined to do. This particular reaction does not apply to all interactions between *o*-NQ and proteins. The interaction recorded here took place with cysteine residues, and the interaction planned to be observed by the present study occurs primarily on lysine residues [5] [6].

The prevalence of *o*-NQ in the environment and common household products presents the need for any deleterious effect to be noted. The following studies were conducted between RNase and *o*-NQ to clearly bring light to any biological activity that the quinone has. Showing any effects in such a ubiquitous protein as RNase would bring implications that many life forms on this planet are being effected by the presence of this quinone in ambient air, among other sources. By investigating *o*-NQ, a more detailed account of its toxicity and the toxicity of naphthalene can be documented. This known effect might then contribute to safe protocol for use of the substance in the future.

3.2: Results of Reactions between *o*-NQ, RNase, and Metals

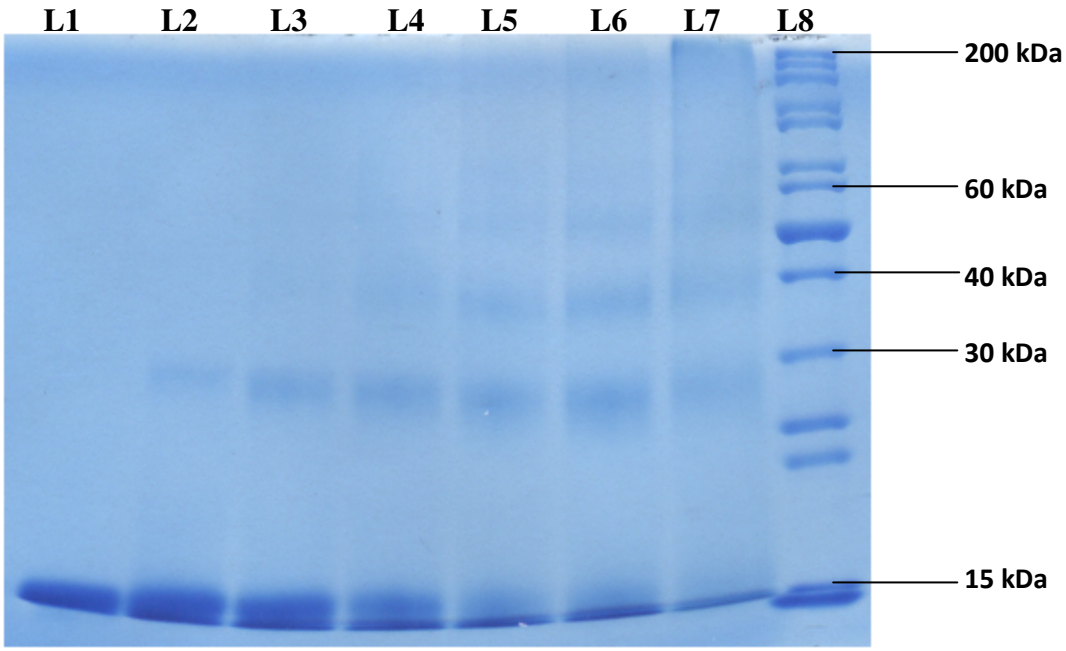
3.2.1.1: Reaction of *o*-NQ (5.0 mM) and RNase (0.10 mM)

This reaction of *o*-NQ and RNase was carried out at a physiologically relevant condition (pH 7.0, 37 °C). The incubation was conducted in a time dependent manner, which is reflected in the resulting gel. The following results section shows the SDS-PAGE gel by which modification was documented. After running the gel, it was clear that this quinone does induce protein modification when in the presence of RNase.

3.2.1.2: Synopsis of Findings

At as early as 10 mins of incubation, it is easy to see a band on the gel that occurs just below 30 kDa. By 1 hr, an additional band has formed around the 40 kDa level. At 2 hrs, banding takes place just over the 50 kDa level. When the incubation had been in progress for 24 hrs, darkening can be seen at the top of the lane. The progression of the banding supports the idea that dimers, trimers, and eventually aggregates are being built. The darkening at the top of the gel is representative of proteins that are 200 kDa or larger. This could mean protein aggregates are present at the top of the gel, and were simply unable to move down the lane because of their extremely high molecular weights.

Figure 3.2.1: SDS-PAGE of Time-Dependent Modification of RNase (0.10 mM) upon exposure to *o*-NQ (5.0 mM) at pH 7.0 and 37 °C



L1: Control 24 hr of RNase only

L2: 10 min with *o*-NQ and RNase (all consecutive lanes will have this)

L3: 30 min

L4: 1 hr

L5: 2 hr

L6: 3 hr

L7: 24 hr

L8: Molecular marker

3.2.1.3: Analysis of ImageJ Quantification

To generate this data, the area of each lane was totaled. The total area assigned to each lane was then divided by the area of each lane. This was the normalization technique used to create percentages. This normalization was done individually for each lane, because each lane could have been subjected to a slightly different amount of systematic error through sample loading into the wells. A relative trend was developed for each band, which can easily be seen in Table 3.2.1 below.

Table 3.2.1: ImageJ Quantification of Figure 3.2.1

Table 3.2.1: Normalized ImageJ Analysis of Figure 3.2.1, Given as percentage of protein in each band					
Molecular Weight Bands	Band 1	Band 2	Band 3	Band 4	Band 5
L1	100	0	0	0	0
L2	93.0	7.00	0	0	0
L3	83.4	16.6	0	0	0
L4	73.7	21.8	4.46	0	0
L5	66.4	21.7	10.4	1.51	0
L6	49.1	23.8	12.4	3.99	10.7
L7	42.1	23.0	8.16	3.98	22.8

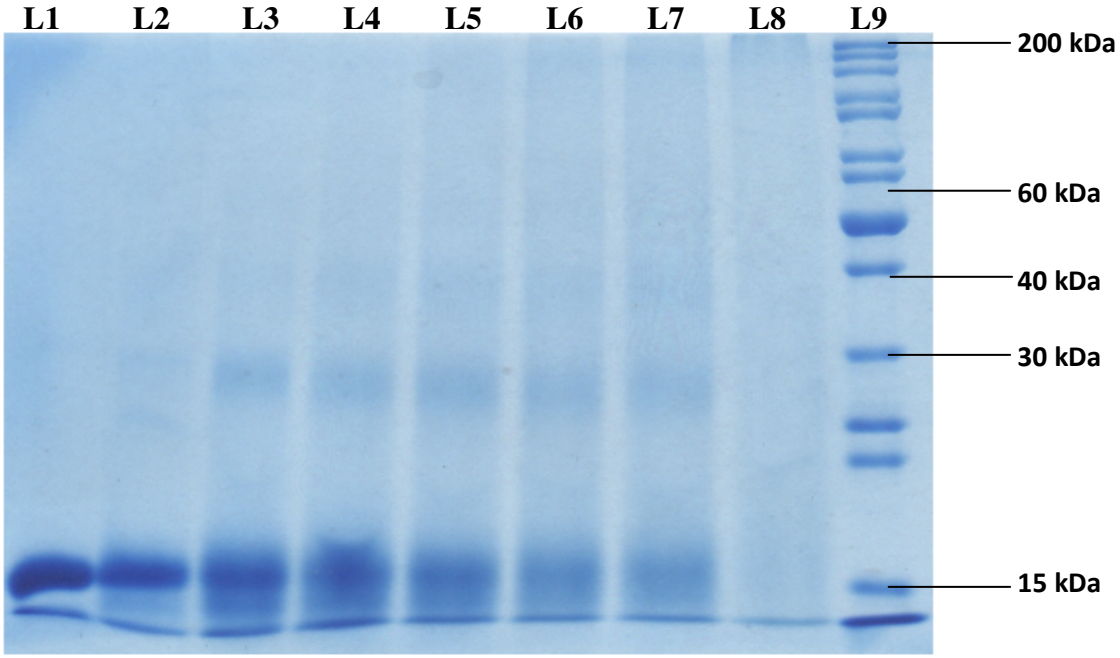
3.2.2.1: Reaction of *o*-NQ (5.0 mM), RNase (0.10 mM) with Cu^{+} (6.0 mM)

