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S-Nitrosothiols: Formation, Decomposition, Reactivity and Possible
Physiological Effects

by

Moshood Kayode Morakinyo

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
in
Chemistry

Dissertation Committee:
Reuben H. Simoyi, Chair
Gwendolyn Shusterman
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Linda George

Portland State University
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ABSTRACT

Three biologically-active aminothiols cysteamine (CA), DL-cysteine (CYSH) and DL-homocysteine, were studied in this thesis. These aminothiols react with nitrous acid (HNO_2), prepared *in situ*, to produce S-nitrosothiols (RSNOs): S-nitrosocysteamine (CANO), S-nitrosocysteine (CYSNO) and S-nitrosohomocysteine (HCYSNO). They also react with S-nitrosoglutathione (GSNO) and S-nitroso-N-acetylpenicillamine (SNAP) through a transnitrosation reaction to produce their corresponding RSNOs. A detailed kinetics and mechanistic study on the formation of these RSNOs and their subsequent decomposition to release nitric oxide (NO) were studied.

For all three aminothiols the stoichiometry of their reaction with nitrous acid is strictly 1:1 with the formation of one mole of RSNO from one mole of HNO_2 . In all cases, the nitrosation reaction is first order in nitrous acid, thus implicating it as a nitrosating agent in mildly acidic pH conditions. Acid catalyzes nitrosation after nitrous acid has saturated, implicating another nitrosating agent, the nitrosonium cation, NO^+ (which is produced from the protonation of nitrous acid) as a contributing nitrosating species in highly acidic environments. The acid catalysis at constant nitrous acid concentrations suggests that the nitrosonium cation nitrosates at a much higher rate than nitrous acid. Nitric oxide itself was not detected as a nitrosant. Bimolecular rate constants for the nitrosation of CA, CYSH and HCYSH were deduced to be 17.9, 6.4, 0.09 $\text{M}^{-1} \text{s}^{-1}$ for the nitrosation by nitrous acid and 8.25 x

10^{10} , 2.89×10^{10} and $6.57 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ for the nitrosation by nitrosonium cation respectively. A linear correlation was obtained between the rate constants and the pKa of the sulfur center of the aminothiols for nitrosation by NO^+ .

The stabilities of the three RSNOs were found to be affected by metal ions. They were unstable in the presence of metal ions, with half-lives of few seconds. However, in the presence of metal ion chelators, they were found to be relatively stable with half-lives of 10, 30 and 198 hours for CYSNO, CANO and HCYSNO respectively. The relative stability of HCYSNO may be an advantage in the prevention of its metabolic conversion to homocysteine thiolactone, the major culprit in HCYSH pathogenesis. This dissertation has thus revealed new potential therapeutic way for the modulation of HCYSH related cardiovascular diseases.

DEDICATION

*This dissertation is dedicated to
the LORD, JESUS CHRIST, for
His unfailing love*

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First of all I will like to thank God for His grace, strength and wisdom to do this work.

I would like to express my deepest gratitude towards my advisor, Professor Reuben H. Simoyi for his encouragement, suggestions and effective supervision of this work from beginning to end. I am particularly grateful towards his invaluable efforts in recruiting so many students from Africa to pursue advanced degrees in the United States. He personally came to Obafemi Awolowo University, Ile-Ife, Nigeria, in 2003 to recruit me to Portland State University. His efforts has helped many of us achieve our dreams in academia. One of the greatest things I have learnt from Prof (as we usually call him) is the ability to bring out the best in any student no matter his/her background. This inspired me to become a leader in research and mentorship. I also want to thank Professor Simoyi's wife, Mrs. Shylet Simoyi, for her care, encouragement and support.

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LIST OF ABBREVIATIONS

NO	nitric oxide
RSH	thiol
RSNO	S-nitrosothiol
CA	cysteamine
CANO	S-nitrosocysteamine
CYSH	L-cysteine
CYSSCY	cystine
CYSNO	S-nitrosocysteine
HCYSH	DL-homocysteine
HCYSSHCY	homocystine
HCYSNO	S-nitrosohomocysteine
HTL	homocysteine thiolactone
GSH	glutathione (reduced)
GSSG	glutathione (oxidized)
GSNO	S-nitrosoglutathione
NAP	N-acetyl-D-penicillamine
SNAP	S-nitroso- N-acetyl-D-penicillamine
SOD	superoxide dismutase
NOS	nitric oxide synthase

CHAPTER 1

INTRODUCTION

1.1 History of Nitric Oxide

Nitric oxide is a paramagnetic molecule which exists as a radical in its ground state. As a free radical, nitric oxide ($\cdot\text{N}=\text{O}$, abbreviated as NO) has interesting chemistry and has been the focus of many research laboratories in recent years; probably due to the realization that it could be synthesized by mammalian cells and act both as a physiological messenger and as a cytotoxic agent.¹ Although it was discovered by Joseph Priestley more than two centuries ago when he reacted nitric acid with metallic copper (R1.1), little was known about NO until its real biological importance was announced to the world in 1987.²⁻⁶

Since then, more than 20,000 articles have been published on the physiological roles of this small but highly important molecule. Nitric oxide is known to inhibit aggregation of platelets and their adhesion to vascular walls⁷ and is also involved in host defense by killing foreign invaders in the immune response.⁸ It acts as a messenger molecule; effecting muscle relaxation not only in the vasculature, but also in the gastrointestinal tract.⁹ It is able to function as a muscle relaxant because of its ability to increase guanosine 3',5'-cyclic monophosphate (cGMP) in smooth muscle tissue.^{10,11} NO has also been shown to act as a carcinogen,¹² a neurotransmitter in the

brain and peripheral nervous system¹³ and is involved in the regulation of gene expression and modification of sexual and aggressive behavior.¹⁴ The role of NO as an important biochemical mediator of penile erections¹⁴ stimulated a pharmaceutical company (Pfizer) in 1998 to start the production of Viagra, a popular drug whose mechanism of action is based on the nitric oxide-guanosine 3',5'-cyclic monophosphate (NO-cGMP) system, which revolutionized the management of erectile dysfunction.¹⁵ Also, a decrease in NO bioavailability has been implicated as a major factor responsible for so many diseases such as atherosclerosis, diabetes, hypertension, inflammation, migraine, meningitis, stroke, sickle cell disease and septic shock.¹⁵⁻¹⁹

In recognition of the many amazing physiological roles of nitric oxide and the rate at which the attention of many scientists shifted to its research, the popular journal, *Science*, proclaimed nitric oxide as the molecule of the year in 1992.²⁰ In 1998, three scientists were honored with the Nobel Prizes for their discovery of nitric oxide as a signaling molecule in the cardiovascular system.²¹ They are Robert Furchgott of the State University of New York, Louis Ignarro of the University of California at Los Angeles, and Ferid Murad at the University of Texas Medical School.²¹ These scientists were able to establish that the endothelium-derived relaxing factor (EDRF) is NO.^{2,4,22,23} The following year, Salvador Moncada, of the University College, London, who was generally believed to have contributed immensely to the field of nitric oxide research, was recognized as the most cited United Kingdom biomedical scientist of that decade.¹⁵

Nitric oxide is relatively unstable in biological systems due to its reaction with oxygen (O₂), superoxide anion (O₂^{•-}) and haem proteins.^{24,25} Nitrite (NO₂⁻) is the major end-product of its reaction with oxygen (R1.2) and nitrite can also release NO on acidification.^{26,27} Nitrites and even nitrates have been used for ages in the preservation of meat.¹ They have the advantages of killing the bacterium responsible for botulism and deepening the red color of meat.

1.2 Synthesis of Nitric Oxide in the Physiological Environment

It is now a well established fact that nitric oxide can be synthesis *in-vivo* via the oxidation of L-arginine to L-citrulline by the three isoforms of the enzyme, nitric oxide synthase (NOS).^{1,26,28} A clear understanding of the biosynthetic pathway of this inorganic free radical is very important, as it will enable us to decipher its numerous physiological roles.

The three major isoforms of nitric oxide synthase that have been identified, characterized, purified and cloned are neuronal NOS (nNOS or NOS-I), inducible NOS (iNOS or NOS-II) and endothelial NOS (eNOS or NOS-III).²⁹⁻³⁴ The location, sources and activation of these isoforms are outlined in **Table 1.1**. The first of these NOS to be identified and described was neuronal NOS. It was identified from rat cerebella and was fully characterized in 1989.³⁵ Apart from NOS, co-factors/co-enzymes that play significant roles in nitric oxide synthesis are molecular oxygen, the

reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH₄), calmodulin, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN).^{26,29,30,36}

Table 1.1: Description of the nitric oxide synthase isoforms

Isoform	Molecular Weight kDa	Intracellular Location	Human Cellular Sources	Presence	Activation
nNOs (NOS I)	161	Membrane associated	Neurons, adrenal medullary	Constitutive	Ca ²⁺ increase leading to calmodulin binding
iNOs (NOS II)	131	Cytosolic	Astrocytes Macrophages, monocytes, leukocytes,	Inducible	Transcriptional Induction, Ca ²⁺ independent, calmodulin always bound
eNOs (NOS III)	133	Membrane (inactive) Cytosolic (active)	Endothelium, platelets, smooth muscle	Constitutive	Ca ²⁺ increase leading to calmodulin binding

The synthesis of nitric oxide as presented in **Scheme 1.1** consists of a two-step process.^{36,37} The first step involves a two-electron NOS oxidation of the amino acid, L-arginine in the presence of molecular oxygen, NADPH and BH₄ to an intermediate species, N-hydroxy-L-arginine. The second step corresponds to a three-electron NOS oxidation of N-hydroxy-L-arginine to form L-citrulline and nitric oxide. The final organic product of these reactions, L-citrulline can be converted back to L-arginine as part of the urea cycle. This recycling takes place via a reaction of L-citrulline, adenosine triphosphate (ATP) and aspartate to form L-arginine and fumarate through

the intermediate L-arginosuccinate. It has been shown, through isotopic labeling of the terminal guanidine nitrogen atoms and molecular oxygen atoms, that the nitrogen atom as well as the oxygen atom in the synthesized nitric oxide was derived from these labelled positions respectively.²⁶

Scheme 1.1: Pathway for the biological production of nitric oxide (NO).

1.3 Peroxynitrite Formation: The Toxic Metabolite of Nitric Oxide

The toxicity effects observed with nitric oxide has been attributed to its diffusion-limited reaction with superoxide anion ($O_2^{\cdot-}$) to produce the powerful and deadly oxidant, peroxynitrite ion ($ONOO^-$).^{38,39} Superoxide anion is produced as one of the intermediates in the reduction of molecular oxygen to water (R1.3 – R1.6).

This reactive oxygen species, $O_2^{\cdot-}$, could be scavenged by superoxide dismutase (SOD) and thereby protect nitric oxide from forming peroxynitrite. Thus, there is competition between nitric oxide and superoxide dismutase for superoxide anion (Scheme 1.2).⁴⁰

Scheme 1.2: Reactions of superoxide anion.

Nitric oxide has been found to be the only biological species that can be produced in high enough concentrations (micro molar) to out-compete superoxide dismutase for superoxide anion.⁴¹ Reaction of $O_2^{\cdot -}$ and NO occurs at such a high rate ($6.7 \times 10^9 M^{-1} s^{-1}$) that nearly every collision results in irreversible formation of $ONOO^-$.^{41,42} The concentration of NO, like many free radicals, determines its roles (beneficial or toxic) in the physiological environment. It is known to perform most of its beneficial roles, such as vasorelaxation, at low concentrations on the order of 5 – 10 nM because at these concentration ranges it cannot effectively compete with superoxide dismutase whose concentrations are approximately on the order of 4 -10 μM .⁴² It plays a toxic role when its concentration rises to micro molar levels. There is need, therefore, for processes that can reduce its concentrations to harmless levels in the physiological environment. These processes include its reactions with red blood cells (hemoglobin),^{43,44} and physiological thiols.^{26,45,45,46} NO reacts with oxyhemoglobin and deoxyhemoglobin to produce nitrate plus methemoglobin and S-nitrosohemoglobin (SNOHb) respectively.^{43,47,48} Its reaction with thiols produces S-nitrosothiols. Formation of S-nitrosohemoglobin is believed to occur via transnitrosation involving a direct transfer of NO^+ from S-nitrosothiols to deoxyhemoglobin.^{26,43,48-50} Thus, hemoglobin can deliver NO to where it is needed for various physiological functions and in a way remove excess NO which may be responsible for the pathogenesis of many diseases such as Alzheimer's disease and Parkinson's disease.⁵¹ It is reasonable to assume that reversible incorporation of nitric oxide into compounds that can transport it from donor or site of production to target

cells can prevent or diminish nitric oxide toxicity. This further stresses the physiological importance of S-nitrosothiols, which can protect nitric oxide against oxidation and releases it during contact with target cells.

1.4 Biological Thiols

Thiols are organosulfur compounds with sulfhydryl functional group (-SH). A good number of them are relevant in the physiological environment, and the aim of this thesis is to elucidate the mechanistic basis for the activities of some of these biological thiols with respect to their reaction with nitric oxide. The most relevant thiols in biological chemistry are cysteine, homocysteine and glutathione. Glutathione is the most abundant low-molecular-mass non-protein thiol in mammals, with an adult human having about 30 g glutathione widely distributed in most tissues.^{52,53} The levels in the kidney and liver, however, are significantly higher.⁵⁴ In general, thiols are readily nitrosated in the presence of reactive nitrogen species including nitric oxide to form S-nitrosothiols.

Thiols are highly nucleophilic with a relatively electron-rich sulfur center. This property is fundamental to the critical role of thiols in protecting cells against oxidative and nitrosative damage as well as in DNA repair mechanisms (R1.8 – R1.12) and detoxification of even ordinary drugs such as in acetaminophen poisoning.⁵⁴⁻⁵⁷

Table 1.2: Therapeutic Indications of some selected thiols

Organosulfur Compound	Structure	Therapeutic Indications
Cysteine	$ \begin{array}{c} \text{H} \quad \text{O} \\ \quad \\ \text{H}_2\text{N}-\text{C}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{SH} \end{array} $	<p>Protection of cells from free radical damage.^{58,59}</p> <p>Detoxification of chemicals and heavy metals.⁶⁰</p> <p>To increase antioxidant glutathione⁵⁹</p>
N-acetylcysteine	$ \begin{array}{c} \text{O} \quad \text{H} \quad \text{H} \quad \text{O} \\ \quad \quad \quad \\ \text{H}_3\text{C}-\text{C}-\text{N}-\text{C}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{SH} \end{array} $	<p>Treatment of acetaminophen over dosage.^{61,62}</p> <p>Treatment of HIV infection^{63,64}</p> <p>To increase intracellular cysteine and glutathione levels.⁶¹</p>
Cysteamine	$ \begin{array}{c} \text{H} \\ \\ \text{H}_2\text{N}-\text{C}-\text{H} \\ \\ \text{CH}_2 \\ \\ \text{SH} \end{array} $	<p>Prevention of hypothyroidism and enhancement of growth in patients with nephropathic cystinosis.⁶⁵⁻⁶⁷</p> <p>Used in topical eye drops to dissolve corneal cystine crystals.⁶⁸</p>
Taurine	$ \begin{array}{c} \text{H} \\ \\ \text{H}_2\text{N}-\text{C}-\text{H} \\ \\ \text{CH}_2 \\ \\ \text{SO}_3\text{H} \end{array} $	<p>Treatment of congestive heart failure.^{69,70}</p> <p>Controlling of diabetes.^{71,72}</p> <p>Inhibition of platelet aggregation.^{71,73}</p>
Methionine	$ \begin{array}{c} \text{H} \quad \text{O} \\ \quad \\ \text{H}_2\text{N}-\text{C}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{CH}_2 \\ \\ \text{S}-\text{CH}_3 \end{array} $	<p>Treatment of acetaminophen poisoning.^{74,75}</p> <p>Ethanol detoxification.⁷⁶</p> <p>Prevention of fatty liver via transmethylation to form choline (lack of choline contributes to liver cirrhosis and impaired liver function).^{77,78}</p>

Conjugation of xenobiotics with nucleophilic thiols is a major pathway by which the body eliminates drugs.⁵⁷ A few selected examples of thiols that are being used in medicine as therapy to cure some specific human diseases are presented in **Table 1.2**.

A thorough examination of thiols shows that the most important parameter associated with their physiological role is the nature of the reactivity of the sulfur-center.⁷⁹ In this study, this thesis will therefore try to show that the reactivity at the S-center is extremely useful in predicting the effect of ingestion and/or inhalation of sulfur-based compounds, either intentionally as therapeutic drugs or unintentionally as xenobiotics such as second hand cigarette smoke.

1.5 S-Nitrosothiols

Nitric oxide should, just like most common radicals such as hydroxyl radical and superoxide anion ($\text{O}_2^{\cdot-}$), not be able to exist freely in the physiological environments. It is a short-lived free radical that reacts once it forms (half-life of 0.1 - 6 s).⁸⁰ For NO to fully perform its physiological roles, therefore, there is a need for endogenous carriers that can easily release it whenever it is needed. Metal- and thiol-containing proteins and low molecular weight thiols have been found to be the main target sites for nitric oxide within the cell.²⁶ Nitric oxide readily reacts with

physiological thiols to produce S-nitrosothiols. Examples of some synthetic and naturally occurring S-nitrosothiols are listed in **Figure 1.1**.

Figure 1.1: Examples of some synthetic and naturally occurring *S*-nitrosothiols.

S-Nitrosothiols, with the general structure, RSNO (where R is mainly an organic group), are an emerging class of both endogenous and exogenous nitric oxide donors. The existence of natural thiol-containing proteins and peptides such as cysteine,^{81,82} glutathione⁸³ and albumin,⁸³⁻⁸⁵ and their nitrosation led to the general belief that S-nitrosothiols are endogenous reservoirs, transport and delivery carriers for nitric oxide. It has been claimed that the bulk of nitric oxide circulates in blood plasma as S-nitroso form of serum albumin (SASNO), the S-nitroso derivative of serum albumin which is believed to be the most abundant S-nitrosated protein ($\sim 7.0 \mu\text{M}$).⁸⁶ The concentration of circulating nitrosothiols, however, may bear no relationship to intracellular concentrations that are likely to contribute more to physiological reactions.^{46,87-89} There is also evidence that seems to suggest that endogenously produced RSNOs are active intermediates responsible for the pharmacological effects of nitroglycerin and other nitro-vasodilators that release NO in the vasculature and platelets.⁹⁰⁻⁹²

1.5.1 Biological and Physiological Functions of RSNOs

RSNOs have been shown to perform the same physiological role as nitric oxide itself.^{49,93-95} For example, just like nitric oxide, N-acetyl-S-nitrosopenicillamine is known to inhibit platelet aggregation and can also act as a protective agent against intestinal damage induced by endotoxins.^{90,96} Platelets and neutrophils are both involved in the inflammatory process and their interaction might be of importance in vascular injury. They have been found to inhibit the adhesion of neutrophils to both

platelets and endothelium through regulation of adhesion molecule expression.⁹⁶ It has been reported that S-nitrosoglutathione (GSNO) and oxidized glutathione (GSSG) can stimulate L-arginine transport in human platelets, providing another potential mechanism by which RSNO could affect platelet aggregation.⁹⁷

RSNOs are responsible for vasodilation of arteries and veins and S-nitrosohemoglobin has been implicated in regulation of blood flow via the release of NO. Once formed, NO diffuses to the underlying smooth muscle cells, where it binds rapidly to the heme group of the enzyme guanylate cyclase which in turn stimulates the production of cyclic guanosine monophosphate (cGMP). This sets up a sequence of events eventually resulting in relaxation of the vessel.^{91,98,99} This mechanism of operation is supported by claims that even low molecular weight RSNOs such as S-nitrosocysteine (CySNO) are unlikely to permeate cell membranes readily because they exhibit negligible distribution from aqueous solution to non-polar phases at physiological pH.⁹¹ Cell-surface electron transport chains make this action possible through a one-electron reduction of RSNO to release NO to other thiol-containing species on the cell surface through transnitrosation and subsequently regeneration of the parent thiol.

RSNOs have been synthesized and administered clinically.¹⁰⁰ For example, GSNO is already being administered in clinical trials (at lower concentrations than those required for vasodilation) for use during coronary angioplastic operations where it can be used to reduce clotting.⁸⁸ GSNO has also been successful in treating the HELLP (hemolysis, elevated liver enzymes and low platelets) syndrome in pregnant

women.⁸⁷ The hypertension associated with this syndrome is believed to be associated with a deficiency in NO production. Clinical trials with other RSNOs are limited due to the rarity of the disorder. Irradiation of GSNO with visible light in the presence of leukemia cells has shown that it has cytotoxic effects to these cells thus phototherapy with GSNO is a potential treatment for leukemia.⁸⁷

RSNOs have also been shown to improve end-organ recovery in models of ischemia/reperfusion injury in the heart and liver.⁸⁷ Ischemia is a condition where the oxygen-rich blood-flow to a part of the body is restricted, and the surge of blood into tissues when this restriction is removed is what is termed reperfusion. Reperfusion of ischemic tissue leads to inflammatory responses and endothelial cell dysfunction through a sudden overproduction of reactive oxygen species such as hydrogen peroxide.⁹⁸ Cardiac ischemia occurs when an artery becomes narrowed or blocked for a short time, preventing oxygen-rich blood from reaching the heart. If ischemia is severe or lasts too long, it can cause a heart attack and can lead to heart tissue death. Hemoglobin has also been shown to exist in the S-nitrosated form and it is believed that S-nitrosation occurs either in the lungs from reaction with NO produced there or via transnitrosation^{46,88} (e.g. with GSNO). Due to the allosteric changes which accompany the oxygenation of hemoglobin, transnitrosation is very likely. The nitrosyl group in S-nitrosohemoglobin (HbSNO) may be reduced to NO under certain conditions, to induce relaxation of pre-capillary vessels and to inhibit platelet aggregation. Existing evidence suggests that S-nitrosation favors the formation of a high oxygen affinity state of hemoglobin, and therefore transnitrosation from HbSNO

to GSH is thermodynamically favored in the deoxygenated state which allows for GSNO formation in low-oxygen conditions.^{97,99,101} Transnitrosation between GSNO and the high molecular weight anion exchange protein then transduces the NO signal out of the red blood cell.

Like NO, RSNOs appear to have a role to play in host defense, affecting both viruses and bacteria. Levels of RSNOs in respiratory pathways are affected by diseases such as asthma and pneumonia and it is believed that RSNOs act as bronchodilators and relax bronchial smooth muscle and thus should have a role to play in the treatment of these diseases.⁸⁷ RSNOs, together with NO, have also been implicated in suppression of HIV-1 replication by S-nitrosylation of the cysteine residues in the HIV-1 protease.⁸⁷ Nitrosylation of another protease enzyme in the human rhinovirus that causes the common cold is also believed to be a possible way of using RSNOs to cure colds. Thus, RSNOs have a potential role in the treatment of asthma and as agents for treatment of infectious diseases ranging from the common cold to AIDS.

RSNOs have also been implicated in regulation of ion channels, and among several NO-sensitive channels, cyclic nucleotide-gated channel (CNG) is the only one that has been shown to directly open due to S-nitrosylation.¹⁰²⁻¹⁰⁴ Many such channels have oxidizable thiol groups that may play a role in the control of channel activity *in vivo* and their presence could suggest that regulation by S-nitrosothiols may occur.^{96,97}

1.5.2 Formation and Decomposition of S-Nitrosothiols

It has been suggested that the formation and degradation of S-nitrosothiols is a dynamic process that is highly influenced by the existing redox environments.¹⁰⁵ Therefore, in order to understand mechanism of formation and degradation of S-nitrosothiols, it is important to examine the basic chemistry of nitric oxide with respect to its reaction with molecular oxygen, the most abundant oxidant in the physiological environment. Reaction of nitric oxide with oxygen produces nitrites (R1.2) via a cascade of intermediate reactions (R1.11 – R1.15)^{26,27} These intermediate reactions produce reactive nitrogen species (RNS), which include NO₂, NO₂⁻, N₂O₄ and N₂O₃.²⁶ RNS play significant roles in the formation of S-nitrosothiols.

1.5.2.1 Endogenous Formation of S-Nitrosothiols

It is well established that S-nitrosothiols are readily formed in the physiological environment via the interaction of NO-derived metabolites with thiols.¹⁰⁶⁻¹⁰⁸ While there are thousands of articles and reviews on the importance of S-nitrosothiols as a signaling paradigm, their *in vivo* mechanisms of formation, which

are vital prerequisites for the proper understanding of their physiological roles, remain a topic of debate.

S-nitrosothiols can be formed from the reaction of nitrous acid (HNO₂) with thiols. Nitrous acid is a weak acid with pK_a value of 3.15 at 25 °C.²⁶ It is easily formed in acidic aqueous solution of nitrite ion. In excess nitrite, N₂O₃ is formed through a condensation reaction of two molecules of nitrous acid (R1.16) and in excess acid, HNO₂ is protonated to form H₂NO₂⁺ (R1.17), which can decompose to release nitrosonium ion, NO⁺ (R1.18).^{109,110}

HNO₂, N₂O₃, H₂NO₂⁺ and NO⁺ readily react with thiols to form S-nitrosothiols (R1.19 – R1.22)

Reactions between thiyl radicals (RS[•]) and [•]NO has also been shown to produce S-nitrosothiols in the physiological environment (R1.23).¹⁰⁹ In addition to these

mechanisms, S-nitrosation can also occur by reaction of a thiol with an already formed S-nitrosothiol. This process is called transnitrosation (R1.24), which often results in the modification of physiological thiols.¹¹¹

The biological importance of reaction R1.24 is that low molecular weight S-nitrosothiols are able to modify protein cysteinyl residues and also convert protein S-nitrosothiols back to the original protein thiols.^{112,113}

Experimentally, synthesis of S-nitrosothiols using acidified nitrite is only suitable for low molecular weight thiols such as glutathione and cysteine, but is unsuitable for proteins because of acid denaturation. Non-thiol functional groups in proteins such as amines, alcohols and aromatics are also susceptible to modification by acidified nitrite.⁹⁷ Spontaneous transfer of the nitroso group from a low molecular weight RSNO such as CySNO to a protein thiol is the most often used method for synthesis of protein S-nitrosothiols.¹¹⁴ When transnitrosation occurs from a stable thiol to an unstable one, release of nitric oxide is facilitated, more so in the presence of metal ions.¹¹⁵ Because of the vast excess of GSH over every other thiol in the cytoplasm, it is likely that the transfer of NO^+ will be effectively unidirectional from other S-nitrosothiols to glutathione.^{46,116-118} Ascorbate has also been shown to promote NO formation in blood plasma based on this reaction. Such transnitrosation reactions

provide possible pathways for the nitrosonium functional group to cross biological membranes and enter cells.¹¹⁷

1.5.1.2 Stability and decomposition of S-nitrosothiol

S-nitrosothiols, RSNOs, differ greatly in their stabilities, and most of them have never been isolated in solid form. Their degradation to release NO is believed to occur both enzymatically and nonenzymatically (**Scheme 1.3**).²⁷ Apart from NO, the disulfide of the corresponding parent thiol is also produced as one of the major products of RSNO decomposition (R1.25).

While nonenzymatic decomposition is known to be influenced by factors such as heat, UV light, certain metal ions, superoxide anion, ascorbic acid and thiols,¹¹⁹⁻¹²² enzymatic decomposition can be influenced by the enzymes glutathione peroxidase,¹²³ γ -glutamyl transpeptidase¹²⁴ and xanthine oxidase.¹²⁵ Seleno compounds mediated non-enzymatic degradation of RSNO is another important pathway that easily occurs in physiological environments.⁹⁶ Selenium is an essential mineral in mammals whose deficiency is closely associated with many heart diseases such as myocardial necrosis and atherosclerosis.⁹⁶ People with these disorders are given diets which have a higher amount of selenium thus making the selenium catalyzed decomposition important. It has been shown that glutathione peroxidase, an essential selenium containing

antioxidant enzyme, potentiated the inhibition of platelet function by RSNOs and also catalyzed the metabolism of GSNO to liberate NO in the presence of peroxide.¹¹⁶

Scheme 1.3: Mechanism of S-nitrosothiol decomposition

Homolytic cleavage

R1.26

Heterolytic cleavage

The mechanism of decomposition generally involves both homolytic (R1.26) and heterolytic (R1.27) cleavage of the sulfur-nitrogen bond of the RSNO. Homolysis of the S-N bond occurs both thermally and photochemically, but, generally, these processes are very slow at room temperatures and in the absence of radiation of the appropriate wavelength.

Many of the physiological roles of RSNO have been attributed to homolytic cleavage of the S-NO bond, with estimated bond dissociation energy of between 22 and 32 kcal/mol.¹²⁶ Decomposition via transnitrosation has been shown to occur by a heterolytic cleavage in which NO^+ group is transferred to another thiol.⁴⁶ This may eventually result in the release of NO if the resultant compound is more susceptible to decomposition than the parent S-nitrosothiol compound.⁴⁶

Catalytic effect of copper on the decomposition of S-nitrosothiols: Since human body contains 0.1 g copper per 75 kg body weight widely distributed in the blood, bone, and muscle, copper ion-catalyzed decomposition of RSNO has drawn the most attention.^{96,118} Presence of trace amounts of Cu(II) or Cu(I) can efficiently catalyze

RSNOs to disulfide and nitric oxide. The characterization of the S-N bond is crucial in determining the stability of RSNOs. Unfortunately, very little work has been done in this area. The little crystallographic data available on isolable RSNOs indicate that the S-N bond is weak, sterically unhindered, and elongated at between 0.17 – 0.189 nm¹²⁷ and exists as either the cis (syn) or trans (anti) conformers (R1.28).²⁶ These conformers are nearly isoenergetic but separated by a relatively high activation barrier of $\sim 11 \text{ kcal mol}^{-1}$.¹²⁸

CHAPTER 2

INSTRUMENTATION, MATERIALS AND METHODS

2.1 INTRODUCTION

The study of rates of chemical reactions is unique and quite different from other disciplines of science in that it differs from case to case depending on the particular combination of the relevant reactants. The uniqueness becomes well pronounced when dealing with non-linear reactions such as those observed with sulfur and its compounds.¹²⁹⁻¹³¹ Reaction dynamical studies involving sulfur and its compounds have been shown to involve intermediate reactions as well as other non-linear kinetics features such as autocatalysis, autoinhibition, free radical mechanisms, polymerizations, and a wide range of pH values over which such reactions are viable.¹³²⁻¹³⁵ For example, oxidation of cysteine, $\text{H}_3\text{N}^+\text{CH}(\text{COO}^-)\text{CH}_2\text{SH}$, an essential amino acid, by acidic bromate was shown to produce cysteic acid, $\text{H}_3\text{N}^+\text{CH}(\text{COO}^-)\text{CH}_2\text{SO}_3\text{H}$, as the most stable final product via cysteine sulfenic acid ($\text{H}_3\text{N}^+\text{CH}(\text{COO}^-)\text{CH}_2\text{SOH}$) and cysteine sulfinic acid ($\text{H}_3\text{N}^+\text{CH}(\text{COO}^-)\text{CH}_2\text{SO}_2\text{H}$) intermediates.¹³⁶ When the same sulfur compound (cysteine) was reacted with nitrous acid S-nitrosocysteine ($\text{H}_3\text{N}^+\text{CH}(\text{COO}^-)\text{CH}_2\text{SNO}$) was produced, which subsequently decomposed to form cystine, a disulfide of cysteine as the most stable final product.^{117,137} A better understanding of this kind of complex reaction behavior can only be possible after the kinetics of the individual elementary steps have been studied and the mechanisms of the relevant reactions have been characterized. This requires accurate determination of stoichiometry, identification of intermediate(s) and the final

reaction products. There is need, also, for the measurement of as many properties of the system as possible, such as concentration, pH and rate dependence. All these require a careful choice of analytical techniques and specialized instrumentation, guided by the speed of reaction under consideration.

The kinetics of a typical reaction can be monitored by among others, the following analytical techniques: nuclear magnetic resonance, electron paramagnetic resonance, ultraviolet and visible spectroscopy, chemical trapping, gas chromatography, and mass spectroscopy. For example, conventional methods like UV/Vis spectrophotometry are only adequate for monitoring kinetics of slow reactions with half-lives of at least several minutes. A fast reaction can be followed by stopped-flow techniques, which involve the fast flowing together of separate solutions of the reactants. This rapid mixing is usually coupled to a rapid-response method for monitoring the progress of reaction with a half-life of as low as 10^{-3} s.¹³⁸

2.2 INSTRUMENTATION

2.2.1 Conventional UV/Vis Spectrophotometry

Reactions with half-lives of at least several minutes were followed on a conventional Perkin-Elmer UV-VIS Lambda spectrophotometer featuring a double beam, all-reflecting system within the range of 190 nm to 1100 nm. For this instrument, the visible region uses a halogen lamp and the UV region uses deuterium lamp.¹³⁹

The spectrophotometer is interfaced to a Pentium III computer and uses the Perkin-Elmer UV WinLab software¹³⁹ for data acquisition, storage and analyses. Path length of the cuvette is 1 cm and a constant temperature of 25 °C is maintained during the duration of data acquisition by using a Neslab RTE-101 thermostated water bath.

2.2.2 Stopped-flow Spectrophotometry

Most of the processes in this study are too fast to be monitored by the conventional UV/Vis spectrophotometric techniques. Some of the reactions are completed within 1.0 s or less. For example, some reactions of cysteamine with nitrous acid started in less than 0.5 s and completed within 10 s. There is a need for an instrument that can combine rapid mixing with a rapid analytical response to monitor this type of reaction. One of such instruments that is capable of doing this is a stopped-flow spectrophotometer. A Hi-Tech Scientific SF61-DX2 double mixing stopped-flow spectrophotometer (**Scheme 2.1**) was used for all rapid kinetics reported for these studies. It consists of sample handling unit (**Scheme 2.2**), control unit, stepper support unit and data acquisition system. Small volumes of solutions are driven from high performance syringes through high efficiency mixer(s). The sample handling unit facilitates both the single mixing of two reagents, using only one of the drives, or double mixing of three reactants by a push-push mode of operation. Double mixing mode enables transient species formed by mixing reactants in A and B to be subsequently mixed with a third reactant, C, after a delay period. Reactant reservoir D is reserved for the buffer and this will push the premixed solutions A and B (from mixer 1) to a second mixer (mixer 2) where it reacts further with reactant C.

Figure 2.1: Schematic diagram showing all components of the SF-61DX2 Hi-Tech KinetAsyst stopped-flow spectrometer.

[Courtesy of Hi-Tech Scientific operator's Manual]

Figure 2.2: Sample Handling Unit (SHU) flow circuit diagram for the SF-61DX2 Hi-Tech KinetAsyst stopped-flow spectrometer.

[Courtesy of Hi-Tech Scientific operator's Manual]

The resultant mixture passes through a measurement flow cell and into a stopping syringe where the flow is stopped. Just prior to stopping, a steady state flow is achieved. The solution entering the flow cell is only milliseconds old. The age of this reaction mixture is also known as the dead time of the stopped-flow system. As the solution fills the stopping syringe, the plunger hits a block, causing the flow to be stopped instantaneously and triggering the observation cell optics. Using appropriate techniques, the kinetics of the reaction can be measured in the cell.

The radiation source is a 12 V, 50 W quartz tungsten halogen lamp. The instrument can also be operated with a 75 W light noise xenon arc lamp or a 100 W short arc Mercury lamp as radiation sources. The monochromator used is a Czerny-Turner type mounted on a rail. There are two photomultipliers, a PM-60e (an end-on $\frac{1}{2}$ inch Hamamatsu R1463) and a PM-60s (a side-on 1-1/8 inch Hamamatsu R928 HA). The passage of light from the monochromator to the photomultiplier is through a 600-micron pure silica optic fiber. The optical cell is made from fused UV silica. It measures 10 mm x 1.5 mm x 1.5 mm. The path length can be 10 mm or 1.5 mm depending on the position of the optic fiber.

2.2.3 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) is the most versatile and widely used type of elution chromatography. It is an excellent technique for the separation and determination of species in a variety of organic, inorganic and

biological materials. In HPLC, the mobile phase is a liquid solvent containing the sample as a mixture of solutes.

HPLC analysis in this study was performed on a Shimadzu Prominence HPLC system controlled by a web browser through a static IP address using the CBM-20A Prominence BUS Communication Module (Columbia, MD). The system consists of an LC-20AT Prominence LC (Kyoto, Japan) containing four LC-600 pumps, a DGU-20A5 Prominence degasser (Kyoto, Japan) and an SIL-10AD VP auto-injector (Columbia, MD). Detection was performed using an SPD-M10A Diode Array Detector (Columbia, MD) and an RF-10A XL fluorescence detector (Columbia, MD). HPLC grade solvents were filtered on 0.5 μm FHUP (organic) and 0.45 μm HATF (aqueous) Millipore isopore membrane filters (Bedford, MA) before they could be passed through the degasser. Injection volumes ranged from 10 μl to 100 μl and separations were carried out on a 5 μm particle size, 250 x 4.6 mm Supelco Discovery (Bellefonte, PA) C18 column (reverse phase, 100 Å pore size) at flow rates ranging from 0.5 ml/min to 1 ml/min. Solvents used were acetonitrile, methanol and water. 1 % trifluoroacetic acid (TFA), 1 % formic acid (FA) and other buffered solvents were incorporated whenever the need arose and either isocratic or gradient elutions were utilized. To curb the interaction of the protonated amines on the analytes with the silanol groups on the stationary phase (which was causing tailing of peaks) the sodium salt of 1-octanesulfonic acid (0.005 M) was incorporated into the aqueous mobile phase. This was sufficient to neutralize the protonated amines and produce good

resolution while eliminating peak tailing. Data were acquired and analyzed using the EZ-Start 7.3 Chromatography Software for Windows (Columbia, MD).

2.2.4 Mass Spectrometry (MS)

Most of the reactions being studied are new, and this makes product identification very important. In conjunction with HPLC, mass spectrometry analyses were used to identify the products of the reactions. The mass spectrometer used is a Micromass QTOF-II (Waters Corporation, Milford, MA) quadrupole time-of-flight mass spectrometer (qTOF MS). Analyte ions were generated by positive-mode electrospray ionization (ESI). Samples were dissolved in 50/50 acetonitrile/water and pumped through a narrow, stainless steel capillary (75 – 150 μm i.d.) at a flow rate of between 1 $\mu\text{L}/\text{min}$ and 1 mL/min . A voltage of 3 or 4 kV was applied to the tip of the capillary, which was situated within the ionisation source of the mass spectrometer. The strong electric field, aided by a co-axially introduced nebulizing gas (nitrogen) flowing around the outside of the capillary, dispersed the solution emerging from the tip into an aerosol of highly charged droplets. The source block was maintained at 80 $^{\circ}\text{C}$ with the desolvation gas (nitrogen) maintained at 150 $^{\circ}\text{C}$ and a flow rate of 400 L/h . The warm flowing nitrogen assisted in solvent evaporation and helped direct the spray towards mass spectrometer. The charged sample ions, free from solvent, passed through a sampling cone orifice into an intermediate vacuum region and from there through a small aperture into the analyser of the mass spectrometer which was held under high vacuum. The lens voltages were optimised individually for each sample.

The settings varied with each set of experiments. Tandem mass spectrometry (MSMS) data was generated by collision-induced dissociation (CID) with argon. Visualization and analysis of data was done using the Micromass MassLynx 4.0 software suite for Windows XP (Waters Corporation, Milford, MA).

2.2.5 Nuclear Magnetic Resonance Spectrometry

Nuclear Magnetic Resonance (NMR) is the study of the properties of molecules containing magnetic nuclei by applying a magnetic field and observing the frequency at which they come into resonance with an oscillating electromagnetic field.¹⁴⁰ In this study, Proton NMR (^1H NMR) was used. It is an important analytical tool that can be used to determine the number of distinct types of nuclei as well as obtain information about the nature of the immediate environment of each type of hydrogen nuclei in the organic products of the reactions. During oxidation of an organosulfur compound, the environment around the S-atom changes and the protons bonded to the adjacent carbon, nitrogen or oxygen atoms also change in terms of chemical shifts. This change in chemical shifts, together with deshielding effects make it possible to follow the progress of the oxidation of a sulfur center.

Proton NMR spectra were obtained using a Tecmag-modified Nicolet NM 500 spectrometer. The spectrometer was operated using a low power pre-saturation pulse at the water resonance position during the 2 s relaxation delay. A total of 8192 complex data points were collected for each spectrum, with a spectral width of ± 3000 Hz in quadrature for 16-128 transients, depending upon individual sample signal-to-

noise. To enhance the signal-to-noise ratio, the data points were typically treated with an exponential decaying function, leading to a 1 Hz line broadening before zero-filling twice, prior to applying a Fourier transformation. The data were processed using Swan-MR software. Spectra were referenced to 0 ppm via internal trimethylsilane (TMS) or via the water resonance (4.76 ppm at 298 K). Final visualization and analyses of the 2-D data sets were performed using NMR View or Swan-MR.

2.2.6 Electron Paramagnetic Resonance (EPR) Spectroscopy

EPR was discovered in 1944 by E.K. Zavoisky.¹⁴¹ It is a non-destructive analytical technique used for studying molecules with one or more unpaired electrons. In EPR spectroscopy the energy differences, ΔE , due to interaction of unpaired electrons in the sample with a magnetic field produced by a magnet are studied. Absorption occurs when ΔE equals the microwave energy, $h\nu$. Equation 2.1 and Figure 2.3 describe the general principle of EPR.

$$\Delta E = h\nu = g\beta H \quad 2.1$$

where h is Planck's constant, ν is microwave frequency, β is Bohr magneton (a constant related to electron charge and mass), H is magnetic field at which resonance occurs, g is a spectroscopic factor, which is a characteristic of a given paramagnetic center. The shape and width of the spectral line as well as the hyperfine (splitting) and area under absorption curve are other important parameters in EPR spectroscopy.

Figure 2.3: The general principle of EPR spectroscopy.

The area which is the double integral of the 1st derivation curve is proportional to the amount of a given paramagnetic center (spin) present in the resonator cavity of the EPR machine.¹⁴¹

For all EPR studies, a Bruker e-scan EPR spectrometer (X-band) was used for the detection of radicals and to confirm the release of NO upon the decomposition of RSNO (equation 2.2). Since direct detection of NO is difficult due to its broad EPR spectrum,¹⁴¹ EPR spin trapping technique was employed. EPR spin trapping is a process whereby a short-lived radical like NO is intercepted by a spin trap resulting in the formation of a radical-spin trap adduct which is stable enough to be detected and characterized by EPR spectroscopy. The intensity of the EPR signal is proportional to the amount of adduct formed. NO-specific spin trap, *aci*-nitroethane, was used for the detection of NO¹⁴². In alkaline environments, nitroethane ($\text{CH}_3\text{CH}_2\text{NO}_2$) forms *aci* anion (equation 2.3) which can effectively scavenge NO to form long-lived NO-adduct (equation 2.4).

The following settings were used for a typical run: microwave power, 19.91 mW; modulation amplitude, 1.41 G; receiver gain, 448; time constant, 10.24 ms; sweep width, 100 G and frequency, 9.78 GHz. All measurements were taken at room temperature.

2.3 MATERIALS

2.3.1 Sulfur Compounds: All the sulfur compounds (thiols, mostly) used in this work (**Table 2.1**) were used without further purification. Choice of these compounds was based on their known physiological effects.

2.3.2 Ionic Strength: Sodium perchlorate (from Fisher Scientific) was used to maintain an ionic strength of 1.0 M in all kinetic experiments.