The reaction of *o*-NQ, RNase, and Cu^{+} was again carried out at a physiologically relevant condition (pH 7.0, 37 °C). The incubation was conducted in a time dependent manner, which is reflected in the resulting gel. The following results section shows the SDS-PAGE gel by which modification was documented. The resulting gel indicates that the Cu^{+} played a role in changing the reaction between the quinone and protein.

3.2.2.2: Synopsis of Findings

The greatest difference ubiquitously present through the gel is the increased level of smearing featured in the lanes. In a more focused view, a great standpoint for comparison of the change that Cu^{+} induced is the 24 hr lane. In Figure 3.2.1, the 24 hr lane has three distinct bands present, along with darkening at the top of the gel. In Figure 3.2.2, it can be seen that the banding in the 24 hr lane is not present. This is a significant comparison to be made, because it offers an overview of the overall differences in the reaction.

Figure 3.2.2: SDS-PAGE of Time-Dependent Modification of RNase (0.10 mM) upon exposure to *o*-NQ (5.0 mM) and Cu^{1+} (6.0 mM) at pH 7.0 and 37 °C



L1: 24 hr negative control (RNase only)

L2: 24 hr positive control (RNase and Cu^{1+})

L3: 10 min, RNase, Cu^{1+}, *o*-NQ (all subsequent will contain all components)

L4: 30 min

L5: 1 hr

L6: 2 hr

L7: 3 hr

L8: 24 hr

L9: Molecular Marker

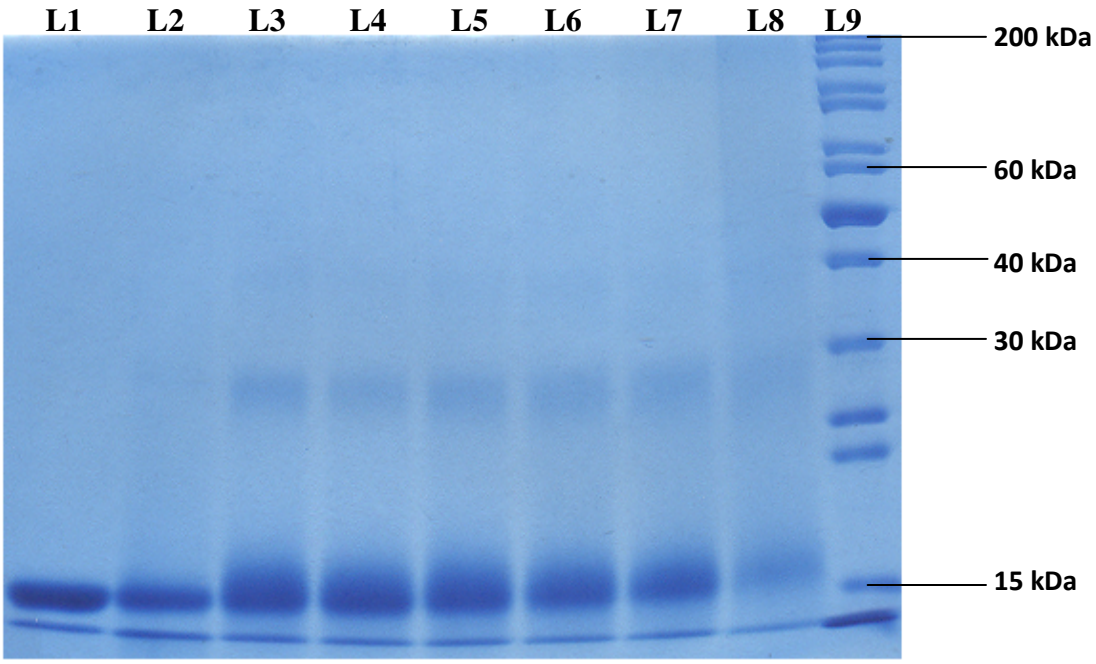
3.2.3.1 Reaction of *o*-NQ (5.0 mM), RNase (0.10 mM), and Fe^{2+} (6.0 mM)

The reaction of *o*-NQ, RNase, and Fe^{2+} was also carried out at a physiologically relevant condition (pH 7.0, 37 °C). The incubation was conducted in a time dependent manner, which is reflected in the resulting gel. The following results section shows the SDS-PAGE gel by which modification was documented. The effect that Fe^{2+} had on the reaction is not as obviously viewed as the Cu^{+} results, but there are still some changes.

3.2.3.2: Synopsis of Results

The smearing effect that was created with Cu^{+} was created again with Fe^{2+} ions. The assays containing Fe^{2+} did not exhibit the same accelerated banding that the Cu^{+} samples did. Going back to the 24 hr lane standpoint for comparison, the gel from the Fe^{2+} assay does not show the darkening at the top that the Cu^{+} gel did. Although the appearance of the banding is not the same as the noticed effect of Cu^{+}, it still shows some differences from the appearance of the gel with no metals present. At this level, it seems that the two different metals have differing effect.

Figure 3.2.3: SDS-PAGE of Time-Dependent Modification of RNase (0.10 mM) upon exposure to *o*-NQ (5.0 mM) and Fe^{2+} (6.0 mM) at pH 7.0 and 37 °C



L1: 24 hr negative control (RNase only)

L2: 24 hr positive control (RNase and Fe^{2+})

L3: 10 min (RNase + *o*-NQ + Iron- all subsequent wells contain this)

L4: 30 min

L5: 1 hr

L6: 2 hr

L7: 3 hr

L8: 24 hr

L9: Molecular Marker

3.3 Discussion of Results

3.3.1: Discussion of Reaction of *o*-NQ (5.0 mM) and RNase (0.10 mM)

It is clear that *o*-NQ exhibits activity toward RNase. In all three gels it is very apparent that protein crosslinking is taking place, based on the multiple levels of banding in higher molecular weights. This idea is also supported by the darkened appearance at the tops of the later time periods in the gels. This darkening is representative of proteins that are 200 kDa or higher. When unmodified RNase's molecular weight is around 14 kDa, it is hard to imagine that protein crosslinking is not taking place given the supporting evidence.