2.3.3 Nitrosating Agent: The nitrosating agent of choice for the investigation of kinetics and mechanisms of nitrosation of the selected sulfur compounds in this work is nitrous acid (HNO_2), which is generated *in situ* during the kinetic experiments via a reaction of sodium nitrite and perchloric acid from Fisher Scientific.

Table 2.1: Organosulfur Compounds

Chemical Name	Acronym	Source
Cysteamine hydrochloride	CA	Sigma-Aldrich
Cystamine dihydrochloride	-	Sigma-Aldrich
L-Cysteine	CYSH	Sigma-Aldrich
L-Cystine	CYSSCY	Acros Organics
DL-Homocysteine	HCYSH	Acros Organics
Homocystine	HCYSSH CY	Acros Organics
Glutathione	GSH	Acros Organics
N-acetyl-D-penicillamine	NAP	Sigma-Aldrich

2.4 EXPERIMENTAL TECHNIQUES

2.4.1 Nitrosation Reactions: All nitrosation experiments were carried out at 25.0 ± 0.1 °C and at a constant ionic strength of 1.0 M (NaClO₄). The reaction system studied is essentially the HNO₂-organosulfur compound reaction. The progress of this reaction was monitored spectrophotometrically by following the absorbance of *S*-nitrosothiols (RSNOs) at their experimentally determined absorption peaks. Three vessels were used for the mixing of reactants. Sodium nitrite and sodium perchlorate were mixed in the first vessel, perchloric acid solutions in the second vessel and Organosulfur compound in the third vessel. Kinetics measurements were performed on the Hi-Tech

Scientific double-mixing SF61-DX2 stopped-flow spectrophotometer. Stock solutions of thiols and nitrite were prepared just before use.

2.4.2 Stoichiometry Determination: Stoichiometric determinations were performed by varying NO_2^- and keeping both thiols and acid constant and testing for the presence of any remaining NO_2^- as the nitrous acid (HNO_2).¹⁴³ The presence of NO_2^- was quantitatively analyzed (gravimetrically) as the orange chloride salt of *p*-nitroso-*N,N*-dimethylaniline that was formed when the solution was reacted with *N,N*-dimethylaniline. Reaction was guaranteed to proceed to completion by maintaining both acid and nitrite in excess over thiols. Due to the well-known decomposition of nitrosothiols, product reaction solutions were not allowed to stand for more than an hour before the addition of *N,N*-dimethylaniline.¹⁴⁴ The salt formed, however, was allowed to settle for 24 h before being filtered, dried, and weighed.

2.4.3 Test for Adventitious Metal Ion Catalysis: Water used for preparing reagent solutions was obtained from a Barnstead Sybron Corporation water purification unit capable of producing both distilled and deionized water (Nanopure). Inductively coupled plasma mass spectrometry (ICPMS) was utilized to quantify the concentrations of metal ions in this water. ICPMS analysis showed negligible concentrations of copper, iron, and silver (less than 0.1 ppb) and approximately 1.5 ppb of cadmium as the metal ion in highest concentrations. Control experiments were performed in distilled deionized water treated with the chelators EDTA or

deferroxamine to establish the possible catalytic effects of adventitious metal ions. The reaction dynamics were indistinguishable from those run in distilled deionised water.

2.4.4 Computer Simulation: The proposed step-by step mechanisms of the kinetic reactions in this study were computer simulated to check for plausability. These simulations are important as they allow detailed models to be developed and tested as data accumulate. They provide a means of evaluating various hypotheses for further experimental investigation. These include reaction mechanism, with appropriate rate constants and a set of parameter values describing the initial conditions which are similar to those used in the experiment. The models give concentrations of the chemical variables as a function of time, which can be compared to the experimental traces. Usually, guessed variables like rate constants are altered until the simulated traces are comparable to the experimental traces. Those rate constants derived from literature values are not altered.

Kintecus software developed by James C. Ianni was used for carrying out simulations in this study.¹⁴⁵ It is a reliable compiler used to model the reactions of chemical, biological, nuclear and atmospheric processes using three input data files: a reaction spreadsheet, a species description spreadsheet and a parameter description file. In addition, one can fit/optimize almost any numerical value (rate constants, initial concentrations, starting temperature, etc.) against an experimental dataset. Normalized sensitivity coefficients are used in accurate mechanism reduction, determining which

reactions are the main sources and sinks (network analysis) and also shows which reactions require accurate rate constants and which ones can have essentially guessed rate constants. Kintecus software is a user friendly package requiring no programming or compiling skills by the user.

CHAPTER 3

KINETICS AND MECHANISMS OF NITROSATION OF CYSTEAMINE

3.0 INTRODUCTION

Cysteamine (2-aminoethanethiol) is an important biological molecule, consisting of a sulfhydryl group and an amino functional group.

cysteamine

Medically known as Cystagon, it can be used as oral therapy for prevention of hypothyroidism and enhances growth in patients with nephropathic cystinosis.^{67,146} This is a lysosomal storage disorder which was long considered primarily a renal disease, but is now recognized as systemic disorder which eventually affects many organ systems in children.¹⁴⁷ This disorder is characterized by cystine accumulation within cellular lysosomes, and cysteamine converts this disulfide into cysteine and a mixed disulfide, cysteamine-cysteine, which can easily be eliminated from the cystinotic lysosomes, thus effecting depletion of cellular cystine.¹⁴⁸ Lysosomes are a major intracellular site for the degradation of a wide variety of macromolecules including proteins, nucleic acids, complex carbohydrates, and lipids. Cysteamine and its disulfide, cystamine, can also be used in topical eye drops to dissolve corneal cystine crystals.^{66,68}

In addition to protection against radical damage in DNA, cysteamine can also act as a repair agent for DNA through the formation of the protective $RSSR^{\cdot-}$ which

then reacts with the DNA^{•+} radical ion to regenerate DNA and form cystamine, the cystamine disulfide.⁵⁴⁻⁵⁶ The ability of cystamine to reversibly form disulfide links with the sulfhydryl groups at or near the active sites of enzymes is also important in regulation of several essential metabolic pathways.^{149,150}

Figure 3.1: Structure of Coenzyme A showing the cysteamine residue.

Cysteamine is known to be produced *in vivo* by degradation of coenzyme A, where it forms its terminal region (Figure 3.1). Coenzyme A is a cofactor in many enzymatic pathways, which include fatty acid oxidation, heme synthesis, synthesis of the neurotransmitter acetylcholine and amino acid catabolism.¹⁵¹ Recently, a second mammalian thiol dioxygenase was discovered to be cysteamine dioxygenase.¹⁵² The first thiol dioxygenase to be discovered was cysteine dioxygenase.¹⁵²⁻¹⁵⁴ The discovery of this new enzyme, cysteamine dioxygenase, further implicates cysteamine as being

as important as cysteine and glutathione in the physiological environment. Thus, knowledge of the kinetics and mechanisms of the metabolism of cysteamine is paramount to understanding its physiological importance. Although many studies have been conducted on metabolism of this thiol and its metabolites with respect to oxidation by oxyhalogen species,¹⁵⁵⁻¹⁵⁷ nothing in-depth has been done on the elucidation of its mechanism of nitrosation to form S-nitrosocysteamine. In this thesis results are presented on the kinetics and mechanism of formation and decomposition of S-nitrosocysteamine.

3.1 RESULTS

The progress of the nitrosation reaction was monitored spectrophotometrically by following the absorbance of S-nitrosocysteamine (CANO) at its experimentally determined absorption peak of 545 nm where an absorptivity coefficient of $16.0 \pm 0.1 \text{ M}^{-1}\text{cm}^{-1}$ was evaluated. Although CANO has a second absorption peak at 333 nm with a much higher absorptivity coefficient of $536.0 \text{ M}^{-1}\text{cm}^{-1}$, its maximum absorption at 545 nm was used due to lack of interference from the absorption peaks of nitrogen species (NO_2^- , NO^+ and HNO_2) (see Figure 3.2).

The major reaction studied was a simple nitrosation of cysteamine through *in situ* production of nitrous acid as the nitrosating agent. The yield of CANO varied, based on order of mixing reagents (see Figure 3.3). The highest yield, which gave quantitative formation of CANO, was obtained by pre-mixing nitrite with acid and incubating the solution for approximately 1 s before subsequently reacting this nitrous

acid with cysteamine in a third reagent stream of the double-mixing stopped-flow spectrophotometer. The value of the absorptivity coefficient of CANO adopted for this study of $16.0 \text{ M}^{-1} \text{ cm}^{-1}$ was derived from this mixing order. Pre-mixing CA with acid before addition of nitrite gave a lower yield of CANO (trace (c)). The available protons are reduced by the protonation of CA:

Figure 3.2: UV/Vis spectral scan of (a) pure CA (0.01 M), (b) nitrite (0.001M), (c) nitrous acid (0.001 M) and (d) CANO (0.001 M).

Figure 3.3: *Effect of order of mixing reactants (a) addition of 0.1 M CA to a mixture of 0.005 M NO_2^- and 0.1 M H^+ (b) addition of 0.1 M H^+ to a mixture of 0.005 M NO_2^- and 0.1 M CA, and (c) addition of 0.005 M NO_2^- to a mixture of 0.1 M H^+ and 0.1 M CA.*

This subsequently reduces the amount of immediately available nitrosating agent, HNO_2 . The depression in CANO formed is exaggerated, as is the case in Figure 3.3 when CA concentrations exceed acid concentrations. In overwhelming excess of acid over CA, the yields are nearly identical for both mixing formats. Most of these reactions were run at pH conditions below 3.0; and at these conditions, it is assumed that the CA exists in the protonated form at the nitrogen center. The second

protonation on the thiol center is more relevant at even lower pH's. Protolytic reactions, in general, are very rapid and essentially diffusion-controlled. Retardation of nitrosation and decrease in product for trace (c) is derived from the decrease in protons as dictated, mathematically, by the equilibrium constant of reaction R3.1. On the other hand, nitrous acid is a weak acid, and initial addition of acid to nitrite ensures that protonated nitrite, the nitrosation species, remains in overwhelming majority over unprotonated nitrite.

3.1.1 Stoichiometry

The stoichiometry of the reaction involves the reaction of 1 mol of cysteamine (CA) with 1 mol of nitrous acid to produce 1 mol of S-nitrosocysteamine (CANO) accompanied by the elimination of a water molecule and with the reaction occurring solely at the thiol center (R3.2).

The stoichiometry was derived by mixing excess solutions of perchloric acid and nitrite with a fixed amount of cysteamine such that cysteamine was the limiting reagent. The reaction was allowed to proceed to completion while rapidly scanning the reacting solution between 200 nm and 700 nm. The maximum absorbances of the resulting CANO at 333 nm and 545 nm were noted and concentration of CANO was deduced from its maximum absorption at λ_{max} of 545 nm where there was no

possibility of interference from the absorption peaks of nitrogen species (NO_2^- , NO^+ and HNO_2) (Figure 3.2).

3.1.2 Product Identification

Product identification and verification was achieved by complementary techniques: UV/vis spectrophotometry, HPLC, quadrupole time-of-flight mass spectrometry and EPR spectrometry. CANO was identified by its distinct spectral absorption peak at 333 nm with extinction coefficient of $536 \text{ M}^{-1}\text{cm}^{-1}$ and by another peak at 545 nm with a smaller extinction coefficient of $16.0 \text{ M}^{-1}\text{cm}^{-1}$. It has a pink color, which is consistent with the characteristic of primary S-nitrosothiols.¹⁵⁸ ^1H NMR analysis could not be used in the identification of this S-nitrosothiol as spectra of CA and CANO were very similar, showing that the carbon skeleton of CA was not disturbed by the nitrosation.

Time-of-flight mass spectrometry was used to prove that identity of product of nitrosation was CANO and that of decomposition of CANO to be cystamine, a disulfide of cysteamine. Scheme 3.1 shows all possible products of reaction. A resolvable mass spectrum is taken at low acid concentrations, and this generally will show both the reagent, CA, and the single product, CANO. This mass spectrum is shown in Figure 3.4a. There are no other peaks that can be discerned from the reaction mixture, apart from those belonging to the reagent and the product.

Scheme 3.1 Possible reactions of nitrous acid with cysteamine

Incubation of the product of nitrosation overnight shows a final conversion of the CANO into the disulfide, cystamine (Figure 3.4b), $\text{H}_2\text{NCH}_2\text{CH}_2\text{S-SCH}_2\text{CH}_2\text{NH}_2$.

The most important deduction from these spectra is the absence of any product from possible reaction of the primary amine in CA with nitrous acid (Scheme 3.1). This is a well known reaction in which nitrous acid reacts with a primary amine to yield alcohols, alkenes and nitrogen via the formation of diazonium salt intermediate.^{159,160} The reaction conditions as well as high nucleophilicity of S-center may probably be unfavorable for the formation of diazonium ion.

Figure 3.4: Mass spectrometry analysis of the product of nitrosation of CA showing (A) formation of CANO, $m/z = 107.9999$. (B) Product of decomposition of CANO after 24 hours. Cystamine, a disulfide of CA was produced with a strong peak, $m/z = 153.0608$

EPR Analysis: Nitric oxide can be trapped and observed by EPR techniques using a nitroethane trap¹⁴². The aci-anion of this trap, generated in basic environments, generates a long-lived spin adduct with NO, which is detectable and quantifiable by its distinct ESR spectrum pattern and g-value:

The adduct $[\text{CH}_3\text{C}(\text{NO})(\text{NO}_2)]^{\cdot-2}$ is EPR-active. No peaks were obtained in the EPR spectrum when the nitroethane trap was utilized during the formation of CANO,

indicating that NO is not released during the S-nitrosation and that it is not a major nitrosation agent in this mechanism that involves *in situ* production of HNO₂. During the decomposition of CANO, however, a strong EPR spectrum indicating the presence of NO is obtained, proving stoichiometry R3.3. Figure 3.5 shows a series of EPR spectra that proves presence of NO in the decomposition of CANO. The first three spectra show that, on their own, nitroethane, CA, and NO₂⁻ cannot generate any active peaks that can be attributed to NO.

Figure 3.5: EPR spectra of NO radical generated during the decomposition of CANO using 0.5 M nitroethane (NE) in 1.0 M NaOH solution as spin trap. (A) 0.5 M NE (B) 0.01 M NO₂⁻ (C) 0.04 M CA; [CANO] = (D) 0.01 M (E) 0.02 M (F) 0.03 M and (G) 0.04 M.

In overwhelming excess of trap, the heights of the spectral peaks can be used, after a standardization, as a quantitative measure of the available NO. NO has a very poor solubility in water, and so the technique can be limited to only low NO concentrations.

3.1.3 Oxygen Effects

Figure 3.6 shows two nitrosation reactions at equivalent conditions except one was run in solutions that had been degassed with argon and immediately capped.

Figure 3.6: *Effect of oxygen on the nitrosation of CA: 0.005 M CA + 0.07 M NO₂⁻ + 0.08 M H⁺ ((a) Bubbled with argon (oxygen absent) and (b) Oxygen present). Neither initial rates nor the final amounts of CANO formed are altered by the absence or presence of oxygen.*

Argon's heavier mass (over nitrogen), ensures that it can cover the top of the reagent solutions, thereby ensuring that no further oxygen can enter into the reaction solution. Similar results were obtained for these reactions. This might be expected when nitrosation was not carried out via a direct reaction of nitric oxide and thiol, in which oxygen reacts with nitric oxide to form nitrosating species (N_2O_3 and N_2O_4).^{161,162} These results agreed with what was obtained by Stubauer and co-workers in their studies on nitrosation of bovin serum albumin (BSA) where yield of the S-nitrosothiol formed was the same in the absence and presence of oxygen.¹⁶²

3.1.4 Reaction Kinetics

The reaction has a simple dependence on cysteamine concentrations (see Figures 3.7a and b). There is a linear first order dependence on the rate of nitrosation with CA concentrations with an intercept kinetically indistinguishable from zero, as would have been expected. The reaction kinetics are more reproducible in the format utilized in Figure 3.7 in which concentrations of acid and nitrite are equal and in overwhelming excess over CA concentrations. Effect of nitrite is also simple and first order (see Figures 3.8a and b) when nitrite concentrations do not exceed the initial acid concentrations such that effectively all N(III) species in the reaction environment are in the form of protonated HNO_2 . As expected, acid effects are much more complex and are dependent on the ratio of initial acid to nitrite concentrations. The nitrosation's response to acid differs, depending on whether initial proton concentrations exceed initial nitrite concentrations.

Figure 3.7a: Absorbance traces showing the effect of varying CA concentrations. The reaction shows first-order kinetics in CA. $[\text{NO}_2^-]_0 = 0.10 \text{ M}$; $[\text{H}^+]_0 = 0.10 \text{ M}$; $[\text{CA}]_0 =$ (a) 0.005 M , (b) 0.006 M , (c) 0.007 M , (d) 0.008 M , (e) 0.009 M , and (f) 0.010 M .

Figure 3.7b: *Initial rate plot of the data in Figure 3.7a. The plot shows the strong first –order dependence of the rate of formation of CANO on CA.*

The most important parameter affecting rate of nitrosation is the concentration of HNO_2 . The kinetics traces shown in Figure 3.9a encompass a proton concentration variation that ranges from concentrations that are lower than nitrite to those that exceed. A simple plot of added acid to the rate of nitrosation shown in Figure 3.9b clearly displays this discontinuity at the point where acid exceed nitrite concentrations. By utilizing the dissociation constant of HNO_2 , the final concentrations of H_3O^+ , NO_2^- , and HNO_2 can be calculated for each set of initial concentrations. These data are shown in Table 3.1 for the input concentrations used for Figure 3.9c. Close examination of the data in Table 3.1 shows that the kinetics of reaction are vastly different depending on whether initial acid concentrations exceed initial nitrite

concentrations. When nitrite is in excess (the first four readings), there is a sharp nonlinear increase in rate of nitrosation with nitrous acid concentrations (Figure 3.9c), and a saturation in rate with respect to the actual proton concentrations despite the recalculation of the concentrations undertaken in Table 3.1. Figure 3.9c would strongly indicate second order kinetics in nitrous acid, and a plot of nitrosation rate vs nitrous acid to the second power is linear (Figure 3.9c insert). Table 3.1 shows that, in the series of data plotted for Figure 3.9c, nitrous acid concentrations and excess proton concentrations increase while nitrite concentrations decrease; and the final observed on rate of nitrosation is derived from these three species concentration changes and not isolated to nitrous acid. At acid concentrations that exceed initial nitrite concentrations, there is a linear dependence in rate on acid concentrations (Figure 3.9d), but a saturation would have been achieved in nitrous acid concentrations as would be expected in this format in which the nitrous acid saturates at 50 mM. The increase in nitrosation rate with increase in acid after saturation of HNO_2 indicates that other nitrosants exist in the reaction environment apart from HNO_2 .

Figure 3.8a: Absorbance traces showing the effect of varying nitrite concentrations. The reaction shows first-order kinetics in nitrite. $[CA]_0 = 0.10\text{ M}$; $[H^+]_0 = 0.10\text{ M}$; $[NO_2]_0 =$ (a) 0.005 M , (b) 0.006 M , (c) 0.007 M , (d) 0.008 M , (e) 0.009 M , and (f) 0.010 M .

Figure 3.8b: Initial rate plot of the data in Figure 3.8a. The plot shows strong dependence on initial rate of formation of CANO on nitrite.

Figure 3.9a: Absorbance traces showing the effect of varying acid concentrations. $[CA]_0 = 0.05\text{ M}$; $[NO_2^-]_0 = 0.05\text{ M}$; $[H^+]_0 =$ (a) 0.01 M , (b) 0.02 M , (c) 0.04 M , (d) 0.05 M , (e) 0.06 M , and (f) 0.07 M .

Figure 3.9b: Plot of the raw kinetics data in Figure 3.9a for the effect of added acid on the rate of nitrosation. Acid concentrations plotted here are those experimentally added, and not calculated.

Figure 3.9c: Plot of initial rate versus nitrous acid concentrations of the experimental data in Figure 3.9a and calculated in Table 3.1. This plot involves the region at which initial nitrite concentrations exceed acid concentrations.

Table 3.1: Input species concentrations and final reagent concentrations for acid dependence experiments. (units of M)

$[\text{H}^+]_{\text{added}}$	$[\text{NO}_2^-]_{\text{added}}$	$[\text{HNO}_2]_{\text{initial}}$	$[\text{NO}_2^-]_{\text{final}}$	$[\text{HNO}_2]_{\text{final}}$	$[\text{H}_3\text{O}^+]_{\text{final}}$
0.01	0.05	0.01	4.01×10^{-2}	9.86×10^{-3}	1.38×10^{-4}
0.02	0.05	0.02	3.04×10^{-2}	1.97×10^{-2}	3.63×10^{-4}
0.03	0.05	0.03	2.08×10^{-2}	2.92×10^{-2}	7.90×10^{-4}
0.04	0.05	0.04	1.18×10^{-2}	3.82×10^{-2}	1.82×10^{-3}
0.05	0.05	0.05	5.03×10^{-3}	4.50×10^{-2}	5.03×10^{-3}
0.06	0.05	0.05	2.22×10^{-3}	4.78×10^{-2}	1.22×10^{-2}
0.07	0.05	0.05	1.29×10^{-3}	4.78×10^{-2}	2.13×10^{-2}
0.08	0.05	0.05	8.93×10^{-4}	4.91×10^{-2}	3.09×10^{-2}

Figure 3.9d: Plot showing the linear dependence of rate of formation of CANO on $[H_3O^+]$ in high acid concentrations where initial acid concentrations exceed nitrite concentrations. Nitrous acid concentrations are nearly constant in this range.

Catalytic Effect of Copper

Copper is a trace element that is highly essential for life by being a cofactor for the function of many enzymes such as cytochrome *c* oxidase, dopamine monooxygenase and the enzyme involved in the removal of reactive oxygen species (superoxide dismutase).^{163,164} Depending on the redox environment, copper can cycle between two states, reduced Cu^+ and oxidized Cu^{2+} , allowing it to play its numerous physiological roles.¹⁶⁵ It has been implicated in many reactions involving organosulfur compounds. $Cu(I)$ is known to be much more active than $Cu(II)$. It is

easier to assay for Cu(II) and addition of Cu(II) to organosulfur reactions involves its initial conversion to the more active Cu(I).^{166,167} Figure 3.10a shows that addition of Cu(II) rapidly increases the nitrosation reaction. Cu(II) ions, however, also catalyze the decomposition of CANO. A plot of rate of nitrosation versus Cu(II) concentrations shows the sigmoidal rate increase followed by an abrupt saturation, which is typical of catalytic mechanisms (Figure 3.10b). No nitrosation occurs in the presence of nitrite without acid (which is necessary to generate the HNO₂). Figure 3.10c shows that, even in the absence of acid, Cu(II) ions can still effect the nitrosation of CA, even though this nitrosation is slow. Cu(I) ions, however, could not effect nitrosation in the absence of acid. Figure 3.10d shows the catalytic effect of Cu(II) ions on the decomposition of CANO. CANO was prepared *in situ* from two feed streams of the stopped flow ensemble. Cu²⁺ was next added from a third feed stream (buffered at pH 7.4) after complete production of CANO. The two control experiments (traces a and b) show the slow decomposition expected from CANO solutions at pH 7.4. Trace b is treated with EDTA to sequester all metal ions in the reaction solution. This trace is identical to the one without EDTA confirming the absence of metal ions in reagent solution which can affect the decomposition kinetics. Addition of micromolar quantities of Cu(II) ions effects a big acceleration in rate of decomposition. Higher Cu(II) concentrations gave a strong autocatalytic decay of the nitrosothiol (trace f in Figure 3.10d).

The catalytic effect of Cu(II) ions on the decomposition can also be evaluated through the rate of production of nitric oxide which is known to be the major product,

together with the disulfide, of nitrosothiol decompositions. Figure 3.10e shows EPR spectra using the nitroethane trap, of the enhanced production of NO with addition of Cu(II) ions. All three spectra were taken after the same time lapse from CANO incubation, and thus the peak heights of the NO-induced spectra can be correlated with extent of reaction.

Figure 3.10a: Absorbance traces showing the effect of varying Cu^{2+} concentrations on the rate of formation of CANO. There is a progressive increase in the rate of CANO formation with increase in Cu^{2+} concentration. $[\text{CA}]_0 = [\text{NO}_2^-]_0 = [\text{H}^+]_0 = 0.05 \text{ M}$; $[\text{Cu}^{2+}]_0 =$ (a) 0.00 M , (b) $5 \mu\text{M}$, (c) $15 \mu\text{M}$, (d) $100 \mu\text{M}$, (e) 1 mM , and (f) 2.5 mM .

Figure 3.10b: A plot of initial rate of nitrosation of CA versus Cu(II) concentrations showing sigmoidal increase in the rate of formation of CANO with increase in the initial concentrations of Cu(II).

Figure 3.10c: Absorbance traces showing the formation of CANO via a reaction of CA, nitrite and copper (II) without acid. The amount of CANO formed increases with increase in Cu^{2+} concentrations. No formation of CANO was observed with Cu^+ . The result shows catalytic effect of Cu^{2+} on the rate of formation of RSNO. $[\text{CA}]_0 = [\text{NO}_2^-]_0 = [\text{H}^+]_0 = 0.05 \text{ M}$; $[\text{Cu}^{2+}] =$ (a) 0.0025 M , (b) 0.0030 M , (c) 0.0035 M , (d) 0.0040 M , (e) 0.0045 M , and (f) 0.0050 M .

Figure 3.10d: Effect of Cu^{2+} on the stability of CANO in a pH 7.4 phosphate buffer. This reaction involves the reduction of Cu^{2+} to Cu^+ , which causes the decomposition of CANO. $[\text{CANO}]_0 = 0.028 \text{ M}$; $[\text{Cu}^{2+}]_0 =$ (a) 0.00 M (pure CANO), (b) 0.00 M (pure CANO in 0.00001 M EDTA), (c) $5 \mu\text{M}$, (d) $10 \mu\text{M}$, (e) $20 \mu\text{M}$, and (f) $30 \mu\text{M}$.

Figure 3.10e: EPR spectra of NO radical showing the catalytic effect of Cu^{2+} on the rate of decomposition of CANO. The intensity of the spectra increases with increase in Cu^{2+} concentration. $[\text{CANO}]_0 = 0.01 \text{ M}$, $[\text{Cu}^{2+}]_0 = (a) 0.00$ (b) $1.0 \times 10^{-4} \text{ M}$ (c) $2.0 \times 10^{-4} \text{ M}$.

Transnitrosation

The most abundant thiol in the physiological environment is glutathione, GSH. A pertinent reaction to study would be the interaction of any nitrosothiol formed in the physiological environment with glutathione. This is due to GSH's sheer abundance which overwhelms the other thiols. A crucial question is the lifetimes of the nitrosothiols formed. If thiols are carriers of NO from point of production to point of its usage, the nitrosothiols formed should be stable enough and have a long enough half-life to enable them to perform this activity.

Figure 3.11a: Superimposed spectra of four well known nitrosothiols (a) CysNO, (b) CANO, (c) GSNO and (d) SNAP (nitrosothiol of penicillamine). Three of them nearly have the same ϵ at 545 nm. The observed separation in the absorbances of these three at 545 nm is due to staggering the time lag before acquiring the spectrum.

Spectrophotometric study of transnitrosation is rendered complex by the fact that many nitrosothiols seem to absorb at the same wavelength; with similar absorptivity coefficients. Figure 3.11a shows a series of superimposed spectra of four well-known nitrosothiols which show nearly identical spectra. Figure 3.11b shows the

spectrophotometric characterization of possible transnitrosation in mixtures of CANO and glutathione, GSH. GSNO has a higher absorptivity coefficient than CANO at the same wavelength, and this explains the initial increase in absorbance on addition of GSH to CANO. LC-MS data have shown that the reaction mixture can contain any of at least 6 components at varying times and concentrations: CANO, GSNO, GSH, CA-CA (cystamine), GSSG (oxidized glutathione) and CA-GS (mixed disulfide). The rate of transnitrosation will be difficult to evaluate due to the presence of the mixed disulfide. Figure 3.11b shows that, relatively, CANO is more stable than GSNO due to the rapid rate of consumption of GSNO upon its formation as compared to the much slower CANO decomposition with or without EDTA. Figure 3.11c shows a GC-MS spectrum of mixtures of CANO and GSH. This involved a 3:5 ratio of CANO to GSH, which is also trace f in Figure 3.11b. The remarkable aspect of this spectrum is the absence of the oxidized glutathione, GSSG, whose precursor is GSNO. Thus GSNO, when formed, rapidly decomposes by reacting with CA to form the mixed disulfide and releasing NO. The cystamine formed and observed in the spectrum is derived from the normal decomposition of CANO (R3.5).

In order to demonstrate more clearly the transnitrosation reactions between CA and other RSNOs, S-nitroso- N-acetyl-D-penicillamine (SNAP) was employed because it absorbs at different wavelength (590 nm) from CANO, which absorbs at 545 nm.

Quantitative formation of CANO was obtained within 1 min in the reaction involving excess CA and SNAP. In the visible regions of the spectrum the reaction mixture showed decomposition of SNAP at 590 nm and simultaneously formation of CANO at 545 nm.

Figure 3.11b: The effect of glutathione (GSH) on the stability of CANO in a pH 7.4 phosphate buffer. All experimental traces have $[CA]_0 = 0.03\text{ M}$, $[NO_2^-]_0 = 0.03\text{ M}$, and $[H^+]_0 = 0.05\text{ M}$. $[GSH] = (a) 0$, $(b) 0$, $(c) 0.01\text{ M}$ $(d) 0.02\text{ M}$ $(e) 0.03\text{ M}$ and $(f) 0.05\text{ M}$. Trace a and b are the control. Trace a has no EDTA. Trace b is the same as trace a with $10\text{ }\mu\text{M}$. EDTA. No significant difference between traces a and b, indicating that the water used for preparing reagent solutions did not contain enough trace metal ions to affect the kinetics. $10\text{ }\mu\text{M}$. EDTA is added to reactions in traces c, d, e and f.

Figure 3.12a shows visible multiple spectra scan taken at every 1.0 seconds. The effect of CA on the transnitrosation reaction is shown in Figure 3.12b.

Figure 3.11c: A LC-MS spectrum of a 3:5 ratio of CANO to GSH; trace f in Figure 3.11b. Final products were predominantly the mixed disulfide and cystamine with no evidence of GSSG.

The amount of CANO produced is proportional to the amount of SNAP decomposed (R3.6 - R3.8).

Plots of initial rate of formation of CANO at 545 nm (Figure 3.12ci) and decomposition of SNAP at 590 nm (Figure 3.12cii) gave linear plots indicating first-order dependence on the initial concentrations of CA. In another series of studies, the effect of varying SNAP on its reaction with CA to form CANO was investigated. As expected, the rate of transnitrosation increases with increase in the initial concentrations of SNAP (Figure 3.12d). First-order kinetics was obtained upon plotting the initial rate of formation of CANO versus the initial concentrations of SNAP (Figure 3.12e).

A further confirmatory evidence for the transnitrosation was achieved by electrospray ionization mass spectrometry (ESI-MS) technique. Figure 3.12f shows that the product of the transnitrosation contains a mixture of components including CANO, N-acetyl-D-penicillamine (NAP) disulfide, and mixed disulfide of CA and NAP.

Figure 3.12a: Repetitive spectral scan of the reaction of SNAP with excess cysteamine (CA) at pH 7.4 showing consumption of SNAP at 590 nm and formation CANO at 545 nm. $[CA] = 0.025\text{ M}$; $[SNAP] = 0.0025\text{ M}$.

Figure 3.12b: Absorbance traces showing the effect of varying CA concentrations on its transnitrosation by SNAP. The plot shows both formation of CANO at 545 nm (dotted lines) and decomposition of SNAP at 590 nm (solid lines). $[SNAP]_0 = 0.0049$ M; $[CA]_0 =$ (a) 0.005 M, (b) 0.0075 M, (c) 0.01 M, (d) 0.02 M, (e) 0.03 M, (f) 0.04 M and (g) 0.05 M.

Figure 3.12ci: Initial rate plot of the data in Figure 3.12b. The plot shows linear dependence on initial rate of formation of CANO on CA in the transnitrosation of CA by SNAP.

Figure 3.12cii: Initial rate plot of the data in Figure 3.12b. The plot shows linear dependence on initial rate of decomposition of SNAP on CA in the transnitrosation of CA by SNAP.

Figure 3.12d: Absorbance traces showing the effect of varying SNAP concentrations on its transnitrosation of CA. The plot shows both formation of CANO at 545 nm (dotted lines, a1-e1) and decomposition of SNAP at 590 nm (solid lines, a-e). $[CA]_0 = 0.03\text{ M}$; $[SNAP]_0 = (a) 0.00503\text{ M}$, $(b) 0.00626\text{ M}$, $(c) 0.00700\text{ M}$, $(d) 0.00808\text{ M}$ and $(e) 0.00883\text{ M}$

Figure 3.12e: Initial rate plot of the data in Figure 3.12d. The plot shows linear dependence on initial rate of the formation of CANO on SNAP.

Figure 3.12f: A LC-MS spectrum of a 1:3 ratio of SNAP to CA; trace e in Figure 3.12d.

MECHANISM

The overall rate of nitrosation is dependent on the rate of formation and accumulation of the nitrosating agents. A number of possibilities have been suggested, and these include HNO_2 , NO , NO_2 , N_2O_3 and the nitrosonium cation, ^+NO . Some nitrosants cannot exist under certain conditions: for example, the nitrosonium cation cannot exist at high pH conditions and NO_2 cannot exist in strictly anaerobic conditions except in aerobic environments where it can form from NO and subsequently N_2O_3 will also be formed¹⁶⁸.

Both NO_2 and N_2O_3 are potent nitrosating agents. There is a slightly higher rate of nitrosation in aerobic environments compared to anaerobic (Figure 3.6). This is a reflection of the low dissolved oxygen concentrations available in aqueous solutions at approximately 2×10^{-4} M. Thus, though the formation for these nitrosants is favored, their viability is hampered by the low concentrations of oxygen as well as NO , which is only produced during the decomposition of the nitrosothiol. No NO was detected during the formation of CANO . In this aqueous environment utilized for these experiments, the reaction of aqueous N_2O_3 to nitrous acid would be overwhelmingly dominant. This is a rapid equilibrium reaction that favors the weak acid, HNO_2 :

Early studies by Bunton and Steadman ¹⁶⁹ had erroneously estimated a value for $K_{\text{H}}^{-1} = 0.20 \text{ M}^{-1}$; which would indicate appreciable amounts of $\text{N}_2\text{O}_3 (\text{aq})$ over nitrous acid. A more accurate evaluation by Markovits *et al* ¹⁷⁰ gave a more realistic value of $K_{\text{H}}^{-1} = 3.03 \pm 0.23 \times 10^{-3} \text{ M}^{-1}$. Most nitrosation studies in aerobic NO environments neglect the contribution to nitrosation by NO_2 and only concentrate on NO and N_2O_3 nitrosations. NO_2 concentration will always be negligibly low because of reaction R3.10, which is two orders of magnitude greater than the rate of nitrosation by NO_2 ; $1.1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ vs $2.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ respectively ^{171,172}.

All nitrosation kinetics show first order dependence in both the thiol (CA, in this case), and nitrite and a slightly more complex dependence on acid. Data in Figures 3.7 and 3.8 show this strong first order dependence on CA and on nitrite. In conditions in which acid concentrations are less than initial nitrite concentrations, the effect of nitrite is more difficult to interpret since a change in its concentrations concomitantly induces a change in both acid and HNO_2 concentrations, and thus its sole effect cannot be isolated. First order kinetics in nitrite strongly suggests that the major nitrosant, at the beginning of the reaction is HNO_2 since there is a direct correlation between nitrite concentrations and HNO_2 , especially when there is consistently excess acid over nitrite. After performing calculations for the relevant reactive species in the reaction medium (Table 3.1), the plot of initial rate of nitrosation vs. nitrous acid concentrations that shows a parabolic dependence

erroneously suggests second order kinetics in HNO_2 (Figures 3.9b and 3.9c) for the region in which nitrite concentrations exceed acid. As mentioned in the Results section, data in Table 3.1 show that HNO_2 is not changing in isolation. Both nitrite and acid are also concomitantly changing, and so the effect observed in rate is not solely due to changes in HNO_2 concentrations. When acid is in excess over nitrite, the amount on HNO_2 saturates, but as can be seen in Figure 3.9d, the rate keeps increasing in a linear way with increase in acid; indicating the presence of multiple nitrosating agents apart from HNO_2 . The simplest rate law that can explain this nitrosation rate involves the nitrosonium cation (NO^+) and HNO_2 . The nitrosonium cation can be formed from the protonation of HNO_2 :

($\text{H}(\text{OH})\text{N}=\text{O}^+$ is a hydrated nitrosonium cation, $^+\text{NO} + \text{H}_2\text{O}$)

Overall rate of reaction, using these two nitrosants gives:

$$\text{Rate} = \left(\frac{[\text{H}^+][\text{N(III)}]_T}{[\text{H}^+] + K_a} \right) [\text{CA}] [k_1 + k_2 K_b [\text{H}^+]] \quad (1)$$

Where $[\text{N(III)}]_T$ is the total of nitrous acid and nitrite concentrations as calculated in Table 3.1, columns 4 and 5 and $[\text{H}^+]$ is the calculated acid in Column 6. K_a is the acid dissociation constant of nitrous acid and K_b is the equilibrium for the protonation of nitrous acid (Reaction R3.12). k_1 is the bimolecular rate constant for the nitrosation of

CA by HNO_2 and k_2 is the bimolecular rate constant for the nitrosation by the nitrosonium cation. K_a was taken as $5.62 \times 10^{-4} \text{ M}^{-1}$; and thus, at high acid concentrations, a plot of $\text{Rate}/[\text{N(III)}]_{\text{T}}[\text{CA}]$ vs. $[\text{H}^+]$ should give a straight line with slope k_2K_b and intercept k_1 . This type of plot only applies to conditions of excess acid over nitrite (Figure 3.9d). One can derive values for k_1 and product k_2K_b from these series of plots. A value of $k_1 = 17.9 \text{ M}^{-1} \text{ s}^{-1}$ was derived from a series of several complementary experiments. k_2 was deduced as $8.25 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ after assuming a K_b value of $1.2 \times 10^{-8} \text{ M}^{-1}$.

Decomposition of CA: S-nitrosothiols, RSNOs, differ greatly in their stabilities, and most of them have never been isolated in solid form. Homolysis of the S-N bond occurs both thermally and photochemically, but, generally, these processes are very slow at room temperatures and in the absence of radiation of the appropriate wavelength. In vivo decomposition of RSNOs is likely to proceed through other pathways apart from these two. Presence of trace amounts of Cu(II) or Cu(I) can efficiently catalyze RSNOs to disulfide and nitric oxide (see Figures 3.10 a – d):

CANO is a primary S-nitrosothiol, and primary and secondary nitrosothiols are generally known to be unstable compared to tertiary nitrosothiols. S-nitroso-N-

acetylpenicillamine is the most well known stable tertiary nitrosothiol ¹⁷³. The mechanism of decomposition of nitrosothiols was believed to proceed through the homolytic cleavage of this weak S – N bond. Computed S-N bond dissociation energies for nitrosothiols show very little variation, however, between primary, secondary and tertiary nitrosothiols; resulting in very similar homolysis rates of reaction at elevated temperatures for all nitrosothiols. The computed activation parameters for thermal homolysis to occur are prohibitively high for this to be a dominant pathway under normal biological conditions. For example, assuming an approximate S-N bond energy of 31 kCal, a rough calculation will indicate that if decomposition was to proceed solely through homolysis, the half-life of a typical nitrosothiol would be in the regions of years instead of the minutes to hours that are observed ¹⁷⁴. This analysis does not support the work reported by Roy et al ¹⁷⁵ in which they established homolysis as the major decomposition pathway. They also managed to trap the thiyl radical, RS·. Close examination of their reaction conditions show high trap concentrations (0.2 M DMPO) and nitrosothiol (0.1 M). Such conditions would catch even very low concentrations of thiyl radical, but would not necessarily indicate that this was the dominant pathway and whether it was derived solely from homolytic cleavage of the S-N bond. Nitrosothiol decompositions in the absence of metal ions are mostly zero order (see traces a, b in Figure 3.10d); and this is inconsistent with an S-N bond homolysis-driven decomposition ¹⁷⁶; which would have been expected to be first order. In the presence of excess thiol, however, decomposition of nitrosothiols proceeds via a smooth first order decay process ¹⁷³.

For a series of nitrosothiols, the homolysis-dependent decomposition rates are not dependent on the bulkiness of the substituent groups attached to the nitrosothiols, which seems to enhance calculations that showed no discernible difference in the S-N bond energies irrespective whether the nitrosothiol was primary, secondary or tertiary^{177,178}. In aerated solutions, the decay was autocatalytic, thus implicating N₂O₃ as the autocatalytic propagating species (see reactions R3.9 + R3.10)¹⁷⁷. Nitrosothiol decompositions were much slower in solutions in which NO was not allowed to escape by bubbling argon; implicating NO as a possible retardant of nitrosothiol decomposition. This can be explained by the recombination of the thiyl radical with NO in the reaction cage after initial cleavage. Elimination of NO from the scene by bubbling air, forces reaction R3.14 to the right. No mechanism has yet been proposed for the implication of N₂O₃ in the autocatalytic mechanism. One can assume that N₂O₃ should assist in the cleavage of the S-N bond; thus accelerating rate of homolytic decomposition of RSNO's by lowering S-N bond energy and subsequently the activation energy.

In aerated solutions, NO then undergoes reactions R3.9 + R3.10 to yield N₂O₃.

The incorporation of the NO_2^- group in the nitrosothiol would weaken the S-N bond; leading to its easy cleavage and re-generation of the N_2O_3 molecule which will proceed to further catalyze the decomposition.

The sequence of R3.14 – 3.16 will deliver quadratic autocatalysis in N_2O_3 .

Recent results have suggested a novel pathway for the decomposition of nitrosothiols which involves an initial internal rearrangement to form an N-nitrosation product¹⁷⁹. Typical nitrosothiol decomposition products after such an internal rearrangement would include thiiranes and 2-hydroxy-mercaptans. This mechanism has been used successfully to explain the production of a small but significant quantity of 2-hydroxy-3-mercaptopropionic acid (apart from the dominant product cystine), in the decomposition of S-nitrosocysteine. GC-MS spectrum shown in Figure 3.4, however, gives a dominant signal for dimeric cystamine, with very little evidence of any other significant products, indicating very little initial N-transnitrosation.

The copper-catalyzed decomposition of nitrosothiols has been well-documented. Other metal ions have also been implicated in this catalysis. The varying rates of nitrosothiol decompositions reported by different research groups can be traced to adventitious metal ion catalysis from the water used in preparing reagent solutions. Even in this case, with the maximum metal ion concentrations of 0.43 ppb in Pb^{2+} , small differences can be observed between EDTA-laced reaction solutions

and those that are not (see traces a, b in Figure 3.10d and traces a, b in Figure 3.11b).