These results suggest that the quinone exhibited the activity as in the proposed mechanism given in Figure 3.3. This figure is an adaptation of what was presented by Kim and coworkers, just with *o*-NQ instead of *p*-BQ. In the gel, the evidence of aggregation is easy to see, but the evidence of adduct formation is impossible to visualize. Adduct formation would not elevate the molecular weight of the protein enough to show an evident increase on the gel. For this interaction to be quantified, an instrument such as a mass spectrometer would be needed. Although it cannot be quantified through the present method, there is still a great likelihood that it is taking place.

The ImageJ analysis showed that as time went on, the band at the bottom of the gel representing the unmodified protein decreases. Along with this, it was a noticeable effect that the other bands went up as a function of time. This quantification supports the

claims made above that modification is occurring, and furthermore confirms that it occurs as a function of time.

3.3.2 Discussion of Metal Ion Assays

In these reactions, an interesting effect was created with the introduction of the metal ions. The results of the Mn^{2+} incubations are not pictured, because there was no noticeable difference between this and the first physiological gel. Even though Mn^{2+} did not alter the course of the reaction, the other metals did.

The first noticeable effect is the increased level of smearing through the other two metal gels. In Figure 3.2.1, the banding is simply more distinct and defined. When looking at Figure 3.2.2 and Figure 3.2.3, it is easy to see that the lanes are much more smeared. This could be an effect of adduct formation in these incubations. Along with this common effect, there were some significant differences in the Cu^{+} and Fe^{2+} building of dimers, trimers and tetramers.

In Figure 3.2.2, it can be seen that the Cu^{+} addition seems to hasten modification of RNase. At the 24 hr standpoint highlighted in the synopsis of this result, it was noted that the banding found in Figure 3.2.1 was no longer present. There could only be two possible explanations for this lack of apparent bands. The first explanation could be that the modification was stunted in some way, but this is not supported by what the gel documented. If modification had been stunted, a much more prominent band would have formed at the bottom of the gel where the unmodified protein appears. This is not the effect observed. Instead, there is a more prominent darkening at the top of the gel, where high molecular weight proteins would collect. This qualitative evaluation of the gel is

what leads to the thought that the reaction is hastened due to Cu^{+}. It is also important to note that even though the addition of Cu^{+} hastens the overall reaction, at the lower time allotments the modification is very similar the effect seen with no metal additions. This could be a result of slightly delayed kinetics.

Fe^{2+} addition resulted in a different banding pattern than the original assay and the Cu^{+} assay. It was hypothesized that metal ions added to the experiment would stimulate the reaction with RNase, and both should have occurred in a similar fashion. Again, this was not the observed effect. Going back to the 24 hr lane again, in Figure 3.2.3 a significant band can be seen at the bottom of the gel near where unmodified proteins should collect. This is a different than what is pictured in both Figure 3.2.1 and 3.2.2. Comparing each lane of the gel to the others before, it seems that banding into the higher molecular weights might actually be stunted as a result of the introduction of Fe^{2+}.

A possible explanation for the differences in modification after the addition of the metals could lie in the oxidative ability that they possess. As mentioned in the methods section, the metal samples were flushed with argon after being divided out into single use containers. However, it could be possible that even with the most of precaution, some of the metals will start to go through redox reactions with the air. This scenario might include the introduction of a fully reduced metal into solution with the quinone. If Fe^{2+} had been reduced by the air or surrounding solution, it might mean that the quinone oxidized this species rather than the lysine residues on the protein. This would then give a less active quinone, less redox cycling and thus less protein crosslinking. It is plausible that Cu^{+} remains a better oxidizing agent through the sample preparation process than

Fe^{2+} has the ability to, and thus stimulated the oxidative power of the quinone much more efficiently.

An alternative explanation is that maybe the two metals interact differently with protein in question. The properties of Cu^{+} and Fe^{2+} are different, and these differences might affect how they behave in solution with other molecules. Perhaps, the Fe^{2+} directly interacts with RNase when Cu^{+} does not. Maybe both of the metals interact with the protein directly, but in different ways. Without further investigation, it will be impossible to pinpoint the precise mechanism by which each component interacts with another, and why their end result is so different.

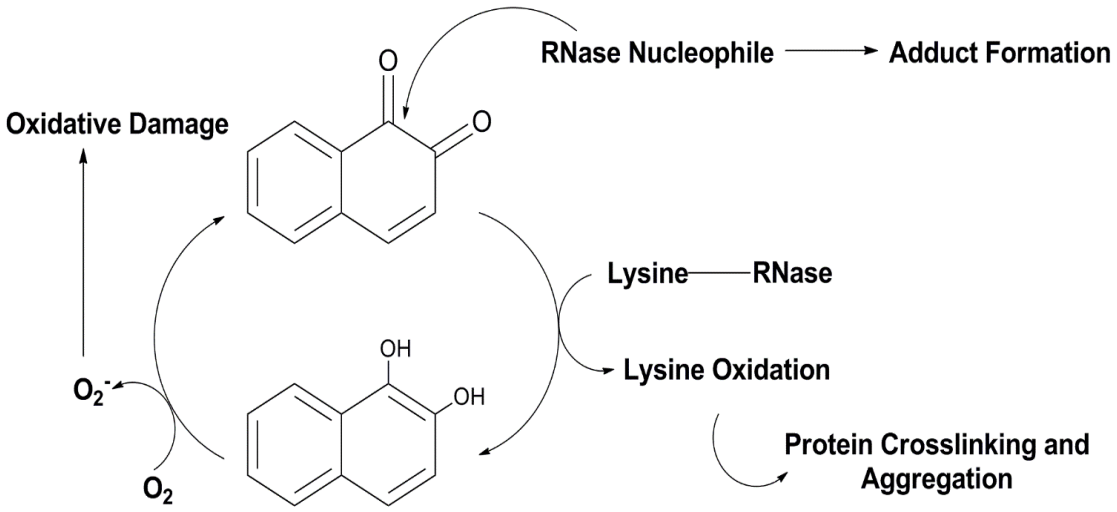
3.3.3: Conclusions

The findings of this study show that there is a much greater risk imposed by exposure to naphthalene and *o*-NQ than a simple sore throat. Protein crosslinking and aggregation result in an altered protein that may lose the integrity of its full function, which is significant. The function of RNase is very important, especially as a defense mechanism. By cutting ribonucleic acid (RNA) some diseases can be prevented, and possible loss of this function could be devastating [7].

This finding also supports that *o*-NQ could be contributing to amyloidosis. To help confirm this, more studies are needed that use an amyloidal protein in a model system. As mentioned before in Chapter 1, RNase is not amyloidal, but it can serve as a model to other proteins that might go through the same mechanism. This is a significant finding because it supports the idea that quinones may be contributing to the incidence of

neurodegenerative diseases. If this is the case, then it seems relevant to society to limit how much this chemical and naphthalene are used.

Figure 3.3-1: Mechanism of *o*-NQ with RNase



Adapted from Reference 3

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Chapter 4: Modification Induced by 2-Hydroxy-1,4-Naphthoquinone

4.1: Introduction

HNQ was investigated in much the same way as *o*-NQ, starting with a background, followed by looking at protein modification ability. The way that people are exposed to HNQ is very different than *o*-NQ, thus the numbers and subset of people affected are different. These aspects are introduced in this background, to show the significance of looking at protein modification induced by this quinone.