Nitrosothiol decompositions have also implicated other metal ions and not just specifically copper¹⁸⁰. Though Cu(II) ions are used to catalyze nitrosothiol decomposition, the active reagent is Cu(I). In this catalytic mechanism, Cu redox-cycles between the +2 and +1 states. Only trace amounts of Cu(I) are needed to effect the catalysis, and these can be generated from the minute amounts of thiolate anions that exist in equilibrium with the nitrosothiol:

The cascade of reactions R3.17 – R3.20 continues until the nitrosothiol is depleted and converted to the disulfide. The complex formed in reaction R3.19 is well known and justified through density functional theory that reports that Cu^+ binds more strongly to the the S center than to the N center of the nitrosothiol¹⁸¹. This preferential binding weakens and lengthens the S-N bond, thus facilitating its cleavage. It is difficult to extrapolate this proposed mechanism into the physiological environment, but some experimental results have shown that protein-bound Cu^{2+} catalyzed nitric oxide generation from nitrosothiols, but not with the same efficiency as the free, hydrated

ion ¹⁸². Further studies are needed to evaluate the efficiency of this catalysis especially in copper-based enzymes.

Effect of Cu(II) on Nitrosation of Cysteamine (Figure 3.10c)

The fact that Cu(II) ions can assist in nitrosating CA from nitrite in the absence of acid while Cu(I) ions are unable to effect this can be explained by recognizing that nitrosation is an electrophilic process, where NO effectively attaches as the (formally) positive charge, while eliminating a positively charged proton. Cu(II), in this case, acts by complexing to the nitrite and forming a nitrosating species which will release the Cu(II) ions after nitrosation.

Further Experiments on Effect of Nitrous Acid

Since it appears that nitrous acid is the major nitrosation species, another series of experiments were undertaken in which the effect of nitrous acid can be unambiguously determined. For the data shown in Figure 3.13a, nitrite and acid were kept at equimolar concentrations and varied with that constraint. Final acid and nitrite concentrations after dissociation, are also equal for electroneutrality (see Table 3.2). Thus, if our simple mechanism is plausible, a plot of nitrous acid vs initial nitrosation rate should be linear with expected slight tailing at either extreme of the nitrous acid concentrations from nonlinear generation of free acid due to the quadratic equation.

Using the same rate law as Equation (1), a value of k_1 can be derived and compared with values obtained from plots of type Figure 3.9d. Kinetics constants derived from plots of type Figure 3.13b are not expected to be as accurate as those from Figure 3.9d, but should deliver values close to those from high acid environments. In the limit of only nitrous acid as the nitrosating agent (too low a concentration of excess H_3O^+ to effect equilibrium of reaction R3.11), data derived from Figure 3.13b delivered an upper limit rate constant for k_1 that was higher than that derived from Figure 3.9d-type data, but well within the error expected for such a crude extrapolation. In order to fully establish the observed effect of acid, another set of experiments similar to experiments in Figure 3.13a, but at pH 1.2 in phosphate buffer system were conducted (Figures 3.14a and b). Other sets of kinetics data were derived in which CA concentrations were varied while keeping the nitrous acid concentrations constant. Varying nitrous acid concentrations were used, and for each, a CA-dependence was plotted (data not shown). These were straight lines, through the origin, and with the slope determined by the concentration of nitrous acid and the free protons existing in solution after the dissociation of nitrous acid. For these kinetics data, equation (1) had to be evaluated completely since $K_a \approx [\text{H}_3\text{O}^+]$ and no approximation could be made.

Figure 3.13a: Evaluation of unambiguous nitrous acid dependence by employing $[H^+]_0 = [NO_2^-]_0$ and varying both at the same equimolar concentrations. $[CA]_0$ fixed at 0.025 M. And $[H^+]_0 = [NO_2^-]_0 =$ (a) 0.005 M; (b) 0.010 M; (c) 0.015 M; (d) 0.020 M; (e) 0.025 M; (f) 0.030 M; (g) 0.035 M

Table 3.2: Input species concentration and final reagent concentrations (mol L⁻¹) for nitrous acid dependence experiments with [H⁺]₀ = [NO₂⁻]₀

[H ⁺] _{added} (mol L ⁻¹)	[NO ₂ ⁻] _{added} (mol L ⁻¹)	[HNO ₂] _{initial} (mol L ⁻¹)	[H ₃ O ⁺] _{final} (mol L ⁻¹)	[NO ₂ ⁻] _{final} (mol L ⁻¹)	[HNO ₂] _{final} (mol L ⁻¹)
5.00 x 10 ⁻³	5.00 x 10 ⁻³	5.00 x 10 ⁻³	1.42 x 10 ⁻³	1.42 x 10 ⁻³	3.58 x 10 ⁻³
1.00 x 10 ⁻²	1.00 x 10 ⁻²	1.00 x 10 ⁻²	2.11 x 10 ⁻³	2.11 x 10 ⁻³	7.89 x 10 ⁻³
1.50 x 10 ⁻²	1.50 x 10 ⁻²	1.50 x 10 ⁻²	2.64 x 10 ⁻³	2.64 x 10 ⁻³	1.24 x 10 ⁻²
2.00 x 10 ⁻²	2.00 x 10 ⁻²	2.00 x 10 ⁻²	3.08 x 10 ⁻³	3.08 x 10 ⁻³	1.69 x 10 ⁻²
2.50 x 10 ⁻²	2.50 x 10 ⁻²	2.50 x 10 ⁻²	3.48 x 10 ⁻³	3.48 x 10 ⁻³	2.15 x 10 ⁻²
3.00 x 10 ⁻²	3.00 x 10 ⁻²	3.00 x 10 ⁻²	3.83 x 10 ⁻³	3.83 x 10 ⁻³	2.62 x 10 ⁻²
3.50 x 10 ⁻²	3.50 x 10 ⁻²	3.50 x 10 ⁻²	4.16 x 10 ⁻³	4.16 x 10 ⁻³	3.08 x 10 ⁻²

The values of k_1 and k_2K_b were utilized in equation (1) to recalculate initial rates and slopes with respect to CA concentrations. These values were utilized to check the accuracy of the kinetics constants derived from high acid dependence experiments. It was noted that, within experimental error, these values checked and were generally robust.

Figure 3.13b: Plot of initial rate of nitrosation vs initial nitrous acid concentrations as calculated in Table 3.2 and derived from the data in Figure 3.13a.

Figure 3.14a: Absorbance traces showing the effect of varying nitrous acid concentrations at pH 1.2 in phosphate buffer. $[CA]_0 = 0.05\text{ M}$; $[NO_2^-]_0 = [H^+]_0 =$ (a) 0.005 M , (b) 0.01 M , (c) 0.015 M , (d) 0.02 M , (e) 0.025 M , (f) 0.03 M , (g) 0.035 M .

Figure 3.14b: Plot of initial rate versus nitrous acid concentrations of the experimental data in Figure 3.14a

Simulations:

A very small set of simple reactions was utilized to simulate the nitrosation kinetics. This is shown in Table 3.3. At low acid concentrations, the nitrosation is dominated by HNO₂ as the nitrosant, and as acid concentrations are increased, both nitrous acid and the nitrosonium cation are involved in the nitrosation.

Table 3.3: Mechanism used for simulating the nitrosation of cysteamine. RSH stands for cysteamine.

Reaction No.	Reaction	k_f ; k_r
M1	$H^+ + NO_2^- \rightleftharpoons HNO_2$	$1.10 \times 10^9 M^{-1}s^{-1}$; $6.18 \times 10^5 s^{-1}$ ($K_{a(HNO_2)} = 5.62 \times 10^{-4}$)
M2	$2HNO_2 \rightleftharpoons N_2O_3 + H_2O$	$1.59 \pm 0.50 M^{-1}s^{-1}$; $5.30 \times 10^2 s^{-1}$ ($K_{M2} = 3.0 \times 10^{-3} M^{-1}$)
M3	$HNO_2 + H^+ \rightleftharpoons {}^+N=O + H_2O$	$4.00 \times 10^1 M^{-1}s^{-1}$; $3.33 \times 10^9 s^{-1}$ ($K_{M3} = 1.2 \times 10^{-8} M^{-1}$)
M4	$RSH + HNO_2 \rightarrow RSNO + H_2O$	$17.9 M^{-1}s^{-1}$; ca. 0
M5	$RSH + {}^+N=O \rightleftharpoons RSNO + H^+$	$8.25 \times 10^{10} M^{-1}s^{-1}$; $3.6 \times 10^3 M^{-1}s^{-1}$
M6	$N_2O_3 \rightleftharpoons NO + NO_2$	$8.0 \times 10^4 s^{-1}$; $1.1 \times 10^9 M^{-1}s^{-1}$
M7	$2RSNO \rightleftharpoons RSSR + 2NO$	$5.0 \times 10^{-2} M^{-1}s^{-1}$; ca. 0
M8	$RSH + N_2O_3 \rightarrow RSNO + HNO_2$	$9.49 \times 10^3 M^{-1}s^{-1}$; ca. 0

Even though Table 3.3 includes nitrosation by N_2O_3 ; the lack of oxygen (approximately kept constant at $2.0 \times 10^{-4} M$ and its most favored and rapid reaction to nitrous acid made the nitrosation by N_2O_3 insignificant. Its insignificant concentration ensured that the simulations were very insensitive to the value of the bimolecular nitrosation constant for N_2O_3 ; k_{M8} . Figure 3.15b clearly shows that concentration of N_2O_3 never rises to a level where it would be significant. The rate constants adopted

for reaction M1 were unimportant for as long as they were fast (in both directions), and were connected by the acid dissociation constant, K_a . Increasing the forward and reverse rate constants while maintaining K_a only slowed the simulations, but did not alter the results. Reaction M2 was also a rapid hydration reaction which strongly favored HNO_2 ; thus rapidly depleting any N_2O_3 formed, or merely keeping these concentrations depressed should the tandem of reactions R3.9 + R3.10 have been added to the mechanism. These were not utilized for this simulation because EPR had not detected any NO at the beginning of the reaction, and only detected NO during the decomposition of the nitrosothiol. There are no reliable thermodynamics parameters for reaction M3 since this would be heavily-dependent on the pH of the medium. For the purposes of this simulation and some of our previous estimates, the equilibrium constant for M3 was set at $1.2 \times 10^{-8} \text{ M}^{-1}$. Kinetics constants for M4 and M5 were derived from this study. In the absence of significant concentrations of N_2O_3 ; the simulations are insensitive to the kinetics constants utilized for reactions M6 and M8. Reaction M8 was assumed to be slow, and in the absence of Cu(II) ions or other adventitious ions, this is indeed so (see Figures 3.10a and c). The simulations are shown in Figure 3.15a, which gives a reasonably good fit from such a crude mechanism. Figure 3.15b is provided to show the concentration variation of some of the species that could not be experimentally determined. Of note is that N_2O_3 concentrations are depressed throughout the lifetime of the nitrosation. Even though the nitrosonium cation is also depressed in concentration; this can be explained by its

Figure 3.15a: Simulation of the short mechanism shown in Table 3. Solid lines are experimental data while the symbols indicate simulations. The mechanism is dominated by the value of k_1 . $[CA]_0 = [NO_2^-]_0 = 0.05\text{ M}$.

Figure 3.15b: Results from the modeling that delivered simulations of the two traces shown in Figure 3.15a. These simulations show the concentration variations of those species that could not be experimentally monitored. Neither N_2O_3 nor NO^+ rise to any significant concentration levels.

high rate of nitrosation as soon as it is formed, and an equilibrium that favors its hydration back to HNO_2 once the thiol is depleted.

Conclusion.

This detailed kinetics study has shown that even though there are several possible nitrosating agents in solution, in the physiological environment, with its slightly basic pH and low oxygen concentrations; the major nitrosant is HNO_2 . Furthermore, the lifetime of CANO is not long enough to be able to be effectively used as a carrier of NO. GSH, which has been touted as a possible carrier of NO, appears to have a lower lifetime than CA. However, its overwhelming concentration in the physiological environment might still be a better carrier of NO than other thiols. Protein thiols, it appears would be best carriers by either intra- or intermolecular transfer of NO.

CHAPTER 4

KINETICS AND MECHANISMS OF FORMATION OF S-NITROSCYSTEINE

4.0 INTRODUCTION

Cysteine is a hydrophilic non-essential amino acid which can be synthesized by humans from methionine. It is an aminothiol with three reactive centers: the amino group, the carboxylic acid and the thiol group.

It contains sulfur in the form that can inactivate free radicals.¹⁸³ Cysteine is implicated in antioxidant defense mechanism against damaging species by increasing levels of the most dominant antioxidant, glutathione. It is involved in protein synthesis and is the precursor to the important aminosulfonic acid, taurine. The highly reactive thiol group, which exists as the thiolate group at physiologic conditions, is an excellent scavenger of reactive, damaging species. Cysteine also plays an important role in the metabolism of a number of essential biochemicals including coenzyme A, heparin, biotin, lipoic acid, and glutathione.¹⁸⁴ While its nitrosation is facile¹⁸⁵, there has not been, to date,

an exhaustive kinetics study of its formation to the extent that this thesis is going to present. The kinetics of its formation and decomposition will aid in evaluating if it could be an effective carrier of NO by deducing, from the kinetics, its possible lifetime.

4.1: Stoichiometry

The stoichiometry of the reaction subscribed to other known nitrosation reactions in which one mole of HNO_2 reacts with one mole of the thiol to form the cysteine nitrosothiol with the elimination of a molecule of water.¹⁴³

S-Nitrosocysteine, CYSNO, was not as stable as the known secondary and tertiary nitrosothiols,¹¹⁸ but was stable at physiological and slightly basic environments for several minutes. The lifetime was sufficient to allow for the determination of the UV/Vis spectrum (see Figure 4.1, spectrum e). The spectrum shows that CYSNO has an isolated absorption peak in the visible region at 544 nm. By running a series of experiments in equimolar excess nitrite and acid over cysteine, it was possible to evaluate an absorptivity coefficient of $17.2 \pm 0.1 \text{ M}^{-1} \text{ cm}^{-1}$ for CySNO at 544 nm. This was also confirmed by varying cysteine concentrations and testing the Lambert-Beer law on the basis of expected product formation. CySNO had another peak at 335 nm

with a much higher absorptivity coefficient of $736 \text{ M}^{-1} \text{ cm}^{-1}$; but at this peak, several other species in the reaction medium such as nitrite and nitrous acid also contributed considerably to the absorbance observed at 335 nm. Spectrum f was taken at very low cysteine concentrations in order to produce CySNO which would give an absorbance low enough at 335 nm to be measured on absorbance scale in Figure 4.1. All kinetics measurements were performed at 544 nm.

Figure 4.1: UV/vis spectral scan of reactants. (a), 0.04 M Cys, (b) 0.03 M NaNO₂ (c) 0.04 M Cys + 0.03 M NaNO₂ (d) 0.03 M NaNO₂ + 0.05 M H⁺, (e) 0.04 M Cys + 0.03 M NaNO₂ + 0.05 M H⁺ and (f) 0.004 M Cys + 0.003 M NaNO₂ + 0.005 M H⁺

4.2: Product determination

The relevant mass spectra for product determination are shown in Figure 4.2. Within an hour of preparing CYSNO, QTOF mass spectra give a dominant m/z peak at 150.87 (see Figure 4.2a) which would be the expected peak from CYSNO from the positive mode electrospray technique utilized.

Figure 4.2: Mass spectrometry analysis of the product of nitrosation of Cys showing (A) formation of CysNO, $m/z = 150.87$. (B) Product of decomposition of CysNO after 24 hours, Cystine, a disulfide of Cys was produced with a strong peak, $m/z = 241.00$

All other peaks observed in the spectrum are small and insignificant. Upon prolonged standing, the peak at 150.87 disappeared while being replaced by a peak at 241.00 which is the expected peak for the cysteine disulfide, cystine. Figure 4.2b is a spectrum of the products in Figure 4.2a after 24 hours. Over this period, the CYSNO completely disappeared and the disulfide was quantitatively formed. Several previous workers in this field had also concluded that the ultimate product in S-nitrosation is the oxidation of the thiol to the disulfide.^{117,186,187}

There has been some debate as to veracity of reaction R4.1, with some schools of thought suggesting that the reaction may be strongly influenced by oxygen concentrations, or lack thereof.

In alkaline solutions, nitroalkanes deprotonate to give an aci-anion, $\text{RCH}=\text{NO}_2^-$, which was recently discovered to be an excellent trap for NO, giving signature-type EPR spectra which can be used as evidence for the presence of NO.¹⁴² Figure 4.3 shows a series of EPR spectra which prove the involvement of NO in nitrosothiol chemistry. Pure trap, nitroethane, does not show any peaks without NO (top spectrum), while nitrite with the trap will show no active peaks either (spectrum B), as does cysteine on its own (spectrum C). The last three spectra show nitroethane with varying concentrations of CySNO. The peaks derived from the interaction of the

trap with NO increase with concentrations of CySNO showing that the decomposition of CySNO involves release of NO.

Figure 4.3: EPR spectra of NO radical generated during the decomposition of CYSNO using 0.5 M nitroethane (NE) in 1.0 M NaOH solution as spin trap. (A) 0.5 M NE (B) 0.01 M NO_2^- (C) 0.04 M Cys; $[\text{CysNO}] =$ (D) 0.02 M (E) 0.03 M (F) 0.04 M and (G) 0.05 M

4.3 Reaction Kinetics

Our study involved the simplest form of nitrosation: one that involves the *in situ* production of the nitrosating agent, HNO_2 , in the same aqueous phase as the reactants and products. More complex forms of nitrosation involve the availing of gaseous nitric oxide through a nitric oxide-releasing compound. The biphasic nature of these nitrosations renders the subsequent kinetics and mechanisms complex. Through the use of the double-mixing feature of the stopped-flow ensemble, nitrous acid was initially formed by the mixing of hydrochloric acid and sodium nitrite and aged for about 1 sec before being combined with the thiol in a third feed syringe to effect the nitrosation reaction. By assuming that protolytic reactions are fast, one can assume that the protonation of nitrite as well as other subsequent reactions would have reached equilibrium by the time the thiol is combined with the nitrosating agent. In general the nitrosation kinetics were more reproducible when $[\text{H}^+]_0 \geq [\text{NO}_2^-]_0$. Reliable kinetics data used to evaluate rate constants utilized data in which acid concentrations were equal or slightly greater than nitrite concentrations.

4.3.1 Effect of cysteine concentrations

For the evaluation of the effect of cysteine on the rate of nitrosation, equimolar concentrations of acid and nitrite were used. The final active concentrations of nitrite and acid at the beginning of the reaction were constant and could easily be evaluated from the acid dissociation constant of nitrous acid. Figure 4.4a shows the absorbance traces obtained from a variation of initial cysteine concentrations. There was a linear

and monotonic increase in the final CYSNO formed for as long as cysteine remained as the limiting reagent, which was the case for all the traces shown in Figure 4.4a.

Figure 4.4a: Absorbance traces showing the effect of varying Cys concentrations. The reaction shows first-order kinetics in Cys. $[\text{NO}_2^-]_0 = 0.10 \text{ M}$; $[\text{H}^+]_0 = 0.10 \text{ M}$; $[\text{Cys}]_0 =$ (a) 0.01 M , (b) 0.02 M , (c) 0.03 M , (d) 0.04 M , (e) 0.05 M , (f) 0.06 M and (g) 0.07 M .

Figure 4.4b shows that the effect of cysteine is linear, with a first order dependence. There was a noticeable saturation in cysteine's effect on the initial rate of formation of

CysNO at higher initial cysteine concentrations. Lower concentrations, however, gave a linear dependence with an intercept kinetically indistinguishable from zero as would have been expected in a system in which all cysteine is quantitatively converted to the nitrosothiol.

Figure 4.4b: Initial rate plot of the data in Figure 4.4a. The plot shows strong dependence on initial rate of formation of CysNO on Cys.

4.3.2 Effect of nitrite

Figure 4.5a showed surprisingly simple and reproducible kinetics upon variation of nitrite. Data in Figure 4.5a were derived from varying nitrite in regions where acid concentrations are in excess. A combination of data involving the variation of initial concentrations from below initial acid concentrations to being above the acid concentrations did not retain linearity through the whole range of the plot because of the varying concentrations of HNO_2 formed that are nonlinearly dependent on the initial nitrite concentrations.

Figure 4.5a: Absorbance traces showing the effect of varying nitrite concentrations. The reaction shows first-order kinetics in nitrite. $[\text{Cys}]_0 = 0.10 \text{ M}$; $[\text{H}^+]_0 = 0.10 \text{ M}$; $[\text{NO}_2^-]_0 =$ (a) 0.01 M , (b) 0.02 M , (c) 0.03 M , (d) 0.04 M , (e) 0.05 M , (f) 0.06 M and (g) 0.07 M .

With the conditions utilized for Figures 4.5a and b; the changes in nitrosating agent, HNO_2 , were clearly linearly dependent on the initial nitrite concentrations. Thus the linear dependence on initial rate of formation of CysNO with nitrite concentrations shown in Figure 4.5b was expected.

Figure 4.5b: Initial rate plot of the data in Figure 4.5a. The plot shows strong dependence on initial rate of formation of CysNO on nitrite.

4.3.3: Effect of acid

The effect of acid, as expected, was more complex (see Figure 4.6a). The data in Figure 4.6a involves changes in acid concentrations that spanned initial acid concentrations below nitrite concentrations to those in high excess over nitrite. The initial acid concentrations in Figure 4.6a were increased step-wise by $1.00 \times 10^{-2} \text{ M}$; but the final acid concentrations in the reaction mixtures did not increase by the same constant rate due to the dissociation equilibrium of HNO_2 .

Figure 4.6a: Effect of low to high acid concentrations on the formation of CysNO. $[\text{Cys}]_0 = 0.05 \text{ M}$; $[\text{NO}_2^-]_0 = 0.05 \text{ M}$; $[\text{H}^+]_0 =$ (a) 0.01 M , (b) 0.02 M , (c) 0.04 M , (d) 0.05 M , (e) 0.06 M , (f) 0.07 M , (g) 0.08 M , (h) 0.09 M , (j) 0.10 M , (k) 0.11 M , (l) 0.12 M and (m) 0.13 M .

Thus a new table was created in which the final initial acid concentrations as well as nitrous acid and nitrite concentrations were calculated (see Table 4.1). These were the concentration values utilized in the acid dependence plot shown in Figure 4.6b.

Available acid concentrations are heavily depressed in conditions in which the initial nitrite concentrations exceed the initial acid concentrations (first 4 data points in plot Figure 4.6b).

Figure 4.6b: Initial rate plot of the data in Figure 4.6a showing first-order dependence of the rate of formation of CysNO on acid at high acid concentrations. NO⁺ is the main nitrosating agent.

The next series of data points involve conditions in which the added acid concentrations exceed nitrite. The resulting final free acid concentrations are derived

from the excess acid plus the acid derived from the dissociation of nitrous acid. This dissociation is heavily depressed by the initial presence of excess acid, and thus there is a roughly linear relationship between the added acid and the final acid concentrations obtaining at the start of the reaction.

Table 4.1: Calculations (in mol L⁻¹) of the final reactive species for acid dependence data shown in Figures 6a, b.

$[\text{H}^+]_{\text{added}}$	$[\text{NO}_2^-]_{\text{added}}$	$[\text{HNO}_2]_0$	$[\text{NO}_2^-]_0$	$[\text{H}^+]_0$
0.01	0.05	9.88×10^{-3}	4.01×10^{-2}	1.12×10^{-4}
0.02	0.05	1.97×10^{-2}	3.03×10^{-2}	2.99×10^{-4}
0.03	0.05	2.94×10^{-2}	2.07×10^{-2}	6.54×10^{-4}
0.04	0.05	3.85×10^{-2}	1.15×10^{-2}	1.53×10^{-3}
0.05	0.05	4.54×10^{-2}	4.59×10^{-3}	4.59×10^{-3}
0.06	0.05	4.81×10^{-2}	1.87×10^{-3}	1.19×10^{-2}
0.07	0.05	4.89×10^{-2}	1.07×10^{-3}	2.11×10^{-2}
0.08	0.05	4.93×10^{-2}	7.37×10^{-4}	3.07×10^{-2}
0.09	0.05	4.94×10^{-2}	5.60×10^{-4}	4.06×10^{-2}
0.10	0.05	4.96×10^{-2}	4.50×10^{-4}	5.05×10^{-2}
0.11	0.05	4.96×10^{-2}	3.80×10^{-4}	6.04×10^{-2}
0.12	0.05	4.97×10^{-2}	3.30×10^{-3}	7.03×10^{-2}
0.13	0.05	4.97×10^{-2}	2.90×10^{-3}	8.03×10^{-2}

Table 4.1 shows that when acid exceeds nitrite concentrations ($[\text{H}^+]_0 > 0.05 \text{ M}$), the final acid concentrations are directly proportional to the initial added acid concentrations and that nitrous acid concentrations remain nearly constant in this range while asymptotically approaching 0.05 M. Since initial nitrite concentrations were constant, the constant concentrations of HNO_2 in this range are expected and the effect of acid can thus be isolated. The linear plot observed for the effect of acid in this range suggests first order kinetics in acid for the nitrosation reaction (see Figure 4.6c).

Figure 4.6c: *Effect of acid on nitrosation in the high acid range. The intercept value should give an estimate of the nitrosation effect of nitrous acid without excess acid.*

It is also important to note that in the first four data points in Figure 4.6b, there is a wild variation in both acid and the nitrosating agent, HNO_2 (see Table 4.1). The discontinuity in acid effect occurs when initial acid concentrations exceed initial nitrite concentrations, and final nitrite concentrations after acid equilibrium has been effected become negligible. Table 4.1 can be used to evaluate the effects of each of the relevant species in solution towards nitrosation. This is shown in Figure 4.6d.

Figure 4.6d: An evaluation of the effects of nitrite, nitrous acid and excess acid on the rate of nitrosation. Plot shows that nitrite is not involved in nitrosation, and that after the saturation of nitrous acid concentrations, a new nitrosant emerges, whose formation is catalyzed by acid.

In this figure, rate of nitrosation is plotted against nitrite, nitrous acid and acid concentrations. Increase in nitrous acid concentrations catalyze nitrosation up to 0.05 M (its maximum), but the fact that rate of nitrosation keeps increasing despite static nitrous acid concentrations implies the presence of another nitrosant. Though nitrosation rate is discontinuous in acid concentrations, there is, generally, a monotonic catalysis of nitrosation by acid.

4.3.4: Effect of Cu^{2+} ions

The established experimental data implicates Cu^+ ions in the catalysis of decomposition of nitrosothiols. However, Figure 4.7a shows that Cu(II) ions are extremely effective catalysts in the formation of CysNO when they are in the millimolar concentration ranges and even lower. A plot to quantitatively evaluate the effect of Cu(II) ions is shown on Figure 4.7b. It shows the typical catalyst effect that shows an ever-increasing rate with Cu(II) followed by a saturation. The micromolar Cu(II) concentration ranges are shown in Figure 4.7c. Figure 4.7c involves a longer observation time that encompasses both the formation and the consumption of CysNO. This figure shows that, even though Cu(II) ions catalyze the formation of CysNO, they also catalyze its decomposition. Thus, in the presence of Cu(II) ions, the maximum expected quantitative formation of CYSNO is not observed since the onset of decomposition of CYSNO is strongly enhanced in the presence of Cu(II) ions.

Figure 4.7a: Absorbance traces showing the effect of varying Cu^{2+} concentrations on the rate of formation of CysNO in the absence of perchloric acid. There is a progressive increase in the rate of CysNO formation with increase in Cu^{2+} concentration. $[\text{Cys}]_0 = [\text{NO}_2^-]_0 = 0.05$; $[\text{H}^+]_0 = 0.00 \text{ M}$; $[\text{Cu}^{2+}]_0 =$ (a) 0.001 M , (b) 0.0015 M , (c) 0.002 M , (d) 0.0025 M , (e) 0.003 M , (f) 0.0035 M , (g) 0.004 M , (h) 0.0045 M and (j) 0.005 M

Figure 4.7b: Initial rate plot of the data in Figure 4.7a showing the catalytic effect of copper.

Figure 4.7c: Absorbance traces showing the effect of varying Cu^{2+} concentrations on the rate of formation of CysNO in the presence of perchloric acid. There is a progressive increase in the rate of CyNO formation with increase in Cu^{2+} concentration. High Cu^{2+} concentrations give an early onset of the decomposition of CysNO. $[\text{Cys}]_0 = [\text{NO}_2^-]_0 = [\text{H}^+]_0 = 0.01 \text{ M}$; $[\text{Cu}^{2+}]_0 = (\text{a}) 0.00 \text{ M}$, (b) $1.0 \mu\text{M}$, (c) $2.5 \mu\text{M}$, (d) $5.0 \mu\text{M}$, (e) $10.0 \mu\text{M}$, (f) $50.0 \mu\text{M}$ and (g) $100.0 \mu\text{M}$.

4.3.5: Decomposition of CYSNO

The lifetime of CysNO in the physiological environment is determined by its rate of decomposition, especially at physiological pH conditions. Figure 4.8 shows the

decomposition kinetics of CysNO. Decompositions of nitrosothiols are metal-ion mediated. Trace a shows the slow decomposition of CysNO using normal reagent water and without chelators to sequester metal ions (mostly 0.43 ppb of Pb^{2+} according to ICPMS analysis). Trace b shows the same solution in trace a, but this time with EDTA added to sequester any metal ions.

Figure 4.8: Effect of Cu^{2+} on the stability of CysNO in a pH 7.4 phosphate buffer. $[\text{CysNO}]_0 = 0.01 \text{ M}$; $[\text{Cu}^{2+}]_0 =$ (a) 0.00 M , (b) $0.00 \text{ M Cu}^{2+} + 10 \mu\text{M EDTA}$, (c) $5 \mu\text{M}$, (d) $10 \mu\text{M}$, (e) $20 \mu\text{M}$, (f) $30 \mu\text{M}$, (g) $40 \mu\text{M}$ and (h) $50 \mu\text{M}$.

This shows that the decomposition of CysNO becomes even slower under these conditions; proving that even the very low metal ion concentrations present in reagent water are effective in catalyzing CysNO's decomposition. The rest of the traces show ever-increasing concentrations of Cu²⁺ ions, but still in the micromolar range. There is noticeable autocatalysis in the rate of decomposition of CysNO.

4.4: MECHANISM.

Experimental data suggests a very simple nitrosation kinetics scheme that is first order in thiol and nitrite and slightly more complex dependence on acid. The acid effect has to be derived from its effect on the possible nitrosating agents. There are five possible nitrosating agents: HNO₂, N₂O₃, NO₂, NO and NO⁺. Pure nitric oxide itself, NO, is highly unlikely to be a nitrosating agent in this environment because no thiyl radicals were observed using the DMPO spin trap during the entire lifetime of the reaction. Nitric oxide was observed only as a decomposition product (see Figure 4.3). NO₂ and N₂O₃ are both derived from the autoxidation of nitric oxide.¹⁸⁸

N₂O₃ and NO⁺ are the strongest nitrosating agents out of the five possibilities¹⁸⁹

Formation of NO₂ is dependent on the dissolved oxygen in solution, which is normally at approximately 2.0 x 10⁻⁴ M. NO concentrations are expected to be in the fractions of millimolar in concentrations. Thus, both reactions R4.3 and R4.4 will not be

effective in producing significant nitrosating agents. The elimination of three nitrosating agents leaves HNO_2 and NO^+ , the nitrosonium cation¹⁹⁰ as the predominant nitrosants. These two nitrosants both support first order kinetics in nitrite and thiol.

Addition of reaction R4.6 is supported by the acid effect on the reaction which show a strong acid dependence even when $[\text{H}^+]_0 > [\text{NO}_2^-]_0$. Without reaction R4.6, a quick saturation in acid effect should be observed when acid concentrations exceed nitrite concentrations since further increase in acid concentrations should leave concentration of the single nitrosant, HNO_2 invariant. It is highly unlikely that the nitrosonium cation exists as the naked form in aqueous media, and one would expect it to be hydrated, HN^+OOH . Other lab groups represent it as the protonated nitrous acid; H_2ONO^+ . From the use of these two dominant nitrosants; one can derive an initial rate of reaction:

$$\text{Rate} = \frac{d[\text{CysNO}]}{dt} = \frac{[\text{CysH}][\text{N(III)}]_T[\text{H}^+]}{K_a + [\text{H}^+]} \left(k_6 + \frac{k_5 k_7 [\text{H}^+]}{k_{-5} + k_7 [\text{CysH}]} \right) \quad (1)$$

Where

$$N(III)]_T = [NO_2^-] + [HNO_2] + [RSNO] + [{}^+N=O] \quad (2)$$

Derivation of (1) from (2) was simplified by assuming that at the beginning of the reaction, concentrations of both the thiol and the nitrosonium ion are vanishingly small. Equation (1) can be further simplified by assuming that the forward and reverse rate constants for Reaction R4.6 are rapid enough such that the term $k_7[{}^+N=O]$ in the denominator can be discarded, giving:

$$\frac{d[RSNO]}{dt} = \frac{[CySH][N(III)]_T[H^+]}{K_a + [H^+]}(k_6 + K_5k_7[H^+]) \quad (3)$$

Where K_5 is the equilibrium constant for the formation of the nitrosonium ion. Both equations (1) and (3) are versatile enough to support all the observed kinetics behavior of the system. This supports the observed first order kinetics in thiol and in nitrite concentrations (Figures 4.4 and 4.5), and also supports the observed bifurcation in acid effects shown in Figure 4.6b. These equations do not support second order kinetics in acid, despite the possible dominance of reaction R4.6 in highly acidic environments where the tandem of protonation of nitrite (R4.5) and the further protonation of HNO_2 (R4.6) will be a prerequisite for the formation of the nitrosant, culminating with second order kinetics. At low acid, the second term in (1) (and in (2) as well vanishes, and if low enough, where $K_a \gg [H_3O^+]$; first order kinetics would result. With such a low value of K_a ; in general $K_a = [H_3O^+]$; and acid behavior would display the curving observed in the low acid concentration range of Figure 4.6b. In high acid

concentrations, where initial acid concentrations strongly exceed the dissociation constant of nitrous acid, the second term will display first order kinetics while the first term will be kinetically silent with respect to acid. The first order dependence in acid observed in the high acid region of Figure 4.6b confirms the mathematical form of Equation 2.

An evaluation of bimolecular rate constant k_6 can be made from the data shown in Figures 4.4a and b. In this set of data, $[H^+]_0 = [NO_2^-]_0 = 0.10$ M such that the final acid concentration at the beginning of the reaction are determined by the dissociation constant of 0.10 M HNO_2 . The millimolar concentration of acid present nullify the second term in equation (3), and the slope of the thiol dependence plot can be equated to $[N(III)]_T[H_3O^+]_0/(K_a + [H_3O^+]_0)$. These values will remain essentially constant for the range of the thiol concentrations used for this plot (figure 4.4b). Since the prevailing pH for these experiments is below the isoelectronic point of cysteine, it is reasonable to assume that a variation in thiol concentrations should not alter, significantly, the final acid concentrations. This evaluation gives $k_6 = 6.4 \text{ M}^{-1} \text{ s}^{-1}$.

The linear part of Figure 4.6b can allow for the evaluation of the second bimolecular rate constant, k_7 . Both first and second terms in Equation (3) contribute to the overall rate of reaction, but any increase in rate with acid is solely on the basis of the second term since the first term saturates with respect to acid as the nitrous concentrations become invariant to further increases in acid. The intercept of Figure 4.6b should enable for the calculation of k_6 . The value of the intercept was $1.06 \times 10^{-2} \text{ M s}^{-1}$ and this was equated to $k_6[CySH]_0[N(III)]_T$ to evaluate $k_6 = 6.4 \text{ M}^{-1} \text{ s}^{-1}$. This

was the exact same value derived from thiol dependence experiments shown in Figure 4.4b. The congruence of these values for k_6 ; while derived from two separate approaches, proves the general veracity of the simple proposed mechanism. The slope can only enable for the evaluation of K_5k_7 ; and without an independent evaluation of K_5 , it is not possible to evaluate an unambiguous value for k_6 . From the rapid increase in nitrosation rate in response to acid increase after saturation of HNO_2 , it appears that $k_7 \gg k_6$. The value of K_5 is heavily-dependent on the pH and ionic strength of the reaction medium, and any value adopted for this study will be on the basis of how best this value can support the proposed equilibrium. By utilizing a previously-derived value of $K_5 = 1.2 \times 10^{-8} \text{ M}^{-1}$; k_7 was evaluated as $2.89 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ (no error bars could be calculated for this value).

Effect of Copper.

Cu^+ is well known to be an efficient catalyst for nitrosothiol decompositions by preferentially bonding the S center over the N center of the nitrosothiol.¹⁶² This preferential binding, which in the model compound $\text{HSN}=\text{O}$ has been evaluated at 39.6 kJ in favor of the S-binding; lengthens the S-N bond and facilitates its cleavage.¹⁹¹ Thus addition of Cu^+ ions to reactions involving the formation of nitrosothiols would show a progressive decrease in the yields of nitrosothiols with increasing additions of micromolar quantities of Cu^+ ions up to a saturation. The interpretation of the effect of Cu^{2+} is a little more involved. Figures 4.7a and b clearly show that Cu^{2+} ions are catalytic in the formation of nitrosothiols. Figure 4.7b also

shows the faintly sigmoidal increase in rate followed by a sharp saturation, which is a hallmark of most catalysis reaction dynamics. The general school of thought is that Cu^{2+} ions, in themselves are innocuous in this environment, but that, in the presence of micromolar quantities of thiolate anions, redox-cycling would occur between Cu^{2+} and Cu^+ ; thereby effecting the catalysis.

Data in Figures 4.7a and b were run in the absence of acid to encourage the formation of the thiolate anions. Recent experimental data has shown that this effect of establishing redox cycling is not a preserve of thiolate anions exclusively, but any effective nucleophile in reaction medium such as ascorbate anions, halogens and pseudohalogens can bring about the sequence of reactions R4.9 and R4.10.^{26,83,192} In the absence of acid, Cu^{2+} catalyzes formation of the nitrosothiol, but on observing the long term effect of Cu^{2+} ions (Figure 4.7c), one notices that even though Cu^{2+} catalyze formation of the nitrosothiol, they also catalyze the decomposition of the nitrosothiol. Higher concentrations of copper ions rapidly form the nitrosothiol, and also encourage a rapid onset of decomposition.

Figure 4.8 attempts to simulate the physiological environment by using a phosphate buffer at pH 7.4. The nitrosocysteine was initially prepared and then combined with the various reagents shown in that figure to evaluate the decomposition

kinetics of CySNO. The first two traces are displayed to show that there are negligible metal ions in the distilled water utilized in our laboratories. The solution run with a metal ion sequester displays very similar kinetics to the one without EDTA. The ambient pH is above the isoelectronic point of cysteine, and thus will encourage formation of thiolate anions. The observed autocatalysis can be explained in a similar manner. Decomposition of CySNO will form the dimer, CySSCy, which can form more thiolate anions. Higher thiolate concentrations should increase the turn-over number of the catalyst. Work by Francisco *et al* showed the same autocatalysis in the decomposition and further reaction of formamidine disulfide with nitric oxide which they explained through the formation of a pseudohalogen, SCN^- , which forms NOSCN .¹⁹³

4.5: CONCLUSION

The mechanism of nitrosation, though simple, differs with thiols and with reaction conditions, especially pH. In this study, the two major nitrosants are nitrous acid itself and the nitrosonium ion which is formed from the further protonation of the weak nitrous acid. There was no strong evidence to evoke the possibility of N_2O_3 as a contributing nitrosant at low pH. Its exclusion still gave a kinetics rate law that was strongly supported by the experimental data. Cysteine nitrosothiol does not seem to have a long enough half life in the physiological environment to enable it to be an NO carrier. The general school of thought is that since the EDRF can be linked with NO ,^{4,100} then NO's interaction with protein thiol residues should be the process by

which it expresses its physiological activity. The most abundant thiol in the physiological environment is glutathione, and maybe its nitrosothiol may be more robust in the human body, thus making it the most likely candidate for the transport of NO. This thesis has proved that the nitrosothiol from cysteine is not that stable, decomposing completely in the presence of micromolar quantities of copper within 100 seconds. With the large numbers of metal ions and metalloenzymes in the physiological environment, one expects the lifetime of a cysteine nitrosothiol to be even shorter.

CHAPTER 5

KINETICS AND MECHANISMS OF MODULATION OF HOMOCYSTEINE TOXICITY BY S-NITROSOTHIOL FORMATION

5.0 INTRODUCTION

Homocysteine, HCYSH, is a non-protein forming biologically active organosulfur amino acid. It is primarily produced by the demethylation of the essential, protein forming amino acid methionine. Under normal conditions, the total plasma concentration of HCYSH usually ranges from 5 to 15 $\mu\text{mol/L}$. A higher fasting level is described as hyperhomocysteinemia which is further categorized into moderate and severe hyperhomocysteinemia for the HCYSH levels in the range 16 to 100 $\mu\text{mol/L}$ and $>100 \mu\text{mol/L}$ respectively.¹⁹⁴⁻¹⁹⁶ Research over the decades has established that hyperhomocysteinemia, is a risk factor in a variety of disease states including cardiovascular, neurodegenerative and neural defects.¹⁹⁷⁻²⁰⁰ Hyperhomocysteinemia is believed to arise from abnormal metabolism of methionine particularly in diet with deficiency of B-vitamins such as folate, vitamin B-12 and/or vitamin B-6.²⁰¹ In the human body methionine is used for the synthesis of a universal methyl donor S-adenosylmethionine (AdoMet).²⁰² Upon donation of its methyl group, AdoMet is converted to S-adenosylhomocysteine (AdoHcy) which subsequently hydrolyzed to HCYSH and adenosine.^{202,203} In the physiological environment, HCYSH levels are regulated by remethylation to methionine by the enzyme methionine synthase in the presence of vitamin B-12 and 5,10-methyltetrahydrofolate, and by trans-sulfuration to cystathionine by the enzyme cystathionine β -synthase and vitamin B-6 as co-factor.^{197,203}

It has become evident that an impairment to remethylation and trans-sulfuration metabolic pathways of HCYSH results in an alternative metabolic conversion of HCYSH to a chemically reactive homocysteine thiolactone (HTL).²⁰³ Formation of this cyclic thioester HTL is believed to be one of the major factors responsible for the pathogenesis of HCYSH.²⁰⁴ HTL is a highly reactive metabolite that causes N-homocysteinylolation of proteins leading to the modification of their structural and physiological functions.²⁰⁵ This means that any process that can prevent the oxidation of HCYSH to HTL will alleviate the toxicity of HCYSH. One of such processes is the S-nitrosation of HCYSH to form S-nitrosohomocysteine (HCYSNO). Unlike HCYSH, HCYSNO does not support ring closure that may lead to the formation of HTL.²⁰⁶ It is therefore plausible to assume that HCYSH toxicity could occur when there is an imbalance between the available nitrosating agents such as nitric oxide (NO) and the HCYSH levels in the body.

S-nitrosohomocysteine has been found in human plasma and has been shown by Glow and co-workers to be an important mediator of vasorelaxation.²⁰⁷ Although some authors have suggested the inhibition of S-center of HCYSH via the formation of S-nitrosothiols,²⁰⁶⁻²¹⁰ the mechanism of reactions of NO and NO-derived nitrosating agents with HCYSH to form HCYSNO has however not been clearly elucidated. We therefore report in this doctorate thesis on the kinetics and mechanisms of nitrosation of HCYSH by physiologically active nitrosating agents.

5.1 RESULTS

The progress of the nitrosation reaction was monitored spectrophotometrically by following absorbance of S-nitrosohomocysteine (HCYSNO) at its experimentally determined absorption peak of 545 nm where an absorptivity coefficient of $20.2 \pm 0.2 \text{ M}^{-1}\text{cm}^{-1}$ was evaluated. Although HCYSNO has a second absorption peak at 331 nm, with a much higher absorptivity coefficient ($1040.00 \pm 3.00 \text{ M}^{-1}\text{cm}^{-1}$), its maximum absorption at 545 nm was used due to lack of interference from the absorption peaks of nitrogen species NO_2^- , NO^+ and HNO_2 at this absorption wavelength (see Figure 5.1).