4.1.1: History and Common Usage of HNQ

For approximately 4000 years, Henna dye has been used as a cultural cosmetic. The highest percentage of people in the world that use Henna can be found in India, and they use it for temporary tattooing. Tattooing is done before significant life events such as weddings, and creates red tinted marking on the skin. The red appearance of the tattoos is related to one of the primary substances found in Henna, which is HNQ. The crushed leaves of Lawsonia Alba are the source of henna dye, and HNQ occurs naturally in these plants [1]. This red tint is useful in the cosmetic world for more than just the tattooing. Henna is frequently used in shampoos, permanent hair dyes, and many other red tinted cosmetics [2]

Studies completed by Kraeling and colleagues investigated the retention of HNQ in the skin after use of shampoos and hair dyes containing it. After short interval time spans, the shampoos showed retention levels between 3 and 7% immediately after use. The retention levels from the hair colors were between 2 and 4% after about 1 hr of use [2]. If HNQ is retained in the skin for any amount of time, this is significant to the subject of protein modification.

4.1.2: Hemolytic Anemia Related to HNQ

In a study completed by Munday and colleagues, HNQ was found to be a direct agent in causing hemolytic anemia [3]. Hemolytic anemia is an umbrella term for many diseases or illness that cause defect in red blood cells. Many familiar diseases, such as Sickle Cell anemia, fall under this umbrella term. Sickle cell anemia is an inherited condition, but there are some anemias that are not passed from the parent, including anemia induced by a toxin [4].

Not every person who comes in contact with HNQ has an anemic reaction [3]. There are certain physiological defects that can make a person more susceptible to toxic anemia. Metabolic pathways are responsible for dealing with the intake of all substances, and so if there is some defect in one of these systems, harmful substances can have a pronounced effect. The most common defect in patients who develop anemia after exposure to chemicals with reactive properties such as quinones is in G6PD enzyme function [4].

4.1.3: Understanding Hemolytic Response to HNQ

In rats, exposure to HNQ has been shown to illicit a hemolytic and nephrotoxic response [3]. McMillan and colleagues carried out studies that attempted to identify just how red blood cells are damaged after exposure to HNQ. They found that normal blood cells were not damaged by the presence of the quinone. To eliminate the possibility that a metabolite of HNQ causes the damage, they also tested THN. Again, this molecule had no significant oxidative power over blood cells [1].

Their next step was to look at blood cells with abnormalities. Oxidative stress was found to occur in cells with low levels of GSH. When GSH levels in cells were depleted, even low concentrations of HNQ and THN were able to cause oxidative stress. They also found that when the GSH levels in blood cells were depleted, skeletal proteins that build up to hemoglobin were modified. These skeletal proteins formed adducts with HNQ molecules [1]. This study provides context to investigating protein modification in a more broad scope with this quinone, to clearly see any toxic effects that it has.

4.1.4: Importance of Background with GSH

GSH in the body acts as an antioxidant, and truly exists in two forms. The forms are oxidized GSSG, and reduced GSH. The most probable way that GSH is involved in the protein modification and hemolytic response mentioned in section 2 above is through reduction power [5]. In chapter 3 section 5 earlier in this work, it was mentioned that when *o*-NQ modifies GPDH, GSH reverses this reaction and leaves the quinone in the hydroquinone form [6]. GSH likely provides the same function with HNQ in solution with blood, hindering its oxidative ability.

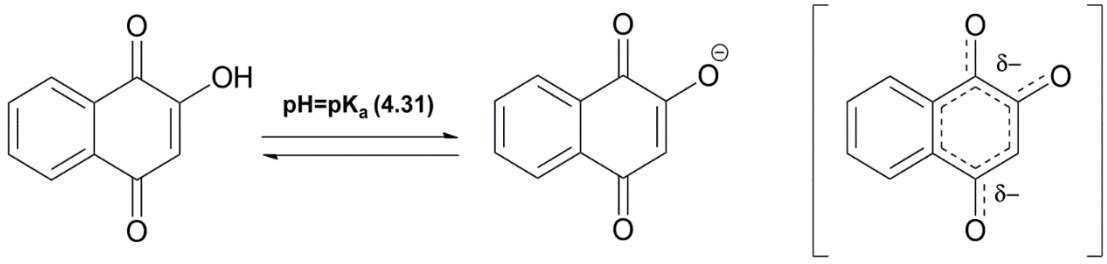
4.1.5: pH Variation

On the subject of differential levels of protonation, pH variation was used in this study. When the 2-hydroxl group of the molecule is protonated, HNQ is neutral and its carbonyl carbon has a partial positive charge. When the pH of the environment reaches the pK_a of the hydroxyl group, HNQ in solution becomes 50% deprotonated. This deprotonation creates a negative charge that is delocalized through the molecule, which makes the carbonyl carbon less electrophilic. Figure 4.1.5 shows this process. This

delocalization yields the carbonyl carbon less susceptible to attack by a nucleophilic amino acid residues in a protein chain.

The pK_a of the hydroxyl group on HNQ is 4.31 [7]. In this context, some assays were run in lower pH values to see if dropping the pH makes any change to the reaction occurring. This method is conceptually acceptable, because some cells in the body exist in lower pH values than approximately seven. For instance, cancer tumors are known to have a low extracellular pH, and given this would be an extreme condition it is still possible [8]. Some organelles in cells are also known to have low pH values as well, like lysosomes. The pH of a lysosome is around 5.0, which is one aspect contributing to how it works to degrade other biological molecules [9]. The pH level of 3.8 is not physiologically relevant, but was chosen as a reference standpoint to see any possible reactivity of HNQ.

Figure 4.1.5: Structure of HNQ Showing Delocalization of Bonds



4.2: Results of Reactions between HNQ, RNase, and Metals

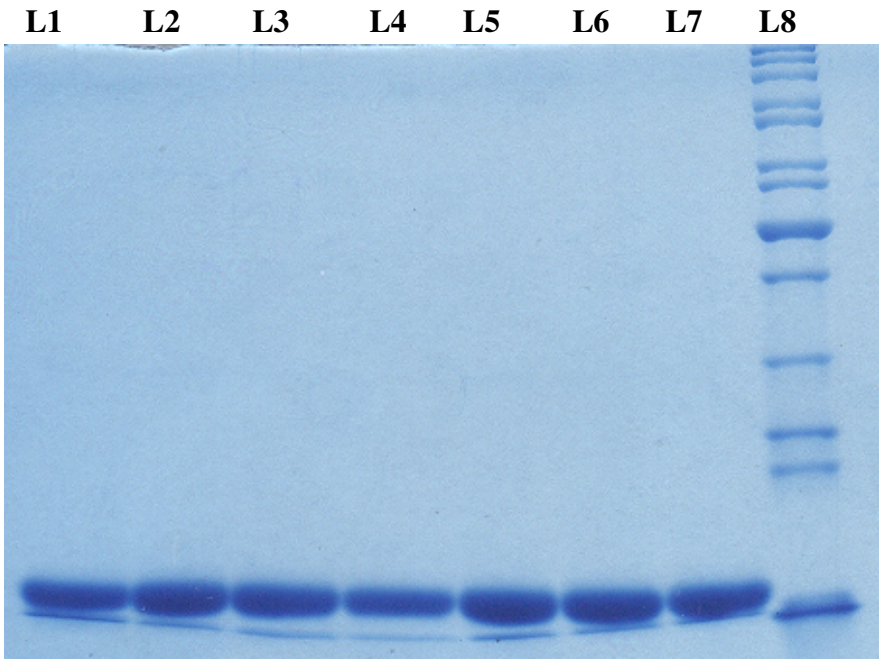
4.2.1.1 Reaction of HNQ (5.0 mM) and RNase (0.10 mM)

This reaction of HNQ and RNase was carried out at a physiologically relevant condition (pH 7.0, 37 °C). The incubation was conducted in a time dependent manner, which is reflected in the resulting gel. The following results section shows the SDS-PAGE gel by which modification was documented. The results of this reaction show that at this condition HNQ is relatively inactive toward RNase.

4.2.1.2 Synopsis of Findings

At no time period in this reaction was a significant gel shift observed. This result indicates that HNQ does not induce the modification of this protein by way of crosslinking. As stated before, it is possible that adduct formation took place in this reaction, and that cannot be quantified through SDS-PAGE analysis. An important feature to take note of is that across the bottom of the gel, the band is identical from the first to the last lane. This suggests that all the protein in solution is most likely unmodified, and a minimal reaction if any took place over the course of 24 hrs.

Figure 4.2.1: SDS-PAGE of Time-Dependent Modification of RNase (0.10 mM) upon exposure to HNQ (5.0 mM) at pH 7.0 and 37 °C



L1: Control 24 hr of RNase only

L2: 10 min with HNQ and RNase (all consecutive lanes will have this)

L3: 30 min

L4: 1 hr

L5: 2 hr

L6: 3 hr

L7: 24 hr

L8: Molecular marker

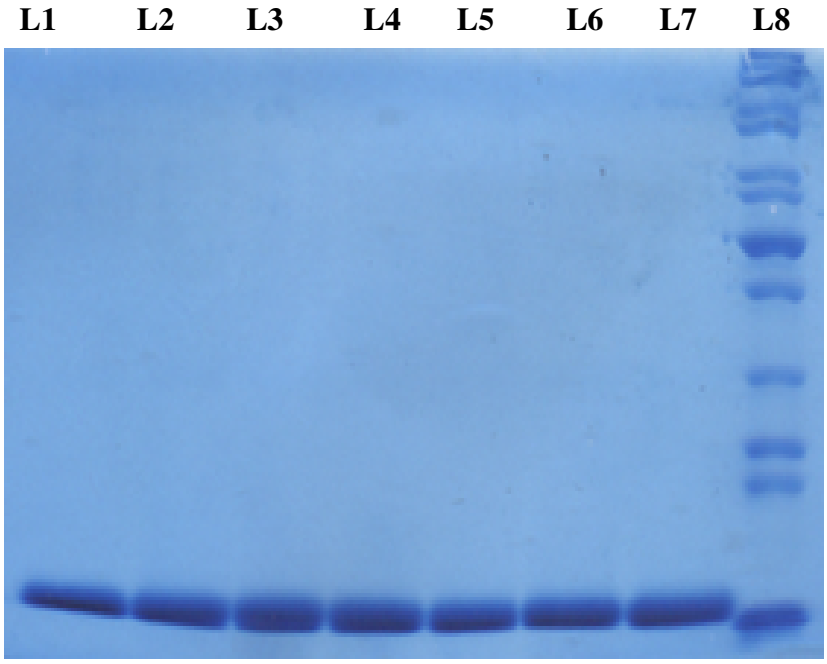
4.2.2.1 Reaction of HNQ (5.0 mM) and RNase (0.10 mM) at Lowered pH Levels

A total of three reactions were carried out at lowered pH levels: 5.8, 4.8, and 3.8. The theory behind the physiological relevance of these pH levels was presented in the background for this quinone. In addition to this physiological relevance of pH 5.8 and 4.8, pH 3.8 was chosen as a reference point for which HNQ would exist primarily in a protonated form rather than an anionic form. This was done in hopes of HNQ existing with an electrophilic carbonyl carbon, and the observance of a reaction occurring. Aside from the altered pH, the conditions of the reaction were maintained from prior reactions. The incubation was still maintained at 37 °C, and carried out in a time dependent manner. It was determined through these reactions that the lowering of pH did not change the interaction documented on the resulting gel.

4.2.2.2: Synopsis of Findings

For the purpose of discussion, the pH 4.8 gel was selected because of it being the central value. None of the three gels showed modification at a lower pH. There was no gel shift of any nature documented across the gels. Along with the absence of gel shift, there was generally no difference at all noted in the results of these variable conditions.

Figure 4.2.2: SDS-PAGE of Time-Dependent Modification of RNase (0.10 mM) upon exposure to HNQ (5.0 mM) at pH 4.8 and 37 °C



L1: Control 24 hr of RNase only

L2: 10 min with HNQ and RNase (all consecutive lanes will have this)

L3: 30 min

L4: 1 hr

L5: 2 hr

L6: 3 hr

L7: 24 hr

L8: Molecular marker

4.3: Discussion of Results

4.3.1: Discussion of Reaction of HNQ (5.0 mM) and RNase (0.10 mM) at pH 7.0 and pH 4.8

Although HNQ has only slight structural differences from *o*-NQ, the reaction that takes place with RNase is radically different. The most likely explanation as to why HNQ is inactive in solution with RNase lies in the delocalization of bonds in this molecule. It was introduced in section 4 of this chapter that when HNQ is at pH 7.0, the hydroxyl group is deprotonated, and this allows for the aromatic bonds to delocalize to this oxygen also. This change in the molecule would make it less susceptible to nucleophilic attack of a protein, and also would lower the molecules ability to oxidize other components in its environment such as the protein RNase.

To combat this difficulty, the lower pH trials were conducted. This was in an attempt to keep the hydroxyl group protonated and possibly stimulate more of an interaction between the quinone and protein. The effect of all lowered pH levels was not inclusive of inducing modification.

4.3.2: Discussion of Metal Ion Assays

They are not presented here, but metal ion assays were conducted for this quinone as well as the other one studied. Again, no real significant change was observed in these gels. The Mn^{2+} trials were consistently found to induce absolutely identical results to those with no added metals. Through this and the result found with *o*-NQ and Mn^{2+}, it can be said that it does not become involved in the reaction in any significant way.

The Cu^{+} and Fe^{2+} results only displayed an increased amount of smearing in the lanes. No banding in higher molecular weights was observed, again confirming that HNQ is a more benign substance in the presence of just RNase. The consistency drawn between the two naphthoquinones and their metal ions assays is the increased amount of smearing that they cause. Even though this is a finding, it doesn't exactly support the idea that the metals hasten a reaction or stunt it. If anything, the smearing might represent an increased amount of adduct formation. With this being said, the metal ions assays with HNQ were inconclusive toward helping to form a general idea about the activity created by the metal additions. This could be caused by the differing properties of HNQ and *o*-NQ, and shows that the addition of metals needs to be examined on a case-by-case basis.