Figure 5.1: UV/vis spectral scan of reactants and product. (a), 0.005 M HCYSH, (b) 0.005 M NaNO_2 (c) 0.005 M HNO_2 , (d) 0.005 M HCYSNO and (e) 0.0025 M HCYSNO.

5.1.1 Stoichiometry determination

The stoichiometry of reaction between HNO₂ and HCYSH was determined as:

Reactions run for the deduction of this stoichiometry were performed by varying the amount of HNO₂ while keeping the amount of HCYSH constant. HNO₂ was generated by mixing equimolar amounts of perchloric acid and sodium nitrite. The reaction was allowed to proceed to completion while rapidly scanning the reacting solution between 400 nm and 700 nm. The concentration of the product HCYSNO was deduced from its maximum absorption at 545 nm. This stoichiometry is the highest HNO₂/HCYSH ratio that produced the maximum amount of HCYSNO. No nitrosation reaction at the N-center of homocysteine was observed. All nitrosation occur at the S-center because it is more nucleophilic than the 2° N-center.

5.1.2 Product identification:

Mass spectrometry was used to conclusively identify the products of the nitrosation reactions. Figure 5.2, spectrum A is from pure DL-homocysteine. Spectrum B, from the product of the reaction, shows a HCYSNO m/z peak at 136.00 which is the expected peak from the positive mode of the electrospray ionization mass spectrometry technique used for this analysis.

The production of NO from the decomposition of HCYSNO (R5.2) was detected by EPR techniques through the use of *aci* anion of nitroethane as a spin trap.²¹¹

Figure 5.2: Mass spectrometry analysis of the product of nitrosation of HCYSH showing (A) pure HCYSH m/z 136.00 and (B) formation of HCYSNO, m/z = 164.87.

In alkaline environments nitroethane ($\text{CH}_3\text{CH}_2\text{NO}_2$) forms an *aci* anion (R5.3) which can effectively scavenge NO to form a long-lived NO-adduct (R5.4).

Figure 5.3: EPR spectra of NO radical generated during the decomposition of HCYSNO using 0.5 M nitroethane (NE) in 1.0 M NaOH solution as spin trap. (A) 0.5 M NE (B) 0.03 M HCYSNO (C) 0.05 M HCYSNO (D) 0.03 M HCYSNO + 2.0×10^{-4} M Cu^{2+} .

Direct detection of NO without a spin trap is difficult due to its broad ESR spectrum.¹⁴¹ Figure 5.3 shows a series of ESR spectra that confirm the production of NO from the decomposition of HCYSNO. The splitting constant is consistent with that expected for a typical *aci*-nitroethane-NO adduct. All spectra were scaled on the same X-Y coordinate range and run under the same conditions. Spectrum A shows that *aci* nitroethane on its own does not show any peaks in the absence of NO. Spectra B and C show nitroethane with varying concentrations of HCYSNO in which the peak height increases with increase in HCYSNO concentrations. Spectrum D has equal concentrations of HCYSNO as in spectrum B but with the addition of copper. It clearly demonstrates the catalytic effect of copper in effecting NO release from S-nitrosothiols. More NO is released in D than in B within the same reaction time as reflected by the peak height, which is directly proportional to the amount of NO.

5.1.3 Reaction Dynamics

The reaction essentially involved the S-nitrosation of HCYSH by nitrous acid, which is generated *in situ* by mixing perchloric acid and sodium nitrite using the double mixing mode of the Hi-Tech Scientific SF61-DX2 stopped-flow spectrophotometer. It is necessary to generate the nitrous acid *in situ* due to its instability to disproportionation reaction (R5.5).

Unlike S-nitrosothiols of most of the primary aminothiols such as cysteine, HCYSNO is surprisingly stable at physiological pH of 7.4. Its half-life was determined to be 198 hours in the absence of reducing agents such as transition metal ions and thiols, which could catalyze its decomposition.

HCYSH dependence: The rate of formation of HCYSNO from the reaction of HCYSH and HNO_2 is significantly dependent on the initial concentration of HCYSH. As long as HCYSH is the limiting reactant, the rate of nitrosation and the amount of HCYSNO formed increases with increase in the initial concentration of HCYSH (Figure 5.4a). The reaction shows a first order dependence in HCYSH with an intercept kinetically indistinguishable from zero (Figure 5.4b).

Nitrite and acid dependence: Change in the initial concentrations of both nitrite and acid (perchloric acid) have a great effect on the dynamics of the reaction. As starting concentration of nitrite increases in excess acid and HCYSH, final concentration of HCYSNO formed linearly increases as seen in traces a-g of Figure 5.5a. Initial rate plot of these kinetics traces displayed a typical first-order dependence on nitrite for a nitrosation reaction in excess acid (Figure 5.5b).

Acid, as expected, exerts a most powerful effect on the rate of formation of HCYSNO. Figure 5.6a shows a series of kinetics experiments in which the concentration of acid is varied from 0.005 – 0.100 mol/L. The concentrations of both nitrite and HCYSH for these experiments were constant and fixed at 0.05 M.

Figure 5.4a: Absorbance traces showing the effect of varying HCYSH concentrations. The reaction shows first-order kinetics in HCYSH. $[\text{NO}_2^-]_0 = 0.05 \text{ M}$; $[\text{H}^+]_0 = 0.05 \text{ M}$; $[\text{HCYSH}]_0 =$ (a) 0.005 M , (b) 0.006 M , (c) 0.007 M , (d) 0.008 M , (e) 0.009 M , (f) 0.010 M and (g) 0.011 M .

Figure 5.4b: Initial rate plot of the data in Figure 5.4a. The plot shows strong dependence on initial rate of formation of HCYSNO on HCYSH.

Figure 5.5a: Absorbance traces showing the effect of varying nitrite concentrations. The reaction shows first-order kinetics in nitrite. $[\text{HCYSH}]_0 = 0.05 \text{ M}$; $[\text{H}^+]_0 = 0.05 \text{ M}$; $[\text{NO}_2^-]_0 =$ (a) 0.005 M , (b) 0.006 M , (c) 0.007 M , (d) 0.008 M , (e) 0.009 M , (f) 0.010 M and (g) 0.011 M .

Figure 5.5b: Initial rate plot of the data in Figure 5.5a. The plot shows strong dependence on initial rate of formation of HCYSNO on nitrite.

Since the nitrosation experiments essentially involve reaction of nitrous acid with HCYSH, a table was created in which the final concentrations of nitrous acid were calculated (Table 5.1). The amount of HCYSNO formed increases as concentration of acid increases until the concentration of the final nitrous acid is approximately equal to the concentration of HCYSH and saturation is attained as shown in traces h-k in Figure 5.6a.

Figure 5.6a: Absorbance traces showing the effect of varying acid concentrations.
 $[HCYSH]_0 = 0.05\text{ M}$; $[NO_2^-]_0 = 0.05\text{ M}$; $[H^+]_0 =$ (a) 0.005 M , (b) 0.01 M , (c) 0.02 M ,
 (d) 0.03 M , (e) 0.04 M , (f) 0.05 M , (g) 0.06 M , (h) 0.07 M , (i) 0.08 M , (j) 0.09 M and
 (k) 0.10 M .

The initial rate plot with respect to the final concentrations of HNO_2 showed a first-order linear plot in the region where the initial concentrations of acid exceeded the nitrite concentration (Figure 5.6b) and a non-linear quadratic second-order kinetics in the region where the final concentrations of HNO_2 were less than the initial nitrite concentration (Figure 5.6c).

Figure 5.6b: Plot of initial rate versus nitrous acid concentrations of the experimental data in Figure 5.6a (traces h-k) and calculated in Table 1 at pH < 2. The plot shows a first-order dependence on the initial rate of formation of HCYSNO on the final nitrous acid concentrations.

Confirmatory evidence for the observed second-order kinetics in excess nitrite was obtained by plotting the initial rates against the squares of the final nitrous acid concentrations. A linear plot was obtained with an almost zero intercept (Figure 5.6d).

Figure 5.6c: Plot of initial rate versus nitrous acid concentrations of the experimental data in Figure 5.6a and calculated in Table 1 at pH 2 and above. The curve fit well into a polynomial of degree two, which is an indication of rate being dependent on the square of the final nitrous acid concentrations.

In order to further establish this observed transition from second-order kinetics to first-order kinetics as the initial concentrations of acid increase from less than to more than the initial nitrite concentrations, a new series of HNO₂-dependence experiments were performed at two fixed pHs 1.2 and 4.0 for two different HCYSH concentrations (Figure 5.7a and 5.7c). The experiments were done under the same conditions. In perfect agreement with our earlier observation, plots of initial rates vs [HNO₂] for data at pH 1.2 gave linear plots expected for first-order kinetics (Figure 5.7b). Also, plots of initial rates vs [HNO₂] for data at pH 4.0 gave a non-linear quadratic curve

expected for second-order kinetics (Figure 5.7d).

Table 5.1: Input species concentrations and final reagent concentrations for acid dependence experiments.

$[\text{H}^+]_{\text{added}}$ (mol/L)	$[\text{NO}_2^-]_{\text{added}}$ (mol/L)	$[\text{HNO}_2]_{\text{initial}}$ (mol/L)	$[\text{NO}_2^-]_{\text{final}}$ (mol/L)	$[\text{HNO}_2]_{\text{final}}$ (mol/L)	$[\text{H}_3\text{O}^+]_{\text{final}}$ (mol/L)	~ pH
0.005	0.05	0.005	4.51×10^{-2}	4.94×10^{-3}	6.16×10^{-5}	4.21
0.01	0.05	0.01	4.01×10^{-2}	9.86×10^{-3}	1.38×10^{-4}	3.86
0.02	0.05	0.02	3.04×10^{-2}	1.97×10^{-2}	3.63×10^{-4}	3.44
0.03	0.05	0.03	2.08×10^{-2}	2.92×10^{-2}	7.90×10^{-4}	3.10
0.04	0.05	0.04	1.18×10^{-2}	3.82×10^{-2}	1.82×10^{-3}	2.74
0.05	0.05	0.05	5.03×10^{-3}	4.50×10^{-2}	5.03×10^{-3}	2.30
0.06	0.05	0.05	2.22×10^{-3}	4.78×10^{-2}	1.22×10^{-2}	1.91
0.07	0.05	0.05	1.29×10^{-3}	4.87×10^{-2}	2.13×10^{-2}	1.67
0.08	0.05	0.05	8.93×10^{-4}	4.91×10^{-2}	3.09×10^{-2}	1.51
0.09	0.05	0.05	6.81×10^{-4}	4.93×10^{-2}	4.07×10^{-2}	1.39
0.10	0.05	0.05	5.50×10^{-4}	4.95×10^{-2}	5.06×10^{-2}	1.30

Figure 5.6d: Plot of initial rate versus square of the final nitrous acid concentrations. This plot shows a second order dependence in nitrous acid at pH 2 and above.

Figure 5.7a: Absorbance traces showing the effect of varying nitrous acid concentrations at pH 1.2 in phosphate buffer. $[\text{HCYSH}]_0 = 0.05 \text{ M}$; $[\text{NO}_2^-]_0 = [\text{H}^+]_0$ = (a) 0.005 M, (b) 0.01 M, (c) 0.015 M, (d) 0.02 M, (e) 0.025 M, (f) 0.03 M, (g) 0.035 M, (h) 0.04 M, (i) 0.045 M and (j) 0.05 M

Figure 5.7b: Combined HNO_2 dependence plots at 0.05 M HCYSH (of the data in Figure 3.7a) and 0.09 M HCYSH showing first-order linear dependence of the rate of formation of HCYSNO on HNO_2 at pH 1.2. These data were utilized in equation 8 for the evaluation of k_6 .

Figure 5.7c: Absorbance traces showing the effect of varying nitrous acid concentrations at pH 4.0 in phosphate buffer. $[HCYSH]_0 = 0.05\text{ M}$; $[NO_2^-]_0 = [H^+]_0$ = (a) 0.005 M, (b) 0.01 M, (c) 0.015 M, (d) 0.02 M, (e) 0.025 M, (f) 0.03 M, (g) 0.035 M, (h) 0.04 M, (i) 0.045 M and (j) 0.05 M.

Figure 5.7d: Combined HNO_2 dependence plots at 0.05 M HCYSH (of the data in Figure 5.7c) and 0.09 M HCYSH showing non-linear quadratic dependence of the rate of formation of HCYSNO on HNO_2 at pH 4.0. These data were utilized in equation 9 for the evaluation of k_4 and k_5 .

Catalytic effect of Copper: Copper is an essential trace element that is indispensable for normal growth and development. It serves as a cofactor for the activity of a number of physiologically important enzymes such as methionine synthase, which plays a key role in the synthesis of methionine from homocysteine, and extracellular and cytosolic Cu-dependent superoxide dismutases ($\text{Cu}_2\text{Zn}_2\text{SOD}$), which play important role in cellular response to oxidative stress by detoxifying the superoxide

anion radical into the less harmful hydrogen peroxide.^{212,213}. Depending on the redox environment, copper can cycle between two states as reduced Cu^+ and oxidized Cu^{2+} ions, allowing it to play its numerous physiological roles. Figure 5.8 shows dual roles of this metal as a mediator of S-nitrosothiol (HCYSNO) formation and decomposition.

Figure 5.8: Absorbance traces showing the effect of varying Cu^{2+} concentrations on the rate of formation of HCYSNO. There is a progressive increase in the rate of HCYSNO formation with increase in Cu^{2+} concentration. $[\text{HCYSH}]_0 = [\text{NO}_2^-]_0 = [\text{H}^+]_0 = 0.05 \text{ M}$; $[\text{Cu}^{2+}]_0 =$ (a) 0.00 M, (b) 1.0 mM, (c) 2.0 mM, (d) 3.0 mM, and (e) 4.0 mM and (f) 5.0 mM.

Trace a represents a control experimental run without copper ions. Traces b-f has the same initial conditions as trace a but with varying amounts of Cu^{2+} ions. The plot shows a progressive increase in the rate of formation of HCYSNO with increase in the initial concentrations of Cu^{2+} ions. High concentrations of Cu^{2+} ions give an early onset of the decomposition of HCYSNO as shown in traces e and f. This observed effect of copper was virtually halted by the addition of excess EDTA which sequesters copper ions.

Transnitrosation: Transnitrosation is a physiologically important reaction between S-nitrosothiols (RSNO) and thiols (R'SH). It involves transfer of NO group from an S-nitrosothiol to a thiol to form a new S-nitrosothiol (R5.6):

In this study, S-nitrosoglutathione (GSNO) and S-nitroso-N-acetyl-D,L-penicillamine (SNAP) were used to transnitrosate HCYSH. They were synthesized as described in the literature.^{214,215} These two S-nitrosothiols have been shown to have excellent clinical applications.²¹⁶ GSNO in particular is believed to be involved in the storage and transport of NO in mammals.^{97,217,218}

Figure 5.9a: Absorbance traces showing the effect of varying HCYSH concentrations on its transnitrosation by GSNO. The plot shows the formation of HCYSNO at 545 nm. The increase in absorbance is due to the formation of HCYSNO which has a higher absorptivity coefficient of $20.2 \text{ M}^{-1} \text{ cm}^{-1}$ than $17.2 \text{ M}^{-1} \text{ cm}^{-1}$ of GSNO at 545 nm. $[\text{GSNO}]_0 = 0.003 \text{ M}$; $[\text{HCYSH}]_0 =$ (a) 0.0 M , (b) 0.005 M , (c) 0.01 M , (d) 0.02 M , (e) 0.03 M , and (f) 0.04 M .

Spectrophotometric studies of transnitrosation between primary S-nitrosothiols such as HCYSNO and GSNO are generally difficult because most nitrosothiols absorb at about the same wavelength ($\lambda_{\text{max}}(\text{GSNO}) = 335 \text{ nm}$ and 544 nm ; $\lambda_{\text{max}}(\text{HCYSNO}) = 331 \text{ nm}$ and 545 nm), but we were able to follow the formation of HCYSNO upon

transnitrosation of HCYSH by GSNO because HCYSNO has a slightly higher absorptivity coefficient ($\epsilon_{545\text{nm}} = 20.2 \text{ M}^{-1}\text{cm}^{-1}$) than GSNO ($\epsilon_{545\text{nm}} = 17.2 \text{ M}^{-1}\text{cm}^{-1}$)²¹⁹. Figure 9a shows the transnitrosation of HCYSH by GSNO to form HCYSNO. Trace a is from pure GSNO without HCYSH. Traces b-f with equal amounts of GSNO show a monotonic increase in amount and rate of formation of HCYSNO with increase in the initial concentrations of HCYSH. Confirmatory evidence for the transnitrosation was achieved by electrospray ionization mass spectrometry (ESI-MS) technique. Figure 9b shows that the product of transnitrosation contains a mixture of components including HCYSNO, GSSG (oxidized glutathione) and mixed disulfide of homocysteine and glutathione.

Transnitrosation by SNAP is simpler and can be monitored spectrophotometrically due to the fact that SNAP and HCYSNO absorb at different wavelengths. Figure 5.10a shows the decomposition of SNAP at 590 nm and simultaneous formation of HCYSNO at 545 nm. Figure 5.10b shows that the effect of HCYSH on its transnitrosation at a constant initial concentration of SNAP is linear, with a first order dependence.

Figure 5.9b: A ESI-MS spectrum of a 1:5 ratio of GSNO to HCYSH. Final products were mixture of GSH, GSSG, HCYSNO, and mixed disulfide of GSH/HCYSH.

Figure 5.10a: Absorbance traces showing the effect of varying HCYSH concentrations on its transnitrosation by SNAP. The plot shows both formation of HCYSNO at 545 nm (solid lines) and decomposition of SNAP at 590 nm (dotted lines). $[SNAP]_0 = 0.0049\text{ M}$; $[HCYSH]_0 =$ (a) 0.005 M , (b) 0.0075 M , (c) 0.01 M , (d) 0.02 M , (e) 0.03 M , (f) 0.04 M and (g) 0.05 M .

The intercept ($4.24 \times 10^{-3}\text{ M}$) on the $[HCYSH]$ axis corresponds to the equilibrium concentration of HCYSH that reacts with SNAP to produce HCYSNO in agreement with the expected 1:1 stoichiometry ratio in equation R5.6. The catalytic effect of copper on the rate of transnitrosation was shown in Figure 5.10c. Trace a is a reaction

mixture without Cu^{2+} ion. The rate of formation of HCYSNO increases with increase in Cu^{2+} ion as we have in traces b-e until saturation at f.

Figure 5.10b: Initial rate plot of the data in Figure 5.10a. The plot shows linear dependence on initial rate of formation of HCYSNO on HCYSH in the transnitrosation of HCYSH by SNAP.

Figure 5.10c: Absorbance traces showing the effect of varying Cu^{2+} concentrations on the rate of decomposition of SNAP at 590 nm and simultaneous formation of HCYSNO at 545 nm (solid lines) during transnitrosation reactions. $[\text{HCYSH}]_0 = 0.03 \text{ M}$; $[\text{SNAP}]_0 = 0.01 \text{ M}$; $[\text{Cu}^{2+}]_0 =$ (a) 0.00 M , (b) $5 \mu\text{M}$, (c) $10 \mu\text{M}$, (d) $50 \mu\text{M}$, (e) $100 \mu\text{M}$, (f) $500 \mu\text{M}$, (g) $1000 \mu\text{M}$ and (h) $2000 \mu\text{M}$.

5.2 MECHANISM

The proposed mechanism for the formation of S-nitrosohomocysteine from the reactions of homocysteine and acidic nitrite should accommodate the involvement of multiple nitrosating agents in the nitrosation process (Scheme 5.1).

Scheme 5.1: Reaction pathways, showing the involvement of multiple nitrosating agents in the nitrosation of HCYSH to form HCYSNO by acidic nitrite.

The first step is the formation of nitrous acid (R5.7):

This is followed by the production of other nitrosating agents through the bimolecular decomposition of HNO₂ to form dinitrogen trioxide, N₂O₃ (R5.8), and protonation to form nitrosonium cation, NO⁺ (R5.9):

These nitrosating agents are electrophilic and attack the highly nucleophilic sulfur center of homocysteine to form S-nitrosohomocysteine (R5.10, R5.11 and R5.12).

The order of increasing electrophilicity of these nitrosating agents is:²²⁰ $\text{HNO}_2 < \text{N}_2\text{O}_3 < \text{NO}^+$.