4.3.3 Conclusions

It is very clear through the background presented for HNQ that it does have some oxidative ability. However, it seems that in such simple situations as the reaction conditions of this study the oxidative activity is not present. This indicates that the mechanism that HNQ goes through in the body to cause hemolytic anemia and some protein modification is more complex than the scope of this study.

Overall, finding that HNQ does not exhibit a great amount of activity toward RNase is a positive finding. Millions of people use Henna dye and are exposed to HNQ frequently. Determining that HNQ damaged proteins in a very serious way would create the need for it to be removed from common cultural use. Indian culture has been using this substance for approximately 4000 years, which means that its removal would significantly impact those people. Often times, when people have been using something

for extended periods of time with minimal effect, they will be highly unlikely to change its place in the culture. In this way, HNQ's lack of activity is a wonderful thing to see.

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Chapter 5: Conclusions about Findings

Chapter 5: Conclusions about Findings

In the context of gaining knowledge about the activity of these naphthoquinones, the conducted experiments were successful. The reactivity of *o*-NQ has now begun to be investigated, and HNQ was found to be relatively benign under physiological conditions. Though insightful data has now been collected, more work is needed to fully understand the mechanisms these molecules go through with RNase. The next steps in this investigation will help fully elucidate the specific interaction that the protein and quinone go through.

5.1: Future Directions

In order to fully examine the activity of these naphthoquinones in the way *p*-BQ was investigated, fluorescence spectroscopy, UV-Vis, and mass spectroscopy analysis need to be performed. Fluorescence was a main contributor to the understanding of the behavior of the reaction, and how the conformation of the protein can be changed. Also, where SDS-PAGE gives a more qualitative view into the activity of the quinone, fluorescence offers a more quantitative look. This is important for validating the data, because showing the same result though multiple methods builds a strong correlation.

Mass spectroscopy has recently been employed in the way of studying these modification pathways by Kim and colleagues. This method has confirmed the presence of adduct formation and dimerization of RNase as a result of exposure to *p*-BQ. This would be a great direction to move in when looking at both *o*-NQ and HNQ, because this interaction cannot be documented very well with SDS-PAGE alone. HNQ was reported as benign with the SDS-PAGE analysis completed in this study, but that's not to say that

it is completely unreactive. Adduct formation could still be taking place as a result of exposure to HNQ, and this reaction would still be significant when one considers that this molecule may sit in a person's skin for approximately a week. Although not confirmed, some of the integrity of a protein's function may be lost upon adduct formation, and this represents the importance of looking at this interaction.

On the subject of employing alternate methods, native conformation SDS-PAGE could be carried out. All the methods used in this study used many denaturation methods to prepare the protein for being run in the gel. Using a native gel might reveal more information about any modification that the two quinones induce on RNase.

Zooming in just on the investigation of *o*-NQ, it would be a very useful viewpoint to look at any modification induced by *p*-NQ. As stated in the background of Chapter 3, *p*-NQ is a sister molecule to *o*-NQ through metabolic pathways. Looking at this modification might give some insight to the difference that positioning of the oxygen atoms makes in activity. Of course, *p*-NQ would need to be subjected to all the same methods used for *o*-NQ in all experiments to draw appropriate comparisons.

For the specific reactions with HNQ, more work is really needed to understand just what role it plays in the body. In the type of reactions studied in this work, a next step could be to look at a more basic pH level. This would alter the protonation of the protein yet again in a way that has not been investigated. This could either shed light on the activity of the molecule, or just support that it is not very active.

Overall, the more work completed in this field can only offer a greater understanding of substances to be avoided. Improving the health of all organisms in the

environment should be a paramount goal for science to accomplish. Through defining unknown toxicities, the studies completed and those to follow have contributed to this goal.

Appendix A

Table 1: Incubation of Time-Dependent Modification of RNase (0.10 mM) upon exposure to *o*-NQ (5.0 mM) at pH 7.0 and 37 °C

0702, [Q] 5 mM pH 7.0	FW	mmol	mg	solution		[Q]i	[Q]f	Incub Vol	Q vol
20 mM/10ml stock, oNQ	158.16	0.20	31.63	in 10%MeOH/buffer		20	5	540	135
Actual oNQ									
(uL)Final RNas->0.1 mM			mg	ml				RNas vol	74.4
Stock RNase	0.726		19.9	2					
	Total	Ctr 24h	10	30	1h	2h	3h	24h	MM
0.734 mM St RNase (uL)	74.4	13.8							
Protein (ug)	739.8	137.0	82.2	82.2	82.2	82.2	82.2	82.2	
Phosphate buffer (uL)	330.6	86.2							
Incub Time		0 min	10 min	30 min	1h	2h	3h	24h	
Incub Temp	37°C								
20 mM Q stock, [Q]f 5 mM	135	0.0							
Final Incubate Vol (uL)	540	100							
Aliquot Taken (uL) X 4			60	60	60	60	60	60	
		4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	
LANE		L1	L2	L3	L4	L5	L6	L7	L8
		Ctr 24h	10	30	1h	2h	3h	24h	MM
Loading Dye		10	10	10	10	10	10	10	0
Incub Aliquot of Total		15	15	15	15	15	15	15	0
Loading Vol (uL)		12	12	12	12	12	12	12	15
Protein loaded (ug)		10	10	10	10	10	10	10	

Table 2: Incubation of Time-Dependent Modification of RNase (0.10 mM) upon exposure to *o*-NQ (5.0 mM) and Cu^{1+} (6.0 mM) at pH 7.0 and 37 °C

0710, [Q] 5 mM pH 7.0	FW	mmol		mg	solution		[Q]i	[Q]f	Incub Vol	Q vol
20 mM/10ml stock, oNQ	158.16	0.20		31.63	in 10%MeOH/buffer		20	5	540	135
Actual oNQ										
(uL)Final RNas->0.1 mM				mg	ml				RNas vol	74.4
Stock RNase	0.726			19.9	2					
20mM/10mL Stock, Cu(I)Cl	99	0.2		19.80	Phosphate Buffer		20	6	540	162
	Total	Ctr 24h	Ctr Cu	10	30	1h	2h	3h	24h	MM
0.734 mM St RNase (uL)	74.4	13.8	13.8							
Protein (ug)	739.8	137.0	137.0	82.2	82.2	82.2	82.2	82.2	82.2	
Phosphate buffer (uL)	168.6	86.2	56.2							
Incub Time		0 min		10 min	30 min	1h	2h	3h	24h	
Incub Temp	37°C									
20mM Cu stock	162		30.0							
20 mM Q stock, [Q]f 5 mM	135	0.0	0.0							
Final Incubate Vol (uL)	540	100	100							
Aliquot Taken (uL) X 4				60	60	60	60	60	60	
		4 x 15		4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	
LANE		L1	L2	L3	L4	L5	L6	L7	L8	L9
		Ctr 24h	Ctr Cu	10	30	1h	2h	3h	24h	MM
Loading Dye		10	10	10	10	10	10	10	10	0
Incub Aliquot of Total		15	15	15	15	15	15	15	15	0
Loading Vol (uL)		12	12	12	12	12	12	12	12	15
Protein loaded (ug)		10	10	10	10	10	10	10	10	

Table 3: Incubation of Time-Dependent Modification of RNase (0.10 mM) upon exposure to *o*-NQ (5.0 mM) and Fe^{2+} (6.0 mM) at pH 7.0 and 37 °C

0702, [Q] 5 mM pH 7.0	FW	mmol		mg	solution		[Q]i	[Q]f	Incub Vol	Q vol
20 mM/10ml stock, oNQ	158.16	0.20		31.63	in 10%MeOH/buffer		20	5	540	135
Actual oNQ										
(uL)Final RNas->0.1 mM				mg	ml				RNas vol	74.4
Stock RNase	0.726			19.9	2					
20mM/10mL Stock, Fe(II)Cl	126.75	0.2		25.35	Phosphate Buffer		20	6	540	162
	Total	Ctr 24h	Ctr Fe	10	30	1h	2h	3h	24h	MM
0.734 mM St RNase (uL)	74.4	13.8	13.8							
Protein (ug)	739.8	137.0	137.0	82.2	82.2	82.2	82.2	82.2	82.2	
Phosphate buffer (uL)	168.6	86.2	56.2							
Incub Time		0 min		10 min	30 min	1h	2h	3h	24h	
Incub Temp	37°C									
20mM Fe stock	162		30.0							
20 mM Q stock, [Q]f 5 mM	135	0.0	0.0							
Final Incubate Vol (uL)	540	100	100							
Aliquot Taken (uL) X 4				60	60	60	60	60	60	
		4 x 15		4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	
LANE		L1	L2	L3	L4	L5	L6	L7	L8	L9
		Ctr 24h	Ctr Fe	10	30	1h	2h	3h	24h	MM
Loading Dye		10	10	10	10	10	10	10	10	0
Incub Aliquot of Total		15	15	15	15	15	15	15	15	0
Loading Vol (uL)		12	12	12	12	12	12	12	12	15
Protein loaded (ug)		10	10	10	10	10	10	10	10	

Table 4: Incubation of Time-Dependent Modification of RNase (0.10 mM) upon exposure to HNQ (5.0 mM) at pH 7.0 and 37 °C

0513, [Q] 5 mM pH 7.0	FW	mmol	mg	solution		[Q]i	[Q]f	Incub Vol	Q vol
20 mM/10ml stock, HNQ	174.15	0.20	34.83	in 10%MeOH/buffer		20	5	540	135
Actual HNQ									
(uL)Final RNas->0.1 mM			mg	ml				RNas vol	72.5
Stock RNase	0.745		20.4	2					
	Total	Ctr 24h	10	30	1h	2h	3h	24h	MM
0.734 mM St RNase (uL)	72.5	13.4							
Protein (ug)	739.8	137.0	82.2	82.2	82.2	82.2	82.2	82.2	
Phosphate buffer (uL)	332.5	86.6							
Incub Time		0 min	10 min	30 min	1h	2h	3h	24h	
Incub Temp	37°C								
20 mM Q stock, [Q]f 5 mM	135	0.0							
Final Incubate Vol (uL)	540	100							
Aliquot Taken (uL) X 4			60	60	60	60	60	60	
		4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	
LANE		L1	L2	L3	L4	L5	L6	L7	L8
		Ctr 24h	10	30	1h	2h	3h	24h	MM
Loading Dye		10	10	10	10	10	10	10	0
Incub Aliquot of Total		15	15	15	15	15	15	15	0
Loading Vol (uL)		12	12	12	12	12	12	12	15
Protein loaded (ug)		10	10	10	10	10	10	10	10

Table 5: Incubation of Time-Dependent Modification of RNase (0.10 mM) upon exposure to HNQ (5.0 mM) at pH 4.8 and 37 °C

0630, [Q] 5 mM pH 4.75	FW	mmol	mg	solution		[Q]i	[Q]f	Incub Vol	Q vol
20 mM/10ml stock, HNQ	174.15	0.20	34.83	in 10%MeOH/buffer		20	5	540	135
Actual HNQ									
(uL)Final RNas->0.1 mM			mg	ml				RNas vol	74.4
Stock RNase	0.726		19.9	2					
	Total	Ctr 24h	10	30	1h	2h	3h	24h	MM
0.734 mM St RNase (uL)	74.4	13.8							
Protein (ug)	739.8	137.0	82.2	82.2	82.2	82.2	82.2	82.2	
Acetic Buffer (uL)	330.6	86.2							
Incub Time		0 min	10 min	30 min	1h	2h	3h	24h	
Incub Temp	37°C								
20 mM Q stock, [Q]f 5 mM	135	0.0							
Final Incubate Vol (uL)	540	100							
Aliquot Taken (uL) X 4			60	60	60	60	60	60	
		4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	
LANE		L1	L2	L3	L4	L5	L6	L7	L8
		Ctr 24h	10	30	1h	2h	3h	24h	MM
Loading Dye		10	10	10	10	10	10	10	0
Incub Aliquot of Total		15	15	15	15	15	15	15	0
Loading Vol (uL)		12	12	12	12	12	12	12	15
Protein loaded (ug)		10	10	10	10	10	10	10	10

Table 6: Screening Test Incubation of RNase (0.10 mM) upon exposure to *o*-NQ (3.0 mM) and Mn^{2+} (0-6.0 mM)

0612, [Q] 3 mM pH 7.0	FW	mmol		mg	solution		[Q]i	[Q]f	Incub Vol	Q vol
20 mM/10ml stock, oNQ	158.16	0.20		31.63	in 10%MeOH/buffer		20	3	100	15
Actual oNQ										
(uL)Final RNas->0.1 mM				mg	ml				RNas vol	13.4
Stock RNase	0.745			20.4	2					
20mM/10mL Stock, Mn(II)Cl	197.92	0.2		39.58	Phosphate Buffer		20	3	100	15
[Mn] final				0	6	0.5	3	6		
			Ctr -	Ctr Q+	Ctr Mn+	1h	2h	3h	MM	
0.734 mM St RNase (uL)			13.4	13.4	13.4	13.4	13.4	13.4		
Protein (ug)			137.0	137.0	137.0	137.0	137.0	137.0		
Phosphate buffer (uL)			86.6	71.6	56.6	69.1	56.6	42		
Incub Time			24h	24 h	24h	3h, 24h	3h, 24h	3h, 24h		
Incub Temp										
20mM Q Stock, [Q]f 3mM			0.0	15	0	15	15	15		
20 mM [Mn] stock					30	2.5	15	30		
Final Incubate Vol (uL)			100	100	100	100	100	100		
Aliquot Taken (uL) X 3				45	45	45	45	45		
				3 x 15	3 x 15	3 x 15	3 x 15	3 x 15		
LANE			Ctr -	Ctr Q+	Ctr Mn+	1	3	6	MM	
Loading Dye			10	10	10	10	10	10		
Loading Vol (uL)			12	12	12	12	12	12	15	