The mechanism of reaction can be explained by each of the nitrosating agents, whose degree of involvement in the process of nitrosation significantly depends on the pH of their environment. For example, at pH conditions around 1.3, HNO_2 and NO^+ are the most relevant nitrosating agents. Protonation of HNO_2 to form NO^+ is favored in these highly acidic conditions. NO^+ is expected to be the more powerful nitrosating agent. On the other hand, at less acidic pHs around 3.8, for reactions in excess nitrite as observed in traces a-e of Figure 5.7a, HNO_2 and N_2O_3 are the main nitrosating agents with the rate of nitrosation by N_2O_3 being higher than that by HNO_2 in accordance with the electrophilic trend. The overall rate of reaction, on the basis of rate of formation of HCYSNO , is given by:

$$\begin{aligned} \text{Rate} &= \frac{d[\text{HCYSNO}]}{dt} \\ &= k_4[\text{HCYSH}][\text{HNO}_2] + k_5[\text{HCYSH}][\text{N}_2\text{O}_3] + k_6[\text{HCYSH}][\text{NO}^+] - k_{-6}[\text{HCYSNO}][\text{H}^+] \quad (1) \end{aligned}$$

Since, initially, there is no accumulation of HCYSNO at the beginning of the reaction, the last term in equation (1) can be ignored, giving:

$$\begin{aligned}\text{Rate} &= \frac{d[\text{HCYSNO}]}{dt} \\ &= k_4[\text{HCYSH}][\text{HNO}_2] + k_5[\text{HCYSH}][\text{N}_2\text{O}_3] + k_6[\text{HCYSH}][\text{NO}^+]\end{aligned}\quad (2)$$

Equation (2) can be further simplified by applying steady state approximation on both N_2O_3 and NO^+ since they are transient intermediates with negligible concentrations at any time during the reaction. They react as soon as they are produced.

$$[\text{N}_2\text{O}_3] = \frac{k_2[\text{HNO}_2]^2}{k_{-2} + k_5[\text{HCYSH}]} \quad (3)$$

$$[\text{NO}^+] = \frac{k_3[\text{HNO}_2][\text{H}^+]}{k_{-3} + k_6[\text{HCYSH}]} \quad (4)$$

Substituting equations (3) and (4) into equation (2) and simplifying gives the following rate equation:

$$\begin{aligned}\text{Rate} &= \frac{d[\text{HCYSNO}]}{dt} \\ &= [\text{HCYSH}][\text{HNO}_2] \left(k_4 + \frac{k_3 k_6 [\text{H}^+]}{k_{-3} + k_6 [\text{HCYSH}]} \right) + \left(\frac{k_2 k_5 [\text{HCYSH}][\text{HNO}_2]^2}{k_{-2} + k_5 [\text{HCYSH}]} \right)\end{aligned}\quad (5)$$

where:

$$[\text{HNO}_2] = \frac{[\text{N(III)}]_{\text{T}}[\text{H}^+]}{K_{\text{a}} + [\text{H}^+]} \quad (6)$$

where $[\text{N(III)}]_{\text{T}}$ from mass balance equation for the total N(III) containing species is:

$$[\text{N(III)}]_{\text{T}} = [\text{NO}_2^-] + [\text{HNO}_2] + [\text{HCYSNO}] + [\text{N}_2\text{O}_3] + [\text{NO}^+] \quad (7)$$

HCYSH is the sum of the protonated and unprotonated HCYSH.

The proposed rate equation is valid from low to slightly acidic pH. At low pH conditions, for reactions run in excess acid, the bimolecular decomposition of HNO_2 to form N_2O_3 becomes irrelevant and the last term in equation (5) can be ignored, resulting in first-order kinetics in nitrous acid with a rate equation given by:

$$\begin{aligned} \text{Rate} &= \frac{d[\text{HCYSNO}]}{dt} \\ &= [\text{HCYSH}][\text{HNO}_2] \left(k_4 + \frac{k_3 k_6 [\text{H}^+]}{k_{-3} + k_6 [\text{HCYSH}]} \right) \end{aligned} \quad (8)$$

Rate equation (8) is supported by the kinetics data shown in Figures 5.6a, 5.6b, 5.7a and 5.7b. On the other hand, for reactions in excess nitrite, when the final perchloric acid concentrations tend to zero, second-order kinetics, which fitted well into a polynomial of degree two were observed. By ignoring the term containing $[\text{H}^+]$ in equation (5) we will obtain a rate equation (9):

$$\text{Rate} = \frac{d[\text{HCYSNO}]}{dt} = k_4[\text{HCYSH}][\text{HNO}_2] + \frac{k_2 k_5 [\text{HCYSH}][\text{HNO}_2]^2}{k_{-2} + k_5 [\text{HCYSH}]} \quad (9)$$

Equation (9) is supported by the kinetics data shown in Figures 5.6a, 5.6c, 5.6d, 5.7c and 5.7d.

As shown in equations 8 and 9, there are five kinetics constants to be evaluated.

Equilibrium constants $K_2 = 3.0 \times 10^{-3} \text{ M}^{-1}$ and $K_3 = 1.2 \times 10^{-8} \text{ M}^{-1}$ as well as the rate constant $k_{-2} = 5.30 \times 10^2 \text{ s}^{-1}$ have already been established.^{26,221,222} The data in Figures 5.7a-d provide rate constants for direct reaction of HCYSH and HNO_2 of $9.00 \times 10^{-2} \text{ M}^{-1}\text{s}^{-1}$, HCYSH and N_2O_3 of $9.49 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and HCYSH and NO^+ of $6.57 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$. These values agree with the order of electrophilicity of these three nitrosating agents ($\text{HNO}_2 < \text{N}_2\text{O}_3 < \text{NO}^+$).

5.3 COMPUTER SIMULATIONS

A network of eight reactions as shown in Table 5.2 can adequately model entire nitrosation reactions. Kintecus simulation software developed by James Ianni was used for the modeling.²²³ The rate constant for the formation of nitrosating agents (HNO_2 , N_2O_3 and NO^+ , Reactions M1 – M4) were taken from literature values.^{26,221,222} The forward and reverse rate constants used for M2 and M3 fit well for the modeling and agreed with the literature values for the equilibrium constants $3.0 \times 10^{-3} \text{ M}^{-1}$ and $1.2 \times 10^{-8} \text{ M}^{-1}$ for reactions R5.8 and R5.9 respectively. Experimental data for reactions in highly acidic pH 1.2 (Figure 5.7a) and mildly acidic pH 4.0 (Figure 5.7c) were

simulated using the bimolecular rate constants, k_4 , k_5 and k_6 determined according to our proposed mechanism. In highly acidic pH the simulations were mostly sensitive to the values of rate constants k_{M1} , k_{M2} and k_{M6} . The rate of acid catalysed decomposition of HCYSNO was found to be halved as the initial concentrations of nitrous acid doubled. Reactions M1, M3, M4, M7 and M8 are the most relevant reactions in mildly acid pH. Figures 5.11a and 5.11b show a comparison between the experimental results (traces a and b in Figures 5.7a and 5.7c) and our simulations. Reasonably good fits were obtained and the simulations were able to reproduce the formation of HCYSNO in high to mild acidic pHs by the reaction of homocysteine and the nitrosating agents.

Table 5.2: Mechanism used for simulating the S-nitrosation of homocysteine. RSH stands for homocysteine.

Reaction No.	Reaction	k_f ; k_r
M1	$H^+ + NO_2^- \rightleftharpoons HNO_2$	$1.10 \times 10^9 M^{-1}s^{-1}$; $6.18 \times 10^5 s^{-1}$ ($K_{a(HNO_2)} = 5.62 \times 10^{-4}$)
M2	$HNO_2 + H^+ \rightleftharpoons {}^+N=O + H_2O$	$4.00 \times 10^1 M^{-1}s^{-1}$; $3.33 \times 10^9 s^{-1}$ ($K_{M3} = 1.2 \times 10^{-8} M^{-1}$)
M3	$2HNO_2 \rightleftharpoons N_2O_3 + H_2O$	$1.59 \pm 0.50 M^{-1}s^{-1}$; $5.30 \times 10^2 s^{-1}$ ($K_{M2} = 3.0 \times 10^{-3} M^{-1}$)
M4	$N_2O_3 \rightleftharpoons NO + NO_2$	$8.0 \times 10^4 s^{-1}$; $1.1 \times 10^9 M^{-1}s^{-1}$
M5	$RSH + HNO_2 \rightarrow RSNO + H_2O$	$9.0 \times 10^{-2} M^{-1}s^{-1}$; ca. 0
M6	$RSH + {}^+N=O \rightleftharpoons RSNO + H^+$	$6.57 \times 10^{10} M^{-1}s^{-1}$; $3.6 \times 10^3 M^{-1}s^{-1}$
M7	$RSH + N_2O_3 \rightarrow RSNO + HNO_2$	$9.49 \times 10^3 M^{-1}s^{-1}$; ca. 0
M8	$2RSNO \rightleftharpoons RSSR + 2NO$	$5.0 \times 10^{-2} M^{-1}s^{-1}$; ca. 0

Figure 5.11a: Comparison of experimental (solid lines, traces a and b from Figure 5.7a) and computer simulations (dotted lines) using the mechanism shown in Table 2 for the formation of HCYSNO in highly acidic environment (pH 1.2). $[HCYSH] = 5.00 \times 10^{-2} \text{ M}$; $[HNO_2] = (a) 5.00 \times 10^{-3} \text{ M}$ and $(b) 1.00 \times 10^{-2} \text{ M}$.

Figure 5.11b: Comparison of experimental (solid lines, traces a and b from Figure 5.7c) and computer simulations (dotted lines) using the mechanism shown in Table 5.2 for the formation of HCYSNO in mildly acidic environment (pH 4.0). [HCYSH] = 5.00×10^{-2} M; [HNO₂] = (a) 5.00×10^{-3} M and (b) 1.00×10^{-2} M.

5.4 CONCLUSION

This study has shown that the conversion of HCYSH to the highly toxic homocysteine thiolactone can be prevented by the inhibition of the sulfur center of HCYSH by S-nitrosation. Nitrosation by dinitrogen trioxide and transnitrosation by other S-

nitrosothiols such as S-nitrosoglutathione (GSNO) are the most effective mean of S-nitrosation in mildly acid pHs to physiological pH 7.4. The high stability of HCYSNO at physiological pH, which rival GSNO, is an advantage in the modulation of HCYSH toxicities by S-nitrosation.

CHAPTER 6

SUMMARY, CONCLUSIONS AND FUTURE PERSPECTIVES

6.0 SUMMARY

The research profile in this dissertation has shown that S-nitrosothiols, S-nitrosocysteamine (CANO), S-nitrosocysteine (CYSNO) and S-nitrosohomocysteine (HCYSNO), can be produced by the reaction of acidic nitrite and corresponding parent amino thiols. These S-nitrosothiols can also be formed by transnitrosation by other S-nitrosothiols at physiological pH 7.4. Formation of these RSNOs is consistent with evidence in literature supporting formation of RSNO by reaction of thiols and nitrosating agents.^{82,106,137} Their kinetics and mechanisms of formation and decomposition were elucidated. The S-nitrosothiols studied are primary nitrosothiols with characteristic pink color and absorbance in the UV/vis in the 333 – 335 nm and 544-545 nm regions (see Table 6.1).

Table 6.1: Absorbance maxima and extinction coefficients for the S-nitrosothiols

S-nitrosothiols	Color	UV/Vis Absorption Maxima (nm)	Molar Extinction Coefficients (M⁻¹cm⁻¹)
S-nitrosocysteine (CYSNO)	Pink	544	17.2
S-nitrosohomocysteine (HCYSNO)	Pink	545	20.2
S-nitrosocysteamine (CANO)	Pink	545	16.0

Five nitrosating agents were identified in the reaction of nitrite and acid (Scheme 6.1). They are NO, NO₂, N₂O₃, NO⁺ and HNO₂. The particular nitrosating agent that will be involved in the process of nitrosation was found to be dependent on pH of medium. The proposed mechanism in this thesis has shown that, in highly acidic environments, HNO₂ and NO⁺ are the main nitrosating agents and in mild to physiological pHs, N₂O₃ and HNO₂ are the most relevant nitrosating agents. NO and NO₂ are radicals and hence can function as effective nitrosating agents either by a direct reaction with thyl radicals or by an indirect reaction with thiols via the formation of a new nitrosating agent N₂O₃ in an aerobic environments (R6.1 and R6.2).

Under acidosis or acidic conditions of the stomach, nitrite is rapidly protonated to form HNO₂ which can then decompose to NO and NO₂.^{224,225} HNO₂ can also react with excess acid to produce NO⁺.²²⁶

Kinetics of RSNO formation: Results from this study have shown that a slight change in substitution at the β-carbon of aminothiols has a great impact on their reaction dynamics. Electron density around S-center plays a significant role in the reactivities of the parent thiols. An electron-donating group increases the electron density on S-

center of the parent thiol as well as the corresponding RSNO. In this study, cysteamine, with the most nucleophilic S-center of the three aminothiols (cysteamine, cysteine and homocysteine), has the highest rate of nitrosation. The order of the rate of formation of the three RSNOs at pH 1.2 is CYSNO < HCYSNO < CANO (see Figure 6.1 and Table 6.2).

Scheme 6.1: Reaction scheme for the nitrosation of thiols

Figure 6.1: Comparison of rate of formation of HCYSNO, CYSNO and CANO at pH 1.2.

Table 6.2: Correlation of first-order rate constant of the S-nitrosothiols with pKa of the aminothiols.

S-nitrosothiols	Structure	pKa	k _{obs} (s ⁻¹)
S-nitrosocysteine (CYSNO)		8.30	1.12
S-nitrosohomocysteine (HCYSNO)		10.00	2.54
S-nitroscysteamine (CANO)		10.75	3.19

The result, as presented in Figure 6.2, shows that acid dissociation constants (pKa values) of the –SH group of the parent thiols strongly influence the rate of formation

of S-nitrosothiol. The rate of formation increases linearly with increase in the pKa values of the thiol. This observed linear correlation between pKa values and rates of formation of S-nitrosothiol is due to the increase in nucleophilicity with increase in pKa values.²²⁷ This indicates that the higher the pKa value, the higher the electron density around S-center and the faster the rate of S-nitrosation of primary aminothiols.

Figure 6.2: Linear dependence of Pseudo first-order rate constants, k_{obs} on the pKa of sulfhydryl group of the parent aminothiols of (a) HCYSNO, (b) CYSNO and CANO.

Equation 6.1 shows a generalized rate equation that can be applied to the S-nitrosation of aminothiol by acidic nitrite at low to mildly acidic pHs.

$$\begin{aligned} \text{Rate} &= \frac{d[\text{RSNO}]}{dt} \\ &= [\text{RSH}][\text{HNO}_2] \left(k_4 + \frac{k_3 k_6 [\text{H}^+]}{k_{-3} + k_6 [\text{RSH}]} \right) + \left(\frac{k_2 k_5 [\text{RSH}][\text{HNO}_2]^2}{k_{-2} + k_5 [\text{RSH}]} \right) \end{aligned} \quad (6.1)$$

k_2 , k_3 , k_4 , k_5 and k_6 are rate constants for the following equations:

At low pH conditions, reactions R6.3, R6.5 and R6.8 predominate and the terms with k_2 , k_{-2} and k_5 in equation 6.1 becomes negligible, resulting in first order kinetics in nitrous acid with a rate equation given by:

$$\begin{aligned} \text{Rate} &= \frac{d[\text{RSNO}]}{dt} \\ &= [\text{RSH}][\text{HNO}_2] \left(k_4 + \frac{k_3 k_6 [\text{H}^+]}{k_{-3} + k_6 [\text{RSH}]} \right) \end{aligned} \quad (6.2)$$

In mildly acidic pHs, when reactions R6.3, R6.6 and R6.7 become the most relevant, equation 6.1 predicts second order kinetics with respect to nitrous acid, with a rate equation given by equation 6.3.

$$\begin{aligned} \text{Rate} &= \frac{d[\text{RSNO}]}{dt} \\ &= k_4[\text{RSH}][\text{HNO}_2] + \frac{k_2k_5[\text{RSH}][\text{HNO}_2]^2}{k_{-2} + k_5[\text{RSH}]} \end{aligned} \quad (6.3)$$

Transnitrosation: Transnitrosation involves a direct transfer of the NO group from one thiol to another without the release of NO. It has been shown to proceed by an attack of a nucleophilic thiolate anion on an electrophilic S-nitrosothiol through a nitroxyl disulfide intermediate (Scheme 6.2)²²⁸⁻²³⁰.

Scheme 6.2: Transnitrosation reaction mechanism

The reaction is first-order in both thiol (R'SH) and S-nitrosothiol (RSNO). The results as presented in Figure 6.3 for the transnitrosation of cyseine, homocysteine and cysteamine by SNAP were found to be related to the dissociation constant of the sulfhydryl group of these thiols. The higher the pKa the faster the rate of

transnitrosation, which means that rate-determining step for this reaction is the nucleophilic attack of thiolate anion on the nitrogen atom of the nitroso group of SNAP. Plot of the measured first-order rate constant, k_{obs} versus pKa was linear (Figure 6.4). The intercept on the pKa axis of 7.57 is very significant as it seems to be the minimum pKa of aminothiols that can be transnitrosated by SNAP. One can infer that any aminithiol with pKa less than the physiological pH of 7.4 cannot be transnitrosated by SNAP.

Figure 6.3: Comparison of transnitrosation of HCYSNO, CYSNO and CANO.

Rate of transnitrosation increases with increase in pKa of the parent aminothiols

Figure 6.4: *Correlation of first-order rate constants of transnitrosation of the aminothiols with pKa. Rate of transnitrosation increases with increase in pKa of the parent aminothiols*

Stability: The stability of RSNO is crucial to its expected roles as the carrier, transport form and reservoir for NO bioactivity in the physiological environments. The S—NO bond stability is believed to be modulated by the structure of the specific RSNO.²³¹ For example, in this study the lengthening of the alkyl chain as in HCYSNO was

found to enhance its stability when compared with CYSNO and CANO (Figure 6.5). This suggests lengthening of the alkyl chain strengthens the S—N bond. Dissociation energies for the S—N bond for cysteine and homocysteine have been previously determined by Toubin et al as 29.5 and 35.8 kcal/mol respectively.¹⁹¹

In this thesis, copper ions were found to catalyse the decomposition of RSNO. The rate of decomposition increases with increase in copper concentrations. Figure 6.5 shows a comparison of the rate of Cu (II) ion catalyzed decomposition of the three RSNOs, CANO, CYSNO and HCYSNO.

Figure 6.5: Effect of Copper(II) on the stability of HCYSNO, CANO and CYSNO.

HCYSNO was the most stable of the three RSNOs.

The intercepts on the initial rate-axis are very significant as they give the initial rate of decomposition of the RSNOs in the absence of the metal ions. The fact that HCYSNO intercept is kinetically indistinguishable from zero is an indication that HCYSNO is relatively stable at physiological pH 7.4. The order of stability is CYSNO < CANO < HCYSNO. Table 6.3 shows the half-lives of these RSNOs at physiological pH 7.4 in the presence of metal ion chelator (EDTA) and in the presence of copper (II) ions without metal ion chelator. CYSNO has the shortest half-life of 0.5 s in the presence of copper. A half-life of 2.0 s has been previously reported for CYSNO in plasma.²³² The rapid metabolism of CYSNO and CANO to release NO may make these compounds advantageous as vasodilators in medical applications.

Table 6.3: Half-lives of the RSNOs in the presence and absence of metal ions

RSNO	Half-life, $t_{1/2}$ (s)	
	Presence of EDTA as metal ion chelator	Presence of Copper (II) ions
CYSNO	3.58×10^4 (or 10.00 hrs)	0.50
CANO	1.10×10^5 (or 30 hrs)	0.84
HCYSNO	7.13×10^5 (198 hrs)	16.5

The general instability of RSNOs in the presence of copper ions is due to the lengthening of the S—N bond and shortening of N—O bond as observed in the copper-RSNOs intermediate complex, $[\text{RS}(\text{Cu})\text{NO}]^+$ (equation R6.9). For example S—N bond lengths of S-nitrosocysteine are 1.87 Å and 2.11 Å in the absence and presence of copper ions respectively.¹⁹¹ The corresponding N—O bond lengths from quantum mechanical studies are 1.18 Å and 1.14 Å in the absence and presence of copper ions respectively.¹⁹¹

6.1 CONCLUSIONS AND FUTURE PERSPECTIVES

This study has shown that the rate of S-nitrosation and transnitrosation of thiols to form S-nitrosothiols and subsequent decomposition of S-nitrosothiols to release nitric oxide depend on a number of factors which include the dissociation constant (pKa) of the sulfuryl group, pH of their environments, nature of substitution at β-carbon, length of the alkyl chain and copper ion concentrations. The larger the pKa of the sulfuryl group of the thiol the faster the rate of nitrosation by acidic nitrite and transnitrosation by other S-nitrosothiols. Also the longer the length of the carbon chain the higher the stability. The stability of S-nitrosohomocysteine even in the presence of metal ions at physiological pH is particularly intriguing, since it is very important in the modulation of homocysteine related diseases. This dissertation has

thus revealed new therapeutics way for hyperhomocysteinemia, which is a risk factor for many diseases such as cardiovascular and neurodegenerative diseases, through S-nitrosation of homocysteine.

Results from this study have shown that RSNOs can be used as NO donors. They can be utilized in the development of NO-releasing compounds for various biomedical applications. For example, NO has been demonstrated as a facilitator of wound healing for people with diabetes.²³³⁻²³⁵ The only factor that may limit their biomedical applicability is their rapid decomposition rates at physiological pH 7.4, especially in the presence of metal ions as observed in this study. In order to overcome this deficiency, there is a need for modification of the structure of RSNOs in a way that prolongs their stability. This can be achieved by developing hydrophobic macromolecules in which RSNO will be covalently incorporated into the polymer backbone. Results from this dissertation will serve as a base for the design of such polymers. Also, catalytic effect of copper, as observed in this study, in stimulating the formation of RSNO and its subsequent decomposition to release NO can be an advantage in the development of biomedical devices where continuous generation of NO is desirable. This can offer a potential solution to the blood-clotting problems often encountered in blood-containing medical devices,²³⁶ since NO is an excellent inhibitor of blood platelets aggregation.⁷ Too many platelets can cause blood clotting,²³⁷ which may obstruct the flow of blood to tissues and organs (ischemia), resulting in stroke and/or heart attack. Bioactive polymer such as poly vinyl chloride (PVC), polyurethane (PU) and polymethacrylate doped with copper (II) ligand sites

can therefore be used as blood platelets aggregation resistant materials. The copper (II) sites within the polymer can catalyze nitrosation of thiols by nitrite and reduced to copper(I). The copper (I) sites in the polymer can then catalyze decomposition of RSNO to NO and thiolate anion. Provided that the blood contacting the polymer has some level of naturally occurring thiols like cysteine and cysteamine, and nitrite at all time, generation of NO at the blood-polymer interface should continue in a cycling manner as presented in Scheme 6.3.

Scheme 6.3: Redox cycling copper catalyzes continuous generation of NO from the reaction of nitrite and bioactive thiols. Using copper (II) doped polymer for coating biomedical devices will enhance continuous production of NO.

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