SDS-PAGE of Screening Test of Modification of RNase (0.10 mM) upon exposure to *o*-NQ (3.0 mM) and Mn^{2+} (0-6.0 mM) at pH 7.0 and 37 °C

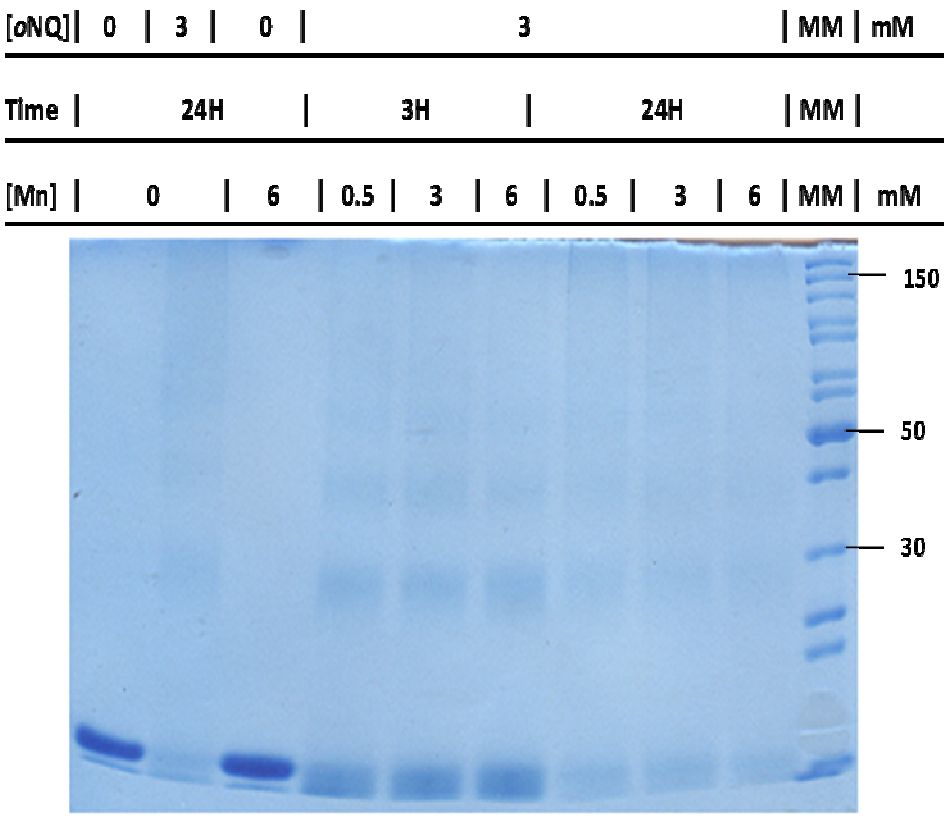
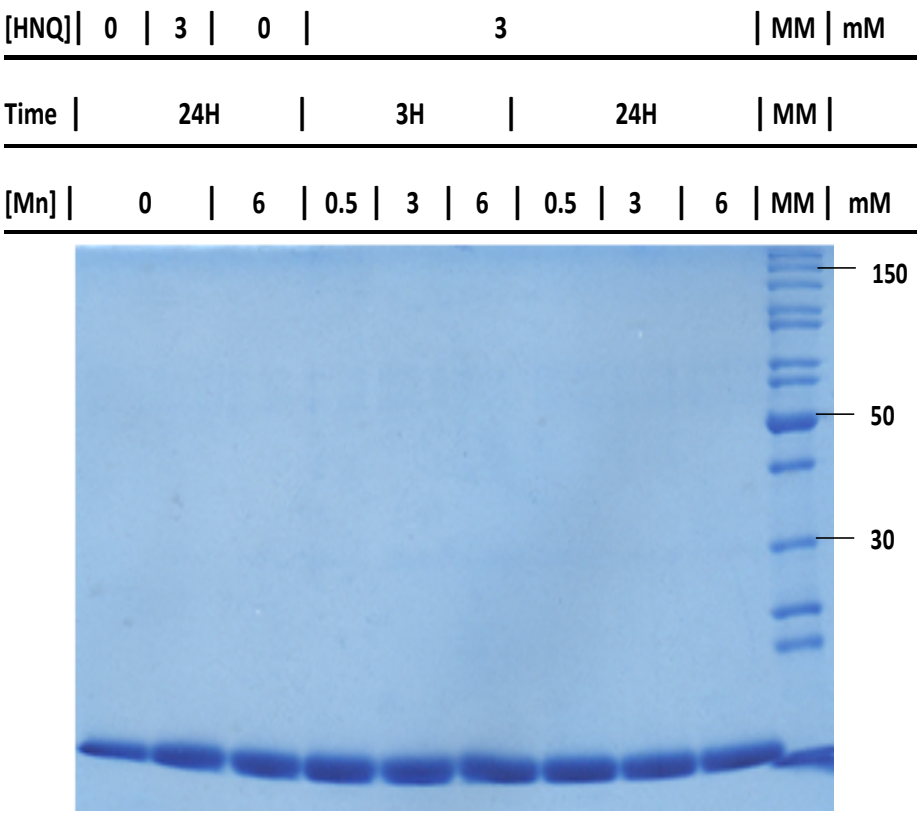


Table 7: Screening Test Incubation of RNase (0.10 mM) upon exposure to HNQ (3.0 mM) and Mn^{2+} (0-6.0 mM)

0610, [Q] 3 mM pH 7.0	FW	mmol		mg	solution		[Q]i	[Q]f	Incub Vol	Q vol
20 mM/10ml stock, HNQ	174.15	0.20		34.83	in 10%MeOH/buffer		20	3	100	15
Actual HNQ										
(uL)Final RNas->0.1 mM				mg	ml				RNas vol	13.4
Stock RNase	0.745			20.4	2					
20mM/10mL Stock, Mn(II)Cl	197.92	0.2		39.58	Phosphate Buffer		20	3	100	15
[Mn] final				0	6	0.5	3	6		
			Ctr -	Ctr Q+	Ctr Mn+	1h	2h	3h	MM	
0.734 mM St RNase (uL)			13.4	13.4	13.4	13.4	13.4	13.4		
Protein (ug)			137.0	137.0	137.0	137.0	137.0	137.0		
Phosphate buffer (uL)			86.6	71.6	56.6	69.1	56.6	42		
Incub Time			24h	24 h	24h	3h, 24h	3h, 24h	3h, 24h		
Incub Temp										
20mM Q Stock, [Q]f 3mM			0.0	15	0	15	15	15		
20 mM [Mn] stock					30	2.5	15	30		
Final Incubate Vol (uL)			100	100	100	100	100	100		
Aliquot Taken (uL) X 3				45	45	45	45	45		
				3 x 15	3 x 15	3 x 15	3 x 15	3 x 15		
LANE			Ctr -	Ctr Q+	Ctr Mn+	1	3	6	MM	
Loading Dye			10	10	10	10	10	10		
Loading Vol (uL)			12	12	12	12	12	12	15	

SDS-PAGE of Screening Test of Modification of RNase (0.10 mM) upon exposure to HNQ (3.0 mM) and Mn^{2+} (0-6.0 mM) at pH 7.0 and 37 °C



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